ADVANCES IN CLINICAL CHEMISTRY

Volume 5

Harry Sobotka &
C. P. Stewart

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VOLUME 5

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Advances in
CLINICAL CHEMISTRY

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FOREWORD TO THE SERIES

A historian of science in years to come may well be astonished at the explosive burst of scientific activity round about the middle of the twentieth century of our era. He will be puzzled by the interrelationship between the growth of population and the rise of the standard of living; he will be interested in the increased percentage of scientists among the population, their greater specialization and the resulting fragmentation of science; he will analyze the economic and the psychological motivation of scientists; he will compare the progress of knowledge with the broadness of the current of scientific publication.

Living as we do in the midst of these events, we are hardly aware of their relatively rapid rate. What we notice is a doubling of the scientific output every ten years, regardless of contemporary political events. It is this climate which has engendered the appearance of series of reviews in dozens of disciplines. It may be with yearning or with a feeling of superiority, that we look back at such annual compendia as "Maly's Jahresberichte der Thierchemie" of one hundred years ago, which encompassed the annual progress in the zoological half of biochemistry within 300 to 400 pages.

Nowadays, that number of pages would not suffice to record the complete annual increment of knowledge in a single specialized division of the subject such as Clinical Chemistry. Media already existing furnish a comprehensive list of publications and an encyclopedic summarization of their contents; the present series of "Advances in Clinical Chemistry"—like other "Advances" series—attempts something different. Its aim is to provide a readable account of selected important developments, of their roots in the allied fundamental disciplines, and of their impact upon the progress of medical science. The articles will be written by experts who are actually working in the field which they describe; they will be objectively critical discussions and not mere annotated bibliographies; and the presentation of the subjects will be unbiased as the utterances of scientists are expected to be—*sine ira et studio.*

The bibliography appended to each chapter will not only serve to document the author's statements, it will lead the reader to those original publications in which techniques are described in full detail or in which viewpoints and opinions are expressed at greater length than is possible in the text.

The selection of the subjects in the present and in future volumes will include discussion of methods and of their rationale, critical and com-

parative evaluation of techniques, automation in Clinical Chemistry, and microanalytical procedures; the contents will comprise those borderline subjects, such as blood coagulation or complement chemistry, which are becoming more chemical with increasing knowledge of the underlying reactions; in some instances the discussion of a subject will center around a metabolic mechanism or even around a disease entity.

While recognizing that the elaboration and testing of methods is of the greatest importance in a subject, part of whose function is to provide reliable, accurate diagnostic and prognostic procedures, the new series will take cognizance of the fact that Clinical Chemistry plays an essential part in the progress of medical science in general by assisting in elucidating the fundamental biochemical abnormalities which underlie disease. The Editors hope that this program will stimulate the thinking of Clinical Chemists and of workers in related fields.

HARRY SOBOTKA
C. P. STEWART

PREFACE TO VOLUME 5

The Editors have striven, as in previous years, to include in the present volume reviews on greatly diversified subjects, all of timely importance. The article on mellituria in Volume 4 has been supplemented by a survey of galactosemia, and we expect to follow in future volumes with reviews of other inborn errors of metabolism or, in modern parlance, of molecular diseases. Likewise, the article on peptiduria supplements that on aminoaciduria in Volume 2 and that on microbiological assay of vitamins extends previous summaries on the nucleogenic vitamins. The haptoglobins lie on the borderline of hematology.

The splendid reception received by this series of Advances confirms the Editors' conviction that clinical chemistry should not be treated merely as a branch of analytical biochemistry but that it be viewed as a natural forum where clinician and chemist meet for the exchange of ideas and experiences.

On the occasion of the fifth anniversary of this enterprise, we should like to thank the publishers for their painstaking care and sympathetic cooperation.

September 1962

HARRY SOBOTKA
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INHERITED METABOLIC DISORDERS: GALACTOSEMIA

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I. Introduction

Galactosemia is a serious disease leading, in many cases, to death in infancy. Almost all the untreated survivors suffer from mental retardation, cataracts, and varying degrees of damage to liver and kidneys. Yet, provided the diagnosis is made early enough and suitable treatment given, all these ill effects can be avoided. The greatest obstacle to diagnosis of galactosemia has been, in the past, unawareness of the main clinical, biochemical, and genetic features of the disease. The first reported case was in 1908 (V1), yet 40 years later the disease was still looked on as a rare curiosity; few pediatricians kept galactosemia in mind in examining infants who failed to thrive, and the laboratory diagnostic tests available left much to be desired. It is only in the last 10 years that it has been clearly realized that galactosemia belongs to the group of inborn errors of metabolism, resembling the conditions so described by Garrod in 1908 (G2).

The association of cirrhosis of the liver with an abnormal galactose tolerance has been known since the work of Bauer in 1906 (B4). It is, perhaps, not surprising that there was doubt as to whether the grossly abnormal galactose tolerance found in galactosemia was secondary to liver damage or whether the abnormally high concentration of galactose in the blood caused the liver damage. Mason and Turner (M2) suggested that the primary defect was an impairment of the ability of the liver to convert galactose to glycogen. Gorter (G8), in 1951, first clearly stated that galactosemia must be considered as an inborn error of metabolism.

In this chapter, the remarkably widespread and variable clinical features of galactosemia are briefly described, followed by an account of experimental galactosemia in animals. The normal metabolism of galactose, the nature of the enzyme defect in galactosemia, the biochemical consequences of this defect, and its correlation with the clinical aspects are next dealt with. Laboratory procedures for the diagnosis of galactosemia in the newborn and later in life, for the monitoring of treatment, and for the detection of carriers are briefly treated. Like the other inborn errors of metabolism, galactosemia is a genetically determined disease; the genetics of galactosemia, and the very important part the biochemist plays in the study of the genetical aspects, are described next. The dietary treatment of galactosemia and the different diets proposed or used are discussed. Finally, conditions that may be confused with galactosemia are mentioned. Throughout the chapter gaps in our present knowledge are emphasized in the hope that they may prove starting points for yet further advances in our understanding of this condition.

2. Clinical Features

2.1. Failure to Thrive

Failure to thrive is one of the commonest features of galactosemia. Severely affected infants cease sucking within a few days of birth and begin to lose weight. There is listlessness, vomiting, and often signs of hepatic dysfunction. Diarrhea is common. A hemorrhagic tendency sometimes occurs. Galactosemia is frequently fulminating, and the severely affected infant can go rapidly downhill and die, often in liver failure or from intercurrent infection, within a few weeks unless given a galactose-free diet.

Some infants survive untreated for several months, and the presenting complaint may be a feeding difficulty or a failure to gain weight satisfactorily or anemia. The anemia may be hemolytic (H10). On examination the characteristic cataracts, mental retardation, and enlargement of the liver may be noticed. A few children even survive for several years without treatment; in some of these cases a milk-free diet was fed because of "milk allergy" and this may have been lifesaving. These older children are often underweight. There does not seem to be any general tendency for survival or nonsurvival to run in particular families, thus one child, in whom the diagnosis was made at the age of 19 months, had two galactosemic sibs who died at 8, and 39 days, respectively (C5).

2.2. Liver Function

Jaundice is a common and early sign of the disease. It was noted in 58% of Hsia's cases (H10). In Göppert's case (G7), there was severe jaundice by the second day of life, but in general, jaundice is first noticed when the infant is from 3 to 14 days old. There is considerable variation in the depth of jaundice, some infants are never noticeably jaundiced while others are very deeply jaundiced within a few days of birth. The jaundice often fades after a week or two, but sometimes persists for months. The circulating bilirubin is, in some jaundiced cases, almost entirely unconjugated (reacting by the indirect van den Bergh test) and in others conjugated (direct reacting). This suggests that in some the conjugating mechanism of the liver is malfunctioning, in others the permeability of the liver cells is altered. Plugging of the bile canaliculi and intrahepatic biliary atresia, probably secondary to damage to the parenchyma cells, have been described. Holzel (H3) has described a case in which a bilirubin stone blocked the bile duct, causing a recurrence of jaundice in spite of treatment with a low-lactose diet. Komrower *et al.* (K13) report a rise in the serum bilirubin when a child,

who had been on a galactose-free diet for 8 months, was once more given galactose.

Enlargement of the liver is almost invariable in untreated cases (D4), and in older children or adults, is often accompanied by splenomegaly. Histologically the liver shows, in the early stages, fatty infiltration, varying degrees of parenchymal necrosis, and distortion of the lobular structures; later changes closely resemble those of Laennec's cirrhosis (T3), with diffuse fibrosis as the most prominent feature and only a moderate degree of fatty infiltration (C5, D4). It is probable that only the milder cases survive to develop fibrosis, so that the liver changes in older patients may not represent later stages of the changes seen in younger patients. However, Cox and Pugh (C5) consider that most of the damage to the liver is caused during the first few weeks of life and that the more mature liver can better resist the toxic action of galactose. The early changes are reversible by treatment with a galactose-free diet, with histological evidence of active repair to liver tissue (B6, C5).

In many early cases the conventional liver function tests are abnormal (C5, E1, T3). The concentrations in the blood of albumin and prothrombin, normally synthesized in the liver, are reduced in some cases of galactosemia; hypoalbuminemia leads to ascites and edema, and hemorrhages occasionally occur because of the low prothrombin content. There is a marked aminoaciduria which is, however, largely, but not entirely, due to renal dysfunction. The galactose tolerance test is not without hazard; it is considered below (Section 4.2). Liver function tests, with the exception of the galactose tolerance test, tend to be normal in older children even without treatment. This suggests either that the liver recovers many of its functions, even though it frequently remains enlarged and more or less cirrhotic, or that only the less severely affected survive.

2.3. Renal Function

The urine frequently contains casts and amorphous debris, but rarely any considerable number of red blood corpuscles. Proteinuria and aminoaciduria are found in nearly all untreated patients from a very early age. The proteinuria is usually reported as "albuminuria" in the literature, but in some cases the urinary protein has been shown by electrophoresis to consist largely of α-globulin and other relatively low-molecular-weight proteins (B21). A similar urinary pattern occurs in a number of diseases of the renal tubule. The proteinuria is often only moderate in degree, e.g., < 50 to 150 mg protein per 100 ml of urine, but is easily detected by the conventional tests for protein, such as salicylsulfonic acid. Excretion of protein can rise to nearly 1 g/100 ml in some cases (H8, L7).

Electrophoresis of the urine in these cases with more massive proteinuria shows a protein pattern resembling plasma (K13).

Aminoaciduria was found in galactosemia by Holzel *et al.* (H9) and independently by Bickel and Hickmans (B12). Aminoaciduria is a not unexpected finding in severe liver disease, and it would be natural to attribute one to the other. However, both groups of workers, on the basis of the patterns obtained on paper chromatography, considered it unlikely that this was an "overflow" aminoaciduria caused by aminoacidemia following liver dysfunction, but rather tentatively suggested a failure of renal tubular reabsorption of amino acids. Holzel *et al.* (H9) quoted Dent as comparing the aminoaciduria of galactosemia to that found in Wilson's disease. Kowrower (K12) later found that the α-amino nitrogen content of the blood was normal in galactosemia, strongly suggesting a renal mechanism for the aminoaciduria. Dent and co-workers (C9) then investigated a galactosemic boy who had been on a galactose-free diet for 2 years. On this diet his amino acid excretion was normal. When he was given 20 g galactose per day for 15 days his plasma α-amino nitrogen clearance rose gradually from 0.5 ml/min to 2.0 ml/min; the concentration of α-amino nitrogen in his plasma rose from 3.33 to 5.53 mg/100 ml over the same period, remaining within normal limits. The urinary amino acid pattern, on two-dimensional paper chromatograms, changed from normal to a marked aminoaciduria, with large increases in the excretion of the low-molecular-weight amino acids such as serine, glycine, threonine, and alanine, and lesser increases in valine, the leucines, and tyrosine. The rise in plasma α-amino nitrogen concentration was so slight that there can be no doubt that this aminoaciduria was very largely renal in origin. Even so, the fact that the plasma α-amino nitrogen level rose gradually on feeding galactose is evidence that the ability of the liver to metabolize amino acids was being impaired.

In some cases, the amino acid pattern on a paper chromatogram is very similar to that found in diseases of the renal tubules, such as cystinosis, where there is a failure to reabsorb all amino acids from the glomerular filtrate and, in consequence, the urinary amino acid pattern resembles that of plasma (W6, W9). In other cases the aminoaciduria is less marked and the amino acids found in greatest excess are glycine, alanine, serine, threonine, and glutamine. In some cases no aminoaciduria has been detected.

Perry *et al.* (P7) showed that the uptake of L-histidine by segments of chick gut was inhibited by galactose. Galactose in a concentration of 0.5% behaved remarkably like $10^{-3}M$ 2,4-dinitrophenol in its inhibitory effect on the gut. The intestine and the renal tubule behave similarly in

absorbing amino acids (M5, M6) and this work of Perry's throws light on the cause of the aminoaciduria.

If the patient is treated with a diet free from galactose, proteinuria vanishes and excretion of α-amino acids becomes normal within a few days. The rapidity of this response is striking. Although the urinary excretion of α-amino acids becomes completely normal, in some cases moderately large amounts of β-aminoisobutyric acid and ethanolamine continue to be excreted for some months; this probably represents a reduced ability of the liver to metabolize these compounds (W9). Darling and Mortensen (D2) report a case of galactosemia in which the excretion of amino acids was not completely normal for over 8 months after starting a galactose-free diet. The same case showed a transient glucosuria when the diet was started, possibly further evidence of renal tubular dysfunction. Glucosuria, in addition to galactosuria, has been found in other cases. If galactose is once more fed to a patient on a galactose-free diet, proteinuria reappears within half an hour, and the general aminoaciduria returns after a week or 10 days.

Hyperchloremic acidosis has been noted in some cases (B7, K13); this is presumably due to defective tubular reabsorption of bicarbonate. Phosphate-losing rickets or marked hypokalemia have not as yet been reported in galactosemia, but some cases show roentgenological evidence of osteoporosis (M2), and Holzel *et al.* (H8) record low levels of serum potassium.

The proteinuria and aminoaciduria, and acidosis and glucosuria where they occur, are probably caused by reversible inhibition of some functions of the renal tubule. There would appear to be no structural damage to the kidney. However, 2 children developed nephrolithiasis while being treated with a low-lactose diet (B7, C5). The time course of events, when galactose is withdrawn from and returned to the diet, suggests that some metabolite of galactose accumulates in the cells of the renal tubules and has an inhibitory effect on the reabsorption of a number of substances.

2.4. Eye Changes

The great majority of untreated galactosemic subjects who survive more than a few weeks develop lenticular cataracts. These first appear as a faint milkiness of the anterior capsule of the lens between the fourth and eighth week. Opacity of the lens increases rapidly unless the infant is fed a galactose-free diet. On such a diet, early cataracts regress but rarely vanish completely, though in the best cases vision is little affected (B16, B19, H10, K13, W4). If treatment has been delayed and, in consequence,

the whole lens is opaque, the improvement on a galactose-free diet is usually only slight and surgical intervention is necessary. Goldbloom and Brickman (G6) suggest that dietary treatment must be started before the nucleus of the lens is completely formed, i.e., in the first 3 months of life, for it to be effective; however, others find that some opacities of the lens persist even when the diet is started at the age of 6 weeks (A4).

Galactose, if fed in large amounts, causes cataracts in a number of other species, even though the other features of galactosemia are absent. These animal experiments are discussed below (Section 2.7) and enzymic mechanisms possibly related to the development of cataract in Section 3.3.3.

Three out of 45 galactosemic patients studied by Hsia and Walker (H10) had retinal detachment and one had intraocular hemorrhage.

2.5. Mental Deficiency

Almost all surviving untreated galactosemic children are mentally retarded. The degree of retardation varies, most cases lying between I.Q. 30 and I.Q. 70. Some previously undiagnosed older galactosemics were found by examining inmates of institutions for mental defectives (B9). There are only 6 recorded cases of untreated galactosemics with normal intelligence (D10, H10, H16, R1).

The mental deficiency characteristic of galactosemia is obviously caused by some inhibiting effect of galactose, or more probably galactose-1-phosphate, on the normal functioning of the brain. The nature of this inhibition is unknown, but is probably concerned with glucose utilization (see below, Section 3.3). Hypoglucosemia may also play a part in some cases, but this is not proven, and the clinical features of galactosemia differ from those of hypoglycemia. Hyperbilirubinemia may cause kernicterus, but probably only in a minority of cases. The effect of galactose or its metabolite on brain metabolism can be reversed by withdrawing galactose from the diet; in older children this does not always lead to complete mental normality, either because some irreparable structural damage to the brain has been caused, or because the child has several years of retardation behind him and starts that much behind his contemporaries, or for both reasons. If such a diet is started in early infancy, the mental development is normal (H3, H10). Donnell et al. (D4, D6) noted that, of 15 treated galactosemics, 4 were mentally defective, only 3 had an intelligence quotient over 100, and there was a negative correlation between age at diagnosis and present intelligence quotient. Clay and Potter (C2) discuss the cause of mental deficiency

and the need for early diagnosis and treatment. Hsia and Walker (H10), in a series of 34 treated galactosemics, found that nearly 2/3 had normal intelligence and that the I.Q. was negatively correlated with the age at which treatment began.

Apart from the mental deficiency, there is little evidence of cerebral damage. In patients dying early in infancy of the fulminating form of galactosemia the brain is edematous, but no abnormality has been found in the brains of several older patients in spite of careful examination (H5). These older patients are presumably mostly those who were less severely affected in infancy. In a very full neuropathological examination of one 8-year-old patient, Crome (C8) found that the brain was small with marked gliosis and scarring of the white matter, some loss of myelin and a deficiency of Purkinje cells in the cerebellum. Changes in the globus pallidus resembled those of kernicterus and this child had had severe jaundice as an infant (bilirubin 26 mg/100 ml serum). This child was, during life, very severely retarded, with an intelligence quotient of only 21 (Griffiths scale), far less than in most untreated galactosemics who survive infancy.

Fits occur in a few patients (B2, C2, C5, H10) and one developed a transitory choreiform disturbance some years after starting treatment (G6). Electroencephalographic abnormalities were noted in one 9½-year-old mentally retarded boy by Holzel (H3) and in 3 affected infants by Ballabriga *et al.* (B2). The latter authors noted that dogs and chickens given galactose intra-arterially or orally developed abnormalities of the E.E.G. In the children the E.E.G. abnormalities persisted even after a galactose-free diet had been given for some years, but they were never very severe. One untreated patient, with an I.Q. of 64, showed evidence of organic brain damage on a Rorschach test (T3). By comparison with some other inborn errors of metabolism, such as phenylketonuria or maple syrup urine disease, galactosemia appears to cause remarkably little neurological abnormality or detectable structural damage to the brain. This is probably a false picture, due to the examination of those who survived infancy and who were, therefore, the less severely affected.

2.6. Hypoglycemia

The concentration of glucose in the blood is often low in galactosemia (B19, M2). The total sugar content of the blood is very high, the bulk of the sugar being galactose, and this tends to obscure the hypoglucosemia when reductometric methods of assay are used. Giving galactose leads in many but not all cases of galactosemia to a marked fall in the con-

centration of glucose in the blood (K13, S17). It is reported that normal adults respond to an intravenous, but not an oral, dose of 60 g galactose with a fall in blood glucose concentration, to less than 40 mg/100 ml (D8).

Two explanations have been put forward: (a) that a raised concentration of galactose in the blood causes release of insulin, just as glucose does (F2, P10); and (b) that galactose-1-phosphate interferes with conversion of liver glycogen to glucose by competitive inhibition of phosphoglucomutase and glucose-6-phosphatase (I5, S17). Neither theory accounts for the occasional galactosemic whose blood glucose concentration does not fall after a test dose of galactose. Hyperplasia of the islets of Langerhans has been noted in one case (C5), and this tends to support the insulin-release hypothesis. However, more work is necessary to settle this point.

2.7. Experiments with Animals

As early as 1935 Mitchell (M7) found that young rats given a diet containing 35% galactose developed cataracts which became mature in from 12 to 37 days. Such lenses were opaque and contained more water and calcium, and less protein and mucoprotein, than the normal lens. Development of cataracts was slower when only 25% galactose was fed or when the galactose was replaced by lactose which formed 70% of the diet. Mitchell and Cook (M8) described in detail the changes in the lens when the high galactose diet was started and also the stages of regression of the cataracts when the rat was put back on a normal diet. The rate and degree of regression varied with the age of the animal and how long it had been on a high-galactose diet, but a nuclear opacity usually remained.

Bannon *et al.* (B3) showed that cataracts developed *in utero* if a pregnant rat was given a diet containing 25% galactose. The fetus was normal up to 15 days, then the lenses began to show vacuolation, degeneration of fibers, and replacement of fibers by a noncellular mass. The central fibers which form the nucleus were the most sensitive, and regions undergoing differentiation rather than growth were affected. The final size of the lens was normal.

Other species of mammal also develop cataracts on a high-galactose diet and, of other sugars investigated, xylose has been found to be cataractogenic when fed in large amounts. It is well-known that cataracts occur in diabetes mellitus; Patterson (P3) has pointed out that galactose, xylose, and glucose are the three sugars whose cell permeability is

affected by insulin. He suggested that galactose and xylose might cause cataracts by hindering glucose metabolism in the lens.

Schwarz and Golberg (S8) found that the lenses of galactose-fed rats contained 10 times the normal concentration of galactose-1-phosphate and Lerman (L4) suggested that this inhibited the normal enzymic activity of the lens.

Craig and Maddock (C6) investigated other effects of a diet high in galactose. A group of rats 21 days old were given a diet containing 70% galactose. After 75 days they were compared with glucose-fed controls. The galactose-fed rats weighed only 50 to 60% of the control weight but, apart from cataracts, were well. There was corneal vascularization but no scarring in the galactose-fed group. The only abnormal organs, apart from the eyes, were the kidneys. The kidneys in the two groups were about equal in weight, the galactose-fed group showing hydronephrosis with a remarkable dilation of the pelvis. Apart from small deposits in the loops of Henle of some material staining with Best's carmine, the kidneys were histologically normal. The liver was histologically and macroscopically normal.

Dam (D1) found that if chicks were fed a diet containing 54.6% galactose, they became severely ill, lost weight, developed spasms, and died after a few days. Blood glucose concentration was normal but the liver was severely depleted of glycogen. Perry *et al.* (P7) also reported epileptoid convulsions in chicks on a normal diet with 10% galactose added to the drinking water. The birds lost weight and died after a mean duration of 4 days. On 5% galactose they gained a little weight but all died within 26 days (mean: 12 days). On 2.5% galactose some survived 35 days (mean: 21 days). Those on 5% and 2.5% galactose did not have fits. No gross or histological abnormality was detected in the liver, pancreas, kidney, cardiac, or skeletal muscle. The liver contained cells filled with glycogen which was shown to be a polyglucose, and hence presumably normal. The blood glucose concentration was normal or high.

One feature of human galactosemia that has never been duplicated in animal experiments is the effect on the liver. It seems likely that some hepatotoxic agent, probably galactose-1-phosphate, is at work in the human liver. Most of the damage to the liver probably occurs very early in infancy, perhaps during the first week (C5), but most experiments have been carried out on older animals (E1). It seems probable that the more mature liver is better able to resist the toxic action of galactose. The present author in collaboration with Dr. Cox and Dr. Bodian, has fed pregnant rats a high-galactose diet without any detectable effect

on the liver of the newborn rat in most cases (a small proportion of the fetuses died *in utero* and underwent maceration). The metabolism of fetal liver is different from that after birth, and in consequence, fetal liver may be less susceptible to poisoning than in the newborn.

3. Biochemical Aspects

3.1. Normal Metabolism of Galactose

When galactose is fed to a normal animal or individual it is converted to glycogen. The conversion takes place mostly in the liver, to a lesser degree in many other tissues. This has been known since 1911 (M11) but it is only recently that the individual metabolic steps have been worked out.

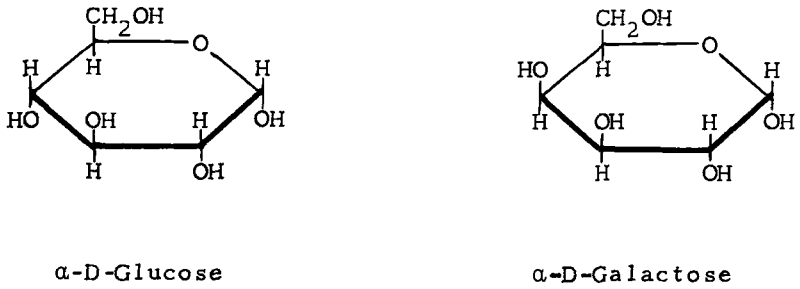

Fig. 1. Structural formulas of glucose and galactose.

Since glycogen is a polyglucose, the essential step is the conversion of a hexose unit with the galactose configuration to one with the glucose configuration, as shown in Fig. 1. The 2 sugars differ only in the orientation of the groups attached to carbon atom 4.

Topper and Stetten (T2) fed rats on galactose isotopically labeled with C^{14} on carbon atom 1. They found that the glycogen formed in the liver was isotopically labeled and 70% of the C^{14} was on C-1 of the glucose units. They concluded that galactose could not be split into 2 triose units which would then recombine, but that a Walden-type inversion must occur at C-4.

Kosterlitz (K14) found that galactose-1-phosphate accumulated in the livers of rabbits fed a high-galactose diet. Kosterlitz (K15) also found that extracts of a galactose-adapted strain of *Saccharomyces cerevisiae* fermented galactose-1-phosphate and glucose-1-phosphate at equal rates; he therefore concluded that the metabolic path of galactose lay through galactose-1-phosphate which was converted to glucose-1-phosphate.

Garner and Grannis (G1), working with the yeasts *Saccharomyces fragilis* and *Saccharomyces marxianus* which ferment galactose, postulated the existence of a "phosphogalactoisomerase" which catalyzed the conversion of galactose-1-phosphate to glucose-1-phosphate. A number of authors suggested that there was a failure to synthesize "phosphogalactoisomerase" in galactosemia.

Most of our present information on the conversion of galactose to glucose has come from the study of microorganisms, particularly the yeast *Saccharomyces fragilis*. However, there is ample evidence that the same metabolic paths are followed in mammalian tissue.

3.1.1. *The Galactose-1-Phosphate Uridyl Transferase Pathway*

Leloir and his associates (T4) showed in 1948 that there occurred in *Saccharomyces fragilis* an enzyme, galactokinase, which catalyzed the reaction of galactose with adenosine triphosphate to produce galactose-1-phosphate. These workers showed that galactokinase, though analogous to hexokinase, was distinct from this enzyme. In 1950 Leloir and coworkers (C1) made the key discovery that the coenzyme for the conversion of galactose-1-phosphate to glucose-1-phosphate was a new type of nucleotide, uridine diphosphate glucose (UDPG). They proved the structure of this new nucleotide by degradative reactions (L3, P1); very mild acid hydrolysis released glucose and left a tribasic acid with a new secondary acid group. Further acid hydrolysis released one phosphate radical and left uridine monophosphate which was identified by X-ray crystallography, paper chromatography, and the rate of hydrolysis with acids and nucleotidase. Pyrophosphatase acted on the original UDPG to release glucose-1-phosphate and uridine monophosphate, hence the 2 phosphate groups were linked as a pyrophosphate. Glucose, released by very mild acid hydrolysis, was identified by paper chromatography, fermentability, and color reactions; since UDPG is nonreducing and the molecule has only 2 acidic hydrogen atoms, both primary, the point of attachment of glucose must be at carbon atom 1, and it must be attached as a phosphate ester. The structure assigned was therefore as shown in Fig. 2.

UDPG was the first of a whole new class of nucleotides, in which the uridine diphosphate group is attached to a monosaccharide. Two important members of the class are uridine diphosphate glucuronic acid, the intermediate in the synthesis of glucuronides and of many other compounds, and uridine diphosphate *N*-acetylglucosamine which is an intermediate in the synthesis of mucopolysaccharides. Uridine diphosphate muramic acid was isolated by Park (P2) from *Staphylococcus aureus*

which had been treated with penicillin, but its structure was not completely known for some time.

The richest source of UDPG is bakers' yeast, which does not ferment galactose. However, this nucleotide has many functions besides the metabolism of galactose-1-phosphate, and has been found in animal tissues. The apoenzyme for the conversion of galactose-1-phosphate to glucose-1-phosphate was obtained from *Saccharomyces fragilis* (L2), and this, with UDPG, was considered to form the enzyme "galacto-waldenase" which converted galactose-1-phosphate to glucose-1-phosphate. Kalckar and co-workers (K8) further clarified the mechanism of the conversion by showing that 2 different enzymes, both derived from *Saccharomyces fragilis*, were involved. The first, a uridyl transferase,

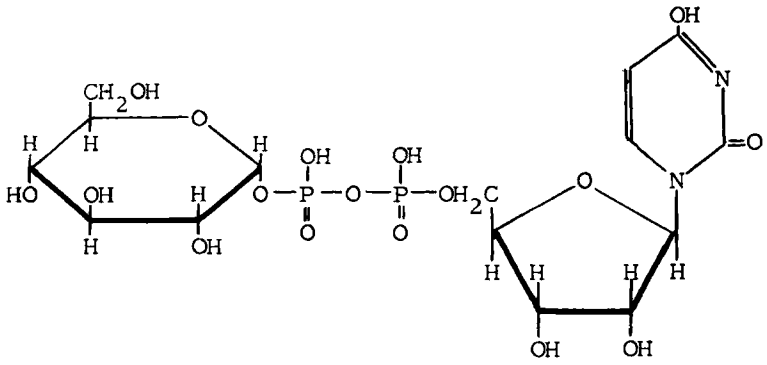

FIG. 2. Uridine diphosphate glucose.

catalyzed the reaction of galactose-1-phosphate with UDPG to give glucose-1-phosphate and uridine diphosphate galactose (UDPGal). The second, "galacto-waldenase," reversibly converted UDPGal to UDPG by an internal rearrangement. At equilibrium, 75% existed as the glucose derivative and 25% as the galactose derivative. The whole series of reactions in converting galactose to glucose is shown in Table 1.

