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VOLUME 18

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Advances in CLINICAL CHEMISTRY

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PREFACE

As they have in the past, the Editors consider that the clinical biochemist must keep constantly abreast, not only of technological advances in his field, but also of newer information concerning the abnormal biochemistry of those conditions and diseases which the clinician meets in his practice and hospital. The Editors have constantly sought to obtain reviews that fulfill each of these functions and hope that the present volume continues to meet these criteria.

The last review of mucopolysaccharides appeared in *Advances in Clinical Chemistry* in 1964. Since then, the various aspects of this field have been greatly developed, and Kennedy now presents a substantial and most comprehensive review of the chemical and biochemical aspects of glycosaminoglycans and proteoglycans, a nomenclature which he considers superior to the older terms. Of particular interest is his very extensive account of the roles of these compounds in health and disease.

The present status of the laboratory diagnosis of thyroid disorders is presented in an extensive review by Wellby, while at the same time the roles of the various parameters in normal and abnormal thyroid function are well described. This review is followed by that of Hall and Gomez-Pan on the hypothalamic regulatory hormones and their clinical applications, a very rapidly growing field which needs repeated surveillance.

In his review on uric acid metabolism in man, Balis considers the recent advances and present status of this important and interesting field. The side clinical effects of oral contraceptive agents have been of concern for several years, and the physiological and biochemical aspects have been studied to elucidate the mechanisms underlying these effects. Anderson *et al.* consider one aspect of these mechanisms, namely, the effects of oral contraceptives on vitamin metabolism. This is a problem of particular interest with regard to developing countries where the use of contraceptive agents is being encouraged and where vitamin intakes may not be optimal. Lead poisoning has become a matter of considerable social and medical concern, and, in the final review on the biochemistry and analysis of lead, Christian considers in some detail the methods available for the analysis of this element.

As in the past, it is a great pleasure to thank our contributors and publisher for their excellent cooperation in making this volume possible.

OSCAR BODANSKY
A. L. LATNER

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CHEMICAL AND BIOCHEMICAL ASPECTS OF THE GLYCOSAMINOGLYCANS AND PROTEOGLYCANS IN HEALTH AND DISEASE

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1. Introduction

1.1. General

The molecular architecture on which the chemical and biological aspects of our bodies are based is not only a scientific challenge, but also

a subject of great fascination. Indeed when King David wrote (D8) in one of his Psalms three thousand years ago that we are "fearfully and wonderfully made" he was more accurate than he probably realized or imagined. Few, if any, can avoid a wonderment for the complexity and yet harmony that exists within ourselves. As the advances of chemistry and biochemistry have permitted some insight into and understanding of our chemical natures and processes, new complexities, and complexities within complexities have been encountered. One such group of complexities are the proteoglycans and glycosaminoglycans, which together constitute a group of macromolecules that clearly are important and essential, if not major, components of our bodies, but whose structures and functions are complex and far from being understood completely. Yet by virture of this importance, an aspect that will be developed in this article, the proteoglycans and glycosaminoglycans demand the attention of the chemist, biochemist, biologist, and clinician. One of the purposes, therefore, of this article, in addition to those evident from the title, is to illustrate how important it is for such persons to depend more upon one another for a better understanding of the processes of health and disease. This will substantiate the view that the ultimate goal of work such as studies of the proteoglycans and glycosaminoglycans should be the benefit of mankind in delineation of the process of life in its material aspects and the early diagnosis and curative treatment of the diseases with which society is blighted. Such a goal should not be regarded as an aspiration to immortality or an attempt to equal the Divinity, since it is accepted that the human body is mortal (P1), but rather as a combat against the diseases that cause distress in, or early termination of, life, not the least of which are the diseases that man has generated himself in modern society.

In answer to the question of why we investigate proteoglycans and glycosaminoglycans, the reply is simple. As will be developed later, these macromolecules occur extensively in almost all, if not actually all, mammalian tissues along with collagen fibers whose positions they maintain and with which the tissue structure, type, and rigidity is produced and maintained. Therefore they are of prime importance in health and disease.

In spite of their importance, the chemical and biochemical aspects of the proteoglycans and glycosaminoglycans have not been the subject of a full-length review in the last decade, but the reviews have either been included as minor, short, or fringe sections in reviews on glycoproteins and polysaccharides, or, if they stand on their own, cover specific portions of the aspects of these macromolecules: distribution and biological role of glycosaminoglycans (J8); isolation and structure of glycos-

aminoglycans (J2); preparation of glycosaminoglycans (J3–J7, S13) and of proteoglycans (S12); separation of glycosaminoglycans (B35, N7, S16); separation, structure, and physical structure of glycosaminoglycans (J10); structure of glycosaminoglycans (C26); structure and biosynthesis of glycosaminoglycans (A8); structure and metabolism of proteoglycans (S32); structure of glycopeptide linkages of proteoglycans (L22); physical chemistry of glycosaminoglycans (B28); conformations of glycosaminoglycans in solution (S43); glycosyltransferases in biosynthesis of glycosaminoglycans (O3); biosynthesis and metabolism of amino sugars and heterosaccharides containing them (W4); metabolism of glycosaminoglycans (S46); metabolism and interactions of glycosaminoglycans (J9); glycosaminoglycans with respect to the molecular biology of the intercellular matrix (B8); and chemical modification of glycosaminoglycans (K16, S13). Only three useful and general reviews (B41, B42, S35), including one (B41) in this Series had been published by 1964, but most of these many reviews, although up to date at the time of their publication, are now considerably out of date.

Recently, the chemistry and biochemistry of the proteoglycans and glycosaminoglycans have been the subject (K13, K21) and major subject (K15) of miniature quick-reference reviews suitable both for expert interest and general instruction at undergraduate and postgraduate level. The biosynthesis of these macromolecules has been similarly treated (P4).

The interest in the field in Britain alone justified and stimulated the formation of the Mucopolysaccharide Club in 1966, and analogous societies have now been in existence for some time in other countries. European Symposia have been held on the subject of connective tissue research, the proceedings of the second symposium, dealing with the biochemical and pathophysiological aspects, having been published (F1) and republished in a somewhat updated form (F9). Proceedings of an earlier symposium were also published (Q1).

1.2. Scope

So great has the importance of proteoglycans and glycosaminoglycans been regarded to be, and so extensive has the interest in them been in the fields of chemistry, biochemistry, and medicine, that the number of papers published on them runs into many thousands, and each year several hundred relevant papers appear. The occurrence of proteoglycans in virtually all mammalian tissues besides tissues of other animals and some bacteria has provided great scope for studies of their isolation, structure, biosynthesis, and properties. This scope has been widened considerably by the fact that the glycosaminoglycans are very amenable

to labeling with radioactive tracers *in vivo* and *in vitro*. Clearly on the grounds of page space alone, this article cannot possibly cover all these papers. This article therefore gives a comprehensive but condensed coverage of the principal established chemical and biochemical facts, the recent important developments, and the currently held theories. The article therefore is intended to be a useful compendium of general purpose, to be of interest to and informative for the nonspecialist, and to commend itself to the specialist since not only are up-to-date papers considered, but references have been selected to include those that are keys to a larger number of less important but interesting papers.

So that the reader is informed of the types of chemical structures involved, general structural information is given at an early stage (Section 3), but the complex fine structural detail has been reserved to subsequent locations, where its discussion in relation to specific phenomena is more appropriate. The coverage of this article would have been restricted to human material, but in many cases the biosynthetic work on the human has not yet been done, and therefore it has been necessary to take advantage of work on nonhumans in order to present a more complete picture, and to permit the human situation to be predicted by analogy. However, in keeping with the overall emphasis of this Series, the principal sections, on healthy processes (Section 6) and the involvement of glycosaminoglycans and proteoglycans in disease (Section 7), are rightfully restricted to human aspects. In this article, in contradistinction to many of the reviews already cited, it is considered most inappropriate to discuss the glycosaminoglycans under headings of the separate glycosaminoglycan names since (1) many properties of the individual glycosaminoglycans are similar and it is essential to compare and contrast directly minor differences between them, (2) heterogeneity and discontinuity in the repeating structures of the macromolecules gives rise to overlap between the defined molecular types.

In essence, this article provides a review of the history of the chemical and biochemical aspects of glycosaminoglycans and proteoglycans in the last decade. It is not concerned with the details of the early structural work, which was excellently reviewed in 1964 (B42), nor with the earlier work on their involvement in disease, which was reviewed in 1964 (B41) in this series. In general, papers published before 1960 have therefore not been considered. In reading or making use of this article, readers should not approach with preconceived ideas nor should they focus on, for example, one particular glycosaminoglycan structure or mucopolysaccharidosis type: it is most important to keep clear of the limited and restricted vision of the field promulgated by some, but to see the involvement of this macromolecular class as a whole in life.

For the sake of completeness, readers are advised that all references on chemical and biochemical aspects of glycosaminoglycans and proteoglycans are now being covered annually in Specialist Periodical Reports (K10, K11, K14), and that this series will in future serve to update this present article on a regular basis. Furthermore the Science Citation Index (S15) is of use in this respect.

So far as the nomenclature and structural representation of monosaccharide carbohydrates (from which glycosaminoglycans are built) is concerned, there has been much confusion in the past with the result that many compounds even today are incorrectly named or improperly or incompletely described. In this article, the recommendations of the International Union of Pure and Applied Chemistry (C7) are followed, and therefore systematic nomenclature is used, other currently used names being included at the first point of major mention to permit comparison with less up-to-date works. The nomenclature of the macromolecules has been in a very bad state of confusion. One further purpose of this review, therefore, is to standardize the various terms that are being used in the field (Section 2), and for this reason the author takes an uncompromising view of terminology. Readers with experience will have no difficulty in recognizing the well-defined, generally accepted, structures on the basis of structure rather than nomenclature, whereas the nonexpert and general readers are taught the up-to-date names. Chair forms are used for depicting monosaccharide structures since these indicate the relative spatial disposition of the substituent groups, an aspect which is not given by the Haworth-type formula.

2. Nomenclature of Glycosaminoglycans and Proteoglycans

So far as definitions are concerned, *proteoglycans* consist of a protein poly(amino acid) chain comprised usually of a hundred or more amino acid units which can be a random (speaking generally, but not mathematically correct) arrangement of any of the twenty or so naturally occurring L-α-amino acids. This protein chain forms the backbone of the molecule and the carbohydrate part of the molecule takes the form of *polysaccharide chains* which are pendant and covalently bound to the protein chain (Fig. 1). These *polysaccharide chains* are linear and fairly regular, possessing alternating monosaccharide sequences which generally involve (a) acidic monosaccharide [D-glucuronic acid (see Section 3 for structures) or L-iduronic acid] (or in one case a neutral monosaccharide), and (b) basic monosaccharide (2-amino-2-deoxy-D-galactose or 2-amino-2-deoxy-D-glucose). The basic units are *N*-acetylated or *N*-sulfated at the amino groups and are in most cases *O*-sulfated at one of the hydroxyl

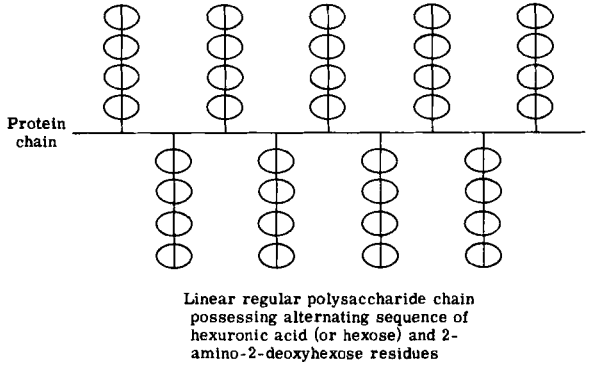


FIG. 1. General representation of proteoglycans.

groups. These polysaccharide chains are therefore strongly acidic, and on the basis of systematic nomenclature are termed *glycosaminoglycans*. No specific name is attributable to the protein chain of the *proteoglycan*. Thus as far as this review is concerned, just two classes of macromolecules are concerned—*proteoglycans* and their constituent *glycosaminoglycans*.

In view of the aforementioned confusion and lack of consistency in the nomenclature in this field, it is necessary to discuss a number of other relevant terms. The introduction in 1938 of the term *mucopolysaccharide* (M33) was to describe collectively 2-amino-2-deoxyhexose-containing polysaccharide materials of animal origin occurring either as free polysaccharides or as their protein derivatives. However, with the various subsequent discoveries of other types of carbohydrate-containing macromolecules, the term has come to be used in so many ways that it is now in a sense quite vague. Since the *glycosaminoglycans* have always come within the *mucopolysaccharide* category irrespective of the way in which that term has been used, they were described widely as *acidic mucopolysaccharides* on account of their highly cationic nature. However, this nomenclature arose at a time when it was not realized that the *glycosaminoglycans*, as we call them today, are attached covalently to protein, and at a time when the polysaccharide was isolated with some amino acid units attached. Thus, *acidic mucopolysaccharide* means the *glycosaminoglycan* of a *proteoglycan* plus (sometimes) a few amino acid units, whereas *glycosaminoglycan* means purely the polysaccharide part of a *proteoglycan*. The term *acid mucopolysaccharide*, used to a lesser extent, is synonymous with *acidic mucopolysaccharide*, whereas *acidic polysaccharide* applies to any polysaccharide containing acidic groups. The term *aminopolysaccharides* was also coined some time ago to describe collectively blood group-specific substances, *acidic mucopolysaccharides* and chitin.

The general terms *polysaccharide–protein* and *mucopolysaccharide–protein complex* and the specific terms, e.g., *chondroitin sulfate–protein complex,* were introduced when it was realized that the *glycosaminoglycan* is associated with protein, but when it (soon) became apparent that the association is stronger than that of a noncovalent complex, the general name *protein-polysaccharide* and specific name *chondromucoprotein* were employed to replace the three foregoing terms. These terms were all forerunners of *proteoglycan* (general) and, e.g., *chondroitin 4-sulfate proteoglycan* (specific).

It is unnecessary in this article to review the historical aspects of the development of all these terms. It is sufficient to conclude that the terms *acid mucopolysaccharide, acidic mucopolysaccharide, aminopolysaccharide, mucopolysaccharide, mucopolysaccharide–protein complex, mucoprotein, polysaccharide–protein,* and *protein polysaccharide* are misleading, historical, and redundant, and that we can well do without them.

On account of the apparent regularity of the polysaccharide chains in proteoglycans and the early belief that the protein present in preparations of the polysaccharide parts represented impurity, greatest attention has been given to the glycosaminoglycan chains themselves rather than to proteoglycans as a whole. Thus the glycosaminglycans have been named individually, but not so much according to their component monosaccharides and their simplified disaccharide repeating structures (these were often unknown at the times of original isolation), but according to trivial reasoning, e.g., by naming after the source.

In all, eight glycosaminoglycans of essentially different chemical structures have been identified. Through the times, these glycosaminoglycans have been individually named in a number of ways, as shown in Table 1. Most of these names are used currently, and so the reader will find it useful to have this table available when consulting the primary literature. Where the term chondroitin sulfate appears in the more recent literature, this can mean chondroitin 4-sulfate or chondroitin 6-sulfate or a mixture of the two. The terms keratan sulfate I and keratan sulfate II are sometimes used to denote keratan sulfates of corneal and skeletal origin, respectively, there being some differences between the two.

However, it has been accepted by many that, pending a complete systematization of polysaccharide nomenclature by the various nomenclature committees (a situation that in fact may never be realized), only one name for each should be regarded as the one which is up-to-date. As will be seen, some of the names in being altered have been systematized to include the terminal "an." This review supports strongly the names given in the first column of Table 1, and these will be used. (The term chondroitin sulfate will be used when no distinction between

TABLE 1
NOMENCLATURE OF THE GLYCOSAMINOGLYCANS

Modern name	Original name	Other names
Chondroitin	Chondroitin	—
Chondroitin 4-sulfate	Chondroitin sulfate A[a]	Chondroitin sulfate
Chondroitin 6-sulfate	Chondroitin sulfate C[a]	Chondroitin sulfate
Chondroitin 6-sulfate	Chondroitin sulfate D[b]	—
Dermatan sulfate	Chondroitin sulfate B[a]	Chondroitin sulfate β-Heparin Dermatan[c]
Heparin	Heparin	—
Heparan sulfate	Heparin monosulfate	Acetylheparan sulfate Heparitin[c] Heparan[c] Heparin sulfate Heparin monosulfuric acid Heparitin sulfate
Hyaluronic acid	Hyaluronic acid	
Keratan sulfate	Keratosulfate	Keratan[c]

[a] Known originally collectively as chondroitin sulfuric acid.

[b] Regarded by some as a separate glycosaminoglycan, but is really a supersulfated chondroitin 6-sulfate and is therefore classified as such.

[c] Names used incorrectly irrespective of system used.

the 4-sulfate and 6-sulfate isomers is given.) It is recommended that only these names should be used and that use of the others, which are misleading to the uninformed, should be discontinued completely forthwith. As will be seen later, the glycosaminoglycans contain acidic (anionic) groups that are capable for forming salts. However, the salt type is not usually indicated when naming a particular glycosaminoglycan since it is not necessarily known which cation of those available throughout the extraction and purification processes was finally assimilated by the glycosaminoglycan. However, where the salt form is known, this may be specified, e.g., sodium hyaluronate, potassium chondroitin 4-sulfate.

For the reasons of lesser emphasis invested and the general irregularity of the sequences already discussed, the protein parts of proteoglycans have not been named, nor for that matter have they been adequately chemically identified for names to be applied to them. This is therefore reflected in the absence of names for the proteoglycans themselves other than to indicate the type of glycosaminoglycan(s) involved, for example dermatan sulfate proteoglycan and chondroitin 4-sulfate-keratan sulfate proteoglycan, but such are nondescriptive of the protein.

In conclusion to the nomenclature aspects, some of the nonrecom-

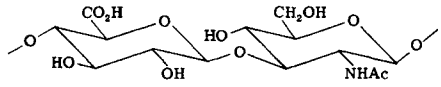
mended terms mentioned have been used by only a few authors. It really is high time the fashion for introduction of new names was stopped until full international agreement can be reached on a completely systematic system.

3. Generalized Primary Structures of Glycosaminoglycans and Proteoglycans

At this stage, it is essential to give a description of the glycosaminoglycans and proteoglycans in terms of chemical structures.

It is well established that carbohydrate structures and sequences can be much more complex than amino acid sequences. Whereas amino acids are generally only involved in linkages via their single amino and carboxyl groups, the carbohydrate may be linked in the pyranose or furanose forms (two anomeric forms each) and at any two of the five free hydroxy groups in such forms. However, the number of variations of linkage types in the glycosaminoglycans is small. Thus there is this limited number of glycosaminoglycan structures as opposed to the large variety of structures that arise, for example, in glucans. Most of the glycosaminoglycans, if not all, can be represented in terms of repeating disaccharide structures which in regular sequence give the polymeric carbohydrate. Many of these disaccharide repeating structures have been known for some time, and since their discovery and derivation have been reviewed *in extenso* (B42), so it is neither relevant nor necessary to review such work here. However, to provide a working or background knowledge for the following sections, the repeating structures will be described, but before this is done, the reader must be alerted to the fact that some problems do exist with these repeating-type sequences. These sequences have been oversimplified by earlier workers, since some irregularities are now known to exist in the structures (see later). Such irregularities constitute only small proportions of the total chain, and therefore the major structures now described provide an adequate basis for the material discussed in this article.

Starting with the simplest of the glycosaminoglycan structures, hyaluronic acid has a disaccharide repeating structure (I) composed of the

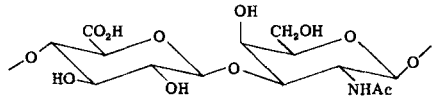


(I)

monosaccharides D-glucuronic acid and 2-acetamido-2-deoxy-D-glucose. Both these monosaccharides are in the pyranose form and have the β-D-anomeric configuration. In describing the linkage types between the component monosaccharides, polysaccharide and oligosaccharide struc-

tures by convention are written such that the nonreducing terminal (the chain end at which C-1 of the last unit is involved in glycosidic linkage to the penultimate monosaccharide unit) is written on the left, and such that the reducing terminal (the chain end at which C-1 of the last unit is not involved in a glycosidic linkage to the penultimate monosaccharide unit) is written on the right. This therefore permits the description such as "the linkage between (of) the D-glucuronic acid residue and (to) the 2-acetamido-2-deoxy-D-glucose residue" to be definitive since it is applied in the sequence left to right only. The linkage between the D-glucuronic acid and the 2-acetamido-2-deoxy-D-glucose residues in hyaluronic acid is β-D-(1 → 3); i.e., the uronic acid has the β-D-anomeric configuration at C-1 from which it is linked glycosidically to position 3 of the 2-acetamido-2-deoxyglucose residue (I). The linkage between the 2-acetamido-2-deoxy-D-glucose residue and the D-glucuronic acid residue is also of the β-D-configuration and involves a β-D-(1 → 4) linkage to the 4-position of the uronic acid residue. Thus altogether hyaluronic acid is regarded as a polysaccharide having the repeating structure -*O*-β-D-glucopyranuronosyl-(1 → 3)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-.

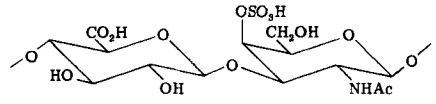
Hyaluronic acid and chondroitin are the only nonsulfated glycosaminoglycans occurring naturally and the structure of chondroitin is analogous to hyaluronic acid in that the repeating structure is isomeric with it. Chondroitin has a disaccharide repeating structure (II) composed of



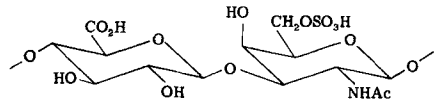
(II)

D-glucuronic acid and 2-acetamido-2-deoxy-D-galactose, both these residues being present in the pyranose form and in the β-D-anomeric configuration, the linkages between them being the same as for hyaluronic acid. Thus altogether chondroitin is regarded as having the repeating structure -*O*-β-D-glucopyranuronosyl-(1 → 3)-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1 → 4)-, and is therefore different from hyaluronic acid only in the orientation of one hydroxyl on every other monosaccharide unit along the chain.

Chondroitin 4-sulfate and chondroitin 6-sulfate, as their names imply, are sulfated varieties of chondroitin. The sulfate group is located at the 4- and 6-positions, respectively, of the 2-acetamido-2-deoxy-D-galactose residues, giving repeating structures of -*O*-β-D-glucopyranuronosyl-(1 → 3)-*O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl 4-(or 6-)sulfate)-(1 → 4)-, (III) and (IV), respectively. Variants of chondroitin sulfate



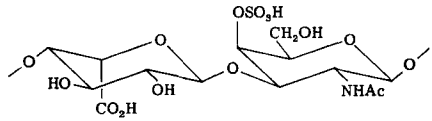
(III)



(IV)

in which the sulfate content is greater than the standard 1 mole per mole of disaccharide repeating unit are known, but have no specific name. The name chondroitin sulfate D was introduced to cover a similar situation appertaining to a glycosaminoglycan preparation obtained from shark cartilage, but this was simply an oversulfated variant of chondroitin 6-sulfate.

Dermatan sulfate is an isomer of chondroitin 4-sulfate in which the D-glucuronic acid units have been replaced by L-iduronic acid units, the linkage positions and absolute orientations of the glycosidic linkages remaining the same. The repeating unit (V) is therefore *O*-α-L-idopyranuronosyl-(1 → 3)-(2-acetamido-2-deoxy-*O*-β-D-galactopyranosyl 4-sul-

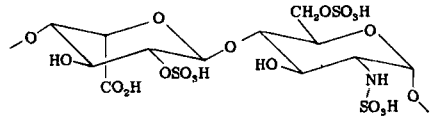


(V)

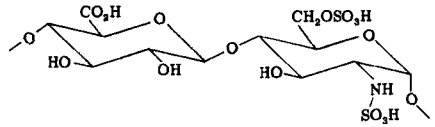
fate)-(1 → 4)-, and since L-iduronic acid is the C-5 epimer of D-glucuronic acid, the structure is different from chondroitin 4-sulfate only in the orientation of the carboxyl group on every other monosaccharide unit along the chain. Dermatan sulfate chains contain, as irregularities, one or two D-glucuronic acid units in place of L-iduronic acid units at random intervals. Dermatan itself, that is, the nonsulfated polysaccharide which is an isomer of chondroitin in which the orientation of the carboxyl group has been changed, and an isomer of hyaluronic acid in which the orientation of the carboxyl group and one hydroxyl group of the repeating unit have been altered, has not been detected in nature. This does not exclude the possibility of its existence, but it has been prepared synthetically by desulfation of dermatan sulfate. The name dermatan sulfate is somewhat inconsistent with chondroitin 4-sulfate; a preferable expression might be dermatan 4-sulfate. Dermatan 6-sulfate is also unknown in nature.

Heparin baffled structural chemists until the late 1960s in spite of extensive investigations since the nature of the uronic acid residues and

the locations of the sulfate groups proved difficult to assign. However, the monosaccharide sequence has now been recognized as a repeating disaccharide unit of L-iduronic or D-glucuronic acid and 2-amino-2-deoxy-D-glucose. In this disaccharide unit there are *O*-sulfate hemiester groups, and the amino groups are not *N*-acetylated as in the foregoing polysaccharides but are *N*-sulfated. The overall sulfate content of heparin lies in the range 2–3 moles sulfate per disaccharide repeating unit. Heparin is regarded as a mixture of principally two disaccharide repeating units: -*O*-(α-L-idopyranuronosyl 2-sulfate)-(1 → 4)-*O*-(2-deoxy-2-sulfamido-α-D-glucopyranosyl 6-sulfate)-(1 → 4)- (VI) and -*O*-α-D-glucopyranuronosyl-(1 → 4)-*O*-(2-deoxy-2-sulfamido-α-D-glucopyranosyl 6-sulfate)-(1 → 4)-(VII). Thus, although heparin contains monosaccharide component



(VI)

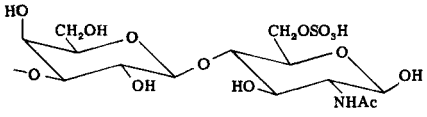


(VII)

analogies with hyaluronic acid and dermatan sulfate, it is particularly different from them on account of the α-anomeric configuration of the D-glucopyranuronosyl and 2-amino-2-deoxy-D-glucopyranosyl units.

That there is a similarity between heparan sulfate (formerly known as heparin sulfate, etc.) and heparin, as may be supposed from the suggestion in the name, in that the former is a further sulfated version of the latter, is incorrect. Although heparan sulfate appears to have backbone carbohydrate structures identical or similar to those of heparin, it differs from heparin in its *N*-sulfate and *N*-acetate contents, the location of its biosynthesis (see Section 4.6), and in its manifestation (see, e.g., Section 7.6, pp. 60–61).

Keratan sulfate is very much the odd one out in the polysaccharides embraced by the term glycosaminoglycan since it contains no uronic acid. "In place of" the uronic acid residue in other glycosaminoglycans, there is a neutral hexose unit—D-galactose. The second component monosaccharide is "normal" being a 2-acetamido-2-deoxyhexose. The repeating disaccharide unit of keratan sulfate is -*O*-β-D-galactopyranosyl-(1 → 4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl 6-sulfate)-(1 → 3)- (VIII) and may therefore be regarded as a sulfated polymer of the well known



(VIII)

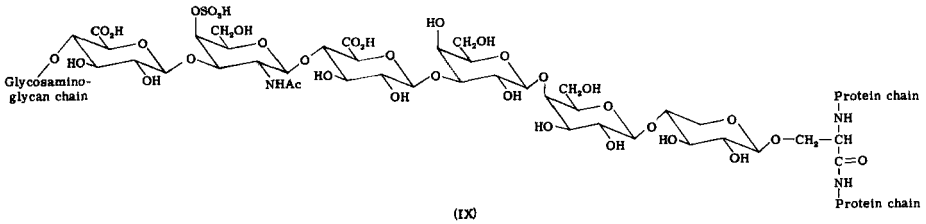
disaccharide *O*-β-D-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucopyranose (*N*-acetyllactosamine). It will be noticed therefore that the linkage types are the reverse of the glycosaminoglycuronans, where the linkages are -*O*-glycopyranuronosyl-(1 → 3)-*O*-(2-acetamido-2-deoxyglycopyranosyl)-(1 → 4)-.

Variants of keratan sulfate in which other neutral sugars occur and in which there is more than 1 mole of sulfate ester per disaccharide repeating unit are known. Keratan sulfate has also been found to contain smaller amounts of neutral monosaccharides, such as D-mannose and L-fucose, and 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid.

The structures show that all the glycosaminoglycans have normal-type polysaccharide structures. It is also noticeable that the structures of the glycosaminoglycans are all different and that none is the mirror image of another. Whereas it cannot be stressed too greatly that these repeating structures are somewhat simplified versions of actuality, they represent the major portions of each of the glycosaminoglycan sequences; the small sequence irregularities and complexities that occur have not been investigated as yet from the viewpoints of health and disease. The present structures, therefore, serve as a basis for discussion.

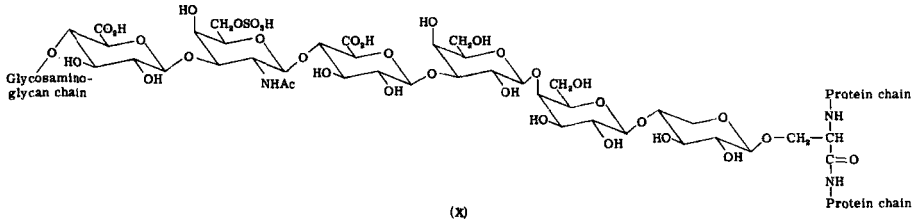
In the overall proteoglycan, the glycosaminoglycans are linked to the protein chain via a glycopeptide linkage. In most cases, this linkage involves monosaccharide units different from those of the main polysaccharide chain. These linkage region monosaccharides are linked glycosidically, the reducing terminal being linked to a side chain of an amino acid in the protein chain.

The chemical aspects of the glycopeptide linkages of proteoglycans have been reviewed (L22). Proteoglycan containing chondroitin-4-sulfate was afforded early attention in this area, and a number of studies led ultimately to the assignment of structure (IX) to the overall structure in the glycopeptide linkage region and commencement of the repeating



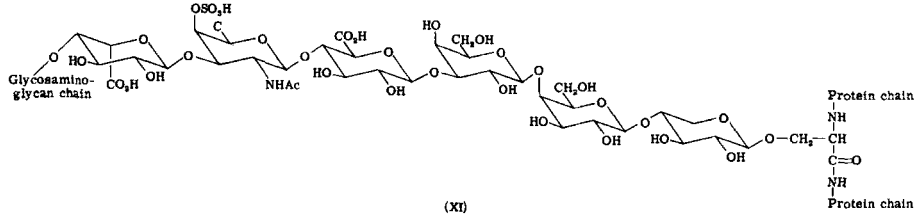
(IX)

disaccharide unit (A4, B40, K4, L21, M45, R15). The structure of the special glycopeptide linkage to which the first repeating disaccharide unit is attached is, therefore, -*O*-β-D-glucopyranuronosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-xylopyranosyl-L-serine. The actual linkage of chondroitin 6-sulfate to protein is identical to that for chondroitin 4-sulfate (A4, H18), and therefore the overall structure in the glycopeptide linkage region and commencement of the repeating disaccharide unit is (X). Dermatan sulfate has also been



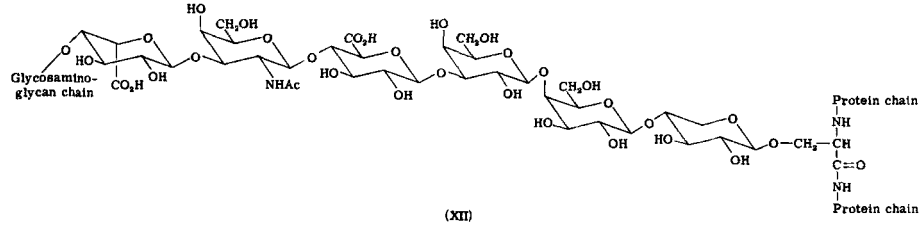
(X)

found to conform to this general pattern in its glycopeptide linkage, including the D-glucopyranuronosyl unit (XI) (B21, F6, S39, S40).



(XI)

It is unclear whether heparin occurs as a proteoglycan, but all heparin samples (L24) contain amino acids and again the glycopeptide linkage acid unit has the D-*gluco*-, not the L-*ido*-, configuration (XII) (C15,

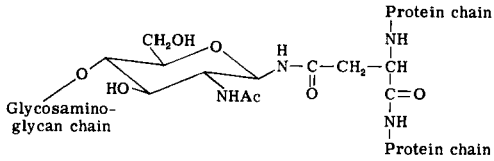


(XII)

L14–L17, L19, L20). The presence of D-galactose and D-xylose in preparations of heparan sulfate (K32, L28) suggest that the glycopeptide linkage of this glycosaminoglycan may be analogous to the foregoing.

Corneal keratan sulfate is apparently unique among the glycosaminoglycans in its glycopeptide linkage in that the glycopeptide structure somewhat represents that which occurs in many glycoproteins (XIII)

(B4, S48). The structure of this glycopeptide linkage is therefore -2-acetamido-1-(L-aspart-4-oyl)-1,2-dideoxy-β-D-glucosylamine. It appears that neutral monosaccharide units are adjacent to the 2-acetamido-1,2-dideoxy-D-glucopyranosylamine unit (B7). In contrast, skeletal keratan sulfate glycopeptide linkage involves 2-acetamido-2-deoxy-D-galactose (which does not occur in corneal keratan sulfate) units linked to L-serine and L-threonine units (G15, S17), but this may not be the only linkage type in this glycosaminoglycan (H11, H13).



(XIII)

Work on the structure of the glycopeptide linkages of chondroitin and hyaluronic acid has progressed least of all. That of chondroitin may be assumed to be identical with that for chondroitin 4-sulfate, etc., whereas that of hyaluronic acid may involve D-glucose, L-arabinose, D-xylose and/or D-ribose (H6, K5, S37, V9, W2, W3), and L-serine (C9), but the various reports do not agree.

No particular structure may be assigned to the protein since no regular amino acid sequence is evident, as is the general case for proteins. On the basis of the foregoing, the general compositional structure of the proteoglycan is that shown in Fig. 2. Whereas all this structural detail

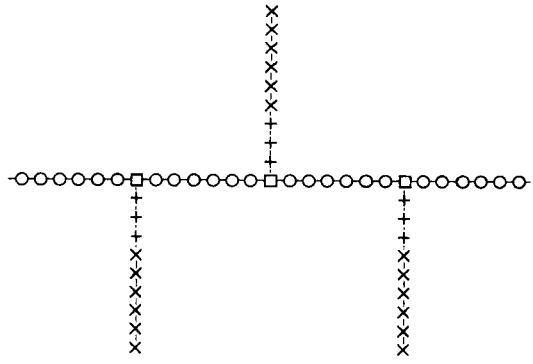


FIG. 2. Compositional structure of a proteoglycan. ○, Regions in the amino acid sequence of the protein backbone which do not bear polysaccharide. □, Amino acids in the amino acid sequence of the protein backbone which are involved in glycopeptide linkage. +, Monosaccharide units atypical to the glycosaminoglycan main structure, but involved in region of glycopeptide linkage. ×, Monosaccharide units of the glycosaminoglycan main chain.

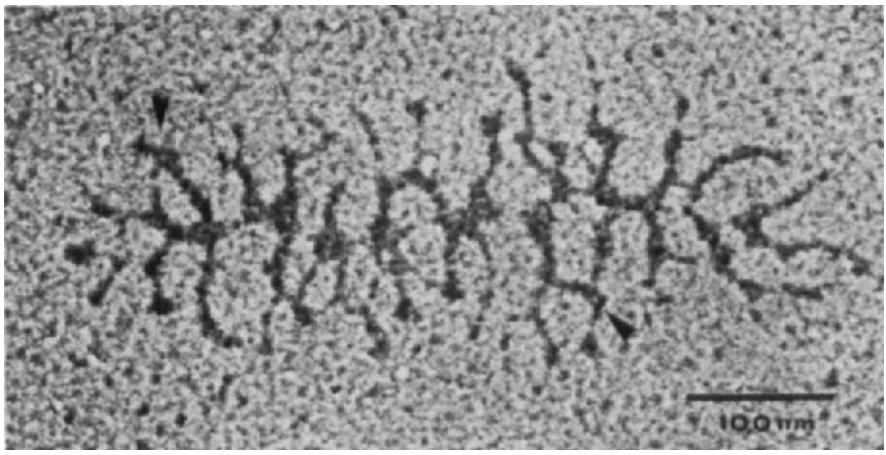


FIG. 3. The format of a proteoglycan molecule showing the protein chain (long axis) and the glycosaminoglycan chains (side chains). Some glycosaminoglycan side chains appear to be intertwined with one or another neighboring chain (left arrowhead); other side chains are bridged by short strands that run perpendicularly from the middle of one glycosaminoglycan chain to another (right arrowhead). Electron micrograph, ×270,000.

is precisely definitive and very much necessary in order to describe the molecules and to give the appropriate basis for the understanding of their participation in health and disease, as is generally the case for macromolecules, there are far fewer data available on the overall shape and appearance of the proteoglycan molecule. The facets of shape and appearance are usually derived from physicochemical studies (e.g., see H23, S2), which most frequently give three-dimensional structures for the crystalline solid, which of course is not the situation appertaining *in vivo*. Such physicochemical studies are outside the scope of this review, but in order to give the reader the overall picture of the shape of proteoglycans, one of the few electron micrographs obtained (R17) is shown in Fig. 3.

4. Biosynthesis of Proteoglycans and Glycosaminoglycans

Considerable advances in the understanding of the biosynthetic processes underlying the formation of proteoglycans have been made recently. However, it must be borne in mind that in both human and nonhuman instances, many of the biosynthetic studies have been carried out on nonstandard or abnormal material including pathological specimens, and cultured cell lines in which the biosynthetic processes may have changed during storage and subculture. Furthermore, as will be

evident from the subsequent sections on biosynthetic control, (Sections 4.5 and 5), the biosynthetic processes are very sensitive to alteration, and experiments conducted *in vitro* will not necessarily reflect accurately the situation appertaining *in vivo*.

Portions of recent reviews on the various aspects of 2-amino-2-deoxyhexoses (W4) and glycoproteins (C19, O3, S24, S32) are relevant to this section since they deal with the general pathways for the activation of monosaccharides in polysaccharide formation and with the general biosynthesis of protein. Further reviews recount the progress of studies in the biosynthesis and metabolism of proteoglycans to various dates in the last decade (N5, P4, R14, R16, S21, S46) including one (N8) with particular reference to the biosynthesis of polysaccharide from glycopyranosyl esters of nucleoside pyrophosphates (sugar or monosaccharide nucleotides). Antigenicity studies have contributed to elucidation of biosynthetic aspects (M47) with particular reference to the protein chains of proteoglycans. Some of the enzymes, transferases and synthetases, which are involved in proteoglycan biosynthesis are listed in the Enzyme Nomenclature handbook (E4), but in many instances it must be recognized that the enzymes responsible for the biosynthetic steps have not been identified, and some of those which have been identified have not yet been characterized to an adequate degree to allow classification.

Essentially a biosynthetic step involves (a) an acceptor molecule, which will be the growing chain biosynthesized, (b) the donor molecule which contains the next unit to be added to the growing chain, and (c) a transferase, that is an enzyme that will transfer catalytically the unit from the donor to the acceptor. Exactly how this is achieved is yet to be discovered, although it is reasonable to assume that all three entities must be in close association to facilitate the transfer. Biosynthesis takes on a block unit-type addition to the growing chain; i.e., a presynthesized unit is added as a whole rather than each unit being biosynthesized totally on the end of the growing chain. The units in the case of glycosaminoglycans are monosaccharides, amino acids, and sulfate ion. Clearly a number of parameters will affect the route and rate of biosynthesis, and operators will provide stimuli and feedback controls. Furthermore effects measured are always subject to influence by the degradation acting on the proteoglycan molecules.

Although a number of enzymes which degrade or cleave glycosaminoglycan chains are known and used, it is somewhat surprising that most of those identified are endogenous only to nonmammalian systems. Some mammalian enzymes active on glycosaminoglycans are known, but in general they have not been studied to any great degree with respect to their action pattern. Therefore at this moment it is not possible to

deduce a degradation sequence for glycosaminoglycans and proteoglycans. Accordingly, the discussion on degradation in this article is disperse and has been included in the context in which the studies were made.

4.1. Formation of the Protein Chain

Various studies (J11) of the incorporation of D-[6-^{14}C]glucose, L-[^{14}C]lysine, and [^{35}S]sulfate into the chondroitin sulfate proteoglycan of chick embryonic cartilage show that the carbohydrate and protein moieties are synthesized at different relative rates. Further approaches to the order of biosynthesis of the proteoglycan moieties have been made (e.g., B17, M13, M16, O6; see also Section 5, p. 36) by a comparison of their rates of formation in the presence of a specific inhibitor of the biosynthesis of one of them, and all the observations accord with protein synthesis preceding polysaccharide elaboration.

Apart from such inhibitive and general effects, the protein biosynthesis of the proteoglycans has not been investigated. It may be presumed to occur via the normally accepted methods of protein synthesis. This control of the protein structure will be quite complex on account of the number of different protein structures involved in proteoglycans from different and from the same source(s). Any specialities in this amino acid sequence will be apparent only when sequence data are available.

It is deducible, however, that certain amino acid sequences code for the attachment of the glycosaminoglycan chain, and that this code must contain L-serine for the majority of glycosaminoglycans, or L-asparagine for some keratan sulfate chains, because these units are part of the glycopeptide linkages (see Section 3, pp. 13–15). The code must involve more than the introduction of these amino acids into the protein chain since not all L-serine or L-asparagine residues are involved in glycopeptide linkages. Furthermore, those not utilized in such linkages cannot be so used subsequently, since intact proteoglycan does not serve as an acceptor molecule for the addition of further glycosaminoglycan chains.

4.2. Addition of Carbohydrate to the Protein Chain

Once the protein sequence has been set up and the protein chain has been produced in intact form, carbohydrate units may be added to complete the formation of a proteoglycan molecule. The biosynthetic aspects of the glycosaminoglycan chain have been studied in considerable detail.

The carbohydrate components of glycosaminoglycans have long been known to be derived from D-glucose without alteration of the carbon

skeleton (T11). The discovery of nucleotide monosaccharides in the biosynthesis of polymeric carbohydrate, and the finding that a great many interconversions between the various monosaccharides required for polysaccharide chain synthesis occur at nucleotide, high energy or activated level, are the bases for the synthesis of glycosaminoglycan chains as well as for other polysaccharides and oligosaccharides. Those required for glycosaminoglycan synthesis are the UDP derivatives of D-glucuronic acid, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, D-xylose, D-galactose (and GDP-L-fucose, UDP-D-mannose, CMP-5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acids for keratan sulfate "additional components").

In the formation of the monosaccharide nucleotides, two pyrophosphorylase reactions (see P4) account for the whole variety of monosaccharide nucleotides. UDP-D-Glucose arises from D-glucose that is first converted by D-glucokinase (EC 2.7.1.2) and ATP to D-glucose 6-phosphate and thence in the presence of D-glucose and phosphato-D-glucomutase (D-glucose-cofactor) (EC 2.7.5.5) to D-glucose 1-phosphate. Direct conjugation of the latter with UTP by a pyrophosphorylase glucose-1-phosphate uridylyltransferase (EC 2.7.7.9) gives UDP-D-glucose. UDP-D-Glucose gives rise to all of the D-monosaccharide nucleotides with the exception of the 2-amino-2-deoxyhexoses and 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acids. UDP-2-Acetamido-2-deoxy-D-glucose arises by a more complex pathway—the D-glucose 6-phosphate is converted by D-glucose-phosphate isomerase (EC 5.3.1.9) to D-fructose 6-phosphate, which is then enzymatically aminated by L-glutamine, N-acetylated by acetyl-CoA, interconverted to the 1-phosphate, and only then conjugated by a specific pyrophosphorylase, UDPacetamidodeoxy-D-glucose pyrophosphorylase (EC 2.7.7.23), to form UDP-2-acetamido-2-deoxy-D-glucose. The range of monosaccharide nucleotides principally required for glycosaminoglycan chain biosynthesis arise as follows. UDP-D-Galactose comes directly from UDP-D-Glucose by the action of an epimerase which acts at C-4 of the D-glucose ring—UDP-D-glucose 4-epimerase (EC 5.1.3.2). UDP-D-Glucuronic acid arises by dehydrogenation of UDP-D-glucose by a four-electron oxidation process catalyzed by UDP-D-glucose dehydrogenase (EC 1.1.1.22), and UDP-D-xylose by decarboxylation of UDP-D-glucuronic acid by UDP-D-glucuronate decarboxylase (EC 4.1.1.35). UDP-L-Iduronic acid arises, allegedly, from an epimerization at the C-5 position of UDP-D-glucuronic acid by UDP-D-glucuronate 5′-epimerase (EC 5.1.3.12) (J1), but UDP-L-iduronic acid has not been isolated (see H10), and it may be that inversion occurs at the polymer level, at least in heparin (L23); this will be discussed in detail later. UDP-2-Acetamido-2-deoxy-D-galactose is formed by the

action of UDP-acetamidodeoxy-D-glucose 4-epimerase (EC 5.1.3.7) on UDP-2-acetamido-2-deoxy-D-glucose.

After the production of the monosaccharide nucleotides which provides the basic material for biological polymerization of the glycosaminoglycan chain, the reactions to form the glycosaminoglycan chains (P4, S46) can be considered to occur in three stages: chain initiation, chain propagation, and chain termination.

Using a cartilaginous preparation, it has been demonstrated (R13; see also G12) that for a chondroitin 4-sulfate chain an enzyme exists that catalyzes transfer (and incorporation) of D-xylose from UDP-D-[^{14}C]xylose to macromolecular proteinaceous acceptor with the production of an *O*-β-D-xylopyranosyl-(1 $\rightarrow$ L-serine) bond, and clearly this is the chain initiation reaction (S45). Native proteoglycan protein (B6), prepared from chondroitin sulfate proteoglycan by periodate oxidation, reduction, and acid hydrolysis, also acts as an acceptor for D-xylose on catalytic transfer by the D-xylosyltransferase. However, it is evident that parts of the protein in addition to the L-serine residues are involved since free L-serine will not act as an acceptor in this reaction. It appears that the transfer to proteoglycan protein is to the hydroxyl group of specific L-serine residues (B5). Intact proteoglycan and proteoglycan from which glycosaminoglycan has been removed using, e.g., hyaluronidase (EC 3.2.1.35), are inactive acceptors, and this demonstrates that certain L-serine residues are not utilized in growing glycosaminoglycan chains on the protein. (The proteoglycan protein produced via periodate oxidation will expose the hydroxyl groups of the utilizable L-serine residues, whereas that produced via enzymic hydrolysis will retain the carbohydrate units of the original glycopeptide linkage regions.)

Continuing with the biosynthesis of a chondroitin sulfate chain, the additions of the two D-galactose residues each occur by a specific D-galactosyltransferase (H18, H19). In the first addition of a D-galactose unit, *O*-β-D-xylopyranosyl-(1 $\rightarrow$ L-serine) and such structural units incorporated in proteoglycan protein are acceptors, the transfer of D-galactose from UDP-D-galactose being catalyzed by the specific D-galactosyltransferase. The terminal nonreducing D-xylose residues must be in the pyranose form. D-Xylose itself also acts as an acceptor for the catalytic transfer (H14), but since the occurrence of D-xylose in mammalian tissues is limited almost exclusively to the glycopeptide linkage regions of the proteoglycans, any terminal nonreducing D-xylopyranosyl residues that the D-galactosyltransferase encounters *in vivo* is most likely to be a part of a growing proteoglycan molecule. It is therefore sufficient for the enzyme to recognize only the monosaccharide structure of the "complete" acceptor molecule. However, *O*-β-D-xylopyranosyl-(1 $\rightarrow$ L-serine) is a

better acceptor than D-xylose, and presumably the natural partial proteoglycan is an even better one. However, once this first D-galactose residue has been added, the newly formed molecule which contains an *O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-xylopyranosyl-(1 → L-seryl) unit does not serve as a substrate for the same D-galactosyltransferase. It is not until this first D-galactose residue has been added that the second specific D-galactosyltransferase can operate, but it will act on *O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-xylopyranosyl-(1 → L-seryl) structures to give the sequence *O*-β-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-xylopyranosyl-(1 → L-seryl). In contrast to the first D-galactosyltransferase, the second transferase absolutely requires an acceptor structure larger than monosaccharide. Transfer will occur to 4-*O*-β-D-galactopyranosyl-D-xylose and to *O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-xylopyranosyl-(1 → L-serine), but not, for example, to 3-*O*-β-D-galactopyranosyl-D-galactose. In this case the enzyme needs to recognize not only the terminal monosaccharide acceptor unit, but also the penultimate unit. This high degree of specificity is necessary in order to limit the addition of D-galactose units to two. The product structure *O*-β-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-xylopyranosyl-(1 → L-seryl) is not an acceptor for either of the D-galactosyltransferases.

The first D-glucuronic acid residue of the first disaccharide repeating unit of the glycosaminoglycan chain is added by a D-glucuronosyltransferase (H20), and of the various oligosaccharides and glycopeptides representing the glycopeptide linkage formed thus far, the *O*-β-D-galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-xylopyranosyl-(1 → L-seryl) unit is the most effective acceptor. The D-gluronosyltransferase involved is specific for this task, being unable to add subsequent D-glucuronic acid units in chain propagation. The action of such a D-glucuronosyltransferase also applies to dermatan sulfate, since the first hexuronic acid unit added possesses the D-*gluco* configuration (see Section 3, p. 14). At this stage the initiation of the chain growth is complete.

In view of the structural identity of the glycopeptide linkage regions for the attachment of chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, heparin, and heparan sulfate, it would appear that the one biosynthetic pathway as discovered for chondroitin 4-sulfate is utilized for the initiation of all these glycosaminoglycan chains. To date, the biosynthetic glycosaminoglycan chain initiation has not been investigated for these other glycosaminoglycans apart from heparin. In the case of heparin it has been shown that D-xylose is transferred from UDP-D-xylose to L-serine residues of protein acceptor (see G12), and from various data (e.g., H14) it has been possible to build up a complete formulation (H15) of the mechanism of biosynthesis of the heparin glycopeptide

linkage region, a mechanism identical to the one for chondroitin 4-sulfate. Again the first hexuronic acid unit is added by a specific enzyme (H15), which is not involved in subsequent chain propagation. The mechanism of the biosynthesis of such glycopeptide linkages therefore involves a number of stages the sequence of which is known (H15). It is noteworthy that although heparin contains predominantly L-iduronic acid with minor amounts of D-glucuronic acid the first hexuronic acid residue added to the glycopeptide linkage possesses the D-*gluco* configuration (but see later). No work on the biosynthetic mechanisms of chain initiations for the keratan sulfate types of chains has yet been reported, and biosynthetic studies on the glycopeptide linkage in hyaluronic acid proteoglycan must await definitive elucidation of its structure.

The biosynthetic steps giving rise to chain propagation are less well known, and the question arising from the common glycopeptide linkage structure for a number of the glycosaminoglycans as to how the propagation of a particular glycosaminoglycan is selected remains unanswered. Early studies indicated the formation of a low-sulfated polysaccharide chain on incubation of a cartilaginous preparation with UDP-D-glucuronic acid and UDP-2-acetamido-2-deoxy-D-galactose, and some information came from studies on hyaluronic acid production in streptococci (S47). At that time the question remained as to whether the chain was propagated by the addition of single monosaccharide units or preproduced disaccharide units to the growing chain. Enzymes that incorporate radioactivity from UDP-D-[^{14}C]glucuronic acid and UDP-2-acetamido-2-deoxy-D-[^{3}H]galactose into growing chondroitin sulfate chains have been recognized (O8) and it is now clear that the polymer results from the concerted action, on the glycopeptide linkage acceptor of two glycosyltransferases, of a D-acetamidodeoxygalactosyltransferase and an D-glucuronosyltransferase, which alternately add the two respective monosaccharide component units of the repeating disaccharide unit directly to the chain without participation of a free disaccharide unit. Transfer of 2-acetamido-2-deoxy-D-galactose to terminal nonreducing D-glucuronic acid units takes place with sulfated, as well as nonsulfated, acceptor oligosaccharides, containing terminal nonreducing β-D-glucopyranuronosyl residues, from chondroitin, chondroitin 4-sulfate, chondroitin 6-sulfate, and hyaluronic acid. The fact that the hyaluronic acid hexasaccharide *O*-β-D-glucopyranuronosyl-(1 $\rightarrow$ 3)-[(2-acetamido-2-deoxy-*O*-β-D-glucopyranosyl)-(1 $\rightarrow$ 4)-*O*-β-D-glucopyranuronosyl-(1 $\rightarrow$ 3)-]$_2$-2-acetamido-2-deoxy-D-glucose acts as an acceptor has led to the claim (R14) that the D-acetamidodeoxygalactosyltransferase has a simple substrate specificity with a requirement only for the correct terminal nonreducing unit of the acceptor, the identity of the penultimate unit being unimportant. How-

ever, it is difficult to ratify this view with the lack of occurrence of the two 2-acetamido-2-deoxy-D-hexoses in any one hyaluronic acid or chondroitin sulfate-type molecule, which suggests that the penultimate unit exerts an influence. Furthermore, a decasaccharide, which contains a terminal nonreducing α-L-idopyranuronosyl unit, from dermatan sulfate is also an acceptor for the D-acetamidodeoxygalactosyltransferase (M2). Nevertheless it is clear that for production of chondroitin, chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate chains a D-acetamidodeoxygalactosyltransferase exists. It is reasonable to assume that a D-acetamidodeoxyglucosyltranferase activity exists for the biosynthesis of mammalian hyaluronic acid.

For the polysaccharides considered thus far, the 2-acetamido-2-deoxy-D-hexose unit has been transferred in such a way as to be in the β-D-configuration in the product. However in the case of heparin the unit must be added so as to be in the α-D-configuration in the product. This is effected by a separate D-acetamidodeoxyglucosyltransferase activity, and evidence for such comes from the ability of a microsomal fraction from a heparin-producing mastocytoma to transfer 2-acetamido-2-deoxy-D-glucose from UDP-2-acetamido-2-deoxy-D-glucose to an exogenously added glycopeptide derived from heparin (H17). Pretreatment of the acceptor with β-D-glucuronidase (EC 3.2.1.31) removed the terminal nonreducing β-D-glucopyranuronosyl unit, resulting in loss of acceptor activity.

The D-glucuronosyltransferase which catalyzes the transfer of D-glucuronic acid from UDP-D-glucuronic acid to the growing polysaccharide chain appears to possess, in addition to a specificity for a terminal nonreducing 2-acetamido-2-deoxy-D-hexose unit, a specificity for the anomeric configuration of this unit. In the case of chondroitin sulfate, the D-glucuronosyltransferase requires a terminal nonreducing nonsulfated or 6-sulfated 2-acetamido-2-deoxy-β-D-galactopyranosyl unit as acceptor; no transfer occurs to a 4-sulfated residue (R14). In the case of heparin, the D-glucuronosyltransferase which transfers D-glucuronic acid to a terminal nonreducing 2-acetamido-2-deoxy-D-glucose unit is specific for this terminal unit in the α-D-configuration (H16). The transferase is inactive when a hyaluronic acid-type pentasaccharide possessing a terminal nonreducing 2-acetamido-2-deoxy-β-D-glucopyranosyl unit is offered as acceptor. The reason for such specificity is uncertain since all the hexuronic acid units are added to growing glycosaminoglycan chains as D-glucuronic acid (the case of occurrence of L-iduronic acid units will be dealt with a little later) and in the β-D-configuration irrespective of the glycosaminoglycan type apart from keratan sulfate. Although the β-D-configuration has been certain for hyaluronic acid and the chondroitin sulfates for some time, the biosynthetic addition of D-glucopyranuronosyl unit

in the β-D-configuration in heparin (which contains largely L-iduronic acid) has been more recently demonstrated (H16) in that heparin fragments with terminal nonreducing 2-acetamido-2-deoxy-D-glucose units acted as acceptor for UDP-D-[^{14}C]glucuronic acid in conjunction with enzyme present in a heparin-producing mastocytoma. That the D-glucuronosyltransferases are specific for terminal nonreducing 2-acetamido-2-deoxy-D-hexosyl residue demonstrates the need for a separate D-glucosyltransferase for the transfer of the first D-glucuronic acid unit to the glycopeptide linkage structure.

Originally it was considered that the biosynthesis of L-iduronic acid units was analogous to that of D-glucuronic acid units, but there is no evidence for the existence of either UDP-L-iduronic acid or a L-idopyranuronosyltransferase, and indeed such an enzyme appears to be absent (e.g., H17, L23). Important information on the biosynthesis of L-iduronic acid units comes from work with a microsomal fraction of a heparin-producing mastocytoma (L23), incubation of which with UDP-D[^{14}C]glucuronic acid and unlabeled UDP-2-acetamido-2-deoxy-D-glucose resulted in incorporation of radioactivity into endogenous polysaccharide. When adenosine 3′-phosphate-5′-sulfatophosphate (a precursor of sulfate groups, see Section 4.3) was included in the incubate, the product polysaccharide contained L-[^{14}C]iduronic acid as well as D-[^{14}C]glucuronic acid. Pulse-chase experiments revealed that D-[^{14}C]glucuronic acid was incorporated into the polymer during the pulse period (in the absence of adenosine 3′-phosphate-5′-sulfatophosphate) and in the bound form was subsequently converted to bound L-[^{14}C]iduronic acid during the chase period (in the presence of adenosine 3′-phosphate-5′-sulfatophosphate). Such experiments lead to the conclusion that in glycosaminoglycan chains the L-iduronic acid residues are formed by epimerization of D-glucuronic acid residues at the polymer level. It must be noted that the epimerization involves only inversion of configuration of C-5 in the D-glucuronic acid unit—this gives an L-configuration, and the C-5 epimer of D-glucuronic acid is L-iduronic acid. No alteration of the anomeric configuration in the glycosidic linkage is involved—the change from β-D- to α-L- is purely one of nomenclature standardization. Such a plausible explanation of epimerization is congruent with the finding that structures in other polysaccharides involve enzymic modification at the polymer level; it does, however, raise a number of questions including those of the effect of sulfation on polymer extension, the incomplete conversion of D-glucuronic acid residues in dermatan sulfate, heparin, and heparan sulfate and the greater degree of epimerization in dermatan sulfate, and the identity of specificity of the epimerase. It is conceivable that more than one epimerase exists. The epimerase for production of the dermatan

sulfate structure would appear to be specific for chondroitin 4-sulfate structure since dermatan and "dermatan 6-sulfate" are not known to occur naturally. Such a specificity in converse explains why the two latter polysaccharide structures are not formed. However, since dermatan sulfate arises from the action of the epimerase on chondroitin 4-sulfate, the occurrence of a few remaining D-glucuronic acid residues is understandable. The mechanism of the epimerase must also be investigated—it appears at the moment that the enzyme acts as the polysaccharide chain is growing rather than after a length of chain has been completed; but it will have to be decided why only certain chondroitin 4-sulfate chains are converted—it is remarkable that there are very very few examples of hybrid chondroitin 4-sulfate and dermatan sulfate structures within the same polysaccharide chain other than very small amounts of the former in dermatan sulfate. Biosynthetic aspects of the buildup of keratan sulfate chains are unknown, they but can be assumed to follow the general lines of the other glycosaminoglycan chains.

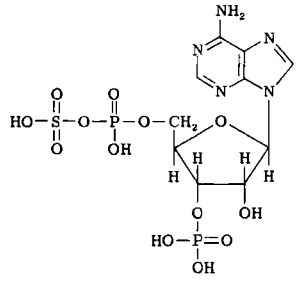
For rat epiphysial cartilage, the monosaccharide nucleotides and other intermediates (H8) in glycosaminoglycan biosynthesis have been isolated and quantitated. Collation of many of the data on the biosynthetic initiation and propagation of glycosaminoglycan chains has permitted the presentation of an overall scheme (K28), which is given in Section 4.3, p. 30.

The chain termination aspect of the glycosaminoglycan chain biosynthesis is not understood at all—indeed it is not known whether such a stage is necessary. Since a statistical distribution of molecular weights is always found for hyaluronic acid proteoglycan species, and since very high molecular weights are attained, it would seem that there is no termination process but that molecular length is limited simply by the turnover and degradative processes. On the other hand, in the case of chondroitin 4-sulfate proteoglycan there is evidence that the polysaccharide chain length is remarkably constant, suggestive of some process leading to uniformity. A number of termination mechanisms may be suggested, including inhibition of polymerization by the growing chain and specialized modification of the ultimate terminal unit. Chain termination may also be considered in conjunction with the overall regulation of proteoglycan biosynthesis, which is discussed subsequently (Section 4.5).

4.3 Addition of Sulfate to the Glycosaminoglycan Chain

The incorporation of radioactivity from [^{35}S]sulfate ion into sulfated glycosaminoglycans by *in vitro* and *in vivo* systems has been known

for a long time. The fixation of radioactive sulfate depends upon a specific biosynthetic process associated with glycosaminoglycan formation (A3), not upon an exchange with the sulfate groups already present in the glycosaminoglycan already formed (B23). The biologically active form of sulfate (R9) is adenosine 3′-phosphate 5′-sulfatophosphate (XIV) which is formed (R10) in two stages:



(XIV)

$$ATP + SO_4^{2-} \rightleftharpoons \text{adenosine 5′-sulfatophosphate (APS)} + 2\ PO_4^{3-}$$

$$APS + ATP \rightarrow \text{adenosine 3′-phosphate 5′-sulfatophosphate (PAPS)} + ADP$$

which are catalyzed by sulfate adenylyltransferase (EC 2.7.7.4) and adenylylsulfate kinase (EC 2.7.1.25), respectively. Although endogenous UDP-2-acetamido-2-deoxy-D-galactose 4-sulfate (T13), which arises from UDP-2-acetamido-2-deoxy-D-galactose by the action of a sulfatotransferase in conjunction with adenosine 3′-phosphate 5′-sulfatophosphate, has been identified, it is not apparently utilized in glycosaminoglycan biosynthesis. Similarly UDP-2-acetamido-2-deoxy-D-galactose 4,6-disulfate and UDP-2-acetamido-2-deoxy-D-galactose 6-phosphate 4-sulfate are known (T13) but do not seem to serve glycosaminoglycan biosynthesis. Thus the sulfate groups must be transferred to the monosaccharide units at some stage after the attachment of these units to the growing proteoglycan molecule. This transfer must be effected by a sulfatotransferase and the evidence is that the transfer occurs directly from the adenosine 3′-phosphate 5′-sulfatophosphate to the carbohydrate (e.g., S54) and the characteristics of the sulfatotransferase have been investigated (S54).

In answer to the question as to at what stage in the proteoglycan glycosaminoglycan chain biosynthesis *O*-sulfation occurs, the discovery of chondroitin was hailed as evidence for the occurrence of sulfation after the chain propagation is complete (e.g., D9). Demonstration of the formation of glycosaminoglycan from UDP-D-glucuronic acid and UDP-2-acetamido-2-deoxy-D-galactose *in vitro* in the absence of sulfating activity and the results of other studies (see S46) have been taken

as supporting the occurrence of sulfation after polymerization. Certainly chondroitin (G17), dermatan sulfate [and also *N*-desulfated heparin and *N*-desulfated heparan sulfate (J17)] and chondroitin 4- and 6-sulfates (S54) will act as sulfate acceptors in the sulfatotransferase-catalyzed reaction. In the case of chondroitin the enzyme is a chondroitin sulfatotransferase (EC 2.8.2.5). However, the specificity of the sulfatotransferase involved is in some doubt (M31), but it would seem that specificity for certain glycosaminoglycans is recognizable (G14, S55). For example, presumably the biosynthesis of keratan sulfate would require a different sulfatotransferase (for the D-galactose units) from the chondroitin sulfates (for the 2-acetamido-2-deoxy-D-galactose units). Sulfation of the polysaccharide chain after propagation is complete is not necessarily the normal route of *O*-sulfation (G14, J17). A careful examination (D18) of various chondroitin 4-sulfate fragments obtained from rabbit ear cartilage showed that they contained stoichiometric amounts of sulfate hemiester groups, suggesting that a close relationship can exist between extension and sulfation of chondroitin chain. Such stoichiometry is less likely to be achieved with sulfation following completion of the polymer. The results of various experiments (D17, M32, S22, S23) including some with the chondroitin sulfate proteoglycan type (K29, K31) are consistent with the theory that sulfation can occur concurrently with polysaccharide propagation/elongation and this route is accepted as the normal one followed. However, it does seem that polysaccharide synthesis will continue without sulfation, as also judged from the results of biosynthesis of glycosaminoglycan chains in the presence of exogenous L-ascorbic acid where sulfation is inhibited but polysaccharide formation from D-[^{14}C]glucose continues (K7) (see Section 5, p. 39), and therefore that this is an alternative mechanism invoked in special circumstances. This alternative may therefore be a safety valve to avoid a stoppage of sulfation affecting proteoglycan synthesis completely. Undoubtedly the sulfatotransferases have some specificity for introduction of 4-*O*-sulfate and 6-*O*-sulfate groups, etc. The *O*-sulfation and epimerization of D-glucuronic acid residues in the biosynthesis of a dermatan sulfate chain are interrelated (L18, L23; see also Section 4.2, p. 24), sulfation preceding epimerization of D-glucuronic acid units. Some information indicates the occurrence of a conformational inversion in the polysaccharide chain during the biosynthetic processes involving sulfation.

Heparin and haparan sulfate also possess *N*-sulfate (sulfamido) residues, and some biosynthetic route must exist for their production. It is very unlikely that 2-amino-2-deoxy-D-glucose residues are available in the polysaccharide chain since this would require the utilization of

UDP-2-amino-2-deoxy-D-glucose in biosynthesis, a nucleotide that has not been shown to be a glycosaminoglycan precursor. However, when adenosine 3′-phosphate 5′-sulfatophosphate (and the appropriate enzymes) is present during glycosaminoglycan chain biosynthesis, or when it is added subsequent to polymer formation, the *N*-acetyl content of the isolated polysaccharide is 30–50% lower than when the polysaccharide is formed in the absence of adenosine 3′-phosphate 5′-sulfatophosphate (S46). Under the conditions in which *N*-acetyl groups are lost, D-glucuronic acid does not disappear, indicating that polysaccharide degradation does not occur at this stage. The resultant polysaccharide possesses a high sulfate content while that portion of the polysaccharide which was not de-*N*-acetylated appears to be similar to the nonsulfated glycosaminoglycan formed in the absence of adenosine 3′-phosphate 5′-sulfatophosphate. Thus *N*-sulfation involves de-*N*-acetylation of an integral 2-acetamido-2-deoxy-D-glucose unit followed at some stage by *N*-sulfation of the exposed amino group (S23). How many enzymes are involved in such a conversion is unknown, but initially a cleavage of the N-C bond of the *N*-acetyl group is necessary, the N-S bond then being formed between the exposed amino group and the incoming sulfate entity. It is most likely that the resubstitution of the amino group is immediate since there is no evidence for the presence of free amino groups in the polysaccharide chain, and indeed *N*-sulfation may involve a direct substitution reaction. It seems that the *N*-sulfation takes place at the completed polymer level—two reasons for this can be offered at present. First, the 2-amino-2-deoxy-D-glucose residues close to the glycopeptide linkage in both heparin and haparan sulfate are *N*-acetylated and the *N*-sulfation of such polysaccharide chains is not regular, and second a sulfatotransferase isolatable from bovine lung catalyzes the transfer of [^{35}S]sulfate from adenosine 3′-phosphate 5′-[^{35}S]sulfatophosphate to de-*N*-sulfated heparan sulfate (F5), 75% of the incorporated radioactivity residing in the *N*-sulfate groups. The sulfatotransferase will catalyze the transfer of sulfate from adenosine 3′-phosphate 5′-sulfatophosphate not only to de-*N*-sulfated heparan sulfate, but also to de-*N*-sulfated heparin, dermatan sulfate, and heparan sulfate (J17).

Heparin and hyaluronic acid will not act as acceptors for the enzyme (E1, J17), whereas chondroitin 4-sulfate is at best a very poor acceptor (E1, J17). The Enzyme Nomenclature Handbook (E4) quotes two sulfatotransferases concerned with heparan sulfate structures: desulfatoheparin sulfatotranferase (EC 2.8.2.8) and heparan sulfate sulfatotransferase (EC 2.8.2.12), but the contents of the relevant references quoted in the Handbook (E1 and S55, respectively) do not permit any distinction between the two supposed enzymes, nor do the reports discussed

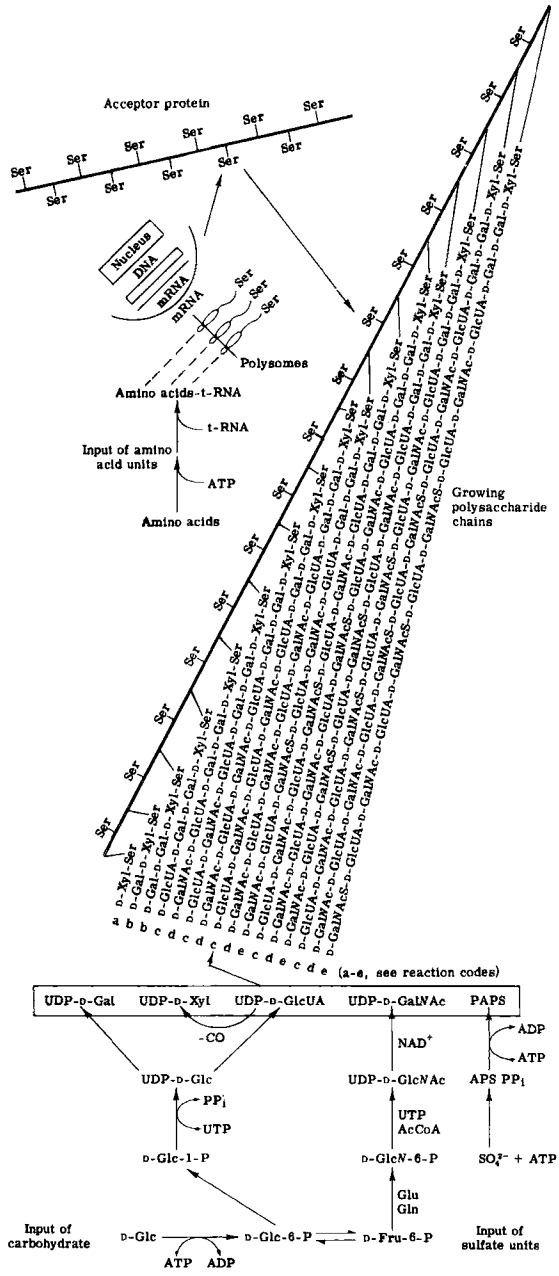
above. A general investigation of heparin sulfatotransferase activity showed that activity (*N- and O*-sulfate producing?) was heterogeneous (R8). So far there is no evidence for or against N-sulfation also being concurrent with chain elongation.