Reaction (1) is irreversible; the enzyme galactokinase has been found in mammalian liver, brain, and erythrocytes, as well as in certain yeasts and other microorganisms. UDPGal, the main product of reaction (2), is the intermediate by means of which the body incorporates galactose into cerebrosides and, probably, other galactolipids, mucopolysaccharides, and lactose; galactose-1-phosphate uridyl transferase occurs in the liver, to a lesser extent in red cells, and probably in other tissues.

Reaction (3) is, from the theoretical standpoint, the most interesting. It involves inversion of configuration at a carbon atom attached to 4

relatively unreactive groups, one of which must be split off and replaced by a similar group. It was shown that neither carbon-carbon bond was broken (T2), and it was therefore believed for a time that the carbon-oxygen bond must be broken, a new hydroxyl group entering the molecule from the opposite side of the carbon atom simultaneously with rupture of the carbon-oxygen bond, thereby producing a Walden inversion of configuration. Leloir (L3) discussed the possible reactions that UDPGal would have to undergo for inversion at C-4 to occur. He eliminated a process of esterification with a carboxylic acid and subsequent hydrolysis because in hydrolysis of carboxylic esters it is always the acyl-oxygen bond which breaks, not the alkyl-oxygen bond, and this would leave the

TABLE 1
REACTIONS INVOLVED IN CONVERTING GALACTOSE TO GLUCOSE

Reaction	Enzyme
(1) Gal + ATP → Gal-1-P + ADP	Galactokinase
(2) Gal-1-P + UDPG ⇄ G-1-P + UDPGal	Galactose-1-phosphate uridyl transferase
(3) UDPGal ⇄ UDPG	Uridine diphosphate galactose 4-epimerase
(4) UDPG + PP ⇄ G-1-P + UTP	Uridine diphosphate glucose pyrophosphorylase

Abbrevations used: GAL, galactose; ATP, adenosine triphosphate; Gal-1-P, galactose-1-phosphate; ADP, adenosine diphosphate; UDPG, uridine diphosphate glucose; G-1-P, glucose-1-phosphate; UDPGal, uridine diphosphate galactose; PP, inorganic pyrophosphate; UTP, uridine triphosphate.

configuration unchanged. However, in hydrolysis of esters of strong acids, such as *p*-toluenesulfonic acid, it is the alkyl-oxygen bond which breaks. He considered it possible that a phosphate ester was formed at C-4, such an ester could be hydrolyzed in acid or neutral solution with rupture of the carbon-oxygen bond. He also mentioned dehydration-rehydration and oxidation followed by reduction as possible mechanisms for the conversion. The position had to be reassessed when Maxwell (M3) showed that nicotinamide adenine dinucleotide (NAD) [name and abbreviation approved by Commission on Enzymes of the International Union of Biochemistry, 1961, formerly termed diphosphopyridine nucleotide (DPN)], was an essential coenzyme for reaction (3), suggesting that this was an oxidation-reduction reaction. In oxidation the carbon-hydrogen bond would be broken, producing a carbonyl group which could then be reduced back to a carbinol with the opposite configuration from the starting material, resulting in epimerization. In consequence,

the enzyme for reaction (3) is nowadays termed an epimerase rather than a Waldenase. Robichon-Szulmajster (R2) has discussed theoretical aspects of the reaction; he suggests that neither the carbonyl compound nor the reduced nicotinamide adenine dinucleotide are ever free, but that they react, together with the uracil moiety, undergoing both stages of the oxidation-reduction while bound to the apoenzyme.

It is reaction (3), the epimerization of UDPGal, which is the rate-limiting step in the over-all utilization of galactose. Anything increasing the activity of the epimerase increases the metabolism of galactose in the body. An increase in the concentration of nicotinamide adenine dinucleotide phosphate (NADP) [formerly termed triphosphopyridine nucleotide (TPN)] increases the activity of the epimerase, as does the addition of oxidized glutathione or other substances that can generate NADP, but an increase in the concentration of reduced nicotinamide adenine dinucleotide ($NADH_2$) [formerly the reduced form of diphosphopyridine nucleotide (DPNH)] depresses the epimerase activity. Ethanol increases the amount of $NADH_2$ in the tissues, and this probably accounts for the inhibition by ethanol of the metabolism of galactose by liver homogenates (I17, I18). Addition of pyruvate and lactic dehydrogenase, a system which oxidizes $NADH_2$, overcomes the inhibiting effect of ethanol. Ethanol has a profound effect on the galactose tolerance curve in normal subjects (S22); Tygstrup and Winkler (T5) found that the normal liver removed 90% of galactose from blood flowing through it, but only 39% after the subject had taken 20 g of ethanol. This effect on galactose metabolism *in vivo* is probably related to the inhibition by ethanol of the epimerase.

Segal and Blair (S13) have shown that aldehydes reduce NAD to $NADH_2$ in the presence of aldehyde dehydrogenase; progesterone, menthol, and menthone inhibit this enzyme and this may account for the stimulatory action shown by these substances on galactose metabolism *in vitro*.

The key metabolite, UDPG, is largely synthesized by reaction (4), the enzyme being abundant in many tissues. Since reactions (3) and (4) are reversible, the body does not need exogenous galactose to form UDPGal and, hence, the many important compounds made from it.

The galactose-1-phosphate uridyl transferase pathway is by far the most important in galactose metabolism; it accounts for about 5/6 of the total galactose metabolized in mammals. It is less active in the newborn than in the adult, e.g., the liver of newborn rats has only 1/5 the adult amount of galactose-1-phosphate uridyl transferase per milligram of protein (I3).

3.1.2. *Other Pathways*

Isselbacher (I3, I4) has shown that mammalian liver and brain, but not red cells, contain an enzyme, UDPGal pyrophosphorylase, which catalyzes the following reaction:

$$\text{UTP} + \text{Gal-1-P} \rightleftharpoons \text{UDPGal} + \text{PP} \qquad (5)$$

A similar reaction had previously been known to occur in *Saccharomyces fragilis* (K8) and in bean seedlings (N3). This is an alternative route to UDPGal, but the activity of the enzyme in newborn liver is very low; even in the adult it is only one-sixth that of galactose-1-phosphate uridyl transferase (I3), as shown in Table 2.

TABLE 2
SPECIFIC ENZYMATIC ACTIVITY OF RAT LIVER AT DIFFERENT AGES[a]

Age	Gal-1-P-uridyl transferase	UDPGal-pyrophosphorylase
	(μMole of reactants converted/mg protein/min)	
Fetal (18 day)	3.4×10^{-4}	0.45×10^{-4}
Neonatal (1 day)	3.9×10^{-4}	0.8×10^{-4}
Adult (60 day)	19.7×10^{-4}	3.8×10^{-4}

[a] From (I3).

It has recently been shown that erythrocytes from galactosemics accumulate galactose-6-phosphate and galactose-1,6-diphosphate, in addition to much larger amounts of galactose-1-phosphate, when incubated with galactose (I2). It is probable that galactose-1-phosphate is first formed and a part is slowly converted to galactose-6-phosphate by phosphoglucomutase, galactose-1,6-diphosphate being an intermediate (P9). There is evidence that galactose-6-phosphate can be oxidized by the hexose monophosphate shunt (I2). This would provide a means of metabolizing galactose independent of uridine nucleotides, but this pathway can play only a minor part in the over-all metabolism of galactose.

3.2. NATURE OF THE ENZYMATIC DEFECT IN GALACTOSEMIA

Schwarz *et al.* (S10) showed that the erythrocytes of galactosemic subjects accumulated large amounts of galactose-1-phosphate, both *in vivo* and *in vitro*. This indicated that the metabolic block in galactosemia occurred at reaction (2) or reaction (3). Kalckar *et al.* (K7) and Isselbacher *et al.* (I9) later showed that normal erythrocytes contained all 4 enzymes needed for reactions (1), (2), (3), and (4), and that in galactosemia there was virtually complete absence of galactose-1-phos-

TABLE 3

ACTIVITY OF GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE IN HUMAN LIVER AND RED BLOOD CELLS[a]

Subject	Liver homogenate (μmoles 1-C^{14}-Gal-1-P incorporated into nucleotides/g liver/hour)	Erythrocyte hemolyzate (μmoles UDPG utilized/ml red blood cells/hour)	*In vivo* galactose metabolism (g/hour)
Normal adult	> 15.0	1.0–3.0	1.7–2.0
Normal infant	> 25.0	2.0[b]	—
Galactosemic adult[c]	1.2	$0 (< 0.02)$	0.012–0.017
Galactosemic infant	$0 (< 0.3)$	$0 (< 0.02)$[b]	—

[a] From (A1) and (E2).

[b] Cord blood.

[c] All 3 determinations on the same individual who was given 1 g of menthol before determining *in vivo* galactose metabolism (see E3).

phate uridyl transferase, the other enzymes being normal, as shown in Table 3.

This has been confirmed for all cases of galactosemia so far examined. If blood from a galactosemic is mixed with normal blood, the ability of the normal blood to metabolize galactose is not inhibited, showing that in galactosemia there is a true lack of galactose-1-phosphate uridyl transferase and that the inactivity of this enzyme is not due to the presence of some inhibitory substance. If the normal blood is first heated to 70°C, it cannot metabolize galactose either alone or when mixed with galactosemic blood; this shows that the substance missing from galactosemic blood is heat-labile and is presumably the apoenzyme. The enzyme was also found to be absent from biopsy specimens of liver of an adult or an infant with galactosemia, using C^{14}-labeled galactose-1-phosphate and measuring the radioactivity of the uridine nucleotide fraction (A1). While there was no detectable incorporation of C^{14} into uridine nucleotides in the infant, in the adult a small amount was incorporated, about 4 to 5% of the amount incorporated in normal liver. The existence of the UDPGal pyrophosphorylase pathway [reaction (5)] in the adult, but not in the infant, probably accounts for this difference.

TABLE 4

PERCENTAGE CONVERSION OF INTRAVENOUS GALACTOSE-1-C^{14} TO $C^{14}O_2$ PER HOUR[a]

Subject	Age	Without menthol	With menthol[b]
Normal	Adult	5.8	5.8
Galactosemic	8 years	0.33	2.8
Galactosemic	12.5 years	0.0	1.0

[a] Data from (E3).

[b] 13 mg DL-menthol per kg body weight given orally over 36 hours preceding injection of galactose-1-C^{14}.

UDPGal pyrophosphorylase provides an alternative metabolic pathway for galactose even in galactosemia. Galactose-1-phosphate can react with uridine triphosphate, in the presence of this enzyme, producing UDPGal which is then transformed by the epimerase to UDPG (see above, Section 3.1.2). However, the activity of the pyrophosphorylase is very low in early infancy and this may explain why newborn galactosemics can tolerate less dietary galactose than later in life; even in galactosemic adults the activity of the pyrophosphorylase is too low to prevent accumulation of large amounts of galactose-1-phosphate. Anything which removes UDPGal or pyrophosphate will cause reaction (5) to proceed from left to right, increasing the rate of metabolism of

galactose. Increased activity of UDPGal epimerase has this effect. Administration of steroid hormones, or menthol or menthone causes a slight increase in the rate of conversion of C^{14}-labeled galactose to $C^{14}O_2$ or to C^{14}-glucuronic acid in the galactosemic subject, as shown in Table 4 (E3, P8). This effect is not seen in normal individuals. Progesterone has a protective action in young rats on a high-galactose diet, delaying the appearance of cataracts (P8). Segal and co-workers (S13, S14) discuss the mechanism by which progesterone and menthol act in stimulating galactose metabolism in the galactosemic. The question turns on whether, in the galactosemic, the rate-limiting step in the utilization of galactose is the pyrophosphorylase-catalyzed reaction between uridine triphosphate and galactose-1-phosphate or the action of UDPGal-4-epimerase; it is unusual for stimulation of a step other than the rate-limiting one to affect the over-all rate of a reaction. It is probable that further work will elucidate this problem.

3.3 Consequences of the Enzymatic Defect

All the varied biochemical and clinical features of galactosemia are, beyond any reasonable doubt, caused by the absence of this one enzyme, galactose-1-phosphate uridyl transferase. Yet it is not possible, at present, to identify all the steps by which the different effects are brought about.

3.3.1. *Accumulation of Galactose and Galactose-1-phosphate*

The blocking of reaction (2) and the absence of the transferase leads to accumulation of its substrate, galactose-1-phosphate. Schwarz (S5) has shown that untreated galactosemic infants have 300 to 400 μg of galactose-1-phosphate per milliliter of red blood cells. If such cells are incubated in 0.02% galactose, they accumulate galactose-1-phosphate at the rate of about 50 μg/ml/hour (I9, S5); normal red blood cells accumulate between 0.5 and 7 μg/ml/hour (S5). Very high concentrations of galactose-1-phosphate are found in other tissues of galactosemic infants (S5, S6), and an adult with galactosemia who was fed a test dose of C^{14}-labeled galactose retained labeled galactose-1-phosphate in all tissues except muscle and bone (E2). The cataractous lenses of rats fed on a high-galactose diet contain 10 times as much galactose-1-phosphate as normal control lenses (S8).

About 10% of the acid-soluble phosphorus of the red blood cells in galactosemia is accounted for by galactose-1-phosphate. Since all this is derived from adenosine triphosphate by reaction (1), it represents the tying up in a metabolically useless form of a high proportion of the high-energy phosphate of the erythrocyte. Untreated galactosemics have

less adenosine triphosphate and diphosphoglyceric acid, and more inorganic phosphate in their erythrocytes than the normal individual or galactosemic on a galactose-free diet (P5, S5), though this difference is not very marked (S4), and may not be caused only by the tying up of high-energy phosphate in galactose-1-phosphate (K1, P6).

The accumulation of galactose-1-phosphate reaches a limiting value when it is about 60 times the normal. This suggests inhibition of galactokinase by its product (H5, S5). Such inhibition would explain the high concentration of free galactose in the blood and urine, and the excretion as free galactose of the bulk of a test dose of galactose fed to an untreated galactosemic, but more experimental evidence is needed on the mechanism and kinetics of the reaction. Two other enzymes catalyzing the conversion of hexoses to their monophosphoric esters are inhibited by products of the respective reactions, hexokinase by glucose-6-phosphate (C7), and fructokinase by fructose-1-phosphate (W5); this lends support to the hypothesis that galactokinase is inhibited by high concentrations of galactose-1-phosphate, leading directly to galactosemia and galactosuria.

3.3.2. *Inhibition of Other Enzymes*

Schwarz *et al.* (S10) found that the oxygen uptake of red blood cells of galactosemics was inhibited by galactose, even in the presence of excess glucose, but that of normal red cells was stimulated. They suggested specific inhibition of phosphoglucomutase by galactose-1-phosphate. This was further elaborated by Sidbury (S15), and Ginsburg and Neufeld (G5).

Phosphoglucomutase is present in all tissues, both in the normal and the galactosemic. It catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate, a reaction essential for the metabolism of all glucose-requiring tissues. Glucose-1-phosphate is the primary product of the breakdown of glycogen, and only after conversion to glucose-6-phosphate can it react further, by the Embden-Meyerhof pathway, to give pyruvate and hence carbon dioxide and water. Phosphoglucomutase has one atom of phosphorus per molecule of enzyme; in the first stage of the reaction with glucose-1-phosphate, this phosphate group is transferred to give glucose-1,6-diphosphate and dephosphoenzyme. In the second step the dephosphoenzyme reacts with glucose-1,6-diphosphate to give regenerated enzyme and glucose-6-phosphate (A3, N2, S18). Posternak and Rosselet (P9) showed that this enzyme would also react with galactose-1-phosphate, converting it to galactose-6-phosphate, and Sidbury (S17) showed that this reaction also went through the hexose

diphosphate. Thus phosphoglucomutase catalyzes all 4 reactions, A, B, C and D:

$$\text{G-1-P} \overset{A}{\rightleftharpoons} \text{G-1,6-diP} \overset{B}{\rightleftharpoons} \text{G-6-P}$$

$$\text{Gal-1-P} \overset{C}{\rightleftharpoons} \text{Gal-1,6-diP} \overset{D}{\rightleftharpoons} \text{Gal-6-P}$$

The over-all equilibrium gives 5% hexose-1-phosphate and 95% hexose-6-phosphate. The rate-limiting step is probably the reaction of hexose diphosphate with dephosphoenzyme. The rate of conversion of galactose-1-phosphate to galactose-6-phosphate is only 1/400 of the rate for glucose-1-phosphate, and this large discrepancy would be expected to lead to competitive inhibition. In fact, competitive inhibition by galactose-1-phosphate of the reaction of phosphoglucomutase with glucose-1-phosphate has been demonstrated (G5, S16). Galactose-6-phosphate is also a competitive inhibitor but is only 1/20 as active as galactose-1-phosphate. The mechanism of inhibition is that galactose-1-phosphate reacts with the enzyme, producing dephosphoenzyme and galactose-1,6-diphosphate, stage C. These products react only very slowly by stage D and the dephosphoenzyme therefore accumulates; it cannot react with glucose-1-phosphate until the enzyme has been rephosphorylated, which can be done efficiently only by glucose-1,6-diphosphate or mannose-1,6-diphosphate. It follows that, if the concentration of galactose-1-phosphate in the cell is comparable to that of phosphoglucomutase, the main route for the utilization of glucose-1-phosphate is blocked. The dephosphoenzyme reacts with any glucose-1,6-diphosphate present, thus regenerating the enzyme, but glucose-1,6-diphosphate is soon depleted. Although glucose-1,6-diphosphate can be made from glucose-1-phosphate by 2 other reactions, these are relatively slow and may themselves be partly inhibited by galactose-1-phosphate (N1, S19).

Free galactose has no inhibitory effect on the *in vitro* activity of purified phosphoglucomutase, supporting the suggestion that it is galactose-1-phosphate which is damaging. The best evidence, however, comes from the study of *Escherichia coli* (K2). Kurahashi (K17, K18) has shown that, in the wild type of this organism, galactose is metabolized by the same route as in mammals, similar enzymes being present. There exist mutant strains lacking galactose-1-phosphate uridyl transferase, and other mutants lacking galactokinase. Neither group can metabolize galactose. In the former, which resemble human galactosemics in their missing enzyme, if galactose is added to the medium, galactose-1-phosphate accumulates and there is a significant retardation of growth. In the latter group, galactose has no harmful effect and galactose-1-phosphate does

not accumulate. However, glucose-1,6-diphosphate does not correct the growth inhibition caused by galactose-1-phosphate (K1). Krooth and Weinberg (K16) made similar observations with cells derived from the skin of galactosemic individuals and grown in tissue culture; unlike normal cells, these cells could not oxidize galactose-1-C^{14} to $C^{14}O_2$, and addition of galactose to the medium inhibited growth of the galactosemic cells but not of the normal cells. Related to this is the immunity toward galactose shown by normal infants. The concentration of galactose in the blood of normal infants rises, after a feed, to about 40 mg/100 ml, yet they show no ill effects. Presumably, the concentration of galactose-1-phosphate in the tissues stays low enough to prevent damage, and the high concentration of galactose in the blood reflects the delay in crossing cell membranes and being converted to galactose-1-phosphate.

Glucose-6-phosphatase is another enzyme competitively inhibited by galactose-1-phosphate (I5). The inhibition of hepatic glucose-6-phosphatase is probably important in relation to hypoglycemia.

An important metabolic path of glucose in the lens is via glucose-6-phosphate to 6-phosphogluconic acid, catalyzed by glucose-6-phosphate dehydrogenase. The activity of this enzyme is low in cataractous lenses from galactose-fed rats (L4). Lerman (L4) has claimed that in normal lenses *in vitro*, a significant reduction in the activity of this enzyme is caused by adding galactose-1-phosphate. However, Weinberg and Segal (W2) could find no inhibition by galactose of glucose-6-phosphate dehydrogenase in leucocytes from a galactosemic individual, even though galactose-1-phosphate accumulated. If erythrocytic glucose-6-phosphate dehydrogenase is inhibited by galactose-1-phosphate, one would expect to see Heinz body formation on incubation of the blood from a galactosemic with galactose, glucose, and acetylphenylhydrazine, by analogy with cases of congenital glucose-6-phosphate dehydrogenase deficiency (B11).

3.3.3 *Correlation with Clinical Effects*

It seems established that galactose-1-phosphate is an enzyme poison, blocking the cell's most important source of energy. The failure to thrive characteristic of galactosemia is thus explained; the less severely affected infants were possibly exposed to lower concentrations of galactose-1-phosphate, either because of their diet or because of some peculiarity of constitution.

The renal tubular dysfunction of galactosemia is very similar to that caused by, e.g., heavy metal poisoning; in both cases it seems likely that the inhibition of enzyme systems prevents the cells of the renal tubule

from carrying out such energy-requiring operations as the reabsorption of amino acids from the glomerular filtrate (W9).

A number of enzymes in the liver are known to be present in low concentration in the newborn period, when the liver is most sensitive to the action of galactose or, more probably, galactose-1-phosphate. Attempts have been made to incriminate some specific reactions in the hepatotoxicity of galactose. Thus bilirubin is normally conjugated in the liver with glucuronic acid. Bilirubin is thereby converted from the "indirect" to the "direct" reacting form (B14); the presence of "indirect" bilirubin in the blood indicates a failure of the conjugating system in the liver. The glucuronic acid moiety of the bilirubin conjugate is derived from uridine diphosphate glucuronic acid, the transfer being catalyzed by an enzyme in the microsomes (B13, D11). The uridine diphosphate glucuronic acid is itself derived by oxidation of UDPG (S23). There is no clear evidence on how the bilirubin conjugating mechanism is interfered with in galactosemia; Sidbury (S17), and Najjar and McCoy (N1) have suggested that (1) the synthesis of glucose-1-phosphate is inhibited by the galactose-1-phosphate which accumulates in galactosemia (see above); (2) this lack of glucose-1-phosphate leads to a lack of UDPG, which, in turn, leads to (3) a lack of uridine disphosphate glucuronic acid to conjugate with bilirubin, which therefore circulates causing jaundice. However, this theory ignores the fact that in many, perhaps the majority, of jaundiced galactosemic infants, the circulating bilirubin is conjugated. So many aspects of liver function are disturbed in galactosemia that it seems likely that a general cytotoxic effect, such as the action of galactose-1-phosphate on phosphoglucomutase, reduces the energy supply to the cell and that therefore many of its functions fail. More experimental evidence is necessary to help explain the special sensitivity of the liver in the first few days of life.

UDPG is the starting point for many of the body's syntheses, e.g., of cerebrosides (via UDPGal), and of mucopolysaccharides (via uridine diphosphate mannose and uridine diphosphate *N*-acetylgalactosamine). It has therefore been suggested that interference with this key compound by galactose-1-phosphate causes the mental deficiency by interfering with myelin formation, and cataracts by preventing the synthesis of some of the substances in the lens (N1, S17). However, there is as yet no evidence of deficient myelination in most cases of galactosemia and the defects in the lens occur at the intracellular level during differentiation. It seems more probable that both the mental effects and the effects on the lens are due to an interference with the normal utilization of glucose.

The hypoglycemia of galactosemia may be due partly to the action of

galactose causing insulin release (P10) and partly to the effect of galactose-1-phosphate on phosphoglucomutase and glucose-6-phosphatase in the liver. The concentration of glucose in the blood is maintained by breakdown of glycogen in the liver to glucose-1-phosphate which is converted by phosphoglucomutase to glucose-6-phosphate and this is hydrolyzed by glucose-6-phosphatase to glucose. Since galactose-1-phosphate inhibits both these enzymes, it is not surprising that hypoglucosemia results when a galactosemic is given galactose. Some galactosemics do not develop hypoglucosemia when given galactose (S17); this finding cannot be satisfactorily explained at present. Mason and Turner (M2) and Kalckar (K1, K5) found that galactosemics responded to epinephrine with a normal rise in blood glucose concentration, suggesting that the phosphoglucomutase in the liver was normally active; further experimental evidence is required on this point, particularly on the effect of age.

Fructose intolerance is a condition showing a number of close similarities to galactosemia (F3, W5). It has been shown that, in fructose intolerance, fructose-1-phosphate accumulates and inhibits a number of enzymes. Marked hypoglucosemia follows ingestion of fructose, but insulin is almost certainly not involved (F1, P10).

4. Laboratory Diagnostic Procedures

The diagnosis of galactosemia cannot be made without aid from the laboratory. When an infant is seriously underweight, vomiting, jaundiced or with an enlarged liver, examination of the urine for reducing substances is both the simplest and the most valuable single diagnostic test that can be carried out. [It must be stressed that a reductometric test should be used and not a test specific for glucose such as "Clinistix" (Ames Co.) or "Testape" (Eli Lilly)]. However, a negative result for reducing substances does not exclude galactosemia, and a positive result is not conclusive evidence for the diagnosis, hence further laboratory work is necessary. In addition to diagnosis of the disease, some of these tests are valuable in the control of treatment and the detection of heterozygous carriers of galactosemia.

Laboratory procedures specific to galactosemia are dealt with below, but a number of other tests are also of value. Liver function tests, though sometimes helpful, are not always informative (see above, Section 2.2). Protein is usually present in the urine in untreated cases, but this occurs in other diseases. Aminoaciduria is a very common finding in galactosemia, though Holzel (H3) states it is absent in some older children with a mild form of galactosemia; aminoaciduria occurs in other diseases and is frequently accompanied by proteinuria and glucosuria.

4.1. Galactose Content of Blood and Urine

The presence of significant amounts of reducing sugar in the urine of a young infant should always make one suspect galactosemia. The tests used may be heating the urine with Benedict's solution or treating it with "Clinitest" (Ames Co.), the tabletted form of Benedict's solution. Galactosemia is far commoner than diabetes mellitus in this age group, and tests specific for glucose, such as "Clinistix" and "Testape," are correspondingly less suitable than reductometric tests. Some galactosemics excrete a moderate amount of glucose as well as galactose (D2, P5, V1), hence identification of glucose in urine does not exclude galactosemia. The method of identifying urinary sugar must be quantitative or semiquantitative as well as qualitative.

4.1.1. *Paper Chromatography*

Paper chromatography is the simplest and probably the best laboratory tool for identifying and roughly estimating galactose in urine and blood. It must be realized, however, that in paper chromatography one is measuring a single physical property—the R_f value. It is wise to confirm the identity of galactose by some independent method such as osazone formation or resistance to fermentation by bakers' yeast. The amount of galactose present in the fluid actually applied to the paper should lie between 5 and 50 μg. In the case of urine a preliminary Benedict's test is advisable to ensure that the amount applied probably lies within these limits. Urine from a galactosemic individual on a diet containing milk usually is very heavily loaded with galactose, so that very little urine need be applied to the chromatogram and the salts present are unlikely to interfere. If the galactose content is lower, correspondingly more urine must be used, and the salts present may affect the running, distorting the spots, and altering their R_f values. Urine can be desalted by treatment with ion-exchange resins (W7); a mixture of a strong acid resin, such as Dowex-50 (Dow Co.), Zeo-Karb 225 (Permutit Ltd.), or Amberlite IR-120 (Rohm & Haas), in the hydrogen form, and of a weak base resin such as De-Acidite-G (Permutit Ltd.) or Amberlite IR-45 (Rohm & Haas) in the hydroxyl or the bicarbonate form, is used. The resins should be in the form of standard beads, about 14 to 52 mesh, regenerated and washed, mixed in equal volumes, and saturated with carbon dioxide. Suitable ready mixed (monobed) resins can also be used, e.g., Amberlite MB-4, but strong base resins and mixtures containing them should be avoided or, if none other is available, converted to the bicarbonate form. The mixed resins are shaken with the urine, using about 1 ml of the mixed resins for 5 ml of urine and shaking for 3 min-

utes; carbon dioxide is usually given off. The mixture is allowed to settle and is centrifuged. All salts, bases, and acids are removed by the mixed resins; proteins, if present in small or moderate amounts, are often rendered insoluble and can be removed by centrifugation.

The supernatant is applied to the starting line on the paper chromatogram in 5 or 10 µl portions, drying after each application and repeatedly applying the treated urine to the same spot till the required volume has been applied. Standard solutions of galactose (10 µg), glucose (10 µg), and lactose (30 µg) are applied alongside the urine to give reference spots. For critical identification, the same volume of urine is applied to 3 separate but neighboring points on the starting line; to one of these is added galactose and to a second glucose, these being the sugars hardest to distinguish. One-dimensional chromatography is adequate since the number of different sugars present in a specimen of urine is small (W6), but glucose and galactose have the same R_f values in a number of different solvents and it is necessary to use a solvent containing pyridine. A mixture of ethyl acetate (12 volumes), pyridine (5 volumes), and water (4 volumes) (J1, S20) gives a very good separation of glucose and galactose from each other and from all other sugars. Butanol (6 volumes), pyridine (2 volumes), and water (3 volumes) has also been recommended (L7). Either ascending or descending chromatography can be used; the ascending technique of Williams and Kirby (W3) is particularly convenient and, with a fast paper such as Whatman 54 and the ethyl acetate-pyridine-water solvent, gives good separations in a few hours, sometimes a point of considerable importance.

After running, the chromatogram is dried without heat by hanging in the fume cupboard. The paper is sprayed with aniline phthalate reagent freshly prepared as follows: 0.55 g phthalic acid is dissolved in 20 ml glacial acetic acid with the aid of heat; the hot solution is rapidly cooled to supersaturate it; and 0.3 ml pure aniline is added and mixed. After spraying, the chromatogram is dried and then heated to 98–100°C in a moist atmosphere for 5 minutes. Glucose, galactose, and lactose give yellow-brown spots on an almost white background, the pentoses and some glucuronides give reddish-brown spots, other sugars give spots of various shades of brown. In ultraviolet light (3650 Å) the spots due to glucose, galactose, and lactose fluoresce, extending the lower limit of detectability to well below 1 µg (W6); if there is much sugar present the spot appears dark with a fluorescent border. Many other spray reagents have been described.

Identification of galactose is primarily by position and color. For semiquantitative estimation of the amount present the spot can be compared

visually with reference spots of known amounts of galactose run alongside the urine on the same chromatogram. It may be necessary to repeat the chromatography, using different amounts of urine, to get an accurate estimate. Because the intensity of the spot is sensitive to development conditions and the nature of the sugar, the galactose spot in the urine should be compared only with galactose standards run and developed simultaneously with the urine.

Blood should be deproteinized by some technique which leaves no extra salt, acid, or alkali in the supernatant. Some suitable techniques are with tungstic acid, with ethanol (B10), or with zinc sulphate and barium hydroxide (S21). The supernatant is desalted in the same way as urine and, if necessary, concentrated before applying to the paper. Subsequent technique is as for urine.

If much more glucose than galactose is present, the spots tend to overlap, making it difficult to detect galactose. Glucose can be removed with bakers' yeast or glucose oxidase before deproteinizing or desalting the blood or urine.

The mere presence of galactose in urine or blood is not necessarily diagnostic of galactosemia. The technique is so sensitive that the galactose excreted in the urine by a normal infant is easily detected. A normal milk-fed infant excretes up to 40 mg galactose per 100 ml urine, occasionally more (W10). A galactosemic infant fed an appreciable amount of milk usually excretes more than this amount of galactose, often much more, up to a gram or more of galactose per 100 ml. There is some overlap between the upper limit for normal and the lower limit for galactosemic infants, particularly if the galactosemic has not taken milk for some time, e.g., if the urine is an early morning specimen. However, the sensitivity of paper chromatography is not a drawback if its quantitative as well as its qualitative aspects are made use of. Paper chromatography is probably the best way of investigating any urine that reduces Benedict's reagent; its use has enabled the diagnosis of galactosemia to be made or excluded in a very considerable number of cases.

4.1.2. *Other Methods of Identifying Galactose*

The mucic acid test is now only of historical interest. It depends on the oxidation of galactose or saccharides containing a galactose residue, such as lactose, with nitric acid to yield mucic acid. Mucic acid is highly insoluble in water, while the isomeric dicarboxylic acids yielded by other sugars are soluble. Mucic acid may be identified by its characteristic thallium salt.

The osazone method is still of use as a confirmatory test. The urine

is warmed with phenylhydrazine, acetic acid, and sodium acetate. The crystals formed are examined under a low-power microscope, each sugar giving a characteristic crystal shape which is compared with the published pictures of osazone crystals, or with standards prepared from pure specimens of the different sugars. The preparation of pure osazones with sharp melting points and correct optical rotation is difficult in small quantities. Levene and LaForge (L6) recommend for the differentiation of glucosazone and galactosazone the observation of mutarotation of a freshly prepared solution which runs for glucosazone to more dextrorotatory values and for galactosazone to less dextrorotatory values. The osazone test is useful only for identifying *which* sugar is present, the failure to form crystals is not by itself adequate evidence of the absence of sugar.

Galactose is not fermentable by bakers' yeast; if urine retains its power to reduce Benedict's solution after incubation with bakers' yeast, any sugar present is probably galactose, lactose, or a pentose. The production of carbon dioxide is evidence that glucose, fructose, or sucrose is present, but does not exclude the simultaneous presence of galactose, lactose, or pentose. Certain precautions are necessary: the yeast must be a pure strain of bakers' yeast unable to ferment galactose; it must be thoroughly washed by decantation before use; the same urine and same yeast must be simultaneously incubated with the addition of glucose and of galactose separately to show that glucose is, and galactose is not, fermented under the conditions used; fermentation must be allowed to proceed to completion. Fermentation can be very suitably combined with paper chromatography.

The determination of galactose in blood is usually made reductometrically after removal of glucose by incubating with bakers' yeast or glucose oxidase. Bakers' yeast has the theoretical advantage that fructose and mannose are removed at the same time as glucose, but in practice the 2 methods give very similar results. The same precautions are necessary in using bakers' yeast with blood as with urine, and the anticoagulant used must be free from fluoride, the blood being refrigerated. By determining blood sugar before and after fermentation, the glucose concentration in the blood is obtained. It is important to use a "true sugar" method of determination, both for fermented and unfermented blood; the essence of a "true sugar" method is that nonsugar reducing substances are removed, either by precipitation with zinc sulfate and barium hydroxide (S21) or by treatment of a tungstic acid filtrate with mixed acidic and basic ion-exchange resins (C3, W7) before heating with the alkaline copper or ferricyanide solution, or using *o*-aminodiphenyl (T1).

"True sugar" methods are distinct from "true glucose" methods such as the use of glucose oxidase, peroxidase, and *o*-dianisidine (H15). Some other "true sugar" methods are less suitable than the two mentioned above, being based on a balancing of errors that works well enough for the determination of glucose in normal or diabetic blood, but is less applicable to the determination of galactose. Nonsugar reducing substances would be estimated as "galactose," hence the importance of removing them.

The discovery by Cooper *et al.* (C4) of a galactose oxidase extractable from *Polyporus circinatus*, Fr., leads to the hope that a specific enzymatic method of estimating galactose in biological fluids may be evolved. The enzyme uses atmospheric oxygen to oxidize galactose to γ-galactonolactone, hydrogen peroxide being formed simultaneously. By analogy with glucose oxidase, it may prove possible to estimate the hydrogen peroxide with peroxidase and *o*-dianisidine, thus getting an estimate of the amount of galactose present, and to get urine dip-sticks specific for galactose. Sugars other than galactose are acted on to a negligible extent or not at all.

4.2. Galactose Tolerance Tests and Their Dangers

In a condition of which the chief characteristic is the inability to utilize galactose, the galactose tolerance test seems, at first sight, an obviously useful diagnostic measure. The occurrence of a number of unfortunate incidents has revealed the danger of giving very sick infants large amounts of a substance that can be converted to a powerful enzyme poison. Even in older children and adults, a test dose of galactose can induce a dangerous attack of hypoglycemia. Holzel (H3) records the case of a boy aged 5 years who was known to have galactosemia but was apparently well, having been on a low-galactose diet since the age of 3½. When this boy was given a test dose of galactose he became drowsy and semicomatose, and developed marked fetor hepaticus; methyl mercaptan and dimethyl sulfide were found in his exhaled breath. He recovered consciousness after being given glucose intravenously, but the fetor hepaticus remained noticeable for several months. Hudson *et al.* (H14) record a case of a galactosemic baby, satisfactorily maintained on a galactose-free diet, who was ill for 2 weeks after a galactose tolerance test.

If the galactose tolerance test is used, it must obviously be applied with discretion. Glucose for intravenous administration and adrenaline for subcutaneous injection should be on hand. It is probably wiser not to carry out this test on young infants who may have galactosemia.

Stenstam (S22) has described several modifications of the galactose tolerance test. The form of test originated by Bauer (B4), in which the urine is tested for galactose, has been replaced by the method of Maclagan (M1) in which the concentration of galactose in the blood is determined at half-hourly intervals and a blood galactose curve constructed, or a "galactose index" calculated. It is usual to give an adult 40 g galactose by mouth, with proportionately less for children. The concentration of galactose in the blood is determined by one of the methods described in Section 4.1.2 and glucose should always be determined in the same blood specimens. The blood galactose curve, in cases of galactosemia, rises to a greater peak value, and remains elevated for longer, than in any other disease. Peak values of 200–300 mg galactose per 100 ml blood, sinking to 100–150 mg/100 ml after 5 hours, are common, but these figures depend, to some extent, on the dietary history immediately previous to the tolerance test. If the patient has been on a galactose-free diet for some time, the peak blood concentrations of galactose are lower (D9), but the curve remains grossly abnormal. The concentration of glucose in the blood often sinks very considerably when a galactosemic is given a galactose tolerance test but in some cases it remains constant (S17).

Galactose tolerance tests have also been used in attempts to detect heterozygous carriers for galactosemia (D5, H6); this is dealt with in Section 5.3.1. Some heterozygotes are made ill by the galactose load (D3, H3, H16). Galactose tolerance tests have now been replaced, for this purpose, by the more specific and sensitive method of assay of enzyme activity.

In the diagnosis of galactosemia itself, the galactose tolerance test, with its dangers and somewhat ambiguous answers, has been largely replaced by assays more closely related to the primary metabolic error, such as the determination of galactose-1-phosphate in the blood or the direct assay of enzymatic activity. Diagnosis of galactosemia and detection of the heterozygotes are only two of the problems presented by galactosemia, and the galactose tolerance test with suitable technical improvements may yet give very useful information in the study of this condition.

4.3. The Determination of Galactose-1-phosphate

It was demonstrated by Schwarz *et al.* (S10) that red blood cells of a galactosemic individual, on incubation with galactose either *in vivo* or *in vitro*, accumulate galactose-1-phosphate. This has been made the basis of a diagnostic test for galactosemia (S6, S9). Red blood cells are

incubated with galactose for 2 hours, then the galactose-1-phosphate estimated. The test can be carried out on blood from the umbilical cord or on venous blood obtained later, but 2 ml is required.

Galactose-1-phosphate can be identified and semiquantitatively determined by paper chromatography of suitably treated extracts of blood (H1). The technique is difficult, requiring prewashing of the chromatography paper with chelating agents, and the results tend to be unsatisfactory, the sugar phosphates sometimes giving elongated spots of indeterminate R_f.

A better method has been described by Schwarz (S6). Washed red blood cells are lysed, precipitated with trichloroacetic acid below 0°C and the supernatant quickly neutralized. Speed and low temperatures are necessary to prevent hydrolysis of galactose-1-phosphate which is very sensitive to acid. Barium acetate and ethanol are added, and the precipitated barium salt of galactose-1-phosphate washed with 80% ethanol. The barium salt is then hydrolyzed by heating with dilute hydrochloric acid, acid and salts removed with mixed ion-exchange resins, and the galactose estimated by paper chromatography as described above. It is probably better to avoid the Amberlite MB-1 resin used by Schwarz and, instead, to use a weak base resin mixture, such as Amberlite MB-4. Recovery of added galactose-1-phosphate should be determined simultaneously.

An enzymatic method (K11) of estimating galactose-1-phosphate in red blood cells is simpler and less time-consuming than the paper chromatographic method, though it requires more expensive reagents and apparatus. This method closely resembles methods of assaying enzyme activity and it is, therefore, described with them in Section 4.4.1.

4.4. Assays of Enzyme Activity

The enzyme galactose-1-phosphate uridyl transferase is, in normal individuals, distributed throughout most or all of the tissues, though in widely varying concentrations. All the tissues of galactosemic individuals, who have a genetically caused inability to synthesize it, are devoid of this enzyme. The red cells of normal blood contain considerable amounts of galactose-1-phosphate uridyl transferase and are by far the most convenient "tissue" to examine. Measurement of the activity of this enzyme in hemolyzates of red blood cells is the most direct way of establishing or excluding a diagnosis of galactosemia. A number of different assay procedures have been devised, and are described below, and listed in Table 5. The hemoglobin content of the hemolyzate is sometimes measured and results expressed as units of enzyme activity per gram of

TABLE 5

METHODS OF ASSAYING ACTIVITY OF GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE IN ERYTHROCYTES

Purpose	Method	Special features	Apparatus required	References
1. Diagnosis	O_2 consumption	Very simple and economical; not quantitative	Glass capillary tubing	(S7)
2. Diagnosis	UDPG consumption	Specific and reliable for diagnostic purposes	UV Spectrophotometer	(A2)
3. Diagnosis	UDPGal formation	Modification of 2., more specific; requires UDPGal-4-epimerase	UV Spectrophotometer	(M4)
4. Diagnosis	UDPG consumption	Modification of 2., giving visible color change; can be applied to technics 3., 5. and 9.	Absorptiometer (400 mμ)	(N4)
5. Detection of heterozygote	UDPG consumption	Measures rate of reaction; can combine with estimation of UDPGal formation; good discrimination heterozygotes from normals	UV Spectrophotometer	(B17)
6. Detection of heterozygote	O_2 consumption	Distinguishes galactosemics, heterozygotes and normals with minimum overlap	Warburg apparatus	(K10)
7. Detection of heterozygote	O_2 consumption	Modification of 6.; similar results	Warburg apparatus	(H12)
8. Determination of enzyme	O_2 consumption	Measures true enzyme activity; good discrimination heterozygotes, normals and galactosemics	Warburg apparatus	(S12)
9. Monitoring of treatment	UDPG consumption (no Gal-1-P added)	Resembles 2.; estimates Gal-1-P present in erythrocytes	UV Spectrophotometer	(K11)

hemoglobin; in other cases the activity is given per milliliter of hemolyzate (which may or may not be diluted) or per milliliter of blood. The units may be micromoles of UDPG consumed, or microliters of oxygen taken up, or a percentage of some other enzymatic activity. This multiplicity of modes of expressing results makes it difficult to compare different methods. Use of the specific activity as defined by the Commission on Enzymes of the International Union of Biochemistry, micromoles of galactose-1-phosphate transformed per minute per milligram of protein, would give a standard in which rate of formation of active enzyme was directly related to the rate of formation of total red cell proteins. Some of these tests have also been applied to the detection of heterozygous carriers for galactosemia; the results are discussed in Section 5.

Improvements in these methods are likely to take the form of improved specificity for galactose-1-phosphate uridyl transferase and improvements in preparing the red cell hemolyzates. Sensitivity and precision of the assays are now good enough to reveal a puzzling feature; some galactosemics appear to have a very small amount of enzymatic activity in their red cells. This is discussed further in Section 5.

4.4.1. *UDPG Consumption Tests*

These tests depend on the reaction of galactose-1-phosphate with UDPG, which is catalyzed by galactose-1-phosphate uridyl transferase. After the reaction has proceeded for a fixed time, it is stopped and the residual UDPG estimated by reduction of NAD, or of thionicotinamide adenine dinucleotide (TNDPN), catalyzed by UDPG dehydrogenase. The success of all the UDPG consumption methods depends on the fact that the UDPGal-4-epimerase of the red cells is rapidly inactivated in the hemolyzates by destruction of its coenzyme, NAD. Five-minute incubation of the hemolyzate at 37°C suffices to inactivate UDPGal-4-epimerase completely.

Anderson *et al.* (A2) were the first to describe a UDPG consumption assay for galactose-1-phosphate uridyl transferase. The washed red cells from 0.25 ml blood are hemolyzed and mixed with galactose-1-phosphate and UDPG in tris buffer, pH 8.1, made up to 0.6 ml. The mixture is incubated for 30 minutes at 37°C. A control, with galactose-1-phosphate omitted, is run simultaneously. A second control has inorganic pyrophosphate substituted for galactose-1-phosphate; this serves to show that the enzymatic activity of pyrophosphorylase is intact, and hence that the hemolyzate has not been maltreated. The reaction is stopped by

adding 1 ml water and heating to 100°C for 2 minutes with stirring. The tube is centrifuged and 0.3 ml of the supernatant incubated for 30 minutes with NAD and UDPG dehydrogenase, in glycine buffer (pH 8.7) and cysteine, made up to a final volume of 1 ml. The optical density is read at 340 mμ against the similarly treated control.

The method gives a very sharp distinction between galactosemics and normals; there is no overlap between galactosemics (homozygotes for galactosemia) and normals (homozygotes for the normal gene), and hardly any overlap between galactosemics and heterozygous carriers for galactosemia. There is, however, a very considerable overlap between the heterozygous carriers and normals. Anderson *et al.* (A2) could detect no difference between heterozygous carriers and controls, but Hsia and his co-workers (H11, H12) found a statistically highly significant difference between the results for a group of heterozygous carriers and those for a group of normals, though the status of individuals could not be determined. Kalckar (K6), and Kirkman (K9) point out that the special features of this method that made it so suitable for diagnosing galactosemia made it less satisfactory for detecting the heterozygote and quite unsuitable for determining the amount of enzymatic activity actually present. For these one would need a method giving zero-order kinetics and measurement of the initial reaction velocity.

A modification of this test (M4) enables the UDPGal formed, as well as the UDPG consumed, to be measured, thereby increasing the specificity. The reaction is carried out as usual (A2) and the residual UDPG estimated with NAD and UDPG dehydrogenase as usual. At the end of this second reaction, when the optical density has reached a steady level, UDPGal-4-epimerase is added to the cuvette. The UDPGal formed in the first reaction is converted to UDPG which reacts further with NAD and so can be estimated.

In an attempt to improve the detection of heterozygous carriers, Bretthauer *et al.* (B17) introduced the modified UDPG consumption test. Higher concentrations of UDPG and galactose-1-phosphate, and an incubation time of only 10 minutes were used. In effect, this modification measured the rate of the reaction catalyzed by Gal-1-P uridyl transferase over the linear part of the curve. In this way the small differences between normals and heterozygous carriers were exaggerated, but at the cost of narrowing the gap between galactosemics and heterozygotes so that there was now a certain amount of overlap (H12), though Donnell *et al.* (D7) found no overlap in 14 families. The sensitivity of the assay of residual UDPG was increased by reducing the concentrations of some reactants, and reading the optical density at 340 mμ at intervals over

90 minutes, extrapolating back to zero time. UDPGal formed was also estimated by Donnell *et al.* (D7) by the method of Maxwell *et al.* (M4).

Walker *et al.* (W1) used a UDPG consumption test combining the features described by Bretthauer *et al.* (B17) and by Anderson *et al.* (A2). Overlap between the values for heterozygotes and for normals found by Walker *et al.* was intermediate between the amount of overlap found by the method of Bretthauer *et al.* and by that of Anderson *et al.*

In a further modification, Nordin and Bretthauer (N4) assay the residual UDPG by incubation with TNDPN. The final optical density is read at 400 mμ, making an ultraviolet spectrophotometer unnecessary.

Kirkman and Maxwell (K11) have described a modification of the UDPG consumption test for estimating galactose-1-phosphate in the erythrocytes. It is of use in monitoring the dietary treatment of galactosemics. Washed red blood cells of the patient and of a normal donor are hemolyzed by repeatedly freezing and thawing, the hemolyzates incubated separately at 37°C for a few minutes to inactivate UDPGal-4-epimerase, then the hemolyzates are mixed and incubated with UDPG in tris buffer as usual, but without the addition of galactose-1-phosphate. The normal hemolyzate is also incubated alone with UDPG and buffer to provide a blank. The normal hemolyzate provides galactose-1-phosphate uridyl transferase. After all the galactose-1-phosphate has reacted, the residual UDPG is estimated as usual. Erythrocytes from a normal adult or child contain 0.0 ± 0.8 mg galactose-1-phosphate per 100 ml, normal cord blood contains 1.7 ± 0.8 mg per 100 ml; it is provisionally considered that dietetic control is satisfactory if the erythrocytes contain not more than 2.8 mg galactose-1-phosphate per 100 ml.

4.4.2. *Gasometric Assays*

Kirkman and Bynum (K10) developed an assay of galactose-1-phosphate uridyl transferase based on oxygen uptake when hemolyzates were incubated with galactose-1-phosphate. The washed red blood cells were hemolyzed and 0.3 ml incubated in Warburg flasks with UDPG, glucose-1,6-diphosphate, phosphoglucomutase, nicotinamide, NADP, cysteine, and methylene blue. The gas phase was air and any carbon dioxide evolved was absorbed in potassium hydroxide in the center well. After equilibration, galactose-1-phosphate was tipped in from the side arm and, later, glucose-1-phosphate similarly added to a control flask. The rate of uptake of oxygen in microliters per hour was measured and allowed the enzymatic activity of the hemolyzate to be calculated.

This test enabled a sharp distinction to be made between galacto-

semics, heterozygous carriers, and normals (H12, K9, K10). There was hardly any overlap between heterozygotes and normals, and none between galactosemics and heterozygotes.

Hsia *et al.* (H12) modified the original manometric method by omitting glucose-1,6-diphosphate and phosphoglucomutase, and using more UDPG. They obtained almost exactly the same results as by the original technique. However, the correlation between galactose-1-phosphate determined by a manometric method and by a UDPG consumption test was only fair (correlation coefficient 0.8). These workers and also Schwarz *et al.* (S12) noted a lag period before oxygen was taken up by the incubation mixture with galactose-1-phosphate but not by that with glucose-1-phosphate, and Hsia *et al.* (H12) noted a similar lag period with Bretthauer's modification (B17) of the UDPG consumption test. The lag period could be abolished by adding glucose-6-phosphate dehydrogenase (H12). In a more recent paper, Hsia and Walker (H10) recorded an unexplained discrepancy, in some galactosemic individuals, between the results of manometric assays and of UDPG consumption tests for galactose-1-phosphate uridyl transferase.

Schwarz *et al.* (S12) have described a different manometric procedure for determining galactose-1-phosphate uridyl transferase activity. Concentrations of reagents were about 4 times as high as those used by Kirkman and Bynum (K10), and the oxygen consumption of a given amount of erythrocytes about twice as high. Schwarz *et al.* explored the various factors affecting oxygen uptake by hemolyzates metabolizing galactose-1-phosphate. The conditions adopted gave maximum oxygen uptake, equal to that on glucose-1-phosphate for normals. Kalckar (K5, K6) has briefly outlined a somewhat similar method of determining the activity of the transferase under conditions of zero-order kinetics during the first 5 or 10 minutes of reaction. The method of Schwarz *et al.* probably measures a quantity more nearly related to the amount of enzyme present than does that of Kirkman and Bynum, but the latter method allows one to discriminate slightly better between heterozygotes and normal homozygotes.

Schwarz (S7) has devised a greatly simplified form of the gasometric assay with extremely simple home-blown glassware and relatively inexpensive reagents in place of a Warburg apparatus. The red cells of 0.2 ml blood are thoroughly washed and hemolyzed. Three portions of the hemolyzate are incubated with nicotinamide, methylene blue, NADP, and magnesium sulfate in a phosphate buffer (total volume 25 µl), with the addition of water to one (to test spontaneous respiration), galactose-1-phosphate and UDPG to the second, and glucose-1-phosphate to the

third. A hemolyzate of normal blood is simultaneously incubated with galactose-1-phosphate. The apparatus consists of a small bulb blown on the end of a capillary tube and is siliconed. A drop of 5 *N* potassium hydroxide solution is introduced into the capillary to seal it, and the movement in millimeters of the potassium hydroxide solution, after incubating at 37°C overnight, is measured. The test is intended to be used for the diagnosis of galactosemia, and is not intended to try to distinguish heterozygotes from normals. None of the quantities need be measured very accurately, and they are kept to a minimum for economy. In galactosemia the meniscus moves ± 0.5 mm in the galactose-1-phosphate tube, but —5 to —13.5 mm with glucose-1-phosphate, while with normals movement is about equal in the two tubes.

4.5. Diagnosis in the Newborn

Since most of the damage to liver, brain, and eyes occurs in the first few days or weeks of life, early diagnosis is of the greatest importance in order that the affected infant can be put on a galactose-free diet at the earliest possible opportunity. Even a single milk feed may do damage which is difficult to repair. The diagnosis is best made before any milk at all is given to the infant, and this can now be done by enzymatic assay on the cord blood. While the test is going through, the infant is fed on glucose and water or one of the commercially available galactose-free preparations. It is obvious that the procedure will be justified only where the baby is a member of a "high risk" group, e.g., the sib of a patient known to have galactosemia.

The test of choice is probably the simple gasometric method of Schwarz (S7), or the UDPG consumption test of Anderson (A2, M4). The manometric assays of Kirkman and Bynum (K10), and Hsia *et al.* (H12) would also serve, but require more elaborate apparatus and technique, and more expensive reagents. The modified UDPG consumption test of Bretthauer *et al.* (B17) has no advantage over the unmodified test for the diagnosis of galactosemia. Measurement of the accumulation of galactose-1-phosphate by the red blood cells on incubation with galactose *in vitro* (S6, S9) has the advantage of requiring no expensive or unusual equipment or reagents, but needs 2 ml of blood and takes 3 days to complete, during which time the infant must be given a galactose-free diet; it is also laborious compared with some of the other techniques but is valuable if an unprepared laboratory is asked to carry out a diagnostic test for galactosemia.

All the above methods can be carried out on blood from the umbilical cord; only a small volume of blood is required for some, and blood from

a heel prick will suffice if none was obtained from the umbilical cord. The baby should not have had an exchange transfusion before the assay—in this case the assay would show the presence of galactose-1-phosphate uridyl transferase in the red cells, but this enzyme could be absent from the liver and other tissues, and the baby could have galactosemia with apparently normal blood enzymes. If an exchange transfusion has been given, the best procedure is to put the infant on a galactose-free diet for 2 or 3 months, and then test the blood. In all the enzymatic assays, the blood must be very fresh and refrigerated immediately after collection, but not frozen. The freshly drawn heparinized blood should be cooled to 0°C, spun down at 0°C within 4 hours, and the plasma and white cells removed as completely as possible; the red cells should be resuspended in ice-cold normal saline and spun down again, this washing being repeated twice with equal volumes of saline, or once more if 10 volumes of saline are used each time. Walker *et al.* (W1) allow heparinized blood to settle for 10–12 hours and do not wash the red cells before freezing. The packed washed red cells can be frozen and will keep up to 2 weeks in the deep freeze; the whole operation from drawing the blood to freezing the cells should be carried out as quickly as possible.

The above procedures are justified when there is a family history suggestive of galactosemia. The problem of detecting the first affected child in a family is more difficult, particularly if one wishes to screen a large population. Berry (B10) uses the filter paper diaper test as a screening procedure. All mothers in the population being studied are issued with pieces of filter paper for insertion in the baby's diaper. When the filter paper is wetted with urine, it is air-dried and posted to the central laboratory. A portion of the filter paper is tested with aniline phthalate for hexoses. If a positive result is obtained, a further portion is eluted and the sugars examined by paper chromatography. Other portions of the filter paper are used for other tests.

5. Genetics of Galactosemia

The occurrence of more than one affected child in many of the families with galactosemia made it appear highly probable that this was a genetically determined disease. The mode of inheritance was worked out by the conventional methods of human genetics and, later, confirmed by biochemical studies of the families. The direct assay of enzymatic activity in heretozygotes for galactosemia must rank as one of the major contributions of biochemistry to the study of human genetics.

5.1. Mode of Inheritance

All cases of galactosemia recorded in the literature up to 1959 were analyzed by Hugh-Jones *et al.* (H16). One hundred and twenty-four cases occurred in 93 families, 36 of them families with a single child who had galactosemia. In 4 families the parents were recorded as being consanguineous (as this datum is not always given, there may have been more consanguineous marriages). There is no recorded case of both parent and child having galactosemia. There is an excess of affected males over females (H10). Statistical analysis of the published data (H16) strongly supports the widely held view that galactosemia is recessively inherited, the trait being carried by a single autosomal gene (C5).

On this view each parent of a galactosemic child has one gene for galactosemia and one allelomorphic normal gene, i.e., each parent is a heterozygote. Since the gene for galactosemia behaves as a Mendelian recessive, the parents are clinically and biochemically normal. These genes are on autosomes, i.e., not on the sex chromosomes. If the gene for galactosemia is represented **g** and the normal allele **G**, then the galactosemic can be represented **gg** (abnormal homozygote), the heterozygote **Gg**, and the normal **GG** (normal homozygote). There is a one in four chance that a child of 2 heterozygotes will have galactosemia, a one in four chance that it will be normal, and a two in four chance that it will be a heterozygote, i.e., a three in four chance that the child will be outwardly normal, as shown in Fig. 3.

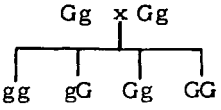

Fig. 3. Inheritance of galactosemia.

It must be emphasized that there is a one in four chance of having galactosemia for *each* child of 2 heterozygotes. This is a statistical concept and does *not* mean that only one child in four will be affected in any real family; several families are recorded in which all or the majority of the children have galactosemia, though these are balanced by other families in which fewer than one child in four is affected. Every pregnancy in such a family must be regarded as having a 25% probability of producing a child with galactosemia.

5.2. The "One Gene One Enzyme" Principle

Garrod (G3, G4) suggested, over 50 years ago, that an inborn error of metabolism was caused by the inherited absence of an enzyme. This

work, far in advance of its time, has been confirmed and extended, notably by Beadle (B5) working with the fungus *Neurospora*. The "one gene one enzyme" hypothesis states that the normal gene synthesizes an enzyme, while the mutant gene lacks this ability. It follows that an individual with 2 mutant genes, i.e., an abnormal homozygote, cannot make the enzyme, but the one normal gene possessed by a heterozygote synthesizes all he needs of the enzyme.

Later work, particularly on abnormal hemoglobins (I1), has slightly modified this view. The gene is now seen as part of a DNA chain, perhaps 1000 nucleotide residues long, which synthesizes "messenger RNA" (M10). The messenger RNA leaves the nucleus, attaches itself to a ribosome in the cytoplasm, and is responsible for the synthesis of a specific polypeptide chain. The amino acid sequence in this polypeptide is defined by the nucleotide sequence in the RNA which is, in turn, controlled by the nucleotide sequence in the DNA. A chemical change in one nucleotide residue of the DNA constitutes a mutation of the gene and leads to a change in the polypeptide. If this polypeptide is part of an enzyme, the mutation will have changed the enzyme, possibly sufficiently to make it inactive or reduce its activity.

It is often difficult to distinguish a mutation, which causes a completely inactive protein to be produced instead of an enzyme, from an effective deletion of the gene, when neither protein nor enzyme is produced. If, however, a mutation causes a protein with weak enzymatic properties to be produced, the abnormal homozygote will be able to carry out the reaction, though at a lower rate than the normal; the product of such a mutation is termed a "leaky gene."

5.3. Gene Expression in the Heterozygote

With better understanding of the mechanism of gene action, the terms "recessive" and "dominant" are losing their importance. It is, however, convenient to continue to use these terms: e.g., "recessive mode of inheritance" indicates that an individual must be homozygous for the relevant gene if he is to develop a particular condition. The normality, in most cases, of the heterozygote for galactosemia indicates that his one active gene produces enough galactose-1-phosphate uridyl transferase for his normal needs. However, by feeding the heterozygote a large loading dose of galactose, it is possible to demonstrate, in some cases, a reduced ability to metabolize this sugar. Direct assay of galactose-1-phosphate uridyl transferase activity in the red cells of the blood has given, in many cases, significantly different results for heterozygotes and normals.

5.3.1. *Results of Galactose Tolerance Tests*

Holzel and Komrower (H6) were the first to use the galactose tolerance test in attempts to detect the heterozygote. They investigated 20 members of 5 families in which cases of galactosemia had occurred. They found both the parents, in one family, had a reduced tolerance for galactose, but in each of the other 4 families one parent had normal galactose tolerance and the other reduced galactose tolerance. Donnell *et al.* (D5) investigated 39 members of 13 families, slightly changing the technique. The results gave highly significant differences between the means for the heterozygous group and for normals, but there was so much overlap that the method was considered unsuitable for deciding whether an individual was heterozygous or homozygous normal.