The data available on the biosynthesis of chondroitin 4-sulfate proteoglycan has been sufficient to permit the presentation in schematic form of many of the stages in the transformation from amino acid right through to complete glycosaminoglycan (K28) (Scheme 1).

4.4. Metabolic Heterogeneity of Glycosaminoglycan Chains

The assembly of chondroitin chains on microsomal protein primer at least in the case of chick cartilage is a rapid and organized process (R7) and preferential formation of full length (i.e., polysaccharide length) chondroitin chains is indicated rather than the random addition of monosaccharide units to all the (potential) glycosaminoglycan chain sites on the protein. However, there is evidence for the existence of several pools of proteoglycan with different metabolic activities within a location (O9), for example four pools of chondroitin sulfate proteoglycan have been demonstrated in calf rib cartilage by labeling experiments (K30). Similar experiments (H25) on topochemical aspects of glycosaminoglycan biosynthesis in bovine arterial tissues indicate that the rate of biosynthesis is selectively regulated according to very much localized functional requirements. On the other hand, the various sizes of the proteoglycan macromolecules are all formed at the same time (H9). In bovine aorta, the hyaluronic acid, chondroitin sulfate, dermatan sulfate, and heparan sulfate chains have different turnover rates (B47); a similar situation has been demonstrated by others (S10), who also showed that the two component monosaccharide units of the glycosaminoglycans turnover at the same rate for a particular glycosaminoglycan. The situation is made more complex by the fact that not only is one glycosaminoglycan type subject to such a biosynthetic heterogeneity, but also that the chondroitin sulfate and dermatan sulfate chains of a chondroitin sulfate–dermatan sulfate hybrid proteoglycan are biosynthesized at different rates (B47, K42).

The metabolic, and hence chemical, heterogeneities of chondroitin sulfate proteoglycan are explicable (K28) by differences in size and turnover rates of acceptor protein pools as well as by differences in activities and specificities of the monosaccharide and sulfate transferases, probably localized in different cell compartments. Similar explanations have been offered for the different metabolic behaviors of the different proteoglycan fractions classified according to the ease with which they


Acceptor protein
Ser
Nucleus
DNA
mRNA
Polysomes
Amino acids-t-RNA
t-RNA
Input of amino acid units
ATP
Amino acids
Growing polysaccharide chains
(a-e, see reaction codes)
UDP-D-Gal
UDP-D-Xyl
UDP-D-GlcUA
UDP-D-GalNAc
PAPS
-CO
NAD+
ADP
ATP
UDP-D-Glc
UDP-D-GlcNAc
APS PPi
PPi
UTP
UTP
AcCoA
D-Glc-1-P
D-GlcN-6-P
SO4 2- + ATP
Glu
Gln
Input of carbohydrate
D-Glc
D-Glc-6-P
D-Fru-6-P
ATP ADP
Input of sulfate units

may be extracted (H9). These metabolic heterogeneities are considered to arise either from a partial separation of different types of proteoglycans or from differences in the rates of degradation of molecules of different sizes and compositions due to the nature and specificities of the normal degradative enzymes. Some metabolic heterogeneities may arise by interaction of the glycosaminoglycans with themselves; there is some evidence (W10) for the inhibition of glycosaminoglycan sulfation by hyaluronic acid. Such a situation may be an explanation for any differences which have been observed between the relative amounts of incorporation of [^{35}S]sulfate and D-[^{14}C]glucose into each sulfated glycosaminoglycan of a tissue. Metabolic heterogeneities may also arise from different half-lives of glycosaminoglycan chains (e.g., H8), and the phenomenon of aggregation of proteoglycans.

4.5. Regulation of Proteoglycan Biosynthesis

The regulation of proteoglycan formation *in vivo* is clearly of importance in the healthy processes of the body, but although some work has been done on the controlling effects of administered compounds little successful study has been made of the natural controlling factors. Various attempts have been made with tissues *in vivo* and *in vitro* and in bacteria (see S8) and the overall effects of certain controlling factors have been described (see S8). It is important to realize that there are many steps to the biosynthesis of the complete molecule, and that each step will be subject to control.

However, at the enzymic level two regulatory mechanisms are apparent. In the biosynthesis of 2-amino-2-deoxy-D-hexose, the D-fructose 6-phosphate:glutamine transamidase catalyzed reaction is subject (K39) to feedback regulation by UDP-2-acetamido-2-deoxy-D-glucose, and since this in turn is in equilibrium with UDP-2-acetamido-2-deoxy-D-galactose, the formation of the various glycosaminoglycan chains are affected by the same mechanism. A second feedback mechanism (N6) affects the biosynthesis of UDP-D-glucuronic acid. UDP-D-Xylose is a potent inhibitor of UDP-D-glucose dehydrogenase (EC 1.1.1.27) which oxidizes UDP-D-glucose to UDP-D-glucuronic acid, and since UDP-D-xylose is formed by decarboxylation of UDP-D-glucuronic acid, the levels of both these monosaccharide nucleotides are regulated by a feedback mech-

Scheme 1. The biosynthesis of proteoglycans from D-glucose, amino acids, and sulfate. Reaction code (for reactions that give the particular terminal nonreducing units shown): a = UDP-D-Xyl + D-xylosyltransferase; b = UDP-D-Gal + D-galactosyltransferase; c = UDP-D-GlcUA + D-glucuronosyltransferase; d = UDP-D-Gal*N*Ac + D-acetamidodeoxygalactosyltransferase; e = adenosine 3′-phosphate 5′-sulfatophosphate (PAPS) + sulfotransferase.

anism. In fact, UDP-D-xylose may be regarded as a specific synchronizer of the synthesis of protein and polysaccharide moieties of a proteoglycan since the first effect of diminished proteoglycan protein biosynthesis on the glycosaminoglycan precursors would be the accumulation of UDP-D-xylose. This key position of UDP-D-xylose would set up a chain of reactions/inhibitions until balance was restored. Additional evidence (K31) also substantiates the existence of a control system that relates biosynthesis of the sulfated proteoglycan to the formation of the glycopeptide linkage.

As will be evident from studies on induced biosynthetic effects (Section 5), sulfation of glycosaminoglycans can be blocked selectively, but this does not seem to exert a feedback control since polysaccharide synthesis can still continue without sulfation. It would therefore seem unnecessary for the living system to provide a control at this stage, but that the system can adapt to variations in sulfate availability by use of the alternative, but not normal, sulfation route of sulfation after polymerization of the carbohydrate.

In the consideration of regulation of proteoglycan biosynthesis it must be recognized that the biosynthetic process will be subject to effects of a number of other biosyntheses which are taking place in the same location. Some of these biosyntheses will utilize some of the precursors which are utilized for proteoglycan biosynthesis. Furthermore there will be environmental effects such as concentration changes, alteration in ionic strength, etc. It is of interest that the biosynthesis of sulfated glycosaminoglycans by human fibroblasts *in vitro* is reversibly inhibited progressively as the pH of the growth medium is increased from pH 6.8 to 8.0 (L13). Departmental cellular effects as discussed in Section 4.6 will also exert a control by regulating the flow of partially complete material from one location to another for the next stage of addition. Added to this is the likelihood, for which there is some evidence (H25), that the biosynthesis is selectively regulated according to very much localized functional environments.

Another level of biosynthetic control must derive directly from available genetic information. The genes may exert control in supplying the appropriate information for formation of both structural protein (the proteoglycan protein) and catalytic protein (the specific enzymes responsible for the biosynthetic steps). Tissue culture techniques have also been useful in the examinations of some factors that influence phenotypic characteristics of cells (see S46), and it has been considered (N1) that proteoglycan and DNA biosynthesis are mutually antagonistic. Such a phenomenon would affect cell differentiation. With the technique of somatic cell hybridization, it has been possible to combine the genomes of two strains of fibroblasts that differ in the rate of production of hyal-

uronic acid proteoglycan and collagen (D31) and to study the relationships between the genes involved in these functions.

Undoubtedly there are also a number of nutritional factors that impinge upon proteoglycan biosynthesis, but specific information has yet to be obtained. It appears that the Mn^{2+} ion greatly stimulates several of the enzymic reactions necessary for chondroitin sulfate proteoglycan formation (T5). There are a number of indications in the literature that glycosaminoglycan chain propagation is or is not subject to regulation by various vitamins and hormones (e.g., T6; see also Section 5 and reviews cited therein), and the (implicated) endocrine regulation of this propagation has been reviewed (G1, S8, S57). A review of the differentiation of connective tissues (T9) includes discussion of the various influencing factors with respect to glycosaminoglycan chain biosynthesis.

Interest has recently been expressed in the possible involvement of lipids in proteoglycan biosynthesis, either as potential donors or as environments in which glycosidic linkages can be formed away from the aqueous milieu. D-Galactosyltransferase, but not other glycosyltransferases, is possibly susceptible to phospholipase C (S14); the inactivation is reversible, reactivation being achieved with phosphatidylcholine (lecithin) or detergent. This infers that involvement in the environment of a phospholipid is important for certain glycosyltransferase activity. Much more work is needed to confirm or deny the association of lipid with proteoglycan biosynthesis.

4.6. Cellular and Intracellular Localization of Proteoglycan Biosynthesis

Deductions concerning the loci of proteoglycan biosynthesis come from a considerable amount of work involving radioactive labeling of proteoglycans *in vitro* and autoradiography of the labeled tissue, and from investigation of a number of induced effects upon biosynthesis—some of such work has been reviewed (S46).

The biosynthesis of the proteoglycan molecule is initiated by formation of the core protein at the ribosome. Most of the information on the addition of carbohydrate units to the protein chain suggests that glycosaminoglycan chain initiation occurs with the newly formed protein soon after it leaves the ribosome and enters the cisternal space of the endoplasmic reticulum. It is at this stage that the material becomes effectively extracellular (W15) and the glycosyltransferases which catalyze the specific and sequential addition of the other glycopeptide linkage monosaccharides to the initially glycosylated protein are presumably membrane bound. At a later stage during the movement of the growing molecule

through the smooth membranes to the Golgi complex the addition of the monosaccharide units to give the alternating sequence takes place. The involvement of the Golgi complex is certain, the chain propagation probably taking place on the membranes (O7), and radioautographic studies (R4) indicate that the Golgi body plays a major role in the process of export of the macromolecule to the extracellular regions. It is not clear whether any further biosynthetic step or remodeling takes place after this, but certainly motion must occur, as will be apparent from the locations of proteoglycans in various tissues. For example, heparin is stored in mast cells whereas heparan sulfate is extracellular. There are several pools of, e.g., chondroitin sulfate proteoglycan with different metabolic activities (K30, O9), e.g., in leukocytes, and in fact there may be separate fast and slow synthesizing pools accounting for metabolic heterogeneities. The existence of such pools complicates the picture of location of the biosyntheses.

In addition to the movement of the growing proteoglycan molecule, consideration must also be given to the location of the contributory enzymes. The D-xylosyltransferase and D-galactosyltransferase activities are concentrated in the rough endoplasmic reticulum (H27) whereas the glycosyltransferases for the production of the main chain of chondroitin sulfate at least are located approximately equally in the rough and the smooth membrane fractions. Sulfatotransferase activity occurs predominantly in the smooth membrane fraction. This suggests that the membrane-bound enzymes move slowly in their membranes through the smooth endoplasmic reticulum to the Golgi zone, with a half-time of days (O10).

It can be argued that the transferases involved in proteoglycan biosynthesis, particularly those that generate the glycosaminoglycan chains, derive some of their specificity from their highly localized position. The particulate nature of such enzymes is evident, and in a membrane-bound system they are not free to find out all the possible acceptors, but are forced to deal with those acceptor structures generated by action of neighboring enzymes.

From the foregoing subsections it will be apparent that there are still large gaps in the understanding of the biosynthetic aspects of proteoglycans. Areas to which no attention has been given include the metabolic heterogeneity of the protein chain (different chains have been recognized), and the regulation of biosynthesis of the protein chain.

5. Induced Effects on Proteoglycan and Glycosaminoglycan Chain Biosynthesis

The finding some time ago that glycosaminoglycan chains of proteoglycans can be labeled radioactively *in vivo* and *in vitro* by use of

[^{35}S]sulfate and [^{3}H] or [^{14}C]carbohydrate precursors has prompted numerous experiments in which the rate of incorporation of such precursors has been compared for the presence and the absence of a number of biological and nonbiological additives. Essentially such experiments have proved to be of use for obtaining biosynthetic information, and indeed interpretation of earlier data on induced effects was subject to the very limited understanding at that time of the biosynthetic processes. But more recently, such work has been applied to assessment of drugs for main and side effects and to the monitoring of therapies.

Unfortunately a number of factors, including time and temperature, can influence the data obtained. Whatever system is used, clearly the initial rate of incorporation of labeled precursor, even in the control experiment, will be affected by the amount of endogenous unlabeled precursor and, in the case of incorporation *in vitro,* by the presence of any unlabeled precursor in the culture medium. Furthermore, *in vivo* components of diet may have an affect whereas *in vitro* components of the culture medium themselves may have an effect on the biosynthetic processes. It is often a problem to decide whether an induced apparent increase in biosynthesis is due truly to a greater rate of biosynthesis effected by the additive, and not due to a decrease in the rate of degradation. It will be recognized from a reading of the biosynthesis of proteoglycans (Section 4) that an enormous number of stages are involved in the biosynthesis of a complete proteoglycan molecule and in its turnover and therefore that an additive may have an arresting or accelerating effect at one or more of many stages.

Thus far the precise stage at which an effect takes place has not really been determined in detail for any additive, but some information can be gained from use of additives that, from experiments with other systems, are known to affect a certain biosynthetic process—insofar as proteoglycans are concerned this is restricted to protein, but even then it cannot be certain that the additive does not also affect the polysaccharide chain formation. Clearly the earlier the stage of effect in the route, the more widespread will be the effect, and accumulation of incomplete molecules may occur. It would seem, in view of the progression of biosynthesis first to protein and then to glycosaminoglycan chain, that protein accumulation effects are more likely, whereas in the case of degradation less specificity of order exists and therefore preferential degradation of protein or carbohydrate may occur. Once again greater attention has been given to the glycosaminoglycan units, rather than the whole proteoglycan, on account of the easier distinctive recognition of the polysaccharide via sulfate incorporation and the less distinctive labeling of protein. Thus at the moment it is generally possible only to make an overall comparison for the presence or the absence of additive, and

the fact that a change is recognized for glycosaminoglycan does not necessarily mean that the biosynthesis of the polysaccharide chain has been affected directly. It must also be borne in mind that it also follows, in view of the multiplicity of factors and stages involved, that a biosynthetic effect demonstrable in isolation cannot necessarily be proved or accepted to apply in the natural system.

Sections of reviews of the actions of various agents on the processes of connective tissues, etc., are relevant to this discussion (B29, S8, T9).

Examples of the use of induced biosynthetic effects for the deduction of the processes involved include the use of puromycin and cycloheximide. These compounds, according to experiments with other systems, inhibit protein synthesis. Thus the inhibition of biosynthesis of hyaluronic acid and dermatan sulfate in human fibroblast cultures (M13, M16), of hyaluronic acid in synovial cells (S31), and of chondroitin sulfate (H1) led to the conclusion that the synthesis of protein acceptor is necessary for the polysaccharide chain initiation. Comparison of the effects of 1-butanol on the biosynthesis of chick cartilage chondroitin sulfate and streptococcal hyaluronic acid led to the suggestion that binding of Mg^{2+} ions and the uridine diphosphate moiety results in stabilization of the spatial arrangement of the specific enzymes (S47, T4).

The effect of L-ascorbic acid (vitamin C) on glycosaminoglycan-chain biosynthesis in animal tissues has been recognized for some time (see mini review in G10), and experimental and "natural" scorbuticity and administration of L-ascorbic acid have been given considerable attention. After the original histological experiments, the incorporation of [^{35}S]sulfate into glycosaminoglycans in scorbutic guinea pigs was found to be lower than normal (F8; see also S27), but the biosynthesis of hyaluronic acid is abnormally high and increased amounts of hyaluronic acid are produced (G10, R12). Such data not only demonstrate a differential effect upon the biosynthetic/metabolic rates of the glycosaminoglycan chains, but also indicate that L-ascorbic acid is an important factor influencing the biosynthetic pathway of the proteoglycans.

Similar extensive work has been conducted with vitamin A (see T6, and references therein; also B29). Experimental deficiency in animals reduces the incorporation of 2-amino-2-deoxy-D-[1-^{14}C]glucose and [^{35}S]sulfate into the glycosaminoglycan chains (D16, W16). In this instance the stage of action has been identified, and the effect is due to a block at the first stage of the sulfate activation step (W17, R3, S50). Thus vitamin A is an agent that can activate ATP-sulfurylase and, like L-ascorbic acid, can be an important factor influencing the biosynthetic pathway of the glycosaminoglycans. However, other work

(T6) is contradictory to this supposition in that it was deduced that vitamin A deficiency has no effect upon the biosynthesis of sulfated glycosaminoglycans, but that it does increase their turnover rate. From this it was concluded that vitamin A is not necessary for glycosaminoglycan chain biosynthesis. Excess of vitamin A increases the incorporation of [^{35}S]sulfate (B11). The effects of various derivatives of vitamin A have also been investigated (T6), and citral, a terpene structurally related to vitamin A, also affects glycosaminoglycan biosynthesis (B27).

In vivo injection of hydrocortisone did not alter the hexuronic acid content of the kidney zones in the rat (B36). Addition of hydrocortisone to the culture medium also affects glycosaminoglycan chain biosynthesis in some way, retarding the rate of [^{35}S]sulfate incorporation and decreasing the amount of polysaccharide synthesized (S9). Hydrocortisone will nullify the increased incorporation of [^{35}S]sulfate brought about by vitamin A (B11). Cortisone itself *in vivo* decreases the amount of the sulfated glycosaminoglycan with the exception of heparan sulfate (C10) but does not affect the levels of glycosaminoglycan containing 2-amino-2-deoxy-D-glucose monitored: hyaluronic acid, heparan sulfate. Prednisolone increases the production of hyaluronic acid by skin (S9).

The effect of steroid hormones on the glycosaminoglycans of target connective tissues has been reviewed (S57) and estrogens (B24, K36) and testosterone (K35) affect the biosynthesis of glycosaminoglycan chains. The addition of parathyroid hormone to bone organ cultures increased the incorporation of 2-amino-2-deoxy-D-[^{14}C]glucose into glycosaminoglycan chains, particularly hyaluronic acid (S20). Growth hormone (L6) and peptides therefrom (M9) also affect glycosaminoglycan biosynthesis.

A number of other compounds have been investigated for their influence upon proteoglycan biosynthesis in animals. Cytochalasin B depresses the incorporation of 2-amino-2-deoxy-D-[1-^{14}C]glucose into the glycosaminoglycan chains of various chick-embryo cells (S4), and glycosaminoglycan levels are affected by the presence of tolbutamide and phenformin (P8). The intraperitoneal injection of rats with a phytohemagglutinin induces the biosynthesis of peritoneal fluid hyaluronic acid (G16), and the effect of fluoride ion in rats is to cause either an increase or a decrease of glycosaminoglycan chain biosynthesis according to its concentration (H2). Carrageenan treatment of murine tissue *in vivo* increases the hyaluronic acid content of granulation tissue (C10; also see S27) but does not alter the levels of the sulfated glycosaminoglycans. Carrageenan also has some affects in glycosaminoglycan production in carrageenan-induced granuloma tissue. Dibutyryladenosine 3′,5′-cyclic phosphate and theophylline increase the incorporation of [^{35}S]sulfate

into various glycosaminoglycan structures in cultured fibroblasts (G5).

It is most important that the effect of relevant compounds on the synthesis of proteoglycans in human tissues is investigated. Although little work has in fact been reported, the reason for attachment of importance to such lies in the extensive involvement of the proteoglycans in maintenance of tissue health, stability, and function. Thus if their biosynthesis is affected by a drug, etc., it is likely that the overall effect upon the tissue may be far reaching. Again it is accepted that assessment in terms of glycosaminoglycan monitoring does not reveal the whole story, but it is informative and positive if an effect is revealed. Accordingly, more recently, the ability of tissue to incorporate radioactivity *in vitro* has been adapted to give a standard method for the investigation of drugs etc. for the possibility of induced effects (B9). The method is based on utilization of human skin, since such can be taken conveniently as a biopsy specimen (but the method is equally applicable to other tissues), but this does therefore require a method that can be based on 1 or 2 mg of tissue. In order to put such an assessment on an appropriate quantitative and controlled basis, and in order to investigate the effect of the exogenous compound under investigation on each of the glycosaminoglycan types, rather than measuring an overall increase or decrease of biosynthesis as in many of the above studies, it was necessary to establish methodology for glycosaminoglycan extraction, purification, and separation (B15) in addition to determination of the conditions giving maximum incorporation of radioactive precursor (usually [^{35}S]sulfate) congruent with tissue viability *in vitro*, and of various concentration effects (B9), and a method for estimation of the radioactivity incorporated (K8).

The method established has been applied in particular to the assessment of the effects of L-ascorbic acid, vitamin A, triparanol, and dimethyl sulfoxide on human skin (B9), on account of uses of these compounds in therapy. The effect of triparanol on incorporation of [^{35}S]sulfate into proteoglycans and adenosine 3′-phosphate 5′-sulfatophosphate of the epidermis was reduction in each case, but an increase into those of the dermis, thus demonstrating a serious disruptive effect of the drug on tissue processes.

Although dimethyl sulfoxide does not affect the proteoglycan biosynthesis of the dermis, its effect on their biosynthesis in the epidermis is dramatic in that the degree of [^{35}S]labeling of them and adenosine 3′-phosphate 5′-sulfatophosphate is reduced extensively. In both the foregoing cases, since effects in which reduced incorporation into adenosine 3′-phosphate 5′-sulfatophosphate and glycosaminoglycan was concomitant, it is likely that the effect occurs at the sulfate activation stages.

On the other hand, in the case of hydrocortisone only certain glycosaminoglycan radiospecific activities are affected suggesting that the effect of the additive occurs at later more specific stages. Testing of L-ascorbic acid in the system gave a marked decrease in [^{35}S]sulfate incorporation into all the glycosaminoglycans of both skin layers but the level of labeling of adenosine 3′-phosphate 5′-sulfatophosphate was abnormally high. This indicates that L-ascorbic acid blocks the utilization of the activated sulfate, thus causing it to accumulate. The increased incorporation into the glycosaminoglycans which occurred with vitamin A could have arisen from a decreased rate of degradation since the level of labeling of adenosine 3′-phosphate 5′-sulfatophosphate was normal. On the other hand, when the incorporation of [^{14}C]glucose was examined (K7), a marked increase of incorporation above control was found; this would indicate that the polysaccharide biosynthesis continues in the presence of L-ascorbic acid (a case for sulfation after polymerization), and that any degradative effects such as those of the free-radical mechanism observed for treatment in isolation of hyaluronic acid with L-ascorbic acid (see K20, and references therein) are not deleterious.

The test system described above is also of use for assessment of clinical conditions for proteoglycan disorders (see Section 7, p. 50) and for monitoring therapy. For example, a proteoglycan disorder has been demonstrated in lipoid proteinosis, but the rectification of abnormal [^{35}S]sulfate incorporation was accompanied by clinical improvement (B10).

Local treatment of rheumatoid arthritis with hydrocortisone results in restoration to normal of the qualitative changes occurring in the joint fluids (J15, S52) and increased viscosities and hyaluronic acid contents after administration have been reported (J15). The therapeutic agent is believed to act by influencing the synthesis of hyaluronic acid in the synovial tissue directly or by its anti-inflammatory action (J15, S52). Assessment of the anti-inflammatory effects of aspirin, indomethacin, and prednisone in cases of arthritis by examination of the polydispersity of synovial fluid hyaluronic acid on agarose gels (H28) has shown that each of these drugs causes a decrease in the content of low molecular weight hyaluronic acid in the fluid. This decrease is matched by a clinical improvement.

Therapeutic attempts in cases of genetic hyperglycosaminoglycanuria have been summarized (V2; see also Section 7.6.6). Infusion of naturally occurring corrective factors (D29) and use of hyaluronidase (EC 3.2.1.35) (D22) for the excessive glycosaminoglycan excretion may hold some promise, although treatment by cell infusion, e.g., leukocyte infusion (K33), while giving some improvement has been very unsuccessful.

Regulation of cell metabolism and differentiation may be affected

by the introduction of new genetic material via viral infection or somatic cell hybridization and mutation (G5, H7, I1, S5, T14).

Thus it can be seen that the effects of added compounds upon proteoglycan levels in tissues and fluids can vary from selective to general increase or decrease. As the methods for drug assessment become used more widely, and with more detailed studies, screening of compounds for therapeutic use and selective activity may be expected to progress along with an understanding of how drugs act and of how other endogenous factors influence proteoglycan biosynthesis.

6. Healthy Processes in Relation to Proteoglycans and Glycosaminoglycans

The trend of research in the field of human proteoglycans and glycosaminoglycans has been such that much greater attention has been given to the abnormal, diseased state than to the normal state. This situation has arisen essentially from the ease with which conditions giving rise to excessive urinary excretion of glycosaminoglycans may be recognized by urinary glycosaminoglycan measurement. The more recent upsurge of investigation of these and other conditions for proteoglycan disorders in tissues requires the establishment of more controls, which together with the considerable interest in variation of proteoglycan biosynthesis with age has begun to provide greater information on normal conditions.

This section is related to the function and biosynthesis of proteoglycans. Some deductions may also be made from the data reported for clinical conditions, which are discussed in the following section (see Section 7). It is regrettable that thus far, although their involvement in maintenance of health is extensive, an understanding of how these macromolecules act is very limited and does not yet really go beyond the simple type of observations of their quality and quantity. However, it is important to realize that the involvement of proteoglycans in health goes much further than can be identified by the simple comparison of their levels in a normal subject. Their responses to internal and external influences, growth of tissue, etc., all of which must be interlocked giving a complex system, have yet to be determined. Furthermore, the glycosaminoglycan levels are subject to influence by controls at all the steps of biosynthesis and degradation. When material from a normal is used it can only be taken to represent the situation in the subject at the particular time it was taken. In addition to the recognition that a range of concentrations, rates, etc., will exist in matched normals, it must not be overlooked (as is frequently the case since many assume that subjects should behave near identically) that every subject is an individual whose unique appearance, etc., is an expression of different

qualitative and quantitative chemical aspects of the body. Furthermore, not only are the chemical procedures of health and life of interest, but also the overall progress of the life of a subject and therefore studies must be conducted for all stages from the cradle to the grave.

Until recently, the work in this area has been insufficient to warrant its review, but some papers on the biochemistry and pathophysiology of connective tissue (F9) are relevant. In consideration of the reports available, it must be borne in mind that comparisons made outside a particular circle of experiments may be invalid on account of the different conditions of the subjects, and conditions and methods for isolation, separation, and measurement of the macromolecules. Many such methods both for tissues and fluids have been reported (see reviews cited in Section 1.1), and it is imperative to ensure that the isolation and separation processes are effective (see B15, K12). Microscale methods have been devised to function on a few micrograms of material for component analysis (e.g., B16; see K17), but more particularly for the complete identification of a glycosaminoglycan on a basis of chemical structure (e.g., B13, B14).

Experiments in animals on glycosaminoglycan and proteoglycan turnover rates imply that there are in the tissues enzymes that degrade the proteoglycans (see Section 4, pp. 17 and 31, and M46). Since the proteoglycans are composite macromolecules containing both carbohydrate and protein, the degradative enzymes can be carbohydrases or proteases, and since carbohydrate and protein are metabolized in parallel (G18) their biosynthesis and metabolism are closely related. Based on the average half-life of a glycosaminoglycan chain and the total amounts of glycosaminoglycan in the body, it is calculable (L8) that in an adult about 250 mg of glycosaminoglycan are disposed of daily. Since only a few milligrams of glycosaminoglycan are excreted per day in the urine (D25), it indicates that most of the material is degraded or metabolized, again implicating the action of degradative as well as biosynthetic enzymes in the healthy processes of the human body. Direct tests on injection of glycosaminoglycan to guinea pigs and urinary assessment (R5) also show that urinary excretion is not the major route for the disposal of glycosaminoglycans per se. In fact much of injected chondroitin 4-sulfate is degraded by the liver (W21) in rats.

Some of the common enzymes that may or may not degrade proteoglycans, and therefore are relevant to a discussion on these enzymic processes, have been surveyed (M46, V2). In considering the enzyme-catalyzed breakdown of a proteoglycan, it is to be remembered that breakage may occur in the carbohydrate and protein in any order, and therefore that breakdown does not occur in the reverse order of biosynthesis. From the viewpoint of proteolysis of the proteoglycans, a number of

lysosomal cathepsins of different specificities are known to occur in many tissues (see M46), but considerable work is necessary to see just how these enzymes impinge upon proteoglycan processes. Similarly, a number of glycoside hydrolases exist in tissues (see K10, K11, K14, K18), and these probably have some effect upon breakdown of glycosaminoglycan chains. The activities of some glycoside hydrolases of human aorta have been discussed with respect to glycosaminoglycans (P6). Of the polysaccharidases which are known to degrade glycosaminoglycans, hyaluronidase (EC 3.2.1.35) activity occurs in the human (see M35, M37) and this enzyme is also active against the chondroitin sulfates. It is not yet known how dermatan sulfate, heparan sulfate, heparin, and keratan sulfate are degraded naturally, although their metabolic turnover (e.g., D10–D12) further implies the existence of degradative enzymes for these glycosaminoglycans. However, it appears from fibroblast culture studies (Section 7.6.4) that the glycosaminoglycans are broken down *in vivo* by glycoside hydrolases (and sulfatases) rather than by polysaccharide hydrolases. An enzyme that releases L-iduronic acid from chemically desulfated dermatan sulfate occurs (M19) in human liver and fibroblasts, and the nature of its action pattern also suggests an important role for sulfatases in glycosaminoglycan breakdown. From animal studies (A7) it appears that chondroitin sulfate is degraded more rapidly than dermatan sulfate, but this comparison must be set against the relative proportions of the two polysaccharide chains present in the tissues and the concentration/rate relationships of them and the enzymes. Enzymes that cleave the glycopeptide linkages of proteoglycans and which are endogenous to mammals have not yet been discovered apart from the enzyme, namely 4-L-aspartylglycosylamine amidohydrolase (EC 3.5.1.37), which cleaves the glycopeptide linkage structure that occurs for some keratan sulfate chains. The excretion as chondroitin sulfate of injected chondroitin sulfate proteoglycan by guinea pigs (R5) may be interpreted in terms of the result either of cleavage of the glycopeptide linkage or of proteolysis since the units attached to the reducing terminal of the glycosaminoglycan chains was not identified. Degradation of proteoglycan material provided in excess does not seem to be necessarily automatic since chondroitin 4-sulfate injected into animals is partially excreted in the urine in undegraded form (R5, W21), although such a result is also open to a number of other interpretations.

Since urinary glycosaminoglycans have become a popular interest, it is appropriate to mention that the normal excretion rate of glycosaminoglycans is 3.8 and 6.0 mg per day for women and men, respectively (D25) but with large individual variations. On account of diurnal and excretion rate variations some search has been made for the existence

in human urine of a standard to which glycosaminoglycan excretion can be related with a view to avoiding the need for standardized aqueous intake and 24-hour specimens. Creatinine excretion has been afforded greatest attention in this respect although its magnitude as a standardization value has been both recommended and disclaimed. The relationship of glycosaminoglycan and creatinine excretion has now been examined critically D24) and the measurement of glycosaminoglycans on a single, sporadic urine specimen is not reliable, whether expressed as milligrams per milliliter of urine or as milligrams per gram of creatinine, because of variation in their excretion at different times of a given day. The ratio of milligrams of urinary glycosaminoglycan to grams of creatinine is fairly constant in the same subject only when calculated from the analysis of a complete 24-hour urine specimen.

A correlation between urinary excretion of glycosaminoglycans and 17-hydroxycorticosteroid has been demonstrated in males undergoing lunar flight simulation (K3). Proteoglycans per se are not excreted in the urine, and since the excreted molecules are actually glycosaminoglycans with a few amino acid residues attached, the glycopeptide linkage region being largely intact (W5), it is clear that the material being disposed of by this route has been subjected to proteolysis. However, although some of the glycosaminoglycans are nonultrafilterable (W8), many of the glycosaminoglycan chains have themselves been subjected to degradation (e.g., C20). The weight ratio of glycosaminoglycan material precipitable by cetyl pyridinium chloride (higher molecular weight) to that subsequently isolatable by ion-exchange chromatography (lower molecular weight) is less than unity for adults.

By far the greatest attention in the area of healthy processes and proteoglycans has been given to variation of distribution pattern, biosynthesis, and degradation with age (see V11). Collected papers on connective and skeletal tissue and aging have been published (E3) and the role of proteoglycans in aging has been the subject of an occasional survey (M26). Skin aging with respect to glycosaminoglycans has been reviewed (L26). The hyaluronic acid and chondroitin sulfate content of tissues has been repeatedly found to be reduced in advanced years with contrariwise changes for dermatan sulfate, heparan sulfate and keratan sulfate (K1, M4). The proportion of chondroitin sulfate plus dermatan sulfate among total glycosaminoglycans in human arterial tissue is presumed to increase slightly from childhood to old age (K26), and in fact the total contents of proteoglycans and the glycosaminoglycan fractions decrease in the same manner as their turnover rates (L27), whereas their half-life times increase. There appears (K1) to be no significant change with age of the total glycosaminoglycan concentration

in human aorta, but changes with age of their distribution and composition (M4) and an increase with age in the chondroitin sulfate to hyaluronic acid weight ratio (B27) have been measured.

Correlation between increased dermatan sulfate contents, calcification and decreased flexibility of tissue on aging is explicable in terms of the greater association with calcium of this glycosaminoglycan. Association with divalent cations promotes the number of interchain linkages (A9) thus decreasing the possibility of movement between molecules.

Considerable changes occur in the glycosaminoglycan contents of skin during fetal development and childhood and adult life (B38, B39). Early fetal skin contains only hyaluronic acid and chondroitin 4- and 6-sulfates, but at twenty times the adult level. During gestation this level drops, principally due to a fall in hyaluronic acid content, and at term, when dermatan sulfate is present, the level is only twice the adult level. Further changes in the glycosaminoglycan distribution and concentration occur during childhood, but very little change occurs during adult life. A report (V7) that there is no correlation in human skin between age and hyaluronic acid content and that dermatan sulfate and heparan sulfate decrease with age would appear to be somewhat at variance with all the general trends deduced from a number of papers but are in agreement with other papers dealing with skin (C16, M34, M40). In sclera (B38) the glycosaminoglycan total concentration varies little; dermatan sulfate is the main component in both fetus and adult, but hyaluronic acid and chondroitin sulfate also occur in the adult sclera. Human corneal glycosaminoglycans increase by a factor of less than 2 at late gestation and thereafter remain constant. Fetal cornea contains chondroitin sulfate, chondroitin, and a small amount of keratan sulfate, whereas adult cornea is rich in keratan sulfate and contains more chondroitin than the fetal material.

Young human placenta contains more glycosaminoglycan than term placenta (L9). Although there are similar quantities of hyaluronic acid and chondroitin 4- and 6-sulfates, the younger placenta contains a greater proportion of chondroitin. The chondroitin sulfates of the early tissue also contain a higher proportion of unsulfated disaccharide units, and the dermatan sulfate of the young tissue is more susceptible to hyaluronidase (EC 3.2.1.35) than term tissue.

Some contributions to the intracellular localization of the biosynthesis of glycosaminoglycan chains during the aging of cartilage comes from histochemical and electron microscopy studies (L25) and data for variation of glycosaminoglycan content with age for prenatal cartilage (M23) and bronchial cartilage (M12). The total chondroitin sulfate content of human tracheobronchial cartilage decreases linearly with age (M11)

and age-dependent changes in the chemical heterogeneities also occur. In articular cartilage, the weight ratio of chondroitin 4-sulfate to chondroitin 6-sulfate, the overall chondroitin sulfate content, and the molecular weight of the chondroitin sulfate chains decrease with age (H24). However, all these changes occur before the age of 30, and subsequently chondroitin sulfate content decreases and the keratan sulfate content may rise. In the knee joint cartilage there is a similar picture of decrease of chondroitin 4-sulfate and increase of chondroitin 6-sulfate with advancing years (G13), and in older cartilages there is a greater proportion of oversulfated disaccharide units and a lower proportion of nonsulfated units. As might be expected from such changes of glycosaminoglycan contents with age, the type of glycosaminoglycan components of proteoglycans also vary with age. From a number of studies including those on animals on keratan sulfate proteoglycans and keratan sulfate-containing proteoglycans (B37, G6, G11, K44, M24, R19, S25), in early life the keratan sulfate contents of nucleus pulposus and cartilage and of the constituent proteoglycans increase with age, without a comparable increase in the proteoglycan macromolecular size (B37, S25). For example, the keratan sulfate content of human tracheal cartilage attains a maximum at the beginning of the fourth decade (M10), but thereafter remains constant; similarly compositions of the proteoglycan fractions from human costal cartilage do not vary in the later decades of life (R19) indicating no change in the proportions of chondroitin sulfate and keratan sulfate. On the other hand, the quantity of chondroitin sulfate and keratan sulfate in human knee joint cartilage decreases between the third and ninth decade of life (G15). The progressive decrease with aging in the percentage of total 2-amino-2-deoxy-D-hexose in human nucleus pulposus, annulus fibrosus, and costal cartilage extractable in the lower molecular weight proteoglycan fraction (G11) is in agreement with the decrease in the lighter and increase in the heavier proteoglycan fractions with advancing age (R18). This reciprocal change indicates a progressive decrease in the water solubility of the proteoglycans. The decrease in extractability of the ligher fraction with increasing age may be due to maturation of the proteoglycans involving intermolecular crossbonding of the proteoglycan molecules or bonding of proteoglycan to collagen. Proteoglycans isolated from human costal cartilage show age-dependent changes not only in chemical composition, but also in macromolecular properties (K44). In bone tissue the concentration of glycosaminoglycans is approximately inversely proportional to age in growing subjects, but there is little change in later life (V10).

The urinary excretion of glycosaminoglycan decreases with age (O4); average values are 70 mg per gram of creatinine at birth compared with

10 mg/g after four years (L1, S33). Studies for subjects up to the age of 25 (D33) show that the average ratio of excreted glycosaminoglycan polymers to oligomers as approximately 0.4 and that this ratio is somewhat independent of age and sex for the range 2–20 years. An increase of chondroitin with age correlates with the decrease of chondroitin 4-sulfate and age-dependent changes in the ratio of chondroitin sulfate isomers are operative (M48), chondroitin 6-sulfate becoming progressively relatively predominant, and the relative proportions of heparan sulfate increase with age (T1). The change with age in the excretion rate of keratan sulfate has also been examined (R11). In amniotic fluid the hyaluronic acid concentration decreases with gestation (W9). Chondroitin sulfate is barely detectable at first but ultimately becomes the major glycosaminoglycan(s).

In conclusion to this section, it must be said that the changes occurring in the biosynthetic and degradative processes of proteoglycans as healthy processes are complex. On account of the lack of reports of isolation of macromolecular intermediates in these processes it is likely that they can occur very quickly. Certainly the half-lives are comparatively short. Relatively rapid processes can be imagined to be necessary for the restoration of imbalances in response, for example, to environmental changes and wounds. Future work must seek to ascertain the purposes of these changes and why some of the influencing factors are selective.

7. Involvement of Proteoglycans and Glycosaminoglycans in Disease

On account of the importance and extensive involvement of proteoglycans in the maintenance of tissue structure, etc., it is logical to expect that the defects in many diseases will be related in some way to the proteoglycans. Furthermore, considering the large number of enzymes that must be involved in the biosynthesis and metabolism of the proteoglycans (see Section 4) and the interrelationship and control that exists between them, and between them and other tissue, etc., components, it is clear that an effect upon one of them may have far-reaching results in the reversible or irreversible discontinuation of the maintenance of the production of and function of perfect tissue etc. With this in mind, it is predictable that proteoglycan involvement is spread right across the disease types.

Whereas a considerable number of conditions has been reported to involve proteoglycan disorders in some way or other, the course of development of investigations has been greatly influenced, and dramatically limited to a narrow compass, by various phenomena. First, much of the work reported is derived from studies carried out more than ten

years ago when the proteoglycans as discrete intact molecules were largely unrecognized, attention therefore being focused on the glycosaminoglycans. Second, attention has continued to be focused on glycosaminoglycan rather than proteoglycan since for the reasons for unique character discussed earlier the former is by far more easily discernible in the presence of other tissue, etc., components. Third, many of the clinical conditions originally examined are characterized by excessive urinary excretion of glycosaminoglycan peptides, i.e., molecules in which most of the protein has been lost. Fourth, by and large there has been no systematic investigation of the involvement of the proteoglycans and/or their components—rather discoveries of disorders have come from the recognition of an excess of material, often by histological methods. Since an excess may be many orders of magnitude different from the normal, whereas a decrease has a smaller maximum range and small differences go unnoticed more easily, the diseases involving proteoglycan disorders reported to date are in fact almost exclusively characterized by an overproduction or accumulation of material. Thus the coverage of disorders by the literature relevant to this Section is regrettably limited, and in most instances only an end product of the disorder has been studied, and this usually on a general qualitative and quantitative basis without structural investigation. Since this end product has been determined as a glycosaminoglycan, it is only to be assumed therefore that a proteoglycan disorder is involved. Therefore what is missing from an understanding of many of the disorders involving the glycosaminoglycan chain in some way is, in addition to structural information, the manner in which the whole proteoglycan is affected and at what stage(s) in the biosynthetic and degradative processes the primary disorder occurs. It may be that in some instances the proteoglycan disorder is a secondary effect. Most important is an understanding of the processes so that successful therapy can be devised.

In keeping with the title of this article and the space available, it has therefore been necessary to make some restrictions with respect to the literature on the involvement and possible involvement of proteoglycan in various diseases. First, only chemically based evidence is considered, although a number of other disorders suggested by histochemical studies may ultimately prove on a chemical basis to involve proteoglycans. Second, work on disorders involving enzymes and other tissue, etc., components, such as glycoproteins, lipids, and collagens, which may *in vivo* impinge upon proteoglycans has had to be excluded where no specific reference is made to the proteoglycans or their components. In view of this and the foregoing discussion, the reports reviewed in this Section depend on an identification of the glycosaminoglycans.

Many methods for their identification, distinction, and measurement in tissues and fluids have been reported (see the reviews cited in Section 1.1; see also Section 6). However, in seeking to confirm a qualitative and/or quantitative deviation from normal, it is often difficult to compare results reported from different laboratories on account of the problems of incomplete extraction, incomplete separation, mistaken identity, and the different methods used. In this context it is also important to realize that although methodology has improved considerably in the last decade, not only does much remain unknown, but some of the earlier work will have to be reevaluated with the new microscale techniques. Furthermore, some glycosaminoglycan disorders may have been overlooked, since in many instances total glycosaminoglycan has been measured, whereas separate quantitation might have revealed a balancing effect between altered individual components. In some instances reports are based on data from only one patient. Again the absolute need for chemical identification on a structural basis cannot be overstressed if the goals discussed above are to be realized. Nevertheless, it must not be overlooked that a considerable amount of work is involved in making a proper investigation for a proteoglycan disorder.

It has not been easy to devise a satisfactory structure for this Section, since the information available to date falls far short of permitting any classification of proteoglycan-involving disorders. Sectionalization on the basis of clinical presentation of the disorder or the location of the disorder is impossible since it is uncertain which clinical features are the result of primary disorders and indeed whether or not the proteoglycan disorder is a primary effect. The choice of sectionalization is therefore one based on the location of presentation of proteoglycan disorder.

Regrettably, in view of the fact that much of the work is only at the stage of recognition of a qualitative and/or quantitative difference from the normal, much of this Section must be something of a catalog. However, one group of diseases which are hereditary and involve proteoglycan disorders almost right through the body, and for which some basic defects have been derived from fibroblast cultures, is discussed in a separate section (Section 7.6.). The sole reason for this is to present the studies of these diseases as by far the best, if not the only, example of disorders where some understanding of the inherent disorders has been achieved. On no account must the reader attach too much importance to these disorders, since they do not occur frequently. They have been regarded as something of an academic curiosity, and the amount of attention afforded them is disproportionate to that given to a number of other, more frequent, diseases involving proteoglycan disorders.

Reviews on disorders with respect to proteoglycan components are

confined to those diseases described in Section 7.6, but earlier studies in the field have been reviewed in this Series (B41). Earlier studies on pathological conditions involving hyaluronic acid have also been covered (B42, S35). Changes in the levels of activity of various enzymes, which may not all be directly related to proteoglycans and are known to be altered both in diseases with and without hyperglycosaminoglycanuria, have been reviewed (V2). Some papers (F9) on the biochemistry and pathophysiology of connective tissues are relevant.

7.1. Tissue Presentation of Proteoglycan and Glycosaminoglycan Disorders

In earlier times, a number of tissue conditions were examined, often histologically, for glycosaminoglycan disorders in the skin, including chronic lupus, venous edema, scleroderma, lupus erythematosus, dermatomyositis, lichen sclerosus et atrophicans, poikiloderma, urticaria pigmentosa, and sun-damaged skin (see B41), all of which showed increased glycosaminoglycan in the skin with the exception of scleroderma, which showed a decreased content. Affected skin from a patient with localized pretibial myxedema contained increased quantities of hyaluronic acid and sulfated glycosaminoglycans (W6). In cases of cystic fibrosis the glycosaminoglycan content of the skin is increased (W13) although earlier reports (L4, L5) claimed a decrease in the 2-amino-2-deoxyhexose and hexuronic acid contents of the skin.

Increases in the levels of glycosaminoglycan have been noted in the brain of a case of Niemann–Pick disease (B45). Brain tissues from cases of galactosemia and infantile neurolipidosis contain less than normal amounts of glycosaminoglycans (B46), and at various stages of infancy, cases of protein-calorie malnutrition (kwashiorkor) contain in the brain less glycosaminoglycan overall, but an increased amount of hyaluronic acid (C11). Decreases in glycosaminoglycan (chondroitin sulfate and/or keratan sulfate) contents of the cornea has been noted in corneal disorders (A6), and the presence of dermatan sulfate is considered to be indicative of repair processes. Corneal clouding occurs in cases of genetic hyperglycosaminoglycanuria, which are discussed separately (Section 7.6.2).

The glycosaminoglycans present in abnormally high amounts in amyloid extracts of amyloid-laden organs from cases of rheumatoid arthritis, familial Mediterranean fever, Hodgkin's disease, and tuberculosis are largely dermatan sulfate and heparan sulfate (P7). The glycosaminoglycans are increased in the livers of cases of G_{M_1}-gangliosidosis type I (C2), and keratan sulfate is predominantly in excess. This occurrence

of a keratan sulfate-like substance in G_{M_1}-gangliosidosis (T12) quite probably arises from the deficiency of β-D-galactosidase (EC 3.2.1.23) that characterizes this disease. A case in which the glycosaminoglycan content of the liver was increased (V5) was also characterized by an absence of α-L-fucosidase (EC 3.2.1.51) and in some respects resembled cases of the Hurler disease, one type of genetic hyperglycosaminoglycanuria. Changes in the glycosaminoglycans of the liver occur in chronic hepatic damage (K37). Early suspicions that glycosaminoglycans are involved in arteriosclerosis and atherosclerosis (see B41) have been confirmed, and theories as to their involvement with calcium have been discussed (W1). It has been suggested that glycosaminoglycans promote progression of fibrosis in the human aorta (O5). Aortic chondroitin 6-sulfate levels are increased in atherosclerosis (D1). Glycosaminoglycans were found to be deposited in other cardiac valves of an infant with C/D chromosomal translocation (D7). Excessive amounts of glycosaminoglycans are located in various tissues of cases of genetic hyperglycosaminoglycanuria (see Section 7.6.2).

With a view to providing a method for the systematic investigation of pathological tissues for proteoglycan/glycosaminoglycan disorders, a technique for the assessment of their rates of biosynthesis/degradation and of the related activated form of sulfate, adenosine 3′-phosphate 5′-sulfatophosphate, which utilizes incorporation of [^{35}S]sulfate *in vitro* under standardized conditions, has been devised (B9, B13). The degree of incorporation of radioactivity into the precursor and each of the glycosaminoglycans is assessed quantitatively and compared with normal human material for which the glycosaminoglycans have been identified structurally. The technique has the distinct advantage of being applicable to 1 or 2 mg of tissue and therefore to biopsy specimens. This technique may be extended using radioactive monosaccharide (e.g., K9) and amino acid precursors. Application of the technique to certain clinical conditions has indicated whether there is any involvement of the glycosaminoglycans (B10). In the case of Ehlers–Danlos syndrome the epidermal incorporation profile is normal, but dermal incorporation into the two chondroitin sulfates is high. The glycosaminoglycan disorder demonstrated is presumably associated with the splitting, breaking, and fragmentation of the dermal collagen bundles. In Dupuytren's contracture both skin layers show reduced incorporation into chondroitin 4-sulfate but increased incorporation into dermatan sulfate, which is consistent with the abnormality of the upper dermal layers. Lipoid proteinosis, another skin disorder, shows high incorporation into epidermal keratan sulfate, the rest of the incorporation profile proving normal (B10, M43, M44). The incorporation profile is normalized after administration of predniso-

lone which also results in clinical improvement. On the other hand, skin from a case of icthiosyform erythroderma gives a normal incorporation profile (B10) further substantiating the validity of the method by proving that deviations from the normal are not artifacts of the method. The method has also been applied to actinic elastosis (S49), where increased incorporation into chondroitin 6-sulfate was found.

Deviation from normal control of proteoglycans is exhibited by many tumors, excess of glycosaminoglycan being produced. In early days it was found that chondrosarcoma and chordoma involve production of glycosaminoglycan and that tumors produce only one glycosaminoglycan in excess (M38). Chondrosarcomas biosynthesize either chondroitin 4- or 6-sulfate (A1, A5, S56), whereas hyaluronic acid is exuded by a mesothelioma (B33, M36), the glycosaminoglycan being recognized in the pleural fluid of some cases (see also Section 7.3, p. 54). In the latter disease, production can be reduced by administration of hyaluronidase (EC 3.2.1.35) (S42). Hyaluronic acid is manifest in sera from cases of reticulum cell sarcoma and neuroblastoma (D19). Hyaluronic acid proteoglycan has been isolated and identified from a cyst of cystic mucoid degeneration (E2), and fluids from pseudomucinous ovarian cysts contain hyaluronic acid (J13).

7.2. Cartilaginous and Skeletal Presentation of Proteoglycan and Glycosaminoglycan Disorders

Arthritic hip-joint cartilage is identifiable with an increase in the chondroitin 4-sulfate and a decrease in the keratan sulfate contents (M3). Overall there is only a slight change in glycosaminoglycan content, but over-sulfated chondroitin sulfate is present. Proteoglycan and collagen with respect to osteoarthrosis have been the subject of an occasional survey (M26). In osteoarthritic cartilage, the glycosaminoglycan distribution within chondroitin 4- and 6-sulfates is retained (H24) but there is an overall decrease in glycosaminoglycan content and a trend toward lower molecular weight species. Indeed, in osteoarthritis it appears that the cartilage proteoglycan is subject to accelerated degradation (A2). A number of arthritic cases have been investigated for cartilaginous dysfunction, which is presumed to arise from poor lubricant properties of the synovial fluid. Such aspects are discussed in Section 7.3. Proteoglycan abnormalities have been recognized in the growth cartilage of cases of pseudoachondroplasia (S36). The levels of chondroitin 4- and 6-sulfates, keratan sulfate, and non-sulfate-containing glycosaminoglycans are all decreased in nucleus pulposus of idiopathic scoliosis (P2), but their relative proportions are normal. However, it may be that the lengths

of the chondroitin 4- and 6-sulfate and keratan sulfate chains are shorter than usual since they behave anomalously on column chromatographic analysis.

7.3. Body Fluid Presentation of Proteoglycan and Glycosaminoglycan Disorders

The examination of proteoglycans and glycosaminoglycans in body fluids presents a less formidable problem than examination of those in solid specimens since the extraction procedure can be much simpler. This in turn has permitted ultimately a more critical assessment of the proteoglycan. The concentration and degree of polymerization of hyaluronic acid in hyaluronic acid proteoglycan in synovial fluids of arthritic and degenerative joint diseases have been the subjects of widespread investigations, but sometimes conflicting reports. Originally an increased amount of hyaluronic acid in synovial fluids from patients with rheumatoid arthritis was reported (R1), and a similar result was obtained for degenerative joint disease (S51); it was therefore concluded (B32, R1) that the pathological fluid contained an excess of incompletely polymerized hyaluronic acid. In other reports, including more recent ones, the hyaluronic acid contents of synovial fluids of patients with rheumatoid arthritis have been claimed to be decreased (H5, K34) by as much as 50% or more (S19). The chondroitin sulfate of synovial fluid in rheumatoid arthritis has also been investigated (B12, S18); it is present as a proteoglycan (W20) and its concentration is reduced to 50% of the normal level (S19). No correlation has been found between the concentrations of hyaluronic acid and chondroitin sulfate and the grade of local articular change in cases of rheumatoid arthritis (S19); indeed a high correlation exists between the concentrations of hyaluronic acid and of chondroitin sulfate in synovial fluids from both these patients and normals. Hyaluronic acid from rheumatoid arthritis patients, but not that from the synovial fluid of normals, is associated with a protein analogous to immunogloblin IgG (H29). Clearly this associated material may affect the molecular weight assigned to the hyaluronic acid in the pathological specimen depending on the method of measurement used, but whether or not it is covalently bound was not determined. Since hyaluronic acid is known to contribute to the viscosity of synovial fluid, and since the viscosity of arthritic synovial fluids is low, it became suggested that the changes in viscosity with advance of the disease impair the lubricant properties of the fluid (J18, J19). This in turn causes abrasion of the meeting cartilage surfaces with their concomitant degeneration.

The mean degree of polymerization of hyaluronic acid in synovial fluid is below normal in the pathological condition (B18, B34, B48, H28, J16, R1). The chondroitin sulfate proteoglycan of synovial fluid is also present in a somewhat degraded form in patients with inflammatory joint disease (W20). Observed lower molecular weights of proteoglycan molecules are of course subject to discussion as to whether shortening of the glycosaminoglycan chains or decrease in the size of the protein backbone, or both are involved. These lower molecular weights might arise either from depolymerization at an abnormal rate of the normal proteoglycan macromolecule or from a disturbed biosynthetic pathway. The demonstration (H21) that β-D-acetamidodeoxyglucosidase (EC 3.2.1.30) is present in abnormally high amounts in the synovial membranes in rheumatoid arthritis and allied joint conditions suggests that the depolymerization of the hyaluronic acid could perhaps be brought about enzymically although this enzyme is not an *endo*-polysaccharidase. Evidence has been presented (W20) that the degradation of chondroitin sulfate proteoglycan in synovial fluid of patients with inflammatory joint diseases is effected by a protease.

On the other hand, it is now known that irreversible depolymerization of hyaluronic acid occurs in solution with a number of reducing agents, including L-ascorbic acid, in the presence of molecular oxygen (see K20). The common occurrence of reducing agents in tissues and cells therefore suggested that this reaction may have biological significance in providing a control mechanism for the depolymerization of hyaluronic acid and the related permeability of the related tissues. Taking this argument a little further, it can be seen that an excess of reducing agent would lead to adverse depolymerization. However, it has been demonstrated (C8) that L-ascorbic acid is unlikely to be responsible for hyaluronic acid depolymerization *in vivo* since synovial fluid is not attacked by this reagent to depolymerize the hyaluronic acid. Although it appears that other components of synovial fluid prevent the reaction in this case, the successful use as a topical application of dimethyl sulfoxide which is a radical scavenger to cut down arthritic advance is consistent with a free radical type depolymerization in the pathological condition. Sensitive methodology based on molecular sieving on a porous silica support and automated fluorimetric analysis of the column eluate (C13, K19) permits a rapid assessment of the protein and polysaccharide in synovial fluid specimens and is a very rapid means of identifying any deviation from abnormality.

A number of drugs have been used for the treatment of various arthritic conditions with various and varying degrees of success. The anti-inflammatory drugs—aspirin, indomethacin, and prednisone—which have

been used all cause a decrease of the polydispersity of the hyaluronic acid of synovial fluids of arthritic cases, decreasing the content of low molecular weight glycosaminoglycan (H28). This decrease is matched by a clinical improvement including a decrease in pain; although rectification is only partial, these drugs do prevent advance of the disease. The advantageous effects of dimethyl sulfoxide as a therapeutic agent are outweighed by its undesirable side effects (see B9 and Section 5, p. 38).

A component closely related immunologically to the proteoglycans of cartilage has been detected (S3) in increased amounts in synovial fluids from patients with gout, lupus, rheumatic fever, and septic arthritis. In each case the proteoglycan material is probably released from the articular cartilage during acute inflammation of the joint.

Some investigations have been made of the glycosaminoglycans in sera in clinical conditions, and the presence of hyaluronic acid in sera from several pathological conditions has been reported (D19, D20). The levels of glycosaminoglycan are increased in serum in exophthalmos (W14), and they also increase with the progress of serum bilharziasis (K24). Hyaluronic acid is manifest in sera from cases of reticulum cell sarcoma and neuroblastoma (D19). Determination of [^{35}S]sulfate incorporation into the leukocyte cellular fraction may help to differentiate between acute granulocytic and acute lymphocytic leukemia (L7); in the latter no incorporation into chondroitin sulfate occurs *in vitro* whereas in the former appreciable incorporation occurs *in vitro*.

The higher level of hyaluronic acid in pleural fluids from some patients with repeated pleural effusions corresponded to mesothelioma (H12; see also Section 7.1, p. 51), but the levels were considerably reduced on treatment of the patients with antimitotics. Proteoglycans have been detected in bile from cases of functional disturbance of the liver and infections of the biliary tract (M25).

7.4. Urinary Presentation of Proteoglycan and Glycosaminoglycan Disorders

The excessive urinary excretion of glycosaminoglycans by cases of genetic hyperglycosaminoglycanuria is well known in the field and has been afforded greatest attention. Although this will be discussed subsequently (Section 7.6.1), it is relevant to draw on such work at this juncture with reference to the form of the urinary glycosaminoglycans. This is a degraded form in which the protein has been reduced to a peptide and many of the glycosaminoglycan chains have been degraded. Whereas even in normals intact proteoglycans are not excreted,

degradation has occurred, but it is not so extensive, as can be deduced from molecular weight studies. Thus in genetic hyperglycosaminoglycanuria the urinary material is very much degraded compared with proteoglycans, and it is reasonable to assume that such will be the situation in other conditions of urinary presentation. Such degradation poses a problem in terms of identification of urinary glycosaminoglycan material in that since cetyl pyridinium chloride precipitation techniques are usually used as a first stage in the isolation, very low molecular weight materials will not be recovered and so any disorder which gives rise to such will escape notice in this respect. Furthermore, many studies have been conducted only in terms of total precipitable glycosaminoglycan, i.e., without separation. Problems in quantitating urinary glycosaminoglycan contents have already been discussed (Section 6, p. 42).

In addition to genetic glycosaminoglycanuria a number of conditions give rise to excessive excretion of glycosaminoglycans, but most of these conditions are apparently nonhereditary ones for which no classification pattern has been established. The urinary concentration of glycosaminoglycans is increased in patients with dermatomyositis (M49), polymyositis (M49), aortic syndrome (M49), progressive systemic sclerosis (M49), G_{M_1}-gangliosidosis type II (W18), rheumatoid arthritis (D23, T7), lupus erythematosus (D27), diabetes (C28), leukemia (R6, S28) and other malignant diseases (R6), chronic hepatitis (K6), florid cirrhosis (K6), obstructive jaundice (K6), in various pathological conditions affecting the muscle (M49), the kidney, or the liver (T7), exophthalmos (W14), and systemic infantile lipidosis (W19). Urinary glycosaminoglycan levels also increase in therapeutic enzymic dissolution of the nucleus pulposus and after surgical stress (S41). Both hyaluronic acid and dermatan sulfate were found in excessive amounts in urine from a patient with multiple soft tissue tumors (S53). In many instances, e.g., rickets (C14), cystic fibrosis (C25), and mastocytosis (C17), the levels of glycosaminoglycan excretion are only slightly raised, and then not in all patients. Undoubtedly, in such cases and many others where the disease is severe, excess shedding of tissue materials occurs and hence excessive glycosaminoglycan excretion is a secondary irregular effect. In both systemic infantile lipidosis with G_{M_1}-gangliosidosis (W19) and G_{M_1}-gangliosidosis type II (W18), keratan sulfate is the predominant urinary glycosaminoglycan, and this may be associated with β-D-galactosidase (EC 3.2.1.23) deficiencies. In the latter disease keratan sulfate also occurs in cultured skin fibroblasts (C3). Keratan sulfate excretion is raised in some cases of achondroplasia, rheumatoid arthritis, and dermatomyositis (R11). Furtheir investigations of a number of connective tissue disorders and rheumatoid arthritis (B31) indicated that only patients with lupus erythema-

tosus and only 20% of patients with rheumatoid arthritis show raised levels. In the two latter conditions and in cases of diffuse scleroderma (W7), and in cases of psoriasis and other dermatoses (M39) the levels of both the sulfated and nonsulfated glycosaminoglycans are increased. Glycosaminoglycans with abnormally high and low sulfate contents have been detected in urine from cases of rheumatoid arthritis and chronic lymphatic leukemia, respectively (F10). A large number of infants and children with various clinical conditions have been screened for excessive urinary glycosaminoglycan (P3), but the data obtained will have been influenced by any contribution from glycoproteinaceous material on account of the method of measurement used.

The urinary excretion of glycosaminoglycans by juvenile cases of vitamin A deficiency and of protein-calorie malnutrition (kwashiorkor) is less than normal (M41). The predominant differences are the absence of hyaluronic acid and the presence of chondroitin sulfate of low sulfate content (C12). Treatment of the cases with vitamin A deficiency restored the glycosaminoglycan spectrum to normal. Decreased urinary glycosaminoglycan levels have also been noted in primary hepatoma (K6). It has been suggested that the urinary excretion pattern of individual glycosaminoglycans is pathognomic of certain hereditary bone diseases (T3).

Normal urinary glycosaminoglycan patterns have been reported for cases of nail-patella syndrome and Farber's disease (W7) and for patients with cystic fibrosis (C25, K38; also see above).

7.5. Cell Culture Presentation of Proteoglycan and Glycosaminoglycan Disorders

A different approach to the study of tissues for disorders involving proteoglycan has been the selection of cells from the tissue and their culture *in vitro*. Fibroblasts have been utilized on account of the ease and convenience with which these may be propagated. In some instances the fibroblasts secrete the glycosaminoglycan into the culture medium. Whereas this technique has been developed and applied mostly in connection with genetic hyperglycosaminoglycanuria (see Section 7.6.4), increased levels of glycosaminoglycans occur in cultured fibroblasts of a large number of cases of different disease outside genetic hyperglycosaminoglycanuria (M17). Cultured skin fibroblasts from cases of G_{M_1}-gangliosidosis type II synthesize a keratan sulfate-like compound (C3), and in cultured skin fibroblasts from cases of Fabry's disease there is a markedly elevated glycosaminoglycan content (M21) but the distribution between hyaluronic acid, chondroitin sulfate and dermatan sulfate

is normal. Although the disease is due to a deficiency in β-D-galactosidase and thereby an accumulation of glycolipid, this cannot be correlated with the β-D-galactose units present in the glycopeptide linkages of many glycosaminoglycans. The glycosaminoglycans in fibroblasts cultured from cystic fibrosis patients are increased above normal (M15). This increased accumulation of glycosaminoglycans is not due to their defective degradation (W11), which is in contrast to the situation appertaining in genetic hyperglycosaminoglycanuria.

Disadvantages of cell cultures are the time and expense required for the establishment of tissue culture cell lines. Furthermore they are of no use for rapid diagnoses. Distinction of a disorder by this method will prove difficult in terms of simple measurement of the proteoglycan and glycosaminoglycan levels since many diseases give rise to metachromasia in the fibroblast. Most important perhaps of all is the question of the alteration/mutation of the cells during culture and reculture which may give rise to a chemical situation in the cell which is not representative of its chemical state *in vivo*. Experiments with cultures from rat material (K27) show that the pattern of cell cultures and the glycosaminoglycan distribution can change on subculture. It would therefore appear that only under defined conditions of age, etc., of the cell can any data be made comparable.

7.6. Genetic Hyperglycosaminoglycanuria

By far the greatest attention to the involvement of proteoglycans/glycosaminoglycans in disease has been given to a group of hereditary diseases which are characterized by the excessive urinary excretion of glycosaminoglycan material and have come to be known as "the mucopolysaccharidoses." The term mucopolysaccharidosis is misleading, not only on account of the vague meaning of the word mucopolysaccharide (see Section 2), but also, because it implies that glycosaminoglycans (acidic mucopolysaccharides) are the only storage material. This undoubtedly arises from the fact of the easily determined excessive glycosaminoglycan excretion, but it is apparent that the diseases involve complex storage disorders in which both glycosaminoglycan and other materials are affected and accumulate in tissues. It is difficult to give an expressive term to this group of diseases, but the term genetic hyperglycosaminoglycanuria coined and selected for use here is acceptable although, as will be seen subsequently, the urinary aspects are only some of the initial indications of far-reaching disorders. Nevertheless, as discussed subsequently, there is now evidence that the primary defect

in these hereditary disorders is faulty breakdown of the glycosaminoglycan(s); this therefore permits their distinction from other genetic diseases in which proteoglycan disorders and hyperglycosaminoglycanuria are secondary effects. However, it may emerge that there is also a disorder in proteoglycan protein metabolism in genetic hyperglycosaminoglycanuria since amino acid differences (e.g., O12) as well as carbohydrate differences have been observed.

In considering this group of diseases, the reader is re-referred to the introduction to this Section, where it is explained that these diseases are considered together solely for the purpose of illustrating the technical advances that can be made in the understanding of the abnormal processes underlying a condition. The classification (see later) of a disease as a case of mucopolysaccharidosis or genetic hyperglycosaminoglycanuria can be very arbitrary, since although some disease types are proven hereditary disorders, many diseases give rise to hyperglycosaminoglycanuria although not all of these are proved to be nonhereditary. Furthermore, there are undoubtedly many hereditary disorders that have not been investigated for glycosaminoglycanuria. The term mucopolysaccharidosis has become something of a band wagon with various workers seeking to add to the classification of confirmed hereditary hyperglycosaminoglycanuria additional conditions characterized by hyperglycosaminoglycanuria, but for which no genetic information has been available. In this article, the term genetic hyperglycosaminoglycanuria is reserved for hereditary conditions where faulty proteoglycan metabolism is considered to be a primary defect.

As already indicated, the attention given to genetic hyperglycosaminoglycanuria is out of all proportion to the large range of clinical conditions known to involve proteoglycan disorders. One company has even gone to the extent of marketing urinary test papers for hyperglycosaminoglycanuria. The frequency of genetic hyperglycosaminoglycanuria is claimed (V2) to be relatively high among inherited diseases, although this attitude is to be questioned. The frequency of cases is related to the genetics; e.g., the Hurler syndrome is autosomal recessive whereas Hunter syndrome is X-linked recessive; and this has a bearing on their relative frequency (L31, M28) and the overall frequency in a Western population has been estimated at less than 1 per 10^5 live births (L31). However, it is to be hoped that the consideration given them is paving the way for successful and more rapid and direct investigation of other proteoglycan disorders, particularly with respect to identifying the primary defect.

Considerable details are now known of the clinical features, and the morphological, chemical, and biological aspects of, and the glycosamino-

glycans excreted by, the various types of genetic hyperglycosaminoglycanuria have been reviewed in detail (D32, M29, M30, V2, V3). Unfortunately, many of the conditions are manifested in childhood, causing extensive deformity and mental retardation. In addition to examining the excreted end product of the disorders in genetic glycosaminoglycanuria, the glycosaminoglycans, considerable attention has been given to a search for the primary defect. However, the examination of the likelihood that enzymic defects underlie the various types of genetic hyperglycosaminoglycanuria is hampered since the enzymes in humans that degrade the glycosaminoglycans generally remain unidentified (see Section 4, p. 17). However, it would seem (M46), in the light of certain evidence, that the intracellular metabolism rather than the excretory mechanism of the glycosaminoglycans is defective. There is now evidence for the defective degradation of glycosaminoglycans in genetic hyperglycosaminoglycanuria (see Section 7.6.4, p. 70, and M46), and mutation with respect to the enzymes involved in the biosynthesis and turnover of glycosaminoglycans can result in deviation from normal control and may be responsible for these inherited disorders of connective tissue (D31). A number of theories have been put forward as to how, why, and where the glycosaminoglycans/proteoglycans are initially accumulated and as to why this accumulation is selective for certain glycosaminoglycans (see M46, V2). Much of the evidence to a recent date for the defective degradation or turnover of the glycosaminoglycan chains of the proteoglycans has been collated (M46). The various studies of these conditions that have been made via cell culture experiments have also been reviewed (N5) as have the enzymic defects in the fibroblasts and the corrective factors for some of the diseases (B1, N4). Changes in the levels of activity of many glycoside hydrolases which may or may not be directly related to glycosaminoglycans, in various tissues from cases of genetic hyperglycosaminoglycanuria, have also been reviewed (V2).

The six classified types of cases have been subclassified (M7, M29, M30) on the bases of clinical symptomatology, mode of genetic transmission, and nature of the glycosaminoglycan present in excess in the urine, and have been assigned type numbers (Table 2). However, even acceptance of this somewhat arbitrary classification of types of hereditary glycosaminoglycanuria does not yield complete simplicity since hyperglycosaminoglycanuria is not always present. The best classification of these diseases would be based on the chemical aspects—the primary enzymic defect, but unfortunately this is not yet possible since the specificities of even the enzymes that have been implicated need to be investi-

TABLE 2

CLASSIFICATION OF THE BETTER-ESTABLISHED TYPES OF GENETIC HYPERGLYCOSAMINOGLYCANURIA

	Designation	Clinical features	Genetics	Principal excessive urinary glycosaminoglycans[a]	Compound deficient
Type I H	Hurler syndrome	Early clouding of cornea, grave manifestations, death usually before age 10	Homozygous for MPS I H gene	Dermatan sulfate, heparan sulfate	α-L-Iduronidase (formerly called Hurler corrective factor)
Type I S	Scheie syndrome	Stiff joints, cloudy cornea, aortic regurgitation, normal intelligence, ?normal life-span	Homozygosity for MPS I S gene	Dermatan sulfate, heparan sulfate	α-L-Iduronidase
Type I H/S	Hurler–Scheie compound	Phenotype intermediate between Hurler and Scheie	Genetic compound of MPS I H and I S genes	Dermatan sulfate, heparan sulfate	α-L-Iduronidase
Type II A	Hunter syndrome, severe	No clouding of cornea, milder course than in MPS I H but death usually before age 15 years	Hemizygous for X-linked gene	Dermatan sulfate, heparan sulfate	L-Iduronic acid 4-sulfate sulfatase
Type II B	Hunter syndrome, mild	Survival to 30s to 50s, fair intelligence	Hemizygous for X-linked allele for mild form	Dermatan sulfate, heparan sulfate	L-Iduronic acid 4-sulfate sulfatase
Type III A	Sanfilippo syndrome A	Identical phenotype:	Homozygous for Sanfilippo A gene	Heparan sulfate	Heparan sulfate sulfatase/sulfamidase
Type III B	Sanfilippo syndrome B	Mild somatic, severe central nervous system effects	Homozygous for Sanfilippo B (at different locus)	Heparan sulfate	α-D-Acetamidodeoxy glucosidase

Type IV	Morquio–Ullrich syndrome (probably more than one allelic form)	Severe bone changes of distinctive type, cloudy cornea, aortic regurgitation	Homozygous for Morquio–Ullrich gene	Keratan sulfate	Unknown
Type V	Vacant[b]	—	—	—	—
Type VI A	Maroteaux–Lamy syndrome, classic form	Severe osseous and corneal change, normal intellect	Homozygous for M-L gene	Dermatan sulfate	Compound isolated but not yet identified
Type VI B	Maroteaux–Lamy syndrome, mild form	Severe osseous and corneal change, normal intellect	Homozygous for allele at M-L locus	Dermatan sulfate	
Type VII	β-D-Glucuronidase deficiency (more than one allelic form?)	Hepatosplenomegaly, dysostosis multiplex, white cell inclusions, mental retardation	Homozygous for mutant gene at β-D-glucuronidase locus	Chondroitin 4-sulfate,[c] chondroitin 6-sulfate	β-D-Glucuronidase

[a] For details of other changes in levels of other glycosaminoglycans, see Section 7.6.1.

[b] Due to modification of earlier classifications.

[c] The classification table given in McKusick (M29) records erroneously that dermatan sulfate is the principal excessive urinary glycosaminoglycan (see page 66).

gated in detail. Some sort of enzyme classification is emerging, as indicated in Table 2, but clearly other factors must be involved to give rise to the differences between the diseases that have so far been shown to involve a common enzymic activity.

Conditions that are hereditary and have been erroneously termed "mucopolysaccharidoses without mucopolysaccharidoses" fall outside the scope of this article and have been reviewed elsewhere (V2, V3).

7.6.1. *Urinary Presentation*

The problems of collection of representative samples and of expression of excretion amounts and rates on a quantitative basis have been discussed (Section 6, p. 42). Urinary screening tests for glycosaminoglycanuria have been discussed in detail (see L11), and routine screening of all referred cases is being carried out for the English Midlands area (L12, T10). Some test papers for such screening have been put on the market. Standard methodology, which may be followed on obtaining a positive screening test, is based on ion-exchange chromatography (K22) and electrophoresis on cellulose acetate (L10, L11), these being particularly applicable to quantitative and qualitative aspects, respectively. Although the cetyl pyridium chloride precipitation method is convenient for separation of glycosaminoglycans from urine, its application for quantitative studies is not without problems since low molecular weight glycosaminoglycan is not precipitated. Similarly dialysis is inappropriate for purification. However, the problem can be overcome by using ion-exchange chromatography to remove the remaining nonprecipitated glycosaminoglycan from the urine (D28), and the ratio of precipitable to nonprecipitable glycosaminoglycan for urine from cases of genetic hyperglycosaminoglycanuria is greater than unity, whereas the ratio is less than unity for normals. Some studies have unfortunately only been taken as far as determination of the total precipitable hexuronic acid-containing material. It would also seem to be overlooked by many that keratan sulfate, the glycosaminoglycan presented by the Morquio syndrome, does not respond in the carbazole-sulfuric acid assay for hexuronic acid since keratan sulfate contains no hexuronic acid. In some instances, separative techniques such as ion-exchange chromatography have been applied, but in some of these cases it is quite evident from component analysis and further separations that the fractions are heterogeneous (e.g., G8).

The major glycosaminoglycans excreted in excess by the various types of hereditary glycosaminoglycanuria are given in Table 2, and since the recognition of the excretion of these goes back more than a decade the relevant literature which is covered adequately in the reviews cited

will not be re-cited here. In patients suffering from genetic hyperglycosaminoglycanuria there is a close correlation between the types of glycosaminoglycan excreted in the urine and the clinical picture (L10, L11). The cellulose acetate electrophoresis method permits distinction between the following syndromes: Hunter + Hurler, Sanfilippo A, Sanfilippo B, Morquio-Ullrich, Scheie, and normal. For comparison, in normals the greatest proportion of the urinary glycosaminoglycans is chondroitin 4- and 6-sulfates some fractions of which may be undersulfated (V8). Heparan sulfate, dermatan sulfate, keratan sulfate and hyaluronic acid together comprise less than 10% of the total normal excretion spectrum. In genetic hyperglycosaminoglycanuria the amount of polysaccharide may exceed 100 times normal (expressed in terms of the disaccharide repeating units of chondroitin 4-/6-sulfate/dermatan sulfate) (M27), and it seems that the amount of the excess may decrease with age of the patient.

Heparan sulfate isolated from the urine of patients with the Hurler syndrome is heterogeneous (K32). Chemically, the urinary molecules are fragments that may represent segments of a single parent molecule similar in structure to the heparan sulfate from normal human aorta. Ion-exchange chromatography of the heparan sulfate gives a different elution profile from the heparan sulfate excreted by cases of the Hunter syndrome (G8, M5). Thus, in spite of the close relationship of various aspects of the two syndromes including their common involvement of α-L-iduronidase (see Section 7.6.4, p. 70), it appears that the anomalous degradation processes could differ. Both the heparan sulfate and dermatan sulfate fractions are of lower than normal molecular weight and deficient in glycopeptide linkage constituents (C20, D14, D30, K32) and vary in molecular size (G8, S44) and degree of sulfation (G8); therefore they probably represent a series of degradation products of the parent glycosaminoglycans in the two syndromes. In fact since all the heparan sulfate and most of the dermatan sulfate excreted by cases of Hurler syndrome have been severely degraded (e.g., C23) (average molecular weights 2300 and 9000, respectively), a majority of the molecules may be considered to be dialyzable. This is congruent with a further report (K32) that gives a molecular weight of 2700–5500 for the heparan sulfate. However, it appears that in both these syndromes glycosaminoglycan is excreted to a certain extent in forms that have molecular weights higher than those for normal urinary glycosaminoglycans since the degradation ratio (D29), which is an expression of high molecular weight to low molecular weight glycosaminoglycan, is of the order of 2 for Hurler and Hunter material, whereas the value for normals is 0.4 (D33).

Gel filtration profiles for the glycosaminoglycans excreted by the two syndromes are distinct (C23). A disulfated disaccharide containing L-iduronic acid, which is absent from urine from normals and other types of genetic hyperglycosaminoglycanuria, occurs in urine from patients with the Hunter syndrome (C27), and it may be that this disaccharide is the substrate of the enzyme deficient in such cases. In addition to the major occurrence of dermatan sulfate and heparan sulfate in these cases, the urinary chondroitin 4- and 6-sulfate levels are also increased above normal levels, which are very low (O11). The chondroitin sulfate levels are also increased for Hurler patients (D14), and the relative proportions of chondroitin sulfate, dermatan sulfate, and heparan sulfate are the same as those found for the excessive glycosaminoglycan deposited in the tissues. Although the amounts are very small, raised excretion levels of keratan sulfate by Hunter and Hurler cases have also been reported (R11) on the basis of selective measurement of D-galactose. Raised levels of non-sulfate-containing glycosaminoglycan also occur in urine from Hurler cases (S7). The raised levels of chondroitin sulfates, etc., may explain the distinct third glycosaminoglycan band that has been found repeatedly in critical analytical electrophoresis of Hunter and Hurler urines (L10, L11). The clinical features of Hurler syndrome are sometimes present without hyperglycosaminoglycanuria (L3, S38).

Patients with the Scheie syndrome also excrete excessive amounts of heparan sulfate and dermatan sulfate (C22), and in electrophoretic analysis only two glycosaminoglycan bands are found (L10, L11); these correspond in position to two of the bands arising from Hurler and Hunter urines. Molecular weight distributions of the heparan sulfate excreted by cases of Sanfilippo syndrome (S44) give an average molecular weight of one-third normal and both the heparan sulfate and dermatan sulfate are heterogeneous in sulfate content (G8), but the degradation ratio (see above) is 2 or more (D33). This heparan sulfate is separable (S44) into two fractions with different molecular weights, sulfate contents, and primary structures. On the basis of physicochemical and other criteria, one of these fractions possesses a conformational structure similar to that found in the glycosaminoglycan from normal tissues but with a lower than normal *O*-sulfate content; the other fraction resembles conformationally heparin and has a high *N*-sulfate content. Raised levels of keratan sulfate have also been found for Sanfilippo cases (R11) although the absolute amounts are small. On the basis of the enzymic defects identified (K43; see later), Sanfilippo cases may be divided into two types, and this has recently been shown to be manifested in the urinary glycosaminoglycan pattern since cellulose acetate electrophoresis gives distinctly either one band or three bands (L10, L11). In the latter instance, it would appear that a glycosaminoglycan in addition to

heparan sulfate is excreted. Certainly the above-mentioned physicochemical data are subject to reinterpretation in terms of a multiplicity of glycosaminoglycan types.

The presentation of the Morquio syndrome can also be in one of two forms, the distinction proposed being with and without hyperglycosaminoglycanuria (M29). This has been confirmed by the electrophoretic examination of the urinary glycosaminoglycans, which show either an abnormal, two-band pattern or a normal, three-band pattern (L10, L11). In the latter case, the Morquio–Brailsford syndrome, the urines gave negative results in the standard screening tests and the absence of hyperglycosaminoglycanuria has also been found by others (B30). This absence of hyperglycosaminoglycanuria also explains and confirms the report (J12) of some related cases of Morquio–Brailsford syndrome who do not excrete excessive amounts of glycosaminoglycan. The glycosaminoglycan reported to be excreted in excess (D35) in the Morquio-Ullrich syndrome, i.e., Morquio syndrome with glycosaminoglycanuria, is keratan sulfate (B30, M29, R11), but it would appear from the above-mentioned electrophoretic data that two glycosaminoglycan species are present in the urine. There may be some heterogeneity within the syndrome since some patients excrete in addition to keratan sulfate raised levels of hexuronic acid-containing glycosaminoglycan (L29). Indeed, the low hexose to 2-amino-2-deoxyhexose molar ratio observed for the excreted polysaccharide and the fact that 50% of the 2-amino-2-deoxyhexose content possesses the D-*galacto*- rather than the D-*gluco*-configuration has prompted the suggestion that the material contains chondroitin sulfate as well as keratan sulfate (K2), as could be expected from a cartilaginous-type proteoglycan. Further characterization of the urinary glycosaminoglycans has shown that there are equal amounts of chondroitin 6-sulfate, containing a high proportion of L-serine, and keratan sulfate-peptide, in which L-serine and L-threonine are the principal amino acids (B44). These two glycosaminoglycan chain types appear to be in part if not totally attached to the same peptide (D14) since they are only separable after proteolysis. In a case with the clinical characteristics of Morquio–Ullrich syndrome chondroitin 4-sulfate and chondroitin 6-sulfate copolymerized with dermatan sulfate were the major glycosaminoglycans excreted (O12). Amino acid analyses showed the total amino acid content of the glycosaminoglycans to be abnormally low, with L-serine as the predominant amino acid. A series of related cases of Morquio–Ullrich disease revealed only a slight increase of glycosaminoglycan excretion (C18), but significant changes were observed in the proportions of hyaluronic acid, chondroitin 4- and 6-sulfates, dermatan sulfate, and heparan sulfate. Particularly significant was the proportionally increased excretion of nonsulfated glycosaminoglycans. There is

therefore considerable heterogeneity within the disease and the latter variant has been proposed as new classifiable type of genetic glycosaminoglycanuria (M27), but for the formalization of such, insufficient chemical detail is available.

Urine from patients with the Maroteaux–Lamy syndrome contains excessive amounts of dermatan sulfate (M8), but partially on account of the comparatively recent recognition of this disease, chemical studies have not been conducted.

A number of other conditions that have not been included in the classical table of genetic hyperglycosaminoglycanuria types (M29) are nevertheless eligible for such a classification. Cases of the Marfan syndrome excrete excessive amounts of glycosaminoglycans, including hyaluronic acid and chondroitin sulfate (B26), but there is essentially a normal distribution (B25), mainly between chondroitin 4-sulfate, chondroitin 6-sulfate and dermatan sulfate.

Urine from children with Marfanoid syndrome reacted positively in the cetyl trimethylammonium bromide precipitation screening test, but the glycosaminoglycan levels are within normal limits (G7). More recently a syndrome in which chondroitin 4-sulfate is excreted in great excess has been recognized (C4, P5, S34, T8). In another case, which is possibly a hereditary disorder, chondroitin 6-sulfate was excreted in excess (S11), and in a further case both isomers of chondroitin sulfate were excreted in excess (B22, H4, S30). Cases of excretion of the two isomers in small excess appear to be due to a β-D-glucuronidase (EC 3.2.1.31) deficiency (H4, S30). Excretion of chondroitin 6-sulfate containing less than stoichiometric amounts of sulfate has also been reported (D21, M42). Dermatan sulfate and heparan sulfate were excreted by a patient whose clinical presentation could not be fitted to any of the tabulated disease types (H26). Urinary glycosaminoglycan levels are raised in cases of hereditary deforming chondrodysplasia (diaphysial aclasis) (L30), and keratan sulfate is excreted in excess by cases of athetosis (M6). Clearly in this list of additional disorders involving urinary glycosaminoglycans, there is some overlap by those described in Section 7.4. (p. 55), but this is due to the present uncertainty as to whether some of those disorders are hereditary hyperglycosaminoglycanuria.

7.6.2. *Tissue Presentation*

Early workers in the field of genetic hyperglycosaminoglycanuria sought to trace the rate and the location of occurrence of the excess glycosaminoglycan, and the various tissues have been analyzed for storage of excess glycosaminoglycan. That a number of parts of the body can be affected

severely by the disease is apparent from the tissue abnormalities and skeletal deformities that occur to various degrees and classify the types on a clinical basis. However, often the symptoms are disguised, and for this reason a chemically based diagnosis is necessary—in the first place by urinary analysis. Methods for investigation of glycosaminoglycan in tissues have already been discussed (see Section 5, p. 38 and Sections 6 and 7.6.5).

The glycosaminoglycans of the tissues are similar to those excreted in the urine, and the results of the earlier studies have been given in the reviews already cited (e.g., V2). Hurler tissue has been afforded the greatest attention, being one of the earliest studied and recognized types of genetic hyperglycosaminoglycanuria. On account of urinary presentation, the kidney was investigated, and excessive amounts of both dermatan sulfate and heparan sulfate occur in this organ (C24) and in the liver (D14, G9, K22) in cases of both Hurler and Hunter syndromes; hyaluronic acid and heparan sulfate are predominant in the normal kidney. The relative amounts of dermatan sulfate and heparan sulfate in the tissues may be determined quantitatively (K22). Raised levels of these glycosaminoglycans also occur in the Hurler spleen (e.g., D14), and the relative proportions of dermatan sulfate, heparan sulfate, and chondroitin sulfate are the same for the liver, spleen, and urine from such cases (D14). As in urinary material, the Hurler tissue glycosaminoglycans are degraded forms of the normal macromolecules (e.g., D14), and these low molecular weight forms can be deficient in glycopeptide linkage components (D14, D30, K32). The heparan sulfate and dermatan sulfate of the liver and spleen at least are polydisperse (D14, G9), and this polydispersity can arise from multiples of degraded glycosaminoglycan chains being attached to a small peptide (D14). In the case of liver and spleen material from Hurler patients, the material consists of single chains of molecular weight of 5×10^3 together with multiples of up to four such chains attached to peptide moieties. The heparan sulfate isolable from both the liver and urine of patients with Hurler syndrome is also heterogeneous on chemical analysis (K32), and the nature of the fragments (molecular weights 2700–5500) is such that they may represent a single parent molecule not structurally unlike the heparan sulfate from normal human aorta. Dermatan sulfate with an average of more than one sulfate group per disaccharide unit has been isolated from the spleen of a Hurler case (C1).

In the Sanfilippo syndrome, glycosaminoglycan accumulates in ocular material (J14), and heparan sulfate is by far the predominant glycosaminoglycan accumulated in the liver (G9), and here the glycosaminoglycan is known to be polydisperse. Heparan sulfate also accumulates

in the brain (G2, T2), but the total glycosaminoglycan level is not abnormally high, the levels of chondroitin sulfates being low. In the brain tissue at least, the chondroitin 4-sulfate sulfatotransferase is absent (G2), whereas the dermatan sulfate and heparan sulfate sulfatotransferase activities are normal. The relative transfer of sulfate by the heparan sulfate sulfatotransferase is twice that of normal and this indicates that *N*-sulfation may be more predominant than *O*-sulfation in the pathological tissue—a situation that would correlate with the accumulation of heparan sulfate. Increased deposition of glycosaminoglycan in the liver has been described for Maroteaux–Lamy disease (V6).

In consideration of these various accumulations of glycosaminoglycans in tissue, it must be realized that the effects most certainly are more far reaching into other tissues than those indicated in the foregoing. Many variations of lysosomal enzymes, particularly glycoside hydrolases, occur in tissues of cases of genetic hyperglycosaminoglycanuria, but it is questionable whether these constitute the primary defect (V4), and it is probable that these alterations are secondary effects (K23, K25). Further discussion of the possible enzymic defects in genetic hyperglycosaminoglycanuria arises from fibroblast culture studies (Section 7.6.4).

7.6.3. *Body Fluid Presentation*

Genetic hyperglycosaminoglycanuria is known to affect a number of the body fluids, including ocular fluids and cerebrospinal fluid, as was discovered at an early date. For example, dermatan sulfate and heparan sulfate occur in abnormally high levels in cerebrospinal fluid from cases of Hurler and Hunter syndromes (C21).

The glycosaminoglycans of amniotic fluid have also been investigated, on account of the potential use of the material for diagnoses (see Section 7.6.5, p. 73). The glycosaminoglycans may be isolated from the fluid as complexes with Alcian Blue (W9), and certainly increased levels are observed for some cases, for example, amniotic fluid from a pregnancy which resulted ultimately in delivery of an infant with Hurler's syndrome contained very high levels of glycosaminoglycan (M22). The glycosaminoglycan mixture contained predominantly heparan sulfate. However, as has been observed for the Hurler and Hunter (M20) and Sanfilippo (F2, M20) syndromes, normal glycosaminoglycan levels can still deliver affected infants.

7.6.4. *Cultured Cell Presentation*

The advantages and disadvantages of cell cultures for study of the underlying defects in diseases discussed in Section 7.5 apply

equally well to the study of genetic hyperglycosaminoglycanuria. In the latter disease, the situation is perhaps even more acute since the clinical symptoms are often disguised, other unrelated abnormalities may be present, and many diseases give rise to metachromasia in fibroblasts. Thus, in selecting cells for study of genetic hyperglycosaminoglycanuria, it must be made quite certain that any defect in the cell composition and function is the result only of such a disease. Furthermore, cell cultures do not necessarily distinguish between homozygous and heterozygous individuals.

Considerable use has been made of fibroblast cultures to study the underlying chemical and biochemical defects in genetic hyperglycosaminoglycanuria, and such work has been reviewed in detail (N5). In many instances the glycosaminoglycan(s) produced by the fibroblast is secreted into the medium. Marked increases occur in total glycosaminoglycan production by skin and ocular fibroblasts from Hurler and Scheie syndromes whereas the increases are only slight for the Maroteaux–Lamy, Morquio, and Sanfilippo syndromes (D2). In the latter disease (K43), and in the Maroteaux–Lamy syndrome (B20), increased glycosaminoglycan accumulation in cultured skin fibroblasts is also evident from radioactive incorporation studies. In the Hunter syndrome, the elevation is marked in skin fibroblasts and slight in ocular fibroblasts, but the skin fibroblast dermatan sulfate contains a few additional sulfate residues on the L-iduronic acid units (B2). However, the material formed by fibroblast cultures often differs from that which accumulates in the viscera or which is excreted in the urine. In cultured Hurler lymphoid fibroblasts dermatan sulfate levels are abnormally high (D6), whereas hyaluronic acid is absent from both the patient and normal cells. Cultured Hurler skin fibroblasts synthesize hyaluronic acid more rapidly than do normal cells (G4), and there is an increased biosynthesis of both sulfate- and non-sulfate-containing glycosaminoglycans in these cells (S7); but the Hurler and Scheie syndromes seem indistinguishable by fibroblast cultures (M29). In the Sanfilippo syndrome heparan sulfate is accumulated in the fibroblasts (K41). In Marfan's syndrome the culture fibroblasts contain large amounts of hyaluronic acid (L2, M14), and in another disease, which is possibly a case of genetic hyperglycosaminoglycanuria, chondroitin 4- and 6-sulfate are accumulated in the fibroblasts (H4, S30).

Radioactive incorporation studies of cultured fibroblasts from cases of Hurler syndrome show (G3) that whereas the incorporation rate of [^{35}S]sulfate into the sulfate-containing glycosaminoglycan fraction is abnormally high, the rate of incorporation of D-[2-^{3}H]glucose into sulfate-containing glycosaminoglycans is normal.

The size of the glycosaminoglycan stored by fibroblasts from affected individuals also differs from the heterogeneous assortment in normal cells. In Hunter and Hurler fibroblasts, the stored glycosaminoglycan is relatively large (F7) whereas in Sanfilippo fibroblasts there is a considerable predominance of very small molecular size species (K41).

It must be remembered that the glycosaminoglycans produced by fibroblasts cultured from patients may not necessarily represent precisely the situation in the patient, particularly if the cells have been recultured a number of times, but in spite of this the cultured fibroblasts have provided evidence for the degradative insufficiency in genetic hyperglycosaminoglycanuria (F7). The strength of this evidence is enhanced by the harmony of defective degradation of glycosaminoglycans, a situation unlikely to arise via mutation of the cells in culture. Much of the evidence comes from successful correction tests on the defective fibroblasts, correction of these pathological cells to apparent normality being achieved by certain factors. By cross-correction tests (see N3, N5) with fibroblasts from a range of patients a number of these factors have now been recognized. Hunter fibroblasts lack one specific factor (C5) and Hurler fibroblasts also lack one factor (B19), but this is different from the one for the Hunter cells. Fibroblasts from Sanfilippo patients can be subdivided into two groups (A and B) each lacking one specific factor (K43) and fibroblasts from patients with the Maroteaux–Lamy syndrome similarly lack one specific factor (B20), whereas Scheie fibroblasts appear to be deficient in the same factor as Hurler fibroblasts (W12). At first the characteristics of the corrective factors were unknown. The Hurler (B3, B19), Hunter (C6), Sanfilippo A (K41, K43), Sanfilippo B (F3, K43), and Maroteaux–Lamy (B20) corrective factors were purified from normal human urine and found to be macromolecular and proteinaceous. A Hunter corrective factor has also been purified (C5) from normal human skin fibroblast cultures, and a Sanfilippo B corrective factor has been purified from normal human placenta (O2). Furthermore in cases of Sanfilippo A or B syndromes, the abnormality in cultured cells can be corrected by a macromolecular factor that occurs in the secretions of fibroblasts of different genotypes (K43). Thus secretions from fibroblasts from one of the Sanfilippo syndromes contain a macromolecular factor that normalizes glycosaminoglycan metabolism in fibroblasts from the other Sanfilippo syndrome, the urine of the patients being deficient in only one of these two corrective factors. The enzymic activities of the various factors that have been identified are as follows: Hurler and Scheie, α-L-iduronidase (B3); Sanfilippo A, heparan sulfate sulfatase/sulfamidase (K41); Sanfilippo B, α-D-acetamidodeoxyglucosidase (F3, O1, O2); Hunter, L-iduronic acid 4-sulfate sul-

fatase (B2); but the Maroteaux–Lamy factor remains to be characterized. That the missing enzyme in the Sanfilippo A syndrome is actually a sulfamidase is evident from the fact that the fibroblasts are virtually inactive against [^{35}S-sulfamido]heparin (M18), whereas normal and Hurler, Hunter, and Sanfilippo B fibroblasts exhibit significant activity, releasing [^{35}S]sulfate from the substrate. In the disease in which chondroitin 4- and 6-sulfates are accumulated in the fibroblasts (see above), the abnormal metabolism has been corrected (H4) by the addition of exogenous β-D-glucuronidase (EC 3.2.1.31) to the culture medium. Corrective factors are not confined to normal human urine; "corrective factors" for the Hunter and Hurler fibroblasts have been found in animal tissues (N4). In fact, these enzymes probably have a widespread occurrence in human tissues and in mammals.

The identification of the enzymic activities of the correction factors has permitted the assessment of the fibroblasts cultured from cases of genetic hyperglycosaminoglycanuria for such enzymic activities (see Table 2). The levels of α-L-iduronidase activity in fibroblasts from Hurler and Scheie patients and a Hurler–Scheie-compound patient are not detectable (H3), but the levels in the cells from the heterozygote parents are 20–95% of the mean normal value. The heparan sulfate sulfate ester hydrolase activities of skin fibroblasts from Hurler, Hunter, Sanfilippo A and B, and Maroteaux–Lamy syndromes are normal or raised (K40). However, these fibroblasts from Sanfilippo A disease only are strikingly deficient in heparan sulfate sulfamidase activity. An interesting difference exists between the dermatan [^{35}S]sulfates of cultured fibroblasts from Hurler and Hunter syndromes—that from the former terminates at the nonreducing end in an α-L-idopyranuronosyl residue whereas that from the latter and from normal fibroblasts terminates at the same end in a 2-acetamido-2-deoxy-β-D-galactopyranosyl sulfate residue (S26). This also is consistent with the Hunter, but not the Hurler, syndrome, being due to a deficiency of a sulfatase specific for L-idopyranuronosyl sulfate residues, i.e., an L-iduronic acid 4-sulfate sulfatase and the deficiency of this enzyme in Hunter fibroblasts has been demonstrated (B2). However, it appears that there are two pools of glycosaminoglycans in fibroblasts—free and bound, and it is therefore questionable whether the degradation of the bound pool is defective in at least the Hurler and Hunter fibroblasts (D26).

Cultured skin fibroblasts from a case in which chondroitin 4- and 6-sulfates are excreted in excess (see p. 69) are severely deficient in β-D-glucuronidase activity (H4), these fibroblasts showing an excessive accumulation and lengthened turnover time of sulfate-containing glycosaminoglycan. The abnormal glycosaminoglycan metabolism can be cor-

rected by addition of β-D-glucuronidase (EC 3.2.1.31) including that of human urinary, plasma and platelet origin (S29). In Marfan's syndrome the accumulation of hyaluronic acid in cultured fibroblasts is due to a greater rate of synthesis (L2), rather than a decreased rate of breakdown.

Interests in prenatal diagnosis of genetic hyperglycosaminoglycanuria have prompted the examination of amniotic fluid cells via *in vitro* culture for enzyme deletions (H3; see also Section 7.6.5, p. 73), but data are not yet available. Peripheral leukocytes from patients with Sanfilippo A syndrome are deficient in heparan sulfate sulfamidase (K40).

7.6.5. *Diagnosis*

The earliest diagnostic tests for genetic hyperglycosaminoglycanuria were based on urinary assessment, and such tests have now been developed to be quite selective. Screening and analytical tests have been described in detail (see L11), and electrophoretic analyses (L10, L11, N2) and ion-exchange chromatographic methods (K22) are very suited to qualitative and quantitative identification of the individual glycosaminoglycans, respectively. Such methods will continue to be of use on account of the ease of collection of a specimen; in the English Midlands area, all referred cases are being screened. Many papers dealing with the measurement of urinary glycosaminoglycan from the point of view of diagnosis have been published (e.g., D34, S33). The cetyl pyridinium chloride turbidity test is claimed (V1) to give an early diagnosis of genetic hyperglycosaminoglycanuria, but this test has been proved to be quite unreliable (L11). The weight ratio of glycosaminoglycan species precipitable from urine by cetyl pyridinium chloride to glycosaminoglycan species subsequently recoverable by ion-exchange chromatography is greater than unity for cases of genetic hyperglycosaminoglycanuria (D28, D29, D33) whereas for normals it is less than unity, and this has been proposed as a means of diagnosis, particularly where 24-hour specimens are not available. Sample mass screening using the toluidine blue-filter paper test has been carried out for infants at 20 days with repeated application of the test to those showing initial positive reactions (S1). It is clear, however, that at such an early age along with the method used many difficulties arise with respect to diagnosis; undoubtedly this is due, in part at least, to immaturity of the enzyme levels in the subject.

Serum enzyme levels may provide a route for diagnosis, and some data (F4) for cases of Sanfilippo B syndrome show promise. Since the α-D-acetamidodeoxyglucosidase activity of serum from these cases is low (F4), it is possible to effect a diagnosis via measurements of the enzyme

level and to distinguish thereby between homozygous, heterozygous, and normal individuals. Measurements of glycosaminoglycan levels in amniotic fluid (e.g., D34) hold some promise for prenatal diagnosis, although normal levels can still deliver affected infants (see below).

The use of fibroblast cultures is more suited to the identification of an enzymic defect on account of the culture time and other disadvantages (see Section 7.5) associated with them, but identification of the enzymic defect in this way can be diagnostic. Furthermore the heterozygote parents of patients with Hurler and Scheie syndromes show in their fibroblast cultures α-L-iduronidase activities which range from 20–95% normal (H3), although the overlap between normal and heterozygote levels can be reduced by expressing the α-L-iduronidase activity relative to the β-D-galactosidase activity. Skin fibroblasts from patients with I-cell disease are also deficient in lysosomal α-L-iduronidase (EC 3.2.1.76) and β-D-glucuronidase (EC 3.2.1.31) activities (H22). Analysis of fibroblast glycosaminoglycan could be used diagnostically, but increased glycosaminoglycan levels do occur in cultured fibroblasts from cases of a number of disease types outside genetic hyperglycosaminoglycanuria (M17), and there are difficulties in distinguishing between heterozygous and homozygous individuals. However, analysis of fibroblast glycosaminoglycan is useful for distinction between the Hurler and Hunter syndromes, which excrete excesses of the same two glycosaminoglycans, since the terminal nonreducing residues of the accumulated dermatan sulfates are α-L-idopyranuronosyl and 2-acetamido-2-deoxy-β-D-galactopyranosyl sulfate units, respectively.

At first it also seemed that assays of amniotic fluid were likely to provide a good basis for antenatal diagnosis, the glycosaminoglycans being isolated, for example, with Alcian Blue, but it soon became clear that on account of variation of the normal levels with gestation age a diagnosis on glycosaminoglycan levels alone is unsound unless the levels are excessively high (C29, W9). Furthermore, normal glycosaminoglycan levels in amniotic fluid can still deliver affected infants, as has been found for Sanfilippo, Hunter, and Hurler syndromes (F2, M20). This situation has prompted some discussion of the hazards of antenatal diagnosis of the Hurler syndrome by such techniques (B43). In view of this, attention has been turned to cultured amniotic fluid cells; but cultured amniotic fluid cells from normal pregnancies have less than half as much α-L-iduronidase activity as fibroblasts from normal adults (H3), and this might cause problems in distinguishing between heterozygous and affected fetuses by enzymic assay alone. Thus the outlook for diagnosis of genetic hyperglycosaminoglycanuria via cultured amniotic cells on the basis of α-L-iduronidase activity alone is doubtful. Thus,

at this stage it seems that a positive antenatal diagnosis via amniotic fluid is possible only where there is a great excess of glycosaminoglycan, but that such a diagnosis should be backed up by qualified evidence from cultured fibroblast cells. Therefore, at the moment it would seem (e.g., F4, O1) that enzyme deficiency tests may provide the most useful precise tool for diagnosis including prediction of the likelihood of producing an affected infant. Such tests may also permit determination of heterozygosity and monitoring of therapy.

7.6.6. *Therapy*

The literature to a recent date on the attempted treatment of genetic hyperglycosaminoglycanuria has been tabulated (R2). The therapy of genetic hyperglycosaminoglycanuria has been attempted with fresh plasma infusions, but generally this has been without clinical success. Plasma infusion and whole blood transfusion of cases of Hurler, Hunter, and Sanfilippo syndromes did not alter the glycosaminoglycans excreted or the molecular weight distribution thereof (D15), and similar infusion of a case of the Hurler syndrome gave no change in the qualitative distribution of the excreted glycosaminoglycans (D5), the total amount of glycosaminoglycan excreted, or the ratio of high to low molecular weight glycosaminoglycan species, and no clinical improvement (D4). However, others (D29), who devised the molecular weight assessment test used in the latter reference, have reported that infusion of Hurler and Hunter cases with normal human plasma causes a decrease in urinary excretion of glycosaminoglycans of relatively large molecular weight and by an increased excretion of their products of degradation. Among the latter, products of degradation of dermatan sulfate and heparan sulfate could be demonstrated. Infusion of the patient with a fraction of normal human serum caused disappearance of a disulfated disaccharide containing L-iduronic acid, which had been found to occur specifically in the urine of a case of the Hunter syndrome (C27). Human plasma infusion in cases of Sanfilippo syndrome (D13) (A or B type undefined by the authors) gave an immediate increase in hexuronic acid output, and most of this increase was attributable to polymeric glycosaminoglycan material. A number of other changes occurred, e.g., the molecular weight spectrum of the nonprecipitable glycosaminoglycan and the molar ratio of sulfate to hexuronic acid contents of the glycosaminoglycans, but levels and values returned gradually after treatment to preinfusion levels. The predominance of heparan sulfate in the urine was unchanged by the treatment. A clinical response was obtained when a case which excreted excessive amounts of chondroitin 4- and 6-sulfates (see p. 66), and whose fibroblasts were deficient in β-D-glucuronidase (EC 3.2.1.31) was infused with fresh human plasma (S29).

The overall results of such therapies are disappointing, particularly as, for example, the abnormally high rate of incorporation of [^{35}S]sulfate into sulfate-containing glycosaminoglycans in cultured fibroblasts from cases of Hurler syndrome can be normalized (G3) by a "corrective factor" preparation, from normal human urine, which contains α-L-iduronidase (EC 3.2.1.76). But further work on examination of therapy, particularly with the urinary "correction factors," may hold potential for success.

Administration of vitamin A has been proposed for the treatment of cases of the Hunter and Hurler syndromes, but this therapy seems to be detrimental (M1), the urinary glycosaminoglycan excretion rate being increased in a case of the Hurler syndrome (D3). L-Ascorbic acid (vitamin C) increases the level of dermatan sulfate in cultured Hurler fibroblasts (S6, S7), but not in the normal cells. Furthermore, L-ascorbic acid causes selective retention of sulfate-containing glycosaminoglycans within the abnormal cell (S7). However, it is possible that dietary therapy could be of some assistance.

In conclusion to Section 7.6, the complex situation arises from the fact that the types of genetic hyperglycosaminoglycanuria are inborn lysosomal diseases in which the material that accumulates is determined by the specificity of an enzyme(s) present in normals, but missing in the patient. As already indicated, probably more than one enzyme defect or deficiency is involved in some cases. Clearly the underlying genetically defined chemical processes have yet to be defined, and it remains to be seen, for example, if the enzyme deficiencies arise from alterations to the primary structure of the enzymes and if inactive enzyme is in fact produced in the tissues.

In conclusion to Section 7, although the reports relevant to this Section are numerous, they are very limited since most of the diseases in which glycosaminoglycan disorders have been implicated have not been investigated for the phenomenon or component which actually gives rise in the first instance to the disorder, for example, in hereditary diseases the chemistry of the gene. Furthermore the studies to date have been almost exclusively limited to glycosaminoglycans, and further work should be directed more at the whole proteoglycan molecule. As yet the investigations of the majority of the diseases cited have not been studied via cell cultures, etc., and the primary defect is unknown. In terms of screening for proteoglycan involvement, urinary assessment still holds a prime place on the basis of convenience, but results must be faced with an open mind so that when a positive is realized, genetic hyperglycosaminoglycanuria is not assumed. Much more systematic investigation will be necessary to classify the proteoglycan defects on the basis of chemical structure of the abnormal product and the specificity and other characteristics of the enzymes responsible. A further ap-

proach to an understanding of the involvement of proteoglycan in disease may come from simulation of the abnormal processes involved. Achievement of such aims should provide a more logical approach to successful therapy, the primary reason for investigation of the disease, and undoubtedly the field holds great potential for the future.

8. Conclusions

It is hoped that readers by this stage will appreciate on the one hand the extensive involvement of proteoglycans in the processes of health and disease and on the other the fact that chemical and biochemical understanding of such processes is still quite limited. There is therefore considerable scope for pioneer research work in this field, not merely from the academic viewpoint, but more importantly with direct respect to human society.

As the sensitive methods of detection of glycosaminoglycans are extended and applied, as methods are developed for investigating on a microscale proteoglycans, and as the systematic analysis of tissues, etc., is afforded the required, correctly directed emphasis and attention with improved techniques, it may be predicted that such goals may be realized. However, in order to gain the absolutely necessary understanding of the associated reactions of biosynthesis, interaction and function, and degradation, research attention must be extended to the cellular and enzymic processes on which many of these phenomena are dependent.

To those who in the earlier years discovered *a priori* the proteoglycans and/or their components and indicated their importance, we say, "Thank you for starting us off on the pilgrimage"; to those who more recently, enabled by the foundation of the earlier work, have through continuous toil opened up the field and demonstrated some of its important depths, we say, "Thank you for showing us the possibilities and the potential with respect to the alleviation of suffering"; and to those just beginning, we say, "Thank you for joining us; we wish you every success."

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THE LABORATORY DIAGNOSIS OF THYROID DISORDERS

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1. Introduction

Over the last decade or so and since the time of the last general review (C6) on thyroid function tests in *Advances in Clinical Chemistry,* there has been a series of important developments in the area of thyroid physiology with concomitant changes in the whole approach to the clinical chemistry type of tests of thyroid function.

Whereas some of the recent advances in thyroid function tests have been necessitated by the new concepts of thyroid hormone physiology, others have been secondary to major technological advances in clinical chemistry, in particular the introduction of radioimmunoassay (RIA).

This review article will attempt to give an account of some of the more important advances on the physiological front as a background to providing a reasonably comprehensive cover of thyroid function tests with particular reference to those recently devised. It should be stated that the newer tests are not necessarily more complicated than earlier ones, but that there is developing a range of tests that vary in their degree of sophistication. Some of the simple screening tests, which admittedly produce limited information, are just as worthy of consideration in a review article as are the more complex tests involving RIA because of the corresponding range of sophistication in laboratories. Some laboratories may do well to carry out only a simple screening procedure, providing it is an effective one, and refer material, when indicated, to reference laboratories which should have available a comprehensive cover of all the relevant thyroid tests.

2. The Thyroid Gland and Its Hormones: Chemistry and Physiology

The thyroid gland consists of numerous small follicles which are really spherical clusters of cells surrounding a central lumen containing protein or colloid, the main constituent of which is the glycoprotein thyroglobulin. Modern techniques, such as electron microscopy and radioautography, have shown that thyroglobulin is synthesized intracellularly and is rapidly transported through the cell, first to the Golgi zone, where the carbohydrate moiety is added, and then to the apical cell surface. The glycoprotein is then secreted into the colloid, where iodination takes place (D7).

2.1. Biosynthesis of Thyroid Hormones

It is well established that four iodinated amino acids are synthesized within the thyroid gland, namely *l*-triiodothyronine (T_3), *l*-thyroxine

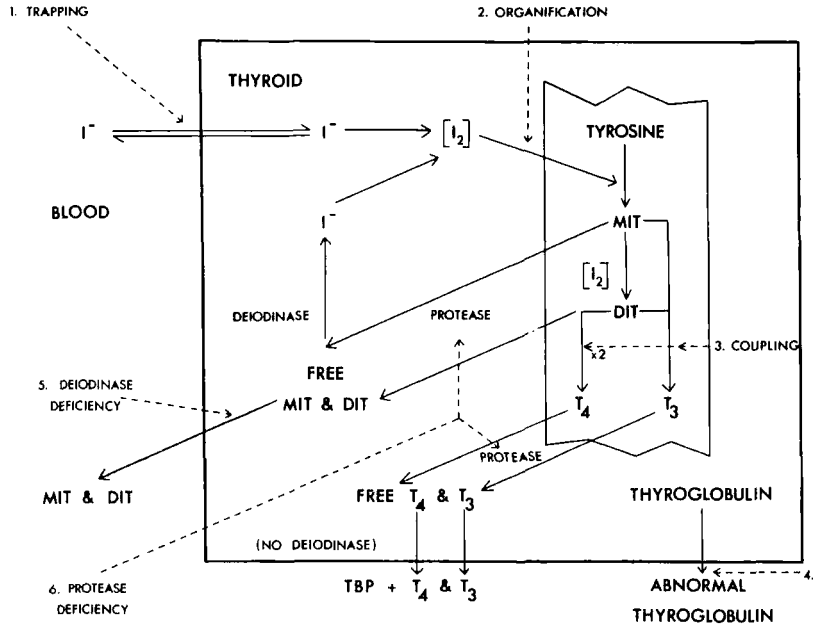

FIG. 1. Schematic representation of the pathways of the biosynthesis of the thyroid hormones. The sites of biosynthetic defects are also indicated. [I_2] = "active iodide," MIT = monoiodotyrosine, DIT = diiodotyrosine, T_3 = triiodothyronine, T_4 = thyroxine, TBP = thyroid hormone-binding proteins.

(T_4), monoiodotyrosine (MIT), and diiodotyrosine (DIT). The orthodox view states that the iodothyronines, namely T_3 and T_4, account for all the organic iodine in normal human serum (P9, W11). However, there are claims that the iodotyrosines, MIT and DIT, also occur in significant amounts in the serum (D5), but if this is true, then they are strongly associated with, or bound to, serum albumin and are not subject to the rapid clearance known for free serum iodotyrosines (W4).

The chemical steps in the biosynthesis of thyroid hormones are also well established and are shown in a simplified form in Fig. 1. There are three main phases in biosynthesis, namely: trapping of iodide and its conversion into an activated form, iodination of the tyrosines (organification), and finally coupling of the iodotyrosines to form iodothyronines. Only a brief outline of the biosynthesis will be given here, as the subject has been well reviewed by others (e.g., D2, P13, S6).

The trapping and transport of iodide occurs at the basal cell membrane. It is an active ATP-dependent process and is probably facilitated by the iodine-binding property of phospholipid. The later stages of biosyn-

thesis take place as an integral part of the thyroglobulin molecule. The iodide is oxidized by thyroid iodide peroxidase to a higher valency form similar to that of iodine, and thereby is activated. "Active" iodine iodinates tyrosine to form both MIT and DIT. Coupling of iodotyrosines to form iodothyronines occurs by another oxidative step dependent upon the availability of a peroxidase and H_2O_2 (D2). Two molecules of DIT couple to form T_4 and one molecule of DIT couples with one of MIT to form T_3.

The iodinated amino acids within the thyroglobulin molecule are stored in the colloid until required, then the colloid is subject to pinocytosis and small fragments are absorbed into the cell and proteolysis of most of the thyroglobulin proceeds. This results in the intracellular release of MIT, DIT, T_3, and T_4. The MIT and DIT are normally broken down by powerful deiodinases, and most of the iodine liberated is reutilized for iodination of further tyrosine molecules, although it is likely that this iodine enters a pool that is distinct from the pool of trapped iodide which has a much more rapid turnover (H8).

There are no thyroid deiodinases for T_3 or T_4, nor are these compounds otherwise metabolized in the thyroid gland. The T_3 and T_4 therefore diffuse rapidly into the blood stream, and this is facilitated by the specific serum binding proteins. The iodide which is not reutilized within the thyroid gland also passes into the blood, and so does a small amount of intact thyroglobulin that arrives via the thyroid lymph channels. In certain diseases, such as congenital goiter, Hashimoto's thyroiditis, and thyrotoxicosis, a much larger proportion of the iodide from intraglandular deiodination is released into the blood. In states of thyroid stimulation, such as thyrotoxicosis (W7) or following thyrotropin (TSH) stimulation (W6), significant amounts of the iodotyrosines are released into the blood, presumably because the thyroidal iodotyrosine deiodinase cannot cope with the extra load of iodotyrosine released during the accelerated proteolysis of thyroglobulin.

2.2. Hormone Transport in Blood

The thyroid hormones in the blood are transported bound to various plasma proteins. These proteins, called collectively T_4-binding proteins (TBP) in this review, include T_4-binding globulin (TBG) discovered in 1952 (G10) and T_4-binding prealbumin (TBPA) discovered by Ingbar (I2) in 1958. It is well known that albumin will also bind both T_3 and T_4, and recently Hoch and Lewallen (H19) studied T_4-binding in specially devised zone electrophoresis on agarose gel and demonstrated low affinity binding by α_1 and β lipoproteins.

There has been a controversy for some time about the precise role of thyroid hormone binding in the serum, and some of the difficulties in interpreting the role have been reviewed by Osorio (O11), Oppenheimer (O5), and Leeper (L9). It is beyond the scope of the present review to further critically evaluate this subject, but a certain amount of material is necessary here, as many thyroid function tests and the whole concept and physiological importance of "free T_4" is interrelated with the carriage of thyroid hormones in the serum. (Reference to the various approaches to free hormone measurements, particularly for free T_4, and also to the methods of quantitatively assessing TBP will be made in Section 3.)

The relationship between serum T_4 and the binding proteins can be shown in the following equation:

$$FT_4 + TBP \rightleftharpoons TBP \cdot T_4$$

where FT_4 is the concentration of free or unbound T_4 in the serum. There is always a certain amount of the T_4 in the FT_4 form exchanging with the protein-bound T_4 ($TBP \cdot T_4$), and some of the free protein-binding sites have no T_4 bound to them (TBP). The equilibrium of the equation is such that there is only a small amount of the total T_4 present as FT_4, namely about 0.04–0.05%. The equation is governed by the law of mass action, so that:

$$(FT_4)(TBP)/TBP \cdot T_4 = K$$

Robbins and Rall (R5) were the first to suggest that although only a very small fraction of the T_4 in the blood is present as FT_4, this nevertheless is the active moiety that is entering cells and stimulating metabolism.

In discussing the role and the nature of TBG, TBPA, and albumin, it is important to first define for each some parameters that can be measured and compared (O11). The "binding capacity" of a protein (for T_4) is expressed as the maximum amount of T_4 that can be bound to the protein contained in 100 ml of serum. The "binding affinity," which cannot be measured in absolute terms, is expressed as the proportion of T_4 bound by each of two proteins with the same binding capacity when the three substances interact. Binding affinity correlates fairly well in an inverse fashion with the value K in the mass action equation. "Binding power" depends upon the binding affinity and the binding capacity for a particular protein in a mixture of proteins and T_4. Binding power is thus equivalent to the mathematical product of binding capacity and binding affinity.

Unfortunately, when studying T_4-binding in *in vitro* systems, binding affinity is highly dependent on pH, ionic composition, and temperature of the buffer medium, although binding capacity is less dependent on these factors (O11). Therefore care has to be taken when extrapolating data from an *in vitro* system to the physiological situation. However, it is quite clear from various studies that TBG has a higher affinity for T_4 than does TBPA, and that TBPA has a higher affinity than does albumin. Albumin has the highest T_4-binding capacity, and TBG the least. There is little doubt that TBG is the main carrier of T_4. It has a high affinity and a capacity that would allow it to carry up to three times the normal concentration of serum T_4. Whereas Osorio (O11) reported work which suggests that TBPA may not bind T_4 under physiological conditions, others (e.g., H6, O5, W18) do ascribe it such a role, and it has been estimated that 15% of the serum T_4 is bound by TBPA (W18.)

The more rapidly acting hormone, T_3, is bound much less firmly than is T_4 and approximately 0.4% of T_3 is present in the free form. It is bound primarily to TBG and secondarily to albumin. Earlier workers considered that T_3 was not bound to TBPA although a recent study demonstrated that TBPA has the same affinity for T_3 as does albumin (L3).

The exact role of thyroid hormone binding is still not completely understood. The most plausible explanation is that the TBP act as a reservoir of T_4 and at the same time buffering the peripheral tissues against the total metabolic effect of the hormones in the blood. Thus they will have a regulatory function, which will deliver more T_4 and T_3 to the tissues on demand. The TBP will also help prevent the loss of T_4 and T_3 through hepatic and renal excretory function. As TBPA has a much lower binding affinity for T_4 than does TBG, and as it has a shorter half-life in the serum (O8), it has been suggested that TBPA provides a labile, rapidly available source of T_4 and T_3 in stressful situations, whereas TBG is a more stable, relatively inert source of hormone. The data concerning T_3 binding are consistent with this theory because T_3 is the more rapidly acting hormone and has a more rapid turnover.

The above theory on the function of TBP may be an oversimplification of the situation as the concept takes no account of active tissue binding for T_4 and T_3 (R5, R6). In particular it is well documented that the liver contains about 30% of the total body T_4, which is in rapid exchange with the T_4 in blood (O6). It is possible that the real role of TBP in addition to regulating the amount of FT_4 in the serum, is not only

to actively transport the hormones to the periphery, but also to actively enhance uptake of hormones by the tissues. If this is the case, it would throw some doubt on the concept that free T_4 and free T_3 are the active moieties. This concept has been questioned by Osorio (O11). He points out that the main basis for the theory, is that free T_4 is invariably proportional to the thyroid status of individuals, but that it is equally possible to prove mathematically that the thyroid status correlates with the protein bound T_4. More recently Harland and Orr (H10, H11) have made observations that strongly suggest that T_4 is not freely exchangeable throughout its distribution space, a finding that is incompatible with the FT_4 concept. They propose an alternative theory in which T_4 is considered to enter a pool called "available T_4" from which it is bound, partly to protein in the plasma and partly to binding sites in the tissues, and furthermore, the plasma bound-T_4 is said to be in equilibrium with liver T_4.

The possibility that FT_4 may not be the moiety determining the rate of delivery of T_4 to the tissues does not detract from the usefulness of FT_4 measurements in thyroid function studies. Sterling and Hegedus (S24) were the first to make a direct estimate of the free T_4 concentration in the serum, basing it on the indirect method of Christensen (C13), and they showed that FT_4 correlated very well with thyroid status. Since that time there has been a series of papers employing a variety of techniques for FT_4, both direct and indirect, which confirm these findings including situations where there are abnormalities in TBP. These papers will be reviewed in Section 3.4.

There is little need to review the various binding abnormalities here, as adequate discussion appears in other reviews (C15, F5, F6, H6, L9, O5, S18). Both TBG and TBPA are subject to either increases or decreases in capacity and these abnormalities may be either primary genetic defects or secondary to many diseases or drugs.

Disturbances in thyroid function are associated with abnormal TBP capacities and a brief review of these is relevant, as the findings have a bearing on the free T_4 concept. Untreated thyrotoxicosis is associated with decreases in both TBPA (B11, C2, I1, I4, V3) and TBG (B11, C2, I1) and myxedema with decreases in TBPA (V3) and increases in TBG (I1). The increase in TBG in myxedema would outweigh the decreases in TBPA, and therefore the overall effect is a decrease in serum T_4-binding in thyrotoxicosis and an increase in myxedema. These findings favor the theory that the important serum T_4 fraction is that attached to TBP rather than the free fraction, as the increased binding capacity in myxedema would help to offset the effects of declining T_4

secretion from the gland by enhancing the delivery of the limited amounts of T_4 to the tissue. This theory also works in converse for thyrotoxicosis.

2.3. Relative Contributions of Triiodothyronine and Thyroxine to Metabolism

It is not the purpose of this section to review the intricacies of the metabolic effects of the thyroid hormones, but rather to compare the relative contributions made by T_3 and T_4, respectively, to overall metabolism. It is only in recent years that the degree of the contribution made by T_3 has been realized, and this has far-reaching consequences not only in regard to the physiology of the thyroid secretion, but also in respect to the recent development and application of thyroid function tests. In addition there are significant pathophysiologic and therapeutic implications.

Whereas T_4 is present in the serum of normal man at a concentration of about 8 μg/100 ml, the concentration of T_3 is only in the order of 140 ng/100 ml. In other words, the concentration of T_3 is only about 1/50 that of T_4 and yet T_3 is said to account for about 65–75% of the metabolic effect of thyroid hormones in man (S19). There are various factors that account for the disproportionately high metabolic effect. First, the distribution volume of T_3 is 43 liters compared with 10 liters for T_4 for a 70-kg man (L5), which means that some of the discrepancy between T_3 and T_4 is made up. Another relevant factor is the more rapid turnover of T_3, 75% per day compared with 10% per day for T_4 (O1), which is due to low affinity binding of T_3 by TBG and TBPA. The total extrathyroidal T_3 has been calculated to be about 75 μg and the extrathyroidal T_4 about 650 μg (H20). The daily production rate of T_3 and T_4 can be calculated from the extrathyroidal hormone and the turnover rate. It amounts to about 40–60 μg of T_3 and 80–100 μg of T_4 (G1, H20, L5). Finally, as T_3 is at least three times as potent as T_4 on a weight basis, it follows that about 65–75% of the total metabolic effect of thyroid hormones in man is due to T_3 (E3, H20).

The importance of T_3 in metabolism is therefore out of proportion to its molar concentration in the serum. The awareness of this fact, together with the recent availability of assay for serum T_3 concentration has resulted in a great deal of attention being recently focused on T_3. The importance of T_3 has been further enhanced by two recent developments, namely, that T_4 has been shown to be deiodinated to T_3 in the blood, and also that states of T_3 hypersecretion are being described.

The peripheral monodeiodination of T_4 to T_3 was first investigated in 1955 by Pitt-Rivers *et al.* (P14) with equivocal results, but the first convincing evidence in man was in 1970 by Braverman *et al.* (B13) and Sterling *et al.* (S22). Other studies have confirmed the T_3 production from T_4 in man (P11) and in animals (S4).

The question arises as to how much of the circulating T_3 is secreted from the gland and how much is derived from peripheral deiodination of T_4. There is no doubt that significant amounts are secreted from the gland in animals, as Taurog *et al.* (T2) demonstrated that the T_3 concentration is much higher in thyroid venous blood than on the arterial side. A more recent study in man (S10) indicated that 28 μg of T_3 were produced per day: 16 μg from thyroid secretion and 12 μg from peripheral deiodination.

Another interesting question which arises is whether the function of T_4 is merely to provide T_3 or whether it has metabolic activity of its own in the peripheral cells. It was suggested from studies with the rat, that virtually all of the T_4 secreted was converted into T_3 (S4). A further study in rat (O7) showed that propylthiouracil (PTU) inhibits the conversion of T_4 to T_3 and is also known to abolish the metabolic effect of injected T_4 but not of injected T_3. This suggests that T_4 needs to be converted to T_3 before it can exert metabolic activity. Such studies have lead to a view that T_4 is only a prohormone, but there are difficulties in extrapolating the data from animal experiments to the human situation. Chopra *et al.* (B19, C11) presented convincing evidence from thyroid function studies in man that T_4 does have intrinsic hormonal activity. An example of their evidence is the frequent finding of normal serum T_3 concentration (both free and total) in patients with untreated primary myxedema where the T_4 levels are invariably markedly subnormal. Data from this group show that of the 110 μg of T_4 metabolized daily, about 26 μg is used to generate 22 μg of T_3, another 22 μg of T_4 is lost in urinary and fecal excretion and the remaining 62 μg is available for hormonal action. Singer and Nicoloff (S10) estimated that 58% of the circulating T_3 comes from thyroid secretion and 42% from peripheral sources and that about 15% of the T_4 secretion is metabolized via intraconversion to T_3.

Studies of serum T_3 levels have stimulated great interest in another clinical setting, namely the T_3-hypersecreting states. The best known of these is T_3-thyrotoxicosis first suspected from the work of Maclagan in 1957 (M1) and documented in 1970 by Sterling *et al.* (S26) and confirmed by others (H21, W1). A state of T_3-hypersecretion relative to T_4 secretion has also been described as a compensatory phenomenon in clinical states characterized by falling serum total T_4 levels. Examples

of this phenomenon are following treatment for thyrotoxicosis with radioactive iodine or surgery (B5, P3, S23), in iodopenic endemic goiter (K8, P6) and dyshormogenetic goiter (F11, G8). Further comments about T_3-hypersecretion as a compensatory phenomenon will be made in Section 6.7.

2.4. Hypothalamic and Pituitary Control of Thyroid Secretion

All phases of the biosynthesis of thyroglobulin and the release of thyroid hormones from the gland are stimulated by thyrotropin (TSH), a glycoprotein synthesized by the thyrotrophes of the anterior portion of the pituitary gland. In common with most other target endocrine glands, the thyroid, through its own secretion, can exercise control over the level of pituitary TSH activity. It has been known for many years that the higher centers, notably in the hypothalamus, also controls the pituitary TSH secretion, but it was only in recent years, since the identification of thyrotropin-releasing hormone (TRH) in 1961, that rapid progress was made in this area. The earlier work has been reviewed by Guillemin (G14) and Leeper (L9).

A simplified version of the feedback control of thyroid secretion is shown (Fig. 2). The main focus of the system is in the secretion of TSH from the thyrotrophes. It is thought that thyrotrophe cell activity is regulated by thyroid hormones through a T_4- or T_3-dependent inhibitory substance produced in the pituitary gland, the concentration of which is dependent upon protein synthesis (R3). The thyroid hormones therefore exert a negative effect at the thyrotrophe level, but the precise form in which the hormone acts there is not known with certainty, that is, whether free or bound hormone or whether T_3 or T_4 or both are active at the pituitary level. However, nuclear binding sites specifically for T_3 have been found in the anterior pituitary of rats (S1). Observations on ^{131}I-treated thyrotoxic patients made in our laboratory suggest that T_3 is relatively unimportant in the negative feedback at the pituitary level (W8).

The inhibitory action of thyroid hormone in the pituitary is opposed by stimulatory effects of TRH. The TRH effect is to lower the sensitivity of the anterior pituitary to the negative inhibitory effect of thyroid hormones.

The presence of TRH in the hypothalamus had been suspected for at least a decade prior to its chemical identification as pyro-glutamyl-histidyl-proline amide in 1969 (N1). It is known to be secreted over a wide area of the hypothalamus, particularly in the region of the supra-optic nucleus, although the exact cellular site of its synthesis is not known (S2). The TRH was first demonstrated in hypophysial portal

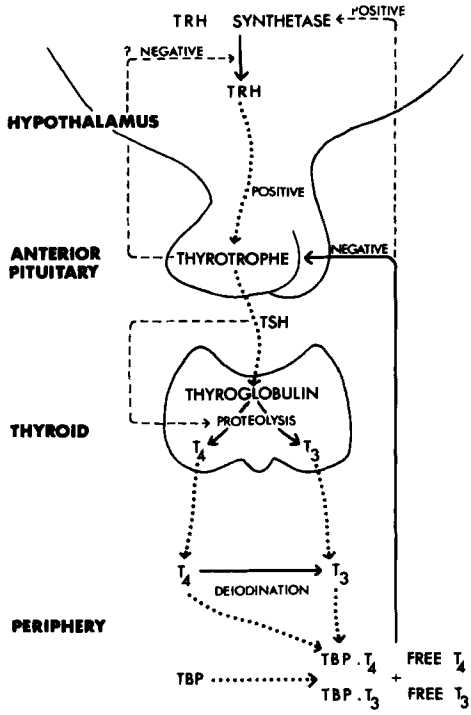

FIG. 2. Schematic representation of the interrelationship between the secretions of the hypothalamus, anterior pituitary, and thyroid glands. TRH = thyrotropin-releasing hormone, TSH = thyroid-stimulating hormone (thyrotropin), T_4 = *l*-thyroxine, T_3 = *l*-triiodothyronine, TBP = thyroid hormone-binding proteins.

blood by Averill *et al.* in 1966 (A13). It is likely that thyroid hormones exert a positive feedback at the hypothalamic level as the synthesis of TRH synthetase has been shown to be stimulated by thyroid hormone (R3). This finding is in accord with the demonstration in man that large doses of T_3 do not completely suppress the thyroidal iodine release unless pharmacological doses of corticosteroids known to suppress hypothalamic function are simultaneously administered (S10).

After traversing the portal venous system through the pituitary stalk, TRH is bound specifically to a receptor site on the cell membrane of the thyrotrophe. Presumably TRH activates adenyl cyclase, resulting in a release of TSH (B19).

3. Specific Chemical Tests on Serum

Prior to 1940 tests of thyroid function were dependent on nonspecific procedures that were based on the effects of the thyroid secretion on

the peripheral tissues, e.g., the basal metabolic rate and serum cholesterol concentration. The first specific measurement of circulating levels of hormone that was suitable for clinical use, namely the protein-bound iodine (PBI), was developed in 1940 by Chaney (C5). However, problems due to iodine contamination, both endogenous and exogenous, led to procedures that employed radioactive counting for the final measurement; for example, total T_4 assay and T_3 resin uptake. This type of measurement did not displace the PBI procedures but merely supplemented them. In recent years the limitations of the existing techniques have become increasingly obvious, particularly in special situations and borderline cases. The advent of radioimmunoassay (RIA) led to a new dimension in thyroid function testing with the development of sensitive techniques for serum TSH, T_4, and T_3 concentration, and today the PBI technique is being rapidly phased out.

3.1. Protein-Bound Iodine and Butanol-Extractable Iodine

The reasons for briefly discussing PBI techniques in this review are minimal except for the fact that many automated units are still operating and because a PBI determination is sometimes useful in conjunction with other tests in delineating the site of a biosynthetic block in thyroid hormone synthesis. The subject was last reviewed in the series *Advances in Clinical Chemistry* by Chaney (C6) in 1958, and probably the most recent review is by Acland in 1971 (A4).

Since all PBI procedures involve determining organically bound iodine, some type of digestion is necessary to convert this to the more easily measured inorganic iodine. The conversion may be either by wet acid digestion or by the more popular dry alkaline ash incineration. These steps naturally follow some form of protein precipitation, usually the Somogyi method using zinc sulfate and sodium hydroxide (B2), which was shown to offer some advantage over the precipitation with trichloroacetic acid (O4). This is such a mild form of protein denaturation that the thyroid hormone remains attached to the precipitated protein. The final phase of any PBI procedure is the quantitation of the liberated inorganic iodine, and this is invariably accomplished with the Sandell and Kolthoff's catalytic procedure. In this, iodine catalyzes the reduction of acid cerate solution by trivalent arsenic and the rate of decoloring of the yellow solution is proportional to the amount of iodine in the solution.

Improvements in the early procedures developed along two main lines, first the colorimetric procedure and, second, the introduction of automa-

tion. The colorimetric iodometric step has been modified by various workers with the addition of substances such as redox dyes, phenanthroline, brucine, and ferri-ferrocyanide, and, last, bromine-bromide. These modifications have resulted in an increase in the sensitivity and, to some extent, in the specificity of the procedure. The introduction of automation to PBI methodology potentially enhances reproducibility, precision, and productivity. Bernotti (B6) introduced automation of the colorimetric step in 1962 and the alkaline incineration technique was fully automated by 1963, with the use of Technicon continuous-flow technology (S28), but this early technique involved the addition of a known excess of iodine to each sample and was cumbersome and insensitive. Later methods obviated this step and also introduced anion exchange resin to remove inorganic iodine from the serum so that precipitation and washing of the serum protein was unnecessary (W15). A semimicro modification of this procedure using 0.5 ml of serum is available (T4). These automated PBI procedures are acceptable in respect to a precision, the coefficient of variation being in the order of 3–5% (V2).

One of the biggest problems with PBI procedures whether automated or otherwise, has been iodine contamination, both in the patient and the operator, as well as in the environment in which the test is carried out. There are several reviews (A4, C1, H6) that adequately list the interferences (including iodine in various forms) with PBI procedure. One of the most comprehensive reviews of the artifactual and physiological factors affecting serum PBI in recent years is the publication by Acland (A4). In a recent study on PBI (P2), iodine contamination was found in 17.5% of euthyroid patients, many of whom had clinically feasible PBI levels. As alternative techniques are now available, it is clear that PBI is an unsatisfactory screening procedure for determining thyroid function.

The problem with iodine contamination had been recognized many years ago and led to the development in 1951 by Man *et al.* (M5) of the serum butanol-extractable iodine (BEI) assay. The principle of this assay is that the serum proteins are precipitated in acidified butan-1-ol and the iodinated compounds are extracted into the alcoholic phase. A very concentrated alkali wash is then used, which removes inorganic iodide and iodotyrosine, if present. The rest of the assay is similar to the PBI technique. Although it was found that the BEI correlates better with clinical status than did the PBI, it was also acknowledged that it did not remove interferences due to organic iodides, such as iodine-containing contrast media (M4), although another study found that the BEI was less susceptible than was PBI to interferences from

iodinated organic compounds (L10). Clearly other techniques for estimating serum T_4 levels were required to overcome the problem of iodine contamination.

3.2. Resin Uptake of Triiodothyronine and Related Procedures

The resin uptake of labeled T_3 (T_3U), an indirect parameter for assessing the levels of thyroid hormone in plasma, is based on an earlier technique, namely, the red blood cell uptake of added ^{131}I-labeled T_3 from plasma, Hamolsky *et al.* (H9) developing the latter technique in 1957. The principle of the test is that T_3 is partitioned between the red blood cells and the plasma, depending on the occupying of specific binding sites on the plasma proteins with endogenous thyroid hormone. In other words, the uptake of T_3 by the cells is inversely proportional to free TBP capacity. It was soon realized that the red blood cell is a variable in the system, which affected the final result (B3) and the cell was replaced with a fixed amount of ion-exchange resin (M13).

Several workers quickly confirmed the usefulness of the resin uptake of T_3 in helping to distinguish patients with disturbed thyroid function from euthyroid subjects (C14, S27, T3, W19). However, this test gives abnormal values in euthyroid subjects with certain extrathyroidal abnormalities, namely increases or decreases in the T_4-binding proteins, particularly in TBG or TBPA and even in hypoalbuminemic states. Certain drugs which increase TBG or which displace T_4 from its binding sites also produce abnormal T_3 uptake values. It is not intended to list all the extrathyroidal factors that affect this test as the subject has been reviewed many times (e.g., C15). The most important of the extrathyroidal factors causing decreases in T_3 resin uptake is seen in pregnancy or in individuals on oral contraceptives.

The test has been modified mainly by varying the nature of the substance used to trap the labeled T_3 not binding to the unsaturated binding sites, e.g., charcoal (B9, B12, G11, H12), resin sponges (M13), and resin-impregnated fiber glass strips (B16). Another technique measures the amount of T_3 bound to the serum, the unbound T_3 being removed with ion exchange resins (S27) or Sephadex columns (C23). Many of the various T_3 uptake methods have been adapted by commercial enterprises into "kits," and the performance of many of these have been reviewed (P1, U1). One source of possible inaccuracy in many of the kit methods is that it is often assumed that there is the same amount of labeled T_3 in each vial and therefore one of the older T_3 resin uptake procedures, in which an initial count of the T_3 added to the vial is made, is likely to be more precise.

Because of the proliferation of commercial "kit" methods there has been little development toward automation in T_3 uptake tests. One automated procedure relies on the assessment of T_3 binding by the serum, the unbound T_3 being removed by continuous-flow dialysis (P15). Although initial assessments were satisfactory, the results comparing favorably with a manual T_3 resin uptake and a PBI procedure, a later report (M7) indicated that the automated procedure was subject to variable error caused by a large and variable proportion of radioiodine in the dialyzate.