These investigations showed that the "average heterozygote" had a reduced ability to metabolize galactose and provided the first direct evidence for the mode of inheritance of galactosemia. The shortcomings of the technique led to the development of direct assay of the enzyme, and galactose tolerance tests are no longer used for this purpose. So many factors control the rate of disappearance of galactose from the blood that interpretation was bound to be difficult, but the greatest uncertainty was caused by the variable rate of absorption of galactose from the gut.

5.3.2. *Results of Enzyme Assays*

Hsia *et al.* (H11), using the technique of Anderson *et al.* (A2), found a statistically highly significant difference between the activity of galactose-1-phosphate uridyl transferase in red blood cells of heterozygotes and of normals, but the overlap was too great to allow all individuals to be assigned to one group or the other. Kirkman and Bynum (K10) achieved a much sharper separation by their manometric method; in their later cases, with improved techniques, there was no overlap between heterozygotes and normals (K9).

Donnell *et al.* (D7), using Bretthauer's modification (B17) of the UDPG consumption test, found only one normal individual with enzyme activity in the heterozygote range. These authors plotted the distribution curves of enzyme activity in parents of galactosemics and normals, and found that each gave a normal (Gaussian) distribution curve with an overlap of only 5% of the area under both curves. Donnell *et al.* calculate that 3% of all normal values lie below 4.0 μmole of UDPG consumed per milliliter of erythrocytes per hour, and 9% of all heterozygote values lie above 4.0. Their data provided the most complete evidence of the

simple Mendelian inheritance of galactosemia and one of the most beautiful demonstrations of the inheritance of any human disease.

Hsia *et al.* (H12) compared 4 methods of determining galactose-1-phosphate uridyl transferase activity. The 2 manometric methods, that of Kirkman and Bynum, and a slight modification, gave very slightly better discrimination between heterozygote and normal than the 2 UDPG consumption tests. More recently, Hsia and Walker (H10, W1), studying

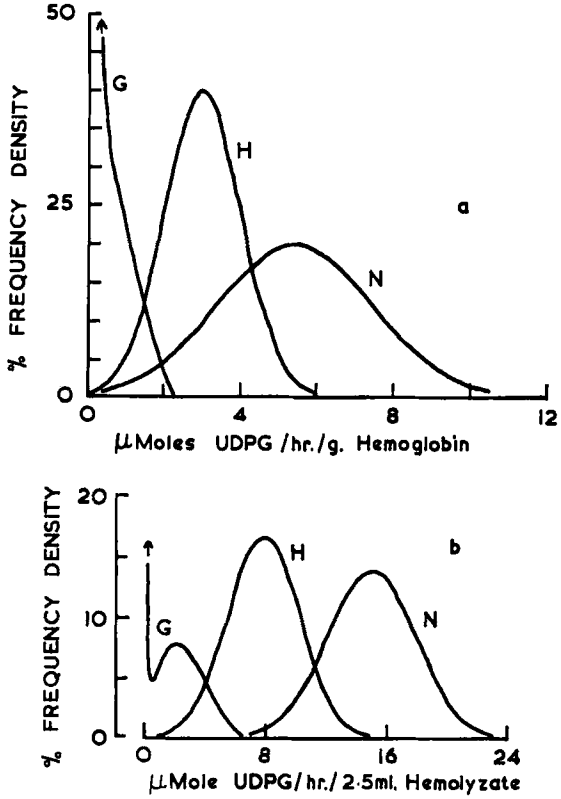

FIG. 4. Discriminatory power of the various methods of estimating erythrocytic galactose-1-phosphate uridyl transferase. *Methods*: (a) Anderson *et al.* (A2); (b) Bretthauer *et al.* (B17); (c) Kirkman and Bynum (K10), and Hsia *et al.* (H12); and (d) Schwarz *et al.* (S12).

G = galactosemics, H = heterozygotes, N = normal controls. The area under a given portion of each curve (product of abscissa and ordinate values) gives the percentage of individuals in the group with enzymatic activity between the limits chosen. By method (b) 69%, and by method (d) 44%, of galactosemics have no detectable enzymatic activity. Data from (A2, D7, H10, H12, K10, S12).

27 kindreds, found that 48 out of 52 parents of galactosemics had Gal-1-P transferase activity below the normal range.

Walker *et al.* (W1) calculated confidence limits for allocating individuals to the heterozygote or the homozygote normal category on the basis of a modified UDPG consumption test. Those with enzymatic activity above 12.73 (or 13.40 in a second group) μmoles/hour/0.6 g hemoglobin had at least a 4:1 probability of being normal, and those with this activity less than 9.67 (or 10.51) had at least a 4:1 probability of being heterozygote. It was calculated that, in a population with equal numbers of heterozygotes and normals, 5% would be misclassified and

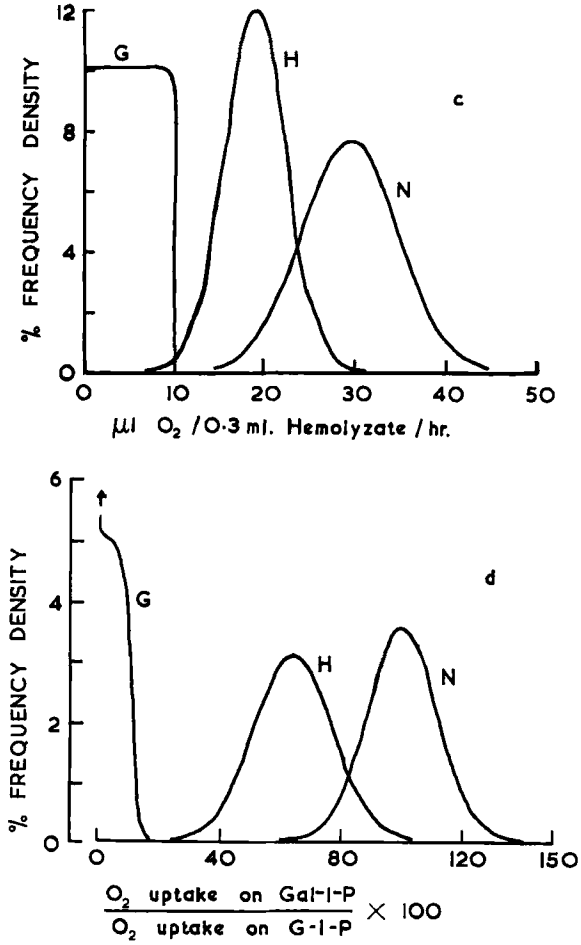

FIG. 4. *(Continued)*

20% unclassifiable (enzymatic activity between the chosen limits). This overlap seems somewhat higher than by other techniques.

Schwarz *et al.* (S12), measuring oxygen uptake by a method giving maximum activity of Gal-1-P-uridyl transferase, found some overlap between normals and heterozygotes, but none between heterozygotes and homozygotes for galactosemia. They conclude that present techniques are adequate to prove the inheritance of galactosemia in a simple Mendelian fashion, and to distinguish the majority of heterozygotes, though in some cases the genetic status may be doubtful. However, present methods are inadequate to enable us to decide whether there are different types of gene for galactosemia—different mutons within the same cistron (K5)—some of them "leaky." Difficulties arise from biological variations in and between individuals, and from differences in techniques of handling the blood, rather than from the precision of the methods of determination (S12). Slight contamination of the hemolyzates with leucocytes may add other enzymes.

The results obtained by a number of workers, using 5 different methods, are represented in Fig. 4. The method of Kirkman and Bynum (K10), and method D of Hsia *et al.* (H12), give such similar results that they are combined. In Fig. 4, normal distribution curves are drawn for normal homozygotes and heterozygotes, using published values for the means and standard deviations (no allowance is made for possible skewness or kurtosis); as far as possible, the same scale is used for all the methods. For galactosemics a smooth curve was drawn from the values published for individual patients.

5.3.3. *Clinical Effects of the Gene in the Heterozygote*

Most heterozygotes for galactosemia show no clinical abnormalities and are distinguishable from homozygous normal individuals only by means of delicate tests such as those above. However, there are a few reports of abnormal sensitivity to galactose in heterozygotes.

Holzel (H3) records the case of a 5-year-old boy who was made to drink milk while away at school but avoided milk at home. He suffered from enlargement of the liver during the school term but his liver returned to normal size during the holidays. When a new baby in the family developed severe galactosemia, the elder boy's tolerance to galactose was determined and found to be grossly abnormal, but at no time did he have mellituria or aminoaciduria. A direct assay of galactose-1-phosphate uridyl transferase in his red blood cells would be particularly interesting.

Hugh-Jones *et al.* (H16) record a family of 6 children of a galactos-

emic, all of whom, in consequence, must have been heterozygotes. Five of these 6 had a reducing substance, shown to be galactose, in the urine, constantly in the case of 3, and during pregnancy in the case of 2. Two were made ill by milk, even one or two pints a day, and preferred to avoid it. This family has other peculiar features which are dealt with below (Section 5.5).

Dawson *et al.* (D3) describe a family with galactosemia in 2 sibs and a family history of milk intolerance over 4 generations. The grandmother of the galactosemic children developed nausea and headache after an oral or intravenous test dose of galactose, as well as after taking milk. The activity of galactose-1-phosphate uridyl transferase in the erythrocytes of 4 generations was determined by the manometric method of Kirkman and Bynum (K10); the results of this assay, which clearly demonstrated the recessive mode of inheritance, did not run parallel to the degree of milk intolerance. Similar findings are recorded by Schwarz *et al.* (S12).

Apart from these possibly extreme cases of heterozygote expression, some heterozygotes have only 15% of the normal activity of galactose-1-phosphate uridyl transferase in their red blood cells (D7). If this accurately reflects the enzyme content of the liver, these individuals would have a notably reduced ability to metabolize any extra load of galactose. Kalckar (K4) has pointed out that during pregnancy a mother is often encouraged to drink a considerable amount of milk and, if she is a heterozygote with a low enzyme activity, the galactose content of her blood would rise and some of the galactose cross the placenta to the fetus. If the fetus is homozygous for galactosemia, galactose-1-phosphate may accumulate and cause damage, perhaps to the brain. This may explain why some galactosemics do not develop normal intelligence even though they are placed on a galactose-free diet at a very early age (C2, K4, K9, L6). Some evidence for this chain of events is provided by Schwarz's finding (S5, S6) of excessive amounts of galactose-1-phosphate in the cord blood of a newborn galactosemic. Hsia and Walker (H10) record that the birth weight of galactosemics is significantly less than that of their unaffected sibs and that some galactosemics are born with signs of cirrhosis of the liver and early cataracts.

5.4. The Prevalence of Galactosemia

Galactosemia is not so extremely rare as to be a mere medical curiosity; any hospital with a large children's department will see a considerable number of cases over the years. Fifty cases have been diagnosed in

Great Britain and Northern Ireland over the last few years (S12), and it is practically certain that not all cases born were diagnosed. Schwarz *et al.* (S12) estimate, from their own data and that of others, that about 1 in 70,000 live births results in a galactosemic infant. This may be compared with an estimated incidence of 1 in 20,000 for phenylketonuria. Hsia and Walker (H10), in Chicago, were able to study a series of 45 galactosemics over a few years. On the basis of the figures of Schwarz *et al.*, which must be considered a minimum estimate, 1 out of every 132 individuals will be a carrier of the gene for galactosemia.

5.5. Some Unsolved Problems

Hsia *et al.* (H12) found, by all 4 techniques used, that the red blood cells of some galactosemics appeared to have some galactose-1-phosphate uridyl transferase activity. This has also been found by Donnell *et al.* (D7), Schwarz *et al.* (S12), and Hsia and Walker (H10). This could be accounted for by some alternative metabolic path for galactose-1-phosphate, but the pyrophosphorylase catalyzing the reaction between galactose-1-phosphate and uridine triphosphate, at that time the only known alternative pathway, is said to be absent from red blood cells (I6). This problem is distinct from that of the metabolism of C^{14}-labeled galactose *in vivo* to produce $C^{14}O_2$ and C^{14}-glucuronide (E2), which might be explained by the presence of the pyrophosphorylase in the liver. A possible explanation of the findings of Hsia *et al.* (H12) would be that the mutant gene produces a protein which, while different in structure from the enzyme, retains some weak enzymatic properties. Direct evidence on this must await techniques for isolating and estimating the enzyme as a protein. Recent evidence that galactose can be metabolized by red cells via galactose-6-phosphate and the hexose monophosphate shunt (I2) would not explain all the above findings since UDPG is not involved. Schwarz *et al.* (S12) also found measurable activity of galactose-1-phosphate uridyl transferase in the red cells of some galactosemics. Their patients fell into 2 distinct groups, those (44%) with no detectable transferase activity and 56% who were consistently found to have 3 to 12% of the normal enzymatic activity. Hsia and Walker (H10) found that, in about one-third of the galactosemics they studied, enzymatic activity was detectable either by the oxygen uptake or the UDPG consumption test but not by both, in a few cases enzymatic activity was found by both methods, and in about half the cases neither method showed any enzymatic activity. The discrepancy, in a third of their cases, between the enzymatic activity found by the 2 methods suggests that some enzymes other than galactose-1-phosphate

uridyl transferase are responsible in these and that in at least five-sixths of galactosemics studied this enzyme is absent or inactive.

Galactosemia is a disease with widely differing degrees of clinical severity. It ranges from the fulminating form, in which the infant is dead within a few weeks of birth, to cases such as those described by Durand and Semach (D10), Hugh-Jones *et al.* (H16), and Hsia and Walker (H10) in which the intelligence was unaffected and the patients led normal lives with minimal or no clinical signs of galactosemia. The question is whether these cases who are little affected are homozygous for the same gene as in the severely affected, or whether we are here dealing with a "leaky gene."

The problem is intensified by the family tree published by Hugh-Jones *et al.* (H16): a highly intelligent man with hepatomegaly and cataracts was found to have galactosemia. The level of galactose-1-phosphate uridyl transferase in his red cells was in the galactosemic range. His 6 living children are healthy, but excrete galactose and are made ill by drinking milk. By a remarkable coincidence, one of these children has married a carrier for galactosemia and they have 2 galactosemic children, the first example of galactosemia in more than one generation of a family. Both these children are of normal or above normal intelligence, both have cataracts, both failed to thrive satisfactorily as young infants. Assays of galactose-1-phosphate uridyl transferase activity in the red blood cells of these grandchildren of the original patient show them to be galactosemics. The remarkable features of this family are the mildness of the disease in the 3 frank cases, and the severity of the manifestations in the heterozygotes in whom the gene for galactosemia cannot be termed purely recessive.

Some other cases are recorded of heterozygotes with marked milk intolerance, very abnormal galactose tolerance curves, and even hepatomegaly on a fairly low milk intake (H3, L6); further work on these might well be rewarding. Donnell *et al.* (D7) record heterozygotes with the activity of this enzyme only 0.9–1.9 μmole of UDPG consumed per milliliter of erythrocytes per hour, 1.25–2.5 standard deviations below the mean for heterozygotes (2.9); the question is considered whether these parents (and sibs) of known galactosemics are not themselves atypical homozygotes with very mild forms of the disease. However, none of them showed milk intolerance or abnormalities in the galactose tolerance test, all were symptomless, and examination of their parents (grandparents of galactosemics) showed that, in each case, one parent had an intermediate or low value for enzyme activity and the other was normal (D7). This is convincing evidence that some

individuals with enzyme activity in their red cells in or approaching the galactosemic range are heterozygotes. Two of the atypical heterozygotes noted by Donnell *et al.*, with the lowest enzyme activities, are mother and son.

Walker *et al.* (W1) discuss five possible causes of the observed variation in enzymatic activity among heterozygotes, with particular reference to high levels of enzymatic activity overlapping those found in normals:

(1) Replication is good enough to eliminate inaccuracies in the assay as a possible cause.

(2) It is possible that, by present techniques, enzymes other than Gal-1-P-uridyl tranferase make an appreciable contribution to the consumption of UDPG or oxygen.

(3) In some families the enzymatic activity differs in different heterozygotes; the gene itself can hardly change within these families, hence variability of enzymatic activity can occur without variation of the gene and "leaky genes" need not be invoked.

(4) It is possible that in some individuals or some families genes at other loci modify the expression of the galactosemia gene to give normal enzymatic activity in the heterozygote.

(5) Environmental influences, e.g., the concentration of progesterone in the blood (P8), may affect enzymatic activity.

Points (4) and (5) of Walker *et al.* are not really separate since the hypothetical other genes may act by altering some feature of the environment; this seems by far the most likely explanation of the very wide range of enzymatic activities found in both heterozygotes and normals.

The extent to which the level of galactose-1-phosphate uridyl transferase activity in erythrocytes reflects the level in the liver is unknown except for a handful of cases. This problem is worth further investigation with particular reference to these 2 groups of heterozygotes, those showing unusual galactose intolerance and those with very low enzymatic activity.

Apart from atypical cases, it is necessary to explain the difference between the infant with the fulminating form of galactosemia and the infant with a more chronic form. It is known that a number of enzyme systems become active either just before or just after birth (B18), and that enzymes become mature at different absolute ages in different infants (B15, L1). Two newborn infants may have livers of very different maturities, one able to withstand the toxic action of

galactose-1-phosphate, the other succumbing rapidly. The possibility of prenatal damage must also be considered.

Male galactosemics recorded number 91, as against 44 females (H10). This is a highly significant excess of males (expected ratio 1.06:1, $P < 0.001$). We can only guess at the explanation. Preferential mortality among affected females *in utero* could account for this excess, but it would have to be very early in fetal life, as the number of recorded miscarriages is too small to account for the excess of males; this appears unlikely. The disease may run a milder course in some females and so not be diagnosed, but study of families with affected boys and girls does not suggest any difference in severity. The unaffected sibs of Hsia and Walker's patients also showed an excess of males, 28 males to 19 females. Walker *et al.* (W1) added further data and concluded that, if both parents were heterozygotes for galactosemia, there was an excess of males in the offspring whether affected or unaffected; if one parent was heterozygous and the other homozygous normal, the sex ratio of the offspring was normal.

A disease such as galactosemia would be expected to die out in a population; its continued existence needs explaining. The gene for galactosemia, in double dose, either is lethal or greatly reduces survival fitness. Because galactosemics, in general, do not reproduce, the gene frequency in the population should slowly drop, generation by generation, to a very low level. There are several mechanisms by which this level could be maintained in equilibrium: (1) by new mutations, (2) by some advantage possessed by the heterozygote, and (3) by germinal selection. At present we cannot decide the relative importance of these 3 mechanisms. The uneven sex distribution in galactosemia may be related to maintenance of the gene in the population.

6. Treatment of Galactosemia

Göppert (G7) recommended in 1917 that lactose should be replaced by other sugars in the diet of galactosemics and that a lactose-free diet should be given from as early an age as possible. These 2 principles have formed the basis of all attempts at treating galactosemia.

6.1. Types of Low-Lactose Diet

There is a remarkable degree of disagreement, among different workers, on the best type of low-lactose diet to feed to galactosemics. The basic principles are clear and generally accepted: since the clinical manifestations of galactosemia are due to intoxication by galactose-1-

phosphate which is derived from galactose, if available galactose in the diet is reduced to a sufficiently low level this intoxication will cease and the infant will recover more or less completely. The differences between different workers largely turn on the maximum permissible galactose content of the diet and on how best to prepare a diet low in available galactose.

Free galactose is a rarity in any natural foodstuff; the milk of all species of mammal examined contains as its main carbohydrate the disaccharide, lactose (β-galactosidoglucose), and this is practically the only dietary source of galactose in early infancy and by far the most important later in life. The mucosal cells of the intestine contain lactase; this hydrolyzes dietary lactose to galactose and glucose which are absorbed into the blood stream. A healthy infant absorbs only traces of unchanged lactose; this is excreted in the urine since lactose can apparently be hydrolyzed only in the cells lining the intestine. Lactose constitutes 7% of human milk, and 4.8% of cow's milk, contributing 41.5 and 28%, respectively, of the total calories. Since half of this lactose can be regarded as being galactose, the galactose intake of a breast-fed infant may be of the order of 35 g per day. Thus a low-galactose diet is, in effect, a diet which is not prepared from milk and thus contains no lactose, or one prepared from milk so treated that the lactose content is greatly reduced.

Cox and Pugh (C5) used a mixture of casein, coconut oil, arachis oil, sucrose, water, mineral salts, and vitamins to produce a synthetic "milk." Casein, as ordinarily precipitated, contains about 1% of lactose so that each ounce of the "milk" contained about 12 mg lactose, i.e., 6 mg of available galactose. Nutramigen (Mead, Johnson), a synthetic "milk" with protein replaced by an enzymatic hydrolyzate of casein, has been widely used for feeding infants with galactosemia. However, this hydrolyzate contains about 0.7% lactose (W8) and Nutramigen itself, in consequence, will supply the infant with up to 200 mg of available galactose per day. Salt *et al.* (S3) recommend a mixture resembling that of Cox and Pugh, but using specially washed casein with a lactose content of between 0.04 and 0.13%, giving 2.8 mg lactose per 8 oz feed; Galactomin (Trufood) is a commercial low-lactose "milk" formulated along these lines. A number of workers have by-passed the problem of removing lactose from milk by feeding soya "milk" (B16, G6, P4). Soya contains the oligosaccharides, stachyose and raffinose (W8), which are α-galactosides, but it seems probable that the human body contains no enzymes capable of releasing galactose from α-galactosides, so that soya probably contains no available galactose. Among commercial

lactose-free soya milks are Mul-Soy (Borden) and Wanderlac (A. Wander Ltd.). Holzel and his co-workers (H2, H7) prepare a special mixture of oatmeal (or other cereal), sucrose, egg, and water, which are cooked together to form a "pudding" similar to that described by Moll and Stransky for feeding infants suffering from gastroenteritis (M9). The available galactose of the various components of "Moll's pudding" has never been determined, but egg contains mucoids, a group of compounds many of which are rich in galactose, and galactocerebrosides (H13). It is rather surprising that so many of the diets used start with a protein derived from milk, increasing the probability of contamination with lactose. The 2 types of diet that do not start with milk use either soya, a plant product with uncertainties about digestibility and availability of essential amino acids, or a thick pudding that is not taken well by some infants. These difficulties and uncertainties can be by-passed by using a preparation based on meat.

As with other more or less synthetic diets, it is necessary to ensure that a child or, more especially, an infant has an adequate intake of all essential dietary substances. Milk is so nearly complete a food that it is usual to assume that a milk-fed infant requires no supplements except vitamins A, C, and D. If a galactosemic infant is receiving a diet that looks like milk, there is a danger of overlooking the need to ensure that the infant is getting enough of the different minerals, the various vitamins, a well-balanced protein (i.e., enough of the essential amino acids in digestible form), fat (including essential fatty acids), and calories. In particular, a galactosemic infant may require additional vitamins such as pyridoxine, riboflavin, thiamine, folic acid, vitamin B_{12}, biotin, inositol, choline, pantothenic acid, nicotinamide, and vitamin K.

The value of any low-lactose diet can be judged only by the effect on the patients. On this basis all the above diets have achieved good results in some cases, none has been completely satisfactory. The results in young and severely ill infants are different from those in older and more moderately affected children, and the two groups are dealt with separately.

6.2. Treatment of the Young Infant

Young infants with galactosemia can be divided into those who are seriously ill before the diagnosis is made on biochemical and clinical grounds, and those who are investigated and detected immediately after birth, before they are given any milk or become ill. Many young infants suffering from the severe form of galactosemia show a dramatic improvement within a few days of starting a low-lactose diet. The

infant's weight, often below its birth weight, starts to rise sharply, the liver softens and decreases in size, jaundice, if present, vanishes, diarrhea and vomiting cease, the infant takes its feeds better, and becomes more alert. This improvement is usually, but not always, maintained; in some infants the liver begins to enlarge again, jaundice occasionally reappears, the infant ceases to thrive and may die after a few weeks, or may slowly recover. This pattern of apparent initial improvement followed by relapse has been observed on several different low-lactose diets. Three possible explanations are: (1) that the insult to the liver during the first few weeks of life caused damage which was slowly repaired, if at all; (2) that the diets used were not low enough in lactose and that galactose-1-phosphate slowly accumulated, causing the liver damage; and (3) that intra-uterine intoxication occurred, damaging the liver before birth.

The group working in Manchester have produced some evidence for the second alternative: a galactosemic infant lost weight on a commercial casein hydrolyzate which was later shown to contain 0.5% galactose (H2). This same infant developed diarrhea when his diet was changed to soya "milk," but did well on "Moll's pudding." As little as 0.5% of galactose can lead to the development of cataracts within 3 weeks (H7). An infant showed definite liver enlargement, vomiting, and weight loss within 3 days of being started on a low-lactose commercial food containing less than 0.1 g galactose per 100 ml (H3). It is calculated that an infant weighing 7 kg would require only 400 mg galactose to reach a toxic concentration of galactose-1-phosphate in his cells, if none were metabolized or lost; this amount would be provided in a few days by a low-lactose "milk" containing only 0.012% galactose (S6). The same workers record a galactosemic infant aged under 2 weeks who improved clinically but failed to gain weight on Wanderlac, a soya preparation; 6 weeks later he was given Nutramigen and began to gain weight very rapidly (S11).

Some other workers have found Nutramigen very satisfactory in spite of its content of available galactose (B16, B19, D6, H14). Some galactosemic infants appear to thrive on Galactomin which contains not more than 0.002 g available galactose per 100 ml, others do better on other diets. Content of available galactose should not be the sole criterion by which a diet is judged; nongalactosemic infants not infrequently have to try a number of infant foods before a formula is found which suits them, and the difficulties of feeding some infants on soya "milk" and Galactomin may be caused by some such nonspecific unsuitability for a particular infant. "Moll's pudding" can cause digestive

upsets (H8, P4). Holzel (H3) states that no matter what low-lactose diet is given, weight gains during the first year of life are never satisfactory. All of Holzel's findings, except one, could be equally well explained by the infants having been exposed for some time before or after birth to hepatotoxic substances as by too much available galactose in the low-galactose diet; the one finding that appears to point unambiguously to too high a galactose intake is the development of cataracts in one infant.

The treatment of infants who have never had milk is, in some ways, simpler. These infants will practically all be the younger sibs or other close relatives of known galactosemic patients. While the diagnosis is being established, preferably by examination of cord blood, the infants should be given glucose and water, or one of the low-galactose preparations. After confirmation of the diagnosis of galactosemia, a suitable low-galactose preparation should be fed. The immaturity of several enzyme systems at birth (B18), and the greater degree of immaturity in some infants than in others (B15), indicates that special care is necessary in treating the newborn; they may be made ill by a concentration of galactose-1-phosphate that would not upset even a slightly older child, and some may be more sensitive than others. This suggests that some preparation as free as possible from available galactose should be fed at first, e.g., a soya "milk" preparation, Galactomin, a meat-base formula, or, if the available galactose is found to be sufficiently low, "Moll's pudding." After a period of, say, 2 or 3 months, it may be desirable to change to some other preparation, such as Nutramigen, which has a slightly higher galactose content but is better tolerated by some infants. Frequent estimations of the galactose-1-phosphate content of the erythrocytes is desirable and may simplify treatment by indicating whether the preparation fed has too much available galactose for a particular infant.

The results of treatment of the newborn are usually very satisfactory. Provided the diet fed is suitable, they gain weight at nearly the same rate as normal infants, their intelligence is normal (D4, D6, H3, H10), they are free from cataracts, liver damage or kidney damage. They grow into children who are normal in every way except that they must avoid milk and tend to be a little smaller than their normal sibs. Hsia and Walker (H10) show that the intelligence quotient of the galactosemic is highly correlated with the degree of dietary control, i.e., the care with which lactose was excluded from the diet.

Lactose can be unintentionally added to the diet in several unexpected ways. Some cereal preparations for baby foods are reinforced with milk

powder. Lactose is very widely used as a tablet base and galactosemic infants have been given excessive amounts of lactose with their vitamin tablets; since lactose is not regarded as an active ingredient of the tablets, its presence is rarely declared in the list of components. No tablet should be fed unless the manufacturers specifically state, or laboratory tests reveal, that it is free from lactose.

6.3. Treatment Later in Life

Older children with galactosemia often benefit markedly from a low-galactose diet. Their rate of weight gain increases sharply as soon as they are put on the diet, even those who seemed little affected by their galactosemia (D9).

Renal calculi occurred in 2 children being treated with a low-galactose diet (B7, C5). In one case it was shown that the serum alkali reserve was low (B7). It is impossible to decide whether this was due to galactose damaging the kidney and causing continued dysfunction after exposure to galactose had stopped, or to some characteristic of the low-galactose diet.

Intelligence also starts to rise in some cases (C5), but in others there is little intellectual improvement, even when the diet is started as early as one month of age (C2). There can be little doubt that the effect on intellectual development depends both on the age at which dietary treatment is started and on the amount of damage to the brain that has already occurred. From Clay and Potter's report (C2), it is evident that irreversible brain damage can occur during the first month of life. This patient has since died in a hospital for mental defectives and a post-mortem examination has revealed extensive scarring of the brain (C8). It is noteworthy that the E.E.G. was still abnormal in some cases in spite of treatment (B2, H3). The position is analogous to that in phenylketonuria where the need for early treatment is recognized, but some older children benefit from a low-phenylalanine diet. Hsia and Walker (H10) consider that the age at which the diet is started in galactosemia is less critical than in the case of phenylketonuria. There is undoubtedly great variation in this respect from patient to patient.

Cataracts in older children rarely regress very appreciably on dietary treatment (W4). If the lenses are sufficiently opaque to interfere with vision, as is generally the case, they usually need discission or extraction, though it may be wise first to try the effect of some weeks of treatment with a low-galactose diet, particularly in the younger patients.

Galactose need not be so rigorously excluded from the diet after the first year of life. The older galactosemic child can have an ordinary

mixed diet, avoiding only milk, foods prepared largely from milk or milk solids, such as ice cream, and lactose (present in such items as vitamin tablets).