The T_3 resin uptake and related procedures are theoretically unaffected by iodine contamination of the specimen or the glassware, as the final measurement is a radioactivity count. This is confirmed in practice with one minor exception, in which the contrast medium, Oragrafin, was found to increase T_3 resin uptake and a resin sponge technique (B10).

It must be stated, however, that T_3 uptake techniques measure the unsaturated T_4 binding in the serum, not the level of thyroid hormone. An overlap is usually found between the values in untreated myxedema and normal subjects, whereas values in thyrotoxic patients are usually separated from normal values. It is largely fortuitous that the T_3 uptake reflects thyroid status so well in the absence of abnormalities in T_4-binding proteins. It is now customary to consider the result of a T_3 uptake in conjunction with the result of a more direct measure of the thyroid hormone, such as a PBI or total T_4 assay (see Section 3.4.2).

3.3. Assays of Total Thyroxine

The term total T_4 assay is currently taken as meaning total T_4 by competitive protein binding assay (CPBA) or by radioimmunoassay (RIA). However, BEI attempts to measure total T_4 assay. Also column chromatography has been used rather widely for serum total T_4 quantitation, and is often referred to as "total T_4 by column." In 1961 Pileggi (P8) developed a method for estimating total T_4 by separating it from contaminating organic iodine-containing compounds with the use of an anion-exchange resin. The T_4 in column eluates is measured by destroying organic matter either by alkaline incineration or by wet digestion and the inorganic iodine so formed is quantitated by its catalytic effect on the ceric–arsenite system. The method is invalidated by many exogenous organic iodine-containing compounds, but contaminating inorganic iodine is said not to affect the reliability of the assay. The original method was modified and improved, making it more sensitive and less susceptible to organic-iodine interference (M10, P7), and semiautomated modifications are available (G15, K4, Y1) that perform fairly well with

a coefficient of variation of about 4% and contain integral screening procedures for eliminating iodine-contaminated specimens.

The most frequently used method of estimating total T_4 in the serum is CPBA introduced by Ekins (E4) in 1960 and developed further by Murphy (M17). In principle, the technique involves the extraction of T_4 from the serum with ethanol, followed by the competitive binding step with TBG. The separation of bound and free labeled hormone is effected with ion-exchange resin.

In spite of its widespread acceptance and use, there are definite limitations in this method of total T_4 assay. There is variability in the efficiency of extraction of the T_4 by the ethanol (B11) and recently an ethanol-extractable substance was discovered which binds sufficient T_4 to cause significant error in the CPBA technique (I5). These factors among others, are probably associated with the underestimation of serum T_4 by CPBA, particularly in patients with thyrotoxicosis (B4, F4, G7, K10).

Some concern has been expressed that the Murphy CPBA system for total T_4 is not operating optimally as a true saturation analysis and that T_4 disequilibrates between TBP and the resin, so that the final result is dependent on the TBP capacity of a serum as well as its total T_4 concentration (M2). However, recent studies have indicated that dissociation of the protein-T_4 complex is too slow to have a significant effect on the resin separation system (C19). In other words, the use of resins in the CPBA system to separate bound and free T_4 is quite valid. Nevertheless, some workers have looked to other media to effect this separation, e.g., Sephadex (C22), but this is not an ideal way, as Sephadex has an affinity for T_4 (C19). Columns of Sephadex have also been used to good effect as a substitute for ethanol in separating the T_4 from the serum (B14, S7), and such techniques are much simpler than the original and furthermore, result in an almost constant 100% recovery of the T_4 in the serum compared with the low and variable recovery with ethanol.

Work simplification in total T_4 by CPBA techniques has proceeded mainly by the "kit" approach rather than by automation, and these depend upon techniques such as Sephadex columns (H24, S7), Sephadex or Sephadex-like granules (B1), fiber strips impregnated with ion exchange resin (T6), polyurethane sponge impregnated with resin (G6). For those who prefer not to be dependent upon kits there are simplified procedures to be set up in the laboratory dependent upon small Sephadex columns (B14) or ion-exchange resin strips (O2). The kit approach can be combined with semiautomation, particularly of the pipetting steps (R4).

There is now little doubt that the original Murphy method of total

T_4 by CPBA based on an alcohol extraction, has been displaced by a variety of techniques which offer the advantages of greater precision and accuracy and in addition are easier to perform and take less of the technician's time. However, during the period of time of these developments, another novel approach in clinical chemistry was evolving, namely RIA. There are two important prerequisites for a satisfactory RIA of T_4, namely a suitable T_4 antiserum and a method for removing the interference of TBP with the antigen-antibody binding reaction.

In 1971 Chopra *et al.* (C8) published details on the production of AB against T_4 (ranging in titer from 1:100 to 1:1000) produced in rabbits after injection of thyroglobulin. Others have produced antibody of much higher titer in rabbits (H13) and sheep (C20). These antisera were found to have satisfactory specificity for *l*-T_4, although they do cross-react with *d*-T_4, but this does not present any difficulty.

The second problem, namely the interference of TBP in the AG-AB reaction, is easily overcome with the use of TBP inhibitors, such as 8-anilinonaphthalenesulfonic acid or sodium salicylate or by the use of heat denaturation of TBP. As a result of these developments, several RIA procedures for total T_4 have been published (C12, C20, D8, H13, L6). These techniques are not difficult although they are better practiced by laboratories with some expertise in RIA.

There are major advantages in the RIA compared with CPBA. First, there is good sensitivity with detection levels down to as low as 10–20 pg, which means that serum volumes as low as 25 μl may be used and therefore RIA is particularly suitable for measuring serum T_4 in neonates. Second, the RIA approach obviates the need for tedious extraction associated with CPBA, which can be quite a problem when handling large numbers.

It is obvious that T_4 by RIA and T_4 by CPBA are unaffected by contaminating iodine. However, it should be stressed that both total T_4 and PBI are uniformly affected by abnormalities in the T_4-binding serum proteins, namely TBG, TBPA, and albumin as referred to in Section 2.2. In summary, where drugs or any other extrathyroidal factor increases any of the binding proteins (usually TBG) there is a concomitant increase in PBI or total T_4 levels in the serum, and a decrease in the T_3 resin uptake. Conversely, decreases in TBP are associated with decreases in PBI and in total T_4, and increases in T_3 resin uptake.

3.4. Free Thyroxine in Serum

In Section 2.2 the free T_4 concept was discussed in the context of whether or not it was the moiety in the serum that was the determining

factor in the rate of delivery of T_4 to the tissues and thereby whether it most accurately reflected the thyroidal status of individual human subjects. Although this question is not fully answerable at this point in time, there is no doubt that serum free T_4 does exist (S24) and does correlate accurately with thyroid status (R5) if we exclude those clinical situations where serum T_3 levels are more important. When there are abnormalities of the T_4-binding proteins, serum T_4 levels and also T_3 uptake are altered, but the free T_4 in the serum will remain constant in these situations. There is a variety of approaches available for assessing the serum free T_4 status and these are detailed in the material that follows.

3.4.1. *Free Thyroxine Concentration Assay*

In 1959 Christensen (C13) reported a method of assessing the free T_4 concentration (FTC) in arbitrary units based on the rate of free radiothyroxine transfer through a dialysis membrane from a serum specimen with added labeled T_4 on one side of the membrane and pure serum on the other. The first published method for measuring free T_4 in absolute concentration was the pioneering paper for Sterling and Hegedus (S24). In this method, serum specimens were enriched with trace amounts of ^{131}I-labeled T_4 and dialyzed against phosphate buffer at pH 7.4. The unbound labeled T_4 moiety in the dialyzate was separated from the contaminating inorganic iodine ^{131}I by column chromatography. The observed mean free T_4 value was 0.11% of the serum total T_4 content for euthyroid subjects. Multiplying the percent free T_4 by the PBI value gave the result for FTC in absolute concentration terms. Marked increases were found in thyrotoxicosis and decreases in hypothyroidism. A very small decrease in FTC was found in euthyroid pregnant subjects. This procedure, although valuable in its day, was very cumbersome and not suitable as a routine assay, and furthermore it produced higher values for FTC than did the later methods. In order to overcome some of these limitations, Sterling and Brenner (S21) modified the technique by introducing carrier T_4 to the dialyzate and employing magnesium precipitation for concentrating the dialyzed T_4. The mean free T_4 was reduced to 0.046% of the total T_4 of normal serum, and there was sharp differentiation between thyrotoxic, euthyroid, and myxedematous subjects. Values in pregnancy (euthyroid) were again slightly reduced, and those in a euthyroid "chronically sick" group were moderately elevated. Similar dialysis procedures for measuring serum FTC have been devised by other workers, the main differences being in the methods used for separating inorganic iodide from T_4 in the dialyzate (I3, L15, S3, S15).

The property of Sephadex gel particles to absorb free T_4 and to act also as molecular sieve has been used to construct assays of FTC. Lee *et al.* (L8) used microcolumns of Sephadex G-25 to fractionate patient's serum enriched with ^{131}I-labeled T_4 into protein-bound and free T_4 moieties. The percentage of T_4 in the free fraction multiplied by the PBI produced an average of 3.1 ng per 100 ml for free serum T_4 concentration in euthyroid subjects. This is a little lower than the value of 4.2 ng/100 ml found by Sterling and Brenner (S21) with the dialysis and magnesium precipitation method. Investigations of the Sephadex method (L8) in the reviewer's laboratory revealed a mean FTC of 5.43 ± 0.99 ng/100 ml in euthyroid subjects, compared with 1.71 ± 0.73 in clinically hypothyroid patients and 19.01 ± 6.25 in patients with untreated thyrotoxicosis (W10). The reasons for the higher values in the latter study are not clear, but there may be a degree of dissociation of T_4 from the Sephadex or differences in the degree of purity of the labeled T_4 solution used. We also found that FTC was only very slightly lowered in euthyroid patients with low TBP and very slightly increased in those with high TBP states. The FTC was within the normal range in 88% of patients with increased binding and in all subjects with decreased binding.

Although the Sephadex column method was very useful in delineating thyroid status, particularly in the presence of T_4-binding abnormalities, it is too time-consuming and capricious for use on a routine basis. Attempts have therefore been made to improve both these aspects, either by employing ultrafiltration (S27) or improved and simplified Sephadex filtration (C3, I6) or cocurrent dialysis (S30). A simplified method (C3) employing Sephadex filtration on a batch basis has been extensively investigated in the reviewer's laboratory for comparison with other techniques (W12, W13). In this method the test serum is tagged with a trace amount of ^{125}I-labeled T_4 and, after equilibration, is diluted in phosphate buffer at pH 7.4 and subjected to treatment with 200 mg of Sephadex G-25 for separation into free and bound fractions. It is simpler to perform than methods involving Sephadex columns, but minor modification is necessary to produce a result as absolute concentration of free T_4. Euthyroid control subjects yield a mean concentration of 4.8 ng/100 ml of serum and results for thyrotoxic and myxedematous patients are each sharply differentiated from those of normal subjects (W12). The performance of the assay in euthyroid patients with T_4-binding abnormalities is reasonably acceptable with 95% of those with elevated binding and 71% of those with low binding being in the normal range.

It must be stated that an FTC measurement, when expressed as an absolute concentration, is dependent on some form of total T_4 measure-

ment and will therefore reflect any inaccuracies in the latter. The earlier publications prior to the development of total T_4 by CPBA, calculate FTC based on PBI measurements and so inevitably some inaccuracies from iodine contamination would occur, as it was not always possible to detect the invalidated specimens. In conclusion, methods for measuring FTC are certainly now fairly easily available but none is particularly suited for routine diagnosis. A good assay, however, would always have potential as a reference method against which the simpler assays may be tested.

3.4.2. *Free Thyroxine Index*

It is now well established that clinically euthyroid patients who have abnormalities in the serum T_4-binding proteins also have abnormalities in the serum total T_4 level and in T_3 resin uptake. Taking the example of the oral contraceptives, TBG is increased, but the abnormalities in total T_4 and T_3 uptake diverge in opposite directions—i.e., total T_4 is elevated and T_3 uptake is depressed. Clark and Horn (C17) took advantage of the inverse nature of these abnormalities and devised a free thyroxine index (FTI) from the mathematical product of PBI and T_3 uptake, basing this on a similar index constructed from the measurement of the uptake of T_3 by red blood cells (O12). The theoretical considerations underlying the FTI are as follows (C17):

$$FT_4 + F\ TBG \rightleftharpoons TBG\ T_4 \tag{1}$$

where FT_4 is free or unbound T_4, F TBG is free T_4-binding globulin, and TBG T_4 is T_4 bound to TBG. At equilibrium

$$(F\ TBG)(FT_4)/TBG\ T_4 = K \tag{2}$$

or

$$1/K \cdot FT_4 = TBG\ T_4/F\ TBG \tag{3}$$

where K is the dissociation constant for the reaction, TBG T_4 can be taken as being equivalent to total T_4 or to PBI, and 1/F TBG can be taken as equivalent to the T_3 resin uptake. Hence:

$$FT_4 = K' \times \text{total } T_4 \times T_3 \text{ uptake} \tag{4}$$

However, it was subsequently shown that the assumption that T_3 uptake is directly related to the reciprocal of F TBG is not quite correct, and

a slightly different calculation, called free T_4 factor, was introduced to account for this discrepancy (G9).

Clark and Horn (C17) found the FTI to be more accurate than either PBI or T_3 uptake alone in confirming thyroid status in patients with untreated myxedema and untreated thyrotoxicosis. Furthermore, whereas the PBI and the T_3 uptake were frequently abnormal in a group of euthyroid women, the FTI was normal in every case. Wellby and O'Halloran (W10) confirmed the usefulness of the FTI in this setting, extending its use to larger groups of patients with low binding status and in addition demonstrated a highly significant correlation between the results of FTI and those of FTC. They also found the FTI to be more useful than PBI or T_3 uptake alone in studying thyroid function in thyrotoxic patients following therapeutic doses of ^{131}I (W9). The excellent correlation between FTI and FTC has been confirmed by others (A9, S17) and there have been many other papers confirming the usefulness of the FTI (G4, G9, H23, K5, L1, M2). The earlier studies had to rely only on the PBI to combine with the T_3 uptake to calculate the index, but the later studies were able to use the total T_4 assay.

There is no doubt that the development of the FTI has contributed greatly to the usefulness of thyroid function studies carried out in routine clinical chemistry laboratories, but it must always be kept in mind that it is a mathematical product of two assays and so any inaccuracy in either assay will be reflected in the FTI result.

3.4.3. *Simple, Sing-Test, Free-Thyroxine Measurements*

It has been established that the combined use of T_3 uptake and total T_4 assays, usually in the form of a free T_4 index (C17), provides a more reliable index of thyroid function than those tests used singly. The FTI is, however, an indirect and empirical parameter subject to errors from both assays required for its calculation. Therefore, the publication by Mincey *et al.* (M11) of a single test of thyroid function which takes into account both the total T_4 of the serum as well as the degree of unsaturation of the T_4-binding proteins, was met with great interest. The test in principle combines a total T_4 by CPBA and a T_3 resin uptake. The T_4 content of the serum is extracted in methanol and reacted with a reference TBG solution to which is bound a trace amount of ^{125}I-labeled T_4. The serum total T_4 displaces radioactive T_4 from TBG, the amount displaced being proportional to the total T_4 in the serum. A second aliquot of 5 μl of patient's serum is added, which provides a small amount of the TBG. The displaced T_4 in the previous step is partitioned into TBG bound and free fractions. The ratio bound in that step depends

upon the amount of unsaturated binding sites in the serum. A fiber strip impregnated with anion exchange resin removes free T_4. The T_4 bound to TBG is counted for radioactivity. Results for individual specimens are expressed as a ratio of the result for an average normal pooled serum. This test, known as the Effective Thyroxine Ratio (ETR) is commercially available in kit form (Mallinckrodt Nuclear, St. Louis, Missouri).

The ETR has received extensive assessment. Mincey *et al.* (M12) concluded that in hyperthyroid and hypothyroid patients and in normal subjects or in euthyroid patients with T_4-binding abnormalities, the ETR performs with a high degree of specificity and is more accurate in confirming thyroid status than either the total T_4 or T_3 uptake. A high degree of correlation between free T_4 index and ETR has also been shown (T5). Another study (M18) of a large group of patients confirms its usefulness and found it comparable with the FTR in diagnostic accuracy and furthermore, it was as useful as a serum total T_4 assay in assessing the adequacy of replacement T_4 therapy and the response to the thyrotropin stimulation test.

Studies from this laboratory (W12, W13) showed the ETR to be as effective as FTI or FTC in differentiating both untreated thyrotoxic and untreated myxedema patients from euthyroid reference subjects. We also found the ETR to have a very small coefficient of variation (3.7% in euthyroid subjects) from which is derived a rather narrow reference range of 0.89–1.05. In patients with low activity of TBP, the diagnotic accuracy of ETR (80.5%) is superior to that of the FTI (62.5%) and that of the FTC (67%). In patients with elevated T_4-binding proteins, the diagnostic accuracy of ETR is 93.5% compared with 83% for FTI and 91% for FTC (W13). An excellent correlation between ETR and FTC using a Sephadex filtration technique was demonstrated (W12). Continuing studies in our laboratory of the relationship between FT_4 and ETR are shown in Fig. 3. Assuming a linear relationship holds, the correlation coefficient is 0.88 and the relationship between the two parameters is highly significant, with $p < 0.001$. However, a second-degree polynomial equation is a slightly better fit and produces a correlation coefficient of 0.94.

Other studies have further confirmed the usefulness of the ETR in routine clinical diagnosis (N2, T7, T9), and it seems that, with some reservations, this type of test should be seriously considered by a laboratory wishing to establish a single and reasonably simple test of thyroid function.

One reservation is in relation to the reference range. Our studies produced a narrow 95% range of 0.89–1.05 obtained in euthyroid volunteers

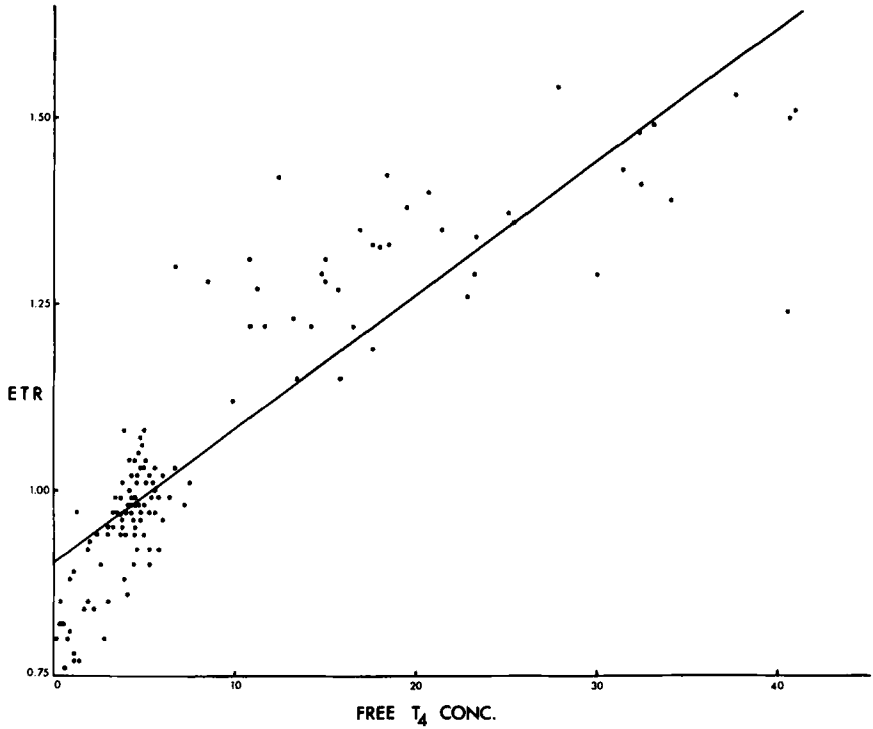

FIG. 3. Correlation of results of Effective Thyroxine Ratio (ETR) and free T_4 concentration (nanograms per 100 ml of serum) in hypothyroid, euthyroid, and hyperthyroid subjects. Refer to text for discussion on correlation coefficient.

and in patients who were proved otherwise to have normal thyroid function. The manufacturers quote a much wider reference range of 0.86–1.13, presumably to account for geographical variation. We quote a reference range between these two, namely 0.90–1.08, on the assumption that this is more realistic than the observed 95% range. The ETR values of patients with untreated thyrotoxicosis very rarely fall below 1.09 although values in untreated myxedema do overlap at the lower end of the reference range. Other workers (M18, W14) suggest that there is overlap at both ends of the reference range and that results should be reported with a reference range and borderline zones. This would alert the clinician to the possibility of requesting further assays such as serum TSH and serum T_3 concentration for results in the lower and upper borderline zones, respectively.

The narrow reference range for ETR is associated with a very low coefficient of variation of the assay, and the question arises whether

this indicates that it has excellent reproducibility or whether the ETR is a rather insensitive test. There are reasons for believing it is comparatively insensitive. For example, the shape of the true mathematical relationship between TER and FTC (Fig. 3) could be interpreted as indicating insensitivity in the ETR. So also could the overlap between the values in hypothyroid patients and euthyroid subjects. This may not be a major problem in the diagnosing of primary myxedema, in which situation an elevated TSH will prove the diagnosis, but is a problem in interpreting low normal values in patients suspected of being hypothyroid after ^{131}I therapy. The finding of an elevated TSH value does not necessarily prove hypothyroidism in this group (see Section 6.7).

Another reservation about ETR is that on theoretical grounds it is not a good parameter to use in patients subject to T_3 suppression or to TSH stimulation tests. A total T_4 is probably more sensitive to these treatments, as the effect of TSH or T_3 treatment on the T_3 uptake is dissociated from the effects on total T_4. The ETR reflects both total T_4 and T_3 uptake and the effect on the total T_4 therefore will be masked by the effect on the T_3 uptake. Fortunately, T_3 suppression and TSH stimulation tests are being phased out. If the limitations of ETR are kept in mind when interpreting a result, they should not detract from its usefulness as a simple, single test of thyroid function.

More recently, alternative procedures for indirectly assessing FTC have become available commercially in kit form, each dependent upon the simultaneous measurement of both the total T_4 by CPBA and the amount of unsaturated TBP. The "Free Thyroxine Equivalent (FTE)" (Ames Co., Division Miles Laboratory, Inc., Elkhart, Indiana) reported by Abreau *et al.* (A1) employs a total T_4 measurement by Sephadex gel filtration at alkaline pH and is adjusted for patients' TBP. It correlates well with FTI but its performance in the presence of binding abnormalities has not been fully assessed. The Normalized Thyroxine (T_4N) (Abbott Laboratories, North Chicago, Illinois) is similar to the ETR in that a dual competitive protein binding analysis incorporating a second aliquot of the patient's serum is made on an alcoholic extract of the serum (A11). Resin sponges are used to effect the separation of free and protein-bound moieties. It is a little less time-consuming as there is no need to evaporate the alcohol extract. The T_4N was found to perform well as a thyroid function test in pregnancy (A12). Another similar procedure, the "Thyopac-5" (Radiochemical Centre, Amersham, Bucks., U.K) utilizes "Sephadex-like" particles to achieve the separation of free and protein-bound T_4 following the dual CPBA common to this type of technique. This procedure produces a value for total T_4, if

required, as well as for FT_4 and has been found to be efficient in confirming the thyroid status during pregnancy (C16). Howorth and McKerron (H24) reported on a similar test, based on columns of Sephadex G-25 (Ames Co., U.K.), which gives a good correlation with clinical thyroid status in a preliminary trial of 45 patients. Presumably at least some of the limitations discussed for ETR apply to these later procedures of indirectly measuring FTI in a single assay.

Other aspects of importance for the laboratory director to consider are the expense and availability of the kits. A possible alternative is for the laboratory to develop their own procedures, in which case the cost would presumably be less, but there might be difficulties in reproducibility of results, and interlaboratory comparisons would be hampered. It is important to realize that, although the free T_4 type of test is the most valid approach for T_4 measurement, it is of limited value in the clinical situations where there is hypersecretion of T_3, where a direct measure of T_3 concentration in serum is also necessary.

3.5. Serum Total Triiodothyronine Assays

3.5.1. *Historical Aspects*

The history of the assay of serum T_3 starts in 1952 with its discovery in the serum and the thyroid gland, and its identification by two groups, Gross and Pitt-Rivers (G13) and Roche *et al.* (R9) using paper chromatographic techniques and radioactivity detection. These methods were comparatively crude and insensitive, however, and very little development took place for a few years. In 1957 Pind (P10) used a chemical assay following paper chromatographic separation to give a value for T_3 of 240 ng/100 ml in normal serum. Further quantitation of T_3 in serum had to wait some years, one of the difficulties being the close chemical resemblance of T_3 to T_4 and the resultant difficulty in separating the two in the systems then available. In the main, these allowed only a semiquantitative assessment of the circulating thyroid hormones in health and disease, although even with these crude techniques, the hypersecretion of T_3 in some cases of thyrotoxicosis was suspected (M1). In 1967 Nauman *et al.* (N3) employed thin-layer chromatography of Sephadex LH-20-purified extracts of serum in a mixture of methanol and chloroform to separate T_3 from T_4, followed by a complex CPBA to quantitate the T_3. The method yielded values of T_3 in normal serum of about 330 ng/100 ml. The method was extremely cumbersome, required large volumes of specimen and, according to Sterling *et al.* (S20),

was subject to falsely elevated levels of T_3 from artifacts produced in the extraction.

3.5.2. *Chromatographic Methods*

A major advance was made in 1969 with the publication of Sterling *et al.* (S20) of a method of assaying T_3 that was dependent on the paper chromatographic separation of T_3 and T_4 in extracts of serum from cation exchange resin followed by quantitation of the separated T_3 by CPBA. With this technique, the mean normal T_3 concentration in serum appeared to be 220 ng/100 ml. A similar value for normal serum, namely 240 ng/100 ml was obtained by another laboratory (W2) using Sterling's technique. The main point of criticism of this technique is the reported conversion of some T_4 to T_3 during the procedure and that T_3 is not completely separated from T_4 in the chromatography (F7, L2). The procedure was modified by Larsen in order to reduce these artifacts (L2), and a mean T_3 concentration of 180 ng/100 ml was obtained. Larsen, however, considered that he did not exclude the possibility of residual artifact in his modified method and suggested that RIA was to be the method of choice for measuring T_3 in serum. In spite of the likelihood of artifact in the Sterling assay, it was a pioneering and very useful method yielding valuable information about T_3 kinetics and, in particular, providing the proof of the existence of T_3-thyrotoxicosis.

At about the same time as the development of the chemical method, a gas–liquid chromatographic (GLC) procedure for T_3 was evolving (H22, N5) which yielded lower values, namely, mean normal levels of 137 ng/100 ml, compared with 68 in hypothyroidism and 510 in untreated toxic diffuse goiter. Even in this procedure, deiodination of T_4 to T_3 occurs to give a falsely high T_3 result, but the amount of such deiodination can be estimated and a correction made to the figures for T_3 (H22). These studies, along with those of Sterling (S19, S20) contributed much to our knowledge of T_3 kinetics, and cases of thyrotoxicosis due to excess T_3 in the serum were identified by this method also. Hollander preferred to call these "T_3-toxicosis" rather than T_3-thyrotoxicosis, perhaps to allow for the possibility that at least some of the excess T_3 was derived from peripheral monodeiodination of T_4. The method has certain advantages over the chemical method (S20). It is more sensitive, using only 1 ml of serum; it is said to be more specific (H22) and, last, simultaneous T_3 and T_4 measurements are feasible. The method, however, is not suited to routine assay because of the expense of the equipment involved. This group were well aware of

this problem and were one of many to develop a RIA, the results of which correlated extremely well with their GLC method (M15).

3.5.3. *Radioimmunoassay of T_3*

A major breakthrough in the assay of T_3 came with the publication by Brown *et al.* (B18) of a method for raising antibodies to T_3. The T_3 was linked to poly-L-lysine by a carbodiimide condensation and injected into rabbits to produce antibodies of acceptable specificity and reasonable activity. This paved the way for a RIA, as one of the main prerequisites for a RIA for T_3 is the ready availability of an acceptable antibody (AB) to T_3. Other prerequisites include a satisfactory method for releasing T_3 from thyroid hormone-binding proteins (TBP), also the inhibition of TBP activity in later phases of the assay. Also necessary is a method of separating AB-bound T_3 from free T_3. Providing the T_3 AB is reasonably specific and in particular does not exhibit significant cross-reactivity with *l*-T_4, there should be no need to separate T_3 from T_4 as a preliminary to the binding step. The RIA therefore has obviously a great advantage over the chemical and GLC assays.

With these antisera to T_3, Brown *et al.* (B17) were able to develop a RIA for T_3, but they found it desirable to make a preliminary separation of T_3 and T_4 as their T_3 antiserum showed significant cross-reactivity with *l*-T_4 (2–5% of the activity for *l*-T_3). The results on normal serum were, however, very low and incompatible with the earlier findings (H22, S20). Many other workers, including this laboratory, tried to repeat this method of raising T_3 AB (B18), but without significant success.

Gharib *et al.* (G2) successfully raised antisera in rabbits against T_3 conjugated with human serum albumin (HSA) using carbodiimide condensation. These antisera and those raised by others have very much higher activity than those of Brown *et al.* (B18) and can be used in assay systems at final dilutions of up to 1:250,000 or more. Furthermore, cross-reactivity with *l*-T_4 is as low as 0.1 to 0.5% and cross-reactivity with other *l*-iodoamino acids is no problem. An alternative method of raising T_3 AB, which has been used by several workers, is to inject thyroglobulin in Freund's adjuvant into rabbits (C8). Some of the injected rabbits produce antisera against T_3, some produce T_4 antisera, and a few produce both T_3 and T_4 antisera. Where antisera against T_3 are raised, the cross-reactivity with other *l*-iodoamino acids, including T_4, is no problem and it is possible to use the T_3 antisera at appropriately high dilution in an RIA system (C9).

There is another problem in RIA of T_3 which has already been referred to, namely the interference by TBP. These proteins, TBG, TBPA, and

albumin, can interfere in two ways; first, by hindering the binding of the T_3 by its AB; and, second, the unbound moiety at the end of the AG:AB reaction step may not be recognized as such in the separating system because of its binding to TBP. There is a variety of ways of overcoming the problem of interference by TBG.

The first way is to add to the initial reaction mixture a substance known to inhibit the binding activity of TBP. Tetrachlorothyronine was probably the first substance to be used for this purpose (M15). It is necessary carefully to assess the optimal amount of blocking agent in each RIA system as an excess could well interfere with the AG:AB reaction or with some other part of the assay (B21). The amount of blocking agent required probably depends a great deal on the avidity of the antiserum being used; in fact some workers have developed successful measurement of T_3 by RIA in unextracted serum without the use of TBG-blocking agents, but using T_3 antisera of such high binding affinity for T_3 as to be in excess of the binding affinity of TBG (H20). Other blocking or displacing agents that have been successfully used include Merthiolate (ethyl mercurithiosalicylate thiomersol) (G3), salicylates (L4), *l*-thyroxine (C9), diphenylhydantoin (L14), and 8-anilinonaphthalenesulfonic acid (ANS) (M14). It has been claimed that ANS is one of the more suitable substances because it displaces T_3 from TBP without impairing its binding by specific antibodies (M14). A recent study (M3) confirms the usefulness of ANS but emphasizes that an inhibitor only partially alleviates the problem of TBG interference and that it is desirable to use T_3 antisera with the highest possible binding affinity for T_3 for the best possible solution to the problem.

An alternative method for removing TBP interference is the recent innovation of Sterling's group (S25, T1) in the use of thermal inactivation of the TBG. It was shown that heating serum to 60°C destroyed most of the binding capacity of TBG, but that TBPA capacity remained virtually unchanged (T1). Subsequently, an RIA procedure was published (S25) dependent on thermal inactivation of TBG in the presence of Merthiolate as the first step, which takes place simultaneously with the AG:AB reaction. The questions that arise are: How effective is this method of removing TBG interference? Does TBPA interfere with the AG:AB binding, and does the heating process interfere with the AG:AB reaction in any way other than by accelerating its speed? Answers to these questions are awaited with interest. It should be noted that Merthiolate is used in this procedure although at a concentration of approximately a tenth of that used by others relying on Merthiolate alone (K1). It is interesting that the heat inactivation technique produces results

for T_3 concentration in normal subjects higher than any other RIA except one (G2); this raises the question whether the method is yielding values that are too high or whether all other methods are too low. However, the authors have carefully validated the technique step by step (S25). It is reassuring that the mean value observed in hypothyroidism is lower than in some other radioimmunoassays.

Ethanol extraction has been used to remove the interference of TBG with T_3-binding by AB in the method of Patel and Burger (P4) in which the ethanol precipitates the serum proteins and solubilizes T_3 and T_4. The ethanol is only partly evaporated to prevent interference by insoluble matter, which would otherwise bind appreciable quantities of labeled T_3. This is a highly sensitive assay, detecting levels of T_3 as low as 6 ng/100 ml and cross-reactivity with *l*-T_4 is 0.2% or lower. A similar method has been published by Werner *et al.* (W16), the main difference in technique being the complete evaporation of alcohol extract to dryness. Another method that separates T_3 and T_4 from TBP in the first step is based on a Sephadex filtration in small columns (S31). It is important to remove all traces of protein in this type of assay, and the latter technique has been challenged on this aspect (S25).

The next aspect of the RIA of T_3 to be considered is the separation of the protein-bound and the free fractions following the AG:AB reaction. The various techniques used have included charcoal particles (E2, P17), dextran-coated charcoal (H26, K1, S31), charcoal combined with methylcellulose particles (M15), polyethylene glycol precipitation (S5, W16) and second antibody precipitation with goat anti-rabbit γ-globulin (C9, G3, M3, P4). Solid phase separations have also been introduced into T_3 RIA in the form of agarose suspension for binding the T_3 antiserum and for facilitating the AG:AB binding reaction and the separation of free and bound T_3 in the one step (S9). Each method presents with certain advantages and disadvantages and the final choice may well depend upon the previous experiences of an individual laboratory, coupled with the ease of availability of the particular material. Although the second antibody seems the most popular, it is a very expensive method unless produced in the laboratory. It has been suggested that the TBG blocking step need not be perfect if the second antibody method is used, particularly if employing T_3 antisera of high binding affinity. However, there is the possibility that the second antibody will interfere with equilibrium kinetics of the first antigen–antibody binding and affect the result (B21). We have found charcoal to vary from batch to batch, necessitating a careful check when changing batches or when making interlaboratory comparisons, and a similar caution is made by another reviewer (B21).

TABLE 1
SUMMARY OF REPRESENTATIVE METHODS FOR T_3 RADIOIMMUNOASSAY

First author	Reference	TBG inactivation[a]	Separation method[a]	Mean T_3 (ng/100 ml) (euthyroid)
Sterling	S25	Heat inactivation	PEG	189
Surks	S31	Sephadex	Dextran charcoal	146
Patel	P4	Ethanol	GARGG	129
Malkus	M3	ANS	GARGG	98
Larsen	L4	Na salicylate	Dextran charcoal	110
Werner	W16	Ethanol extract	PEG	116
Sekadde	S5	ANS	PEG	146
Fang	F3	ANS	GARGG	112
Hesch	H15	Merthiolate	Dextran charcoal	126
Hesch (Evered)	H15	Na salicylate	Dextran charcoal	120
Kirkegaard	K6	Merthiolate	Dextran charcoal	133
Eastman	E2	ANS	Charcoal	120
Kanagasabapathy	K1	Merthiolate	Dextran charcoal	136
Chopra	C9	*l*-T_4	GARGG	—[b]
Hüfner	H26	Merthiolate	Dextran charcoal	120
Postmes	P17	Merthiolate	Charcoal	146
Mitsuma	M15	Tetrachlothyronine	Charcoal and methylcellulose	139
Gharib	G3	Merthiolate	GARGG	218

[a] PEG = polyethylene glycol, GARGG = goat anti-rabbit γ-globulin, ANS = 8-anilinonaphthalenesulfonic acid.

[b] Assay too insensitive to yield mean value in euthyroid subjects.

The number of T_3 RIA procedures published is now growing rapidly and it is impossible to review each one, but a list of many of them is given (Table 1), together with the method of blocking TBG interference and the method of separating free and bound AG. Also listed is the mean value for euthyroid subjects for each method, but it must be emphasized that this parameter is of comparatively little importance when compared with other parameters, such as precision, reproducibility, and sensitivity. Other important points to consider are the range of values obtained in primary hypothyroid patients and the degree of overlap between abnormal thyroid states and the euthyroid state.

We have used a method that depends upon: (1) the production of high-affinity antisera in rabbits injected with T_3-bovine serum conjugate, (2) Merthiolate (final dilution 1:1,000) to inhibit TBG activity, (3) AG:AB reaction at 4°C, (4) dextran-coated charcoal to achieve separation of AB-bound and free AG, and (5) the addition of T_3-free serum

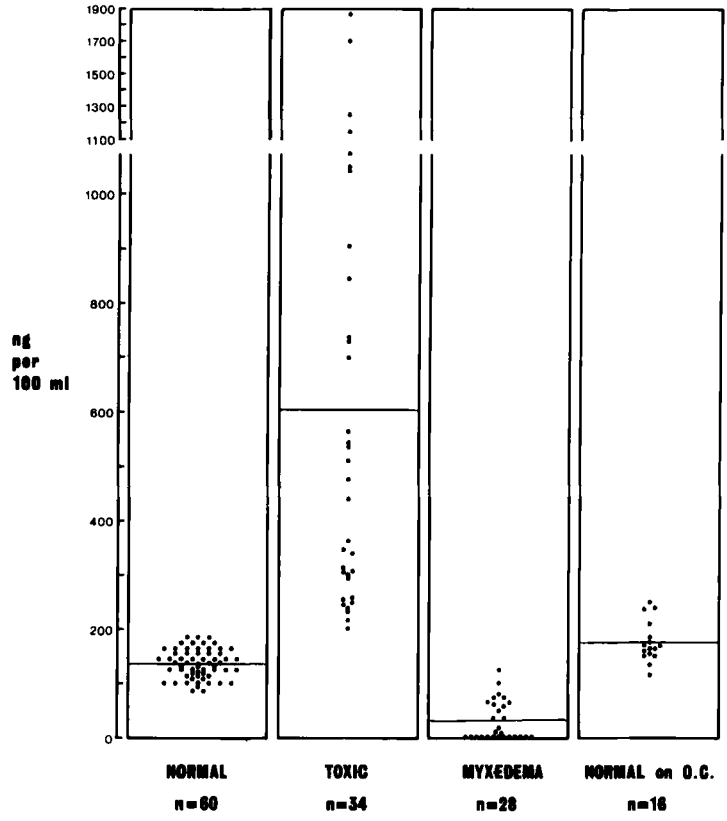

FIG. 4. Results of total triiodothyronine assays (nanograms per 100 ml) in 60 normal subjects, 34 untreated thyrotoxic patients, 28 untreated patients with iodiopathic myxedema, and 16 normal subjects on oral contraceptives (O.C.). The light horizontal line in each panel represents the mean value for the group. Unpublished data of A. S. Kanagasabapathy and M. L. Wellby (1974).

to blanks and standards. It was found essential to add T_3-free serum to all standards to overcome the documented interference of Merthiolate with the AG:AB-binding reaction (K5). A separate blank for each serum specimen is not used in this assay system because it makes the procedure too cumbersome as a routine assay. However, ideally and to increase precision further, such a serum blank should be included to account for individual variation in TBG content, which cause parallel changes in the percent of T_3 bound in the absence of AB (L4). Values for T_3 in 60 euthyroid subjects range from 84 to 188 ng/100 ml, with a mean of 136 (Fig. 4). Significant elevations ($p < 0.001$) are found in euthyroid females with presumed increased TBG levels associated with

pregnancy or with taking oral contraceptives. There is an overlap in the values found in untreated myxedema and normal subjects but values in thyrotoxicosis do not overlap.

It is obvious that there is a choice of a wide range of techniques for T_3 RIA and it is not possible to make recommendations of a particular assay. Laboratories about to set up a T_3 method should take cognizance of the various pitfalls in the methodology and, in their choice of method, be guided by avoiding these if possible and also considering the expense and availability of the raw products. They would also be influenced by the degree of sophistication of their interest in the area of diagnosis of thyroid dysfunction, for example, a nonspecialized laboratory may do well to consider one of the growing number of commercially available "kits," which mostly depend on single step solid phase RIA.

3.6. Serum-Free Triiodothyronine and Urine Triiodothyronine

It is generally agreed that T_3 is carried in the blood bound mainly to TBG (L4) and that the binding affinity of TBG for T_3 is much less than that for T_4 (see Section 2.2). Small amounts of T_3 may be carried on TBPA and albumin. It is estimated that from 0.25% to 0.45% of the total T_3 of the serum is in the free or non-protein-bound form. By analogy with the free T_4 concept, it is possible, although not proved, that it is this small free moiety of T_3 which is the determining factor in the rate of delivery of T_3 to the tissues. It can be calculated that the contributions of free T_4 and free T_3 (FT_3) to the metabolic activity of thyroid hormones are of approximately equal magnitude. If the FT_3 fraction is the important physiological factor, then methods of its estimation are relevant in the diagnosis of thyroid dysfunction.

Development of FT_3 assays has been mainly confined to two groups, one in the United States and one in the United Kingdom. In the United States, Werner's group (N3) reported an FT_3 method in 1967 in which a dialyzing technique was used. They found the dialyzable fraction ($\equiv$ free) to be 0.46% of the total. This is considered to be a valid estimate, but as the total T_3 method used in conjunction with the dialysis was artifactually elevated, their estimate of absolute FT_3 concentration is not valid.

The introduction of RIA for T_3, with its increased sensitivity and specificity, has paved the way for the development of free T_3 assay. There are two approaches possible in exploiting RIA for FT_3 assay. One way is to carry out two separate procedures, namely a RIA of total T_3 and a dialysis or Sephadex manipulation using added labeled T_3 as a marker, to determine the fraction in the free form. Any inorganic

^{125}I-labeled iodine contaminating the labeled T_3 would be a problem with this method. Absolute FT_3 is obtained by simple calculation. The alternative and preferred technique is to dialyze unlabeled T_3 from the serum and carry out an RIA on the dialyzate.

In the United Kingdom, Ekins' group has used the latter technique and found absolute FT_3 to range from 3.9 to 7.4 pg/ml with a mean of 6.0 pg/ml (E5). In another study (C19), an FT_3 concentration was devised based on a Sephadex filtration technique originally set up for FT_4 (C3) with prelabeling of the serum with ^{125}I-labeled T_3 purified immediately before use. Total serum T_3 was assayed by RIA (with this technique). The amount of total T_3 in the free form was in the order of 1.5%, and the euthyroid range of absolute FT_3 concentration is 16–23 pg/ml. This method appears to give values for FT_3 somewhat higher than that which is acceptable, and certainly much higher than those found by Ellis and Ekins (E5). Further assessment and modification of these procedures is necessary to develop a method suitable for clinical diagnosis.

In the meantime it is worth considering urine T_3 excretion, as this should correlate reasonably well with serum free T_3 concentration. There are probably three sources of urine thyroid hormones: first, the hormones conjugated in kidney and liver; second, the unconjugated hormones derived from the serum unbound hormones; and finally, the hormone metabolites (B21). Several groups have independently published methods for measuring urine T_3 excretion (B22, C4, H27) based on RIA of either unextracted or extracted urine specimens. There is considerable interlaboratory variation in values for T_3 excretion, dependent in part on whether or not an extraction of urine is used. Other limitations of the procedure include extrathyroidal factors such as diurnal variation in excretion (H27), renal dysfunction, and the effect of drugs on the excretion of T_3. In spite of these limitations a good diagnostic separation was obtained between hyperthyroid, euthyroid, and hypothyroid patients (B21) although probably most of the above limiting factors were screened out. Clearly urine T_3 measurements should be interpreted cautiously and with due regard to extrathyroidal factors affecting them. Hopefully, free T_3 procedures will be developed sufficiently in the near future as to make urinary T_3 measurements unnecessary.

3.7. Effects of Extrathyroidal and Physiological Factors on Reference Ranges of Thyroid Function Tests

There would be little point in this review in tabulating the reference ranges for all the thyroid function tests referred to, particularly as in

many cases the nomenclature is not standardized and also because normal values for many tests are so method-dependent. However, it is important for the reader to be aware of the modifications that occur in the results of thyroid function tests caused by physiological and other extrathyroidal factors.

3.7.1. *Diurnal and Seasonal Variation*

The thyroid secretion unlike that of the adrenal cortex, is reasonably stable and no significant diurnal variation has been reported. The data are scarce, however, but Surks *et al.* (S31) measured serum total T_3 and T_4 concentrations at frequent intervals throughout a 24-hour period and demonstrated that both hormones are relatively constant throughout the day. Information about the possible effects of stress on thyroid hormone levels in inconclusive. Whereas Hetzel *et al.* (H17) showed rapid changes in PBI level in association with acute stressful life experiences, Volpé *et al.* (V5) failed to find any evidence that the function of the normal thyroid gland is readily affected by severe mental and physical stress. However, seasonal variation in thyroid function has been reported (D9, L13, W3). In a study in Montreal, a city characterized by sharp seasonal temperature gradients, the PBI level was shown to decrease significantly as average monthly temperatures increased (D9).

3.7.2. *Age and Thyroid Function Tests*

Interesting changes have been found in thyroid hormone levels in the neonatal period. It has been known for many years that serum T_4 levels are normally higher in infancy than at any other time. Such elevations could well obscure a diminution in thyroid hormone levels associated with hypothyroidism, particularly partial hypothyroidism associated, for example, with dyshormonogenesis. Early detection of hypothyroidism is essential as treatment is simple and the consequences of delay in treatment are severe and to some extent, irreversible.

Until recently, accurate data on this topic were scarce for the obvious reason that earlier methods required large specimens of blood in relation to the amount of blood available in this age group. Recent studies have helped to clarify the situation, particularly in regard to T_4 levels, but further observations of T_3 levels are necessary because of conflicting results. It is now realized that total T_4 levels are slightly to moderately elevated at birth, as measured in cord blood. Davies *et al.* (D1) report total T_4 concentration ranging from 7.2 to 13.5 μg/100 ml in cord blood, with a mean of 9.9 μg/100 ml, similar to the findings of Abuid *et al.* (A2). This is a little lower than the levels found by others (B15, K9,

O3), which suggest that cord total T_4 concentrations do not materially differ from those in maternal serum. Increases in TBG activity only partly explain the elevated total T_4 concentration as both ETR and FTI were shown to be elevated (B15, O3), and in addition there is probably some increased thyroid activity compared with adults. In the early neonatal period, most workers have observed a sharp rise in both total and free T_4 levels in the serum (A2, A3, D1). In the most comprehensive study (A2) average concentration of total T_4 at birth was 10.9 μg/100 ml and increased to 17.2 μg/100 at 3 days after birth and was back to 10.3 μg/100 ml, that is a high normal level, by 6 weeks of age. Free T_4 increased from 2.2 ng/100 ml to 4.9 ng/100 ml at 3 days and subsided to 2.1 ng/100 ml, that is not significantly different to adult normal by 6 weeks.

The situation in regard to T_3 levels is even more interesting and more complex. Total and free T_3 are present in low concentrations in cord blood when compared with adults (A2, E1, F8), and Fisher *et al.* (F8) showed that total T_3 levels increase sharply within the first few hours of life, presumably associated with the release of TSH occurring at delivery (E1, F9). However, there is a biphasic increase in total T_3 as shown by Abuid *et al.* (A2, A7). At birth total T_3 is 48 ng/100 ml, at 3 days after birth it increases to 125 ng/100 ml, and at 6 weeks it is 163 ng/100 ml. In addition, their earlier studies (A7) demonstrated that the first increase in total T_3 occurs at 24–48 hours and reaches values as high as 262 ng/100 ml. The free T_3 response differs in that it is low at birth and increases more slowly to a maximum at about 3–4 days, and stays at that level for at least 6 weeks. The fact that free T_3 is elevated at 6 weeks is evidence that increased TBG activity is not the explanation for the elevated total T_3 levels. Observations beyond the age of 6 weeks are conflicting, and further work is necessary to ascertain for how long and to what extent, total and free T_3 levels are elevated. Such observations will have obvious diagnostic implications in the interpretation of levels of T_3 concentration in the young age group.

With regard to the question of the possible effect of senescence on thyroid function, Jeffreys *et al.* (J2) reported that whereas PBI appeared to decrease in the aged, the decrease was more often associated with the chronically sick aged patient, together with decreases in TBG, and that FTI as a result, did not change in these groups. There is evidence that the serum T_3 concentration decreases with age (B20, R11), and if this is confirmed, it presumably relates more to a progressive change in the relative output of T_3 and T_4 or to a decrease in peripheral monodeiodination of T_4 rather than to decreases in TBP.

3.7.3. *Altered Thyroid Hormone Binding and Thyroid Function Tests*

The alterations in the normal ranges of thyroid function tests secondary to disturbances in TBP, are very well known and have been referred to in Sections 2.2 and 3.4.2. There is a very large body of literature on the various factors, physiological, pathological, and drug induced, which cause abnormalities in TBP, and it is impossible to review them here. Recent reviews have provided the necessary information including comprehensive tables of factors that cause increased and decreased TBP (C15, H6, H23). Although these factors are of decided interest in relation to the physiology of thyroid hormone transport, they are largely superfluous in the context of thyroid hormone tests, as the FTI, which remains unaffected by these factors, is so firmly established. Likewise, the single-test free T_4 type procedures, such as ETR, are unaffected by these factors.

It must be appreciated that the same factors that affect total T_4 levels have a similar, although less pronounced, effect on total T_3. For example, pregnancy and the administration of oral contraceptives, through their TBG-elevating effect, significantly elevate the serum total T_3 concentration in euthyroid subjects (B21). The reader is also referred to Fig. 4. Unfortunately, the calculation of a free T_3 index is not necessarily a valid procedure, and furthermore, there is as yet, no uniformity in the performances of various free T_3 concentration procedures, nor is any simple enough for routine assay.

3.7.4. *Thyroid Function in Starvation and Chronic Illness*

Abnormalities in thyroid function tests may be encountered in a variety of severe nonthyroidal illnesses, either acute or chronic, and some of the abnormalities are mediated through decreases in TBPA or in TBG, or in both binding proteins. Where the abnormality is due only to decrease in TBG and TBPA, the FTI, FTC, and similar measurements are normal but total T_4 in serum is low and the resin uptake of T_3 is elevated. This is the situation in chronic renal disease and although the FTI and similarly calculated free T_4 "factors" do not give normal results in all patients, the free T_4 concentration is normal in nearly all euthyroid patients (J3).

In other studies in nonrenal chronic illness ("sick euthyroid" patients), the total T_4 levels are found to be low and associated with low TBG activities and either normal or marginally elevated serum-free T_4 concentrations (B7). The mechanisms underlying the thyroid hormone changes (particularly of T_3) were further elucidated in the very recent paper by Carter *et al.* (C1). The most striking abnormality found was a highly significant reduction in both total and free T_3 concentrations in the

serum. Most total T_3 levels were in the range for myxedema patients. The serum total T_4 levels were significantly reduced, but not so dramatically, and FT_4 remained normal. Five of the 75 patients tested with functional tests had normal thyroid reserve, and all patients had normal serum TSH levels. This finding of low free T_3 levels is a most important observation, and it excludes the possibility that decreased activities of the thyroid hormone-binding proteins were contributing to the overall picture. The conclusion is that there is decreased extrathyroidal conversion of T_4 to T_3, which is probably a compensatory mechanism in view of the patients' decreased requirements for thyroid hormone.

In an independent, but closely related, study (P16), nine euthyroid obese volunteers undergoing marked caloric deprivation exhibited striking decreases in both total and free T_3 concentration, together with slight increases in free T_4 concentration and normal levels of serum TSH. Low serum T_3 levels in euthyroid subjects have also been found in hepatic cirrhosis (C10). These papers (C1, C10, P16) have immediate practical application in regard to the interpretation of thyroid function tests and in particular of T_3 assays in the chronic sick patient or in any state of inanition. They also have a bearing on the controversy on whether or not T_4 has intrinsic hormonal activity or is merely a prohormone, and there are implications in respect to the nature of the feedback control of TSH production.

4. Supplementary Tests

4.1. Serum Thyrotropin Assays

Recent reviews on the laboratory aspects of thyrotropin (TSH) assay have been published by Hall (H4) and Raud and Odell (R1), so that it is only necessary here to discuss some aspects of the application of TSH assay. The key role of TSH in the control of thyroid secretion has been outlined in Section 2.4, and it is clear that TSH assays are of importance in the diagnosis of thyroid disorders, especially where thyroid hypofunction is involved. When combined with some form of stimulus, such as thyrotropin-releasing hormone (TRH), TSH levels are also useful in the investigation of thyrotoxicosis.

4.1.1. *TSH Assay in Diagnosis of Primary Hypothyroidism*

As expected, in idiopathic hypothyroidism, because of failure of negative feedback from thyroid hormones on the thyrotrophes, serum TSH levels have been found to be elevated. However, the difficulty arises on attempting to answer the question: What is the normal range for serum TSH? The difficulty, in other words, is in defining what constitutes

an elevation in TSH. Different methods produce different ranges for normal subjects, and different laboratories, using apparently the same methodology, produce different reference ranges. Hall (H4) has tabulated some normal values for serum TSH obtained with different methods. If the standard curve is set up in a TSH-free human serum, the reference range for euthyroid subjects is likely to be in the order of 0–3 μU/ml defined in terms of the M.R.C. Standard A human TSH. Such a range is obtained in the methods of Hall *et al.* (H5) and Patel *et al.* (P5).

The TSH level for individuals is thought to be fairly stable from day to day and a small diurnal variation has been found with a peak immediately after onset of sleep (P5). There is a sharp surge of TSH in the neonate at birth which falls to normal in a few days (F9), producing the first spike of the bimodal serum total T_3 increase (A2, A7). With these methods, TSH levels for patients with idiopathic hypothyroidism range from 5 or 6 μU/ml to very high levels such as 500–600 but patients with fully developed untreated myxedema are unlikely to have TSH values of less than 30 μU/ml. The main value of TSH assay in the setting of hypothyroidism is in the diagnosis of the marginal case of primary hypothyroidism. Irvine *et al.* (I8) claimed that whereas in their hands a plasma TSH of less than 7 μU/ml indicates normal thyroid reserve, levels between 7 and 25 μU/ml are equivocal and a TSH of greater than 25 μU/ml indicates impaired or absent reserve of thyroid function. Hall (H4) has coined the term "subclinical hypothyroidism" to apply to those patients who have normal thyroid function tests, no clinical evidence of hypothyroidism, no evidence of previous treatment for thyroid disorder, but who have a mild to moderate elevation in serum TSH. These patients should be screened for goiter and particularly for autoimmune thyroid disease. Similar TSH elevations may be detected in clinically euthyroid patients who have been treated with ^{131}I for thyrotoxicosis (S11, W8).

4.1.2. *TSH Assay in Other Clinical Situations*

A patient with clinical hypothyroidism who does not have an elevated serum TSH concentration must have hypothyroidism secondary to either hypothalamic or pituitary disease. A finding of an undetectable TSH level per se does not distinguish these patients from normal because of the insensitivity of the assay. A TRH stimulation test may identify the site of the primary dysfunction, whether pituitary or hypothalamus (see Section 5.3).

In patients with untreated thyrotoxicosis, the TSH should be zero, but many normal subjects too have undetectable TSH levels. The com-

bined use of TRH and serum TSH assays is of use in the diagnosis of thyrotoxicosis when the results of conventional thyroid function studies are not decisive (O10) (see Section 5.3).

4.2. Thyroidal Radioiodine Uptake Measurements

Measurement of the thyroid uptake of radioactive iodine has for many years been a useful supplement to thyroid function tests on blood. While thyroid uptake tests are not usually part of the services offered by a clinical chemistry department, they should always be considered in relation to the clinical chemistry tests, and therefore a brief discussion on them is relevant in this review.

The relative importance of thyroid uptake studies has fluctuated somewhat through the years. Probably the most commonly used test is a simple measurement of uptake in the thyroid at 3 or 4 hours and at 24 hours after a standardized dose of ^{131}I. However, some centers favor a more sophisticated iodine clearance study where the thyroidal ^{131}I content is related to the blood content of ^{131}I after intravenous injection. One limitation of the simple procedure was recognized many years ago when marked daily fluctuations of 3-hour and 24-hour uptakes were noted within individual subjects (L11).

Publications in recent years have drawn attention to decreasing normal values for thyroidal radioidine uptake (K2, P12, S16), and these have certainly contributed to the waning popularity of simple thyroid uptake measurements. The reported decreases in thyroid uptake have been found in various countries, and it is possible to relate the 24-hour uptake measurements fairly accurately to the iodine intake for a particular community (S16). It is well recognized that average iodine intake has increased markedly in communities. Not all this increased intake is restricted to certain individuals who consciously take iodine-containing health foods and the like, but much of it is inflicted on the unwary population at large in the form of such substances as calcium iodate in bread and iodophors in milk.

About 5–10 years ago thyroid uptake measurements were becoming more frequently used because of the development of functional type tests such as T_3 suppression (W17) and TSH stimulation (H25), which required the measurements of both thyroid uptake as well as of serum thyroid hormone levels before an after treatment with T_3 or TSH, respectively. This type of test was of great value at the time, particularly for investigation of patients whose thyroid function was either borderline high or borderline low, and where the existing chemical assays on blood were of limited value. Improved procedures were devised for the T_3

suppression test (C7) and because of these and because of the fact that T_3 RIA has become more generally available only in the last year, the investigation of suspected cases of T_3-thyrotoxicosis has had to depend on T_3 suppression tests in many centers.

However, these indications for thyroid uptake have virtually disappeared, first because of the replacement of TSH stimulation test by sensitive TSH assays (H4, I8) and, second, by the replacement of tests of thyroid autonomy, such as the T_3 suppression tests by TRH stimulation tests (see Section 5.3). This restricts the application of thyroid uptake tests mainly to a few special areas, particularly as a key test in the elucidation of patients with biosynthetic goiter. Many of these patients are children, and it is desirable to use ^{132}I in these as it has been shown that the radiation exposure from the standard dose of ^{132}I is approximately 1% of the exposure from the equivalent dose of ^{131}I (H18). Another advantage in using ^{132}I is that repeated studies in the one patient are possible, as its half-life is 2.2 hours compared with 8 days for ^{131}I, and therefore there are no errors from residual activity from a previous dose.

Thyroid uptake measurements may also be of value in the diagnosis of subacute thyroiditis and thyrotoxicosis factitia. In both conditions the "blocked thyroid gland syndrome" may be found with high circulating levels of thyroid hormone and zero or low thyroid uptake.

In vivo radioisotope tests have been used in another area in recent years, namely to assess suppressibility of thyroid activity while the patient remains on antithyroid therapy for thyrotoxicosis. This test was introduced by Alexander *et al.* (A7) in order to predict which patients could come off antithyroid therapy without significant risk of relapse of thyrotoxicosis; suppressibility being equated with remission. This procedure created widespread interest, but in spite of modifications and improvements to the test regime (A8), it has not received full acceptance.

In summary, it would seem that with the major exception of the investigation of dyshormogenetic goiter, quantitative thyroid uptake measurements have been superseded and the only other thyroid studies requiring radioactive isotopes will be in large nuclear medicine scanning units being established. These will, in the main, use alternative isotopes, such as ^{99m}Tc and ^{125}I (R10).

4.3. Tests for Thyroid Autoantibodies

Some reference to testing for thyroid autoantibodies is made in this review for the sake of completeness, but as it is a specialized area that

is normally established by a serology or immunology department rather than by a clinical chemistry laboratory, a brief summary only is given, concentrating mainly on the application of the tests, not on the methodology involved.

The detection of thyroid autoantibodies is important in the diagnosis of chronic lymphocytic thyroiditis (Hashimoto's disease) and the rarer forms of autoimmune thyroid disease, as the earlier reviews showed (D6, H2, O14). There are three antigen–antibody systems to be considered, namely, the two antibodies reacting with thyroid colloid [thyroglobulin and "second colloid antigen" (CA2), respectively], and in addition the antibody reacting with thyroid microsomes.

The main methods of detecting thyroglobulin antibodies are precipitin techniques, which have been modified into latex fixation tests, tanned red cell agglutination (D6), and sensitive immunofluorescence techniques. The older precipitin test was a gel diffusion technique carried out on an Ouchterlony plate. The latex fixation test is slightly more sensitive than the precipitin test, but the sensitivity of the tanned red cell (TRC) exceeds both of the others (A10). This is not necessarily an advantage, however, when testing patients for lymphocytic thyroiditis as it is positive in many symptomless patients with nonprogressive focal thyroiditis. Thyroid autoantibodies can also be found with sensitive techniques in patients without evidence of thyroid disease (H1). Therefore less sensitive techniques, such as the latex slide agglutination and an electroprecipitin test, are probably preferable in the diagnosis of lymphocytic thyroiditis (R2).

In the investigation of mild or incipient forms of idiopathic myxedema, it is advantageous to use the sensitive TRC test and fluorescent antibody tests as well, as a very high proportion will have positive results by one or other of these tests. The finding of thyroid antibodies in the serum of patients with hypothyroidism strongly suggests that the etiology of the hypothyroidism is idiopathic myxedema rather than secondary pituitary hypothyroidism (V1). Furthermore, 80% of Graves' disease patients have positive tests (either TRC or fluorescent antibody), which is a useful observation in the differential diagnosis of thyrotoxicosis and anxiety states.

Some patients with lymphocytic thyroiditis have antibodies directed against the microsomal or cellular fractions only. Others have positive results for the thyroglobulin antibody, but have no antibodies against the microsomal fraction. The microsomal antibody is the most significant in relation to the etiology of thyroiditis as it is cytotoxic to cultured thyroid cells (D6). Microsomal autoantibodies are detected either by complement fixation technique (CFT) or by immunofluorescence. Be-

tween 80 and 90% of patients with lymphocytic thyroiditis will produce a positive result usually in a high dilution of serum, and 70% of patients with Graves' disease have a positive result, but in lesser dilution.

There are indications that lymphocytic thyroiditis is increasing in incidence in all parts of the world, particularly in children (H28). This may be a reflection of increasing awareness of the condition rather than a true increase in its frequency. It is possibly the most common cause of goiter in children. There is a strong possibility that it is genetically determined and sometimes secondary to a dyshormonogenetic defect, particularly the formation of an abnormal iodinated protein (D3).

It is now generally accepted that Graves' disease is one of the autoimmune thyroid diseases, and a variable degree of lymphocytic infiltration may be found in the thyroid gland. As discussed above, antibodies against both the thyroglobulin and microsomal fractions are found in Graves' disease usually in low titer although 5% have positive precipitin tests or positive results in high dilution with more sensitive tests. Thyroid autoantibody tests in Graves' disease have an important practical application. If antibodies are detected in a high dilution or with the comparatively insensitive precipitin test, an alternative treatment to surgery should be considered as these patients have a much higher risk of developing hypothyroidism after subtotal thyroidectomy (H6).

Whereas there has been little recent development in the methods for detecting thyroid autoantibodies and in the diagnosis of lymphocytic thyroiditis, there are interesting advances in the concepts of pathogenesis of this type of disorder, consequent on the rapid increase in knowledge of the science of clinical immunology. In a recent review, Irvine (I7) summarizes recent findings which suggest that a subpopulation of T lymphocytes has an important suppressor effect on the tendency for B lymphocytes to form autoantibodies. When the suppressor T lymphocytes are absent or lacking in function, autoimmunity tends to emerge according to the precise nature of the T cell deficiency.

It is probable that the more recently discovered immunoglobulins, all in the IgG class, such as long-acting thyroid stimulator (LATS) and LATS protector, should be classified as thyroid autoantibodies and discussion on these follows in the next section.

4.4. Thyroid-Stimulating Immunoglobulins

Recent years have seen exciting advances in the recognition of thyroid-stimulating immunoglobulins of the IgG class, which have an extremely important bearing on the etiology of Graves' disease. It would be premature for clinical chemistry departments, except those with special inter-

ests, to consider diagnostic procedures for these substances, as their exact diagnostic role remains to be elucidated and the techniques involved are complex in nature.

Adams and Purves (A6) reported the presence of the abnormal thyroid stimulator, LATS, in the serum of some patients with thyrotoxicosis. LATS stimulates the thyroid activity in humans and in many other species also. Even with concentration procedures it is not possible to detect LATS in the serum of all cases of Graves' disease, and furthermore, LATS activity does not correlate well with thyroidal activity. Fifteen years after the discovery of LATS, Adams and Kennedy (A5) published details of another thyroid stimulator, LATS protector (LATS.P) which is present in the sera of nearly all patients with Graves' disease and which appears to stimulate only the human thyroid. It is therefore probable that, in LATS-negative patients with Graves' disease, LATS.P is the pathogenic agent (I7). Both LATS and LATS.P are considered to be thyroid autoantibodies. It is possible to assay both LATS and LATS.P in a technically complex method of extreme sensitivity as published by Bitensky (B8). This is one of a series of cytochemical assays developed in the Kennedy Institute of Rheumatology in London, U.K.

Another landmark in this field is the demonstration by Smith and Hall (S12) that thyroid-stimulating IgG fractions displace TSH from its binding sites on human thyroid membranes in a system based on radioreceptor assays for TSH in guinea pig thyroid homogenate (M6). Smith and Hall found evidence of TSH being displaced by IgG in the serum of most cases of Graves' disease but only by a small proportion of patients with lymphocytic thyroiditis and malignant thyroid tumors. They concluded that the human specific thyroid stimulator, LATS.P, is probably identical with the activity of the Graves' immunoglobulins responsible for displacing binding of TSH to human thyroid membranes (S12). Clearly this is an area which is rapidly developing and which has potential for diagnosis in the difficult cases of Graves' disease as well as in throwing further light on its etiology.

5. Functional Physiological Tests

The purpose of this section is to summarize those procedures carried out on patients which depend upon certain physiological aspects of thyroid function including the interrelationship of the thyroid gland with the hypothalamus and the anterior pituitary glands. The TSH stimulation and the T_3 suppression tests are only briefly discussed, mainly for historical reasons, as both are becoming redundant because of more recent developments such as TSH and T_3 assays, and the diagnostic use of thyrotropin releasing hormone.

5.1. TSH Stimulation Test

The TSH stimulation is primarily a test of thyroid reserve and has three main applications: (1) to identify those patients with minor impairment in thyroid function where the conventional thyroid function tests produce normal results; (2) to determine whether or not a patient, who is receiving thyroid therapy for presumed idiopathic myxedema, requires this therapy; (3) to distinguish between idiopathic (primary) myxedema from hypothyroidism secondary to pituitary disease.

Normally there is a brisk increase both in the thyroid uptake of ^{131}I and in serum levels of total T_4 following intramuscular TSH injection. If doses of 10 USP units of TSH are given on three consecutive days, a normal subject will respond with a rise in serum total T_4 of at least 3 μg/100 ml from baseline level, blood being taken 8 hours after the last injection. The 24-hour thyroidal uptake should increase by 2- to 3-fold or by an absolute increment of 15–20% of the dose compared with the initial 24-hour uptake. Care must be taken if using this 3-dose schedule in elderly patients or where ischemic heart disease is present.

There is definitely an overlap in responses obtained in normal subjects and those obtained in patients with partial thyroid deficiency, even if the number of injections of TSH is reduced. However, failure of the thyroid uptake and total T_4 to increase after TSH is strong evidence of primary thyroid disease. In the patients receiving thyroid hormone therapy, a significant response to TSH fairly conclusively excludes underlying thyroid failure unless the patient has been taking this therapy in full doses for many years. The test is probably most useful in distinguishing idiopathic thyroid failure from secondary or pituitary hypothyroidism.

A single estimation of serum TSH which is so convenient, has replaced the TSH stimulation test which was inconvenient and sometimes dangerous for the patient. A definitely elevated level of serum TSH, e.g., in excess of 30 μU/ml, is very convincing evidence of impairment of thyroid reserve (H4, I8). A finding of a normal TSH with a definitely lowered FTI and T_3 concentration in the serum, in a clinically hypothyroid patient, is good evidence for pituitary disease. Also see Sections 4.1.1 or 4.1.2 for further discussion on the usefulness of the TSH assay.

5.2. Triiodothyronine Suppression Test

The principle of the T_3 suppression test is that supraphysiological doses of T_3 are administered which normally inhibit TSH production

by the pituitary with consequent reduction in thyroid function, thyroid uptake of ^{131}I, and serum total T_4 concentration. If, however, the thyroid is autonomous and is not under pituitary TSH control, the T_3 administered will in no way affect thyroid activity. Until very recently, the test was most frequently used to help in the diagnosis of borderline hyperthyroid states, as nearly all patients with hyperthyroidism, whether Graves' disease or toxic nodular goiter, have an autonomous thyroid. On the other hand, failure to suppress does not necessarily imply hyperthyroidism but does indicate autonomy as, for example, accompanying euthyroid Graves' disease or autonomous functional ("hot") nodules.

The T_3 suppression test was introduced by Werner (W17), and the usual regime is to carry out a 3- and 24-hour uptake of ^{131}I or ^{132}I and take a blood for total T_4. The T_3 is then given orally in a dose of either 40 μg three times daily, or 20 μg four times daily, depending on the age and cardiac condition of the patient, and after 7–10 days thyroid uptake and total T_4 are measured. Suppression is indicated by the 24-hour thyroid uptake falling to less than 50% of the initial uptake and the total T_4 to approximately 2 μg/100 ml, or less. If no suppression takes place, the thyroid uptake values commonly increase slightly. Modifications of this regime have been suggested, some to make the test safer for the patient (F10) and others to enhance the usefulness of the test in distinguishing between the normal and the autonomous gland (C7).

The T_3 suppression has very recently been displaced by two other tests, namely the estimation of serum T_3 concentration and the TRH stimulation test. A single estimation of serum total T_3 will frequently be helpful where there is clinical hyperthyroidism even if the patient has T_3-thyrotoxicosis. In clinical situations where it is desirable to demonstrate that the thyroid gland is autonomous, the TRH stimulation test has replaced the T_3 suppression test.

5.3. Thyrotropin-Releasing Hormone Studies

It is beyond the scope of this review to discuss, except in brief outline, the hypothalamic substance, thyrotropin-releasing hormone (TRH), and its application in clinical chemistry, although many publications on TRH have appeared over the last five years or so. However, only a limited number of clinical applications of TRH are related primarily to the thyroid gland. Some reviews which include information on TRH have appeared recently (H7, H14, S8). A review on hypothalamic releasing hormones appears in this volume (H6A). Reference to the physiological role of TRH has been made in Section 2.4.

The most obvious clinical response when TRH is administered to man is a sharp rise in serum TSH levels (F1, F12, H14, O9, S14), the shape of the response depending on the dose and method of administration. In most studies, a single bolus intravenous injection of 200 μg TRH has been given, after which TSH rises from a baseline of 0–2 μU/ml in normal subjects, to a peak of about 10–20 μU/ml 20 or 30 minutes later. Females have a greater response than do males. The magnitude is dependent on the TSH methodology, but even in any individual laboratory using the one method, the degree of the normal response is rather variable. Through its TSH effect, TRH injection is associated with a rise in both T_4 (K3, L7) and T_3 (L7, S8), the peak rise in T_3 occurring at about 2 hours after intravenous injection. The rise in T_4 is much less dramatic; it takes several hours to reach the peak and has little diagnostic application.

Shenkman (S8) has suggested that the measuring of T_3 and TSH responses to TRH provides a useful combined test of thyroidal and pituitary reserve. This test may have a place in special areas such as in the investigation of thyrotoxic patients following ^{131}I therapy but usually following the TSH response is sufficient. Hall (H3) suggests that the following standardized test be used: 0 min, blood for TSH; 200 μg TRH in 2 ml of saline rapidly injected; 20 min: blood for TSH; 60 min: blood for TSH.

The main diagnostic applications of TRH are listed below (H3, H14).

1. In hyperthyroidism: Patients with hyperthyroidism either do not respond to TRH or respond in a markedly subnormal fashion. The high circulating levels of thyroid hormone inhibit the normal responsiveness of the thyrotrophes to TSH. Patients with T_3-thyrotoxicosis also fail to respond to TRH. The TRH is thus a useful test to confirm hyperthyroidism when the condition is clinically obvious but the routine thyroid function tests are equivocal.

2. In euthyroid Graves' disease: About 70% of these patients have abnormal suppressibility to T_3 and the TRH test seems a more sensitive and more accurate test to prove autonomy in this group and is more convenient for the patient.

3. In idiopathic hypothyroidism: Usually patients in this group have subnormal levels of thyroid hormones in the blood and markedly elevated serum TSH levels and TRH testing is superfluous. However, in those with early or minimal hypothyroidism or with preclinical hypothyroidism, the TSH level may be minimally elevated and difficult to distinguish from a high normal result. The TRH administration causes an exaggerated response in TSH, which is usually easy to distinguish from the normal response.

4. To separate hypothalamic from anterior pituitary disease: Theoretically, a TRH test seems to be an ideal method for distinguishing these disorders, but anomalous results have been recorded (C1, F2, O13). Discussion on this topic is beyond the scope of this review.

5. In treated Graves' disease: The TRH response in both TSH and serum T_3 concentration may elucidate the thyroid status of this difficult group more readily than do the conventional tests alone. However, TRH responsiveness may not return until some time after the patients are rendered euthyroid (C18). We have made similar observations in our laboratory.

5.4. Thyroid Suppressibility during Drug Treatment for Thyrotoxicosis

Alexander *et al.* (A7) in 1967 suggested that patients whose thyroid was suppressed after 6 months of antithyroid drug therapy, and while still receiving therapy, were likely to be in remission and drugs could be stopped. If there was no suppression, then antithyroid therapy should be continued and the patient be reassessed in a further 6 months. The test is carried out as follows: Patients remain on the appropriate dose of antithyroid drug, and to this is added *l*-T_3 at a dose of 80 μg per day. As the antithyroid drug interferes with the later phases of uptake, it is necessary to perform an early uptake at 10–20 minutes after an intravenous dose, preferably of ^{132}I. In this way, trapping only will be measured, and this is unaffected by the antithyroid drug. Suppression is present if the early uptake is less than 8% of the dose. The advantage of this method of assessing remission is that patients remain on the antithyroid drug throughout the whole procedure. The alternative approach of stopping the drug is difficult for those patients whose thyrotoxicosis exacerbates.

With this technique, the early results were satisfactory and most patients who suppressed on T_3 remained in remission. The work received some support but is not universely accepted. As the group continued their studies, they found that the course of thyrotoxicosis was more variable than that which they had first experienced. This led to a modified regime (A8) in which those patients who showed suppression were continued on antithyroid drugs and T_3 for another 6 months. After that the carbimazole was stopped and the T_3 was continued. The FTI was then used as a parameter to assess whether the patient remained suppressed, in other words, remained in remission. This followup could continue indefinitely with the aid of the patient's local medical officer.

6. The Choice and Uses of Tests in Special Situations

In this section, an attempt will be made to rationalize the use of thyroid function tests in various clinical areas, particularly in those where interpretation can sometimes be difficult. It is superfluous here to discuss in any detail the use of thyroid tests in certain clear-cut areas, such as in confirming the diagnosis in a newly presenting case of Graves' disease or of idiopathic myxedema. What is indicated is a standard regime of either an FTI or ETR (or similar test) or even both. If these measurements are in the overlap region between hypothyroid and normal subjects, a TSH assay is needed. If the routine tests are in the overlap between values for hyperthyroid patients and normal subjects, then a serum T_3 concentration is indicated.

6.1. Dyshormonogenetic Goiter

The steps in the biosynthetic pathway are well known and were briefly discussed in Section 2.1 (Fig. 1). Defects may occur in the various stages of the biosynthetic pathway, leading to a decrease in the amount of available thyroid hormone. The defects may be briefly summarized as follows: The sites are shown on Fig. 1: (1) trapping of blood iodide by the thyroid gland (site 1); (2) "organification," that is, iodination of tyrosine by activated iodine (site 2); (3) coupling, that is, conversion of the tyrosines into T_3 and T_4 (site 3); (4) formation of abnormal forms of thyroglobulin; in this defect the abnormal thyroglobulin (iodinate protein) is released into the blood (site 4); (5) iodotyrosine deiodinase (site 5); (6) thyroglobulin protease deficiency (site 6).

When presented with the problem of diagnosing one of these biochemical lesions in a goitrous child, certain screening procedures should be performed first. These include a measurement of the 2-hour uptake of ^{132}I. It is essential not to administer ^{131}I to young children becaue of the risk of thyroid cancer developing later. If the uptake is very low, a trapping defect is indicated. An elevated uptake indicates any of the other defects, the elevated uptake being one of the compensatory phenomena seen in congenital goitrous hypothyroidism. If the 2-hour uptake is elevated, an oral dose of 1000 mg of potassium perchlorate should immediately be given and the activity over the thyroid measured just 60 minutes after the dose. Perchlorate induces discharge of any iodine accumulated in the gland which has not been organified (M16) and if more than 10% is discharged, there is good evidence of an organification defect.

The other screening tests to be performed are serum total T_4 and PBI estimations. The combination of a high thyroid uptake, negative perchlorate discharge, low total T_4 and a discrepancy between the PBI and total T_4 is likely to be found in either coupling, thyroglobulin or deiodinase defects. The PBI measures both thyroglobulin and the iodotyrosines in addition to T_4. Special procedures are necessary to differentiate these defects.

Chromatographic examination of the serum, or RIA reveals the presence of DIT which would be present in the coupling or deiodinase defects. The deiodinase defect may be detected by following the rate of deiodination of an oral dose of labeled DIT (M9, N4). A thyroglobulin defect can be demonstrated either by RIA of thyroglobulin in the serum or by an indirect chromatographic method on paper, where the DIT from digested thyroglobulin is demonstrated (W5). Finally, a thyroglobulin protease deficiency is demonstrated by special techniques as described by McGirr (M8) in which serum butanol-extractable iodine is shown not to increase after TSH injection.

The ability to identify the exact lesion in a dyshormonogenetic goiter is not as important as the detection of such goiters at the earliest possible opportunity. Patients may have thyroid biosynthetic lesions and remain euthyroid for some time because of compensatory mechanisms, such as enhanced thyroid uptake of iodine (which leads to the goiter) and preferential secretion of T_3 (G8). However, hypothyroidism is likely to occur insidiously and remain unrecognized for a long enough time to cause permanent damage such as mental retardation (F11, W5). Constant awareness of the condition is necessary and, in addition, a satisfactory screening procedure would be worthwhile. Klein *et al.* (K7) suggested the measurement of TSH in cord serum or 3 days after birth, when TSH has reached a stable level, is a suitable screening for congenital hypothyroidism.

6.2. Lymphocytic Thyroiditis (Hashimoto's Disease)

Function tests of thyroid activity have little or no value in establishing the diagnosis of lymphocytic thyroiditis because of the wide spectrum of thyroid activity that may be seen in this condition. Thyroid function is likely to be normal, low normal, or subnormal, but on occasions increased function may be encountered. The diagnosis of this condition rests on a clinical suspicion of its existence, the presence of microsomal or thyroglobulin antibodies at high dilution with purposefully insensitive techniques (as discussed in Section 4.3). Where indicated, the definitive diagnosis is made by biopsy.

However, routine thyroid studies including TSH assay and thyroid uptake of ^{131}I are useful in these patients to ascertain the state of the thyroid function.

6.3. Subacute Thyroiditis (De Quervain's Thyroiditis)

This is a rare but interesting condition in that it is one cause of the "blocked thyroid gland" syndrome, which is characterized by elevated thyroid hormone levels accompanied by zero or very low thyroid uptake of ^{131}I. The block occurs in the first phase of the disease, usually when the patient is complaining of acute pain in the thyroid area. It is caused by thyroglobulin released from the damaged gland breaking down in the periphery and producing the elevated levels of T_3 and T_4 in the serum inhibiting TSH release. It is helpful also to estimate the PBI level, as this is likely to be more elevated than the total T_4 in the serum owing to thyroglobulin in the serum.

Routine studies of PBI, T_4 and thyroid uptake should therefore be carried out in patients suspected of having this condition and be repeated at regular intervals (if the patient is not treated with *l*-thyroxine) to follow the progress of the disease (G5). As the acute phase passes the thyroid uptake will return, and frequently a rebound phase is seen with increased thyroid uptake. Thyroid autoantibodies should be looked for and will be found only in a very low titer (V4) compared with the high titers found in lymphocytic thyroiditis which can sometimes mimic subacute thyroiditis if it presents acutely. Followup studies of total T_4 and thyroid uptake, together with TSH assays, are desirable as some patients gradually go into a state of permanent thyroid failure.

6.4 Differentiation of Idiopathic Hypothyroidism and Hypothalamic or Pituitary Hypothyroidism

If a patient suspected of developing clinical hypothyroidism is investigated only with routine tests, namely total T_4, FTI, and the ETR or a similar estimation, there is no way of determining whether the patient has idiopathic myxedema or hypothyroidism secondary to hypothalamic or anterior pituitary disease. The distinction should be made, however, because it is dangerous to treat pituitary hypothyroidism with thyroid hormone without simultaneously treating with adrenocortical hormones.

In addition to considering the clinical features, the differentiation may be made with a TSH stimulation test as described in Section 5.1, but this has been replaced by a TSH estimation on the serum. The TSH

in pituitary hypothyroidism is undetectable but in idiopathic myxedema it is markedly elevated. Even if there is no goiter, a high percentage of patients with idiopathic hypothyroidism have positive antibodies providing a sensitive technique is used, whereas patients with pituitary hypothyroidism are unlikely to have positive antibodies. If the TSH is low in clinically hypothyroid patients and pituitary disease is therefore suspected, provocative tests of pituitary function are indicated to determine which target organs are involved.

Testing with TRH also distinguishes between idiopathic myxedema and pituitary hypothyroidism because of the exaggerated TSH response in the former compared with the negligible response in the latter (H7, H14, S8). However, for routine clinical diagnosis, the test is not indicated in this setting. The TRH test is also useful for distinguishing hypothalamic from pituitary disease but interpretation is difficult (C21, F2, O13) and is outside the scope of this review.

6.5. Thyroid Hormone Treatment of Hypothyroidism

Recent work has drawn attention to the need for reassessment of the optimum doses required to treat hypothyroidism. Until recently, *l*-thyroxine was commonly given in daily doses of as much as 0.3 mg. It was appreciated many years ago that with doses of 0.3 mg of *l*-T_4 per day, PBI or total T_4 were elevated compared with values obtained in normal subjects. Ranges of total T_4 from 8 to 16 μg/100 ml are expected in the former group whereas the range of values obtained with T_3 uptake is virtually the same as the reference range (W9).

However, the ready availability of RIA for serum T_3 concentration leads not only to an increased insight into the importance of the contribution of serum T_3 to the physiological effects of the thyroid hormones (see Section 2.3), but also to a second method of monitoring thyroid hormone levels in patients on replacement therapy. Furthermore, serum TSH assays are proving useful for this purpose. In 1972 Surks *et al.* (S31) demonstrated that after *l*-T_4 therapy at a dose of 0.2 mg per day, both T_3 and T_4 levels tended to be high normal or just elevated. Evered *et al.* (E8) in 1973 found that the minimum daily dose of *l*-T_4 needed to suppress serum TSH concentration to normal and to relieve symptoms, was frequently as low as 0.1 or 0.15 mg, and no patient required more than 0.2 mg. Almost identical results were obtained by Stock *et al.* (S29), and they showed that nearly all their patients receiving replacement with *l*-T_4 had serum total T_4 levels in the generally accepted range and serum T_3 levels about 130 ng/100 ml, just slightly less than the mean level in normal subjects.

Although clinical judgment is usually very useful in the decision as to whether a patient with idiopathic myxedema is receiving sufficient replacement therapy, there is no doubt that serum TSH and T_3 assays contribute a great deal more. If the patient is receiving *l*-T_3 therapy, only the TSH can be used as serum T_3 levels rise and fall rapidly after each dose. Measurement of TSH is particularly important in treating the elderly patient or a patient with ischemic heart disease, and in these it may not be desirable to suppress TSH to normal levels. The measurement of TSH is also useful in monitoring the *l*-T_4 dose in children, who seem to require a higher dose on a weight basis than do adults.

Patients are sometimes seen who are taking excessive amounts of *l*-thyroxine either as thyrotoxicosis factitia (self-administered) or thyrotoxicosis medicamentosa. These should prove to be no diagnostic problem, as their tests should fall into the category of "blocked thyroid syndrome," and thyroid uptake will be negligible with very high levels of thyroid hormones in the serum, particularly of total T_4.

6.6. T_3-Thyrotoxicosis

The subject of T_3-thyrotoxicosis has already been discussed in Sections 2.3 and 5.2, and is referred to here merely for the sake of completeness. There is only one test required to confirm the diagnosis, namely the total T_3 concentration assay. Any clinically thyrotoxic patient with normal levels of free thyroxine must be investigated with a total T_3 assay. If this is elevated, the patient has T_3-thyrotoxicosis, providing TBG levels are normal. If TBG is elevated, an estimation of the free T_3 concentration in these patients would yield valuable information (C18, E5). If a congenital increase in TBG is suspected, the TBG may be measured either in terms of activity (E6, R7) or as absolute concentration by RIA (L12).

T_3-thyrotoxicosis may have some unusual presentations, for example, the recent case of thyroid storm reported where the total T_4 levels were quite normal, but the T_3 concentration was strikingly elevated (J1).

If doubt remains about the diagnosis of thyrotoxicosis, either T_3-thyrotoxicosis or conventional thyrotoxicosis, a TRH test should be performed. It is well documented that there is either no response or a markedly subnormal response in serum TSH to TRH in any form of untreated thyrotoxicosis. Hall (H3) has recommended a standardized sampling time of 0, 20, and 60 minutes, following the intravenous dose of 200 μg of TRH. However, more frequent and more prolonged sampling may be preferable in the questionably thyrotoxic patients. Our regime includes two baseline specimens taken at 20 and 30 minutes after the

insertion of the in-dwelling needle, which allows the T_3 concentration levels to settle down to a stable baseline. Samples are taken at 10, 20, 30, 60, 120, and 180 minutes after the TRH injection. The monitoring of T_3 levels in the serum sometimes yields useful information, supplementing the TSH data.

6.7. Other Triiodothyronine Hypersecreting States

It is now becoming increasingly recognized that T_3 hypersecreting states other than T_3-thyrotoxicosis exist and can occur in a variety of clinical situations. The common denominator is a slowly decreasing thyroid reserve, which is compensated for, either fully or in part, by a T_3 hypersecretion, which is probably TSH mediated. The total T_4 levels are either low normal or definitely subnormal, and yet none of these patients show any marked evidence of clinical hypothyroidism.

The T_3 compensatory phenomenon has been noted in: (1) iodopenic endemic goiter (D4, K8, P3); (2) dyshormonogenetic goiter (F11, G8, N4); (3) euthyroid endocrine exophthalmos (H16); (4) incipient thyroid failure associated with autoimmune thyroid disease (E7); (5) thyrotoxicosis treated either with antithyroid drugs or with ^{131}I (B5, S23, W8).

These observations are particularly important for the ^{131}I-treated patient. It has long been recognized that there is an unfortunately high incidence of hypothyroidism in this group, and the chances of hypothyroidism probably increase each year after the last dose of ^{131}I (G12). Another relevant point is that quite a high percentage of specimens for thyroid studies sent to a laboratory are from such treated patients. Clearly, to rely on FTI and ETR (or ETR-like) measurements alone in this group would lead to a significant number being classified as hypothyroid when they are in fact euthyroid. The TSH assay by itself does not fully elucidate the situation, as there are a number of recent publications that demonstrate an association of elevated TSH values with low normal or normal T_4 in the serum in clinically euthyroid ^{131}I-treated patients (S11, S13, T8, T10).

All patients in the groups mentioned warrant full investigation with T_3 and TSH assays as well as the routine thyroid tests.

7. Conclusion

There are dangers as well as benefits from a constantly enlarging range of tests available for investigating a patient with suspected thyroid disease. The patient's well-being is threatened if a large body of clinical data is allowed to come between the patient and his physician. There

is a definite risk that the physician will become absorbed in the data, and so overlook symptoms and signs. He may forget to ask the patient whether he feels any better or may forget to put his finger on a thyroid nodule or to take an exophthalmometer reading. No matter how sophisticated is the test, nothing can substitute for an expert clinical appraisal. Clinical chemists and clinical endocrinologists are interdependent in their responsibility of coping with the greatly increasing amount of data for which they need assistance from system analysts and computer programs (R8).

There is also a danger of the patient feeling that no one is taking a personal interest in him. He therefore fails to keep appointments, is lost for "follow-up," and consequently is at risk, either from overtreatment or from undertreatment at a later stage. This problem can be overcome by the automated follow-up registers, such as the one operating successfully from Glasgow (B9).

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Addenda

Addendum 1 (*Section 2.3, p. 111*): Recently Chopra (C7a) demonstrated that 3,3′,5′-triiodothyronine or "reverse" T_3 (rT_3) is present in normal serum in a concentration of approximately 40 ng/100 ml and that there are large increases in rT_3 concentration in the serum of the newborn when serum T_3 concentration is very low. As rT_3 has no physiological activity, he postulated that deiodination of T_4 may switch from T_3 to rT_3 as regulatory mechanism controlling the biological action and metabolism of T_4.

Addendum 2 (*Section 3.7.2, p. 137, after 1st paragraph*): The decreased concentration of serum T_3 in the neonatal period is accompanied by a greatly increased concentration of 3,3′,5′-triiodothyronine or reverse T_3 (C7a).

Addendum 3 (*Section 4.4, p. 145*): Subsequently this group (M16a) demonstrated a reduced frequency of detection of thyroid-stimulating immunoglobulins in thyrotoxic patients following treatment. The reduction following surgical treatment was much greater than treatment with either anti-thyroid drugs or radioactive iodine. Furthermore, there was a significant correlation between the serum thyroid-stimulating immunoglobulin level and some indices of thyroid function, particularly thyroid uptake of ^{131}I at 1 hour. It was also shown that the effects of serum thyroid-stimulating immunoglobulins on the binding of TSH to thyroid membranes correlate well with their effect in activating membrane adenyl cyclase. These observations suggest that thyroid-stimulating immunoglobulins are likely to be responsible for the hyperthyroidism of Graves' disease (M16a).

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THE HYPOTHALAMIC REGULATORY HORMONES AND THEIR CLINICAL APPLICATIONS

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1. Introduction

The hypothalamus controls the function of the anterior pituitary gland by the production of a series of chemical messengers. These are synthesized in areas of the hypothalamus not yet well defined in man. They are transported to the median eminence, probably by a process of axonal flow similar to that involved in the transport of the posterior lobe hormones. They are stored in the median eminence, from which they are released in response to certain stimuli, passing in the portal venous system down the pituitary stalk to the anterior lobe, where they affect the synthesis and release of the pituitary hormones.

There has been some debate as to the terminology of these chemical messengers secreted by the hypothalamus. The authors accept the terminology suggested by Andrew Schally, a pioneer in this field, who describes them as hormones. Since they may either stimulate or inhibit the release and synthesis of the anterior lobe hormones, they are better referred to as regulatory hormones than as releasing hormones. There seems no reason to avoid the term hormone when the active material has been isolated, identified, and synthesized. When the material is merely defined by its biological action in a hypothalamic extract, the term regulatory factor is appropriate.

So far three distinct hypothalamic regulatory hormones have been fully characterized (Table 1), thyrotrophin-releasing hormone (TRH), first identified by Schally and his colleagues and by Guillemin and his group, luteinizing hormone/follicle-stimulating hormone-releasing hormone (LH/FSH-RH), first identified by Schally and his colleagues, and growth hormone-release inhibiting hormone (GH-RIH), first identified by Guillemin and his colleagues.

A variety of other factors have been detected in hypothalamic extracts, and these are listed in Table 1.

The hypothalamic–pituitary system has been likened to a "cascading amplifier" (B1, B2) whereby the hypothalamic signal causes the release of larger amounts of anterior pituitary hormones, which in turn lead to the production of even greater amounts of target gland hormones (Fig. 1).

In this chapter we shall consider only the three regulatory hormones whose structures have been established and which have been studied in man.

2. Thyrotropin-Releasing Hormone (TRH)

Thyrotropin-releasing hormone was the first hypothalamic regulatory hormone to be characterized. Its existence had been postulated since

TABLE 1
THE HYPOTHALAMIC REGULATORY HORMONES

Anterior lobe hormone	Regulatory hormone or factor	Structure
Thyrotropin	Thyrotropin-releasing hormone (TRH)	Tripeptide
Prolactin	Prolactin-releasing hormone (PRH) ?different from TRH	Tripeptide
	Prolactin-release inhibiting factor (P-RIF)	?, dopamine
Luteinizing hormone and follicle-stimulating hormone	Luteinizing hormone/follicle stimulating hormone-releasing hormone	Decapeptide
Growth hormone	Growth hormone-release inhibiting hormone (GH-RIH)	Tetradecapeptide
	Growth hormone-releasing factor	?
Corticotropin	Corticotropin-releasing factor	?
Melanocyte-stimulating hormone	Melanocyte-stimulating hormone-release inhibiting hormone (M-RIH)	Tripeptide ?Active in man
	Melanocyte-stimulating hormone-releasing factor (MSH-RF)	?

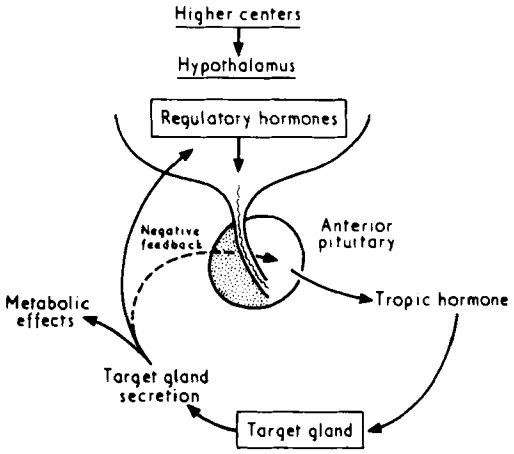


FIG. 1. Relationships between hypothalamus, pituitary, and target organs.

1951 (G14), but it was not until 15 years later that its isolation from porcine hypothalami was achieved (S4).

2.1. STRUCTURE

Elucidation of the structure and subsequent synthesis of porcine (F5) and ovine (B14) TRH established its nature as a tripeptide:

Pyro-Glu-His-Pro-amide

Porcine, ovine, bovine, and human TRH appear to have the same structure, and immunoreactive TRH has been found in hypothalamic extracts from man, pig, rat, hamster, chicken, frog, snake, salmon, as well as in the whole brain of the lamprey and the amphioxus (J1).

2.2. Biosynthesis and Action

Biosynthesis of TRH occurs in a wide area of the hypothalamus and appears to be under the control of a nonribosomal (soluble) enzyme system, TRH synthetase (R5), which is activated by norepinephrine (G15). TRH is stored in the median eminence, from which is secreted into the hypophysial venous portal system to be transported to the anterior pituitary gland (R2). There it is specifically bound to membrane receptors (G13, W3) and activates adenyl cyclase, leading to increased production of cyclic adenosine monophosphate (K1).

2.3. TRH Analogs and Inactivation

A number of TRH analogs with greater activity have been synthesized, although an intact amide group and the cyclic glutamic acid terminus appear to be essential for activity (R4). The presence of a cyclic structure at one end of the TRH molecule, and an amide group at the other, may explain some of its biological characteristics, such as activity after oral administration (P5). TRH is rapidly inactivated in plasma by enzymic cleavage of the amide group (N1) and is excreted by the kidney and the liver (R2). The half-life of TRH is about 4 minutes.

2.4. TRH Actions in Normal Man

Administration of synthetic TRH to humans causes a dose-related release of thyrotropin (TSH) by the pituitary (B10, H1) between the intravenous doses of 15 and 500 μg. Oral, subcutaneous, or intramuscular administration requires bigger doses. The TSH response to intravenous TRH is significant within 2–5 minutes, peaking at 20–30 minutes with a return to basal levels by 2–3 hours. An elevation in thyroid hormone levels in response to TRH is seen, with triiodothyronine (T_3) peaking at 3 hours and thyroxine (T_4) at 8 hours (L1). TRH stimulates the synthesis as well as the release of TSH (M5).

2.5. TRH and the Pituitary-Thyroid Axis

The TRH-induced TSH release is modulated by the circulating levels of thyroid hormones (Figs. 2 and 3). These act as suppressors of TSH

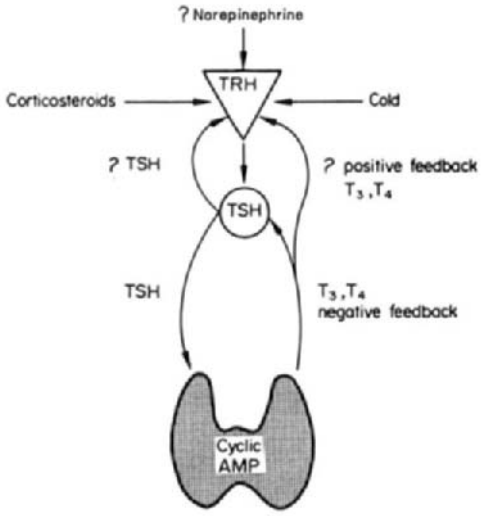


FIG. 2. Hypothalamic–pituitary–thyroid axis. T_3, triiodothyronine; T_4, thyroxine; TRH, thyrotropin-releasing hormone; TSH, thyroid-stimulating hormone.

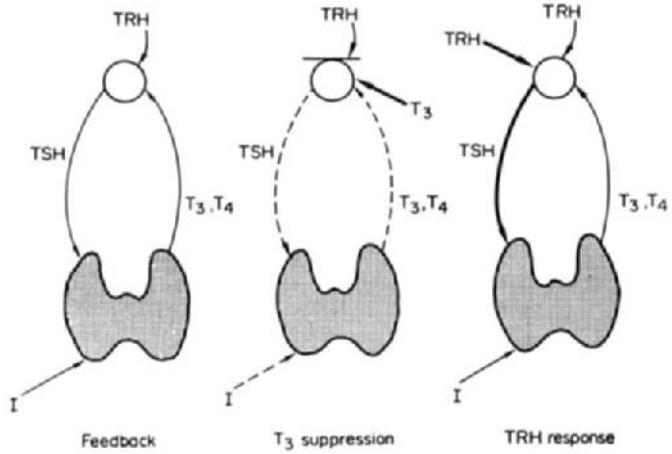


FIG. 3. Normal feedback situation with normal levels of circulating T_3 and T_4 allowing normal action of TRH on TSH release, normal TSH levels act on thyroid to allow normal iodine uptake. T_3 suppression: Administered T_3 suppresses the action of TRH on TSH release, TSH levels fall and thyroidal iodine uptake is reduced—normal T_3 suppression. TRH test: Administered TRH increases TSH release, but in the standard test at 20 minutes there is no effect on thyroid iodine uptake or thyroid hormone release.

release at a pituitary level. Both T_4 and T_3 are important in this negative feedback system, although T_3 appears to have higher affinity for the nuclear receptors in the thyrotroph cell. We have seen patients with elevated levels of T3 and low levels of T4 in whom the TSH response to TRH was exaggerated (G5). This would not be expected if T3 were the sole inhibitor of the TRH-induced TSH release. The blocking effect of T4 and T3 appears to be mediated by induction of the synthesis of a protein suppressor in the thyrotroph. Although it seems clear that the negative feedback of thyroid hormones operates at a pituitary level, their effect on TRH production is still controversial. It has been reported that T_3 and T_4 stimulate TRH synthetase activity (R5), hence exerting a positive feedback at a hypothalamic level, but the experimental work supporting this hypothesis remains to be confirmed. Prolonged exposure of animals to cold increases TRH synthesis (R5) and activates the hypothalamic–pituitary–thyroid axis. This mechanism probably operates in the newborn and explains the higher TSH levels seen in the early stages of extrauterine life. We have not observed this phenomenon in adults after short-term cooling.

2.6. Other Factors Affecting the Thyroid-Stimulating Hormone (TSH) Response to TRH

Administration of theophylline enhances the TSH response to TRH (F1). Administration of estrogen to men also enhances the TRH-induced TSH release (F2), and in the intact human, females exhibit a greater response than males (O2) (Fig. 4). Supraphysiological doses of corticosteroids suppress the TSH response to TRH (W4), and this inhibitory effect is also seen in patients treated with *l*-dopa (S10). The administration of growth hormone-release inhibiting hormone (GH-RIH, somato-

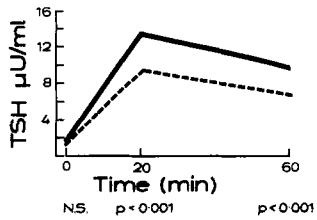


Fig. 4. Mean response to thyrotropin-releasing hormone (200 μg i.v.) in males (---, 20 subjects) and in females (—, 25 subjects). TSH, thyroid-stimulating hormone.

statin) inhibits the TSH response to TRH in a dose-related manner (C1, H3). We do not find obvious differences in TSH response to TRH when matching age groups or referring the response to body surface area.

2.7. Specificity

The hypophysiotropic actions of TRH are not specific for TSH. It consistently releases prolactin (PRL) when administered to man (Fig. 5) and animals (J2). A maximal response is obtained with intravenous doses of 100 μg. It is evident that the mechanism controlling PRL and TSH release after TRH are independent, since PRL release is not affected in isolated thyrotropin deficiency (S1) and the PRL response to TRH is not affected by GH-RIH in spite of complete blockage of TSH release (C1, H3). In men, TRH produces a small but consistent release of follicle-stimulating hormone (FSH) (M10). This response is abolished by estrogen administration whereas the TSH response is enhanced in these subjects (M6). Franchimont reported that TRH releases luteinizing hormone (LH) in some females at mid-cycle (F6). TRH also causes growth hormone (GH) release in some patients with chronic renal failure (G3, G12) and in patients with acromegaly (F3, G11, I1), (Fig. 6), and this effect can be blocked by administration of GH-RIH (G11). In acromegalics showing a GH response to TRH, complete suppression of GH levels with bromocriptine (2-broma-ergocryptine,

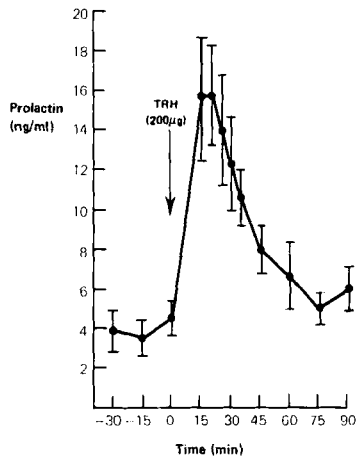


Fig. 5. Effect of TRH on prolactin release (mean ± SEM) of six normal men.

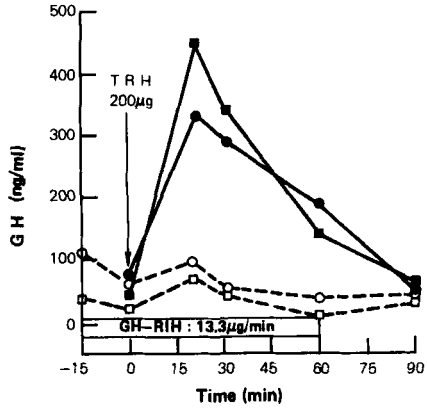


FIG. 6. Effect of growth hormone-release-inhibiting hormone (GH-RIH) on the thyrotropin-releasing hormone (TRH)-mediated GH release in two patients with active acromegaly. ■, ●, control responses; ○, □, responses after GH-RIH.

CB 154) does not abolish the TRH-mediated GH release (G9).

2.8. Clinical Applications

2.8.1. *Diagnostic*

2.8.1.1. *The Standard TRH Test.* Ormston *et al.* (O2) devised a standard intravenous TRH test that has proved to be a valuable tool in the diagnosis of thyroid disease. Synthetic TRH (now commercially available), 200 μg, is given as an intravenous bolus, and blood is sampled for immunoreactive TSH at 0, 20, and 60 minutes (Fig. 7). Basal routine thyroid function tests (T_3, T_4, thyroid hormone-binding capacity) are also estimated in the 0 sample. The test is free from significant side effects, although about half the patients experience a transient metallic taste, deep uretheral sensation, flushing of the face, and mild nausea, all of which pass off within 3 minutes of the injection. Although the 60-minute sample is helpful to detect delayed responses, in routine clinical practice the peak sample (20 minute) is enough to confirm the diagnosis of thyroid disease. It is now our practice to perform the TRH test during an out-patient visit when indicated. Women show a greater response during the follicular phase of the cycle (S2), and in a given subject, the response is greater at 23.00 hours (W1), but these differences may be disregarded when the test is performed on a routine clinical

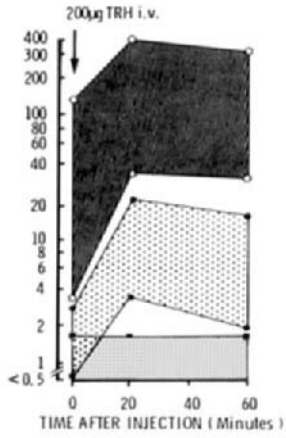


FIG. 7. Serum thyroid-stimulating hormone (TSH) response to thyrotropin-releasing hormone (TRH) in normal subjects and in patients with hyperthyroidism and primary hypothyroidism. Ordinate: serum immunoreactive TSH (μU/ml). ⊠, Normal controls ($n = 45$); ▨, primary hypothyroidism ($n = 26$), serum TSH > 21 μU/ml 20 minutes after TRH; ⊡, thyrotoxicosis ($n = 35$), serum TSH < 1.8 μU/ml 20 minutes after TRH.

visit. Previous therapy with thyroid hormone should be discontinued 3 weeks (for T_4) and 2 weeks (for T_3) before the test is performed.

2.8.1.2. *Results in Primary Hypothyroidism.* Patients with primary hypothyroidism have an exaggerated TSH response to TRH, but the elevated basal TSH is usually enough evidence to confirm the diagnosis, and the TRH test is not required. However, as the TSH response to TRH is proportional to basal levels, the minimally elevated basal TSH levels seen in some cases of subclinical or mild hypothyroidism may be separated from borderline TSH values due to assay artifacts by the use of TRH.

2.8.1.3. *Results in Hyperthyroidism.* Patients with hyperthyroidism fail to respond to TRH because of the elevated circulating levels of T_3 and T_4. The main diagnostic application of the TRH test is in the exclusion of mild or subclinical hyperthyroidism, since a normal response absolutely excludes this diagnosis. However, an impaired or absent response to TRH is not in itself pathognomonic of hyperthyroidism since it can be seen in patients with ophthalamic Graves' disease (O1), autonomous thyroid adenomata (E1), supraphysiological replacement with T_4 or T_3 for hypothyroidism and in some subjects rendered clinically and biochemically euthyroid after treatment of hyperthyroidism (V4). This transient lag in restoration of normal TSH response to TRH makes the

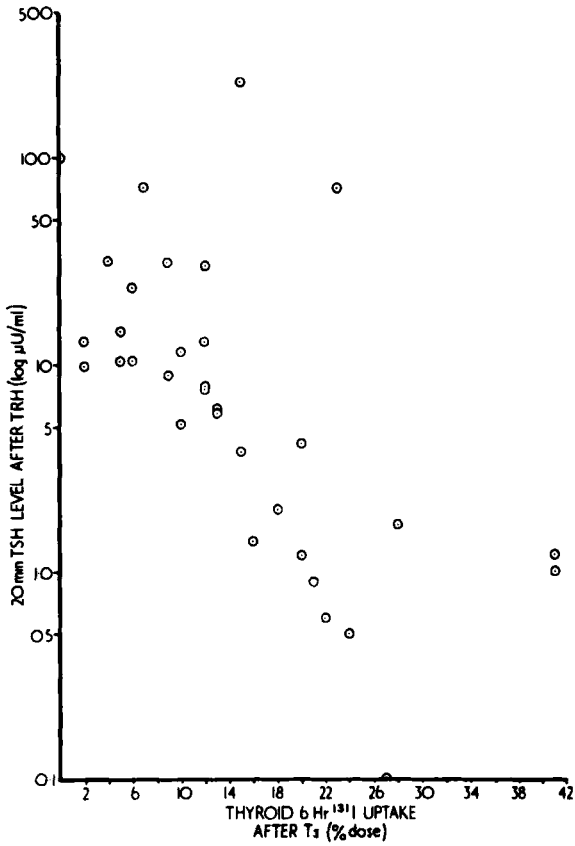


FIG. 8. Relationship between 20-minute level of serum thyroid-stimulating hormone (TSH) after 200 μg of thyrotropin-releasing factor (TRH) i.v. and the 6-hour thyroidal ^{131}I uptake after one week on triiodothyronine (T_3), 100 μg daily.

TRH test unreliable in the follow-up and monitoring of antithyroid therapy for a period of up to 4 months after clinical remission has occurred.

2.8.1.4. *TRH Test and Thyroid Suppressibility.* The TSH response to TRH correlates well with thyroid suppressibility (O1) and has now replaced the outdated T3 suppression test (Fig. 8). It is more rapid, less expensive, safer, and does not require administration of radioisotopes or a potentially dangerous drug to the patient.

2.8.1.5. *Results in Pituitary–Hypothalamic Disease.* Patients with hypothyroidism due to pituitary disease usually fail to respond to TRH. The TRH test is a useful tool in the assessment of pituitary reserve of TSH in patients with pituitary lesions (H2). Patients with hypotha-

lamic disease may show a characteristic response in which the 60-minute TSH value is higher than that obtained at 20 minutes. This "delayed" type of response may also be seen in patients with primary pituitary disease, although the pressure effects of the tumor or the effect of previous therapy may play a role in causing hypothalamic damage. In patients with anorexia nervosa, a "hypothalamic type" of response is often seen, which returns to normal after restoration of normal weight.

2.8.1.6. *Results in Chronic Renal Failure.* The TRH test provides unreliable information in patients with impaired renal function. An exaggerated response may be due to poor renal clearance of TRH (G12), and we have observed impaired, absent, or delayed TSH responses (G3) without any evidence of hypothalamic–pituitary or thyroid disease in a group of patients with chronic renal failure.

2.8.2. *Therapeutic*

So far no definite therapeutic applications have been described for TRH. It has been suggested that it has antidepressant effects, but further controlled studies (M15) did not substantiate the initial reports. The role of TRH as a tool in the treatment of thyroid carcinoma (by increasing uptake of the therapeutic dose of radioiodine by the tumor) (F4) is as yet not confirmed. TRH could be used in the treatment of hypothalamic (tertiary) hypothyroidism, but it does not offer any advantage over thyroid hormone replacement. We have not observed any beneficial effect of TRH in regenerating thyroid remnants after surgery. It has been reported that TRH administration to cows results in an elevation of PRL which increases milk production (K3). Further controlled studies of the applications of TRH in the dairy industry are required.

3. Luteinizing Hormone/Follicle-Stimulating Hormone-Releasing Hormone (LH/FSH-RH)

The elegant experiments of Harris (H4, H5) and his colleagues indicating a hypothalamic control of gonadotropin secretion from the pituitary stimulated an intense and successful search for the factor involved.

3.1. Structure

The isolation of animal hypothalamic extracts with gonadotropin releasing properties (S3) was immediately followed by the determination of structure (M4) and subsequent synthesis (M3) of a decapeptide:

Pyro-Glu-His-Trp-Ser-Trp-Gly-Leu-Arg-Pro-Gly-amide

This remarkable contribution of Andrew Schally's group to clinical endocrinology enabled investigators to carry out extensive clinical evaluation of this hormone. This material, named luteinizing hormone/follicle-stimulating hormone-releasing hormone (LH/FSH-RH), like TRH has a pyroglutamyl group at the amine terminal and an amide group at the carboxyl terminal. This structure is also shared by oxytocin and vasopressin and some other nonhypothalamic oligopeptide hormones. Both the natural and synthetic LH/FSH-RH have identical biological activity and as in the case of other hypothalamic regulatory hormones LH/FSH-RH has no phylogenetic specificity, its effects can be demonstrated in man as well as in a variety of animals.

3.2. Biosynthesis and Actions

LH/FSH-RH causes release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. It has been suggested that a hormone with pure FSH-releasing properties distinct from LH/FSH-RH might exist (B9); however, experiments *in vivo* have not substantiated this hypothesis. There is now increasing evidence that a single hormone, LH/FSH-RH, controls the secretion of both gonadotropins, the differential effects on LH and FSH being explained by changes in pituitary responsiveness induced by feedback control of gonadal steroids. In animal studies, production of antibodies against LH/FSH-RH blocks the release of both LH and FSH and prevents ovulation (A4). It has been impossible to separate LH from FSH-releasing activity in a vast number of LH/FSH-RH analogs synthesized. We therefore accept the concept of a single gonadotropin-releasing hormone of physiological importance as proposed by Schally.

LH/FSH-RH is formed in the hypothalamus, particularly in the basal-ventral area, but the precise location of its origin remains to be elucidated. By the use of immunohistochemical techniques, LH/FSH-RH has been found in the arcuate nucleus and in the median eminence (P1). Electron microscopy studies revealed that some neural axons of the median eminence contain immunopositive LH/FSH-RH (P4). Norepinephrine appears to be the most important neurotransmitter regulating LH/FSH-RH biosynthesis. From this origin, LH/FSH-RH is transported, presumably by axonal flow, to the median eminence, where it is stored. It is secreted into the hypothalamic–pituitary portal system to reach the anterior pituitary gland, where it binds to membrane receptors on the LH- and FSH-producing cells. It has been shown that LH/FSH-RH induces ultrastructural changes in the gonadotropin-pro-

ducing cells (R6), and there is good evidence that the action of LH/FSH-RH is mediated by activation of the adenylate cyclase system in the gonadotrophs (B7). LH/FSH-RH is rapidly degraded in the blood by enzymic cleavage of the pyro-Glu-His group from the amino residue (R1), and it is excreted by the kidney. The half-life of LH/FSH-RH is about 4 minutes.

3.3. LH/FSH-RH Actions in Man

The effects of LH/FSH-RH in man are seen within 2 minutes of an intravenous injection and are dose dependent within the range of 25–100 μg (B3) (Figs. 9 and 10). The increment in LH is greater than that of FSH in adults, and the release of both hormones appears to be simultaneous in response to an intravenous bolus of LH/FSH-RH. When the decapeptide is administered as an infusion, the release of FSH anticipates that of LH, perhaps indicating that the threshold for FSH secretion is lower than for LH. Administration of LH/FSH-RH to prepubertal subjects causes a significant release of both gonadotropins, indicating that the pituitary is responsive and that the hypothalamus (or perhaps higher centers) are primarily responsible for the initiation and maintenance of puberty. The FSH response to LH/FSH-RH predominates over the release of LH in prepubertal subjects. LH/FSH-RH also stimulates the synthesis of LH and FSH (R3).

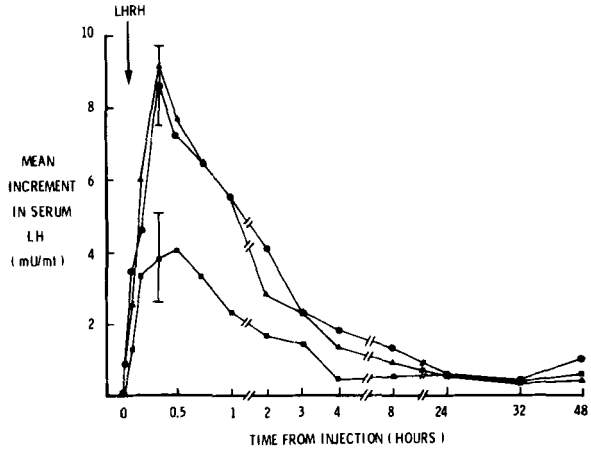


Fig. 9. Effect of LH/FSH-RH on serum LH in four normal men. Dose of LHRH: ●, 100 μg; ▲, 50 μg; ■, 25 μg. Vertical brackets: mean ± 1 SE.

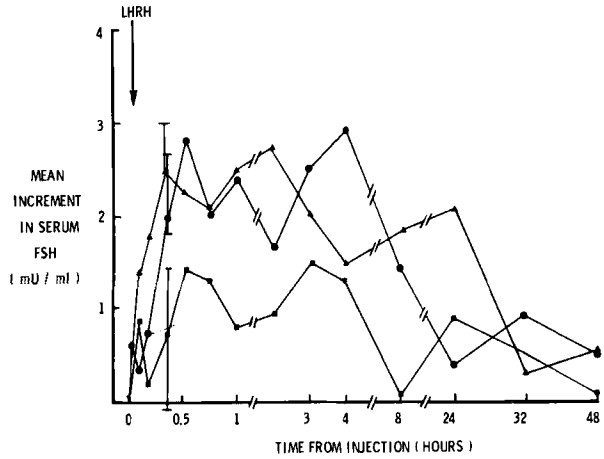


FIG. 10. Effect of LH/FSH-RH on serum FSH in four normal men. Dose of LHRH: ●, 100 μg; ▲, 50 μg; ■, 25 μg. Vertical brackets: mean ± 1 SE.

The time course of release of LH and FSH appears to be the same whether the route of administration of LH/FSH-RH is subcutaneous, intramuscular, or intravenous (M7). The intranasal route is also effective but requires much larger doses.

3.4. LH/FSH-RH AND THE PITUITARY–GONADAL AXIS

The regulatory effects of gonadal steroids upon the gonadotropin response to LH/FSH-RH are as yet poorly understood. In males, administration of estrogens suppresses the response (M8), but large doses of testosterone have only a slight suppressive effect. Chronic administration of testosterone to male subjects has been shown to suppress basal levels of gonadotropins (M13), although the response to LH/FSH-RH was little affected. This may indicate that androgens act at a hypothalamic level exerting a negative feedback, thus reducing the production of LH/FSH-RH, and the minor effect at a pituitary level may be mediated by prior conversion to estrogens. In the female, estrogens may enhance the gonadotropin response to LH/FSH-RH, exerting a positive feedback effect at a pituitary level, but also at the hypothalamus. Arimura and co-workers have observed a rise in immunoreactive LH/FSH-RH at midcycle (A5). Large doses of estrogens may suppress the gonadotropin response, and we have seen impaired responses in women taking the contraceptive pill. Progesterone or a combination of estrogens and pro-

gesterone inhibit the gonadotropin response to LH/FSH-RH (A8). This differential pituitary sensitive to gonadal steroids may be responsible for the modulation of pituitary responsiveness during the cycle. In males it has been demonstrated that a protein hormone, inhibin, produced during the early phases of spermatogenesis, has a negative feedback effect on FSH release. This probably accounts for the finding of men with oligo- or azoospermia who show a normal LH but an exaggerated FSH response to LH/FSH-RH.

In healthy subjects LH/FSH-RH is specific for LH and FSH, but patients with acromegaly occasionally show a GH response to this decapeptide (G11).

3.5. Clinical Applications

3.5.1. *Diagnostic*

3.5.1.1. *The Standard LH/FSH-RH Test.* The diagnostic value of LH/FSH-RH is not as useful as that of TRH. Besser and co-workers (B3) devised an intravenous LH/FSH-RH test that involved the administration of 100 μg of this material with blood sampling at 0, 20, and 60 minutes for estimation of immunoreactive LH and FSH.

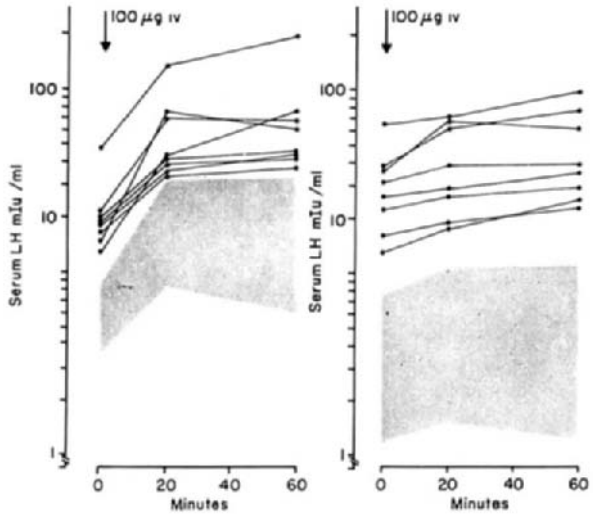


Fig. 11. Effect of 100 μg of LH/FSH-RH on serum LH and FSH in normal controls ⊞ and postmenopausal women ●.

This test is completely free from side effects and can be performed in conjunction with the TRH test and the insulin tolerance test, since there is no interaction between the various hormones released (M10). This procedure provides a test of pituitary reserve of LH, FSH, TSH, PRL, GH, and ACTH.

3.5.1.2. *Results in Primary Hypogonadism.* Patients with primary gonadal failure show an exaggerated response to LH/FSH-RH (Fig. 11), but the elevated basal values are sufficient evidence for the diagnosis, and the LH/FSH-RH test is not usually required.

3.5.1.3. *Results in Pituitary–Hypothalamic Disease.* The standard test (B3) is not of value to differentiate between hypothalamic and pituitary causes of hypogonadism. Mortimer *et al.* (M9) reported that in a series of 155 patients with hypothalamic or pituitary disease, all but 9 showed some response to LH/FSH-RH although 137 were hypogonadal. It is probable that a test employing smaller doses of LH/FSH-RH (e.g., 25 μg) might achieve greater discrimination. However, in combination with the clomiphene test, the LH/FSH-RH test may be helpful in differentiating hypothalamic and pituitary causes of hypogonadism. A negative clomiphene test combined with a positive LH/FSH-RH test is indicative of hypothalamic failure. This pattern is observed in the syndrome of isolated gonadotropin deficiency, where the lesion seems to be located at a hypothalamic level (M1).

3.5.2. *Therapeutic*

LH/FSH-RH is a useful therapeutic agent in cases of hypogonadotropic hypogonadism and infertility. It has been shown to induce ovulation in women with diseases of the pituitary or the hypothalamus (Z2) and in women with anovulation caused by contraceptive steroids (Z1). In males with hypogonadotropic hypogonadism it has recently been shown that prolonged LH/FSH-RH administration in doses of 500 μg at 8-hour intervals subcutaneously results in initiation and maintenance of secretion of gonadotropins, elevation of plasma testosterone, restoration of potency, and induction of spermatogenesis (M12). It is interesting that in this study some patients showed restoration of potency before plasma testosterone was raised to normal levels. This effect of LH/FSH-RH on libido suggests a direct central effect of the decapaptide on behavior. Support for this was obtained by Moss and McCann in animals (M14). After the successful production of anti-LH/FSH-RH antiserum in the early stages of development of LH/FSH-RH radioimmunoassay, it was shown that the administration of this antiserum to rats on the morning of proestrus prevented the elevation of LH and FSH and sub-

sequent ovulation (A4). The application of this observation to the control of fertility in animals remains to be elucidated.

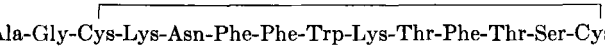
More than 200 synthetic analogs of LH/FSH-RH have now been produced. Incorporation of D-amino acids in position 6 of the decapeptide appears to enhance activity (V2). This increase in activity may be attributable to greater affinity to receptors, higher efficacy in stimulating secretion, slower enzymic degradation, or a combination of these factors. Arginine, tyrosine, serine, and leucine may be concerned with the binding of LH/FSH-RH to the receptors (S6) whereas histidine, tryptophan, and the pyroglutamic acid seem to have a functional role. The N-terminal by itself, however, does not have intrinsic activity. A number of superpotent LH/FSH-RH analogs have been tested in animals and in man for biological activity (K2). Their prolonged action is perhaps the most important advantage, since their administration can sustain elevated gonadotropin levels in patients with hypogonadotropic hypogonadism. Further studies are required to establish the applications of these analogs in clinical endocrinology.

The information obtained during the preparation of the superpotent analogs was useful to prepare some competitive inhibitory analogs. The principle was to find an analog with higher affinity to the gonadotroph receptor but much less intrinsic activity, thus blocking endogenous LH/FSH-RH action. Some of these inhibitory analogs have been synthesized, and their use holds a promise of a new form of nonsteroidal form of contraception (V3).

4. Growth Hormone-Release Inhibiting Hormone (GH-RIH or Somatostatin)

4.1. Isolation and Synthesis

Growth hormone-release inhibiting hormone was isolated from ovine hypothalami by Brazeau *et al.* (B12) and shown to be a cyclic tetradecapeptide:



The peptide was then synthesized, and the product was shown to have the same biological activity as the natural material. Synthesis of the material in the cyclic (native) form is difficult and results in low yields. Schally and his colleagues (C3) have described the solid phase synthesis of GH-RIH in cyclic form in a highly purified state. They have shown that a high molecular weight compound formed during the cyclization

reaction, probably predominantly a dimer, also possesses considerable inhibitory activity.

The synthetic tetradecapeptide, either in linear or cyclized form has been shown to suppress growth hormone secretion in man (H3, P6, S8), in animals (B11), and in isolated pituitary tissue (B8), confirming the lack of phylogenetic specificity seen with the other hypothalamic regulatory hormones.

4.2. Location

Krulich *et al.* (K7) demonstrated a high content of GH-RIH in rat median eminence by bioassay. Pelletier *et al.* (P3), using an immunohistochemical technique at the electron microscope level, have shown that GH-RIH is contained in the secretory granules of many nerve endings located mainly in the external zone of the median eminence of rats. After the development of a sensitive radioimmunoassay for GH-RIH by Schally and his colleagues (A6), the same group made the important discovery that GH-RIH is present in the rat stomach and pancreas in a concentration similar to that found in the hypothalamus. GH-RIH was also found in the duodenum and jejunum but in smaller concentration (A7). They suggested that GH-RIH might be synthesized in the pancreas and stomach in addition to the brain and might be involved in local regulatory mechanisms for pancreatic and gastric secretion as well as for secretion of growth hormone.

4.3. Mechanisms of Action

Borgeat *et al.* (B8) studying isolated rat pituitary tissue showed that GH-RIH inhibited both basal and prostaglandin (PGE_2)-induced cyclic AMP (cAMP) accummulation, suggesting that the tetradecapeptide might act at a step preceding cAMP formation. However GH-RIH inhibits the accumulation of cAMP induced by theophylline, which raises the possibility that GH-RIH may also act at a step following cAMP formation. Such a possibility is supported by the finding that GH-RIH inhibits both the GH and the TSH release induced by N^6-monobutyryl-cAMP. The action of GH-RIH is unaffected by inhibitors of RNA and protein synthesis, suggesting an action independent of new protein formation. Other *in vitro* studies have shown that GH-RIH is able to prevent the barium-induced decrease in number of secretory granules and also the number of exocytic events in rat hemipituitaries (S7).

These findings suggest that GH-RIH might influence exocytic events, possibly reducing the calcium influx associated with the secretory process.

4.4. Forms of GH-RIH and Analogs

Schally *et al.* (S5) have shown the existence of at least three forms of GH-RIH of differing molecular size in porcine hypothalamus, all of which were biologically and immunologically active. The biological significance of this finding is not clear at present.

A number of GH-RIH analogs have been synthesized in an effort to obtain compounds with prolonged biological activity. Brazeau *et al.* (B13) reported that deletion of the N-terminal dipeptide (Ala^1-Gly^2) was compatible with high biological activity in rats and that acylation of the third residue (Cys^3) renders the molecule less soluble and prolongs its action. These workers used an *n*-acetyl analog in the reduced (linear) form and an *n*-benzoyl analog in the oxidized (cyclized) form. These acylated analogs were tested in the native form in two volunteers (Figs. 12 and 13) and a patient with acromegaly (E2). Although

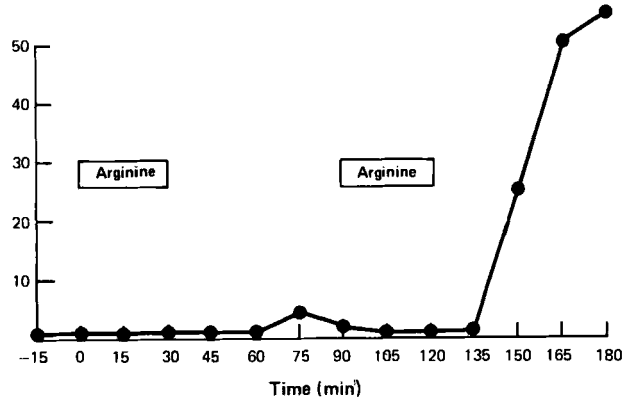


Fig. 12. Effect of *N*-acetyl-des-Ala^1-Gly^2-growth hormone–release-inhibiting hormone on arginine-induced growth hormone release (ordinate: ng/ml).

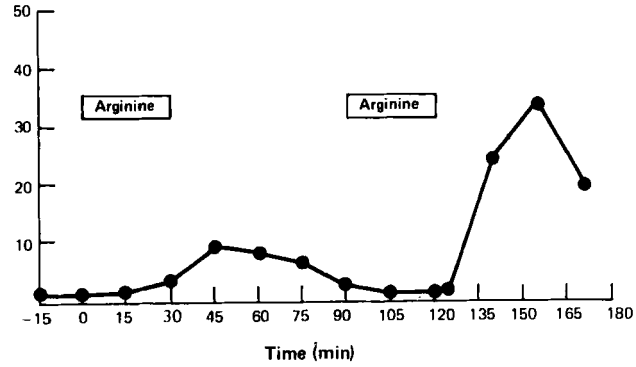


Fig. 13. Effect of *N*-benzoyl-des-Ala^1-Gly^2-GH-RIH on arginine-induced growth release (ordinate: ng/ml).

biologically active in suppressing arginine-induced GH release in the volunteers and basal GH release in the acromegalic and also suppressing insulin release, no apparent prolongation of action was observed, and in addition side effects comprising nausea and malaise were observed.

4.5. Specificity

This will be considered in detail under the headings of individual hormones. However, it is apparent that GH-RIH has a variety of actions at pituitary and extrapituitary levels. At the pituitary it affects the release of GH, PRL, TSH, and ACTH whereas beyond the pituitary it influences the release of renin, insulin, glucagon, gastrin and gastric acid, and pepsin production. It is uncertain whether its variety of pituitary and extrapituitary actions are physiological or pharmacological since they have been achieved both *in vivo* and *in vitro* with large amounts of GH-RIH. At physiological levels of GH-RIH greater specificity may exist, but further work is required to define these levels. The lack of specificity of GH-RIH is not surprising since the other regulatory hormones are also relatively nonspecific. It may imply similarities of pituitary receptors for the regulatory hormones.

4.6. Duration of Action

GH-RIH has a very short biological half-life, probably less than 4 minutes, and to demonstrate its full effect it must be given by intravenous infusion (H3). Single injections given intravenously, intramuscularly, or subcutaneously usually have little effect (B4). Its action can be prolonged by mixing it with gelatin, arachis oil (B4), or protamine zinc, but none of these preparations is very effective. There is an urgent need for analogs with greater duration of action or some new vehicle that prolongs its release before GH-RIH can become a practical therapeutic agent in clinical practice.

4.7. Side Effects and Toxicity

Early studies with the highly purified cyclic form of GH-RIH showed no toxic effects on the blood, kidney, or liver with repeated intravenous infusion, some of which were prolonged for as long as 28 hours. Short infusions of the so-called long-acting analogs did cause side effects in volunteers. More recently we have observed colicky upper abdominal pain, vomiting, and diarrhea after about 6 hours of infusion of GH-RIH

in two hypothyroid patients. These symptoms rapidly resolved without deleterious effects, and there was no rise in serum amylase in either subject. The underlying mechanism for these side effects is not understood at present.

More recently Koerker (K5) has reported that some baboons given repeated injections of the linear form of GH-RIH died of hemorrhage and that there was an associated thrombocytopenia in one. Short-term effects on inhibition of platelet aggregation were also observed 2 hours after administration of a standard dose of GH-RIH. Similar mild inhibition of platelet aggregation has been observed during short-term infusions of the cyclic form of GH-RIH into normal volunteers, but this reverted to normal afterward. None of the subjects studied by us had a reduction in platelet aggregation to dangerous levels and other coagulation studies were normal throughout and after a 6-hour infusion of GH-RIH. Other side effects reported by Parker *et al.* (P2) included arousal from sleep, cramps, and diarrhea using the linear form.

4.8. Actions on the Anterior Pituitary

4.8.1. *Growth Hormone*

GH-RIH suppresses GH secretion in man and in animals both *in vivo* and *in vitro*. It also suppresses GH secretion in response to a variety of stimuli including exercise (P6), insulin-induced hypoglycemia (H3) (Fig. 14), sleep (P2), arginine (M11, S8), *l*-dopa (S8), sodium-pentobarbital (B11), isoprenaline and chlorpromazine (K3), and electrical stimulation of the ventromedial nucleus (M2). In a variety of *in vivo* studies, it has been shown that the circulating GH levels are suppressed

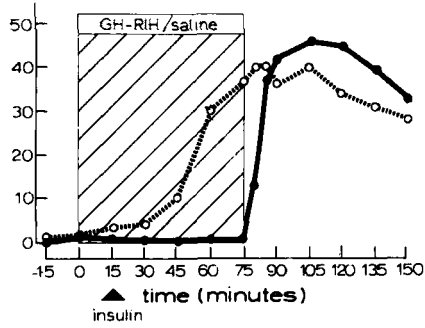


Fig. 14. Growth hormone–release-inhibiting hormone (GR-RIH) effect on GH response (ordinate: ng/ml) to hypoglycemia. Mean of 5 normal men. ●, GH-RIH; ○, saline.

only during the course of a GH-RIH infusion, rising rapidly after the infusion is terminated, usually to levels higher than those observed in control infusions. The results of most experiments support the view that GH-RIH inhibits the release of GH by a direct action on the somatotroph cell without having any action on GH synthesis. Its effect on GH secretion in acromegaly will be discussed later.

4.8.2. *Prolactin*

Prolactin secretion by pituitary cell cultures is inhibited by GH-RIH, but to a lesser extent than GH (V1). GH-RIH does not appear to affect basal PRL levels in normal subjects, but it has been reported to lower PRL release by otherwise normal pituitary cells when its secretion has been elevated by hypothyroidism or by chronic administration of estrogens. It does not affect the fall in PRL produced by *l*-dopa (S8) nor the rise in PRL resulting from insulin-induced hypoglycemia (H3) or thyrotropin-releasing hormone (C1). Further critical studies of the effect of GH-RIH on basal circulating PRL levels are required.

4.8.3. *Luteinizing Hormone and Follicle-Stimulating Hormone*

Basal LH and FSH levels are not apparently altered by GH-RIH (H3, S8), and there is no reduction of the LH and FSH response to LH/FSH-RH (H3). Further studies are required on the effect of GH-RIH on gonadotropin secretion in differing physiological and clinical states.

4.8.4. *Adrenocorticotropin (ACTH)*

Corticotropin secretion is unaffected by GH-RIH in normal subjects (H3) both basally and in response to insulin hypoglycemia. However, Tyrrell *et al.* (T1) have shown a significant fall in elevated ACTH levels in Nelson's syndrome (4 patients) and in Cushing's disease (1 patient) in response to GH-RIH infusions. These results suggest that the mechanism determining release of ACTH by adenoma cells differ from that in normal pituitary cells.

4.8.5. *Thyrotropin*

Basal secretion of TSH in normal men is unaffected by short-term (75-minute) infusions of GH-RIH (C1). Although this might be taken to indicate that basal TSH secretion does not directly depend on TRH drive, longer infusions of GH-RIH are required for further investigation of this matter. The elevated basal TSH levels seen in hypothyroid patients are lowered by GH-RIH given by infusion over several hours (G7), though with prolonged (24-hour) infusions some escape

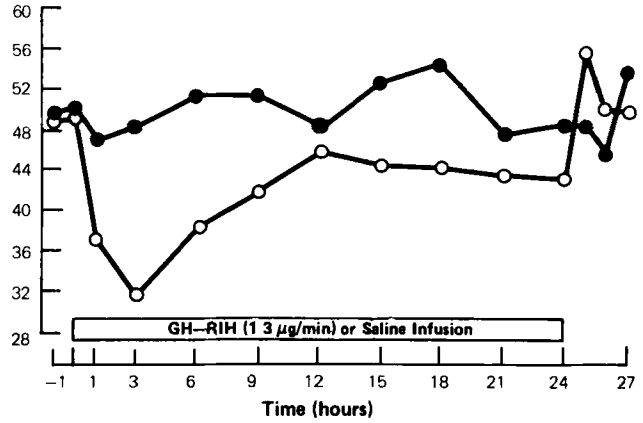


FIG. 15. Effect of GH-RIH on serum TSH (ordinate: mU/liter) in a patient with primary hypothyroidism. ●, saline; ○, GH-RIH.

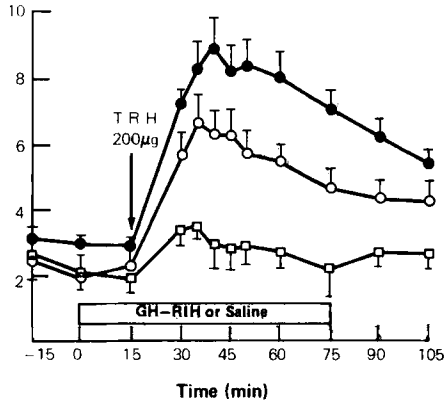


FIG. 16. Effect of growth hormone–release-inhibiting hormone (GH-RIH) on the thyrotropin-releasing hormone (TRH)-mediated thyroid-stimulating hormone (TSH) release (ordinate: mU/liter); mean ± SE of 5 men. ●, saline; ○, GH-RIH, 1.3 μg/min; □, GH-RIH, 13.3 μg/min.

occurs (Fig. 15). A rebound rise in TSH is observed after discontinuation of the infusion. These results suggest that TRH drive is, at least in part, responsible for the maintenance of the elevated basal TSH levels of primary hypothyroidism. The TSH response to TRH is inhibited by GH-RIH both in normal subjects (Fig 16) (C1, H3, S9, W2) and in patients with hypothyroidism (C1). The degree of inhibition depends on the dose of GH-RIH used, being appreciably greater at an infusion rate of 13.3 μg per minute than at 1.3 μg per minute. This contrasts with

the inhibition of GH in acromegaly, where infusions of GH-RIH at these doses are equally effective.

As mentioned earlier, the PRL response to TRH was not inhibited by GH-RIH. This dissociation of the TSH and PRL responses to TRH by GH-RIH suggests different mechanisms for release of TSH and PRL, only the former being affected by GH-RIH.

4.9. Actions on the Pancreas

GH-RIH inhibits basal secretion of insulin and glucagon (C2, D1, G2, M11), glucose-stimulated insulin secretion (A1) and arginine- stimulated glucagon secretion (M11), and the insulin and glucagon response to a meal and to the administration of tolbutamide (G4) (Figs. 17–19).

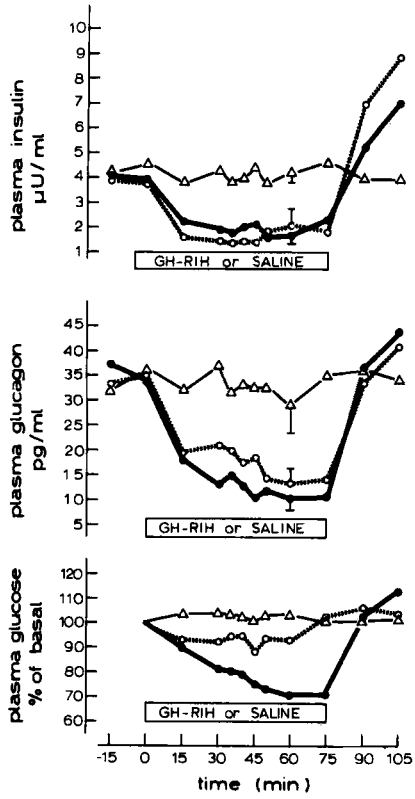


Fig. 17. Effect of growth hormone–release-inhibiting hormone (GH-RIH) on basal insulin, glucagon, and glucose levels; mean ± SE of 6 normal men. △, saline; ○, GH-RIH, 1.3 µg/min; ●, GH-RIH, 13.3 µg/min.