6.4. Treatment during Pregnancy

The intellectual improvement on a low-galactose diet is sometimes disappointingly small, even when the diet was started fairly early in infancy. It has been suggested that this may be due to damage to the brain occurring *in utero* (C2). Schwarz (S5, S6) found a raised level of galactose-1-phosphate in the blood of an infant who had been given no milk, even though the maternal blood had a low galactose content. Donnell *et al.* (D7) found that the mothers of galactosemics may have only 15% of the normal galactose-1-phosphate uridyl transferase activity in their erythrocytes; in late pregnancy the galactose tolerance curve tends to be abnormal (A2); women are encouraged to drink milk during pregnancy. These 3 factors may, it is suggested (K9), together bring about a greatly raised concentration of galactose in the blood of the mother. This may cause a toxic level of galactose-1-phosphate to be reached in a galactosemic fetus. It is therefore recommended that the mother of a galactosemic child should not drink milk during subsequent pregnancies.

Apart from Schwarz's finding (S5, S6) and the induction of cataracts in fetal rats by feeding the pregnant mothers a high-galactose diet (B3), Hsia and Walker (H10) found a significantly lower birth weight for galactosemics than for their normal sibs. The difference was greater for those galactosemics who died in infancy than for those who survived. This is conclusive evidence of intrauterine damage; the correlation, among galactosemics, of low birth weight with death in infancy is especially important. Hsia and Walker point out that the death rate in infancy among galactosemics is still high in spite of improvements in diagnosis and treatment; this may well be explained by an effect on the fetus. In this respect galactosemia would differ from, say, phenylketonuria.

6.5. Other Forms of Treatment

Exclusion of available galactose from the diet is the only successful form of treatment for galactosemia. As with other inborn errors of metabolism, the missing enzyme cannot be replaced. Galactosemia resembles scurvy in this respect; man, other primates, and guinea pigs lack the enzyme which in other mammals oxidizes L-gulonolactone to ascorbic acid and, in consequence, they require exogenous ascorbic acid throughout their lives if they are not to develop scurvy. It would

be rash to predict that we will never find a way of giving man an enzyme he lacks, but this will certainly remain impossible for a long time to come.

The effect of some steroid hormones on galactose metabolism in galactosemics did, at one time, raise the hope that some alternative to a low-galactose diet might be effective (E3). Menthol and menthone have a similar effect (see above, Section 3.2).

This effect has no place in treatment at present. It is, however, worth investigating this curious effect further to try to elucidate its mechanism and to see whether galactosemics vary in their response.

6.6. Effects of Deprivation of Galactose

No ill effects of feeding infants a diet free from galactose have ever been described. Galactose residues are an essential part of cerebrosides in the myelin sheath, gangliosides in the gray matter of the brain, many mucopolysaccharides, and glycoproteins. Before the enzyme defect had been identified, it was feared that the infant could not thrive on a diet completely free from galactose because he would not be able to synthesize these compounds. It is now realized that, in those cases investigated, the galactose residues are incorporated from uridine diphosphate galactose (B20). Since UDPGal-4-epimerase is normal in galactosemia (I9), uridine diphosphate galactose is produced from UDPG and there is therefore no shortage of galactose residues for incorporation into other molecules. It is remarkable that the great bulk of the galactose residues in, say, the cerebrosides of the brain, are normally derived from glucose. It is found that galactosemics do well without any dietary source of galactose. This is in marked contrast to phenylketonurics, who must be fed a small but definite amount of phenylalanine, and a diet for galactosemics is correspondingly easier to formulate.

It has been suggested that one effect of changing a diet is to change the flora of the gut (K3). In infants on a milk diet, lactobacilli are the predominant bacteria in the gut. Lactose stimulates the growth of lactobacilli and some strains are specifically stimulated by oligosaccharides present in traces in human milk. In infants fed a milk-free and lactose-free diet, lactobacilli will tend to be replaced by coliform organisms. Some strains of *E. coli* are pathogenic, hence a galactosemic infant on a low-lactose diet may be more liable to develop gastroenteritis than a normal infant. A further difficulty is that lactobacilli synthesize some of the vitamins the infant requires, so that an infant on a low-lactose diet may require additional vitamins, particularly of the B

group. Galactosemics do not differ, in these respects, from other infants on a lactose-free formula.

6.7. Control of Treatment

However carefully the diet is formulated, biochemical monitoring is necessary to ensure that the diet is adequate, that it is being adhered to, and that no unsuspected sources of available galactose have been included. It is a simple matter to examine the blood or urine for galactose, preferably by paper chromatography, but this reflects recent changes in the diet too well to be of much use as a monitoring technique. If the child is receiving an article of diet too rich in available galactose, but has a galactose-free diet for a short time before the test, no galactose will be found in the blood or urine. A more reliable test, from this standpoint, is the estimation of erythrocytic galactose-1-phosphate (K11, S6); this has the great additional advantage that the substance estimated is believed to be directly responsible for the clinical effects in galactosemia.

7. Conditions Mimicking Galactosemia

The term galactosemia is used throughout this chapter to mean a condition characterized by a lack of the enzyme galactose-1-phosphate uridyl transferase, this enzymatic deficiency being genetically determined. There are a few conditions in which the clinical and biochemical features resemble those of galactosemia more or less closely, making differential diagnosis difficult. Some cases of galactose intolerance can be distinguished from galactosemia only by assaying galactose-1-phosphate uridyl transferase; some of the cases described in the older literature, in which this enzyme was not determined, may have had galactose intolerance not caused by galactosemia.

The main site of galactose metabolism is the liver. Any factor depressing liver function may decrease the over-all metabolism of galactose. This is the basis of the galactose tolerance test, introduced by Bauer (B4) for the diagnosis of cirrhosis of the liver and used by Maclagan (M1) and others, in improved form, for the diagnosis of any parenchymatous liver disease. Liver disease in an infant on a milk diet may lead to a raised concentration of galactose in the blood and consequent galactosuria. The effect on tissue metabolism is difficult to predict, galactose-1-phosphate may not accumulate in most tissues because of the presence of the transferase. Two tissues that are likely to be damaged are the lens, by analogy with galactose-fed rats, and the liver, further damaged by the galactose-1-phosphate derived from

the circulating galactose. Such an infant may well show several of the features of galactosemia. If the cirrhosis is familial, as in the cases described below, the similarity will be even closer.

7.1. Familial Galactose Intolerance

Schwarz *et al.* (S12) briefly record 3 patients with several of the following signs: failure to thrive, jaundice, galactosuria, aminoaciduria, cataracts, a grossly abnormal galactose tolerance curve, and clinical improvement on a galactose-free diet. In 2 of these cases there was a positive family history of siblings that had been jaundiced and had died during the first 3 weeks of life. Yet estimations of galactose-1-phosphate uridyl transferase activity on the red blood cells of these 3 patients revealed a normal value in each case.

7.2. Congenital Hepatic and Renal Dysfunction

A rare condition occurring in several countries has been described as "congenital hepatic and renal dysfunction" (W9), "congenital cirrhosis of the liver with renal tubular defects akin to those in the Fanconi syndrome" (B1), and "atypical tyrosinosis" (S1, S2); the familial cirrhosis described by Berger *et al.* (B8) may be related. The clinical signs are: failure to thrive; often severe and progressive jaundice with onset a few days after birth; hepatomegaly; in many cases death in liver failure a few weeks or months after birth; proteinuria; massive aminoaciduria; and excretion of very large amounts of reducing sugar. However, this sugar is mainly glucose, though large amounts of fructose are also present in infants given a formula containing sucrose; galactose may also be present in moderate amounts. In one specimen 2.5 g glucose and 1.5 g fructose per 100 ml urine were present.

The aminoaciduria is usually far more marked in this condition than in galactosemia, and tyrosine is excreted in particularly large amounts. Hydroxyphenyluria occurs, large amounts of *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyl-lactic acid, and *p*-hydroxyphenylacetic acid appearing in the urine (the last may be an artifact derived from *p*-hydroxyphenylpyruvic acid). The urine reduces Brigg's reagent (strongly acid phosphomolybdate), though it is advisable to precipitate protein first with trichloroacetic acid. The phenolic acids, in the raw urine or in suitable extracts, react with Millon's reagent and can be identified by paper chromatography (B15).

The urinary amino acids reflect both their high concentration in the blood, due to poor functioning of the liver, and failure of renal tubular reabsorption. The phenolic acids and tyrosine in the urine are evidence

of hepatic dysfunction, as is probably the fructosuria in those cases given sucrose. The glucosuria and proteinuria are caused by a failure of renal tubular reabsorption.

In those patients who survive more than a few weeks, the effects of renal tubular dysfunction become more severe. Acidosis and hypophosphatemic rickets are prominent features. The urine is alkaline and gives a strong Rothera reaction. However, the ability to concentrate the urine is never lost and there is neither polydipsia nor polyuria. Aminoaciduria, hydroxyphenyluria, glucosuria, fructosuria, and proteinuria continue. The liver remains large and cirrhotic. Death finally occurs in liver failure, sometimes after several years. There is evidence that some children recover with no residual signs other than a large firm liver.

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THE MALABSORPTION SYNDROME, WITH SPECIAL REFERENCE TO THE EFFECTS OF WHEAT GLUTEN

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1. Introduction

The term "malabsorption syndrome" might be expected to include such conditions as pernicious anemia, but as a rule it is used in a more limited sense to cover a group of patients in whom the absorption of the main nutrients is defective. This commonly results in the passage of bulky, pale, fluid or semifluid, offensive, fatty stools. There may be other features associated with this steatorrhea, such as anorexia, loss of weight, abdominal distension, skin pigmentation, stunted growth, delayed development, or various deficiency states, especially those due to lack of folic acid, cyanocobalamin, iron, or calcium. The etiology of the malabsorption syndrome is complex. One group results from surgical operations on the gastrointestinal tract, a second is associated with constitutional diseases, such as diabetes, a third is due to granulomatous or neoplastic disease of the intestines, a fourth group is attributable

to faulty preparation of food for absorption in the intestinal lumen, and a fifth, which may be termed the "sprue group," includes such clinical conditions as celiac disease, idiopathic steatorrhea (nontropical sprue), and tropical sprue. Many of these forms of the malabsorption syndrome can be definitively diagnosed on the basis of the history, clinical and pathological findings, and course of the disease. No attempt will be made in this paper to discuss the differential and definitive diagnosis of all these forms of the malabsorption syndrome. Attention will be confined to primary diagnosis of the malabsorption syndrome and the differential diagnosis of the sprue group. Reference will also be made to the pancreatogenous malabsorption syndrome, since the recognition of this type of patient is important and tends to be neglected. The main objective of this chapter is to consider the special features of the sprue group with particular reference to the part played by wheat gluten in pathogenesis.

2. Diagnosis

2.1. Primary Diagnosis

2.1.1. *Daily Fecal Fat Output*

The primary diagnosis of the malabsorption syndrome rests most satisfactorily upon estimation of the daily fecal fat output. This can readily be assessed by techniques based on the original method of van de Kamer *et al.* (V3, W3). However, it is wise to express the result as grams of fecal fat per day rather than as a coefficient of absorption, since the origin of much of this fat still remains in doubt (F16). The average figure for daily fecal fat output should be based on the results of not less than 5 consecutive days, preferably 10. If a standard hospital diet is being consumed and the average daily fecal fat output is less than 5 g per day, it can be concluded that the patient is not suffering from the malabsorption syndrome. If the average fecal fat output exceeds 5 g per day, the malabsorption syndrome may be present; the fecal fat output may be much greater than this and can exceed 200 g per day. If the values lie between 5 and 10 g per day, or if severe anorexia is present, further evaluation is usually required (C10, H3).

The daily fecal fat method has one disadvantage; it does not distinguish between unabsorbed dietary fat and that derived from other sources. To do this it is necessary to use a labeled fat. The method that has received most attention in recent years is the use of I^{131}-labeled fat (B7, B12, C12, G10, L7, M8, M10, P8, R13, S17). In the hands of most investigators the results obtained with I^{131}-labeled fat are in accord

with the results of chemical analysis so far as labeled fat in the stools is concerned. However, doubts have been expressed about the stability of the iodine label in the intestinal lumen (C13). With some methods difficulty may arise from contamination of stools with urine. The evaluation of over-all fat absorption from iodine levels in blood or urine (W1) cannot be recommended. Other forms of label, such as C^{13}, have been used by some investigators (B14), but further experience in these methods is still required. There is a need for an effective method using ingestion of labeled fat, but complete reliance cannot be placed on the methods available at the present time. Various microscopic methods of estimating fecal fat have been suggested from time to time (D3, D8, P9); they cannot be expected to give reliable quantitative results. In general, it is wise to keep to the method of complete stool collection and chemical analysis of contained long-chain fats. This provides the information on fecal fat output required for diagnosis and for the assessment of the effects of therapy or the administration of possible deleterious agents, as described later. For these purposes differentiation between dietary and endogenous fat in the stool is not essential.

2.2. Definitive Diagnosis

2.2.1. *Pancreatic Enzymes and Bile Salts*

The presence or absence of pancreatic enzymes can only be satisfactorily decided by intraduodenal intubation and direct examination of samples of small intestinal contents after the administration of a suitable stimulus to pancreatic secretion (F11). It is not sufficient to look at one enzyme only, such as trypsin, since a specific deficiency of lipase can occur (S11). Assessment of the degree of hydrolysis of fat in the stools is quite unreliable as a guide to pancreatic enzyme activity (C10).

Bile salt deficiency must also be directly studied. It may occur in the absence of obstruction or obvious liver disease (R7). The majority of patients with one form or another of the sprue syndrome will be found to have pancreatic enzymes and bile salts within the normal range. Pancreatic enzymes are absent or markedly deficient in patients with pancreatogenous malabsorption syndrome (B17, F13). It is surprising how frequently this necessary step in differential diagnosis is omitted.

2.2.2. *The Rate of Absorption of Index Substances*

The study of the level of absorbed substances in the blood or in the urine may provide a measure of the rate of absorption from the small

intestine. Such methods do not give an assessment of over-all absorption. The absorptive area studied in these tests is usually the upper part of the small intestine. The results can be affected by delayed emptying of the stomach, by destruction of the index substance, or by changes in the rate of its removal from the blood stream during the investigation. It is hardly surprising, therefore, that results obtained are often difficult to interpret.

2.2.2.1. *Glucose Tolerance Curves.* Glucose tolerance curves have been used for many years as a measure of the rate of absorption of glucose from the small intestine. Delay in gastric emptying can be obviated by intraduodenal administration (F25). The main disadvantage of the glucose tolerance curve is that glucose is a normal body constituent and that its level in the blood may vary independently of absorption. Furthermore, the rate of removal of glucose from the blood stream is liable to change during the test. In the malabsorption syndrome associated with enteropathy, the glucose tolerance curve indicates delay and depression in the rate of absorption; in pancreatogenous malabsorption, the glucose tolerance curve is commonly normal (F13, F25, G1, R1).

2.2.2.2. *Xylose Absorption Test.* D-Xylose has the advantage over glucose that it does not occur in the body, it is slowly metabolized and a substantial amount of an administered dose (30% or more) is normally passed out in the urine in 5 hours (B6, B20, B24, C6, F4, H4, L1): a 5 g dose is the most satisfactory (S3). However, the xylose absorption test is not without its problems. The intestinal flora may rapidly destroy xylose; this may sometimes account for unexpectedly low recovery. There may also be poor renal clearance of xylose; this is said not to be the explanation of the poor excretion observed in older patients by Fowler and Cooke (F10). The extent of renal clearance is readily checked by estimating the blood level of xylose; this may be done more rapidly if the glucose is removed with glucose oxidase (R3). Xylose recovery may be low in cirrhotic patients according to Shamma'A and Ghazanfar (S10), although this was not found by Fowler and Cooke (F10). Nevertheless, the xylose absorption test plays an important part in the diagnosis of enteropathy. In malabsorption syndrome with enteropathy affecting the upper small intestine, the amount of xylose recovered from the urine in 5 hours is markedly reduced; in the majority of patients with pancreatogenous malabsorption, xylose excretion is within normal limits.

2.2.2.3. *Fat.* The alimentary hyperlipemia following the administration of a standard fat-containing meal can be measured by various

methods (F23, H7, K1). They give an indication of the rate of absorption of glyceride fat. In the malabsorption syndrome with enteropathy, glyceride absorption is delayed and the rate is depressed; in pancreatogenous malabsorption the alimentary hyperlipemia does not occur unless finely emulsified fat is ingested (F18). The use of fat has the same disadvantage as that observed with glucose; as a normal blood constituent, it may vary independently of absorption. Distinction can be made between absorbed and endogenous fat by the study of the absorption of I^{131}-labeled fats. An ingenious method of differentiating between malabsorption of enterogenous and pancreatogenous origin is to feed I^{131}-labeled oleic acid or I^{131}-labeled triolein (D9, K2). Both tend to be depressed and delayed in enterogenous malabsorption, whereas absorption of triolein is markedly depressed, but oleic acid absorption is normal in pancreatic lipase deficiency.

2.2.2.4. *Vitamin A.* To overcome the difficulty of identification, vitamin A can be used as a label. The changes in the blood vitamin A curve following the administration of vitamin A in oil provide similar information to that given by the chylomicrograph. In malabsorption due to enteropathy the curve is depressed and delayed, and in pancreatic lipase deficiency it is markedly flattened. The use of vitamin A in oil and in aqueous dispersion is similar in principle to the use of labeled triolein and oleic acid (B4, G3, L4).

2.2.2.5. *Other Substances.* Several other index substances (W8), such as carotene, have been used by various workers in this field. There does not seem to be any particular advantage in these other methods. The use of one good water-soluble index substance, such as xylose, and one fat-soluble index substance, such as I^{131} fat or vitamin A in oil, is sufficient for diagnostic purposes.

2.2.3. *Radiography*

Radiographic study of the small intestine may provide useful information (A6, F12, F14, K4, L2, M2, P3). If a simple suspension of barium sulfate is used, the upper small intestine of a normal person usually displays a fine feathery appearance; a similar pattern is seen in most patients with pancreatogenous malabsorption. In the patient with enteropathy, however, the opaque medium appears in massive clumps. This was at one time thought to be due to vitamin deficiencies, but it was demonstrated experimentally that the cause was flocculation of the barium sulfate with excessive secretion of mucus. This clumped appearance may be seen in normal children, possibly due to the

difficulty of providing a long enough preparatory period, and sometimes in older people due to faulty preparation or to the inclusion of mucus-stimulating substances with the opaque medium. If a nonflocculable preparation of barium sulfate is used, no clumping occurs, but an abnormal dilated appearance of the small intestine may be revealed, giving a "ladder" or "stacked coin" pattern (A10, A13, F24). No such appearance is observed in most patients with pancreatogenous malabsorption (F16, H6). The pattern in the sprue group is due to atony, dilation, and possibly edema of the small intestine. Radiographic studies also enable a condition such as regional enteritis to be directly diagnosed if constriction of the lumen or fistula formation is present.

2.2.4. *Small Intestinal Biopsy*

There are now many devices available for the peroral biopsy of the small intestinal mucous membrane following the pioneer work of Shiner (S12) and of Crosby and Kugler (C15). The type of apparatus used will depend upon the nature of the investigations being undertaken. For some purposes it is helpful to be able to cut several pieces of mucous membrane which are delivered to the operator before the tube is withdrawn (B2, F7). On the other hand, for purely diagnostic purposes the Crosby and Kugler capsule may be ideal, since it is relatively easy to swallow. Extensive studies of the small intestinal mucosa have now been carried out. As predicted by Paulley in 1954 (P5) the mucosa has been shown to be abnormal in many patients with the malabsorption syndrome (B15, B23, C16, D2, D7, P10, R8, S14, S15, S20, T4, Y1), but it is usually normal in patients with pancreatogenous malabsorption. The changes in the sprue syndrome may consist of shortening and clubbing of the villi with infiltration of the mucous membrane by lymphocytes and plasma cells. In apparently more severe damage, the villi may be stuck together. In the patient with severe malabsorption syndrome the normal structure of the mucous membrane becomes practically obliterated. This condition is commonly called "villous atrophy." This term may be misleading, since many absorbing cells can be seen buried in the substance of the disorganized mucosa. The identities of the various cell types in the damaged mucosa have been well established by Padykula *et al.* (P1) using histochemical methods of differentiation. They have demonstrated that the more superficial "crypts" are lined by villous epithelium and that the peculiar layer of cells so frequently seen covering the luminar surface of the damaged mucosa are relatively undifferentiated cells that can be readily distinguished from the normal villous epithelium.

It has been largely assumed that villous atrophy would mean gross interference with absorption, since the villus is the essential absorbing unit. However, if the crypts are lined with absorbing cells and if, as seems likely, the crypts open into the lumen, there is no reason why the intestine exhibiting "villous atrophy" should not carry out effective absorptive functions. The shortened, clubbed villus, which looks less severely damaged, might not be any more efficient than those seen in villous atrophy. These features of the mucosal damage, and also differences in the area and amount of the intestinal mucosa involved, may well account for the apparently poor correlation between the severity of the damage and the degree of absorptive incapacity. Fone *et al.* (F9) have claimed that villous atrophy and "clubbing and infiltration of villi" represent two etiologically distinct conditions. This view is not supported by Girdwood *et al.* (G6). It is indeed difficult to see how this could be true. In patients showing complete recovery on a gluten-free diet, villous atrophy may change back to apparently normal villi; when gluten is reintroduced mucosal damage begins to occur and the first changes are likely to be indistinguishable from the appearance of "clubbing and infiltration"; it must be presumed that continuation with gluten in such a patient would eventually bring back the original state of villous atrophy. While it is clear that several factors may be involved in the production of the histological changes in the sprue group, there does not seem to be any satisfactory evidence at the present time to justify attempts at differentiation on histological grounds. While the picture of villous atrophy is fairly distinctive and strongly suggestive that the patient is suffering from enteropathy of the sprue group type, clubbing and infiltration of the villi may occur, perhaps, as an earlier stage of this same process or as a result of infestation (R5), or due to the action of neomycin (J3) and probably may result from many other factors not yet defined.

Histochemical studies indicate a decrease in enzymes in the mucosal cell and electron microscopy shows disappearance of microvilli and other changes (A12, B16, H2, S16, S19, Z1). Further studies are needed before the importance of such changes can be adequately assessed. It is claimed that direct examination of the biopsy under the dissecting microscope may also be informative (H5). Clearly, there is much to be learnt about small intestinal biopsy material. At present histology combined with study under the dissecting microscope appears to be the most useful approach for diagnostic purposes. Various abnormalities of the mucous membrane may be expected in the sprue group, while the majority of patients with pancreatogenous malabsorp-

tion will have a normal mucosa. Peroral biopsy of the small intestine mucosa must now be regarded as a routine diagnostic procedure in this field.

2.3. Summary of Differential Diagnosis of Pancreatogenous and Enterogenous Malabsorption

The main points in differential diagnosis between the sprue group and pancreatogenous malabsorption are summarized in Table 1. Occa-

TABLE 1

A Guide to Differential Diagnosis of Pancreatogenous and Enterogenous ("Sprue Group") Malabsorption

	Normal	Pancreatogenous Malabsorption	"Sprue group"
Fecal fat (g/day)	< 5	>50	> 10
Pancreatic enzymes	Normal	Deficient	Normal
Absorption rate			
Glucose (mg/100 ml increment)	> 40	>40	< 40
Xylose (% in urine in 5 hours after 5 g orally)	> 30	>30	< 30
Fat (Maximum chylomicrons per standard field after 30 g fat)	>150	<25	<100
Vitamin A curve	Normal	Flat	Depressed
Radiography			
Flocculable media pattern	Feathery	Feathery	Clumped
Nonflocculable media pattern	Feathery	Feathery	Dilated; "ladder" appearance
Biopsy			
Small intestinal mucosa	Normal	Normal	Abnormal

sionally patients are seen in whom enteropathy and pancreatic enzyme deficiency are associated, in which case a mixed picture is seen.

3. Differentiation of the "Sprue Group"

The syndrome observed and the changes demonstrable in the intestinal mucosa are similar in celiac disease, idiopathic steatorrhea (nontropical sprue), and tropical sprue. This has led some investigators to conclude that these 3 conditions are manifestations of the same underlying fault (A2). There are, however, sound reasons for doubting the homogeneity of the sprue group (F19). The arguments in favor of differences in etiology can be conveniently developed from con-

sideration of 4 important features which will be seen to have different etiological significance in the various conditions that make up the sprue group. These common features are electrolyte imbalance, wheat gluten intolerance, folic acid and cyanocobalamin deficiency, and the effects of intestinal flora.

3.1. Electrolytic Imbalance

3.1.1. *Water and Salt Balance*

Chronic diarrhea is likely to result in a disturbance of water and salt balance. This has been shown to be so in the malabsorption syndrome (C9, F5). These changes, especially potassium deficiency, may cause some aggravation of the intestinal situation and increase of abdominal distension. Some improvement in absorption may consequently occur on appropriate rehabilitation. However, a residual defect of absorption will remain until more specific therapy is instituted.

3.1.2. *Adrenal Cortical Function*

Adrenal cortical insufficiency was suggested by Verzár (V6) as a possible basic fault in the sprue group. However, studies in sprue (P2) and in idiopathic steatorrhea (nontropical sprue) (B5) have shown that the adrenal cortex responds normally to stimulation, although there is some evidence of adrenal cortical insufficiency in some patients of the sprue group (D6), and some of the signs and symptoms resemble those seen in Addison's disease (L3). This effect may have its origin in anterior pituitary deficiency (M6); growth and sexual development may also be retarded in young patients. Treatment with a gluten-free diet in appropriate patients causes these deficiency effects to disappear; the endocrinological effects appear to be secondary to the action of gluten in this group of patients. Corticosteroid treatment of patients with the malabsorption syndrome has been advocated (A1, L5). It is claimed that the patients show great improvement, but this has been questioned (B1, F3). In any case, the effect is only maintained while the steroids are being administered and relapse occurs rapidly after discontinuation of treatment. In view of the much safer and more effective lines of therapy available, the suggestion that corticosteroid therapy is the treatment of choice (M9) cannot be accepted. It may be concluded on present evidence that disturbance of water and salt balance, and adrenal cortical insufficiency are secondary phenomena in the sprue group and do not represent a basic underlying defect.

3.1.3. *Calcium and Magnesium*

Calcium and magnesium deficiency also occur in some patients with the malabsorption syndrome and this may lead to tetany or bone changes. Low blood calcium levels may result from decreased absorption associated with lack of effective compensatory parathyroid activity. In patients in whom secondary hyperparathyroidism is effective, extensive loss of calcium from the bones may occur. The cause of the defective absorption of calcium in patients of the sprue group is complex and not yet fully understood (B3, D1, J1, M7, N1). It is important that complications such as calcium or magnesium deficiency should be corrected before the final steps of definitive diagnosis are attempted. If this is not done, the secondary effects may obscure the results of other tests.

3.2. Wheat Gluten Intolerance

To differentiate the constituent members of the sprue group, it is necessary to consider 2 effects. First, the effect of a wheat gluten-free diet and second, the effects of reintroduction of gluten after a period of remission on a gluten-free regimen.

3.2.1. *The Effect of a Gluten-free Diet*

A strict gluten-free regimen involves the exclusion of many articles of the diet, in addition to the obvious ones such as bread, biscuits, and cakes; it is important that the gluten-free diet should be acceptable to the patient and nutritionally adequate. If a strict gluten-free regimen is instituted, 3 responses are possible: complete recovery, partial recovery, or no effect.

From early days the striking effect of a gluten-free diet on steatorrhea was apparent (A7, A8, F29, R7, V2). A number of investigators have maintained that the mucosal lesions in celiac disease and nontropical sprue are irreversible (R10, R11, R12, S13). The likelihood of small intestinal mucosal lesions being irreversible may be questioned on general biological grounds. Some of the early studies on recovery were not done on patients continuing on a gluten-free regimen; this invalidated them. Subsequent studies have shown that recovery can occur in both children and in adults (A5, F20, F28) with gluten-induced enteropathy, and in patients with tropical sprue in Hong Kong (F18). It is now firmly established that the mucosal lesion in celiac disease, idiopathic steatorrhea, and tropical sprue can be reversed.

It is true, however, that complete recovery does not always occur and an appreciable number of patients show improvement, but high

fecal fat levels and other abnormal features, which may include persistent mucosal damage, continue. Before irreversibility of this type is accepted, it is important that the patient should be kept continuously on a strict gluten-free diet for an adequate period; this may be for a year or longer. If significant abnormalities are still present after a year or more, the situation may be regarded as at least partly irreversible. There may be several reasons for this. First, the patient may not have been taking a strict gluten-free diet. Sometimes unusual foods may contain wheat flour and this may have resulted in accidental intake of gluten, or the patient may not have adhered to the diet. Many patients find it difficult to keep strictly to a diet, especially when it is something of a nuisance. It may be significant that some patients of this type show definite improvement each time they come into hospital and are brought under closer dietetic control. The possibility that small amounts of gluten are causing the persistence of abnormalities cannot be disregarded in a great many of these patients. Cameron *et al.* (C1) claim that the strictness of the gluten-free regimen is not a factor in determining complete or partial recovery. One may, however, have some doubt about the control of any diet outside a metabolic ward. If a strict gluten-free regimen is being maintained and abnormalities still persist, it must be concluded that some other etiological factor is involved in addition to wheat gluten. For the purposes of this discussion the patients can be considered in 2 groups—those that recover completely on a gluten-free diet and those that only show partial recovery. Patients that show no beneficial effect on a gluten-free diet are not relevant to this discussion.

3.2.2. *The Effect of Reintroduction of Gluten into the Diet of Patients in Remission*

The effect of reintroduction of gluten is readily demonstrated in patients who show complete recovery; it is difficult to study, however, in those that do not return to normal. In the first group the reintroduction of wheat gluten or appropriate fractions has been shown to cause many effects including steatorrhea. The other effects of reintroduction of gluten will be discussed in detail later.

The change in daily output of fecal fat forms the basis for the definitive diagnostic test for *gluten induced enteropathy* (F16, F21). This diagnosis is justified if the patient presented with the main features of the malabsorption syndrome, if the fecal fat output fell to normal levels on a gluten-free diet, and if subsequent reintroduction of gluten into the diet caused an unequivocal increase in fecal fat

to abnormal levels. The time taken for this increase to occur in such patients varies from 1–2 days to 1–2 weeks.

As already mentioned, it is difficult to assess the effect of reintroduction of gluten into patients that are only partially recovered, since there are commonly large daily fluctuations in the fecal fat level. For the present it seems wise to retain the term "idiopathic steatorrhea" to cover these patients and to accept the view that they may exhibit varying degrees of gluten intolerance.