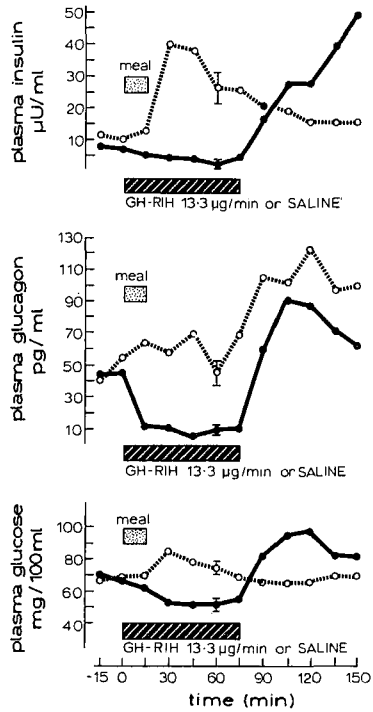


FIG. 18. Effect of GH-RIH on the secretion of insulin and glucagon after a standard meal and the hyperglycemia caused by feeding. ○, saline; ●, GH-RIH, 13.3 μg/min.

The effects on plasma insulin and glucagon result from a direct action of GH-RIH on the alpha and beta cells of the pancreas since they have been demonstrated by direct perfusion experiments with the isolated canine pancreas (A1, I2).

As a result of its action on insulin and glucagon release, GH-RIH has a variety of effects on carbohydrate metabolism (Fig. 17). During glucose tolerance tests the rise in plasma glucose is delayed and peak values are often higher and more sustained, so that glucose tolerance is impaired (M11). It seems likely that the impairment in carbohydrate tolerance during GH-RIH is the result of suppression of insulin secretion. The delay in peak glucose concentration might result from some action of GH-RIH on the speed of glucose absorption from the gut, possibly mediated by an action on one or more gastrointestinal hormones. Certainly in studies with intravenous glucose loads, the glucose peaks were higher after GH-RIH but were not delayed (A1).

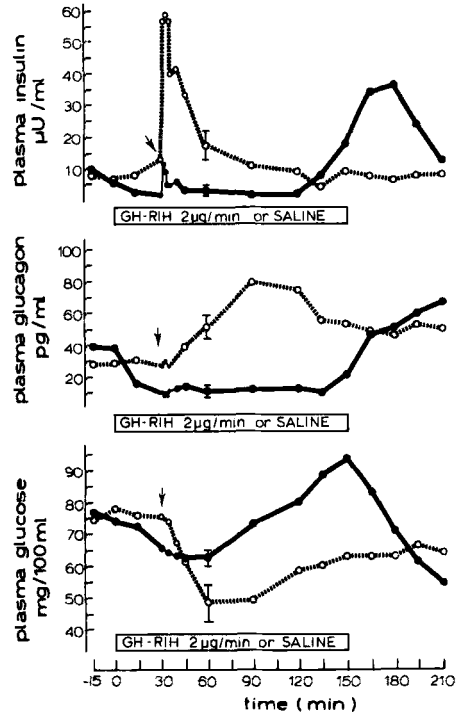


FIG. 19. Effect of GH-RIH on insulin and glucagon secretion after tolbutamide (→, 1 g i.v.): inhibition of the hypoglycemic effect of tolbutamide. ○, saline; ●, GH-RIH, 2 μg/min.

After a brief fast, GH-RIH causes a fall in basal blood glucose in baboons (K6) and in man (G4). It has been shown that this results from inhibition of glycogenolysis. It seems likely that the inhibition of glycogenolysis is due to lowering of glucagon levels by GH-RIH. Evidence for the role of glucagon in the control of the fasting glucose level was provided by Alford *et al.* (A3). They infused glucagon at a rate which did not in itself alter plasma glucose or insulin concentration, when GH-RIH administration resulted in a rise in plasma glucose. They therefore concluded that plasma glucagon plays an important part in the maintenance of the fasting plasma glucose in man. Koerker *et al.* (K6) also showed that infusion of GH-RIH in baboons after prolonged fasting (60–65 hours) caused a comparable fall in plasma glucose, glucagon, and insulin suggesting that the mechanisms involved in gluconeogenesis might also be dependent on glucagon.

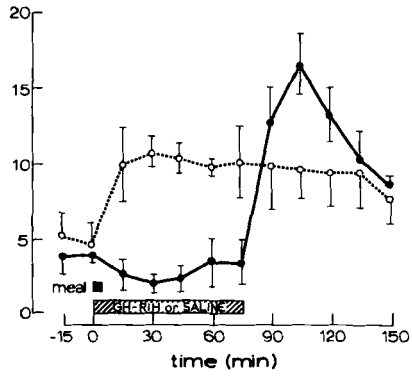


FIG. 20. Plasma gastrin concentrations (ordinate: pmoles/liter) in response to a meal. Effect of administration of growth hormone–release-inhibiting hormone. ○, control; ●, GH-RIH.

4.10. ACTIONS ON THE GASTROINTESTINAL TRACT

4.10.1. *Gastrin*

Bloom *et al.* (B6) were the first to demonstrate inhibition of gastrin secretion in normal men by GH-RIH (Fig. 20). Both basal gastrin and the gastrin release in response to food was inhibited by GH-RIH. Plasma gastrin concentration did not fall to undetectable levels during a GH-RIH infusion, possibly owing to the persistence of other molecular forms of gastrin in the circulation. Similarly the raised plasma gastrin levels seen in patients with pernicious anemia were partially suppressed by GH-RIH (B6).

4.10.2. *Gastric Acid and Pepsin*

Gastric acid secretion fell to almost undetectable levels during a GH-RIH infusion in a patient with the Zollinger-Ellison syndrome, suggesting that suppression of biologically active gastrin levels was responsible for this effect. However Gomez-Pan *et al.* (G8) have shown that GH-RIH inhibits gastric acid and pepsin secretion in response to pentagastrin and the gastric acid response to food and hypoglycemia in cats with gastric fistulas (Figs. 21 and 22). These results indicate that GH-RIH acts directly on the parietal and peptic cells, the first report of a direct action of GH-RIH on exocrine secretion. In view of its high concentration in the stomach, it is possible that GH-RIH might act locally

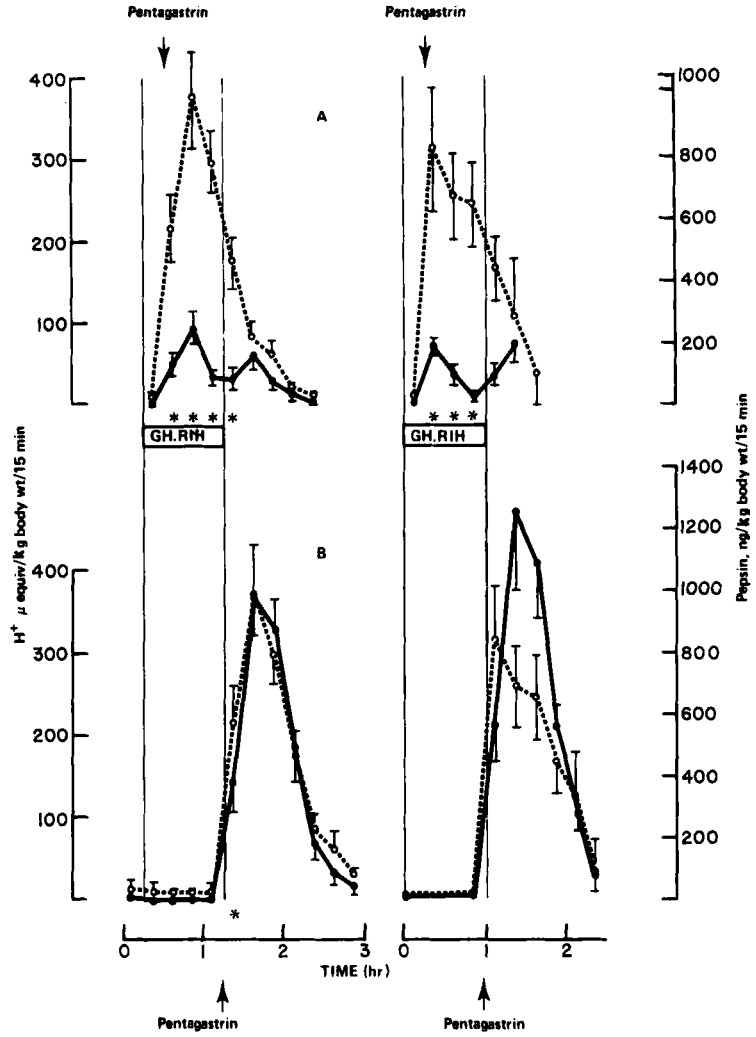


FIG. 21. Effect of growth hormone–release-inhibiting hormone (GH-RIH) on the pentagastrin-induced secretion of gastric acid and pepsin secretion. (A) Pentagastrin (6 μg/kg s.c.) injected during the GH-RIH infusion. (B) Pentagastrin injected immediately after GH-RIH infusion. *, significant; ○, control; ●, GH-RIH.

to coordinate endocrine and exocrine secretion. It is of interest that the gastric acid response to histamine is only blocked by high doses of GH-RIH, supporting the view that gastrin and histamine stimulate acid secretion by different mechanisms (A2).

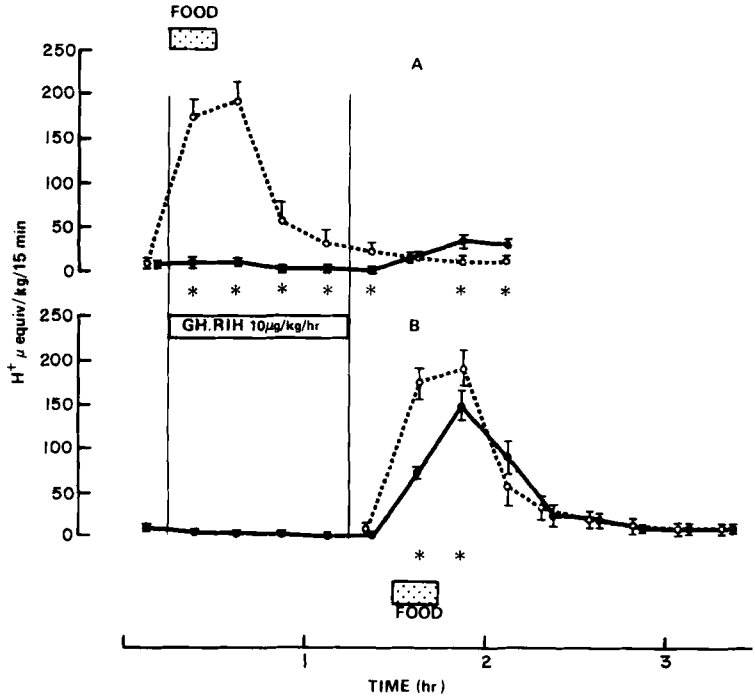


FIG. 22. Effect of GH-RIH on gastric acid response to feeding. (A) Food was administered during GH-RIH infusion. (B) Food was administered immediately after GH-RIH infusion. *, significant; ○, control; ●, GH-RIH.

4.11. ACTIONS ON THE KIDNEY

In four normal men the elevated renin level induced by pretreatment with frusemide was suppressed by GH-RIH infusion, the first demonstration of an action of GH-RIH on the kidney (G10). These results lend further support to the view that GH-RIH inhibits exocytic events mediated by calcium influx.

4.12. ACTIONS IN DIABETES MELLITUS

The hyperglycemia of dogs made diabetic by alloxan or pancreatectomy can be reduced or abolished by GH-RIH infusion, probably owing to its suppression of glucagon whether secreted by the pancreatic alpha cells or by the newly identified gastrointestinal A cells (D2). Similarly in human diabetes mellitus, infusions of GH-RIH cause a fall in plasma glucose that parallels the fall in plasma glucagon (G1). These effects were also obtained in a hypophysectomized diabetic patient, indicating

that they are independent of the suppression of GH. GH-RIH infusions combined with insulin prevented the postprandial hyperglycemia in diabetics more effectively than insulin alone. It was suggested that excessive glucagon secretion accounts for about 25% of the fasting plasma glucose levels in diabetes and that GH-RIH might be a useful adjunct to insulin in treating diabetes by suppression of both glucagon and GH. It is known that the overall secretion of GH and the GH response to stimuli are increased in diabetes, and there is now a considerable body of evidence linking GH and diabetic angiopathy. Long-term studies of GH suppression by GH-RIH in diabetic patients will await the production of a longer-acting preparation of GH-RIH as well as proof that it is lacking in toxicity under these circumstances.

4.13. Actions in Acromegaly

The raised GH levels in patients with acromegaly can be suppressed by GH-RIH (B4, H3, Y1) (Fig. 23). Infusions of 100 μg of GH-RIH over 75 minutes were equally effective as doses of 250 and 500 μg

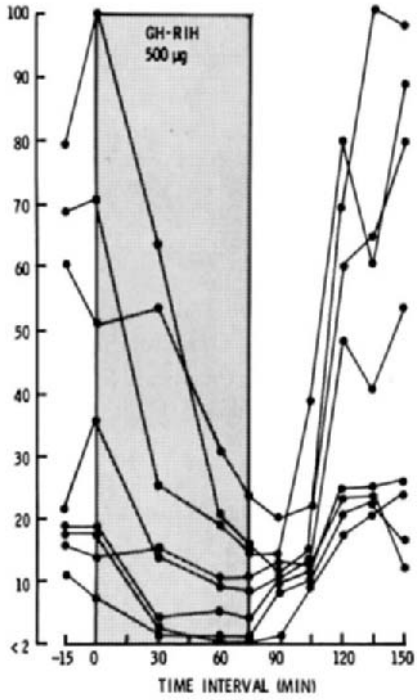


Fig. 23. Plasma GH responses (ordinate: ng/ml) to infusions of 500 μg of growth hormone–release-inhibiting hormone GH-RIH in eight acromegalic patients.

whereas a 10-μg dose over this period was ineffective. A similar dose-response relationship could be achieved by a single intravenous bolus (C2). Constant infusions of GH-RIH at a rate of 1.3 μg per minute for 28 hours suppresses plasma and urinary GH, insulin, and glucagon throughout the period. The effect of a single intravenous injection of GH-RIH lasts for about 30 minutes in acromegalics, and this effect can be prolonged for a few hours by the intramuscular route if GH-RIH is mixed with gelatin, arachis oil, or protamine zinc. Clearly GH-RIH in a long-acting form would offer therapeutic benefit to patients with acromegaly, though its effects on tumor growth would first need to be determined and its safety verified.

4.14. Actions on Hormone Secretion by Tumors

As mentioned previously, GH and ACTH secretion by pituitary tumors can be suppressed by GH-RIH. Secretion of insulin by insulinomas (G6) and glucagon by glucagonomas (M11) can also be inhibited by GH-RIH (Fig. 25). Similarly gastrin secretion by a pancreatic tumor has been suppressed in a patient with the Zollinger–Ellison syndrome (B6) (Fig. 24). Such suppression of gastrin was accompanied by a marked fall in gastric acid secretion suggesting that GH-RIH might have a role in the emergency management of patients with the fulminating variety of the Zollinger–Ellison syndrome. The latest tumor hormone to be suppressed by GH-RIH is the vasoactive intestinal peptide (VIP) (B5). Bloom *et al.* showed that circulating VIP levels were reduced in a patient with the Verner–Morrison syndrome, who presented with profuse diarrhea and hypokalemia. Subsequently a pancreatic tumor-secreting VIP

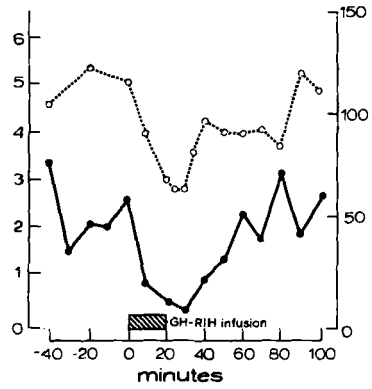


Fig. 24. Effect of growth hormone–release-inhibiting hormone (GH-RIH), 700 μg, on gastric acid output (left ordinate: mmoles H^+/10 min) and plasma gastrin levels (right ordinate: pmoles/liter) in one patient with Zollinger–Ellison syndrome.

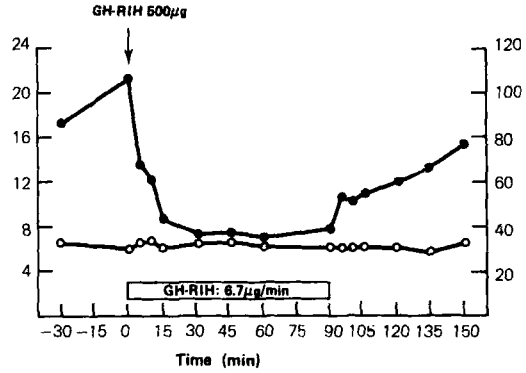


FIG. 25. Effect of GH-RIH on plasma insulin (left ordinate: μU/ml) and glucose levels (right ordinate: mg/100 ml) in one patient with an insulinoma.

was removed from the patient. Suppression of insulin, glucagon, and gastrin by tumors had similar characteristics in that it was only partial in these disorders and also the return to base-line levels after discontinuation of the infusion was not as abrupt as in normals. The latter feature is seen in acromegaly too. Partial suppression may be explained by the persistence of elevated levels of the related hormones in the circulation, namely, big gastrin in the Zollinger–Ellison syndrome and proinsulin in patients with insulinoma. These hormones are not affected by short-term infusions of GH-RIH.

Suppression of hormone secretion by GH-RIH from a variety of tumors suggests that it acts on some common secretory mechanism, possibly mediated by calcium influx. Longer-acting GH-RIH preparations might have therapeutic potential in malignant varieties of these tumors or when the tumor cannot be located.

5. Conclusions

GH-RIH is a remarkably potent agent with a wide variety of actions. Whether it has a physiological role or not remains to be determined. Is it normally a regulator of GH secretion, or does it have wider functions outside the brain as a local regulator of endocrine and exocrine secretion? These questions as well as its potential therapeutic role in acromegaly and diabetes mellitus remain to be answered.

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URIC ACID METABOLISM IN MAN

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1. Background Biochemistry

Uric acid that is produced in man is essentially the product of the action of the enzyme xanthine oxidase on xanthine and hypoxanthine. A tiny amount of uric acid may be ingested as part of the diet, but the great bulk is the result of the action of this enzyme on these two purines. These purines are themselves produced either as a result of the breakdown of cellular material *in toto*, the turnover of nucleic acids in the cells, or as a result of the intermediary metabolism of various purine nucleotide derivatives. These latter compounds are active in the flow of energy, in methyl group transfer reactions, and as part of the functional molecule of many vitamins. There is direct and indirect evidence that some of the uric acid derives from all these sources. Essentially this evidence consists of the demonstration that other parts of the nucleic acids are found in the urine, such as pyrimidine breakdown products (P9) and methylated purines, which are found only in nucleic acids. There is also isotopic evidence that some labeled purines appear in the urine too quickly after administration of radioactive precursors

for them to have come via nucleic acids, but radioactivity is maximal several days later, suggesting that most of the purines have come from nucleic acids (B9, K13). For those who might be interested in the details of the chemistry of purine and purine nucleotide synthesis, a chapter in an earlier volume of this series may be of interest (B2).

It is well known that uric acid is an end product of only a few mammals: man, higher apes, and the Dalmatian coach hound (F5). The Dalmation is an anomaly because its excretion is due to a kidney abnormality (F5). The other mammals that do not excrete uric acid as a primary purine end product, form it as an intermediate, but they possess the enzyme uricase, which degrades uric acid to allantoin, a more highly water-soluble substance than uric acid, and in some cases this is degraded further.

In view of its occurrence in man and the higher apes, many have speculated on the possible correlation of uric acid levels and intelligence. Several papers have been published showing apparent positive correlation between the serum uric acid levels and either direct or indirect measurements of mental ability (D6, S15). In the year 1975, however, it would probably be improper to discuss these correlations since men, in general, have a higher uric acid level than women.

For years the most important property of uric acid in the eyes of clinical investigators has been the limited solubility of it and its sodium salts. As a result excessive production of uric acid can lead to deposition of sodium urate in various parts of the body resulting in a clinical entity known as gout (S12). Gout is not only extremely painful but can cause serious kidney damage. The lower mammals convert uric acid to more soluble products, and thus they avoid this danger. Modern biochemical investigators have found purine metabolism in general to be of great theoretical interest, and in addition to the clinical problem there is hope the study of this metabolic system will provide answers to a number of ancillary enigmas of biochemistry.

2. Various Purines in Urine and Serum

Not only is uric acid found in the urine and the serum, but small amounts of its precursors xanthine and hypoxanthine and a variety of other purines are found in the serum and in the urine. Many of these are breakdown products of the ribonucleic acids which contain methylated purines. When these macromolecules are broken down the methylated purines are not reutilized but are excreted (B13). In addition, a large number of purine derivatives such as theobromine and caffeine are found in various foods.

2.1 Analytical Problems

These latter compounds can be a cause of considerable annoyance in the studies of the metabolism of the less common purine derivatives. In addition several purine derivatives and analogs are used as drugs. Enzymic determination of uric acid is not interfered with by these compounds, although colorimetric procedures are. Determination of those purines found in lower concentrations, for example, hypoxanthine and xanthine, is complicated excessively by these other purines, since they interfere with both enzymic and colorimetric assays. Some of these derivatives are found in quantities quite large compared to the hypoxanthine and xanthine found in serum and urine and have physical and chemical properties sufficiently similar that analysis without prior purification is difficult. It is not easy to separate them by simple paper or column chromatography. Thus, it is often necessary to use highly sophisticated chromatographic equipment or, as was done in the past, use multistep methods, and these have the severe disadvantage that recovery is not complete (W2, W3). These are not insurmountable problems, but they are of sufficient magnitude that investigators must be aware of them.

3. Abnormal Metabolism of Purine Derivatives

There are a number of conditions, some of which have been referred to above, that result in aberrant purine metabolism. Some of these are transient, some of long duration, some are of endogenous, some of external origin. Unfortunately, those we have learned the most about are the least common. Fortunately, our ability to deal with the difficulties is not as limited as our knowledge.

3.1 Gout

The most common clinical problem connected to abnormal uric acid metabolism is gout. As has been mentioned, this is caused by precipitation of sodium urate crystals in various parts of the body. Generally speaking, there is an excessive amount of uric acid or monosodium urate in the serum of the afflicted individual (B2). The disease can be classified as primary gout when the cause is apparently overproduction of uric acid per se or secondary gout when the hyperuricemia is a consequence of another abnormality, such as excessive turnover of cells associated with leukemia and lymphoma and following administration of cytolytic drugs in the course of therapy for certain malignancies (K14, K15). It can also be brought about by impairment of the natural mechanisms

for the elimination of uric acid through the kidney. These can be secondary to diabetes (P1), acute alcoholism (L5), glucose-6-phosphate dehydrogenase deficiency (J1), and excess lactic acid (Y6). These and other factors have been widely discussed elsewhere (B2, G7) and is not the subject of this review, which will concern itself with more recent developments in this area of purine metabolism.

3.2. Xanthinuria

The simplest abnormality of purine metabolism to the biochemist is xanthinuria. This is a disease in which the enzyme xanthine oxidase is absent (D5, E5). At first it would appear that this would be advantageous; however, xanthine, which is the principal urinary purine in xanthinuria, is at best no more soluble than uric acid, and probably is less so under physiological conditions (K15); as a result, most xanthinurics develop xanthine gout (S4). It is difficult to make too many generalizations in regard to this subject since the number of xanthinurics is rather limited. Probably fewer than a dozen have been thoroughly studied. It had been believed at one time that the abnormality was more common than realized since most xanthinurics are asymptomatic. It had been assumed that there would be no way of detecting subjects and that they would escape in the general population. However, many institutions have in recent years instituted almost routine assays of serum uric acids levels, in conjunction with the introduction of automatic clinical analyzers, and very few, if any, new xanthinurics have been turned up as a result of this rather extensive determination of uric acid levels. Furthermore, the large-scale screening done by Stetten and his associates during World War II on uric acid levels (S15) in Army inductees, and the many studies carried out on hyperuricemia on large numbers of individuals have not shown any instances of xanthinuria (F4, H1, M9).

Ayvazian and Skupp (A3) on the basis of studies of the fate of orally administered AMP and GMP concluded that 90% to 100% of the guanine bases of RNA were converted to xanthine and excreted, whereas the bulk of the adenine appeared to enter into the metabolic pool. Direct measurements of this were carried out in a second xanthinuric patient to whom radioactively labeled hypoxanthine and xanthine were administered simultaneously (B15). It is possible to distinguish the carbon-6 from the carbon-8 of oxypurines by oxidizing them to uric acid and then degrading the uric acid to alantoin, which results in the liberation of the carbon-6 as CO_2. Thus, the fate of these two compounds, hypoxanthine and xanthine, could be studied simultaneously by using one containing radiocarbon in position 6 and the other containing radiocarbon in position 8. The rate of decay of these two compounds could be studied

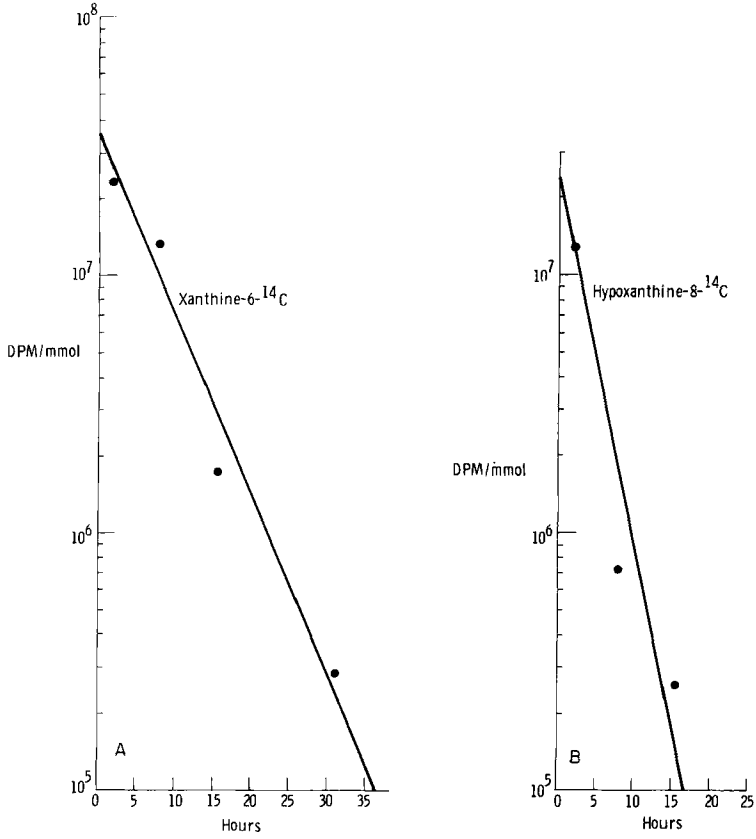

FIG. 1. Turnover of xanthine and hypoxanthine. Excretion of hypoxanthine-8-^{14}C (A) and xanthine-6-^{14}C (B), in urine of xanthinuric patient following administration of mixture of xanthine-6-^{14}C and hypoxanthine-8-^{14}C. From Bradford *et al.* (B15)

simultaneously in a patient by determining the radioactivity in the urinary hypoxanthine and xanthine. From the semilog plot of these values, the pool sizes, rate of turnover, and extent of elimination of each can be calculated. This could not be done in a subject with functioning xanthine oxidase (Fig. 1).

As a result of these studies it was found that the bulk (ca 80%) of

TABLE 1
OXYPURINE POOL SIZES (B3)

Parameter	Hypoxanthine	Xanthine
Pool size (mg)	118	73
Daily turnover (mg)	960	276
Fraction in urine	0.05	0.79

the urinary purine was derived from xanthine and 20% from hypoxanthine (Table 1). It follows that in patients who have xanthine oxidase, 80% or so of the uric acid is derived from xanthine and 20% from hypoxanthine. On the other hand, the data also showed that there is a much larger synthesis of hypoxanthine than of xanthine. The great bulk of the hypoxanthine is reincorporated into purine derivatives while most of the xanthine formed is an end product and is eliminated in the urine, normally as uric acid. It was rather surprising to find that in the one elderly patient studied, approximately 1 g of hypoxanthine was formed per 24-hour period.

3.2.1. *Formation of Xanthine from Guanine*

The question of the origin of the xanthine has not been completely answered. Animal studies would suggest that it is derived not from xanthine or a xanthine nucleotide, but rather from guanine. The evidence for this, shown in Table 2, is based upon the fact that when psicofuranine, a compound known to inhibit the conversion of xanthosine phosphate to guanosine phosphate (F8, S10) was administered, a great decrease in the amount of xanthine excreted was seen while no appreciable change was observed in the amount of hypoxanthine excreted. Thus, there was no general effect on oxypurine excretion, cell breakdown, or *de novo* purine synthesis. The most likely explanation is that this antibiotic reduces the formation of guanylic acid, and a certain fraction of the guanylic acid pool is normally subject to degradation. When the guanylic acid pool is depleted, there is less guanine or guanosine available for leakage from the cells and degradation to xanthine. The inhibition of the amination of xanthylic acid and accumulation of xanthylic acid

TABLE 2
EFFECTS OF PSICOFURANINE ON URINARY PURINES OF THE RAT

Day	Avg. wt.	Xanthine	Hypoxanthine
1	118	5.82	0.99
2	110	3.30	0.57
3	103	2.31	0.74
4	100	0.91	0.59
5	99	0.60	0.82

[a] All animals were receiving allopurinol in their diet (0.075%). They received 125 mg of psicofuranine per kilogram of body weight per day. Xanthine and hypoxanthine values are in milligrams per days.

should have resulted in a large increase in the amount of xanthine excreted if urinary xanthine were derived directly from cellular xanthylic acid. Bearing in mind the danger of extrapolating from rat to man, it is nevertheless, highly probable that the xanthine, and hence the uric acid, found in the urine of man is primarily derived from the breakdown of guanine nucleotides.

This is consistent with the known fact that injections of guanine derivatives into experimental animals results in essentially all of that material being excreted as uric acid, not in its incorporation into cellular guanine nucleotides (B19, P6). Nevertheless, enzymes that are capable of converting gaunine into guanine nucleotides do exist in most cells since if the guanase inhibitor aminoimidazole carboxamide (AIC) is given to experimental animals along with guanine, then guanine is incorporated into nucleic acids of intact animals (M3). In addition, tissue breis incubated with excess guanine do convert it to nucleic acid guanine (B1).

Furthermore, when very large quantities of guanine are administered to experimental animals so that the guanase is saturated, there is incorporation of guanine. Thus, it would appear that guanine and its derivatives are more rapidly degraded than they can be reincorporated into nucleotides and the primary source of uric acid in the urine is from guanine derivatives which are converted to xanthine.

3.2.2. *Hypoxanthine Active Intermediate*

Hypoxanthine, on the other hand, which accounts for only a fifth or so of the urinary uric acid is an active intermediate. It is degraded to xanthine and then to uric acid by xanthine oxidase. This enzyme is found mainly in liver, kidney, and bowel, while guanase is widely distributed and would quickly deaminate any guanine formed. The product xanthine is a poor substrate for hypoxanthine phosphoribosyltransferase (HPRT). Most of the hypoxanthine formed is reutilized by conversion to inosinic acid. Similar conclusions were reached by Ayvazian and Skupp in 1965 when they administered ^{14}C-labeled purines to patients (A2). Furthermore, these studies and those earlier studies show that the xanthine is converted to hypoxanthine, presumably at the nucleotide level, and on the basis of what we know about microorganisms, we would assume it to be via guanine nucleotides (M2). Since label was found in urinary 7-methylguanine as early as 4 hours after administration of ^{14}C-labeled purines, and since methylation of RNA occurs at the macromolecular level (B13), interconversion must be rapid and incorporation of some of these products into nucleic acids must also occur quickly.

One of the interesting observations made in the study of xanthinurics

is the fact that the amount of oxypurines excreted is less *in toto* than the amount of uric acid that is normally excreted. A similar observation was made when the total oxypurine output of gouty subjects given allopurinol was studied (K8, R6–R8, W6, Y5). In these cases, the reduction in urinary uric acid (see below) brought about by this inhibitor of xanthine oxidase was balanced partially by an increased excretion of xanthine and hypoxanthine into the urine. The major reason for this appears to be reutilization of xanthine and hypoxanthine by the body and the concurrent repression of *de novo* synthesis. Similar observations have been made in mice, in which it was shown that blocking degradation of purines by the concurrent administration of allopurinol permits the utilization of exogenously administered hypoxanthine (P7) and xanthine (P8) for nucleic acid synthesis and concurrent depression in *de novo* synthesis of purines.

3.2.2.1. *Reactions of Hypoxanthine.* The fact that hypoxanthine is an active intermediate in normal cells directs attention to the three chemical reactions hypoxanthine can undergo in the mammal (Fig. 2). It can be converted to inosine by reaction of the purine with ribose 1-phosphate catalyzed by purine nucleoside phosphorylase. This reaction is probably primarily a phosphorolytic reaction, *in vivo*, and converts inosine to hypoxanthine and probably does not function to convert hypoxanthine to inosine. There does exist a limited concentration of

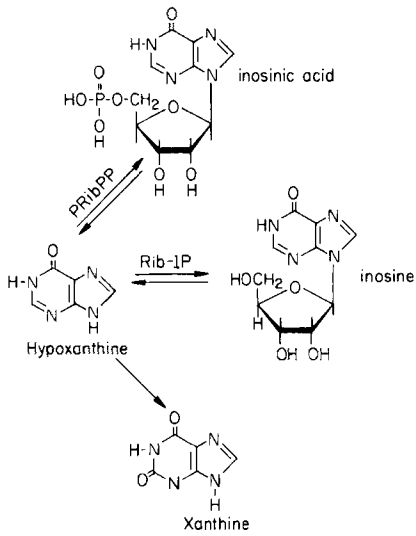

FIG. 2. Reactions of hypoxanthine. PRibPP, phosphoribosylpyrophosphate; Rib-1P, ribose 1-phosphate.

the enzyme inosine kinase that can catalyze the conversion of inosine to inosinic acid, but the activity of this enzyme is so small compared to the phosphorolytic activity of inosine phosphorylase that very little inosinic acid is formed by this route (P2). The main reaction that hypoxanthine undergoes, and the one that must be responsible for the large pool of hypoxanthine being constantly formed and converted to usable cellular products, is the reaction of hypoxanthine with phosphoribosylpyrophosphate (PRibPP) to form inosinic acid. This reaction is catalyzed by the enzyme hypoxanthine phosphoribosyltransferase (HPRT).

3.2.2.2. *Hypoxanthine Phosphoribosyltransferase (HPRT).* Purine phosphoribosyltransferases have been known for a long time. They have been shown to exist in a large number of bacterial and animal species (B4, M13). The one that converts hypoxanthine to inosinic acid also acts on other 6-hydroxypurines and their analogs to form the corresponding ribosylphosphates. In some microorganisims there are two, or perhaps even more, enzymes which convert various 6-hydroxypurines to ribonucleotides. In mammals that have been studied, and certainly in man, one enzyme converts xanthine, hypoxanthine, and guanine, the three naturally occurring 6-hydroxpurines, to their 5′-phosphoribsyl derivatives. In all the species studied a variety of analogs of these compounds are converted to analogs of the natural ribonucleotides. One class of such compounds are the 6-mercaptopurines. They have a mercapto group in place of the hydroxy group and pharmacologic activation of these compounds requires conversion to their ribonucleotides (B5, B18). In fact, cells that lack this enzyme are resistant to the action of these drugs. There also is an enzyme that converts 6-aminopurines to their ribonucleotides. This enzyme, adenine phosphoribosyltransferase (APRT), has been demonstrated in almost all tissues that have been examined. It, too, acts on analogs of the naturally occurring substrate. The most widely studied are 2,6-diaminopurine and azaadenine. Absence of this enzyme leads to resistance to these purine analogs. These compounds have been of less clinical importance than have the analogs of 6-hydroxypurines (B4). There are enzymes in man capable of inactiviating both hypoxanthine and guanine, the natural substrates of HPRT. Hypoxanthine is the normal *in vivo* substrate because the enzyme guanase, which inactiviates guanine, is found in all tissues examined while xanthine oxidase, on the other hand, is localized only in a few tissues (H8, W4). As a result guanine at physiological concentrations is more rapidly converted to xanthine than to guanylic acid. Xanthine, a poor substrate for HPRT thus is an end product (K6). Hypoxanthine is well converted as is shown by the fact that only a small fraction of the daily pool ends up as urinary end products.

3.3. Lesch–Nyhan Disease

As mentioned above, one of the chemical reactions that hypoxanthine can undergo is the conversion into inosinic acid. Data have accumulated that suggest that the amount of hypoxanthine that goes through this route is of the order of magnitude of a gram per day. It follows that, if this reaction could not occur, that is, if an individual were unable to convert hypoxanthine to inosinic acid, then this gram of hypoxanthine would be excreted either as such or as a degradation product of hypoxanthine. In a person who had normal amounts of xanthine oxidase, the gram of excess hypoxanthine would be converted to a molar equivalent of uric acid. This excessive production of uric acid would be expected to lead to hyperuricosuria, eventually hyperuricemia and gout. Despite the biochemical obviousness of this reasoning, investigators had not predicted that such a kind of gout would exist. The discovery that it is a real entity came about despite, rather than because of, the available biochemical information. In 1964 Lesch and Nyhan (L4) described a genetic disorder of uric acid metabolism which had neurological manifestations. They studied two brothers who excreted four times the amount of uric acid normally produced by patients of their age and weight. The administration of labeled glycine was followed by a rapid excretion of very highly labeled uric acid. This indicated that the overproduction of uric acid was related not to the excessive destruction of some long-lived cell constituent, but to rapid synthesis of purines, which were soon converted into uric acid. The most striking aspects of the clinical history of these patients were choreoathetosis and a tendency toward self-mutilation. The latter characteristic was most bizarre and striking in that one of the patients had partially amputated one joint of one finger and both of them had severely chewed away parts of their lips.

Since the description of these first patients, more than a hundred have been discovered around the world, and there is no correlation (N1) in terms of race or geographic or economic background. It was early noted that the disease was one of males only, and studies of the history of several families showed it to be X-linked (S7); that is, the disease is transmitted through the mothers but is expressed in only 50% of the male offspring. Female children are carriers of the disease in 50% of the cases. Early reports have maintained that the children were slightly retarded (K7). However, there has been at least one report of a patient having essentially normal intelligence (S3). In consensus, it would appear to be that in view of the overtones of the nature of the physical conditions and the very limited ability to speak that is found in most of these children at an early age, it is difficult to measure

their intelligence. However, certainly either because of the disease per se or because of secondary factors, these children are generally retarded. It is not possible to say whether it is a primary result of the biochemical lesion.

Similarly, there have been reports of behavioral differences among the patients, and although this has not been well documented it does appear that at a given institution the patients tend to be extremely difficult or aggressive or spiteful children, while in other institutions, they are more manageable and not as ill-tempered. A number of attempts have been made to determine whether any physical changes are consistent with this disease. Although some have reported pathological changes (G9), in general, aside from the obvious damage caused by deposition of sodium urate, no consistent change has been found (F3, G8, G10). We must therefore conclude that there is no general histologically demonstrable pattern of destruction in the brain.

A wide range of symptoms have been described, and it is not clear whether there are stages of the disease that differ, or whether it can take many courses, or whether there is a family of diseases. In some cases neurological abnormalities were present at birth. Other patients appeared normal until the ages of 6–8 months. Some at the age of 1–2 years began to self-mutilate. There are reports of patients who did not begin to self-mutilate until their teens. Some have been diagnosed as having cerebral palsy until they were into their late teens, at which time gouty symptoms led to further studies and they were classified as Lesch–Nyhan patients (B3).

3.3.1. *Metabolic Aspects*

Examination of the urine of patients with this disease reveals in addition to the high uric acid an elevated hypoxanthine to xanthine ratio (L4). Furthermore, it was found that upon the administration of allopurinol, an inhibitor of xanthine oxidase that is widely used in the treatment of gout, the total output of purine is not reduced. This is in contrast to the reduction in total oxypurine output seen in normal or most gouty individuals. In addition, the ratio of hypoxanthine to xanthine is not reduced; i.e., Lesch–Nyhan patients still maintained elevated hypoxanthine to xanthine ratios. These data have suggested an interference with hypoxanthine metabolism or reutilization, since the reduction in purine output after allopurinol therapy is due to anabolism of the hypoxanthine to inosinate (B4). It was shown by Seegmiller and his associates that there was an absence of hypoxanthine phosphoribosyltransferase in the red cells of subjects with Lesch–Nyhan syndrome.

The initial studies suggest that there is complete absence of the enzyme in the cells of the patients. More recently, however, evidence has begun to accumulate that suggests that, in some cases, there is a small amount of residual activity. Studies of skin fibroblasts have suggested that a small amount of hypoxanthine is incorporated. With somewhat modified assay procedures lysates of the fibroblasts of patients have been shown to convert some hypoxanthine to inosinic acid (F7, K4). In addition, one patient who was classified as having Lesch–Nyhan syndrome was shown to have an enzyme that was active but required unusually high levels of cosubstrate PRibPP (M5). The enzyme was detected in several strains of fibroblasts, as mentioned above, but it appeared to be a relatively unstable enzyme (F7, R5). This led the authors to postulate that perhaps the enzyme was present in greater amounts than was generally accepted, but owing to an instability was not detectable under the usual assay conditions. These involve the use of red cells, most of which are old and in samples that sometimes were not available for assay immediately after they were obtained from the patient. On the other hand, in view of the widespread disagreement in the details of the description of the symptoms of the disease it is also possible that there are a variety of diseases, some of which have enzymes with altered kinetic properties, some with small amounts of unstable enzyme, and some in which there is no enzyme present whatsoever.

3.3.2. *Enzymic Defect*

Whether the disease is due to the production of small amounts of partially functional enzyme, or no functional enzyme, does not answer the question whether the genetic defect is due to reduced enzyme production or to synthesis of normal amounts that are structurally ineffective. The fact that some patients were demonstrated to have small amounts of an altered enzyme does not answer the question, although it does suggest that in some cases at least some enzyme protein is made. One approach to this problem is to purify the enzyme from normal individuals and then prepare antibodies against this enzyme in another animal species. If the enzyme is made in patients with Lesch–Nyhan disease and the alteration that leads to an inactive enzyme does not change the immunological site of the enzyme too extensively, there is a real possibility that immunologically cross-reacting material would be found in the cells of patients with Lesch–Nyhan disease. The absence of cross-reacting material would not prove that the enzyme was not made in an inactive form, but the presence of cross-reacting material would establish the synthesis of an inactive enzyme. Such a study was carried out (R5). The enzyme was purified from pooled

blood bank erythrocytes by conventional precipitation and chromatographic techniques. Antibodies were prepared to it, and it was shown that the erythrocytes of five Lesch–Nyhan patients examined all contained cross-reacting material.

Despite the fact that all the patients' erythrocytes contained the same amount of cross-reacting material, there was evidence of heterogeneity. The neutralization curves of the lysate of erythrocytes of Lesch–Nyhan patients were different even though the amount of serum that completely neutralized antibody was the same (B6). Thus, various immunological as well as genetic and stability data suggest that there are a number of mutations that can lead to the absence of functional HPRT and the clinical entity known as Lesch–Nyhan syndrome.

3.3.3. *Secondary Enzymic Changes*

As a result of an extensive screening program, a family was found that had a genetically regulated deficit in APRT (K3). The enzyme produced did not appear to be abnormal in electrophoretic mobility, pH optimum, Michaelis constants, or heat stability (H6). The four subjects studied did not suffer any apparent physical disability attributable to the lack of the enzyme.

Of perhaps greater interest was the observation that patients with Lesch–Nyhan syndrome have elevated levels of APRT in their red cells (S5). It is not clear at this time how general this increase is in the various tissues of the patients.

3.3.3.1. *Studies of Adenine Phosphoribosyltransferase (APRT).* APRT catalyzes the condensation of adenine with PRibPP to form adenylic acid (AMP) and pyrophosphate. Like HPRT, APRT is widely distributed in nature. Both enzymes are found in most, if not all, tissues of rats, mice, cattle, monkeys, and man as well as a variety of bacteria and yeasts. In one of the earlier studies of tissue distribution, Murray (M12) measured the levels of both enzymes in a large number of mouse and rat tissues. There seemed to be a suggestion that activities, in general, correlated with mitotic activity and there was a low level in erythrocytes of both mouse and rat. He was struck by the variation in the ratio of the two enzymes among tissue types, the HPRT ranging from 0.4 to 12 times the APRT. This led to the almost obvious conclusion that each enzyme may have a specific tissue-related function. In particular, the brain ratio of 11.7 in the mouse is highly suggestive in view of the neurological overtones of HPRT deficiency. However, in the rat the ratio is 1.7 so that these facile conclusions are probably of limited significance.

In more recent work, the levels in human autopsy material were ex-

amined (R2) and the brain HPRT:APRT ratios were severalfold higher than those seen in any other tissues. Other studies with surgical specimens did not support this and did not show any unusual brain ratios (B3). More recently, with improved techniques or a fresher cadaver, the first group did another autopsy and got the same results seen with surgical specimens (B14).

APRT is a unique protein easily distinguished from HPRT by its heat lability K11). APRT has two naturally occurring substrates for the purine site on the enzyme—adenine and aminoimidazolecarboxamide (F2, K12). That there is one enzyme involved was established by the constant ratio of binding of the two substrates during purification of the enzyme (F2). The synthetic purine analog 2,6-diaminopurine is an excellent substrate (G1, K16).

Although APRT catalyzes a reaction superficially analogous to that catalyzed by HPRT, the two enzymes differ in several striking ways. The synthesis of HPRT is controlled by a gene on the X-chromosome, APRT by one on chromosome 16 as shown by gene elimination studies on mouse–human cell hybrids (T1). The APRT from human red cells has a molecular weight of 26,000 (Y1), while that of HPRT is about 70,000 (O1). More interesting is the fact the HPRT has a substrate, hypoxanthine that is produced enzymically, but no enzymic source of adenine has ever been demonstrated in man. Thus, APRT could well be an enzyme without a natural substrate.

3.3.3.2. *Bases for Aberrations in APRT.* Not only was there a greater amount of APRT in the red cells of Lesch–Nyhan patients, but the enzyme was apparently more heat stable. In an effort to probe this phenomenon further, Rubin *et al.* studied the half-life- of APRT in circulating erythrocytes (R4). This is a relatively simple procedure since these anucleated cells do not continue to synthesize protein (M4), and as they age their density changes, which permits density fractionation according to age (P4). By means of ultracentrifugation in an isosmotic discontinuous gradient after the method of Piomelli (P4), red cells were fractionated and the amounts of HPRT and APRT were assayed in each age group. The HPRT had the same half-life in normal and patients' red cells, that is, 88 days. On the other hand, the APRT of normal subjects had a half-life of 34 days whereas that of the patients with Lesch–Nyhan disease had a half-life of 86 days. In addition, it was shown that the amount of enzyme in the freshly released reticulocytes was the same in both normal and Lesch–Nyhan subjects.

These facts showed that the increased amount of APRT is not due to an increased rate of synthesis, but to posttranscriptional stabilization of the enzyme. Since earlier workers had shown that PRibPP stabilizes

APRT (H10, M15), and since PRibPP concentrations are higher in red cells of Lesch–Nyhan patients, it is logical to assume that the increased substrate is responsible for the increased APRT levels. One puzzling aspect of this work was the observation that the APRT in crude lysates of very young cells seemed more heat labile than that in older cells. This problem was resolved by the later observation that PRibPP levels in young erythrocytes are only one-sixth those in more mature cells (Y2). It is interesting to speculate that these changes are related to ATP concentrations and that the latter is concerned with the fixed lifespan of the erythrocyte.

In contrast to the observation that as cells aged HPRT catalytic capacity decreased but HPRT antigenic activity was constant, in the same cells APRT catalytic and antigenic activity decreased in parallel. This may explain why subjects with decreased APRT catalytic activity have not been shown to have an inactive immunologically cross-reacting material. It may mean that the inactivation of APRT is much more extensive than simple denaturation.

Despite the fact that APRT is coded for by a gene on chromosome 16, there seemed to be differences between APRT from normal and Lesch–Nyhan subjects in crude lysates. As the enzymes are purified, these differences lessen both in terms of heat stability, chromatographic and antigenic activity (R3, R4). Any differences must be reflective of secondary changes in the cell milleu and could be lost on purification.

3.4 Partial Hypoxanthine Phosphoribosyltransferase Deficiency

One of the more interesting aspects of HPRT is that in the circulating red cell of normal subjects it is inactivated as a first-order reaction and its half-life can be determined (R4). The enzyme has a half-life of 88 days in the red cells of normal subjects; that is, the catalytic property of the enzyme has a half-life of 88 days. Interestingly, if one determines the half-life of the enzyme in terms of the immunological properties, it turns out to be infinite, that is, despite the fact that the enzyme is denatured as a catalyst, the antigenic properties are not destroyed during the life of the red cell (Y2). Obviously, this is why the inactive enzyme in the erythrocytes of the deficient patients is the same as that in normal subjects since there is complete cross reactivity of catalytically active and inactive enzyme.

In addition to the Lesch–Nyhan patients who have little, if any, enzyme activity, there exists a small group of patients who have some activity which is considerably less than normal (K2, K5, Y3). A systematic study

of a rather large number of hyperuricemic patients with a history of acute gouty arthritis or uric acid urolithiasis, or both, revealed the fact that not more than 2% of the gouty population can attribute its gout to this partial deficiency. Several of these patients are members of large families that have been thoroughly studied and it is quite clear that this defect is X-linked and expresses itself only in certain of the male offspring. Although only a small fraction of gout patients have this defect, there are probably tens of thousands of them in total.

As one would expect, since these patients owe their overproduction of uric acid to excessive release of hypoxanthine, they often do not respond well to allopurinol unless great care is taken in adjusting the dose. These patients, like Lesch–Nyhan patients, have limited ability to anabolize hypoxanthine to inosinate. Production of xanthine, which is no more soluble than uric acid, is not to the patients' advantage (K15). Proper regulation of the allopurinol results in a beneficial distribution of the purines into three products, uric acid, xanthine, and hypoxanthine and resolves the solubility problem.

3.4.1. *Range of Enzyme Levels*

A large range of abnormalities has been observed. In some cases no detectable enzyme is found in the red cells (D3). In other cases the amount of activity is about 30% of normal (Y3). Families have been reported with 1–3% of normal enzyme levels. No simple correlation between behavior and enzyme level is apparent. Some patients have concurrently elevated APRT, some do not. In some, but not in all, there is increased heat stability of the APRT. In one study (E2) eight patients from five kindreds were examined in detail. No correlation was found between clinical manifestations and the level of HPRT in red cell lysates. These authors seemed to find the line between partial deficiency gout and the symptoms of Lesch–Nyhan disease a question of degree, not of kind.

3.4.2. *Stability of HPRT*

In one family that was studied rather extensively, two sisters each had an affected son. One of these children had been diagnosed as having Lesch–Nyhan syndrome because he was mentally retarded, had seizures, and no enzyme was detected in his red cells. His cousin was neurologically normal, was a better than average student, and also had no detectable enzyme in his red cells. However, an examination of the white cells revealed that there was considerable enzyme activity and that they were not Lesch–Nyhan victims (D3). Further study revealed the reason

for the apparent low activity or nondetectable activity in the red cell even though there was considerable activity in the white cell. The enzyme is an extremely unstable variant. Storage at 4°C resulted in almost complete loss of activity in 96 hours, while the normal enzyme actually appears to be increased in activity under these conditions. Thus, the enzyme was quickly lost as the red cells circulated, and assays of these cells were very misleading.

3.4.3. *Role of Phosphoribosyl Pyrophosphate (PRibPP) Levels*

There have been suggestions that the HPRT of Lesch–Nyhan patients is also unstable, but since it is absent in immature blood cells, this is probably not a factor in the expression of the syndrome (D2). Study of the activity of this enzyme emphasizes another feature which, though known, is often overlooked. Normally enzymes are assayed in cell lysates under assay conditions with saturating substrate concentrations. Under these conditions the enzyme in the white cell lysates of these patients appears to be about 20% of normal. However, the enzyme can also be assayed by using intact cells and incubating them with a concentration of hypoxanthine that is essentially equal to that found in physiological fluids. Under these conditions it was shown that the enzyme activity was normal. These findings suggested that PRibPP is the rate-limiting substrate in the endogenous reaction. It is obvious that if several enzymes use the same substrate and they do not all have the same K_m, then endogenous substrate concentrations for some reactions cannot be half-maximal.

Estimates of PRibPP levels from studies with fresh cell lysates indicate that the concentration is about 8×10^{-6} M which is well below the K_m of the enzyme 2×10^{-4} M (D3, H5). The assay techniques are indirect. In one, ^{14}C-labeled adenine is added along with excess purified APRT. The amount of AMP-^{14}C formed is a measure of the PRibPP present. In a second assay, carboxy-labeled orotic acid is added together with orotidylate pyrophosphorylase and orotidylate decarboxylase. The liberated $^{14}CO_2$ is a measure of the orotidylic acid which is a measure, in turn, of the PRibPP concentration.

Normally a fraction of the cellular enzyme complement is sufficient to maintain proper function and the regulation of activity is a function of substrate level. In the case of those subjects with partial deficiency of HPRT, it is possible to demonstrate the superiority of the intact cell assay as a measure of the true *in vivo* situation. In addition, these studies demonstrate the advantage of using an intact cell over the crude lysate in looking for an index of actual enzyme performance in tissues in general.

4. Regulation of Carrier's Response to Defect

As mentioned above, the gene that regulates HPRT production is located on the X-chromosome. Therefore, heterozygotic mothers, those with one mutant and one normal X-chromosome, are carriers. Their male offspring have a 50% chance of inheriting the defect. The mothers of Lesch–Nyhan children do not exhibit any of the neurological symptoms of the disease. They are not "partial" Lesch–Nyan patients. This is true despite the fact that in each female cell only one X chromosome is functional. This raises many questions of regulation in the whole subject versus that seen in *in vitro* enzyme studies and permits an approach to the evaluation of many intriguing problems of human biology.

4.1. Lyon Hypothesis

According to the Lyon hypothesis (L9), one X-chromosome in each cell is randomly inactivated in an early stage of embryonic development. As a result one half of the cells of a carrier of this enzyme deficiency should have normal amounts of the enzyme and the other should possess the amount that the individual with the defect has. Half of the cells of a mother of a Lesch–Nyhan patient should have no HPRT and half should have the normal amount; the carriers of defects that result in 10% enzyme activity in the effected male offspring should have half of their cells normal and half with 10% of the enzyme. In addition, one would expect that the average value of the enzyme in a sample of red cells drawn from heterozygotes should have contained 50% normal activity in the case of the carrier of Lesch–Nyhan syndrome and the appropriate proportion in the carrier of a partial defect.

4.1.1. *Selection of Cells*

By the use of a very simple assay for the enzyme in red blood cells, it was shown that the heterozygotes for Lesch–Nyhan disease did not have the expected decreased concentration of enzymes in the peripheral blood cells (B11, D1). Concurrently, other investigators were able to demonstrate by plating techniques that there were in fact two populations of cells in fibroblasts obtained from proved heterozygotes (M8, S1). In addition, mosaicism was demonstrated by analysis of the hair roots of patients with HPRT deficiency (G2, S9). Investigation into the behavior of lymphocytes was extended by Nyhan *et al.* (N2) through use of a double marker. They analyzed glucose-6-phosphate dehydrogenase (G6PD) activity in the lymphocytes of members of a Lesch–Nyhan kindred that was also heterozygous fo G6PD. This

enzyme had previously been shown to segregate in peripheral blood cells in accordance with the Lyon hypothesis. Starch and polyacrylamide gel electrophoresis of lysates revealed that these cells possess only the B variant of G6PD and only the normal HPRT. However, when fibroblasts were evaluated, both A and B variants of G6PD were recognized and thus, the enzymic expression of the fibroblast seems to be representative of the true gene type. The peripheral blood cell data give evidence of some selective event that probably occurs in the marrow or stem cells (D1).

The association of loci for HPRT deficiency and G6PD abnormality on the X chromosome has permitted mapping studies. The crossover frequency seen in tissue culture and clinical situations suggests that these genes, though on the same chromosome, are not very closely linked (D3, M10, N2).

A number of investigators have shown that the urinary purine excretion pattern and the white and red cell enzyme patterns of mothers of Lesch–Nyhan patients are completely normal. The explanation that has been offered, and it seems most logical, is that the abnormal lymphocyte is at a disadvantage during embryological development, so that normal cells are selected for propagation (D1). An alternative explanation is that the defective cell has an abbreviated life-span, and thus circulating lymphocytes in the heterozygote are predominantly normal. In the hemizyogote, the abnormal cell would not be competitively excluded and an increase in lymphopoietic activity could compensate for the shortened survival. It may also be that some factor associated with being female in some way protects or enhances the activity of the defective enzyme in the circulating lymphocyte, and that this factor is not operative in tissue culture. The currently available data do not permit selecting among these possibilities. Despite the fact that no heterozygosity of red cells was detected among the mothers of Lesch–Nyhan patients, this is not the case among the mothers of patients with the partial defects. Several reports exist in literature of heterozyogotes with partial deficiency demonstrating intermediate activity (D3, D4, E3, E4, K10). It is intriguing to speculate on the possible reasons why a partial defect results in no selective advantage whereas the complete defect does. It may well be related to the fact that most cells with partial defect are functionally normal, as is evidenced by the absence of the severe neurological symptoms seen with Lesch–Nyhan disease. One might almost be tempted to say that a criterion of Lesch–Nyhan disease, as distinguished from the partial defect, is the absence of mosaicism in the heterozyogote. In this regard, it should be mentioned that not only were the two cousins reported by Dancis *et al.* (D3) devoid of any demonstra-

ble activity in their red cells, but a 2-year-old male child of a family that carried a gene for partial disease in enzyme activity showed absolutely no detectable enzyme in his peripheral blood cells and showed no evidence whatsoever of the classical neurological symptoms of Lesch–Nyhan disease (Y4).

4.1.2. *Metabolic Cooperation*

An alternative explanation for the unusual absence of mosaicism in the circulating blood cells of Lesch–Nyhan heterozyogotes is the phenomenon referred to as metabolic cooperation. This is a form of cell-to-cell communication in which the mutant phenotype of certain enzyme-deficient cells is corrected, at least in culture, by contact with normal cells (B21, C2, C3, P5, S16). The most striking and most relevant example is the demonstration that cells deficient in HPRT are unable to incorporate radioactive hypoxanthine into cellular material. On the other hand, normal cells are able to incorporate this precursor into intercellular nucleotides and polynucleotides, a fact that can be demonstrated at the cellular level by radioautography. When certain mutant cells deficient in HPRT are grown in close contact with normal cells, they also incorporate radioactivity that is not removed by acid washing. The biochemical nature of this transfer has been shown by isotopic exchange experiments to be due to transfer of radioactive nucleotides or derivatives thereof from normal to mutant cells. This has been demonstrated (C2) by showing that in the presence of inhibitors of protein synthesis the transfer continues; therefore, it is not due to the transfer of some stimulatory substance that results in the production of enzyme molecules in the normally enzyme-deficient cells. Furthermore, in the presence of inhibitors of messenger RNA synthesis, there is, again, transfer of labeled material from competent to deficient cells. Therefore, it is not the transfer of some informational ribonucleic acid that is responsible for the transfer. If cells that have been grown in contact are separated, the enzyme-deficient geneotype immediately loses the ability to pick up labeled material. Therefore, no enzyme has been transferred, for under these conditions the half-life of the enzyme has been shown to be many hours. Thus, we know that cell-to-cell contact is necessary and cell-to-cell contact is in many cases sufficient to permit transfer of nucleotide derivatives from competent to deficient cells. The transfer does not go on between all cells of all kinds and all species, but it does go on between most human cells that have been examined (C4), and therefore it would be a suitable, though not unique, explanation for the absence of neurological abnormalities in the heterozygotic mothers, half of whose cells would be expected to be deficient in this

enzyme. It is not possible to say whether this is why mothers who carry the defective gene do not have half their nerve cells damaged. It may be that the explanation is more closely related to the absence of mosaicism in the circulating blood cells, or the reason may be an entirely different one, but it is a possible explanation.

5. Role of Phosphoribosyl Pyrophosphate

The first step in purine synthesis is usually considered to be the reaction of PRibPP with glutamine to form phosphoribosylamine. The reaction is catalyzed by the enzyme amidophosphoribosyltransferase (H4), an allosteric enzyme subject to complex feedback regulation. It follows quite logically that the rate of purine synthesis might be regulated, in part, by the concentration of PRibPP. If excess PRibPP were present, excessive purine synthesis might result. Obviously, in the absence of PRibPP, purine synthesis could not occur.

5.1. Rate of Synthesis in Gout

Early studies to evaluate the rate of PRibPP synthesis in gouty subjects were based on attempts to trap the compound with imidazole acetic acid. The results did not suggest that overexcretors of purine were in general overproducers of PRibPP, although some were (J2). More direct measurements of the synthesis of PRibPP in isolated red cells also suggested that in some gouty subjects there was a potential for excess production of PRibPP (H7). On the other hand, other workers found no correlation between either PRbiPP concentrations or PRibPP synthetase activities in normal and gouty subjects (M7).

5.1.1. *PRIbPP Synthetase*

That the model is potentially realistic was demonstrated by a recent isolation of a cultured cell line that overproduces purines and excretes large amounts of them into the culture medium. The cell was further shown to have an altered PRibPP synthetase that overproduced PRibPP. The mutant enzyme has normal catalytic properties but was not subject to feedback control by AMP, ADP, or ATP (G5).

Three cases of abnormal PRibPP synthetase have been reported in the literature (B8, S14, Y4). Sperling *et al.* reported an elevated and altered synthetase in the erythrocytes of a gouty overproducer of uric acid. No other enzymic abnormality was noted. The synthetase was not only able to catalyze the excessive production of PRibPP, but did so only at low concentrations of inorganic phosphate. At concentrations

of phosphate that gave maximal activity, the production of PRibPP was normal. However, the authors pointed out that *in vivo* concentrations are much lower than the optimal and the response to phosphate is sigmoidal in normal lysates. At physiological phosphate concentrations, the enzyme of this patient had excessive activity and its response to phosphate levels was rectangular hyperbolic. The authors further proposed that the overproduction of uric acid in this patient was due to this aberrant enzyme.

The other two reports of altered synthetase are also based on assays of red cell lysates. These authors report optimal phosphate concentrations higher than those found by Sperling *et al.*, and the response to phosphate was different. These later papers report results consistent with that to be expected if excess synthetase, not altered synthetase, were present. Sperling's observations are esthetically more intriguing and more like the usual structurally altered enzyme seen in inborn errors. Further work may reveal that the excess enzyme seen by Becker *et al.* (B8) and Yu *et al.* (Y4) have their own specific peculiarities.

6. Adenosine Deaminase (ADA) Deficiency

There have recently been reported a small number of patients with an absence or very low level of ADA (G4, H9). This enzyme is responsible for the conversion of adenosine to inosine. The enzyme is normally found in most animal tissues (B16). Although the symptoms seen in patients with the ADA deficiency are severe and life threatening, there have been few clear biochemical data to indicate the enzyme's importance. For some time, adenosine has been known to cause dilation of the coronary arteriole, and it had been thought that the role of ADA was to control adenosine levels (B12, C1).

6.1. Toxicity of Adenosine

More recently adenosine has been shown to be toxic to fibroblasts and lymphocytoid lines in culture (G6). The toxicity is dependent upon conversion of adenosine to adenylic acid (AMP) since a line devoid of adenosine kinase activity is much less susceptible to adenosine toxicity (I1).

Two hypotheses as to the basis of adenosine toxicity are most common. The first is related to pyrimidine synthesis. Since the toxic effects of adenosine even at fairly high levels can be prevented by the addition of uridine, Ishii and Green (I1) suggested that a proximal step in the

toxic sequence was the blockade of endogenous uridylate synthesis. They further showed that the conversion of aspartate to uridine nucleotides was reduced with the concomitant accumulation of orotate. This is not readily interpretable in specific molecular detail without further data.

Cells exposed to adenosine, not surprisingly, have elevated levels of ADP and ATP. The changes in guanine nucleotides is less pronounced; thus it may be that the balance between adenine and guanine derivatives might become distorted in ADA deficiency (G6). This could lead to imbalance in those regulatory steps that normally are controlled by combinations of adenylic and guanylic acid. This second possible mechanism is a corollary of the reports that adenosine can cause changes in cyclic AMP levels in several animal tissues *in vitro* (F1, S8).

One of the more interesting aspects of this enzyme is the fact that it seems to exist in several forms. There is a basic low molecular weight (35,000) portion found in erythrocytes (E1). In other tissues complexes have been reported with molecular weights up to 435,000 (A1, E1). These forms have different kinetic and physical properties. Evidence by Nishihara *et al.* (A1) indicates that the various forms of the enzyme are interconvertible. They isolated a heat-labile protein from lung tissue (MW 139,000) that was free of ADA activity but was able to convert lower molecular weight forms of ADA to the large complex normally found in lung (M1). The specific function of the conversion factor remains ambiguous. The effects on *in vivo* stability and susceptibility to various allosteric effectors remains to be investigated. Patients with congential ADA deficiency do have the conversion factor in normal amounts, so that it is probably not under control of the same gene that regulates synthesis of the ADA per se.

7. Theoretical Considerations

The enzyme-catalyzed reactions that lead from the free purines to the ribonucleotides have been referred to as a "salvage pathway." In view of the major recycling of hypoxanthine shown in patients with genetic blocks and the severity of clinical manifestations of some such individuals, the term salvage appears to be a major misnomer. An attempt has been made to integrate the known reactions of purine derivatives into a logical sequence of steps that might explain the magnitude of the amount of hypoxanthine and suggest a basis for the consequences of interruption. Figure 3 outlines such a scheme, which is an expansion of a cycle previously proposed (B3).

Each of the reactions in the figure has been shown to occur in almost

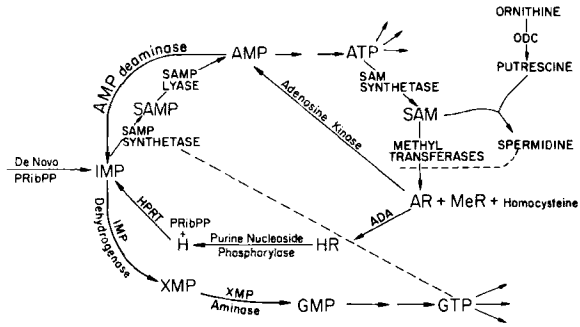

FIG. 3. Reactions of purine recycling. ODC, ornithine decarboxylase; ADA, adenosine deaminase; HPRT, hypoxanthine phosphoribosyltransferase; SAM, S-adenosylmethionine; SAMP, adenylsuccinate; PRibPP, phosphoribosylpyrophosphate.

all normal cells. There are isolated exceptions, e.g., the absence of adenylsuccinate lyase in the human erythrocyte (L7, L8). In the preceding sections it has been shown that there is a large rapidly renewed pool of hypoxanthine that in the absence of HPRT is excreted in the urine as hypoxanthine or its oxidation products.

Inosine is the only potential precursor of hypoxanthine, and it could be formed in some cells from inosinate by phosphatases. However, such a blind cycle is teleologically unsatisfactory, and furthermore, the nucleotide phosphatases are membrane bound (S13) and inhibited by the normal intracellular concentrations of ATP (M14). Thus, it must be assumed that inosine is formed from adenosine.

Inosine can be converted directly to inosinate by inosine kinase. This enzyme has been shown to exist in human cells, but at a low level (P2). It undoubtedly does not play a major role. Inosine phosphorylase cleaves inosine to hypoxanthine and ribose 1-phosphate (K1). Inosine is formed by the deamination of adenosine catalyzed by adenosine deaminase, an enzyme found in varying concentrations in essentially all normal mammalian cells examined. Studies with an inhibitor of adenosine deaminase, ribosyl-4-amino-5-imidazole carboxamide, on several strains of *Escherichia coli* revealed a major role for the enzyme. In cells with a block in purine synthesis a condition of guanine deprivation occurred after growth with adenine as purine source, and there was a derepression of the enzymes that convert IMP to XMP and XMP to GMP (K17).

In another direction one must consider that adenosine accumulation could lead to other biochemical effects. It has been known for some time that adenosine can stimulate cyclic AMP synthesis in brain slices

(S2, S8). Others have reported that adenosine inhibits adenylate cyclase from brain, adipocytes, and lung tissue (F1, M6, W1). Brain tissues of most animals have very little adenosine deaminase. In other tissues the role of breakdown products must be further explored. The fact that cyclase in intact slices is stimulated while that of broken-cell preparations is inhibited may be related to the so-called "latent" adenosine deaminase that is mitochondria-bound and inactive in assays of adenosine deaminase in homogenates (M16).