Since intolerance to gluten may arise as a result of mucosal damage, it would not be surprising if other patients with conditions associated with changes in the mucosa might not also exhibit gluten intolerance. This may be the explanation of the improvement described in some chronic sprue patients on a gluten-free diet (C2) and a similar effect reported in a patient with regional enteritis (C4). A patient with mesenteric reticulosarcoma also showed great improvement, but in this case there may have been an underlying gluten-induced enteropathy (B13). The early sprue patients studied in Hong Kong do not appear to be affected by wheat gluten and on recovery they show no adverse response to a gluten-containing diet (F19). Most sprue patients in Puerto Rico seem to be unaffected by dietary gluten (A11).

3.3. Deficiency of Folic Acid and Cyanocobalamin

3.3.1. *Folic Acid*

Severe folic acid deficiency is an inconstant feature of patients with gluten-induced enteropathy (F26). It is generally regarded as unusual in children, but more common in adults. It seems to be essentially a late manifestation. The folic acid deficiency gives rise to all the usual features and can be readily demonstrated by various tests. If the patient is given folic acid, the signs of deficiency may clear up dramatically, but the steatorrhea is not substantially altered until a gluten-free regimen is instituted. If, however, a gluten-free diet is introduced first, the folic acid deficiency may show spontaneous remission without supplementation with folic acid. Folic acid deficiency does not appear to be a major factor in the development of gluten-induced enteropathy; it is secondary to the effect of gluten.

In tropical sprue, both in Hong Kong and in Puerto Rico, folic acid deficiency is a constant and early feature (B25, F18). Its importance in sprue was first demonstrated by Spies and his colleagues (S18) in the West Indies. However, it cannot be concluded that tropical sprue is simply due to dietary folic acid deficiency. In Hong Kong the condition may develop in a previously healthy individual on an adequate

diet within a few months of arriving in the area. Studies to show the beneficial effect of folic acid have been largely carried out in hospitalized patients; it is, thus, a double treatment and involves removal of the patient from the local environment associated with the development of the disease. Furthermore, folic acid therapy fails to cure a significant number of patients with sprue; in these people steatorrhea may continue indefinitely (F18). Complete recovery occurs in sprue patients from Hong Kong, but this does not seem to be achieved in Puerto Rico (G2, R6). Whereas the folic acid deficiency in gluten-induced enteropathy seems to be largely due to interference with absorption, there are some indications that there may be a fault in folic acid metabolism in tropical sprue (C7, F22). It is possible that a dietary factor, such as the products of oxidative rancidity of fats, may exert some action on conversion of folic to folinic acid (F19). Such an effect might explain the rapid development of folic acid deficiency in some patients in Hong Kong. This should prove to be a fruitful field for further investigation.

3.3.2. *Cyanocobalamin*

Deficiency of cyanocobalamin is not a common feature of gluten-induced enteropathy, possibly because the damage to the mucosa may be mainly duodenal and jejunal rather than ileal. It is not observed in patients with sprue in Hong Kong, probably because the history is usually too short for the depletion of normal cyanocobalamin stores. In some patients in the sprue group, however, it is possible to demonstrate interference with the absorption of isotopically labeled cyanocobalamin (F8, F30, G8, K3, O1). In such patients, it might be expected that cyanocobalamin deficiency would eventually occur and this is sometimes observed. The cause of the faulty absorption of cyanocobalamin may be due to lack of intrinsic factor, but more frequently the addition of intrinsic factor does not cause improvement in these patients (G7). Alternative causes of faulty absorption are ileal damage, bacterial action (H1), or interference with calcium absorption (G9). In long standing cases of sprue, cyanocobalamin deficiency is more common. It seems likely that this is due to interference with ileal function. It is interesting that a number of Hong Kong sprue patients with persistent steatorrhea have shown some abnormality of ileal function. Whatever may be the cause of cyanocobalamin deficiency in these patients, there seems to be little doubt that it is secondary to other factors.

3.4. Effects of Intestinal Flora

In the malabsorption syndrome some increase in the numbers and activity of intestinal bacteria in the lower bowel might be expected,

since there is an increase of the available food supply. This active flora seems to account for several of the properties of the classical stools of the malabsorption syndrome. Thus, the bulk, fluidity, pallor, offensive odor, and high volatile fatty acid content can be largely ascribed to bacterial action (F16). The long chain fats may also be modified in various ways (J4, S1). These effects are essentially secondary to the malabsorption, since they disappear when the enteropathy is corrected.

In some patients with tropical sprue, however, as already mentioned, steatorrhea may persist. In such patients folic acid, especially when given by mouth, may aggravate the steatorrhea. A dramatic cure is brought about by antibacterial therapy. The effect is thought to be due to action upon the flora, but this has not yet been conclusively proved. Although in some patients with malabsorption *Proteus* (C14) or *Giardia* (C11, W2), or other abnormal organisms may play some part in maintaining steatorrhea, there is, at present, no evidence of abnormal organisms in sprue patients with persistent steatorrhea. The precise cause of the persistent steatorrhea remains unknown.

The type of antibacterial therapy used is important. No effect need be expected from the use of a single chemotherapeutic agent. Successful use has been made of a triple combination of sulfonamide, chloramphenicol, and chlortetracycline (A8). It would seem advisable to avoid neomycin (F1, J2), since this can itself cause a malabsorption syndrome.

3.5. Summary of Differentiation of the "Sprue Group"

The main points of differentiation are summarized in Table 2. It will be seen that the group can be divided up into gluten-induced enteropathy, "acute sprue," "chronic sprue" and idiopathic steatorrhea. As the cause of the sudden development of severe folic acid deficiency

TABLE 2
Summary of Etiological Factors in the "Sprue group"

	Gluten-induced enteropathy	"Acute sprue"	"Chronic sprue"	Idiopathic steatorrhea
Electrolyte imbalance	(±)	(±)	(±)	(±)
Wheat gluten intolerance	++	—	(±)	—
Folic acid deficiency	(±)	++	(±)	(±)
Floral changes	(±)	(±)	++	(±)
Other factors (possibly dietary)	—	?+	—	?+

++, Major etiological factor. —, No significance.
(±), Secondary factor. ?+, Possible etiological factor.

in acute sprue is not known and the mechanism of action of the flora in chronic sprue is equally obscure, it seems advisable to keep to these terms acute sprue and chronic sprue for the time being. There is no fundamental reason why sprue should be confined to, or why gluten-induced enteropathy should not occur in, the tropics. Geographical classifications tend to be artificial, but it seems inadvisable to attempt to alter these terms until something better, founded on a clearer understanding of etiology, can be put in their place.

4. The Mechanism of the Effects of Gluten in the Malabsorption Syndrome

Two types of patient will be considered: first, those that are suffering from gluten-induced enteropathy as already defined; second, those that do not conform to the criteria required for the diagnosis of gluten-induced enteropathy, but are gluten-intolerant in that they show some improvement on a gluten-free diet.

4.1. Effects in Patients with Gluten-induced Enteropathy

A fruitful field for investigation is the study of the effects of reintroducing gluten after remission of all signs and symptoms has been achieved on a strict gluten-free diet. This is a necessary step in the diagnostic procedure. The main effects that have been examined are steatorrhea, radiographic changes, mucosal damage, increase in blood peptides, increase in titer of circulating antibodies, complement fixation, change in growth rate, anorexia, and vasomotor effects. In addition, gluten and certain fractions have been shown to inhibit the peristaltic reflex in isolated animal preparations. These various effects of gluten will be considered in detail.

4.1.1. *Steatorrhea*

The daily output of fecal fat may be regarded as the simplest quantitative measure of the effect of gluten on these patients. The evidence available suggests that the extra fat is derived from dietary fat and mainly represents interference with absorption. There is usually a disproportionate increase of saturated as compared with unsaturated fats (W4). The reason for this is not clear. There may be selective rejection of longer chain and more saturated fats; there may also be increased hydrogenation of unsaturated fatty acids (S1). The increase of fat output may occur within a day or 2, or it may be delayed for 10 days or more. There are many possible explanations for this delay. It may require many small insults to the mucous membrane

before the stage of interference with absorption is reached; alternatively, the delay might be related to the building up of the antibody titer; again, the lag period might be dependent upon changes in the intestinal flora. There is a need for further work on the steatorrhea response to determine more precisely the mechanism involved. This, in turn, might help to elucidate the mode of action of gluten.

4.1.2. *Radiographic Changes*

Return of the characteristic clumping pattern and dilatation on reintroduction of gluten was reported in early studies (A7). The phenomenon was studied in greater detail by Breton *et al.* (B19), who showed that gluten caused increased mucous secretion and clumping in patients with gluten-induced enteropathy, but not in normal individuals, or those with other forms of the malabsorption syndrome. This would seem to indicate an irritant effect of gluten on the intestinal wall in these patients. Increased mucous secretion is also observed in patients with tropical sprue, in whom gluten intolerance is not a major factor.

4.1.3. *Mucosal Damage*

On reintroduction of gluten into the small intestine of a patient with gluten-induced enteropathy in complete remission, some signs of damage to the mucous membrane were observed, but at present the evidence is not completely convincing. The attempt to demonstrate early damage and edema of the mucosa by histological methods is far from satisfactory. Rubin (R9) has demonstrated damage to the lower end of the ileum when gluten was introduced in a patient with a damaged upper intestine, but previously normal ileum. The production of steatorrhea by gluten is accompanied by decreased recovery of xylose. It seems probable that the effects on absorption that can be demonstrated when gluten is reintroduced into the diet in these patients is accompanied by mucosal damage. The effect of gluten on the mucosa might be a direct toxic effect on the intestinal cells, or it might be due to some form of antigen/antibody reaction (F15). It is not possible at the present time to decide between these 2 alternatives; indeed, both may occur.

There are differing reports on the effect of gluten on the small intestine of the rat. Ribeiro *et al.* (R2) claim that more fat and nitrogen are passed by a rat on a gluten-containing than on the gluten-free diet; this may have been partly due to differences in food intake. However, Althausen and Grodsky (A3) were unable to

show any deleterious effect from feeding a high dosage level of wheat gluten to rats. In any case, there is no doubt that in the normal animal or human subject wheat gluten has a negligible effect, compared with the patient with gluten-induced enteropathy in whom quite small doses of gluten cause major pathological changes. There seems to be little doubt from the demonstration of an increased titer of circulating antibodies, the occurrence of complement fixation, and the heavy infiltration of the intestinal wall with plasma cells, that antibody formation is probably occurring at an enhanced rate in this group of patients as compared with normal subjects. However, this does not mean that antibody formation contributes to pathogenesis. The antigen/antibody reactions might be a concomitant of mucosal damage and might play no part either in the causation or the maintenance of damage. The beneficial effect of corticosteroids might suggest that an antigen/antibody reaction contributes to steatorrhea production, but it could equally well be due to an anti-injury or anti-edema effect. If an antigen/antibody reaction does play a part in the pathogenesis of the mucosal lesions in gluten-induced enteropathy, the damaging effect would appear to be largely confined to the small intestine, since skin tests appear to be negative (C8) and incidence of other allergic reactions is not increased in these patients (F15).

4.1.4. *Increase in Blood Peptides*

Weijers and van de Kamer (W6, W7) were the first to show that there was an abnormal blood peptide curve after the administration of gluten or gliadin to patients with celiac disease. The increase of bound glutamine in the blood was greater in celiac than in normal children. Since it is known that the absorption of all index substances is delayed and decreased in these patients, it was apparent that the increased blood level was probably due to faulty handling of the peptides concerned. This phenomenon has also been observed by other investigators (A4, F17, G11, P7, S5, V7). However, Gerrard *et al.* (G4, M1) could not demonstrate changes in blood glutamine, although they demonstrated a significant increase in urinary glutamic acid in celiac patients following gliadin administration. The differentiation of the bound-glutamine curves in celiac and normal children was not clear-cut and there are indications that the phenomenon may occur in other forms of intestinal disease. There is no evidence that the glutamine-containing peptides found in greater quantity in the blood have any deleterious effects. It may, however, be concluded that these observations provide some evidence for a faulty barrier function of the intes-

tinal mucosa in these patients. It is of interest that several enzymes have been shown to be deficient in damaged mucosa, and in tropical sprue sucrase may be sufficiently reduced in the mucosa to allow sucremia to occur (S2, S4). The relationship between increased blood peptides and mucosal damage has not yet been satisfactorily defined. If the penetration of peptides is still found in patients with histologically normal mucosa, it would be suggestive evidence of the existence of some basic enzyme defect; however, peptide penetration may simply be one of the manifestations of mucosal damage.

4.1.5. *Increase in the Titer of Circulating Antibodies*

Using the tanned red cell technique, Taylor *et al.* (T1) have shown that there is an increase in the titer of circulating antibodies to gluten and certain gluten fractions if they are administered to patients with the malabsorption syndrome as compared with subjects who are not gluten intolerant. Some of these patients also showed an increase in the titer of circulating antibodies to certain milk proteins. Unfortunately, the precise status of the patients studied is not recorded, so that it is not known whether they were suffering from gluten-induced enteropathy or whether they should be placed in the idiopathic steatorrhea category with or without gluten intolerance. It is also uncertain whether the mucous membrane was damaged or recovered. Since many of the patients were probably not on a strict gluten-free regimen, mucosal damage may be presumed. However, precise information on these points is important if any serious attempt is to be made to decide whether the increase in titer of circulating antibodies is simply an expression of a faulty barrier function of the mucosa, or whether it has some greater significance in the causation and/or maintenance of the syndrome. It would be particularly interesting to know whether the pattern of circulating antibodies is different in patients with gluten-induced enteropathy in whom other food proteins seem to have no etiological significance and in those with idiopathic steatorrhea in whom gluten intolerance may not be the complete answer.

4.1.6. *Complement Fixation*

Berger (B8, B9) showed that both normal and celiac children form antibodies to a number of food proteins. These antibodies did not cause any deleterious effect in most of these individuals, but it is claimed that they have a different form in celiac children, in whom some antigens may assume a damaging role. In the celiac child certain cereals such as wheat gliadin have been shown to cause a reduction in

other circulating antibodies; this is claimed to indicate complement fixation by the gliadin. This effect of gliadin was unaltered by peptic/tryptic digestion, but it was abolished by incubation with intestinal mucous membrane extract (B10). There is no reason why the observations reported should not be a result, rather than the cause, of mucosal damage. The relationship of these reactions to the status of the individual patient and the situation in the small intestine needs to be clarified before their significance can be interpreted.

4.1.7. *Changes in Rate of Growth*

It is well known that celiac children often show stunted growth. Sheldon (S11) has studied this and demonstrated the dramatic effects on growth rate of the removal of gluten from the diet and its reintroduction. The effect on growth occurs more quickly than any demonstrable change of gastrointestinal function (F17). While interference with nutrients probably plays some part in the over-all effect on growth, there seems to be evidence for a more direct effect of gluten. It is conceivable that this may link up with the suggestion that there is interference with anterior pituitary function (M6) in some of these patients. It is a fact that many patients with celiac disease show decreased activity of the adrenal cortex (D6), depressed growth rate, and delayed sexual development, both male and female. All these effects are modified by the establishment of a gluten-free regimen. This is an aspect of gluten-induced enteropathy that might be more extensively studied.

4.1.8. *Anorexia*

Loss of appetite is a striking feature in children with gluten-induced enteropathy and it largely accounts for wasting and weight loss. Patients with pancreatic enzyme deficiency often lose more calories in the stools, but maintain weight well by increasing calorie intake. Severe anorexia also occurs in patients with tropical sprue, but in this condition the loss of appetite appears to be closely linked to folic acid deficiency. Anorexia is one of the first effects observed in patients with gluten-induced enteropathy when gluten is reintroduced into the diet. Appetite seems to return more quickly than demonstrable improvement in the intestinal mucosa or regression of other gastrointestinal signs and symptoms, and anorexia is induced by the reintroduction of gluten into the diet before there is demonstrable steatorrhea. However, the level of intestinal change required to bring about a reflex anorexia is unknown. Anorexia may be accompanied by nausea and vomiting.

4.1.9. *Vasomotor Changes*

Vasomotor changes also occur when gluten is reintroduced. The patient may feel hot, show flushing or pallor, or have attacks of sweating. These have been reported by a number of observers (H8, K5, K8, W5). Detailed studies on the precise nature and/or cause of these vasomotor effects do not appear to have been carried out. They have sometimes been referred to as "gliadin shock." They might repay further study.

4.1.10. *Effect on the Peristaltic Reflex*

The evidence on the motility changes in these patients is rather conflicting. There is no doubt that the intestine is usually dilated and it is common experience that movements in the upper intestine after administration of a barium meal are greatly reduced as compared with normal subjects. Hypomotility was demonstrated in patients with the malabsorption syndrome by direct methods many years ago (M3). The radiographic appearances of the intestine may return to normal on a gluten-free diet and the reintroduction of gluten results in the reappearance of dilatation. Ardran *et al.* (A9) were unable to demonstrate any difference in the pattern of movement in patients with malabsorption syndrome and normal subjects using short periods of cineradiography. However, these patients were not given gluten and they were not all proved cases of gluten-induced enteropathy. It is not known what part hypomotility may play in the pathogenesis of the malabsorption syndrome. Some studies suggest that hypomotility per se does not give rise to a malabsorption syndrome (C17). However, Paulley *et al.* (P6) have shown recently that induced hypomotility may lead to changes in intestinal flora. There may be a great difference in the effect of hypomotility on a normal intestine and one in which a considerable area is damaged; again, the time relationships between the induction of hypomotility and the development of malabsorption may be critical. There is clearly a need for more work in this field.

Schneider *et al.* (S6, S7, S8) have demonstrated that wheat gluten and certain gluten fractions cause inhibition of the isolated small intestine of the rat. In the Trendelenburg preparation, as modified by Bülbring *et al.* (B21), inhibition of the peristaltic reflex occurred if the material was placed outside the intestine so that it could reach the muscle without traversing the mucous membrane. If it was placed inside the lumen no inhibitory effect was seen. If the gluten or gluten fractions were incubated with rat mucous membrane extract, complete inactivation of the inhibitory effect was obtained. This action of mucous

membrane extracts was thermolabile. Further analysis of the inhibitory effect on the peristaltic reflex revealed that inhibition also occurred in the coaxially stimulated guinea pig ileum preparation (P4). This suggested that the effect might be due to interference with acetylcholine release. This was shown to be so by direct estimation of liberated acetylcholine. The fraction obtained after incubation with mucous membrane extract did not cause any reduction of acetylcholine release.

The inhibitory effect on the peristaltic reflex in the rat has been used as an assay system to help in further fractionation and purification of this agent in wheat gluten, and it has also been used for the study of human intestinal biopsy material (S9). Intestinal mucous membrane extracts from normal subjects rapidly inactivated the peristaltic inhibitory factor. Inactivation was also obtained with mucous membrane from patients with malabsorption not associated with gluten intolerance. Patients with gluten-induced enteropathy with damaged mucous membrane did not inactivate the peristaltic inhibitory factor within 20 minutes, the limit of the time period so far studied. When the mucosa recovered, however, the ability to inactivate the antiperistaltic factor returned. In spite of this, the patient showed other effects when gluten was reintroduced, including steatorrhea. This suggests that the induction of hypomotility may not be the key factor in the induction of steatorrhea when gluten is reintroduced into the diet in a patient with gluten-induced enteropathy in remission. These effects are still under close study and no conclusions about their significance in relation to the whole syndrome can be reached at the present time.

4.2. Effects in Patients with Idiopathic Steatorrhea and Tropical Sprue

In some patients with idiopathic steatorrhea (nontropical sprue) a gluten-free diet did not appear to result in a return to normal. There might be some improvement, but steatorrhea and other features of the syndrome, including mucosal damage, might persist. As already discussed, this might be due to failure to establish a *strict* wheat gluten-free diet. It might also be due to other cereals, such as rye or some varieties of oats, if these were not satisfactorily controlled. If, however, the strictness of the gluten-free diet could be guaranteed and the patient still failed to return to normal, it must be concluded that some other factor was concerned in the maintenance of the syndrome. It has been suggested by a number of people that sensitivity to other food materials might be involved (C5, K9). It is known that circulating antibodies

to various food proteins occur in normal people as well as patients with the malabsorption syndrome, although in the latter the titer may be higher. It has also been claimed that the exclusion of other foods, especially milk products, may result in great improvement in some of these resistant patients. This evidence is not conclusive, however. It is necessary to reintroduce the particular articles of diet after a period of remission and demonstrate that they can cause the steatorrhea and other persistent features. There are other possible explanations of the beneficial effect of removal of milk products. Thus, it has been shown that steatorrhea can be improved by exchanging saturated for more unsaturated fatty acids (F2). This might occur when milk fats are replaced by other fats. It is also possible that the change of diet may influence the intestinal flora. It is of interest that lamblia have a requirement for glutamic acid, so that a gluten-free diet might be expected to have some beneficial effect in giardiasis (V1). It would seem unwise at present to attempt to arrive at any conclusion about the possible contribution that sensitivity to other food materials might make to the maintenance of mucosal damage and steatorrhea.

In tropical sprue, gluten does not appear to play much part in relatively early cases, such as those studied in Hong Kong. There is evidence, however, that patients with chronic sprue of many years' duration may show some improvement on a gluten-free diet (C2). This may be a secondary phenomenon, due to general reduction of the enzymes in the small intestinal mucosa; other mucosal enzymes have been shown to be reduced or ineffective in these patients. The important question is whether such patients will still react to gluten when the mucous membrane is brought back to normal by appropriate therapy, if this can be achieved. The rehabilitation of a malnutritional state induced over a period of many years is an extremely complex problem.

4.3. The Nature of the Deleterious Agent(s) in Wheat Gluten

4.3.1. *Other Cereals*

Early studies following the pioneering observations of Dicke (1950) (D4) indicated that wheat and rye gluten occupied a special place in the pathogenesis of celiac disease (A7, D5). The Dutch group found that oats were deleterious (V2), but this was not found to be so in Britain (M11, R7). The reason for this discrepancy remains a mystery. Patients with gluten-induced enteropathy appear to tolerate all other foods well, once the enteropathy has been brought under control. Chemical treatment of gluten with flour improvers is not responsible

for this effect in celiac patients; extensive studies have been made using untreated wheat gluten.

4.3.2. *Fractionation of Wheat Gluten*

Complete acid hydrolysis of the gluten results in loss of its deleterious properties. Deamidation by acid or by treatment with papain (K6) also markedly reduces its toxic action on patients with gluten-induced enteropathy. However, peptic/tryptic digestion does not significantly reduce toxicity (F27). This might be expected from the fact that

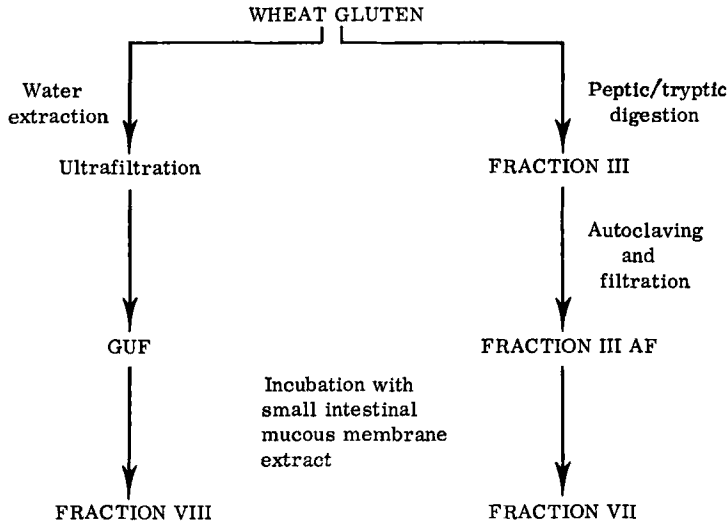

FIG. 1. Some fractions of wheat gluten used for the study of the deleterious action in gluten-induced enteropathy.

patients with gluten-induced enteropathy usually have normal gastric and pancreatic enzymes (F14, M4, T2). The peptic/tryptic hydrolyzate (Fraction III) can be autoclaved and filtered to give Fraction III AF without loss of toxicity. It can also be treated with performic acid to render the peptide substantially a straight chain compound without apparent loss of potency (V4, V5). If, however, the peptic/tryptic hydrolyzate is treated with an extract of pig intestinal mucous membrane, a new fraction is obtained (Fraction VII) which is no longer deleterious. This modification does not involve deamidation. Other types of fractionation have been used. Thus, gliadin, which is an extract of gluten with 70% ethanol, has the same potency as gluten. An aqueous extract can be made from gluten and ultrafiltered (Fraction GUF), which has marked antiperistaltic potency. If this is incubated

TABLE 3

THE EFFECTS OF WHEAT GLUTEN AND WHEAT GLUTEN FRACTIONS ON PATIENTS WITH GLUTEN-INDUCED ENTEROPATHY AND ISOLATED TISSUE PREPARATIONS[a]

	Gluten or gliadin	Fraction III	III AF	VII	GUF	VIII
Response of gluten-sensitive guinea pig tissues	+	+	—	0	0	0
Steatorrhea in patients with gluten-induced enteropathy	+	+	+	—	—	0
Inhibitory effect on peristaltic reflex in isolated rat intestine	+	+	+	—	+	—
Increase of blood peptides	+	+	0	0	0	0
Complement fixation	+	+	0	—	0	0
Increased titer of circulating antibodies	+	+	0	0	0	0

+, Positive response. —, Negative response. 0, Not yet tested.

[a] For details of gluten fractions see Fig. 1.

with intestinal mucosal extract a further fraction is obtained (Fraction VIII), which is comparable to Fraction VII in the other fractionation series. The origin of these main fractions is indicated in Fig. 1.

The effects of these various fractions have been or are being studied. The main results available at the present time are shown in Table 3. It will be seen that Fraction III still caused a response in the tissues of a gluten-sensitized guinea pig, but this effect disappeared in Fraction III AF after autoclaving and filtration. Since Fraction III AF was active in patients with gluten-induced enteropathy, this suggested that simple sensitization to gluten was not the cause of the deleterious effect. There was also a difference between Fraction III AF and Fraction GUF in that the latter did not cause steatorrhea at the dosage level or within the time interval that such a response occurred with Fraction III AF. This suggested that the antiperistaltic factor and the steatorrhea-inducing factor might be separate entities. Finally it was apparent that incubation with small intestinal mucous membrane caused inactivation of all the deleterious effects so far studied. Further investigation along these lines should provide valuable information and help in the final elucidation of the nature of the deleterious agent or agents in gluten.

4.3.3. *Chemical Nature*

The most recent studies on the nature of the antiperistaltic factor suggested that it was a small peptide, or possibly glycopeptide (R4). It might be a group of substances rather than a single substance. The possibility of using the antiperistaltic assay system as a means of detecting active fractions has greatly facilitated the isolation and identification of active factors. So long as the only means of identification is the demonstration of a deleterious effect in patients, progress is likely to remain slow.

4.4. Why Do Patients with Gluten-induced Enteropathy React to Gluten?

4.4.1. *An Enzyme Defect?*

It is apparent that patients with gluten-induced enteropathy respond to the administration of gluten differently from other people. It is obvious from consideration of the evidence already presented that the reason for this may lie in inadequacy of the small intestinal mucosal barrier. It is clear that the intestinal mucosa or extracts of it will inactivate the deleterious agent(s) in gluten; this is a thermolabile reaction, presumably enzymatic in nature. Patients with gluten-induced

enteropathy have been shown not to inactivate one of the deleterious agents in gluten, the antiperistaltic factor, in an effective manner. Further evidence is awaited before it can be concluded that this is the mechanism by which the other possible deleterious agents in wheat gluten cause their effects. A peptidase deficiency has been suggested (B22, F17, K7). However, there does not appear to be a deficiency of the common peptidases in the mucosa of celiac children (M5). Such peptidases are, however, widely distributed and it seems more likely that some rather special enzyme needed for the handling of wheat gluten is defective. The suggestion that there is a deamidase deficiency (B11) does not fit in with the observation that inactivation with mucous membrane extract can occur without deamidation.

The inadequacy of the mucosal barrier to wheat gluten might be due to mucosal damage. Thus, it might be comparable to the reduction in other enzymes already demonstrated in some of these patients. If this is so, then the enzymes might be expected to return on recovery of the mucous membrane and gluten intolerance should disappear. This may be the case in some patients who can be regarded as having an acquired and transient gluten intolerance. This also may be true of the inactivating mechanism for the antiperistaltic factor, which may be depressed due to mucosal damage. The steatorrhea-producing effect, however, seems to be longer lasting. Even after many years on a gluten-free diet, a patient may respond to administration of gluten by the development of steatorrhea. It may be significant that many old patients alleged to be recovered do, in fact, display a wide range of abnormalities (G5, L6). It is possible that this indicates a residual sensitization of the tissues or survival of antibodies. On the other hand, it might be indicative of a basic enzyme defect which will always allow the damaging agent(s) in gluten to produce their effects. Such a defect might be genetically determined. It is of interest, therefore, that a number of investigators (B18, C3, T3) have concluded that there is some genetic basis in the incidence of celiac disease. A more precise classification of these patients into those with proven gluten-induced enteropathy and those with less well-defined syndromes might be helpful.

4.4.2. *Faulty Barrier Function; A Possible Vicious Circle Mechanism*

A summary of the etiological situation so far as present knowledge goes is illustrated in Fig. 2. The evidence suggests that mucosal damage is present and that this results in defective barrier action (F22). Consequently, various components of gluten pass through into the body

and bring about several effects. Some of these effects may play a part in the maintenance of mucosal damage and thus a vicious circle is established. This can only be broken by the introduction of a strict gluten-free regimen. Even though no other factor seems to be involved in the maintenance of gluten-induced enteropathy, it does not follow that there is a basic enzyme defect as shown at A; an alternative mechanism might be the initiation of mucosal damage by some other factor such as infection, as shown at B. Once the vicious circle involving gluten is established, it might continue even though the original "infection" has disappeared. One or other of these two possibilities

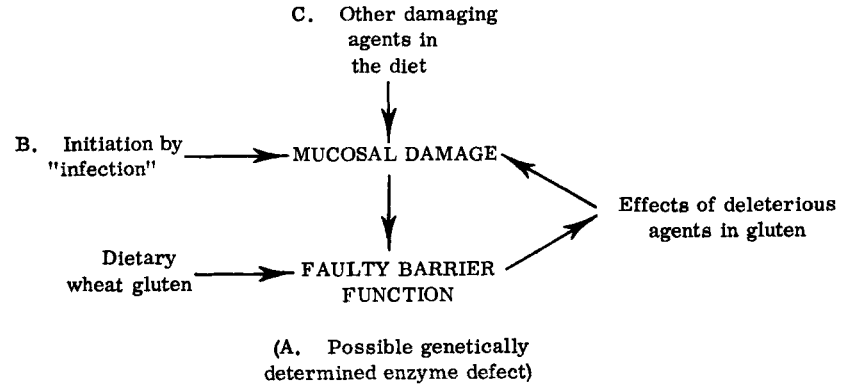

FIG. 2. Summary of the etiological position and possible vicious circle mechanism.

seems the likely picture in gluten-induced enteropathy. In other patients with idiopathic steatorrhea and gluten intolerance, further factors may be involved in maintenance of the syndrome, as shown at C. In such a situation a gluten-free diet might be expected to give only partial improvement; recovery would be dependent upon control of the other factors involved. It is possible that each of these various alternatives may exist among patients belonging to the malabsorption group.

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PEPTIDES IN HUMAN URINE

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1. Introduction

That the urine of pathological subjects may contain certain free amino acids (cystine—Wollaston, 1810; leucine and tyrosine—Frerichs and Staedele, 1854) has long been known. The presence of glycine combined with benzoic acid in the form of hippuric acid was also demonstrated in normal urine at about the same time. For a long time, however, free amino acids were not considered to be components of normal human urine and some sporadic communications indicating the presence of single amino acids in this material were treated as exceptions confirming the general rule. As early as 1911 Andersen (A4) maintained, in one of the chapters of Neuberg's monograph on urine, that "it is not possible to deny that amino acids may be found in normal urine, but this has only been demonstrated beyond question in the case of glycine" [translated from the German article (A4)]. Spaeth also expressed a similar opinion in his monograph published in 1924 (S7).

One of the chief factors contributing to this state of knowledge was the lack of efficient and precise methods suitable for the detection and determination of amino acids. The only procedure then in common use, based on the formation of naphthalenesulfonate derivatives of amino acids, was not satisfactory for the detection of amino acids in small concentrations. Although the formol titration method for the quantitative determination of α-amino nitrogen, adapted by its inventors—Henriques and Soerensen (H4, H5)—to the examination of urine, was known as early as in 1909, this procedure was not very convenient and the precision of the measurements questionable. It was found, however, that α-amino nitrogen accounts for about 0.5–1.5% of the total nitrogen content in urine and that this percentage considerably increases when urine is subjected to the conditions in which decomposition of peptide linkages takes place. This fact drew attention to the existence in urine of some amino-acid complexes of peptide nature rather than to the possibility of the presence of free amino acids. The whole subject was, however, very little studied, because the investigation of polypeptide compounds was even more difficult than the determination of amino acids. The situation remained unchanged even after the development of more adequate methods. The introduction of the Van Slyke gasometric method for determination of α-amino nitrogen (V1) only made possible the confirmation of the presence in normal urine of considerable amounts of amino acids and the marked increase of this amount after hydrolysis of the theoretically existing peptide linkages.

In the third decade of this century, as the methods for detection and identification of single amino acids were improved, the number of observations showing the existence of these compounds in urine increased. Systematic investigations on this problem were, however, still lacking. The only exception was the studies of Kapeller-Adler *et al.* (F1, K1, K2, K3), who demonstrated the existence of histidine in normal urine and found the correlation between the concentration of this amino acid and the different phases of pregnancy. Up to 1940 the problem of free and combined amino acids in urine was in fact passed over.

The improved methods introduced in the years 1940 to 1945 created new possibilities in this field. The Van Slyke method for quantitative determination of amino acids, based on the measurement of the volume of carbon dioxide evolved in the course of the reaction between amino acids and ninhydrin (V2), was much more reliable than the older methods. At the same time the microbiological methods designed for amino acid determinations (D3, S6) made possible the detection of very small concentrations of these compounds. The application of these

methods to the urine examination revealed that the average amount of α-amino nitrogen excreted by a normal man during 24 hours in the form of free amino acids equals 100–180 mg. In this amount, equivalent to about one gram of free amino acids, almost all typical protein components, with the exception of proline and glutamic acid, are contained. Attention was also paid to the fact, particularly relevant to our further considerations, that in addition to the considerable amounts of free amino acids, a large amount of these substances is excreted with urine in the conjugated form represented, at least in part, by slowly dialyzing polypeptides.

The introduction of paper chromatography and column chromatography using ion-exchange resins for urine analysis (D2, M2, M3) was the turning point in the studies on free and combined amino acids present in normal and pathological human urine. Both procedures, much cheaper and more convenient than the microbiological methods still occasionally used, made the examination of the amino acid content of urine available to every laboratory provided with average equipment. The problem of aminoaciduria became a subject of actual interest and the number of papers dealing with it is steadily increasing. Although this problem is loosely connected with the chief subject of this article, only some general reviews concerning it will be briefly mentioned (B4, B11, H3).

Due to the development of chromatographic methods, the existence in urine of amino acids in the conjugated form became once more an actual issue. According to Woodson *et al.* (W3) no more than 50% of the total amino acid content in urine exists in the form of free amino acids. Some amino acids, e.g., aspartic and glutamic acids, proline, valine, and cystine appear in measurable amounts only after hydrolysis of the original, more complex substances. A considerable part of urinary bound amino acids is accounted for by substances of small-molecular weight. Hippuric acid for example, excreted in human urine in amounts ranging from 1–2.5 g/24 hours accounts for 65–75% of the total bound glycine (S8), and half of the bound glutamic acid has been found in the form of phenylacetylglutamine (S10). Moreover, a definite amount of amino acids giving positive ninhydrin reactions is contained in such compounds as pantothenic acid, anserine, and carnosine. The above-mentioned substances which can easily be detected in urine and identified, will not, however, be the subject of our further consideration. Only amino-acid complexes with typical peptide character will be dealt with.

The existence of compounds of this type in urine has long been known. In the classical textbooks and monographs edited at the beginning

of this century the name "albumoses and peptones" was generally used to describe these substances. Different meanings were ascribed to these names by different authors, but it was generally agreed that they represent the intermediate products of protein degradation. The methods then used gave practically no opportunity for precise characterization or detection of these compounds in small concentration. For this reason, in older literature "albumoses and peptones" have been described only as components of abnormal urine derived particularly from subjects with high fever and wasting diseases. Several procedures aiming at the detection of these compounds in urine have been devised, but, being of only historical value, they will not be described here.

Studies on the so-called oxyproteic acids occupied an exceptional position among various investigations concerning low-molecular weight products of protein degradation occurring in urine. The name oxyproteic acids was given by Bondzyński to a preparation obtained from urine in the course of studies carried out with Gottlieb, Dombrowski, and Panek (B6, B7). These substances, precipitated from urine in certain definite conditions with barium salts, were correctly recognized as peptide compounds. Due to the relative convenience and reproducibility of isolation, oxyproteic acids were extensively studied in the first decades of this century, but no consistent picture of their properties was gained. To these substances, which in the light of recent investigations proved to be a well-characterized group of peptide compounds, more space will be devoted on the following pages. It should be mentioned that a substance isolated from urine by Hari (H2) and a polypeptide described by Abderhalden and Pregl (A1) showed some resemblance to oxyproteic acids.

For several decades all these insufficiently characterized urine constituents gained only little interest. The inadequacy of methods, making characterization and quantitative determination of these compounds extremely difficult, was discouraging for investigators. It was only the development and introduction to laboratory practice of paper and column chromatography methods that created new possibilities in this field. The first authors to carry out systematic studies on free amino acids in urine already considered the presence of polypeptides in this fluid. During the last decade some sporadic communications have appeared reporting the isolation of single peptides from normal urine. Though the number of such publications is still relatively small, they show that the way leading to systematic investigations of the subject of peptiduria has already been paved. A new field, abundant in promising possibilities, has been opened in the chemistry of urine.

2. Principal Methods of Analysis

At present there is no general procedure for the identification of all peptides present in urine and for the qualitative and quantitative examination of the bulk of these compounds in such a manner as is now available in the case of free amino acids.

The simplest methods are usually restricted to the estimation of the amount of combined amino acids as a whole or of some definite fraction thereof separated from urine in certain fixed conditions. More efficient separation procedures permit identification of some simple peptides, which represent in many cases the nonphysiological constituents of abnormal urine.

Recently introduced procedures for the examination of peptides in urine, though restricted only to a certain limited peptide fraction, make the separation of this fraction and analysis of its individual components possible.

2.1. Quantitative Determination of Peptides in Urine

The determination of amino nitrogen before and after acid hydrolysis of urine has frequently been used for the quantitative estimation of the amount of urinary peptides (H5, M4). The number of liberated α-amino groups represents, in fact, the whole of formerly combined amino groups, not necessarily attached to a second amino acid partner. Besides, considerable losses connected with decomposition of some amino acids occur in the course of hydrolysis thus limiting the true quantitative value of this procedure.

In Goiffon's method (G3) peptides are precipitated with phosphotungstic acid from a trichloroacetic acid filtrate. The precipitate is dissolved and color is developed by a reaction with Folin's phenol reagent. This method, however, is not only specific for peptides and a separate assay of uric acid has to be made since this substance also reacts with Folin's reagent.

The practically more convenient and less complicated Balikov method (B1, B3) based upon the biuret reaction also exhibits poor specifity toward peptides, and being devoid of the appropriate system of reference standards, it does not permit any absolute evaluation of the amount of these compounds in urine.

For this reason either of these methods is suitable only for a comparative estimation of peptide content in urine.

2.2. Determination of Amino Acids Liberated in the Course of Total Hydrolysis of Urine

Introduction of microbiological methods for the determination of amino acids made possible the estimation of the amount of both free and combined amino acids in urine. Dunn *et al.* (D4), Thompson and Kirby (T1), Eckhard and Davidson (E1), and Woodson *et al.* (W3) estimated the amount of amino acids liberated in the course of acid or, as in the case of tryptophan determination, alkaline hydrolysis. Microbiological and colorimetric methods used for the determination of certain amino acids present very little opportunity for evaluating the proper quantitative relations between free and combined amino acids, since under the applied condition both combined and free amino acids are equally involved in the reaction. In 1949 Albanese *et al.* (A3) applied such methods to the quantitative determination of free and combined amino acids in the nondiffusible fraction of urine, and subjected the procedures to broad criticism from just this point of view.

The column chromatography technique using Dowex 50 ion-exchange resin, introduced in 1951 (M2) and improved in 1954 (M3) by Moore and Stein, first made possible the precise quantitative analysis of amino acids liberated in the course of acid hydrolysis of urine. Similar results were also obtained by Müting in 1954 (M4), who used paper chromatography methods. In this procedure amino acids were quantitatively determined after staining on the paper and elution of the resulting spots.

2.3. Determination of Amino Acid Content of Certain Nitrogen Fractions Isolated from Urine

In 1897 Bondzyński and Gottlieb (B6) isolated from urine a fraction containing substances, which they named oxyproteic acids. The mixture of these compounds was precipitated from the concentrated urine in the form of barium salts insoluble in ethanol. Although they did not ascribe a peptide nature to the isolated substances, they nevertheless considered them as products of protein degradation. Only later, due to the experiments of Browiński and Dombrowski (B12), Gawiński (G1) and Giedroyc (G2), their peptide nature was clearly proved, although other theories concerning the structure of these compounds were also formulated (E2, E3, F2).

Almost at the same time Abderhalden (A1) showed the peptide character of the undialyzable part of urine. By means of the Fischer analysis method he isolated from the hydrolyzate of this mixture certain free amino acids, and for the first time introduced the name "polypeptide" for the analyzed constituent of urine.

The development of modern methods, suitable for the analysis of ampholytes in biological fluids, provided means for isolating from urine some chemically better defined fractions containing peptide compounds. The methods used did not, however, exclude the existence of some other forms of combined amino acids in the fractions studied.

The first fractionation of urinary ampholytes in this way was carried out by Boulanger *et al.* (B10) in 1952 with the use of ion-exchange resins. They had designed this procedure previously for the fractionation of ampholytes in blood serum (B8). According to this method, deproteinized urine was subjected to a double initial procedure aiming at the separation of low-molecular weight substances from macro-molecular ones. One of the methods consisted of the fractionation of urinary constituents by means of dialysis, the second was based on the selective precipitation of urinary ampholytes with cadmium hydroxide, which, as had previously been demonstrated, permits separation of the bulk of amino acids from polypeptides precipitated under these circumstances. Three fractions, i.e., the undialyzable part of urine, the dialyzed fraction, and the so-called "cadmium precipitate" were analyzed subsequently.

From the undialyzable fraction urochrome was extracted with butanol according to the Drabkin technique, and the amino acid composition of the resulting preparation was then examined. The other ampholytes of this fraction were separated into two parts by the use of Deacidite. One part, containing alkaline substances, was not adsorbed on the resin, and the other, exhibiting acid properties, was adsorbed on the column and eluted with 0.25 *N* hydrochloric acid. Following acid hydrolysis, both fractions were roughly characterized with reference to the amino acid composition. The whole undialyzable fraction was also chromatographed on paper using the butanol-acetic acid solvent system, the separated spots localized in ultraviolet light, and the amino acid composition of each spot determined. The dialyzed fraction and the "cadmium precipitate" were examined by these authors in a similar manner (see Fig. 1). With the use of Permutit-50 exchanger each preparation was fractionated into two parts: nonadsorbed initial solution and eluate obtained with 0.3 *N* ammonia. The first was then separated on the Deacidite column into three fractions: alkaline, not adsorbed by the resin, weakly acidic, washed out with 0.1 *N* acetic acid, and more acidic, eluted with 0.25 *N* hydrochloric acid. All three fractions were subsequently analyzed by means of two-dimensional paper chromatography before and after acid hydrolysis. Boulanger *et al.* performed the most precise comparative studies on dialyzing parts of urines derived from normal and pathological subjects, as well as from persons belonging to the same family.

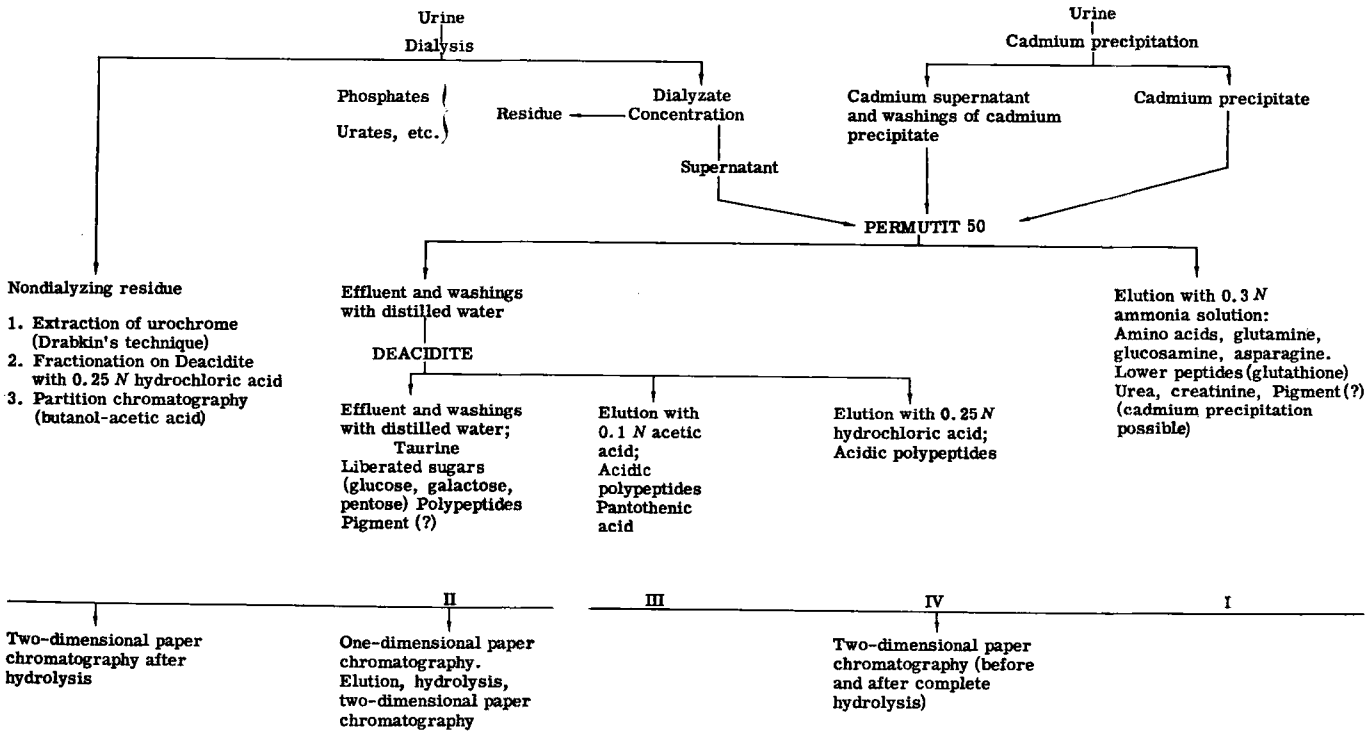

FIG. 1. Fractionation of urinary constituents according to Boulanger *et al.* (B10).

A similar ion-exchange resin method was used by Ling in 1955 (L1) for the examination of combined amino acids in urine. According to this procedure urine was desalted and simultaneously freed from amino acids by using Amberlite IR-112, H^+-form resin. The effluent collected from the column was then fractionated on Amberlite IRA, OH^--form resin, by successive elution with 0.16 *N* acetic acid, 0.08 *N* formic acid, 0.25 *N* formic acid, 0.08 *N* hydrochloric acid, and finally with 0.16 *N* formic acid. The solutions of all acids contained 10% of acetone. The collected fractions were hydrolyzed with hydrochloric acid and the liberated amino acids identified by means of paper chromatography.

2.4. Examination of Low-molecular Weight Peptides Isolated from Urine

The paper chromatography method introduced by Dent in 1947 (D1, D2) for the examination of free amino acids in urine revealed the presence on the chromatograms of additional ninhydrin-positive spots, which were no longer detectable after hydrolysis of the urine. This procedure created incidentally quite a new means for investigation of peptide content in urine. The paper chromatography method, modified subsequently by different authors, permitted both the detection of abnormal peptides in the urine of pathological subjects and, in some cases, the determination of the amino acid composition of single peptide spots eluted from the chromatograms.

The column chromatography technique designed by Moore and Stein in 1951 and 1954 (M2, M3) for the analysis of amino acid mixtures, applied to urine examination (S8), greatly increased the possibilities in the field of urinary peptides. The use of a much greater amount of the material examined made possible the detection of urinary ampholytes in concentration far below the sensitivity limits of paper chromatography methods. The resolving power could be increased still further through repeated chromatography of previously separated fractions.

In 1952 Carsten (C1) developed a method, which allowed him to isolate and characterize several lower peptides contained in normal and pathological urine. According to this procedure, urine was desalted on the Amberlite IR-100 column and the adsorbed substances washed out with 2 *M* ammonia solution. The eluate was then passed through the column of Amberlite IRA-400. This column retained the ampholytes and rejected the weak bases. The former were recovered by elution with 1 *M* hydrochloric acid and the eluate was subsequently fractionated on Dowex 50 resin with 2 *M* and later 4 *M* hydrochloric acid as the eluents. By applying two-dimensional paper chromatography to further analysis of

individual fractions, the presence not only of free amino acids, but also of several substances was demonstrated, which after hydrolysis proved to be di- and tetrapeptides.

Similar, but more detailed studies were carried out by Westall in 1955 (W2). The procedure developed by this author aimed especially at the search for nitrogenous compounds existing in small amounts in urine and liberating free amino acids upon hydrolysis. Taking into account the minute concentration of these urinary constituents, he started with as much as 100 liters of urine, processing it in four successive stages in the following manner: after desalting the urine with ethanol, all ampholytes were adsorbed on Zeocarb 215 resin, and the amino acids and peptides retained on the column eluted with 0.2 *N* ammonia solution. The effluent was then subjected to displacement chromatography on Dowex 2 with the use of 0.1 *N* hydrochloric acid. Every one of 30 collected fractions of 200 ml volume was subsequently analyzed by means of two-dimensional paper chromatography using two different solvent systems: one of them consisted of phenol-ammonia and butanol-acetic acid-water, the second of phenol-ammonia and lutidine-collidine used in two directions, respectively. All fractions were analyzed before and following acid hydrolysis. As an additional means for separation of amino acids, paper electrophoresis was used throughout. Besides routine analyses carried out on all fractions, some of them, especially rich in unknown amino acids and peptides, were subjected to more detailed studies. They were subfractionated accordingly on small columns filled with Zeocarb 215 and Dowex 2 resins or separated with the displacement chromatography technique of Moore and Stein on Dowex 50. The fractions containing non identified substances were hydrolyzed and their amino acid composition determined.

Although the Westall procedure, as well as Carsten's technique, made possible the isolation from urine of certain individual and homogeneous peptides, nevertheless, both methods were restricted to investigation of low-molecular weight substances of this type separated along with the accompanying amino acids.

2.5. Isolation of Polypeptides Contained in Urine

In 1958 Hanson and Fittkau (H1) published their new method designed for the isolation of polypeptide compounds from urine. Using this method, they isolated more than ten peptides starting with 83 liters of normal urine. The general outline of the method was as follows: urine was treated with activated carbon and the adsorbed substances eluted with aqueous acetone containing hydrogen sulfide. The effluent was then

subjected to countercurrent distribution with the use of a *n*-butanol-0.75 *M* aqueous sodium β-naphthalenesulfonate system. As a result of 110 successive extractions, a fraction containing four-fifths of the total nitrogen of peptide character was obtained. This fraction was subsequently separated by means of high-voltage electrophoresis (acetate buffer, pH = 1.9, potential gradient 60 volts/cm) and the resulting electrophoretic fractions analyzed with two-dimensional paper chromatography in phenol-water and *sec*-butanol-formic acid-water solvent systems. The spots corresponding to the individual peptides were eluted from the chromatograms, hydrolyzed, and analyzed for amino acid composition.

For isolation of certain definite peptide groups from urine, Sarnecka-Keller (S1) used a different technique based on a modification of Bondzyński's method, originally used for precipitation of oxyproteic acids from urine (B6, G1). The general outline of this method consisted in removing the majority of anions from the concentrated urine with a saturated solution of barium hydroxide. At the same time the reagent forms salts with a definite group of polypeptides, precipitated with an ethanol and ether mixture. The precipitate obtained was dissolved in water and separated by high-voltage electrophoresis (pyridine buffer, pH = 6.0, strips of Whatman No. 1 paper 59 × 7.5 cm, time of separation 60 min at the potential gradient of 28 volts/cm). Every one of the four fractions obtained—one acid, one neutral, and two alkaline—was then chromatographed on the paper with butanol-acetic acid-water (144:13:43 by volume). This procedure revealed the presence of peptide compounds, which were next hydrolyzed, their amino acid composition was characterized, and in some cases *N*- and *C*-terminal amino acids were determined. The comparative analysis of the peptide fractions, precipitated from the different specimens of urine by means of the above procedure, is now being performed with the "finger printing" method.

3. Results of Studies on Urinary Peptides

3.1. Amino Acids Liberated After Complete Hydrolysis of Urine

According to Stein (S8), 1761–2459 mg of amino acids appears in 24 hours urine collection from normal subjects after its acid hydrolysis. The estimations performed by Müting (M4) showed that the average content of amino acids in 24-hour specimens of urine derived from 20 normal males and the same number of normal female subjects was 2221 and 2288 mg, respectively. The results of these two authors do not, however, represent exclusively the amount of peptide-bound amino acids, but rather the whole of these compounds liberated as a result of the acid

hydrolysis of urine. The experiments of Stein *et al.* (S10) made it clear that from these figures as much as 580 mg of glycine bound in the form of hippuric acid and 550 mg of glutamic acid contained in phenylacetylglutamine should be subtracted. No other nonpeptide-bound amino acids excreted in urine in such amounts have so far been found. It is possible then to assume, in accordance with the present state of knowledge, that the amount of amino acids excreted during 24 hours in normal urine in the peptide-bound form equals about 1000 mg.

TABLE 1

AMINO ACID CONTENT OF ACID HYDROLYZATES OF NORMAL URINE[a]

	Amount excreted (mg per day)					
	Total after hydrolysis			Increase after hydrolysis		
Amino acids	Specimen A	Specimen D	Specimen G_2	Specimen A	Specimen D	Specimen G_2
Taurine	147	302	180	13	8	0
Aspartic acid	192	194	251	192	194	251
Threonine	58	65	98	34	38	45
Serine	77	106	155	42	37	82
Glutamic acid	470	483	640	470	475	640
Proline	67	89	94	67	89	94
Glycine	893	796	1124	750	680	940
Alanine[b]	69	62	116	28	24	45
Cystine	52	61	42	52	50	42
Valine	22	28	40	22	28	40
Isoleucine	26	22	25	5	8	—3
Leucine	19	32	31	5	14	7
Tyrosine	48	68	111	24	20	62
Phenylalanine	24	58	54	10	32	23
Histidine	—	270	450	—	33	130
Methylhistidine	140	107	46	10	3	—1
Lysine	48	57	77	37	39	60
Arginine	<20	<20	<20	—	—	—
	2372[c]	2820	3554	1761[c]	1772	2457

[a] According to Stein (S8).

[b] Uncorrected for aminoadipic acid.

[c] Exclusive of histidine, the determination of which was lost in this case.

The amounts of single amino acids excreted in urine in the conjugated form, as determined independently by Stein and Müting, are given in Tables 1 and 2. According to Stein, glycine, glutamic acid, aspartic acid, histidine, and proline are quantitatively the most important amino acids liberated in the course of urine hydrolysis. Serine, lysine, tyrosine, cysteine and cystine, threonine, alanine, valine, phenylalanine, and leucine are

TABLE 2

PEPTIDE-BOUND AMINO ACIDS IN 24-HOUR SPECIMENS OF URINE DERIVED FROM 40 NORMAL SUBJECTS[a]

	20 Males				20 Females			
	Amount of amino acids (mg)			α-Amino N (calc'd)	Amount of amino acids (mg)			α-Amino N (calc'd)
Peptide-bound amino acids	Lower limit	Average	Upper limit	Average	Lower limit	Average	Upper limit	Average
24-Hour specimen of (ml)	600	1090	1700	—	550	980	1500	—
Aspartic acid	85	141	202	14.80	92	149	207	15.64
Glutamic acid	180	313	561	29.73	217	428	614	40.66
Lysine	84	132	181	12.67	79	134	176	12.86
Arginine	21	31	49	2.54	20	31	50	2.54
Histidine	42	85	119	7.68	38	72	117	6.48
Tyrosine	12	17	26	1.31	13	24	28	1.85
Tryptophan	19	36	54	2.47	21	40	55	2.74
Phenylalanine	0	12	25	1.04	0	10	26	0.85
Oxyproline	0	0	0	—	0	0	0	—
Proline	36	80	107	9.76	28	65	94	7.80
Cystine	77	110	154	12.87	65	90	132	10.53
Methionine	217	365	510	34.31	198	338	482	31.77
Leucine	39	67	94	7.30	34	58	87	6.32
Isoleucine	7	18	26	1.96	6	21	29	2.29
Valine	98	144	172	17.28	82	144	158	13.68
Glycine	190	271	361	50.78	157	229	318	42.62
Alanine	115	162	198	25.43	121	178	201	27.95
Serine	97	139	177	18.07	105	170	212	22.61
Threonine	55	98	134	10.78	61	107	145	12.31
Taurine	0	0	0	—	0	0	0	—
	1374	2221	3150	260.75	1337	2288	3131	261.50

[a] According to Müting (M4).

liberated less abundantly. Only traces of arginine, isoleucine, and methylhistidine appear in urine hydrolyzates.

The data of Stein and Müting differ substantially in the matter of methionine quantity, which, accordingly to the latter, is the quantitatively most important conjugated amino acid of normal urine. A similar opinion was also expressed by Albanese *et al.* (A2) in 1944, but Wallraff *et al.* (W1) reported that methionine appears only occasionally in urine hydrolyzates and in amounts never exceeding 12 mg/24 hours.

Müting detected also much greater amounts of serine, lysine, threonine, cysteine, alanine, valine, leucine, and arginine, and smaller quantities of glycine, glutamic acid, aspartic acid, and histidine than those reported by Stein. He nevertheless allocated the latter four amino acids to the group of amino acids appearing in urine hydrolyzates most abundantly. The occurrence of large amounts of these four amino acids in hydrolyzed urine was also observed by Dunn *et al.* (D4). Pollack and Eades (P2) called attention to the fact that the appearance of large amounts of glutamic and aspartic acids in urine hydrolyzates may originate, in a great measure, from the decomposition of glucuronic acid conjugates of these amino acids.

Thompson and Kirby (T1) reported larger amounts of conjugated arginine than those estimated by Stein. Their data are, however, concurrent with those of Müting. The presence of conjugated tryptophan, demonstrated by Müting, was also confirmed by Woodson *et al.* (W3). According to these authors, tryptophan appeared in urine after alkaline hydrolysis in amounts ranging from 11 to 86 mg/24 hours. Conjugated threonine was found in urine by Sheffner *et al.* (S4) in amounts from 45 to 90 mg/24 hours. This value occupies the mean position between the figures given for this amino acid by Stein and Müting, respectively.

3.2. Amino Acids Liberated in the Course of the Hydrolysis of Certain Peptide Fractions Isolated from Urine

As early as 1905 Abderhalden (A1) isolated from the hydrolyzate of the nondiffusible fraction of human urine four amino acids, i.e., leucine, alanine, glycine, and glutamic acid, and detected two others: phenylalanine and aspartic acid. Some amino acids derived from this fraction have been quantitatively determined by Albanese *et al.* (A3) who found in the amount of the nondiffusible fraction corresponding to one liter of urine as much as 32.8 mg tryptophan, 18.0 mg phenylalanine, 16.2 mg methionine, 15.2 mg cystine, 13.1 mg arginine, 6.7 mg histidine, and 3.9 mg tyrosine.