Adenosine, in addition to its role as a substrate for adenosine deaminase, is phosphorylated to AMP by adenosine kinase. It would appear that in mammalian systems the conversion to the nucleotide predominates over deamination at physiological concentrations of adenosine (S11). The toxic effects of adenosine seen with cells in culture may require initial conversion to AMP by the kinase. Cells deficient in adenosine kinase are unable to convert certain nucleoside analogs to nucleoside phosphates and are, therefore, resistant to these antimetabolites (B10). There is no obvious metabolic deficit in such cells, but this is in no way indicative of whether an animal would suffer if all its tissues lacked adenosine kinase.

Transmethylation yields 1 mole of adenosine for every mole of methylated product. Patients are known with inborn errors that lead to incomplete metabolism of the homocysteine released after transmethylation. Homocysteinurics release about 3 mg of homocysteine per kilogram on unsupplemented diets, more on supplemented (G3, L1). This is minimal, owing to other fates of homocysteine and possible incompleteness of the block (L2). Cystathioninurics have been reported to excrete over a gram of cystathionine per day and up to 0.6 g per gram of creatinine (F6, H3, K9). Since the latter probably have some cystathioninase, and some remethylation of homocystine may occur, the reported values are also minimal. Nevertheless, the moles of homocysteine and its products are of the same order as the amount of hypoxanthine formed per day.

Interference with SAM synthesis has been reported with a number of compounds (L6). The most active of these and also several compounds that block methylation of histones, e.g., adenosine, spermidine, histidine, have all been shown to be ulcerogenic (M11).

Much of the inosinate that is not synthesized *de novo* is formed via adenosine, as outlined above. Some inosinate is produced via adenylate deaminase. The significance of this enzyme in priming the Krebs cycle has been emphasized by Setlow and Lowenstein (S6). The IMP formed can be reconverted to either AMP or GMP. The deaminase is under control of ATP and GTP (B20). Thus this route may be of importance in maintaining the A:G ratio in the cellular nucleotide pool.

Polyamines are formed from ornithine and SAM, thus affording an alternative fate for this purine derivative (P3). The polyamines, as well as adenosine, regulate transmethylation (H11, L3). Ornithine decarboxylase is produced in greater quantity in rapidly dividing cells (B17, D7, R1, R10). Levels of S-adenyosylmethionine decarboxylase are also increased in such tissues (H2, R9). There is evidence that this increase is cyclic AMP mediated (B7). This ties these compounds although indirectly to adenosine concentrations.

These reactions could in theory be the source of the uric acid found in man. They indicate the origin is part, not of random leakage or breakdown, but of an integrated important metabolic cycle. Only additional research can establish the extent to which this cycle is real, and its ultimate significance *in vivo*.

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EFFECTS OF ORAL CONTRACEPTIVES ON VITAMIN METABOLISM

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1. Introduction

The use of oral contraceptive agents (OCAs) is widespread and is being increasingly encouraged in developing countries. Their use has been associated with a number of side effects, in particular, a possible increased risk of thrombotic and embolic vascular disease. There is also evidence that OCAs may affect the metabolism of a number of vitamins. Evidence for deficiency of thiamine, riboflavin, ascorbic acid, pyridoxine, folic acid, and vitamin B_{12}, and for excess accumulation of vitamin A has been reported. This is of particular concern to populations in which vitamin nutrition may already be suboptimal and has been the subject of recent brief reviews (O2, R4, T1, W13).

This paper will review what is presently known about interactions between vitamins and oral contraceptives. It is clearly important to assess carefully the evidence available with regard to each vitamin and to point out that much remains to be learned about normal vitamin metabolism and the metabolic pathways in which they serve as important coenzymes. The normal metabolism of pyridoxine and folic acid will be discussed in some detail, since they have received the greatest attention by investigators.

2. Vitamin A (Retinol)

2.1. Plasma Vitamin A and Carotene Levels in Women Not Taking Contraceptives

The status of vitamin A in women not taking contraceptives may first be noted briefly. Laurence and Sobel (L2) found that plasma vitamin A levels are lowest during menstruation, peak at approximately day 15, decrease slightly and than peak again on day 26 before declining to the low levels found during menstruation. Essentially the same findings were more recently reported by Yeung (Y1). In pregnancy, the plasma vitamin A levels remain fairly constant during the first two trimesters but decrease significantly in the third trimester (B8).

Plasma carotene values are low during menstruation but rise about 17% at the 6th to 10th days of the cycle and remain constant at this level during the remainder of the cycle (Y1). In pregnancy, the mean value of the plasma carotene during the sixth, seventh, and eighth months was significantly higher than that for the first 5 months (B8).

TABLE 1
MEAN FASTING PLASMA VITAMIN A LEVELS IN WOMEN DURING THE MENSTRUAL CYCLE[a]

Days of cycle	Mean values (μg ± SD per 100 ml)		p value for significance of difference between values in columns 2 and 3
	Controls[b]	Woman taking oral contraceptives[b]	
1–5	27.3 ± 0.9	45.6 ± 4.3	<0.01
6–10	33.9 ± 1.4	40.6 ± 3.2	<0.05
14[c]–19	32.3 ± 1.0	46.5 ± 4.5	<0.01
20–24	29.3 ± 0.7	45.4 ± 4.7	<0.01
25–28	30.5 ± 1.1	42.6 ± 2.3	<0.01
Total cycle	30.5 ± 5.0	44.2 ± 14.5	<0.01

[a] Values are those of Yeung (Y1).

[b] Number of controls in each time period ranged from 15 to 22 subjects and totaled 97 for the entire cycle. Number of women taking oral contraceptives in each time period ranged from 11 to 14 subjects and totaled 67 for the entire group.

[c] No values were given for the 11- to 13-day period.

2.2. PLASMA VITAMIN A LEVELS IN WOMEN TAKING ORAL CONTRACEPTIVES

Several reports have indicated that women taking oral contraceptives had significantly increased levels of vitamin A in serum or plasma (B14, G2, W7, Y1). The data of Yeung (Y1) demonstrate this clearly (Table 1). The plasma vitamin A levels in women using oral contraceptives were significantly higher than in control nonusers, both for the entire cycle as well as for individual periods within the cycle. The existence of elevated levels of vitamin A for protracted periods has given rise to the concern that these levels may represent a hypervitaminosis and, when pregnancy is decided upon, result in congenital anomalies in newborn infants (G1, W7).

The evidence for such a danger is tenuous, however. Oral administration to rats of vitamin A in very large doses, namely 35,000 IU daily for 2–16 days, during the period of gestation leads to a lower than normal pregnancy rate but a high incidence, 52%, of malformations in the offspring, as compared with zero percent in controls (C5). In each case, the outstanding malformation consisted in a protrusion of part or all of the forebrain through a variable-sized defect involving the parietal and frontal bones in the upper part of the cranium (exencephaly). Other anomalies, such as cleft palate, shortening of the mandible and maxilla, gross eye defects, and hydrocephalus were also frequently present. Pilotti

and Scorta (P6) described a case of a pregnant woman who had ingested excessive amounts of vitamin A and D (40,000 and 600,000 IU, respectively) daily for a month at the beginning of pregnancy and gave birth to a child who, at postmortem, showed malformations of the urogenital system. However, such an individual case report hardly constitutes evidence of a causal relationship.

Indeed, the administration of substantial doses, 10,000 IU daily, during the last months of human pregnancy may counteract the decrease in the maternal plasma vitamin A which, as has been noted, ordinarily occurs at this time, but does not affect the plasma vitamin A concentration of the newborn infant (L7). Very high doses of vitamin A, 200,000–400,000 IU, given during labor, raised the maternal plasma vitamin A substantially, that is, about to 2- to 4-fold above the normal level, but did not affect the level in the newborn infant. Studies on pregnant guinea pigs yielded similar results, and considerable storage of vitamin A was found in the livers of the newborn guinea pigs. These findings indicate that there is significant transmission of vitamin A through the placenta and, at the doses employed, rapid withdrawal of vitamin A from the plasma and storage in the fetal liver.

These studies would suggest that the moderately elevated plasma vitamin A levels in the blood of pregnant women who have taken OCA are unlikely to adversely affect a fetus if pregnancy did develop. In fact, the mechanism by which excess of maternal vitamin A in the guinea pig leads to congenital anomalies has not been elucidated. It is well to note that exposure of the maternal rat or mouse to unrelated noxious agents such as radiation, trypan blue, and hypoxia may also lead to similar fetal anomalies (C5).

Gal *et al.* (G3) reported significantly higher postpartum concentrations of serum vitamin A in mothers of children with birth defects involving central nervous system than in mothers of normal children. Bubb (B23), however, has suggested that these reported differences may be spurious, since the measurement of vitamin A by the methods used by these investigators may not be specific in the presence of high concentrations of carotenoids in serum.

2.3. Effect of Intramuscular Depot Progestins on Vitamin A Metabolism

The use of an intramuscular progestogen preparation "Depo-Provera" (150 mg of medroxyprogesterone acetate or 6α-methyl-17α-acetoxyprogesterone) every 3 months does not lead to an increase in the level of plasma vitamin A levels (B14). For example, the plasma

vitamin A level, expressed as micrograms per liter of retinol, was 370 ± 50 (SD) for 24 persons treated with "Depo-Provera" as compared with levels of 380 ± 82 (SD) for 88 untreated controls and contrasted with levels of 730 ± 91 (SD) for 48 persons receiving combined oral contraceptives.

3. Vitamin B_1 (Thiamine)

There appears to be only one reference to the effect of OCA on vitamin B_1 (B16). It has been appreciated for some years that the pyrophosphate of thiamine acts as a coenzyme in a number of metabolic transformations and is responsible, among other activities, for the transfer of "active acetaldehyde" and "active glyoxal." Transketolase mediates the reversible interaction of ribulose and ribose phosphates to form glyceraldehyde and sedulose phosphate. A C_2 fragment, active glycoaldehyde, is transferred during this reaction by combination with and release from thiamine pyrophosphate. Briggs and Briggs (B16) studied 20 healthy young women given OCAs during menstrual cycles. Before treatment, the erythrocyte transketolase activity was stimulated by excess thiamine pyrophosphate to the extent of 23 ± 7 (SD) %. After the third cycle, the stimulation was 30 ± 11 (SD) %, which was significantly greater ($p < 0.005$) than during the control period. This could indicate that a relative deficiency of thiamine is sometimes induced by contraceptive steroid combinations. More investigation of their effects on thiamine metabolism is needed.

4. Vitamin B_2 (Riboflavin)

Employing the activity of erythrocyte glutathione reductase as an indicator of riboflavin nutrition, Sanpitak and Chayutimonkul (S3) found that the activity was significantly lower than normal in the erythrocytes of women taking oral contraceptives. In the derivatives, riboflavin 5'-phosphate flavin mononucleotide, FMN and flavin-adenine dinucleotide (FAD), the isoalloxazine ring acts as a reversible redox system, and these two compounds, particularly the latter, act as coenzymes in the action of many reductases. Sanpitak and Chayutimonkul (S3) observed that the *in vitro* stimulation of erythrocyte glutathione reductase activity by the addition FAD to the assay mixture was much less in the control women than in those taking contraceptives. Briggs and Briggs (B14) have reported that the urinary excretion of riboflavin was decreased markedly from a mean value of 38 ± 12 μg of creatinine per mole in a control group of 15 untreated African women to 19 ± 11 (SD) μg of creatinine per mole in 10 African women taking combined contraceptives.

5. Vitamin C (Ascorbic Acid)

5.1. Concentration of Vitamin C in White Cells

The normal concentrations of ascorbic acid in plasma and white cells have been studied by several investigators as a basis for determining deviations in disease or abnormal physiological conditions. Lowry *et al.* (L8) submitted evidence that the concentration of ascorbic acid in the white cells was a measure of the total body content of this vitamin with a maximal value of 4 g. They studied three groups of young, vigorous male volunteers who were in the Royal Canadian Air Force. These subjects received daily intakes of 8, 23, and 78 mg of ascorbic acid for a period of 8 months and, at the end of this period had, respectively, white cell concentrations of 11.9, 12.9, and 24.2 mg per 100 ml of white cells. Upon the administration of large doses, namely, 500, 1000, and 2000 mg, of ascorbic acid daily for a period of 4 days to the group of subjects who had been on 8 mg per day, the white cell concentration rose to maximal values of about 30, 32, and 35 mg per 100 ml, respectively.

These latter values are essentially in agreement with other studies on healthy adults on normal diets, namely, a range of 25 to 38 mg with an average of 34 mg per 100 g of white layer white cells in 7 healthy young adults (B24) and a mean value of 36.1 ± 8.3 (SD) mg per 100 g in 22 healthy young adults (B9).

5.2. Concentration of Vitamin C in Plasma

There is fairly good agreement in the literature with respect to the normal concentration of ascorbic acid in the plasma: 0.75 ± 0.40 (SD) mg per 100 ml in 50 healthy young medical students as calculable from the data by Roe *et al.* (R5); 1.16 ± 0.09 (SE) mg per 100 ml in healthy young women on a supplement of 100 mg of ascorbic acid (D6); 0.79 ± 0.42 (SD) mg per 100 ml in 23 healthy adults (B9); 0.79 ± 0.04 (SE), and 0.73 ± 0.05 mg per 100 ml in two groups, each of about 30 Royal Canadian Air Force men, on a daily dietary intake of 78 mg of ascorbic acid (L8).

As has been pointed out, Dodds *et al.* (D6) in 1950 indicated that the plasma ascorbic acid concentration in healthy young women on a supplementary daily intake of 100 mg ascorbic acid was higher than that in males. Reports on changes during the menstrual period have been conflicting (R2). Using a small number of subjects on a dietary intake of 150 mg of ascorbic acid, Rivers and Devine (R2) reported that fasting plasma levels of ascorbic acid and total ascorbic acid were

increased during the high estrogen and luteinizing hormone secretion phases of the cycle. Total ascorbic acid was determined by using the 2,4-dinitrophenylhydrazine method, and ascorbic acid by titration with 2,6-dichlorophenolindophenol. The increases in the plasma levels 3 hours after a dose of 300 mg were of the same order.

The effects of oral contraceptive agents on ascorbate levels in plasma, leukocytes, and platelets of African women living in the Republic of Zambia have been studied in some detail by Briggs and Briggs (B13–B15). The mean value for the plasma concentration in 15 Zambian women of apparently good health was given as 7.5 ± 2.0 (SD) μg/liter (B14). The "μg" is, however, a misprint (M. Briggs, personal communication and should be 7.5 ± 2.0 (SD) mg/liter, or 0.75 ± 0.2 mg per 100 ml, essentially the same value obtained by Lowry (L8), Roe (R5), and Bodansky *et al.* (B9).

With regard to the concentration of ascorbic acid in leukocytes, Briggs and Briggs (B13) reported a control value of 39 ± 28 (SD) μg per 10^8 cells. On the assumption that the average diameter of the white cells is 12 μm (W9) and that the cell is spherical, the value of Briggs and Briggs (B13) can be calculated to be about 45 mg/100 ml. The mean normal values obtained by Butler and Cushman (B24), Lowry *et al.* (L8), and Bodansky *et al.* (B9) were about 30 mg/100 ml. The administration of oral contraceptives leads to significant decreases in the plasma, leukocytes, and platelet concentrations (Table 2).

TABLE 2
EFFECT OF ORAL CONTRACEPTIVES ON CONCENTRATION (±1 SD) OF ASCORBIC ACID

	Controls	Oral contraceptives	Reference
Platelets (mg/g wet wt.)	0.26 ± 0.11 (10)	0.19 ± 0.13[a] (10)	Briggs and Briggs (B13)
White cells (μg/10^8 cells)	39 ± 28 (31)	26 ± 22[a] (39)	Briggs and Briggs (B13)
Plasma (μg[c]/100 ml)	0.75 ± 0.20[c]	0.45 ± 0.31[c]	Briggs and Briggs (B14)
Urine (mg/100 ml)	3.19 ± 1.34 (7)	1.48 ± 0.87[b] (6)	Harris *et al.* (H6)

[a] P value is <0.01.

[b] P value is <0.05.

[c] These values were given by Briggs and Briggs (B14) as 7.5 ± 2.0 (SD) μg per liter. The term "μg" is a misprint and should be "mg" (M. Briggs, personal communication, February 26, 1975). The value for controls is essentially the same as those obtained for presumably normal Canadian and American adults by Lowry *et al.* (L8), Roe *et al.* (R5), and Bodansky *et al.* (B9).

TABLE 3
EFFECT OF ORAL CONTRACEPTIVE AGENTS (OCA) AND VITAMIN C SUPPLEMENTATION ON THE CONCENTRATIONS OF ASCORBIC ACID (mg/100 g) IN THE LEUKOCYTES OF MATURE WOMEN[a]

Subject	Mean ± SD in Controls	Mean ± SD in Subjects on OCA
Subjects on daily dietary intake of 85 ± 47 mg/day	24.1 ± 14.5 (54)[b]	19.0 ± 7.0 (42)[c]
Subjects with daily ascorbic acid supplement of 50–200 mg/day	35.2 ± 12.1 (8)[e]	19.4 ± 6.1 (19)[d]
All subjects	25.7 ± 14.5 (64)	19.0 ± 6.6[d]

[a] Based on data of McLeroy and Schendel (M2).
[b] Number in parentheses refers to number of subjects.
[c] Significantly lower ($p < 0.05$) with respect to controls.
[d] Significantly lower ($p < 0.01$) with respect to controls.
[e] Significantly higher ($p < 0.05$) with respect to groups of all subjects or subjects on ordinary dietary intake.

A more detailed study of the effect of oral contraceptives on the ascorbic acid level in the luekocytes was submitted by McLeroy and Schendel (M2). The results obtained in 126 sexually mature women are shown in Table 3. It may be noted first that the ascorbic acid concentration was significantly increased in the control subjects who were taking supplements. This may be taken to indicate a state of tissue undersaturation in those taking about 85 mg daily, although concurrent studies of plasma levels and urinary excretions of ascorbic acid would, in accordance with the studies of Lowry *et al.* (L8), have furnished more definitive information on this point. The concentration of ascorbic acid was significantly decreased as the result of administering the contraceptive agents, and it may be noted that the level in those subjects taking ascrobic acid supplements was no higher than in those on the ordinary dietary intake.

6. Folic Acid

6.1. INTRODUCTION

Shojania *et al.* (S11) reported in 1968 that many women taking OCAs have low levels of serum folate, thereby focusing attention on a possible interaction between these drugs and folate metabolism. This was followed by a number of reports of megaloblastic anemia in women using

these agents. Streiff in 1970 described megaloblastic anemia (hematocrits 11–32%) in 7 women who had been taking oral contraceptives for 1.5 years or more (S21). Serum folate levels in these women were low and B_{12} levels were normal. All had been eating normally and had not lost weight. Gastrointestinal studies which included radiographic examination, measurement of D-xylose absorption, stool fat, and serum carotene were normal. Since no other possibilities were evident, OCAs were considered the likely cause of folate-deficiency anemia in these women. A number of similar case reports have appeared (F1, H11, N2, P2, R20, S2, S10, W12). Streiff presented evidence (S21), which will be discussed below, that OCAs may interfere with folate absorption. And there was also evidence at the time that other drugs, diphenylhydantoin in particular, might impair folate absorption (H10).

It is well recognized that patients with occult melabsorption due to jejunal disease, in particular, gluten-sensitive enteropathy (nontropical sprue) may present with a selective nutritional deficiency such as folic acid deficiency (M5). Folate deficiency anemia is very common also in tropical sprue. More recent reports of OCA-associated anemia and folate deficiency suggest that, if jejunal biopsies and follow-up gastrointestinal studies are performed, a significant number of these women will be found to have occult intestinal malabsorption (J3, S10, T2, W12). But it is not clear at present whether or not OCAs do interfere with folate absorption. In interpreting these clinical reports, therefore, it is important to review briefly what is known about folate absorption and disposition in normal individuals and to examine the evidence for changes that may be induced by OCAs.

6.2 Normal Folic Acid Absorption and Metabolism

Folic acid, or pteroylglutamic acid, is present in blood in its free (unconjugated) form. In foods, however, the bulk of folic acid exists as polyglutamate conjugates (B3). Its principal conjugate in most situations is felt to be pteroylheptaglutamic acid, in which the glutamates are joined together by γ-peptide linkages. Folate in foods is often present also as reduced formyl derivatives and as reduced methylfolate compounds. Within cells folate is stored as polyglutamic acid conjugates, while the free or monoglutamate form, reduced to tetrahydrofolate by the enzyme dihydrofolate reductase, is required for coenzyme function (R18).

It is reasonable to expect that deconjugation is required for absorption of folate because pteroheptaglutamate is a large and strongly electronegative molecule that would not diffuse easily across biological mem-

branes (R18). Studies of folate deconjugation during absorption have utilized primarily conjugates purified from yeast. When these are fed to humans, unconjugated folate is excreted in urine (B4, S22). A number of workers have demonstrated that when purified yeast folate is ingested by normal individuals, unconjugated folate appears in the blood (P5, R18). Studies using ^{14}C-labeled folate conjugates have indicated that cleavage to the monoglutamate occurs before or during absorption. If the pteroate portion of the molecule or the first glutamate is labeled, ^{14}C appears in plasma folate; but if the second glutamate is labeled this does not occur, and the label appears as $^{14}CO_2$ in the breath (B25).

Gastric and pancreatic proteolytic enzymes are inactive against conjugated folate, because the polyglutamates are linked by γ-peptide bonds rather than the usual α-peptide linkages characteristic of proteins. The small intestine has been demonstrated to be capable *in vitro* and *in vivo* of deconjugating folate (B3), and it appears unlikely that the liver is responsible for the appearance of free folate in blood after ingestion of conjugates (R18). It has not been definitely established whether hydrolysis of conjugated folate occurs in the intestinal lumen, at the brush border, or intracellularly. The brush border would seem to be a likely site for hydrolysis of folate, because other short-chain molecules, such as peptides and disaccharides, are hydrolyzed by brush border enzymes.

Hydrolysis of ^{14}C-pteroylheptaglutamate occurs in the intestinal lumen during jejunal perfusion in man, and contact with the mucosa is required for this deconjugation to occur (H1). Cell fractionation studies do not show high folate conjugase (γ-glutamyl carboxypeptidase) activity in the brush border fraction, but indicate that highest activity is present in the lysosomal fraction (B3, S19). It is possible that conjugated folate is taken up by epithelial cells and that hydrolysis occurs in lysosomes. The folate conjugase has a low optimal pH and the lysosomes may provide an acidic environment for hydrolysis. Another possibility is that the enzyme is released into the lumen of the intestine as mucosal cells proliferate and are sloughed. At present there is not enough experimental evidence to distinguish these various possibilities and the precise process by which folate is deconjugated during absorption remains somewhat obscure (R18).

The mechanism of absorption of unconjugated folic acid is also incompletely understood. There is some evidence for a specific transport mechanism for pteroylglutamate, but, as discussed by Rosenberg and Godwin (R18), such a mechanism is not accepted by all investigators and a requirement for cellular metabolic energy has not been demonstrated. Furthermore, much folate in the diet is in the form of dihydro- and tetrahydrofolate, which may not be absorbed by the same mechanisms

as unreduced pteroylglutamate. There is evidence that unreduced folate monoglutamate is absorbed largely unchanged (R18), while the dihydro and tetrahydro derivatives are methylated before they appear in peripheral blood (P4). Folinic acid (5-formyltetrahydrofolic acid) is also methylated during absorption. While the intestinal mucosa is clearly capable of methylating folic acid and its reduced forms, there are conflicting experimental data about the precise role of methylation in folate absorption (R18).

The quantitation of folate absorption under both normal and abnormal conditions remains difficult, especially in view of the variety of forms of folate that may be consumed. Absorption of each of these forms has not been studied in detail. Since OCAs have been postulated by some to interfere with folate conjugase (see below), it is especially important for this discussion to compare the efficiency of absorption of conjugated and unconjugated forms of folic acid.

As reviewed in detail by Rosenberg and Godwin (R18), folate absorption has been measured by three basically different methods: (1) measurement of rises in blood folate after an oral dose, (2) measurement of folate compounds in urine after an oral dose, and (3) administration of isotopically labeled folate by mouth followed by measurement of isotope appearing in plasma and excreted in urine and feces. Folate in plasma and urine is assayed with bacteria, usually strains of *Lactobacillus casei* or *Streptococcus faecalis,* which require folate for growth. They differ somewhat in the forms of folate they can utilize, but in general these microbiological assays measure unconjugated folate in either their reduced or unreduced forms.

After oral administration of unconjugated pteroylmonoglutamate, there is a plasma rise and also increased excretion of microbiologically detectable folate in urine. These measurements may be difficult to interpret because the degree of both increases varies markedly with the size of the oral dose and with the size of endogenous folate stores. Giving a prior folate loading dose to saturate tissue stores only partially corrects this variability. Use of radioactive labeled compounds allows more direct measurement of folic acid absorption. Balance studies using ^{3}H-labeled folate in man have shown (A6) that 41–95% of an oral dose is absorbed, if absorption is calculated from the amount of unabsorbed label in feces. The amount of label appearing as folate in urine and the degree of plasma rise varies markedly depending upon whether a prior loading dose of folate is given. With such a loading dose, 25–60% of orally administered label appears in urine (A6, K4, K5) while without a loading dose only 2–22% is so excreted (A6).

There is disagreement among investigators as to whether conjugated

folate is absorbed as well as the monoglutamate (P5, R18). Absorption studies using purified yeast folate (which is mostly a mixture of reduced heptaglutamic folates) indicate that these conjugates are less well absorbed than unreduced pteroylmonoglutamate (B3, P3, P5). There are a number of possible explanations for this: (1) conjugated reduced and unconjugated reduced folate may be handled differently during absorption at least with regard to methylation. Pure reduced folate monoglutamates are well absorbed but are methylated before they appear in plasma (P4). Methylated folate in plasma is detectable by the *L. casei* assay, whereas unmethylated folates (if fewer than 3 glutamic acids are attached) will support growth of both *L. casei* and *S. faecalis*. After ingestion of yeast folate polyglutamates, the folate that appears in plasma will support growth of both bacteria, indicating that reduced folate polyglutamates are only partially methylated during absorption (R18). (2) Yeast folate preparations commonly used to assess folate polyglutamate absorption may contain inhibitors of the folate conjugase enzyme (R18, S22). However, a recent study by Perry and Chenarin (P5) indicated that yeast preparations do not contain material which was inhibitory to folate deconjugation either *in vitro* or *in vivo*, and such inhibitors have never been demonstrated in other folate containing foods. Studies using crystalline yeast heptaglutamate (S22), ^{14}C-labeled polyglutamates synthesized by the solid phase method (B25, H1) and ^{3}H-labeled pteroylheptaglutamate (R18) indicate that the conjugate is almost as well absorbed as free folate. Therefore, while it is clear that deconjugation of folate is an important step in absorption of the vitamin, whether the conjugates in food are less available for absorption than free folate is an important question which awaits further clarification.

6.3. Oral Contraceptives and Plasma Folate Concentrations

As was mentioned earlier, reports by Shojania *et al.* (S11, S12) of decreased plasma and red blood cell folate concentrations in users of OCAs first drew attention to this subject. Luhby *et al.* (L10) also found evidence of folate deficiency in some women taking OCAs, and Wertalik *et al.* (W6) found lower plasma folate levels in women on "the pill" than in controls. It was felt (S12) that a large number of subjects given these agents for long periods of time needed to be studied in order to demonstrate a statistically significant difference, because folate concentrations were seldom profoundly depressed in such women.

A number of subsequent articles described no significant differences in plasma folate levels between users of oral contraceptive and normal

women (C2, K1, M1, M4, P1, P9, S17, S19). Shorter duration of contraceptive use, obtaining controls from different sources, and small numbers of subjects in some of these studies have been cited (S12) as objections to a number of these reports. Variations in serum folate concentrations do not occur with the menstrual cycle (S19), but Stephens *et al.*, do mention that higher plasma folate concentrations might be expected after eating, and in the studies of Shojania *et al.* (S11, S12) the drawing of fasting bloods was not specified. But the latter authors also found greater 12-hour urinary formiminoglutamic acid (FIGLU) excretion after a histidine load in OCA-users in addition to lower plasma and erythrocyte folate concentrations. These parameters often improved after discontinuation of medication (S12).

The possible effects of OCAs on folate metabolism, as indicated by these studies of serum folate concentrations, may therefore be mild but could assume considerable importance in subjects with gastrointestinal disease and decreased absorption of folate, women with marginal dietary folate intake, and those who subsequently become pregnant and then have an increased requirement for folic acid. Studies that followed were warranted by these considerations.

6.4. Oral Contraceptives and Folate Absorption

Impairment of absorption of conjugated folate by OCAs initially appeared to be a likely possibility. Streiff (S21) measured serum folate levels after oral administration of free folic acid in nine normal women and in nine other women who had been taking OCAs for at least one year, and found similar rises in serum folate in both groups. In contrast, when the same women were given yeast folate polyglutamate orally, the rise in serum folate was less in the women taking OCAs. Similar results were reported by Necheles and Snyder (N2). In these two studies, subjects were not given saturating doses of folic acid prior to testing.

The findings of Stephens *et al.* (S19) were similar, but they also reported that if tissues are saturated with folate before testing, folate polyglutamate absorption, as measured by rises in serum folate monoglutamate, was of the same degree in women using oral contraceptives and in those who were nonusers.

In a later study Shojania and Hornady (S10) also failed to find a difference between normal women and users of contraceptive medications when folate and folate polyglutamate absorption was estimated. Subjects were saturated with folate before absorption was studied. A rough correlation was found in both groups of women between original serum folate concentrations and the rise in serum folate after polygluta-

mate administration, and some women were found to have low serum folate levels and subnormal rises in serum folate after oral polyglutamic folate whether or not they were users of oral contraceptives.

These authors previously found (S12) that in all of 16 women who initially had subnormal serum folate concentrations, serum folate concentrations rose within 3 months after OCAs were stopped. Subsequently they reported (S10) 3 women with low serum folate levels while taking OCAs and low folate polyglutamate absorption that persisted after medication was discontinued. It was of interest that one of these subjects developed gluten-sensitive enteropathy a year later and a second had a family history of that disease. Other case reports of folate deficiency and mild intestinal malabsorption in users of OCAs have appeared (J3, T2, W12). It should be emphasized, therefore, that evidence of impaired folate absorption in women taking these agents may suggest the presence of inapparent small bowel disease.

The hypothesis that contraceptive steroids might interfere with folate deconjugation in the small intestine was examined by Stephens *et al.* (S19). Homogenized human jejunal mucosa obtained by peroral biopsy and lysosomal preparations from guinea pig jejunum were utilized. Estradiol, estrone, and progesterone were found not to inhibit folate conjugase in these systems, or to inhibit transport of folate conjugase across lysosomal membranes.

At present, therefore, there seems to be little solid documentation for an absorptive defect of folate polyglutamate in normal women taking OCAs, and furthermore, a mechanism for such an impairment is not apparent.

6.5. Contraceptives and Other Effects on Folate Metabolism

Little is known about other aspects of folate metabolism, such as the factors that determine plasma clearance, in either normal subjects or users of oral contraceptive steroids. As described earlier, maximum serum folate concentrations after oral folate polyglutamate are lower in contraceptive users than in nonusers, when subjects are not presaturated (S19). This may be due to increased clearance from plasma, because poor absorption was not found. Stephens *et al.* (S19) felt that this was probably not due to tissue folate depletion because there was no correlation with initial fasting serum folate concentrations. However, erythrocyte folate concentrations were not measured and might have been a better index of tissue saturation with the vitamin.

A plasma clearance study of injected folic acid in 6 women taking oral contraceptives and 7 controls was recently reported (S13). Plasma

concentrations were first measured 5 minutes after injection, and were significantly lower at this time in the contraceptive users. After the initial 5 minutes there was no difference in clearance rates, suggesting that only the initial distribution volume or the initial tissue uptake was greater in the subjects on medication. Chanarin *et al.* (C4) have shown that within 3 minutes of intravenous injection of folic acid [15 μg/kg, which is the same dose used by Shojania *et al.* (S13)], 60% is cleared from plasma and distributed in intra- and extracellular fluids. Shojania *et al.* (S13) felt that the difference they found between users and non-users of OCAs was too great to be explained by fluid retention.

Plasma folate clearance is increased during pregnancy (C4). This may be due to placental uptake, as is generally believed, or to effects of elevated steroid hormones in pregnancy. Plasma protein concentrations change during pregnancy and during treatment with contraceptive steroids (S14). About half of folate in plasma is reported to be protein bound (M7), and some effects of pregnancy and OCA-use on binding of folic acid to blood proteins have been reported (D1, M6, P1).

Da Costa and Rothenberg (D1) have demonstrated in leukocytes and serum a macromolecular substance that binds unreduced folates and dihydrofolate. This material was present in leukocyte lysates and serum of about half of the pregnant women tested and in the majority of 10 women taking OCAs. This binding factor was immunochemically similar to that found previously in some human leukemia cells, but was not found in normal control subjects (D1). It was identified by binding with added tritium-labeled pteroylglutamate, and might not be detected if it were already saturated with folate. Among 51 pregnant women there was a suggestion that the presence of folate binder in leukocytes was correlated with lower serum folate concentrations, although this correlation could not be definitely established statistically (D1). Further study of folate binding factors in the blood of pregnant women and contraceptive users is clearly needed, and might be helpful in explaining the effects of contraceptive steroids on folate metabolism.

There has been little investigation of tissue metabolism of folate and what effects contraceptive steroids may have. Bovina *et al.* (B10) have reported data indicating that oophorectomy results in decreased tetrahydrofolic acid and increased methyltetrahydrofolic acid in liver. This effect is partially corrected by exogenous estrogen treatment. It is not known whether contraceptive steroids given to normal women affect metabolic interconversions of various forms of endogenous folate. Another interesting postulate is that induction of drug metabolizing enzymes in the liver by exogenous chemicals may increase physiological demand for folate (M13). Human studies indicate, however, that OCAs may impair rather than enhance hepatic drug metabolism (C1).

At present there are not sufficient data to decide whether derangements in folate metabolism unrelated to absorption occur in oral contraceptive users, and further research is needed.

6.6. Conclusion

Users of contraceptive steroids may develop megaloblastic anemia due to folic acid deficiency. This is a rare occurrence, and a significant number of such women can be expected to have previously unrecognized small bowel disease or some other cause of folic acid deficiency. Whether there are significant abnormalities in folate metabolism in a large proportion of women taking OCAs has not been conclusively demonstrated; if such abnormalities do exist they are generally mild, and may be apparent only if large numbers of subjects are studied and compared with controls. Evidence with regard to impairment of folate absorption is conflicting, and recently attention has been given to the possibility that OCAs may increase clearance of folate from blood or change the amounts of binding proteins in plasma and in cells. At least in Western countries, available information regarding interactions between folate and OCAs does not clearly indicate that women on contraceptive steroids require more folate than do other women. More needs to be learned about normal folate absorption and disposition and how they are affected by various metabolic changes.

7. Vitamin B_{12} (Cobalamine)

Vitamin B_{12} status in oral contraceptive users has received less attention than that of folate, possibly in part because there have been no reported cases of megaloblastic anemia with evidence of B_{12} deficiency in OCA-users. All women with apparent folate deficiency anemia in association with use of OCAs (see above) had normal serum B_{12} levels in those instances in which it was measured.

There are several reports (B12, S9, W6), however, that women using OCAs have statistically significant lowering of serum B_{12} levels when compared to normal control women. Wertalik *et al.* (W6) also observed four women before and after starting oral contraception, and noted that all developed reductions in serum B_{12} levels ranging from 24 to 58%. Reduction in B_{12} levels in women using OCAs appears to be seldom severe and has not been associated with decreased tissue (red blood cell) B_{12}, anemia, or hypersegmentation of polymorphonuclear leukocytes (B12, S9, W6). Similar observations have been made in pregnant women (R19).

Some women taking an OCA may have subnormal levels of both folate and B_{12} (W6), suggesting a relationship between these two changes. However, the four women who were reported (W6) to have decreasing levels of serum B_{12} subsequent to starting to take the pill did not have falling folate levels in serum. Two subjects with low B_{12} and folate levels were treated with folic acid only while contraceptive steroids were continued, and they responded with supranormal serum folate levels and no change in serum B_{12}. Another such woman with low serum folate and B_{12} concentrations was treated with oral folic acid and cyanocobalamin (1.0 μg/day) without stopping the OCA; her serum folate rose, but serum B_{12} concentration did not (W6). Therefore, a clear relationship between B_{12} and folate serum concentrations in contraceptive users is not apparent.

African women using OCAs were observed (B12) to have lower serum B_{12} levels than nonusers, while a significant reduction was not found in women given Depo-Provera (medroxyprogesterone acetate) every 3 months, possibly indicating that estrogens have a greater effect on B_{12} metabolism than do progestogens. The mechanism for an effect of any OCA on B_{12} is not clear, however. The Schilling test for vitamin B_{12} absorption was normal in a few women tested while taking OCAs (S9, W6). Unsaturated B_{12}-binding capacity was found to be unchanged in OCA-users in one study (W6). Bianchine *et al.* (B6) found binding capacity to be somewhat increased in women on oral contraceptives but did not report serum B_{12} measurements. It is not known whether OCAs affect plasma clearance, renal excretion, or tissue uptake of B_{12} in plasma.

It can be concluded that the relationships between OCAs and the mild associated decreases in serum B_{12} concentrations which have been reported requires further clarification.

8. Vitamin B_6 (Pyridoxine)

8.1. Introduction

The vitamin B_6 group of coenzymes consists of pyridoxine, pyridoxal, and pyridoxamine and their metabolically active phosphorylated forms. They are striking for the variety of enzymic reactions in which they are important, and many amino acid transformations, including various transaminations and decarboxylations, are vitamin B_6 dependent. Compounds with vitamin B_6 activity are apparently not stored in the body in large amounts, and biochemical evidence of B_6 deficiency can develop quickly if intake is inadequate (S4).

Vitamin B_6 status in man is most commonly assessed by measuring the amounts of xanthurenic acid and other tryptophan metabolites in

urine, most of which are excreted in increased amounts when B_6 is deficient. Other techniques, which are less often used, include the measurement of vitamin B_6 in plasma and urine, quantitation of 4-pyridoxic acid in urine, and measurement of blood (especially erythrocyte) transaminase activity. Methods of assessing B_6 status are in many instances not well standardized, so that it is often difficult to compare results obtained by different laboratories.

As will be discussed in this section, some women taking OCAs have increased urinary excretion of tryptophan metabolites. The pattern of excretion of these metabolites resembles that found in pyridoxine deficiency, and can be corrected by giving large amounts of pyridoxine (R6, T3). This has suggested that OCAs either may produce an absolute deficiency of pyridoxine or increase the body's requirement for this important coenzyme. There is evidence (A1, B1) also that altered pyridoxine status may be associated with the mental depression that sometimes occurs in women taking OCAs.

The tryptophan metabolites found in urine are products of a complex pathway in which a number of enzymic reactions are pyridoxal phosphate (PLP) dependent (Fig. 1). The PLP-dependent enzymes in the pathway differ with regard to their distribution in subcellular organelles, and they are not equally sensitive to vitamin B_6 deficiency (S7). Studies in animals, which will be discussed, indicate that conjugated estrogens may, even in the absence of vitamin B_6 deficiency, displace PLP from apoenzymes to a varying degree and result in a redistribution of PLP among cellular apoproteins. Therefore it is important initially to examine what is known about the normal tryptophan–niacin and the serotonin pathways and how enzyme substrates, apoenzymes, PLP, and endogenous and exogenous steroids interact, in order to interpret the related biochemical changes that have been found to occur in women using OCAs.

8.2. The Tryptophan–Niacin Pathway, the Serotonin Pathway, and Vitamin B_6

Tryptophan is an essential amino acid which is ingested by Americans in quantities that exceed the normal daily requirements for protein synthesis (R1), and considerable amounts are converted to nonprotein substances such as nicotinic acid and serotonin (Fig. 1). The tryptophan–niacin pathway, which is also known as the kynurenine pathway (Fig. 1), is important for production of the vitamin, nicotinic acid, and provides also a means for degrading tryptophan to acetoacetyl-CoA, carbon dioxide, and ammonia (P7). The amount of tryptophan metabolized by the various pathways available depends greatly on the amount of

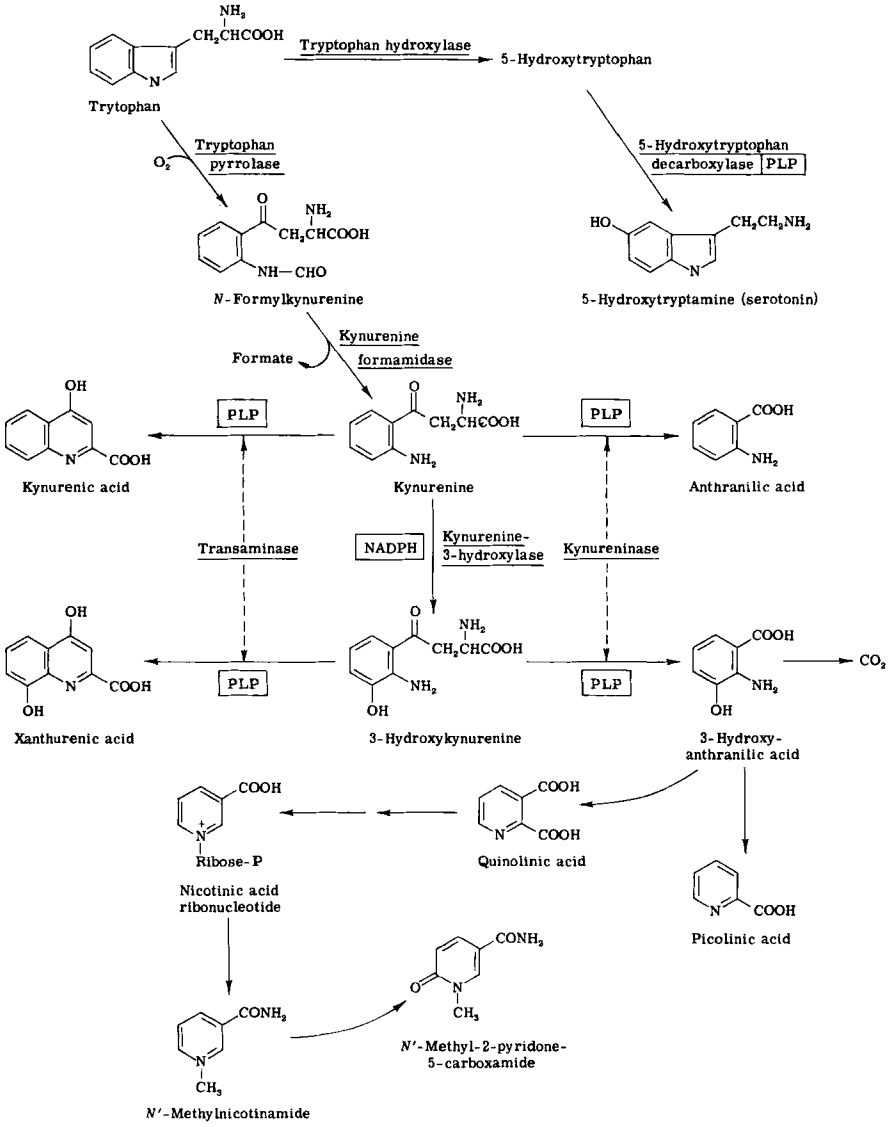

FIG. 1. Metabolism of tryptophan to serotonin (5-hydroxytryptamine) and niacin. Pyridoxal phosphate (PLP) dependent reactions are indicated. Reactions not shown which may result in formation of products excreted in urine include the acetylation of kynurenine and 3-hydroxykynurenine, conjugation of anthranilic acid with glycine (to form *o*-aminohippuric acid) and with glucuronic acid, and the dehydroxylation of kynurenic acid and xanthurenic acid to quinaldic acid and 8-hydroxyquinaldic acid, respectively.

the amino acid in the diet. Isotope studies in man indicate that a major part of administered tryptophan is metabolized to CO_2 and that this proportion is increased by giving a tryptophan loading dose (H5, L4). After a loading dose, intermediates and by-products of the tryptophan–niacin pathway are also excreted in greater amounts (L4). When tryptophan intake is restricted, some conversion of tryptophan to niacin still takes place; and when intake is restored, tryptophan is used first to restore nitrogen balance and then to restore pyridine nucleotides, after which niacin metabolites appear in the urine (N1, V2).

Little of the tryptophan that enters the tryptophan–niacin pathway is actually used to form nicotinic acid ribonucleotide, and 60 mg of tryptophan results in the formation of only about 1 mg of nicotinic acid (C8). Evidence recently reviewed (C8) indicates that this ratio is not a fixed one and shows considerable variation depending upon the amount of tryptophan and preformed nicotinic acid available to the organism and also the amount of PLP present. It is also of interest that nicotinic acid in the form of NADPH is required in one enzymic step in the kynurenine pathway, the hydroxylation of kynurenine to 3-hydroxykynurenine.

Intermediates or by-products of the tryptophan–niacin pathway which are excreted in the urine include kynurenine, 3-hydroxykynurenine, kynurenic acid, xanthurenic acid, anthranilic acid, N^1-methylnicotinamide, and N^1-methyl-2-pyridone-5-carboxamide (2-pyridone) (Fig. 1). Tryptophan metabolites account for the major part of the diazotizable aromatic amines present in the urine (B19, P7) and are relatively easy to quantitate after a loading dose of tryptophan. They are often measured to assess vitamin B_6 status because pronounced changes in their excretion occur in vitamin B_6 deficiency. The advantages of using a standardized tryptophan loading dose has been emphasized (C13, L4, P7), and 2 g seems to be most widely recommended. Leklem (L4) has summarized the effects of various doses of tryptophan and pointed out that 1–6% of a 2-g dose of tryptophan is accounted for in the urine within 24 hours as kynurenine, hydroxykynurenine, kynurenic acid, and xanthurenic acid. At doses above 2 g in normal subjects, a greater proportion is excreted as these metabolites, and kynurenine becomes the predominant one of the four.

Xanthurenic acid was the first tryptophan metabolite found to be elevated in the urine of pyridoxine-deficient animals (L5). When vitamin B_6 is deficient, liver kynureninase (Fig. 1) which is located in the cytosol, becomes rapidly depleted of PLP. However, the transaminases that metabolize kynurenine and 3-hydroxykynurenine to kynurenic acid and xanthurenic acid, respectively, are located in both kidney and liver and

in both the cytosol and mitochondria. The mitochondrial transaminase becomes depleted of PLP less rapidly. This accounts for the diversion of tryptophan metabolism to formation of large amounts of xanthurenic acid in pyridoxine deficiency (O1, R15).

But in assessing the state of vitamin B_6 nutrition, it is often advantageous to quantitate the urinary excretion of as many tryptophan metabolites as possible (P7). For example, in severe deficiency, as occurs in tuberculosis patients treated with isoniazid, the activities of both 3-hydroxykynureninase and 3-hydroxykynurenine transaminase are apparently markedly reduced, such that xanthurenic acid excretion may be normal and excretion of kynurenine and 3-hydroxykynurenine markedly increased (B20, P7).

It might be anticipated that B_6 deficiency would result in deficient pyridine nucleotide synthesis, since 3-hydroxykynureninase is sensitive to PLP depletion. Studies of nutritional B_6 deficiency in man, however, indicate that, although tryptophan metabolism becomes abnormal, urinary output of quinolinic acid still increases after a tryptophan load (R15). Decreased formation of pyridine nucleotides from tryptophan may occur only when PLP depletion is severe (R15).

The initial step in the tryptophan–niacin pathway is felt to be rate limiting and is catalyzed by the heme-containing enzyme tryptophan pyrrolase. Much of the evidence for this is based on studies in both man and animals of the effects of hydrocortisone on the activity of this enzyme and on urinary excretion of tryptophan metabolites. Tryptophan pyrrolase is induced by adrenal glucocorticoid administration (R15). A correlation between the levels of this enzyme in human liver obtained by needle biopsy and the amounts of kynurenine in urine has been demonstrated in a group of subjects with various diseases, some of whom were treated with hydrocortisone (A4). Xanthurenic acid, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid in addition to kynurenine are excreted in increased amounts after hydrocortisone treatment in man. Administration of large doses of vitamin B_6 reduces the excretion of these metabolites to levels equal to or below the levels found before hydrocortisone treatment. These findings suggest, therefore, that kynurenine 3-hydroxylase activity is adequate to metabolize greater than usual amounts of its substrate, but that the PLP-dependent enzyme 3-hydroxykynureninase may not be fully saturated with its coenzyme and that its activity can be potentiated by administering PLP (R10, R14, R15).

Tryptophan loading may have effects on the kynurenine pathway other than simply increasing available substrate and bringing out small deficiencies of PLP which would otherwise be latent. Tryptophan can itself

induce tryptophan pyrrolase activity (R15). Also, it has also been suggested (H13) that tryptophan given in large doses may result in vitamin B_6 deficiency by combining with PLP through the formation of Schiff bases. But formation of Schiff bases with other amino acids might also be expected, and it has been reported (L4) that when 10 g of glycine or lysine are given with 2 g of tryptophan there is no additional change in the excretion of tryptophan metabolites. Vitamin B_6 given with a tryptophan load in normal individuals results in a lower excretion of kynurenine, 3-hydroxykynurenine, and xanthurenic acid, raised excretion of 3-hydroxyanthranilic acid, and unchanged kynurenic acid excretion relative to that found after tryptophan loading alone (L4). This information suggested that under normal conditions PLP-requiring enzymes in the pathway are not fully saturated with the coenzyme (L4).

It should be mentioned also that results of the tryptophan loading test vary to some extent with age and sex. Women excrete more of a tryptophan load as the four metabolites discussed above than do men, and women under 40 excrete a greater percentage of the load as these metabolites than do older women (L4, R8). This sex difference, which is evident only with the higher tryptophan loading doses of 5 g or more, is felt to be due to endogensous estrogens (M3, R8). Further evidence of an estrogen effect in nonpregnant women is the increased excretion of tryptophan metabolites in the urine at the time of ovulation when estrogen formation is greatest (B21, R8).

Tryptophan is also the initial substrate for the 5-hydroxytryptamine (serotonin) pathway (Fig. 1). Administration of hydrocortisone to the rat results in decreased serotonin levels in brain and induction of tryptophan pyrrolase activity in the liver (C16, K6). In the gerbil, in which corticosteroids do not induce hepatic tryptophan pyrrolase, hydrocortisone does not result in any change in brain serotonin concentrations (G6). Although these findings are not conclusive proof, they suggest that if tryptophan pyrrolase is induced, particularly in the liver, tryptophan in brain may be diverted from the serotonin pathway to the kynurenine pathway, resulting in decreased serotonin synthesis in brain (C16).

8.3. The Tryptophan–Niacin Pathway in Pregnancy and during the Use of Oral Contraceptives

Pregnancy results in an increase in the urinary excretion of xanthurenic acid (B21, S18, V1, W1), 3-hydroxykynurenine, kynurenine, acetylkynurenine, 3-hydroxyanthranilic acid, 2-pyridone, and N^1-methylnicotinamide (B21, H8, V1, W1). Large doses of pyridoxine may reduce the urine levels of these metabolites to or near the normal range (W1, R10). The findings in one study (H4), in which women early in preg-

nancy excreted much larger quantities of xanthurenic acid than did nonpregnant controls with similar plasma PLP concentrations, indicate that factors other than vitamin B_6 deficiency may be responsible for the observed changes in tryptophan metabolism. The fact that at term low PLP plasma concentrations in some women have been documented (H4, W2, W3) does indicate that a degree of true PLP deficiency may develop in late pregnancy. This may be due to increased demands by the fetus for vitamin B_6 because at delivery B_6 in cord bloods has been shown to be significantly higher than in maternal blood (B18, C7).

Rose reported in 1966 (R6) that women taking contraceptive estrogen–progestogen tablets had elevated excretion of xanthurenic acid in urine. Subsequent studies showed that excretion of kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, kynurenic acid, acetylkynurenine, N^1-methylnicotinamide, but not 2-pyridone were increased in women taking OCAs; for most of these compounds, this could be demonstrated only after tryptophan loading (P8, R6, R7, R12, T3). The degree of elevation of urinary tryptophan metabolites appeared to increase with the dose of the estrogenic component and with the duration of treatment (R9). These changes returned to normal after treatment with large doses of pyridoxine (L9, P8, R12). There is, however, apparently no information on the effects of pyridoxine treatment on N^1-methylnicotinamide excretion during use of OCAs.

The estrogenic components of OCAs have been linked to these effects. Estrogens given alone produce the same changes in urinary tryptophan metabolites of women as do estrogen–progestogen combinations (B20, P8, R7). An adult male treated with ethynylestradiol (0.1 mg daily) had abnormal levels of tryptophan metabolites in urine, which returned promptly to normal when treatment was stopped (R7). Progestogens, in contrast, have been found not to produce these effects (R9, R10).

Wolf (W10) found that in men given estrogens elevated amounts of N^1-methylnicotinamide and 2-pyridone excreted in urine did not return to normal with vitamin B_6 administration, although other urinary tryptophan metabolites returned to near the normal range. In men given a progestational agent there was suppression of formation of the nicotinic acid metabolites N^1-methylnicotinamide and 2-pyridone (W10). On the basis of these and other observations, it has been suggested that the lack of elevation of 2-pyridone in OCA-users is due to interference with the effects of estrogens on nicotinic acid metabolism, in particular the oxidation of N^1-methylnicotinamide to 2-pyridone, by progestogens (R15).

It is clear, therefore, that endogenous estrogens in nonpregnant or pregnant women and exogenous estrogens in women taking OCAs result in derangements in tryptophan metabolism. These changes occur in

women whose vitamin B_6 intake is apparently normal. Furthermore, Luhby and co-workers (L9) have presented evidence which indicates that a minimal dose of about 25 mg of pyridoxine hydrochloride is required to normalize xanthurenic acid excretion in OCA users. This amount of the vitamin is much more than is present in normal Western diets (1 mg) and is greater than generally recommended daily allowances (2 mg). Absolute deficiency of vitamin B_6, therefore, seems unlikely to be the sole explanation, and a number of additional mechanisms have been explored.

There is evidence that estrogen administration results in increased activity of tryptophan pyrrolase. This enzyme activity has been shown to be elevated in the livers of pregnant rats (A8, G8). There is some evidence also that a mestranol–norethynodrel combination has a greater stimulatory effect on tryptophan pyrrolase than does estradiol, and that progesterone alone is an inhibitor of the enzyme in rat liver (R15).

Induction of tryptophan pyrrolase may result at least in part from the elevated plasma levels of adrenal corticosteroids that are known to occur in the rat and in humans during pregnancy and after estrogen administration (D2, G4, S14a, W4). Although these elevated levels are commonly not regarded as being physiologically important because they are accompanied by a rise in plasma steroid-binding proteins (S14), the sinusoidal capillary bed in the liver is in fact permeable to plasma proteins and substances bound to them. Protein-bound hormones may, therefore, gain access to hepatocytes (K2). Cortisol administration however, results in a less pronounced increase in xanthurenic acid excretion than that which occurs during use of an OCA (W8), indicating that adrenal steroids are not solely responsible for the effects of contraceptive steroids. Estrogens also increase liver tryptophan pyrrolase in adrenalectromized rats, although the increase is suboptimal (B11, B17).

Estrogens may modify the activity of enzymes in the kynurenine pathway other than the rate-limiting enzyme tryptophan pyrrolase. Mason and co-workers (M9, M12) have presented *in vitro* and *in vivo* evidence that estrogens may effect binding of PLP to the apoenzyme of kynurenine transaminase.

Studies *in vitro* with rat kidney tissue preparations showed that estrogen sulfates inhibit kynurenine transaminase in a reversible fashion that can be relieved by dialysis (M10, M11). Competition of sulfate and phosphate esters of estrogens with PLP for the kynurenine transaminase apoenzyme has been demonstrated (S5). An estrogen effect on this enzyme *in vivo* was suggested by the finding of higher enzyme activity in the kidneys of adult male rats than in either females or estrogen-treated males (M9, M11). This difference applied to renal kynurenine transaminase, two-thirds of which is in the soluble fraction and the

remainder mitochondrial, but not to enzyme in the liver, where this enzyme activity is reported to be confined to mitochondria (O1, U1). A comparison of the effects of estrogen treatment and pyridoxine deficiency on the kidney supernatant kynurenine transaminase was made. Both conditions resulted in decreased supernatant enzyme activity, but saturation of apoenzyme with PLP was decreased in pyridoxine deficiency (O1, U2, M9) and increased with estrogen treatment (M9). Renal mitochondrial transaminase was little affected by pyridoxine deficiency, but was considerably reduced by estrogen treatment. These *in vivo* studies in rats suggested that estrogens may cause a redistribution of PLP among kidney apoenzymes (M9).

Estrogens also have effects on kynureninase activity, as assayed by measuring the amounts of anthranilic acid formed from kynurenine (Fig. 1). Liver kynureninase activity is higher in mature male rats than in females, and this difference is abolished by estradiol treatment of males (M11, R12). Activity of this enzyme is not reduced in estrogen-treated female rats, but a mestranol–norethynodrel combination was found by Rose and Brown (R11) to elevate liver kynureninase activity. The mechanism of this enzyme stimulatory effect remains to be clarified, and it is not known whether, like the stimulation of tryptophan pyrrolase by estrogens, it may be mediated in part by adrenocorticoids (R15). These studies using kynurenine as a substrate are probably relevant to the enzyme activity which catalyzes the metabolism of 3-hydroxykynurenine to 3-hydroxyanthranilic acid, although it has not been definitely proved that the same "kynureninase" (Fig. 1) catalyzes both reactions.

Estrogens and progestogens may also affect steps in the tryptophan–niacin pathway after the formation of 3-hydroxyanthranilic acid. Hormones probably do not change tissue levels of NAD in normal animals and man, but they may regulate how much NAD is derived from tryptophan. Wolf (W10) found that in men with prostatic diseases estrogens or an estrogen–progestogen combination increased the proportion of a 2-g loading dose of tryptophan that could be accounted for as N^1-methylnicotinamide and 2-pyridone, while a progestogen alone or hydrocortisone alone resulted in decreases. The metabolism of NAD, nicotinamide, and related compounds is complex (D4), and more needs to be known about the various pathways for pyridine nucleotide synthesis and how they may be regulated by hormones (W11).

8.4. Other Changes in Vitamin B_6 Metabolism Associated with the Use of Oral Contraceptives

Means of assessing vitamin B_6 status other than measuring tryptophan metabolites in urine include the estimation of vitamin B_6 compounds

in blood and urine, measuring levels of B_6-dependent transaminase activity in erythrocytes, and measuring urinary 4-pyridoxic acid excretion (S4, S20). The latter is the major end product of vitamin B_6 found in urine. These methods are of great supplemental value in helping to decide whether changes in tryptophan metabolism are due to absolute B_6 deficiency, but have been less widely used because methodology is often difficult.

A number of methods have been described for estimating vitamin B_6 compounds in blood and tissues (C3, C6, C7, H2, H3, S20). PLP is a major form of vitamin B_6 in blood, and its level in blood has been shown to correlate with other biochemical indications of B_6 nutrition (H2, H3). Plasma levels of B_6 compounds do not vary with the menstrual cycle (C6, L11), but they do often fall late in pregnancy (H4).

Lumeng *et al.* (L11) compared plasma PLP concentrations of women, taking OCAs for 6 months or more, with age-matched controls; they also followed prospectively plasma levels in a group of women who were started on estrogen–progestogen combinations. They noted that PLP levels were lower in contraceptive users than nonusers, but this was less commonly found than was excretion in urine of abnormal quantities of tryptophan metabolites. This supports the idea that the abnormalities in tryptophan metabolism induced by contraceptive steroids do not always reflect absolute B_6 deficiency. Plasma PLP concentrations were often observed to return to normal with continued use of the agents, but in a few subjects remained in the below-normal range. The dose of supplemental PLP which might be required to correct the subnormal plasma PLP concentration was not determined (L11).

There is a rapid decrease in urinary vitamin B_6 when normal subjects are placed on a diet low in B_6 content (K3). Recently Miller *et al.* (M14) reported a study of five women receiving a constant diet of known B_6 content. Three subjects taking OCAs showed most of the expected changes in tryptophan metabolites in urine and some evidence for decreased urinary excretion of B_6. This was in contrast to the earlier findings by Aly *et al.* (A5) that B_6 excretion in urine was unchanged by OCAs, and could have been due to a number of factors including differences in the organisms used to assay urine B_6. Urinary 4-pyridoxic acid output in urine is also decreased quickly on a B_6-deficient diet, and has been reported to be normal in OCA-users (P8).

Transaminase activities can be measured in plasma, erythrocytes, and leukocytes, and erythrocyte levels appear to be most valuable in assessing vitamin B_6 status (S4). A better indication is obtained also if erythrocyte glutamate–pyruvate transaminase (EGPT) and glutamate–oxaloacetate transaminase (GOT) activities are measured before and after the *in*

vitro addition of PLP. The latter produces a greater than normal stimulation of enzyme activity in B_6 deficiency states. There is evidence that women using OCAs have subnormal levels of EGPT and EGOT (D5, R16, R17, S1) and that activity is greatly increased by adding PLP *in vitro* (S1).

These assessments of B_6 nutrition in OCA-users by methods other than studying tryptophan metabolism do indicate that at least some of the women who excrete abnormal quantities of tryptophan metabolites in urine have a degree of true deficiency in vitamin B_6. However, the possibility that contraceptive steroids may affect binding of B_6 compounds to blood proteins, or to proteins in erythrocyte proteins remains to be explored.

A recent study (A7) of normal human plasma indicates that added PLP binds strongly to plasma proteins, especially albumin, that pyridoxal is more weakly bound, and that pyridoxine does not bind. Such information could provide a basis for investigating the possible modifying effects of exogenous steroids.

8.5 Oral Contraceptives and Depression

Depression has been described in women taking oral contraceptives (A1, B1, H9, L6, W8) although a causal relationship has not been convincingly demonstrated (L3, W5). There has been interest lately in depression in OCA-users because these women often manifest abnormalities in tryptophan metabolism and because depressive illnesses may be associated with abnormal brain neuroamine metabolism.

In a recently reported study, Adams *et al.* (A1, A2) selected women on OCAs who had indications of depression as judged by scores on standardized questionnaires. Using a double-blind crossover treatment schedule with pyridoxine and placebo, Adams *et al.* (A1) were able to show that depressed women with biochemical evidence of altered tryptophan metabolism and/or absolute vitamin B_6 deficiency showed a better response to a follow-up questionnaire than those women who were depressed but did not have these biochemical derangements. Because not all women who are depressed while taking OCAs have evidence of vitamin B_6 deficiency (A1), a number of other mechanisms have been suggested to account for the presence of depression in OCA users (W5, W8). It remains to be precisely determined how commonly depression in women using contraceptive steroids can be blamed on these agents, and how frequently it may be linked to changes in B_6 status.

Studies concerning possible changes in neuroamine metabolism associ-

ated with OCA administration are nevertheless of considerable interest and importance. Serotonin levels have been reported to be reduced in the brains of individuals with depressive illnesses (C9, C15). Reduced brain serotonin in depression has been ascribed to possible abnormalities in tryptophan metabolism because, while total plasma tryptophan is normal in depressed patients, unbound plasma tryptophan (which can cross the blood–brain barrier) and spinal fluid tryptophan levels are said to be reduced (C10, C12). Tryptophan concentrations in brain are lower than the Michaelis constant of tryptophan hydroxylase for its substrate (J2), which suggests that reduced availability of tryptophan in brain could result in less serotonin concentrations in brain. There is considerable evidence for (C15, C16, G6, S15), and some against (B2, W8), the possibility that cortisone-induced reduction in brain serotonin might be due to the known effect of cortisone in inducing tryptophan pyrrolase (K6), thereby reducing the amounts of tryptophan available for serotonin formation.

Plasma concentrations of a number of amino acids are decreased in the second half of the menstrual cycle and when contraceptive steroids are given (A5, C14, R13). Reports that plasma tryptophan levels are normal in OCA-users (A5, C11, C14) is evidence against the possibility that less tryptophan is available to the brain for 5-hydroxytryptamine synthesis when women are taking these agents. Plasma tryptophan measurements in depressed women taking contraceptive steroids, however, have not been reported.

It is not surprising that there have been no direct assessments of neuroamine status in the central nervous system of OCA-users, and information is derived from experiments in animals. It should be pointed out also that serotonin content is apparently normal in brains of B_6 deficient animals (B5) and that the effects of contraceptive steroids probably cannot be ascribed solely to absolute B_6 deficiency.

An estrogen–progestin combination was shown (N3) to reduce brain serotonin concentrations in female rats. Similar changes occur with the estrous cycle (G9). Estrogens may induce tryptophan pyrrolase activity in liver (B11), and this could divert tryptophan from the 5-hydroxytryptamine (serotonin) pathway (C16), as was discussed earlier.

By increasing the activity of the kynurenine pathway, estrogens could also increase the requirements for PLP and make less available to act as the coenzyme for 5-hydroxytryptophan decarboxylase, or estrogen conjugates could displace PLP from the decarboxylase coenzyme directly. Even though in nonhuman mammals tryptophan hydroxylase, not the decarboxylase, is thought to be rate-limiting in serotonin synthesis (J1), levels of decarboxylase are said to be so low in human brain

that this enzyme could be rate-limiting (R3). In addition, kynurenine sulfate, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid, which are some of the tryptophan metabolites increased by giving OCAs, are capable of reducing brain serotonin in the rat (G5).

8.6. Conclusion

Contraceptive steroids, especially estrogens, produce abnormalities in tryptophan metabolism in the great majority of women who use them. Evidence of absolute B_6 deficiency is much less common, and the amount of pyridoxine required to correct urinary output of tryptophan metabolites is much higher than what is found in normal diets. The studies reviewed provide at least a partial understanding of the biochemical basis for these phenomena.

Oral contraceptive agents might be regarded as inducing a "relative" or "functional" deficiency of vitamin B_6 or a state of "vitamin B_6 dependency" (L9). A number of human diseases have in fact been described in which clinical benefits is derived from administering large doses of pyridoxine, and it has been suggested that specific apoenzymes are deranged in these disorders such that greater than normal amounts of B_6 coenzyme are required for normal enzyme function (S7).

With regard to users of oral contraceptives it remains to be determined whether supplementation with large doses of vitamin B_6 is beneficial, harmful, or simply unnecessary. If future studies indicate that there is a casual link between contraceptive pills and depression and confirm that vitamin B_6 supplementation is beneficial when depression occurs (A1), then possibly many OCA-users should be so treated. Supplementation with about 30 mg of pyridoxine daily during OCA administration has been recommended (L9) also because studies in animals indicate that exposure to large quantities of tryptophan metabolites may have harmful effects. Many of these were discussed in a symposium published in 1971 (B7), and include the carcinogenic properties of tryptophan metabolites in the bladders of mice (B22), adverse effects on oxidative phosphorylation (Q1) and gluconeogenesis (L1), and the complexing of xanthurenic acid with insulin, which interferes with the effects of the hormone on glucose uptake from blood (K7). There is a preliminary report (S16) that administration of B_6 may improve glucose intolerance in OCA-treated women.

There are, however, potential hazards to widespread administration of pyridoxine in large doses. Vitamin B_6 can increase tyrosine aminotransferase activities in rat liver (G7), and estrogens can increase alanine

and tyrosine aminotransferase (B11, R13). These additive effects on amino acid metabolism may be undesirable in areas of the world where protein intake is sometimes marginal. Erythrocyte riboflavin concentration has also been demonstrated to fall after high doses of pyridoxine (S8). It would be wise, therefore, to await the results of further research before recommending that pyridoxine be given to all women taking OCAs.

9. Vitamin E (Tocopherol)

9.1. Symptoms and Laboratory Findings in Human E Deficiency

The function of vitamin E in man is still doubtful and controversial. The existence of vitamin E deficiency disorders in animal species has been well established, and these include reproductive problems, disorders of the blood, liver, brain, cardiovascular system, and myopathies. In general, tocopherol requirements are a function of certain peroxidizable lipids in the diet and in tissues (M8, S6). In the adult human, prolonged limitation of tocopherol intake does not lead to any distinct symptomatology, but a lowered plasma concentration of tocopherol and an increased susceptibility of erythrocytes to hemolysis by hydrogen peroxide slowly become evident. For example, Horwitt (H12) observed that a group of 19 normal subjects, placed on a basal diet containing approximately 2 mg of vitamin E daily, showed a decrease from an average plasma tocopherol value of 1.4 mg per 100 ml to about 0.5 mg per 100 ml in 2 years and to about 0.2 mg in 5 years. Inversely, the susceptibility to hydrogen peroxide hemolysis increased from less than 5% to a value of 70% in 2 years and to 80–85% in 5 years. Subjects on a basal diet supplemented with 15 mg of α-tocopherol daily showed a much slower decrease of plasma tocopherol, namely, from 1.4 to 1.0 mg per 100 ml in 2 years and 0.6 mg in 6 years. The susceptibility of the erythrocytes to hemolysis rose much more slowly than in the group on the basal diet alone to only 20% in the sixth year.

Clinical manifestations of vitamin E deficiency may be seen in premature infants fed on formulas high in polyunsaturated fatty acids (H7). In a group of 6 infants on a nonfat powdered cow's milk formula with added cottonseed oil plasma vitamin E levels decreased from 0.22 ± 0.04 (SE) mg/100 ml at birth to 0.08 ± 0.03 mg/100 ml at 4 weeks. Inversely, the *in vitro* erythrocyte sensitivity to peroxide hemolysis increased from 18 ± 4 (SE)% at birth to 77 ± 14 (SE)% at 4 to 5 weeks after birth. In general, the clinical symptoms were mild, consisting of edema and skin lesions which appeared at about 4 weeks of age.

In a group of five infants who received the same formula plus a daily oral supplement of 100 mg of mixed tocopherols (60 mg of α-tocopherol), the plasma tocopherol rose from 0.35 ± 0.05 (SE) mg/100 ml at birth to 0.79 ± 0.17 (SE) mg/100 ml at 4 weeks. The susceptibility to peroxide hemolysis remained low and constant, 10 ± 3 (SE)% at birth and 11 ± 6 (SE)% at 4 to 5 weeks. Infants in this group had no clinical symptoms suggesting tocopherol deficiency.

9.2. Oral Contraceptive–α-Tocopherol Interrelationships

The details of this interrelationship have been explored in some detail in female rats by Aftergood and Alfin-Slater (A3). In rats receiving 0.01%, *dl*-α-tocopheryl acetate incorporated into their diet, the daily oral administration of Enovid E was begun at 13 weeks and continued for 28 days. The plasma tocopherol concentration at the end of this period was 0.58 ± 0.09 mg/100 ml and significantly lower than the concentration of 0.77 ± 0.04 mg/100 ml in the control rats who had received only the propylene glycol carrier. The extents of hemolysis were 6.9% and 5.2%, respectively, in the Enovid E and control groups, and were not significantly different from each other. As might be expected, in the groups of rats receiving no supplement of tocopherol, the plasma tocopherol was maximally decreased to zero and the rate of hemolysis was maximally elevated to over 90% even in the absence of OCA treatment, and no further lowering of plasma concentrations of tocopherol or raising the rates of hemolysis by Enovid E was discernible.

The only study in humans appears to be that of Briggs and Briggs (B14). After approximately 2–3 months of treatment with OCA, a group of 10 African women living in the Republic of Zambia had an average plasma tocopherol concentration of 1.18 ± 0.21 (SD) mg/100 ml, not significantly lower than the value of 1.25 ± 0.19 (SD) mg/100 ml in a control group of 15 Zambian women.

OCAs, therefore, may lower α-tocopherol plasma concentrations in rats receiving supplements of α-tocopherol in their diet, but an effect on human α-tocopherol metabolism has not been demonstrated.

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THE BIOCHEMISTRY AND ANALYSIS OF LEAD

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1. The Physiological Concentrations of Lead

1.1. Lead in Blood, Urine, and Tissues

Before describing the various methods available for the analysis of lead in clinical samples, a brief summary should be made of the concentrations of lead to be expected in blood and urine under normal conditions and different clinical situations. This will aid the analyst in the evaluation or development of an analytical procedure and give him an idea of its requirements. He will also be more cognizant of those situations in which an analysis may be suspect or may be borderline and should be repeated.

Most human and animal tissues contain lead, but little is known about this metal in the tissues. It probably occurs as a contaminant rather than a true element possessing a necessary biological function. Lead is absorbed slowly and incompletely from the gastrointestinal tract, and can also be absorbed from the respiratory tract when inhaled (R4). Lead poisoning is usually chronic because the metal is excreted even more slowly than it is absorbed. Consequently, lead in exposed individuals tends to accumulate in the body and is stored by the tissues, especially in the bones. Symptoms of poisoning may follow such storage when, owing to other diseases, the stored lead is released into the general circulation.

TABLE I
LEAD IN HUMAN TISSUES

Tissue	Lead in ash, median[a] (ppm)	Lead in dry tissue[b] (ppm)	Lead in fresh fresh tissue[c] (ppm)	Total lead in tissue[d] (mg)
Aorta	170	—	—	—
Brain	5	3.1	0.2–0.7	0.21
Heart	<5	2.1	—	0.11
Kidney	110	4.2	0.7–3.7	0.10
Liver	130	7.9	0.9–4.6	2.2
Lung	47	4.6	—	—
Ovary	15	—	—	—
Pancreas	53	3.7	—	—
Prostate	10	—	—	—
Spleen	25	5.1	—	0.04
Testis	11	—	—	—
Adrenal	—	782	—	—
Thyroid	—	664	—	—
Jejunum	—	661	—	—
Muscle	—	—	—	3.0
Skeleton	—	—	—	145
Rib	—	—	5.0–12.9	—
Vertebra	—	—	2.6–14.7	—
Femur	—	—	18.2–108	—
Tibia	—	—	15.3–96.5	—

[a] Tipton (T3).
[b] Butt *et al.* (B9).
[c] Thompsett (T2).
[d] Kehoe *et al.* (K3).

The levels of lead and the distribution of lead in normal adult human tissues are shown in Table 1 (B9, K3, T2, T3). Certain tissues, notably the aorta and liver, and especially the adrenal and thyroid glands and the jejunum portion of the small intestine, appear to contain higher portions of lead than others. Kehoe *et al.* (K3) have shown the accumulation of lead in the bones. Sognnaes (S5) has shown that lead in the bulk enamel of teeth is 30 ppm in young teeth to 90 ppm in old teeth. Up to 500 ppm is reported in the peripheral enamel.

Bielecka and Kucharska (B5) in a study of a 7-month exposure of rats to industrial dust, found that lead showed the highest affinity to kidney cells. The levels of lead in lungs, liver, and kidneys were directly related to the concentrations of lead in the atmosphere to which the animals were exposed.

Hasegawa *et al.* (H1) determined lead in healthy subjects between 19 and 60 years of age. The difference in lead concentrations in adjacent hair segments were small in healthy subjects. On the other hand, in

the case of a lead worker or a patient with plumbism, in which the concentration of lead in the hair was markedly higher, the difference in concentrations among segments was significant. Renshaw *et al.* (R3), in estimating exposure to lead, found that the lead concentration increased significantly from the root to the tip of the hair, suggesting that lead entered the hair by deposition on the surface followed by diffusion into the hair structure.

The range of lead in blood is 1–60 μg/100 ml and values under 30–40 μg/100 ml are regarded as the most probable range of "normal" values (R4). The critical blood level is usually considered to be concentrations above 80 μg/100 ml. While 60–80 μg/100 ml indicates abnormal absorption of lead, it is often not to a degree that will give rise to symptoms. Whitlock has noted from other investigators that the ambient lead concentration in the Los Angeles population is 40 μg/100 ml while that of the rural population is 30 μg/100 ml (W6).

Devoto (D7) reported that blood lead concentrations were approximately the same in city and country dwellers, were higher in traffic policemen, and were highest in foundry workers. The mean concentrations were 21, 20, 28, and 78 μg/100 ml in the four categories of subjects, respectively. Increases in lead levels were related to increases in atmospheric lead concentrations and not to lead in water or food ingested by the subjects.

Ninety percent of blood lead is found in the erythrocytes. Symptoms of lead poisoning are due to circulating lead and not that found in fixed deposits.

Lead is excreted in both urine and feces. However, true alimentary excretion is small and most of the lead in feces represents unabsorbed lead, while most absorbed lead is excreted by the kidneys. In normal individuals (no known exposure) the daily urinary excretion is 10–75 μg of lead, with the borderline at 80–100 μg/day (R4). Concentrations of 50 μg/liter or greater generally indicate lead poisoning. Kehoe and co-workers have studied the excretion of lead in normal and lead-intoxicated individuals (K4–K6). Lead affects porphyrin metabolism (G7).

Reiders (R2) has found that the urinary lead excretion following intravenous administration of $CaNa_2$-EDTA is increased significantly more in persons with excessive lead burden than in unexposed individuals, and he has suggested this as a definite diagnostic test. Intoxicated patients or those with increased lead absorption excrete at least 1 mg of lead in the first 24 hours following treatment, while normal individuals excrete a maximum of 700 μg. $CaNa_2$EDTA is frequently used in the treatment of lead intoxication (B4).

About 250 μg of lead is excreted per day in the feces of normal persons (R4). Tipton *et al.* (T4) have studied the patterns of elemental excretion

and dietary intake of humans for over twenty elements, but unfortunately the important element, lead, was not included.

Oberle (O1) commented in the degree of lead poisoning of children in the slums of cities where old paint containing lead is present. Aronow (A7), in a letter, replied that putty was also a likely source of lead in these situations.

2. Destruction of Organic Matter

Depending on the procedure employed for the determination of lead and the particular sample, the organic matter in biological samples may need to be removed prior to analysis. In some cases, simple preparation of a protein-free filtrate for fluid samples is satisfactory. In other cases, the organic matter must be destructively removed.

The two most commonly employed techniques for destruction of organic matter in biological samples is wet digestion and dry ashing. In dry ashing, the sample is oxidized by heating in the presence of air, and oxygen acts as the oxidant. In wet digestion, the organic matter is usually attacked by hot oxidizing acids. Each of these techniques possesses advantages and disadvantages over the other. Dry ashing, although requiring greater overall time, 2 hours or more, utilizes little operator time and is simple. Samples do need to be predried and preashed. Introduction of impurities is minimized. Two dangers exist. First, the inorganic constituents may be retained on the container. Second, certain elements may be lost by volatilization under given conditions.

Digestion is rapid, usually about one-half to 1 hour, and volatility and retention losses are minimal. However, contamination from reagents is always a danger. The purity of present day reagent grade acids is sufficiently high, however, that this is not a serious problem in most cases. In the author's experience, hydrochloric acid is one of the most troublesome with respect to lead impurities.

Gorsuch (G4, G6) has used radiotracers to study the recovery of different elements from biological materials by different ashing techniques. His results for lead are summarized in Table 2. He found that simple dry ashing with or without acids is satisfactory, provided the temperature does not exceed 500°C. At temperatures above this, appreciable amounts of lead are volatilized, especially if chloride is present. Lead is retained on silica crucibles at temperatures greater than 500°C. Very little is retained on platinum, and for this reason the use of platinum is slightly preferable. Gorsuch (G5) demonstrated that significant amounts of lead are lost by heating at 600°C in the presence of large amounts of ammonium chloride (25–45%), but that none is lost in the

TABLE 2
ASHING AND DIGESTION METHODS FOR LEAD (G4)

Method	Lead lost
A. Dry ashing	No
With H_2SO_4	No
With HNO_3	No
With $Mg(NO_3)_2$	No
With $Mg(OAC)_2$	No
B. Wet digestion	
$HNO_3 + HClO_4$	No
$HNO_3 + HClO_4 + H_2SO_4$	Yes
$HNO_3 + H_2SO_4$	Yes

presence of sodium chloride. Hasson and Cherry (H2) have reported large losses of lead from human blood when dry ashed even at relatively low temperatures. This problem has not been found for bone samples, however (H8, H9, P1). In addition, Gleit and Holland (G2) found no loss of lead from blood samples ashed at 400°C for 24 hours.

A mixture of nitric, sulfuric, and perchloric acids in the ratio of 3:1:1(v/v) is a generally useful and very efficient digestion medium for biological materials, but it must be used with caution with samples containing lead. Gorsuch has found that lead is lost when digested in the presence of sulfuric acid by coprecipitation as lead sulfate on other sulfates, usually calcium sulfate. Therefore, a mixture of nitric and perchloric acids is recommended. This mixture must be used with more caution because, in the absence of sulfuric acid, it can be evaporated to near dryness with the danger of explosion.

Pickett (P2) has found that samples can be digested in the presence of sulfuric acid provided that care is taken to dissolve all the sulfates remaining. This is done by boiling with water and a little hydrochloric acid. In dealing with samples rich in calcium, such as bone, it is best to avoid using sulfuric acid.

Low-temperature ashing (G1–G3) offers advantages over conventional dry-ashing techniques. A radiofrequency discharge is used to produce activated oxygen radicals, which are very reactive and will attack organic matter at low temperatures. This minimizes volatility and retention losses. Radiotracer lead in blood samples has been recovered quantitatively by this technique (G2).

Tissues can be solubilized by tetramethylammonium hydroxide (TMAH). Murphy *et al.* (M8) prepared rat liver and kidney homoge-

nates and hair samples for atomic absorption analysis by digestion with an aqueous solution of TMAH. The endogenous tissue levels of lead, the reproducibility of the analyses, and the recovery of added standards compared favorably with the results obtained by standard wet digestion with nitric acid. Faster and safer processing and handling of samples is claimed. Gross and Parkinson (G8) used a similar procedure with alcoholic TMAH to solubilize small quantities of tissues for atomic absorption analysis by either graphite furnace or aspiration methods. Recovery of lead from liver mash samples was 94%, with a coefficient of variation of 6% in the furnace mode.

3. Solvent Extraction

Both spectrophotometric and atomic absorption spectroscopic methods, as well as others, frequently utilize solvent extraction. Because of its importance, a brief description of the principals of solvent extraction techniques is presented.

In performing solvent extraction, the metal ion in aqueous solution is extracted into an organic solvent that is immiscible with water. This is accomplished by shaking the aqueous solution with the organic solvent and then separating the two phases. In order for the metal ion to extract into the organic solvent, the charge on it must first be neutralized. Also, the metal ion must be made "organic-like" in character since it must dissolve in a nonpolar solvent. There are two common ways of accomplishing these requirements. First, the metal ion may be allowed to react with an organic reagent in ionic form (a ligand) in the proper ratio to neutralize the charge. At the same time the product formed is "organic-like." The product is known as a complex, or more often a chelate. In a chelate the organic reagent (a chelating agent) has more than one group that complexes with the metal. Some organic molecules, such as ethylenediaminetetraacetic acid (EDTA) form charged chelates with metal ions, and so these will not be extracted. In fact, EDTA may be used as a masking agent to prevent the extraction of an interfering metal. This works provided the EDTA does not also prevent the extraction of the test element.

The second way by which a metal ion may be extracted is as an "ion-association" complex. A high concentration of a species is added that will associate with the metal and neutralize its charge. When shaken with the organic solvent, the solvent will associate with the complex, making it "solvent-like." An example is the extraction of iron(III) from HCl solution into ether. The ion-association complex is probably similar to $\{(C_2H_5)_2O{:}H^+, FeCl_4[(C_2H_5)_2O]_2\}^-$. Several different elements can

be extracted as ion-association complexes from chloride, bromide, or iodide media, and when applicable, these extractions are extremely simple and efficient. Specific examples are detailed by Morrison and Freiser (M6).

Chelate formation is the more common means used for solvent extraction, and so we will describe the principles involved. Most chelating agents are weak acids that ionize in water. The usual practice is to add the chelating agent to the organic phase. The extraction process can be thought to consist of four equilibrium steps, each with an equilibrium constant. These are represented by the following:

$$(HR)_o \rightleftharpoons (HR)_a \qquad K_{D_{HR}} = \frac{[HR]_o}{[HR]_a} = \text{distribution coefficient of ligand} \qquad (1)$$

$$HR \rightleftharpoons H^+ + R^- \qquad K_a = \frac{[H^+][R^-]}{[HR]} = \text{acidity constant} \qquad (2)$$

$$M^{n+} + nR^- \rightleftharpoons MR_n \qquad K_f = \frac{[MR_n]}{[M^{+n}][R^-]^n} = \text{formation constant} \qquad (3)$$

$$(MR_n)_a \rightleftharpoons (MR_n)_o \qquad K_{D_{MR_n}} = \frac{[MR_n]_o}{[MR_n]_a} = \text{distribution coefficient of chelate} \qquad (4)$$

First, the chelating agent, HR, distributes between the aqueous (a) and the organic (o) phases. Second, the reagent in the aqueous phase ionizes. Third, the metal (M) ion chelates with the reagent anion to form an uncharged molecule, and finally, the chelate distributes between the organic and aqueous phases. The distribution *ratio* for the extraction is defined as the ratio of the concentration of all forms of the metal in the organic layer to those in the aqueous layer. In the absence of complicating side reactions, such as hydrolysis of the metal ion in the aqueous phase or dimer formation of the chelate in the organic phase, the distribution ratio is given by

$$D = \frac{K_{D_{MR_n}} K_f K_a^n}{K_{D_{HR}}^n} \cdot \frac{[HR]_o^n}{[H^+]_a^n} = K \cdot \frac{[HR]_o^n}{[H^+]_a^n} \qquad (5)$$

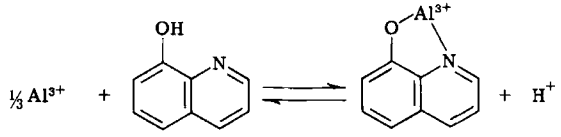
Note that the distribution ratio is independent of the metal ion concentration. Because of this, tracer quantities as well as relatively large concentrations of the metal can be extracted with equal efficiency. This is true provided the solubility of the chelate in the organic phase is not exceeded.

The extraction efficiency can be affected only by changing the reagent concentration or by changing the pH. A 10-fold increase in the reagent concentration will increase the extraction efficiency by the same amount as an increase in the pH of one unit, in other words, a 10-fold decrease

in the hydrogen ion concentration. Each effect is greater as n becomes larger. By using a high reagent concentration, extractions can be performed in more acid solutions. Also, the more stable the chelate (that is, the larger the K_f), the greater the extraction efficiency; this serves as the basis for the separation of many metals and will be discussed in more detail below.

For the most part, the nature of the organic solvent is not too critical in determining the success of an extraction. For multivalent metals with $n > 1$, the solvent may affect the distribution ratio because it affects the relative solubilities of the reagent ($K_{D_{HR}}$) and the chelate ($K_{D_{MR_n}}$). Dithizone and its chelates, for example, are more soluble in chloroform than in carbon tetrachloride, and so extractions with chloroform solvent require a higher pH than those with carbon tetrachloride.

An example of a chelate is the one formed between Al^{3+} and 8-hydroxyquinoline, commonly called oxine. The formation of the chelate is as follows:



(6)

There are three oxine molecules for each aluminum, resulting in an uncharged chelate. It is apparent from the hydrogen ion dependence that more alkaline solutions would favor formation of this chelate and its extraction, as predicted from Eq. 5.

The percent extraction of a complex or a chelate is related to the distribution ratio by

$$\%E = 100D/[D + (V_a/V_o)] \tag{7}$$

where V_a and V_o are the volumes of the aqueous and organic phases, respectively. It is readily shown using this equation that it is more efficient to perform multiple extractions with small volumes of organic solvent than to perform a single extraction with a large volume of organic solvent, if the extraction efficiency is significantly less than quantitative.

The selectivity of an extraction can often be controlled by proper pH adjustment. We see from Eq. 5 that the efficiency depends on the stability of a chelate as well as the pH of the solution. Since different metal chelates have different stabilities, it should be possible then to adjust the pH to a value at which some metals will extract and others will not. Figure 1 illustrates the effect of pH on the percent extracted for a series of metals using the chelating agent dithizone in carbon tetrachloride. These are typical extraction curves. The position of the

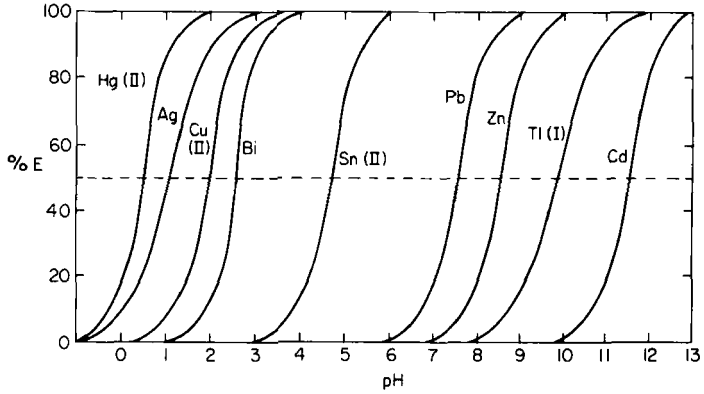


FIG. 1. Qualitative extraction curves for metal dithizonates in carbon tetrachloride. From Morrison and Freiser (M7, p. 161). Reproduced by permission of Elsevier Publishing Co.

curves on the pH axis will depend on the reagent concentrations. We see that there is a pH below which essentially none of the chelate is extracted and a minimum pH above which essentially all of it is extracted. One should be aware, however, that extraction efficiency may in certain cases decrease at higher pH values owing to factors such as hydrolysis of the metal ion. Looking at the figure, we see it would be difficult to separate copper from silver, but it would be relatively easy to separate copper from lead. For example, the pH of the aqueous phase could be adjusted to 5 and all the copper would extract, leaving the lead behind. If it were necessary to obtain the lead in an organic solvent for analysis, the pH could be changed to 10, and then the lead would extract.

Another means of increasing the selectivity of extractions is the adding of masking agents. Masking agents are competing complexing agents which form charged complexes that are more stable for certain metals than is the complex with the extracting agent. EDTA and cyanide ion are commonly used masking agents. For example, even though Cu^{2+} forms a more stable complex with oxine than does VO_2^{2+}, the vanadium may be extracted in the presence of copper by adding EDTA. The EDTA forms a more stable complex with the copper ($Cu\text{-}EDTA^{-2}$) than does oxine.

Frequently, quantitative extraction of an element cannot be achieved in a single step. In these cases, as mentioned above, quantitative separations are usually possible with multiple extractions. However, the extraction need not be quantitative so long as it is reproducible. Most procedures require the preparation of a calibration curve anyway, and when

solvent extraction is involved in the analysis, this step is included in the calibration.

For most extractions, a single-stage or "batch" extraction is performed. In this procedure, the aqueous sample to be extracted is mixed in a separatory funnel with an immiscible organic phase plus chelating agent, if appropriate. The mixture is shaken until equilibrium is obtained. This usually takes a minute or less. In some cases, the shaking must be very gentle to avoid foaming or the formation of an emulsion. Alternatively, centrifuging may be used to speed up the separation of layers.

4. The Determination of Lead

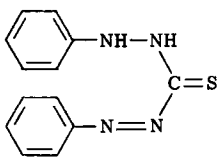
We see from the pevious discussions that a method for the analysis of lead should be able to determine accurately 0.4 ppm in blood and 0.1 ppm in urine. Assuming the availability of 1 ml of blood, this means that one should be able to analyze for 0.4 μg of lead. With urine samples, the limit will fall above this if at least 10 ml are available for analysis.

There are a number of analytical techniques that possess the required sensitivity for these analyses, and we will discuss some of the more important ones with respect to sensitivity, specificity, reliability, and speed.

4.1 Spectrophotometry

Spectrophotometry combined with solvent extraction has probably been the most widely used method for lead analysis. This is because of the wide availability of spectrophotometers in clinical laboratories. Atomic absorption spectroscopy, when the instrumentation becomes more widely available in clinical laboratories, will probably become more and more prominent in these analyses.

The standard spectrophotometric method for determining lead is the diphenylthiocarbazone (dithizone) method. The structure of dithizone is:



It forms a red complex with lead:

$$Pb^{2+} + 2H_2Dz = Pb(HDz)_2 + 2H^+$$

The chelate may generally be extracted with chloroform from solutions at pH 7.5–11.5 and with carbon tetrachloride at pH 7–10. The absorption

maximum occurs at about 510–520 nm. The molar absorptivity is about 60,000–70,000 at 520 nm in carbon tetrachloride, and micro to relatively large amounts of lead can be measured.

The disadvantage of this reagent is that it is not specific for lead, and preliminary separations may be required. By judicious use of masking agents and proper adjustment of pH prior to solvent extraction of the lead complex, many major interferences can be eliminated.

Referring again to Fig. 1(M7), it is apparent that if the extraction is performed at pH 8, Tl(I) and Cd will not interfere while Hg, Ag, Cu, Bi, Sn(II), and to a lesser extent Zn, will extract with the lead. The degree of interference from these will depend on the degree of overlap of their absorption spectra with that of lead and their molar absorptivities, as well as on their relative concentrations.

If solvent extraction is carried out in the presence of cyanide, the zinc group of metals (Zn, Cd, Hg) is complexed and they will not extract. Addition of citrate complexes the elements of the ammonia group (Fe, Al, etc.) so that their hydroxides will not precipitate. The precipitation of the alkaline earth phosphates can be prevented by the addition of hexametaphosphate (J1). Under these conditions, Bi(III), Tl(I), and Sn(II) interfere in alkaline solution. The bismuth can be removed by preextraction at pH 3. Alternatively, if a positive lead test is found, bismuth could be ruled out or confirmed by back-extracting at pH 3. If bismuth is present, it will remain in the organic layer while lead will go into the aqueous phase. Extraction of thallium can be prevented by extracting the lead at pH 6.0–6.4 with chloroform. Or the thallium can be back-extracted at this pH; this requires much closer control of the pH. The oxidation of dithizone by air is catalyzed by the presence of significant amounts of manganese(II).

Rather than adding masking agents or controlling the pH, the lead can be isolated beforehand by extracting lead iodide (ion association complex) or lead diethyldithiocarbamate. The lead iodide can be extracted with methylisobutyl ketone from HCl-acidified solution in the presence of KI (W2) while the diethyldithiocarbamate chelate can be extracted into carbon tetrachloride at pH 7 (L3). The lead can be back-extracted in the former case into an aqueous solution of sodium hydroxide and in the latter case into a solution of pH less than 7. Then it can be analyzed with dithizone. In this way, Zn, Cd, Hg, Ag, Al, and Sn do not interfere.

A dithizone procedure for blood and urine samples is described in Standard Methods of Clinical Chemistry (R4). This is a modification of the procedure of Bessman and Layne (B3). The sample is digested with nitric, sulfuric, and perchloric acids, and the lead is extracted from

alkaline solution in the presence of cyanide, tartrate, and sodium sulfite into 3 ml of chloroform. If bismuth is suspected, this is extracted at pH 3.4 before extracting the lead, but this preextraction is not done routinely. A 2-ml blood sample or a 10-ml urine sample is taken for analysis. The blank is equivalent to about 1 μg of lead or 0.5 ppm in the blood sample and 0.1 ppm in the urine sample. Thus, only lead intoxication is indicated with blood samples.

Thallium, which may give rise to lead intoxication symptoms, would give a positive lead test with this procedure.

4.2. Atomic Absorption Spectroscopy

It is apparent that the spectrophotometric procedures are rather time consuming since several multiple extractions must be performed in order to minimize interferences. Atomic absorption spectroscopy has enjoyed wide popularity in recent years as a trace metal analysis tool. This is due to a number of factors including high sensitivity, selectivity, and ease of sample preparation. With biological fluids, often no sample preparation at all is required, depending on the element analyzed, its concentration and the sample matrix. Because of its advantages, this technique will be treated in some detail.

Atomic absorption involves aspirating a solution of the element to be determined into a flame. The element is converted to free atomic vapor in the flame. This atomic vapor is in the ground electronic state and can absorb light of the appropriate wavelengths, corresponding to the energies of the electronic transitions (the resonance wavelengths). This absorption is sharp line in nature, and the light source employed is usually a hollow cathode lamp that emits the resonance lines of only the element being analyzed (plus lines of a filler gas). Except in rare cases, only the element of interest can absorb these resonance lines, so the method is quite specific. Light scattering or molecular absorption by particles in the flame (from high salt-containing samples) can occur, especially at short wavelengths, but this can be corrected for. Chemical interferences may occur that prevent the formation of atomic vapor in the flame. These can usually be eliminated by simple addition of an appropriate releasing reagent to the solution.

Molecular absorption or light scatter can be corrected for by several methods. If the interfering salt composition is known, it may be possible simply to add the salt or salts to standards and make a direct comparison with calibration curves. It is relatively simple to measure the "background absorbance." One way is to choose a nonresonance line from the lead hollow cathode lamp or another lamp that is not absorbed by lead and which occurs at least two bandpasses from the resonance

line. The background absorbance of this line is then measured. Since the molecular absorption is broad band in nature, it is essentially constant in the region of the resonance line, and so it will absorb the same fraction of the nonresonance line as it does of the resonance line. This absorbance is then simply subtracted from the total absorbance at the resonance wavelength. This method does require, though, two separate measurements.

An automatic correction can be made with double-beam instruments that are equipped with a hydrogen or deuterium continuum light source. For example, in the Perkin-Elmer 303 atomic absorption spectrophotometer, a deuterium arc is time-shared along with the hollow cathode lamp (the instrument operates as a single-beam system when the correction system is used; the reference channel is blocked out). Both beams are passed through the flame and a ratio is taken. The Instrumentation Laboratory 353 atomic absorption spectrophotometer is a dual double-beam instrument, i.e., a two-channel (two monochromator) instrument with double beams in each channel. In the background correction mode, a deuterium lamp is used in the second channel and a hollow cathode lamp in the first channel. In this manner, the double-beam operation is not sacrificed. The nonspecific absorbance in the second channel is automatically subtracted from the line absorbance in the sample channel. In these continuum source corrections, line absorption of the continuum source is negligible compared to the background over the bandwidth of the monochromator.

The application of atomic absorption spectroscopy to biological samples has been reviewed (C7, C11, C12) and is treated in detail in the book by Christian and Feldman (C14). It has been used successfully for the trace analysis of lead in biological fluids.

The most sensitive absorption line of lead is 2170.0 Å closely followed by the 2833.1 Å line. The sensitivity, defined as the concentration for 1% absorption, is 0.5 ppm and a detection limit of about 0.1 ppm can be obtained. The 2833 Å line is often preferred over the 2170 Å line because it is less noisy, less subject to background absorption, and gives better linearity.

Several flames are satisfactory for lead determination. Air-acetylene, air-coal gas, air-propane, and oxyhydrogen have been used. Generally, a cool flame such as air–coal gas is slightly more sensitive, but it is much more subject to interferences. Hence, a flame like air–acetylene is preferred. An oxidizing, nonluminous (lean) flame is best.

In general, lead atomic absorption is enhanced by a rather small amount by addition of water immiscible organic solvents, usually by less than 50% (D1, C3). Solvent extraction into water-immiscible solvents does offer significant enhancement. Chakrabarti (C2) obtained a 3-fold

enhancement by extracting the lead as lead iodide into methylisopropyl ketone, while Dagnall *et al.* (D2) achieved a 6-fold enhancement by performing the same extraction with MIBK.

Willis (W7, W8) first determined lead in urine by extracting with ammonium 1-pyrrolidinecarbodithioate (APCD)[1] at pH 1.5–4.5 into methyl-*n*-amyl ketone. The lead was concentrated more than 100-fold. Although the optimum pH for extraction is 3–4, the extraction efficiency curves for aqueous solutions and urine samples containing lead cross at pH 2.5–3.0. Therefore, calibration curves could be prepared from aqueous standards. However, this places a narrow restriction on the pH range, and Willis preferred to use a method of standard additions. This procedure gave the same results as when samples were first ashed. By extracting 50 ml of urine with only 1.5 ml of ketone, 0.1–0.5 ppm of lead added to urine was quantitatively recovered and results for pathological specimens (0.04–0.7 ppm) agreed with a colorimetric procedure. By taking 100–200 ml of urine, 0.02 ppm of lead could be measured with a standard deviation of 0.002 ppm. There was considerable emulsion when the ketone was shaken with the urine, but most of it could be recovered by centrifuging.

Pierce and Cholak (P3) determined lead in urine in a similar manner, but by extracting 25 ml of urine using 5 ml of MIBK. Water-saturated MIBK had to be used for zeroing the instrument. They noted significant light scatter and corrected for it by reading the absorption at 2204 Å (a nonresonance line from the lead hollow cathode lamp that is not absorbed by lead) and subtracting this from the absorption of the lead resonance line at 2170.0 Å; the cobalt line at 2182 Å can also be used for background correction (T1). The lead could also be determined by dry ashing, adding 5 ml of 10% hydrochloric acid, and aspirating.

Kopito and Shwachman (K7) have described a method for the analysis of lead in either freshly voided or partially decomposed urine. The lead in 25 or 50 ml of urine is coprecipitated on bismuth hydroxide by adding bismuth nitrate and ammonia. After centrifuging, the precipitate is dissolved in acid to a final volume of 5 ml, and this solution is aspirated. With a 25-ml sample, 0.05–0.2 ppm of lead can be determined. The bismuth does not interfere with the lead absorption, and it suppresses interferences from sodium, potassium, calcium, magnesium, and phosphates. This procedure appears to offer sufficient sensitivity and the advantages of freedom from interference and simplicity in operation. Control of pH is not critical. Kopito and Shwachman claim that in

[1] This compound is also widely referred to in the literature as ammonium pyrrolidinedithiocarbamate (APDC). It is added to the aqueous phase.

cases of lead poisoning the concentrations of lead are sufficiently high that urine samples can be aspirated directly.

Segal (S3) found it necessary to apply a background correction to measurements at 2170 Å, using the 2204 Å line as above, either for the direct aspiration of urine samples using standard additions, or using the bismuth hydroxide coprecipitation method of Kopito and Schwachman. Zurlo *et al.* (Z2) described a procedure similar to that used by Kopito and Schwachman. The lead in urine is separated by coprecipitation with thorium in the presence of copper(II). The precipitating solution contains 2 g of thorium nitrate hexahydrate and 2 g of copper sulfate in 100 ml of water acidified with 2 drops of 1:1 hydrochloric acid. One-half milliliter of this added to 12.5 ml of urine at pH 5–6, and the thorium precipitates as thorium phosphate (by reacting with phosphate in the urine). After 5 minutes, the mixture is centrifuged and, after the supernatant has been discarded, the precipitate is dissolved in 0.5 ml of 1:1 hydrochloric acid plus 2 ml of water. Hence, the lead undergoes a 5-fold concentration. The precipitation is quantitative, even from the urine of subjects excreting coproporphyrins or treated with chelating agents, because the added copper liberates the chelated lead. This method is probably to be preferred to the Kopito and Schwachman method, for this reason; in addition, it apparently is free from certain other difficulties inherent in the latter method, such as burner clogging, precipitation of calcium with the lead, etc.

Roosels and Vanderkeel (R5) determined lead in urine in the presence of EDTA by extracting the dithizone complex in the presence of added calcium. The calcium was added presumably to release the EDTA from the lead. In view of the fact that the lead-EDTA chelate is 20,000,000 times more stable than the calcium chelate, it is unlikely that the mechanism is a simple release of the EDTA by the calcium. Few data were given. This procedure is not recommended because a chloroform solution of the lead is aspirated. Dangerous phosgene is produced in flames with this solvent.

Berman (B1) extracted the APDC complex of lead from urine into MIBK after adjusting the pH to 2.2–2.8 with trichloroacetic acid (TCA). Yamauchi (Y1) used a similar procedure, but added 2 ml of 5% Triton X-100 per 100 ml of urine to prevent formation of emulsion. He reported that urinary and blood lead levels in industrial workers were not always parallel.

Lorimier and Fernandez-Garcia (L4) concentrated lead from nitric acid acidified urine by precipitation with a $CaCO_3$–HCl–$(NH_4)_2HPO_4$ solution at pH 9 and then dissolving the precipitate in hydrochloric acid. The lead was extracted with APCD at pH 3 into water-saturated

MIBK for atomic absorption measurement. As little as 0.05 μg/ml of lead could be determined with a precision of 0.004 μg/ml.

Lead has been determined in whole blood by precipitating proteins with TCA and then extracting the lead in the protein-free filtrate with APCD and MIBK (P3, B1). The pH is adjusted to 2.8 with sodium hydroxide. Usually about 5 ml of heparinized blood is taken with 5 ml of MIBK. The limit of the determination is about 0.1 ppm.

Cholak *et al.* (C5) determined 0.05–20 μg of lead in biological materials by extraction with APCD and MIBK at pH 8.5. Potassium cyanide was added to mask the effects of iron, zinc, and copper. Only bismuth and cadmium were found to interfere under these conditions. Mishima *et al.* (M4) deproteinized 5 ml of blood with 10 ml of 5% TCA and extracted the lead with 1 ml of 1% APCD and 5 ml of MIBK. Marumo *et al.* (M2) overcame a reported interference from greater than 220 μg iron in blood by extracting the iron with cupferron into MIBK prior to extraction of the lead with APCD.

Berman, Valavanis, and Dubin (B2) described a method for the analysis of 0.2–3 ppm of lead in 250 μl of blood using a molten-lead hollow cathode lamp which gives an improved signal-to-noise ratio. A 250-μl sample of heparinized blood is treated with TCA, and the protein-free filtrate is extracted with sodium diethyldithiocarbamate and 1 ml of MIBK. The absorbance of the 2833 Å line is measured. The standard deviation of this method is ±0.02 ppm, but results compared with a similar macroprocedure (B1) were ±0.05 ppm. No systematic study has been conducted on this procedure for losses of lead on the precipitated proteins nor on the effect of chelating agents such as EDTA on the extraction of the lead, which might be used in chemotherapy. Roosels and Vanderkeel (R5) claim that the method cannot be used in the presence of EDTA, but they give no supporting data.

Davidow (D3) has experienced no difficulty in the presence of EDTA using the APCD extraction procedure described by Hessel (H5). In the Hessel procedure, removal of proteins and consequent alteration in the pH is avoided by hemolyzing the sample with Triton X-100, prior to solvent extraction of the lead. A 5-ml blood sample is treated with 1 ml of 5% Triton X-100 solution and is extracted with 5 ml of MIBK after adding APCD. Water-saturated MIBK must be used to zero the instrument. Standards are prepared in pooled human blood which is preserved with EDTA, so this chelating agent appears not to interfere in the determination. This procedure is rapid and can be used to readily measure normal blood lead levels, although it does require a fairly large sample. Hessel found that his method gave better recoveries compared with Berman's procedure (B1), and he suggests

that precipitation of proteins may result in appreciable loss of lead; no data were given, however. Westerlund-Helmerson (W4), in using the Hessel procedure, found that results were low if samples were not analyzed when fresh. This problem was overcome by adding heparin and Triton X-100 when the sample was collected. In this manner, the sample could be stored for several months at room temperature. He used aqueous lead standards for greater convenience.

Mitchell, Ryan, and Aldous (M5) described a modification of the v/v) and APCD (2% w/v). This is extracted with 1 ml of MIBK and analyzed by integrating the signal for 4 seconds. A water-saturated MIBK blank is aspirated between samples and standards. They found that lead levels in heparin-treated blood decreased significantly if left at room temperature more than 3 days. Also, addition of 0.002 *M* EDTA to the blood sample did not affect the results.

Zinterhofer *et al.* (Z1) determined lead in undigested blood (6 ml) and urine (40 ml) from patients receiving EDTA therapy, using a method similar to that described by Roosels and Vanderkeel above (R5). The lead was extracted with APCD and MIBK in the presence of EDTA by the addition of excess calcium, which "displaces lead from its EDTA complex." Completeness of extraction was established with ^{210}Pb. In the presence of 3.33 mg of Na_2EDTA per milliliter of blood, extraction of ^{210}Pb averaged 30% without added calcium, but was increased to 100% by the addition of 0.5 mg of calcium per milliliter of blood. A slight loss (5%) occurred if the calcium was added to the system before APCD, but not if added after. For urine samples containing 5.0 mg of Na_2EDTA per milliliter, the addition of 1.8 mg of calcium per milliliter resulted in 100% recovery of lead. These investigators preferred the less sensitive 2833 Å line over the 2170 Å line because it is less noisy and less subject to interference by flame absorption and light scattering.

Farrelly and Pybus (F1) described a simple and reliable procedure similar to that of Hessel in which 2 ml of packed red cells are hemolyzed with one drop of saponin solution (20 μg/20 ml water), 2 ml of formamide is added to prevent emulsion, and the lead is extracted with 0.2 ml of 2% APDC solution and 1.5 ml of MIBK. Aqueous standards were used and lead was unequivocally shown to be completely extracted because results were identical to those obtained on dry-ashed samples. A very good precision of 0.045 μg/ml of red cells was obtained. The authors preferred to use red cells because 90% of the lead is contained within them, allowing more accurate measurement. Also, much larger changes in lead concentrations are noted when lead poisoning occurs. They reported the following concentrations (μg/ml) of red cells: Nor-

mal, <0.40; lead exposure (no clinical symptoms) 0.70–1.6; mild lead poisoning 1.6–2.0; moderate lead poisoning 2.0–2.5; severe lead poisoning >2.5. In the one case of severe lead poisoning, a concentration of 3.7 μg/ml of red cells was found compared to 0.75 μg/ml in whole blood, a much easier change to measure. In view of these results, clinicians might be advised to seriously consider the desirability of routinely analyzing red cells rather than whole blood. To determine lead in urine of patients on chelate therapy, Farrelly and Pybus digested the urine prior to measurement by a modification of Willis' (W7, W8) procedure.

Andreoleti *et al.* (A6) also hemolyzed blood samples with saponin followed by deproteinization in two stages, with 2.5% and 50% trichloroacetic acid, respectively. A dilution factor was determined using a cadmium tracer.

Davis and Christian (D4) have obtained evidence that the formation constant of the lead-APCD chelate is about 5×10^{20} [$Pb(APCD)_2$]. This compares with a formation constant for the lead-EDTA chelate of 1×10^{18}. This 500-fold larger stability for the APCD chelate would indicate that EDTA may not interfere in the extraction if a sufficient excess (100- to 1000-fold) of APCD is present.

It should be possible to apply the coprecipitation methods of Kopito and Shwachman (K7) or Zurlo *et al.* (Z2) to protein free filtrates of blood samples. This would eliminate problems of pH control, and interferences from complexing agents in the latter procedure. The former method has, in fact, been applied to digested blood samples (K8).

Lyons and Quinn (L5) concentrated lead from blood, urine, and tissue by anion-exchange chromatography for atomic absorption spectroscopy measurement.

Although blood lead can be determined using conventional atomic absorption spectroscopy, relatively large samples are usually required or else one must operate near the limit of sensitivity of measurement, so that precision suffers; also, a preconcentration is required. A number of nonaspiration and nonflame atomization systems exist which possess greatly improved sensitivity over conventional flame aspiration systems. Several of these have allowed the rapid determination of blood lead in small samples. The first is the sampling boat system (K1). Up to 0.5 ml of sample is pipetted into a small boat-shaped tantalum vessel, which is then placed near the flame and dried. When the sample is dry (60–90 sec), the boat is inserted into the flame and the element of interest is vaporized, producing a sharp absorption peak, typically of 1 or 2 seconds duration, which is recorded on a strip chart recorder. The peak height is proportional to the element content. The method of standard additions must be used to determine lead in blood and

urine, since aqueous standards result in substantially different absorption (K2). Irreproducible results are obtained in analyzing blood samples directly. Hence, a 0.5-ml sample is diluted to 5 ml with saline solution containing 1% nitric acid, and a 0.2-ml aliquot of this is pipetted into the boat. Eight milliliters of urine are diluted to 10 ml with 5% nitric acid, and 0.2 ml of this is analyzed. A background correction was made with a hydrogen lamp in this work. EDTA in urine caused high results.

Hilderbrand *et al.* (H7) analyzed 0.25-ml blood samples and 0.5-ml urine samples using the sampling-boat technique. Bizollon and Galy (B6) analyzed 0.5 ml of urine, but frequently found inaccurate results. Hence, they diluted samples (and interfering substances) and enhanced the lead concentration with known amounts of added lead. Precise measurements could be made on lead concentrations above 15 g per liter of urine.

Hauser *et al.* (H3) preashed blood samples in the sampling boat in a low temperature asher prior to analysis by the sampling-boat method.

The sampling-boat technique is frequently hampered by poor precision. Delves (D5) described an improved system. He modified the system of White (W5) who used a platinum-wire loop in conjunction with an absorption tube to increase sensitivity. Delves vaporized samples from microcrucibles made of nickel foil into a nickel absorption tube situated in air–acetylene flame. The sensitivity is 1×10^{-10} g lead per 1% absorption at the 2833 Å line. Only 10 μl of whole blood are required. Before analysis, the sample is dried in the nickel crucible on a hot plate at 140°C (ca. 30 seconds) and then is partially oxidized with 20 μl of 30% hydrogen peroxide at 140°C until a dry yellow residue is obtained. Standards of lead added to normal blood are run in a similar fashion.

Fernandez and Kahn (F3) reported that the accuracy and precision of the Delves method are adequate for the determination of normal and excess lead levels in blood and that 30–50 samples could be analyzed in 1 hour. The correlation coefficient between their results and those using a colorimetric dithizone procedure was 0.99. Heinemann (H4) was able to analyze a single heparinized blood sample in less than 5 minutes and 30–40 samples in 1 hour using the Delves procedure. He also analyzed untreated urine. Hicks *et al.* (H6) found a correlation coefficient of 0.96 between the Delves method and conventional macro atomic absorption measurements; the coefficient of variation for the Delves method was 8% at 40 μg of lead per 100 ml. In addition, the analysis of lead in capillary and intravenous blood samples correlated

well (0.93), so that the Delves method can readily be applied to capillary blood for pediatric practice and for screening large numbers of subjects.

Olsen and Jatlow (O2) modified the Delves procedure to permit the direct use of aqueous standards rather than the method of standard addition. They accomplished this by adding a small drop (ca. 2 μl) of a 150 mg/liter albumin solution to just coat the bottom of the cup. The albumin was dried on a hot plate. In the presence of albumin, aqueous standards gave essentially the same response as blood samples. A small nonspecific molecular absorption (equal to about 3 μg of lead per 100 ml) due to blood salts was corrected for by adding 50 m*M* sodium chloride to standards. EDTA had no effect on the analysis. The authors also improved precision by stabilizing the burner mount. Again, the 2833 Å line was preferred over the 2170 Å line because of greater stability and linearity. These authors employed a similar procedure for the determination of lead in 10 μl of urine.

Ediger and Coleman (E1) deleted the chemical oxidation of the blood in the Delves method by ignition of the sample near the burner before the cup is inserted into the flame. This volatilizes most of the organic matter while leaving the lead in the residue. Contamination of samples by lead is reduced as is the analysis time.

Delves and Reeson (D6) described a time delay circuit to eliminate the recording of nonspecific absorption, which is especially useful in single-beam instruments that cannot be equipped with background correction accessories with a continuum source. The device eliminates the recording of all the nonspecific absorption signals from the combustion products of the sample that precede the lead signal. In this manner, the lead signal can be integrated, free from the combustion signal, and results can be calculated automatically.

Rose and Willden (R7) reported improved precision by wet digestion with aqua regia instead of drying in the presence of hydrogen peroxide. An 0.8-ml volume of aqua regia was added to 10 ml of blood in the crucible and evaporated to dryness on a hot plate. Smoke was eliminated, and thus reproducibility was increased. The nickel cups and tube had to be replaced with similar apparatus made of fused quartz (available commercially). Recovery of lead added to whole blood was 100%.

Anderson and Mesman (A3) and Arroyo (A8) have used the Delves method to determine lead in urine. The former investigators reported a standard deviation of 5% in the analysis of untreated urine and a sensitivity 0.020 mg of lead per liter of urine.

Joselow and Singh (J2) noted a loss of sensitivity in the Delves procedure for blood lead analysis due to distortion produced by repeated removal and replacement of the cup in the holder. For this reason,

they recommended that samples be absorbed on filter paper, similar to the procedure of Cernik and Sayers (C1), and punched into the cup without removing the cup from the holder.

The Cernik and Sayers (C1) method is a paper punched disc technique for the collection and determination of lead in capillary blood using the Delves cup as an atomizer. In their procedure, the accurate measurement of microliter volumes was eliminated by spotting the blood onto Whatman No. 4 filter paper (9 cm diameter). The blood was collected from an ear lobe with a heparinized Harshaw tube and expelled by gravity or by blowing. To prevent contamination, the filter papers were enclosed in 6-inch petri dishes and not exposed more than necessary during the spotting of the blood. Eight duplicate samples could be spotted onto one piece of filter paper. The blood was air dried and then an 8.9 mm diameter disc was cut using an ordinary paper punch modified to cut discs of the required size. Such a disc contained 10 μl of blood, suitable for analysis by the Delves cup method. The punched out discs were placed in the nickel crucible without prior treatment and analyzed in the usual manner. The standard deviation of samples containing 50–147 μg lead per 100 ml was 2.81–5.33 μg/100 ml.

Bogden and Joselow (B7) also spotted a drop of blood onto filter paper, dried it, and then burned a standard-sized disc punched from the filter paper in an atomic absorption spectrophotometer. Blood lead analysis by this method was reported to compare favorably with macromethods, and specimen collection was much easier.

A further 10-fold improvement in detection limits for lead is obtained over the above sampling boat and Delves methods by using nonflame atomizers. West and Williams (W3) described a system in which the sample is vaporized from a carbon filament heated, within 5 seconds, to 2000–2500°C in an inert atmosphere by passage of about 100 Å at 5 V. Amos *et al.* (A2) evaluated this atomizing system for atomic absorption and atomic fluorescence measurements. They found detection limits of 2×10^{-11} g and 3×10^{-12} g for lead by atomic absorption and atomic fluorescence, respectively. Lead was determined in 2 μl of blood by atomic fluorescence by diluting the blood 2.5 times with water and preheating on the carbon filament to remove most of the organic material at a temperature just below that at which lead will vaporize. These investigators found that matrix effects on lead were significantly diminished by operating in a reducing atmosphere. This was accomplished by introducing some hydrogen into the inert gas stream flowing over the carbon rod. The flow rate was such that the hydrogen ignited spontaneously when the carbon reached a sufficiently high temperature, and occurred before the lead vapor was produced. Matousek and Stevens

(M3) applied the carbon rod atomizer to the direct atomic absorption determination of lead in 0.5 μl of packed red cells. A blank correction was required using a synthetic blood electrolyte solution. Two microliters of xylene were added to the carbon rod to prevent samples and standards from soaking into the rod.

Anderson *et al.* (A4) found that by limiting the field viewing conditions such that only an area extending 0.5 mm above the carbon filament is irradiated, the sensitivity for lead is improved. Aggett and West (A1) investigated the behavior of the carbon filament for the determination of trace elements extracted from aqueous solutions into organic solvents (benzene, chloroform, carbon tetrachloride, MIBK) by a variety of chelating agents. They concluded that there is no general restriction on the nature of the solvent, as in flame-based techniques, and that the behavior of the organometallic systems is virtually identical to that of aqueous solutions of the metals. Only with the copper(II)–oxine system was a slightly anomalous behavior observed.

Kubasik *et al.* (K10) determined lead in whole blood using the carbon rod atomizer. Only a 3-fold dilution of the whole blood with Triton X-100 (50 ml/liter) was required, and results were comparable to those obtained by flame techniques. Addition of xylene to the carbon rod (M3) did not prevent soaking of the sample into the rod and so blood standards had to be used. Rosen and Trinidad (R8) described a method for the measurement of lead in capillary blood samples, developed in conjunction with a simple micro blood-collecting system. Heparinized, untreated blood (0.5 μl) was injected into the carbon rod, with 0.2–0.3 μl of xylene on either side of the sample. The sample was subsequently dried, ashed, and atomized. The sensitivity was 0.5 μg/100 ml per 1% absorption, and the standard deviation of over 450 blood samples analyzed in duplicate was 0.91 μg/100 ml. Results agreed closely with those obtained using conventional flame methods carried out on simultaneously collected venous samples.

Machata and Binder (M1) reported that, with a graphite atomizer, matrix effects caused by iron in blood caused calibration curves to be rather flat (nonlinear) and results to be imprecise. These matrix effects were avoided by the addition of lanthanum salts.

Related to the carbon rod atomizer is the heated graphite tube furnace atomizer. Here, the sample is injected into the center of a graphite tube, which is resistively heated. Welz (W1) reported the determination of blood lead with such a system in which the sample is dried (20 seconds), ashed, and atomized by stepwise increase in the temperature. The precision was poor, though, being 5–10 μg/100 ml. Norval and Butler (N3) described a graphite tube system for use on any number of atomic

absorption instruments which gave a detection limit for blood lead of 0.5 ng.

Feldman (F2) described a system similar to the heated carbon filament system of West and Williams (W3) except that a tantalum ribbon was used in place of the carbon filament. This has the advantage of requiring less power for heating as well as not requiring a cooling block with water cooling. A limit of detection of 10^{-11} g of lead was reported. Lead was determined in blood using a protein-free filtrate from a 20-μl sample. An automatic background correction was made using a continuum source to compensate for any molecular absorption or light scattering. Hwang *et al.* (H11) determined lead in 100 μl of whole blood using this same atomizing system with the hemolysis and solvent extraction system of Farrelly and Pybus (F1) to obtain the lead in 0.5 ml of water-saturated MIBK. Twenty microliters of the MIBK extract were analyzed. Donega and Burgess (D8) described an atomizing system similar to that of Feldman (F2) except that it could be operated under reduced pressure in an inert atmosphere.

Hwang *et al.* (H10) reported a direct procedure for the determination of blood lead using the tantalum ribbon atomizer. A 25-μl sample of 1:10 water-diluted whole blood was analyzed. The dilution served to facilitate the manipulation of viscous blood samples and to complete hemolysis of red blood cells. An automatic background corrector was used with frequent standardization to eliminate the need for duplicate measurements of the sample and to establish a working curve for each set of unknowns. However, triplicate determinations could be made with as little as 10 μl of blood.

Anderson *et al.* (A5) made an evaluation of eight atomic absorption systems for the determination of lead in capillary blood. The precision of results obtained with the Delves method (4.5–8.4% c.v.) or with a tantalum ribbon atomizer (5.1–9.0% c.v.) was better than with a heated graphite crucible (12.7–15.9% c.v.). Using the Delves cup, results with the filter paper disc technique were higher and less precise than those with liquid blood samples. Schramel (S1) investigated the use of the graphite furnace for the determination of trace elements in an acid digest of a biological standard. He found considerable interferences in the determination of lead from the matrix elements calcium, potassium, sodium, phosphorus, and sulfur.

Davis and co-workers (N2, C4) described a new preconcentrating sampling technique for flameless atomic absorption spectroscopy in which a tungsten alloy wire loop is soaked in the sample solution for a specified period of time. Metal ions are concentrated on the surface of the wire loop, apparently by an ion exchange mechanism. The wire

loop is then used as an electrically heated atomizer. The detection limit for lead was 2×10^{-11} g. In an interference study of 20 cations and 16 anions (C4), foreign cations generally enhanced lead absorption by retarding vaporization; this actually allowed the slow detection system employed to respond more efficiently. This technique has not yet been applied to blood analysis.

Lead in urine has also been determined using the more sensitive atomizing techniques. Kubasik and Volosin (K9) first extracted the lead from urine with sodium diethyldithiocarbamate into MIBK and measured the lead in the extract with a carbon rod atomizer. Excess amounts of several normal urinary constituents had negligible effect on recovery of the lead.

Lead in nitric acid digests of hair has been accurately measured by flame atomic spectrophotometry (S6). Renshaw *et al.* (R3) used nonflame atomic absorption to measure lead in single sections of hair.

4.3. Polarography

The sensitivity of conventional dc polarography is slightly less than 10^{-5} *M*, or about 0.5–1 ppm lead. This is just at or above the limit for analyzing physiological levels of lead, unless fairly large samples are available, but it is suitably sensitive to detect lead intoxication. Polarography offers a high degree of specificity, and modern modifications of conventional polarography are among the most sensitive techniques available for lead analysis.

Polarography can be considered an electrolysis method on a microscale in which the applied potential is varied and the current is recorded (C9, C10). The electrolysis cell consists of a micro working electrode, and the auxiliary electrode is a nonpolarizable electrode whose potential is known and remains constant on the passage of small currents. A salt bridge separates the two electrodes. The microelectrode employed in polarography is the dropping mercury electrode (DME), in which mercury flows through a vertical capillary to form successive drops of mercury which dislodge and fall to the bottom of the cell. The advantages of the DME are severalfold. The surface is continually renewed and so it does not become contaminated or changed by deposited metal or other product. The surface area is very reproducible and can be calculated, if necessary, from the weight of the drop. The most important feature is that mercury has a high hydrogen overvoltage, allowing very negative potentials up to -2 V or more to be achieved before the discharge of hydrogen. By convention, all polarographic potentials are referred to the saturated calomel electrode (SCE), and so this electrode

is commonly used as the auxiliary or reference electrode. Its potential at 25°C is 0.242 V vs the normal hydrogen electrode (NHE). When a potential difference is impressed between the two electrodes, then, the potential that the microelectrode adopts will be equal to the applied potential versus the SCE.

The important feature of this technique is that the working electrode is a *micro*electrode, which restricts the current to a few microamperes and allows limiting currents to be reached. Conditions are maintained so that the current is diffusion controlled; i.e., the only way the electroactive test substance (one that can be reduced or oxidized at the available potentials) can reach the electrode is by diffusion. When the applied potential reaches the decomposition potential of the test substance a current flows, and it increases linearly as the potential is increased, in accordance with Ohm's law (since the resistance of the circuit remains constant). But with a microelectrode and under diffusion-controlled conditions, the number of ions or molecules of test substance that can diffuse to the electrode and maintain the electrolysis current is limited. Thus, with a dilute solution (10^{-3} to 10^{-2} *M* and less) a limiting current is ultimately reached and an S-shaped plot of current versus applied potential results. The limiting current is proportional to the concentration of test substance. The potential at which the current is one-half of the limiting current (called the diffusion current, i_d) is the half-wave potential ($E_{1/2}$) and is independent of concentration. The half-wave potential is characteristic of the particular test substance being electrolyzed.

Diffusion conditions are maintained by adding a high concentration (ca. 0.1 *M*) of an inert supporting electrolyte, such as potassium nitrate. The high concentration of an electrochemically inert salt prevents electrical attraction or repulsion of the test ions by the charged electrode since the large excess of inert ions are preferentially attracted or repulsed by the electrode. The inert electrolyte is not electrolyzed, however, except at very large potentials. The supporting electrolyte also aids in reducing the resistance of the solution, and hence the IR drop of the circuit; thus, the potential at the working electrode is equal to the applied potential.

With solid electrodes, solutions may also be stirred at a constant rate or else the electrode may be rotated or vibrated at a constant rate. The current will be increased, but will still be proportional to the concentration of the electroactive species.

The limit of detection is about 10^{-5} to 10^{-6} *M* of the test substance, and it is governed by the magnitude of the *residual current*. This is due mainly to the charging current and arises from the charged surface of the electrode, which acts as a capacitor and must be "charged" up.

The limit of negative working potential that can be applied is governed by the reduction of the supporting electrolyte cation or the discharge of hydrogen in acid solutions. The positive limit of working potential is restricted by the oxidation of the mercury electrode itself and, in the absence of mercury complexing or percipitating ions, is about +0.4 V vs SCE.

More positive potentials can be attained by employing solid microelectrodes, but then negative potentials often cannot be employed. In 1941 Laitinen and Kolthoff (L1) coined the general term "voltammetry" for current–voltage curves obtained at all microelectrodes, including mercury and solid microelectrodes. Polarography is a special type of voltammetry in which the electrode is the DME. Either oxidation or reduction of a test substance can take place at microelectrodes, depending on the applied potential and the test substance.

When working in the negative potential region, as with lead, oxygen must be removed from the solution since oxygen is electrochemically reduced. This is usually accomplished by bubbling with oxygen-free nitrogen for several minutes. Also, a surface-active agent, such as gelatin or Triton X-100, is sometimes added as a "maximum suppressor." Frequently, a "spike" or maximum appears on a polarographic wave at a DME, and small amounts of surface active agents will eliminate this; larger amounts may suppress the wave itself.

With mercury electrodes, contact with highly acid solutions for more than a few minutes should be avoided in order to prevent acid dissolution of the electrode (C15).

The shape of a polarographic wave is given by

$$E = E_{1/2} + RT/nF \ln [(i_d - i)/i] \tag{8}$$

where R is the gas constant, T the absolute temperature, n the number of electrons involved in the electrode reaction, F the Faraday constant, and i the current at applied potential E on the steep portion of the wave. At 25°C a plot of log $(i_d - i)/i$ vs E results in an intercept of $E_{1/2}$ and, for a reversibly electrolyzed substance such as lead, a slope of $0.059/n$. This is useful in differentiating substances that may be reduced at similar potentials.

The half-wave potential for the reduction of lead in acid (HCl) solution is about −0.49 V vs SCE. Thallium(I) and tin(II) have polarographic waves close to this. The lead wave can be distinguished from the thallium wave by a plot of log $(i_d - i)/i$ vs E. This gives a slope of $0.059/n$ volt and for lead n will be equal to 2 while for thallium it will be unity. Alternatively, the waves can be separated chemically.

The potential of the thallium reduction is practically unaffected by chelating agents. The lead wave is shifted to —0.80 V in sodium hydroxide solution. Tin(II) is reduced at —1.26 V in this medium. Alkaline tartrate is often used as the supporting electrolyte. In this medium, lead is reduced at —0.79 V, thallium at —0.50 V, and tin(II) at —1.20 V. In tartaric acid medium, the half-wave potentials are —0.52, 0.49, and —0.68 V, respectively.

Brezina and Zuman (B8) have summarized some of the applications of polarography to biological samples. Lead in blood can be determined by adding citrate and hydrochloric acid to precipitate proteins, and then running a polarogram directly on the protein free filtrate. The sample is saturated with oxygen before adding the acid in order to prevent the liberation of porphyrines, which give interfering polarographic waves.

In other methods, the sample is first wet or dry ashed. An acid or alkaline tartrate supporting electrolyte seems to be satisfactory for most purposes. Some investigators separate the lead by extraction with dithizone prior to analysis.

For the analysis of lead in urine, the lead is often separated by precipitation as the phosphate in ammoniacal medium or as the oxalate, which is then ignited. The polarogram is finally run in tartrate or citrate medium, either acid or alkaline.

There are a number of polarographic techniques that are much more sensitive than conventional polarography. One of these is pulse polarography, which minimizes the effect of the charging current. In differential pulse polarography, a single rectangular voltage pulse of constant amplitude (usually 5–100 mV) is superimposed for 40–60 msec on a slowly increasing voltage ramp during the last quarter of the drop-life of the DME. Two current measuring periods are used, one immediately before the pulse and the other at the end of the pulse. The measured current represents the difference in the two sampled currents. The plot of this difference as a function of potential is a symmetrical peak, essentially equal to the derivative of the normal S-shaped peak. The charging current decays exponentially with time during each drop life. By making measurements at the end of the drop life, one avoids the period at the beginning of the drop life when the charging current is changing most rapidly. In addition, the measurement is arranged to be made toward the end of the pulse where the charging current is smallest. Hence, the sensitivity is generally 10–100 times greater than in conventional dc polarography. Moreover, in the differential mode, improved resolution is achieved. Sensitivity does, however, suffer for irreversible electrode processes.

In pulsed techniques, synchronization of the drop time of the DME with the frequency of applied pulses is necessary. This is achieved by mechanical drop control using a magnetically controlled "hammer" knocking the capillary and causing detachment of drops from the capillary. The drop time can be synchronized with the application of voltage pulses.

An even more sensitive technique than pulse polarography is anodic stripping voltammetry. In this technique, commonly a hanging mercury-drop electrode (HMDE) is used, where a mercury drop is suspended from the end of a mercury plated platinum wire or a gold wire, sealed in a glass tube. Mercury-coated graphite electrodes are also frequently used. These are more sensitive because the deposited metal is diluted less in the thin layer of mercury. First, the solution is stirred at a reproducible and constant rate, while a constant potential is applied which resides on the diffusion current of the polarographic wave of a metal ion; the reduction product of the metal ion must form an amalgam with the electrode. During this step, the metal ion reaches the electrode surface by diffusion and mass convection (stirring), where it is reduced and concentrated in the drop. After a preselected time, the stirring is stopped and the potential is scanned anodically (toward the more positive potentials). If the electrode process is reversible, the amalgamated metal will be anodically stripped out of the electrode, that is, reoxidized, giving rise to an anodic wave. The wave will be peaked, and because the concentration of the amalgam in the electrode is much greater than that of the metal ion in solution, the peak height will be greatly magnified over that of the normal polarographic reduction wave.

The peak height is proportional to the square root of the rate of potential scan, and so a fairly rapid scan is employed, about 50 mV/second; the voltammogram is thus recorded in about 20 seconds. The peak current is also directly proportional to the time of preelectrolysis, and the time used will depend on the concentration. The upper concentration limit is about 10^{-6} to 10^{-5} M, and the lower concentration limit is 10^{-8} to 10^{-9} M! The lower concentration corresponds to about 0.1 ppb. It is not difficult to use 1-ml volumes in these techniques, and so 10^{-10} g can be determined. This compares favorably with almost any other analytical technique.

For 10^{-6} M, preelectrolysis times of 60 seconds or less are sufficient, and proportionately longer times are used as the concentration decreases. For 10^{-9} M, electrolysis times of 1 hour are usually required.

Anodic stripping pulse voltammetry (C6) is 10-fold more sensitive than dc anodic stripping voltammetry. Down to 10^{-9} M concentrations have been determined, and even analyses of 10^{-10} M solutions have

TABLE 3
pH AND RESISTANCE OF DIFFERENT SOLUTIONS OF TISSUE ASHES (N1)

Sample	G/ml	pH	Resistance (ohms)
0.01 *M* KCl	—	—	770
Skin	1	11.3	500
Liver	1	8.2	185
Blood	1	9.4	572
Muscle	1	10.4	145
Urine	5	10.5	12.5
Urine	10	—	8.8
Brain	1	8.6	135
Lung	1	8.5	188
Kidney	1	8.5	135

been reported. Concentrations which by dc anodic stripping voltammetry may require 30–60 minutes of preelectrolysis, will require only 30 seconds to 5 minutes by differential pulse stripping voltammetry. An additional advantage is that only 10^{-3} *M* or less supporting electrolyte is required. This is important for minimizing impurities.

One of the main problems with a technique as sensitive as these is that of finding a supporting electrolyte that is suitably free of lead. Biological materials contain sufficient quantities of salts that dry-ashed samples can be simply diluted with water at a ratio of 1 ml per 1 g of wet sample (N1). Table 3 lists the resistances of several sample solutiions prepared in this way compared with that of 0.01 *M* potassium chloride (N1). It is advisable in these direct diltuions to employ a standard additions technique for calibration. This will account for any changes in the anodic stripping potentials in the presence of any complexing salts in the samples. Complexing agents have been demonstrated to shift the anodic stripping peak potential (D4).

The application of stripping analysis has generally required prior destruction of organic matter. For example, Roschig and Matschiner (R6) determined lead in 0.2 ml blood by mineralization with HNO_3, $HClO_4$, H_2O_2, and H_2SO_3. Ascorbic acid was added to the electrolytic cell, presumably to remove oxygen; Christian and Davis (C13) have used ascorbic acid to rapidly remove oxygen from solution for polarographic lead determination. Quartz cells and digestion apparatus were used to minimize lead contamination. A standard deviation of 10% was reported for 0.2–2.0 ppm of lead in blood.

Voloder *et al.* (V1) mineralized 0.2 ml of blood with nitric acid and hydrogen peroxide. The residue was dissolved in an electrolyte. They

found a detection limit of 5 μg lead per 100 ml. Traces of remaining nitric acid were reported to interfere in their procedure. Sinko and Gomiscek (S4) determined blood in *serum* by digesting 0.5 ml with 0.5 ml of HNO_3–$HClO_4$ (3:1) in a Teflon autoclave. They were able to detect as little as 0.01 ppm lead in the serum.

Searle, Chan, and Davidow (S2) described a microprocedure for blood lead using anodic stripping coltammetry. Fifty microliters of blood or 0.5 ml of urine were digested for 30 minutes in the polarographic cell with 0.2 ml or 0.4 ml, respectively of 70% perchloric acid. After cooling, 4 ml of water was added and the lead was plated for 20 minutes onto a mercury-coated carbon electrode at —0.8 V vs SCE, followed by anodic stripping and measurement. They analyzed blood samples containing 23–83 μg of lead per 100 ml of blood. Except for low, nontoxic lead concentrations, recoveries for anodic stripping voltammetry and atomic absorption spectroscopy correlated well. The relative standard deviation was somewhat larger by the former method, but the largest differences were at the low concentrations.

Duic, Szechter, and Srinivasan (D9) used derivative pulse stripping voltammetry to determine lead in 0.2 ml of blood digested with 0.4 ml of $HClO_4$ and 0.1 ml of H_2SO_4 and diluted to 7 ml. The method can be applied to samples as small as 50 μl.

When working with polarography, care should be taken to avoid excess exposure to mercury vapors. A well ventilated laboratory should be used (C8).

4.4. Neutron Activation Analysis

This technique is extremely sensitive for many elements. But for lead, it is not as sensitive as more conventional chemical (instrumental) methods. The interference free detection limit using irradiation with a neutron flux of 10^{13} neutrons/cm^2-sec for 1 hour is only as low as 2 μg (G9). By reactor pulse analysis, as little as 0.5 μg can be detected. These sensitivities are satisfactory if sufficient sample is available. This technique appears to offer little advantage, and, in view of the relative unavailability and the expense of facilities, it will not find much use for clinical lead analysis.

4.5. Ion Selective Electrodes

Ion selective electrodes in principle represent the analytical chemist's dream of a simple probe that will, essentially without preparation or manipulation of the sample, give a signal directly dependent on the concentration of the test ion. Unfortunately, for lead the state of the

art has not advanced sufficiently for utilization of these electrodes in the clinical laboratory. Experience in our laboratory with the Orion ion-exchange liquid-membrane lead electrode has shown that it possesses neither the sensitivity nor the selectivity required for measurements in biological fluids (L2). The electrode is actually more responsive to the alkali metals than to lead, which makes it useless for biological applications. According to Orion literature (O3), their solid state lead electrode can be used to measure as little as 10^{-7} M of uncomplexed lead (0.02 ppm). However, copper as well as silver and mercury must be absent. Also, the iron(III) concentration cannot exceed the sample lead level, although the iron can be eliminated by adjusting the sample pH to above 4. In view of the fact that blood contains about 1 ppm of copper, it appears unlikely that this electrode will find use in the clinical laboratory unless a preliminary separation procedure is performed. Rechnitz (R1), however, has reported that the solid state electrode should not be subject to interference by normal ionic constituents of body fluids, although no experimental evidence was presented.

In conclusion, there are a number of techniques that will give reliable results for lead analysis. The spectrophotometric method, while probably the most popular, suffers from lack of specificity, and great care must be taken to ensure that a positive test is due to lead. In addition, the technique is quite tedious, requiring several extractions and pH adjustments, and the use of cyanide solutions always poses a danger in the laboratory. The use of sulfuric acid for digestion in the procedure recommended by Rice *et al.* for blood and urine should be used with caution.

Polarography and atomic absorption spectroscopy each possesses a high degree of specificity. In the anodic stripping analysis mode, polarography is one of the most sensitive techniques available for lead analysis. Atomic absorption spectroscopy has the advantage of requiring minimal sample preparation. Nonflame atomic absorption techniques are sensitive and very rapid and should find increasing use in the clinical laboratory.

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