Considerable amounts of glutamic acid, glycine, and alanine, as well as smaller quantities of aspartic acid, serine, threonine, basic amino acids, leucine, phenylalanine, and cystine have been demonstrated in a total hydrolyzate of the nondiffusible fraction by Boulanger *et al.* (B10). Using Deacidite resin, they separated this material into two polypeptide fractions, acid and alkaline, and found that glutamic acid, aspartic acid, leucine, and certain cystine derivatives were the chief constituents of the former, whereas the latter contained considerable amounts of glycine, basic amino acids, and alanine.

Similar effects of hydrolysis were observed by Boulanger in the case of the polypeptides deposited in the form of so-called "cadmium precipitate" (see Section 2.3). Here again considerable quantities of glycine, alanine, and basic amino acids were found in the alkaline fraction of the hydrolyzate, and chiefly glutamic acid and glycine in the acid fraction. Boulanger *et al.* also discovered that, after hydrolysis of the diffusible fraction of human urine, some new amino acids, not encountered formerly in the free form, appeared. To this group belong: proline, valine, leucine, and phenylalanine. Besides, they demonstrated a marked increase of the concentration of such amino acids as glutamic acid, glycine, threonine, alanine, cystine, tyrosine and, to a lesser extent, aspartic acid.

Using the procedure described above, Ling (L1) obtained five peptide fractions of acid character from urine and determined their amino acid composition after total hydrolysis. Fraction I, eluted from Amberlite IRA 400 with 0.16 *N* acetic acid, contained α- and β-alanine, γ-aminobutyric acid, aspartic and glutamic acids, glycine, proline, sarcosine, serine, and valine; fraction II, eluted with 0.08 *N* formic acid, α-alanine, glutamic acid, aspartic acid, glycine, serine, threonine, sarcosine, proline, valine, leucine and isoleucine; fractions III and IV, eluted with 0.16 *N* and 0.25 *N* formic acid, respectively, glycine and glutamic acid; fraction V, eluted with 0.08 *N* hydrochloric acid, α-alanine, aspartic and glutamic acids, valine, tyrosine, and leucine.

The data obtained by Ling, concerning the amino acid composition of acid peptides, can be compared with the results of Westall (W2), who studied the amino acid composition of the hydrolyzate of a mixture of at least ten acid peptides. He demonstrated in this hydrolyzate the existence of considerable amounts of glutamic acid, aspartic acid, glycine, alanine, valine, leucine, serine, and threonine, smaller amounts of tyrosine, phenylalanine, and proline, as well as traces of β-alanine. Neither Ling nor Westall could find any sulfur-containing amino acid or arginine, histidine or lycine in this mixture. Westall demonstrated, however, small quantities of β-aminoisobutyric acid, not detected by Ling.

3.3. General Remarks Concerning the Raised Level, or a New Appearance, of Some Peptides in Pathological Urine

Balikov *et al.* (B2), using the method which they devised especially for the determination of urinary peptides, demonstrated a marked increase of these compounds in urine of patients suffering from burns. Using the same method, Sarnecka-Keller observed a considerable rise in the amount of urinary peptides in liver cirrhosis and leukemia (S1).

Stein *et al.* found in the course of experiments dealing with free and conjugated urinary amino acids in Wilson's disease (S9) that besides a marked aminoaciduria, almost a twofold increase in the excretion of all bound amino acids could be observed. As compared with normal urine (S8), unusual amounts of conjugated leucine, isoleucine, and valine are excreted in cases of Wilson's disease. Also the increase of glutamic acid, aspartic acid, and phenylalanine after urine hydrolysis is much more distinct in this disease than in normal conditions. Other bound amino acids are at or below normal levels.

In the course of studies on other pathological amino acidurias, the accompanying peptiduria has also been observed by many authors. Rapp de Eston *et al.* (R2) observed a marked increase in the excretion of both free amino acids and peptides in patients with diffuse hepatic necrosis. Using a simplified chromatographic method adapted to clinical purposes and suitable for analysis of amino acids excreted with urine, Skarżyński *et al.* (S5) demonstrated a raised level of a certain peptide which is always present in normal urine in smaller quantities, as well as the appearance of some new peptides in cases of jaundice and liver cirrhosis. Some abnormal peptide spots were also detected on the chromatograms in cases of progressive muscular dystrophy (K4) and in patients irradiated with X-rays (S2).

Boulanger, analyzing with his method the urine of patients with malignant tumors, observed the appearance on the chromatograms of two peptide groups, not present in normal urine specimens. Hydrolysis of the peptide groups eluted from chromatograms revealed that one of them contained considerable amounts of aspartic acid, glutamic acid, serine, glycine, and smaller amounts of threonine, alanine, and cysteic acid. Valine, leucine, alanine, glycine, threonine, aspartic acid, glutamic acid, and lysine were released from the second polypeptide group after its complete hydrolysis (B10).

In 1948 Dent (D2) described the so-called "nephrosis peptide." He observed the presence of this peptide in the deproteinized urine of two patients with nephrosis. However, he could not find this peptide in the

urine of another nephrotic patient, but demonstrated its presence in the blood serum. The majority of amino acids liberated from this peptide after its hydrolysis proved to be identical with the normal protein components. The presence of a peptide of this type in the urine and blood serum of nephrotic patients was confirmed later by Ellis *et al.* (E4), who showed in addition that this peptide exhibits an activity analogous to that of vasopressin.

3.4. Single Peptides Isolated from Normal and Pathological Urine

In the course of studies on aminoaciduria in Fanconi's syndrome, Dent (D1) isolated from the urine of the subject investigated a simple peptide identified as serylglycylglycine. Carsten (C1) found in normal urine several peptides containing in every case one of the dicarboxylic amino acids. He discovered also two tetrapeptides, one of them consisting of equimolar amounts of aspartic acid and glycine, and the second composed of glycine, alanine, and glutamic acid in the ratio 2:1:1. The first of these tetrapeptides was also found in the urine of a patient with rheumatoid arthritis.

Plaquet *et al.* (P1) found in the urine of rachitic children peptides consisting of proline, hydroxyproline, and glycine, which they believed to be the products of collagen degradation. Two similar peptides containing considerable amounts of proline and hydroxyproline were isolated from the urine of a patient with rheumatoid arthritis by Mechanic *et al.* (M1). One of these peptides consisted of three proline, two hydroxyproline, and nine glutamic acid residues, the second one consisted of four proline, four hydroxyproline, and one glutamic acid residues. The *N*-terminal amino acid in the first peptide was demonstrated to be hydroxyproline.

Many different peptides consisting of two or more amino acid residues have been found in normal urine by Westall (W2). The existence of a peptide consisting exclusively of proline and hydroxyproline has also been confirmed by this author. Five other peptides were characterized and the following amino acid content was found after hydrolysis: peptide I—arginine, lysine, histidine, and glycine in equimolar amounts; peptide II—arginine, lysine, glycine, and glutamic acid; peptide III—equimolar quantities of glutamic acid, glycine, lysine, and an unidentified substance giving a positive ninhydrin reaction; peptide IV—glutamic acid, aspartic acid, and lysine; and peptide V—only glutamic and aspartic acids.

All peptides described above were separated, by means of the fractionation technique used by Westall together with free amino acids.

They belong then to a small-molecular weight fraction of urinary constituents.

Special attention should be paid to polypeptides isolated from a nondiffusible fraction of normal human urine by Boulanger *et al.* (B10). By means of paper chromatography they isolated from this mixture seven single polypeptides and determined their amino acid composition. The results of the analyses are given in Table 3.

TABLE 3

RELATIVE PROPORTIONS OF AMINO ACIDS IN BANDS ELUTED FROM THE CHROMATOGRAMS OF THE NONDIFFUSIBLE FRACTION OF URINE SEPARATED WITH BUTANOL-ACETIC ACID SYSTEM[a]

Bands:	1	2	3	4	5	6	7
R_f (mean value):	0	0.05	0.10	0.15	0.25	0.31	0.43
Aspartic acid			10	1		1	
Glutamic acid	3	3	3	4		4	5
Serine		2	3	2			6
Glycine		10					
Alanine	4	3	6	4	++	1	3
Diamines			3	2			1
Hydroxyproline						1?	
Cysteic acid			1				
Valine			2				
Leucine			3				

[a] According to Boulanger (B10).

A group of macromolecular polypeptides was also found in normal urine by Bode *et al.* (B5). These polypeptides, extracted from urine with a mixture of ethanol and ether followed by chloroform, contained also some lipid and carbohydrate components.

3.5. HOMOGENEOUS PEPTIDE GROUPS ISOLATED FROM NORMAL URINE

By means of a procedure described above, Hanson and Fittkau (H1) isolated seventeen different peptides from normal urine. One of them, not belonging to the main peptide fraction, consisted of glutamic acid, and phenylalanine with alanine as the third not definitely established component. The remaining peptides contained five to ten different amino acid residues and some unidentified ninhydrin-positive constituents. Four amino acids, i.e., glutamic acid, aspartic acid, glycine, and alanine, were found in the majority of the peptides analyzed. Twelve peptides contained lysine and eight valine. Less frequently encountered were: serine, threonine, tyrosine, leucine, phenylalanine, proline, hydroxyproline, and α-aminobutyric acid (found only in two cases). The amino acid composi-

tion of individual peptides, as determined by Hanson and Fittkau, is given in Table 4.

In 1961 Ansorge *et al.* (A5), using the same technique as previously, determined the peptide composition of urine derived from four normal subjects, three males and one female. Among twenty isolated peptides, seventeen were found in all specimens of urine, two peptides in three specimens, and the remaining one only in two specimens of urine. The identity of individual peptides was established on the basis of their electrophoretic and chromatographic behavior, as well as the amino acid composition after complete hydrolysis. It should be pointed out, however, that the amino acid composition of the peptides examined differs considerably from that obtained by the same authors in the case of the peptides described in 1958 (H1).

Sarnecka-Keller (S1), by use of Bondzyński's method modified by Gawiński, isolated from normal urine a well-defined peptide fraction easily reproducible without change of qualitative composition. Throughout the isolation procedure all factors which may have caused any changes in peptide structure were taken into account. The isolated preparation was substantially similar in general properties to that obtained originally by Bondzyński *et al.* (B7).

The nitrogen content of this mixture amounted in the case of normal urine to 0.68–1.27% of the total nitrogen, whereas the peptides precipitated under these circumstances constituted 16.1–20.9% of all substances appearing in urine and giving the biuret reaction. The peptide mixtures obtained in identical conditions from six samples of pathological urine (liver cirrhosis and leukemia) exhibited marked differences in the nitrogen and peptide content as compared with those isolated from normal urine.

The further fractionation of this mixture by selective precipitation with Pb^{++} and Hg^{++} ions, undertaken by Bondzyński *et al.* (B7), proved unsuccessful, since no distinct separation of peptides was attained.

High-voltage electrophoresis and subsequent paper chromatography of the fractions obtained made possible the isolation from the analyzed mixture of twenty-two components giving colored spots with ninhydrin and isatin. Among these, fourteen were identified as peptides and their amino acid composition established (Table 5). In the case of eight peptides, also *N*- and *C*-terminal amino acids were determined (Table 6).

All the isolated peptides were composed of at least five different amino acids. The quantitative proportion of amino acids in these peptides was much the same as that given by Stein for urinary-bound amino acids.

TABLE 4

AMINO ACID COMPOSITION OF URINARY PEPTIDES[a]

Fraction No.	Asp	Glu	Ser	Gly	Thr	Ala	Tyr	Val	Leu	Phe	Pro	Hydroxy pro	Cys	Lys	Arg	α-ABA[b]	Unidentified
III_1	+	+		+		+		+	+				+				+
II_{32}	+	+		+		+		+	(+)					+			
II_{51}	+	+		+													
III_2	+	+	+	+		+	+	+	+	+				+			(+)
III_{50}	+	+	+													+	+++
III_{31}	+	+		+		+								+			(+) (+)
II_{81}	+	+		+		+		+	+		+	+		+	+		
II_{34}	+	+		+		+								+			
II_{73}		+		+		+						+		+	+		
III_{43}	+	+	+		+									+			(+) (+)
II_{82}	+	+		+		+		+			+	+		(+)	+		(+)
III_{44}				+		+								+			
III_{32}	+	+		+		+		+	+				+	+			+
III_{51}		+		+	+				+					+	+	+	+(+) (+)
II_{43}	+	+		+		+		+	+					+	+		
III_{46}		+			+	+		+									(+)
N-III		+				(+)				+							

[a] According to Hanson and Fittkau (H1).

[b] α-Aminobutyric acid.

TABLE 5

AMINO ACID COMPOSITION OF URINARY PEPTIDES[a]

Peptide	Cystine + cysteine	Lysine	Histidine	Glycine	Arginine	Aspartic acid	Glutamic acid	Alanine	Proline	Tyrosine	Valine	Leucine	Phenyl-alanine	Un-identified
B_1 II			●●●●	●			●●	●●			●	●		1
B_1 III		●●	●●	●●		●●	●●	●●	●		●●	●●		1
B_1 IV		●●	●●	●●		●●●	●●	●●	●●		●●		●●	
B_2 I		●●●●	●	●●			●●	●			●●	●		1
B_2 II		●●		●●		●●	●●	●●						
N I	●●		●●	●●		●●	●●	●●	●●					
N IV			●●	●●●		●●●	●●	●	●		●●	●●		
N V	●●	●●			●●			●●●				●●		1
N VI		●●		●		●●	●●●	●●	●●			●●		
N VII	●●	●●		●●			●●	●●	●●					
A II	●●●	●●	●	●●		●●	●●	●●	●●	●●●				
A V	●●		●	●●		●●	●●	●●	●●					
A VI	●●	●●	●	●●		●●	●●	●●	●●					
A VII	●●	●●	●	●	●●	●	●●	●●	●					

KEY: ●, traces; ●●, moderate amount; ●●●, large amount; ●●●●, this amino acid is principal component of peptide.

[a] According to Sarnecka-Keller (S1).

Large amounts of glycine, histidine, glutamic acid, aspartic acid, and proline were characteristic for these compounds. Unlike other peptides, or other bound forms of amino acids, they contained a large amount of alanine, which was found in all fourteen peptides. There are marked differences between the peptides described here and those studied by Hanson and Fittkau. No one peptide isolated by these authors contained histidine, whereas this amino acid was found in ten peptides isolated by Sarnecka-Keller. The presence of two peptides, one of which was composed almost exclusively of histidine and the other chiefly of lysine with only small amounts of other amino acids, was especially characteristic. Neither of these two peptides had a free amino group, while the remaining six had glycine or alanine as *N*-terminal residue.

TABLE 6
TERMINAL AMINO ACIDS OF URINARY PEPTIDES[a]

Peptide	*N*-terminal amino acid	*C*-terminal amino acid
B_1 II	—	Histidine
B_2 I	—	Lysine
A II	Alanine	Glycine
A V	Alanine	Glycine
A VI	Glycine	Glycine
A VII	Glycine	Glycine
N IV	Glycine	Glycine
N VII	Glycine	Alanine

[a] According to Sarnecka-Keller (S1).

The urinary pigment urochrome appears to be another distinct polypeptide fraction found in urine. As early as 1912 Browiński and Dombrowski (B12) postulated the peptide character of this compound. Rangier and de Traverse (R1) described urochrome as polypeptide combined with indoxyl. The investigations of Noworytko (N1) indicate that urochrome is not a homogeneous substance, but rather a mixture of peptides combined with some colored component, presumably a dipyrrole pigment. The presence of this type of pigment as a component of urochrome was also suggested by Sattelmacher and Fürstenau (S3).

4. Concluding Remarks

Systematic investigations on the appearance of peptides in urine were initiated no more than ten years ago, when modern analytical methods were introduced. This relatively short period devoted to the studies on peptiduria, as well as the diversity of the methods used, has undoubtedly been the chief reason for the confusion in the state of our knowledge on this subject. Nevertheless, two important facts can now be accepted with-

out any doubt: (a) that peptiduria is a physiological phenomenon and the peptides excreted with normal urine represent as much as 1–2% of the total nitrogen and about 50% of the so-called conjugated amino acids excreted within 24 hours, and (b) that the total amount of peptides excreted in urine and the qualitative composition of these compounds show some variations even in normal subjects, and that marked qualitative and quantitative differences can be observed in certain pathological conditions.

At the moment, no methods making possible the qualitative and quantitative evaluation of the whole of the peptides excreted, are available. The relatively simple method of Balikov gives only some general information concerning the amount of urinary peptides without differentiation. Moreover, the standard used in this method may not be suitable for the identification of many peptides, the nature of which is yet unknown. The methods used by Hanson and Fittkau, and by Sarnecka-Keller permit the identification of peptides within a certain group exhibiting a fairly uniform qualitative composition. However, these groups do not represent the whole of the peptides existing in urine, but only some fraction of it. Moreover, the peptide group isolated by Sarnecka-Keller and that obtained by Hanson and Fittkau certainly differ from each other in respect of peptide composition. The procedure used by Boulanger *et al.* made possible the characterization of only a limited number of peptides appearing in urine and probably different from those described by the previously mentioned authors.

In this situation the further improvement of analytical methods, or even the search for a quite new approach to the problem of peptiduria seems to be necessary. It will not be possible to treat the problem of peptiduria in such manner as is now available in the case of aminoaciduria, until the qualitative characterization of all peptides and the quantitative estimation of single peptides is achieved. Only then will questions be answered concerning the origin of the peptides excreted in urine, their role in the metabolic activity of the human body, and their importance in the diagnosis of pathological conditions. In addition, the extending of the scope of investigations to the peptides occurring in blood plasma would be necessary in order to enable the direct comparison of the peptide content in both fluids.

Most probably some fraction of the peptides appearing in urine represents small quantities of peptide hormones, such as vasopressin, oxytocin, glucagon, and the melanocyte-stimulating hormone, the occurrence of which in small amounts in normal urine has been occasionally reported. As the specific enzymatic decomposition of these hormones takes place

in the organism, some of the peptides contained in urine may be the products of this feedback control of the concentration of peptide hormones in body fluids. The approach to the problem of peptiduria from this point of view should open up quite new aspects in the field of the physiology of peptidic hormones and may lead to solutions, yet unattained with the use of biological methods.

Within recent years, the peptides possessing a pharmacological activity, not produced in special endocrine glands, but originating from plasma or tissue protein degradation, have become of a considerable interest. Some of them, such as bradykinin, hypertensin, and the peptides releasing the inflammatory response described by Menkin (M1a) and independently by Spector (S7a) are now well characterized and, in many cases, recognized by means of biological methods as the constituents of urine. The identification of such peptides together with the other chemically defined urinary constituents would certainly channel the whole subject in new directions.

Finally, some other peptides produced in the body tissues in effect of protein degradation under the action of the enzymes belonging to the cathepsin group, should be mentioned. Presumably, some of the urinary peptides originate indeed from this type of activity. The increased peptiduria, concomitant with the intensified protein breakdown in such pathological conditions as malignancy and radiation injuries, is in accordance with such an explanation for the origin of at least part of the peptides excreted with urine. The peptides described by some authors, containing considerable amounts of proline and hydroxyproline are most likely the products of collagen decomposition. A more careful examination of this problem may create quite new possibilities for the evaluation of some irregularities in protein metabolism on the basis of the analysis of urinary peptides.

In our opinion, investigations in the field of physiological and pathological peptiduria appear to have a very brilliant future. Further methodological advances, primarily in the development of uniform and standardized procedures making the results of different laboratories comparable, are, however, necessary before this trend of research gains an established position in clinical chemistry.

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HAPTOGLOBINS

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1. Introduction

1.1. Historical Remarks

In 1938, while studying how the pseudoperoxidase (J4) activity of hemoglobin (Hb)[1] varied with the experimental conditions, Polonovski and Jayle (P3) fortuitously discovered the occurrence of a substance identified later as haptoglobin (Hp). They found that serum contained a variable amount of a nondialyzable substance which, under certain conditions, made Hb behave like a true peroxidase. Since the substance

[1] The following abbreviations are used throughout this paper: Hb = hemoglobin; HbO_2 = oxygenated hemoglobin; HbOH = hemiglobin(=methemoglobin); HbCO = carboxyhemoglobin; Hp = haptoglobin; HbBC = hemoglobin-binding capacity; HBG = heme-binding β-globulin; Hi = haptoglobin index; S.D. = standard deviation; E.S.R. = erythrocyte sedimentation rate (Westergren).

apparently linked Hb, they called it haptoglobin (P4). Jayle utilized his observations in the elaboration of a micromethod for measuring the Hp "content" or the specific hemoglobin-binding capacity (HbBC) of serum (J5). The capacity, as measured by his method, was expressed in Hi (haptoglobin index) units. One unit corresponded to the mean value found for serum from healthy subjects and could be defined by the hemin iron of a standard solution of horse Hb (J9). Jayle and his biochemical and clinical co-workers made a series of interesting observa-

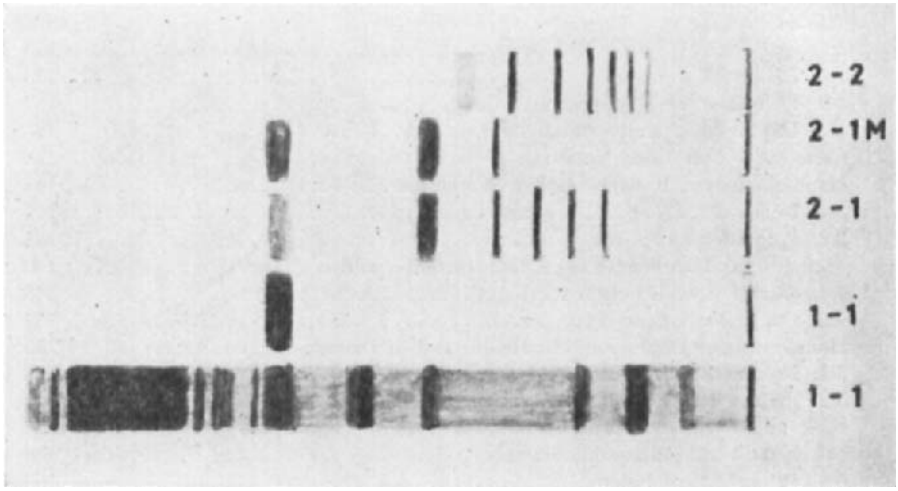

FIG. 1. Schematic presentation of the protein pattern of the common Hp types in pure form after starch-gel electrophoresis. The protein pattern of a normal serum belonging to Hp type 1–1 is given at the bottom. The cathodic part is excluded.

tions on the properties and metabolism of Hp; the Hi was therefore soon accepted by the French-speaking medical world.

A comprehensive, up-to-date review "A Biochemical, Genetic and Physio-pathological Study of Haptoglobin" has recently been presented by Jayle and Moretti (J12). Since the present article is concerned mainly with problems of current interest in clinical work and research, the older literature will be dealt with only in brief outline.

It was soon realized (J10, R5) that Hp did not consist of a single protein, but of a group of proteins with very similar properties. Conclusive evidence of the molecular heterogeneity of Hp was produced by Smithies (S5), who used electrophoresis with a defined starch gel in which the mobility of protein molecules varies with their charge and size (Fig. 1). Smithies and Walker's discovery (S9) of different types of

genetically controlled Hp's in man aroused a world-wide surge of interest in Hp's.—The voluminous literature on the genetic control of Hp's was thoroughly reviewed in 1961 by Baitsch and Liebrich (B1). Our present knowledge of the biochemical properties of Hp was presented in a thesis by Herman-Boussier, "Préparation et propriétés physiques et chimiques des haptoglobines humaines" (H5).

1.2. Definition of Haptoglobins

So far, no attempts have been made to formulate a precise biochemical or biological definition of Hp's. This lack of an exact definition may be explained, at least to some extent, by the molecular heterogeneity of Hp's and by the insufficiency of our knowledge of the homeostasis and of the biological significance of Hp's.

As mentioned above, the name haptoglobin was coined to designate plasma components forming stable Hb complexes in which the Hb has acquired the properties of peroxidase. To describe an unidentified substance simply by the properties of its complexes with only one other substance is unsatisfactory. Hp as well as Hb from different species will presumably vary to some extent in composition, in the stability of their complexes, and in their peroxidase properties. Thus dog Hp, for example, links human or dog Hb, but the latter Hb complex shows only weak peroxidase activity in Jayle's conventional peroxidase test (N5).

We may provisionally regard Hp's as plasma proteins reacting stoichiometrically at physiological pH with Hb and globin, with the formation of stable complexes and with essentially the same antigenic structure within each species. The association constants of the Hb complexes are not known, but it is evident from various observations that the complexes are fairly stable (see Section 3.2.). A point that has not been studied systematically is whether the stabilization of the linkage between heme and globin after reaction with Hp and Hb is a regular occurrence independent of the species from which the Hp is derived.

The vague definition offered above or rather these characteristics appear to be sufficient since no plasma protein capable of forming a strong bond with Hb is known to occur in mammalian plasma except those mucoproteins with the general biochemical properties originally accepted as characteristic of haptoglobins.

Yet confusion exists in the literature since authors have not always made a distinction between hemoglobin- and heme-binding plasma proteins. Nyman (N6) and Aber and Rowe (A1) have stressed the difference between these proteins. A recently discovered heme-binding β-globulin (N1) [originally called α_2-globulin (B6)] with a higher

affinity for heme than other plasma proteins (A7, B6, F1, N1, T1, V2) has been accepted by many authors as hemoglobin binding. This misinterpretation of the findings made in the experimental investigation of the interaction between Hb and plasma proteins can be explained by the fact that those investigators who used the properties of the heme part of the Hb to trace the complexes formed assumed that the linkage between heme and globin is stable, which it is only within a limited pH range and only if the Hb molecules are protected, e.g., as HbCO. Synonyms and abbreviations used for the heme-binding β-globulin (HBG) are hemopexin (A7), cytochromophilin (B6) and the designation β_1B (in the immunoelectrophoretic pattern) (V2), or hemosiderophilin. The name cytochromophilin refers to the supposed affinity for cytochrome c (B6). The protein has also been described as myoglobin-binding (L2, W2). Judging from our control experiments, either myoglobin or cytochrome c is bound to HBG that has captured the heme from its earlier carriers.

In the later discussion of the method for measuring Hp it is considered as established that haptoglobins are the only native plasma proteins which exist or may exist in normal or pathological mammalian plasma with a HbBC above 5 mg/100 ml, i.e., the lowest capacity measurable by routine methods. It is also assumed that native haptoglobins themselves have no affinity for heme. The suggestion by Jones *et al.* (J14) that rabbit Hp has affinity for heme still lacks supportive evidence and can therefore not yet be accepted.

Our experience derives principally from studies with human, pig and dog plasma, and the statements made below refer mainly to problems in human pathophysiology.

1.3. Haptoglobin Units and Synonyms

The serum haptoglobin concentration was originally expressed in Hi units. One unit corresponded to the titer in a peroxidase assay "of a serum giving volume by volume complete saturation of horse Hb *M*/80,000. Following this nomenclature, a serum having one concentration unit contains 1.1 g Hp/litre" (J12). Jayle and Moretti recommend (J12) that serum Hp values should be given in grams per liter for the following reason: "The use of empirical units cannot be justified any more today, since the chemical constitution of haptoglobin as well as that of the complex Hb-Hp is now established." This argumentation would be acceptable if it really were established that Hp's have a known equivalent weight relative to Hb and that this was independent of the Hp phenotype. We do not think that these criteria have yet been satisfied.

Available data on the variation of the plasma Hp concentration in

health and disease have been obtained by various analytical methods standardized with Hb. The molecular weight of Hb has been determined with greater exactitude than that of the Hp type (1-1), which is the type that has received most attention. It would therefore appear wise to adhere to the practice of expressing the Hp concentration as its HbBC, i.e., "the amount of hemoglobin (mg) that can be bound by the haptoglobin present in 100 ml of a solution" (N5). An advantage of this description is that it reminds the examiner that the Hp measured is only the amount of Hp reacting with the Hb.

Approximate conversions. Hi $\times$ 1.1 = HbBC $\times$ 0.013 = g Hp per liter (J12).

Hyperhaptoglobinemia means that the serum HbBC exceeds the upper limit of the normal range of variation.

Anhaptoglobinemia denotes the absence of measurable amounts of Hp, which in practice means that the HbBC is less than 5 mg/100 ml. Conventional methods are not sensitive enough to measure true Hp concentration or the HbBC with any degree of exactitude below 10 mg/100 ml. Immunological techniques will probably prove capable of measuring Hp concentration below 10 mg/100 mg, especially since it is easy to prepare potent antihaptoglobin sera with precipitating antibodies.

2. Genetically Controlled Variation

After the discovery that the molecular heterogeneity of the Hp's is genetically controlled, Smithies and Walker (S9) offered a simple hypothesis for the inheritance of the Hp types. According to this theory two autosomal, allelic Hp genes Hp^1 and Hp^2 exist, and the three different combinations give rise to the phenotypes Hp 1-1, 2-1, and 2-2. This has been largely confirmed by all later investigators. The discovery is of great interest to forensic medicine (B1, G1, G3, H4) and to geneticists. "This system is of unusual interest, then, in relation to the genetic control of protein synthesis, for not only does the heterozygous individual produce proteins different from those in either homozygous type, but also in two of the types the haptoglobins consist of several fractions" (A4). "No other genetic system is known in which a single locus controls the specificity of more than one protein" (A5). The distribution of the phenotypes in different races has been comprehensively reviewed by Baitsch and Liebrich (B1), who also discuss the genetical problems in fair detail. On analysis of the data they had collected from various sources they found Hp^1 to be uncommon in Asia and most common in Africa. In Europe Hp^1 is more common than in Asia, but Hp^2 is the commonest type. Smithies and Connell (S8), and Allison (A4) have developed

Jayle and Boussier's earlier views (J8) further and proposed a polymerization theory to explain the many Hp fractions observed in individuals with the gene Hp^2 in single or double dose. Allison suggests that the protein products of the two Hp genes have the same molecular size (80,000) but that the Hp^2 gives rise to the protein product Hp 2, which, in contrast to Hp 1 with one single combining site, is assumed to have complementary combining sites. Under certain conditions Hp 2 can form a series of polymers. The molecular type Hp 1 formed in the heterozygote combines partially with the complementary site of Hp 2 and thereby stops further polymerization. In the homozygote Hp^1 Hp^1 the prerequisite for formation of polymers is lacking. The theory is useful as a working hypothesis, but the primary polymer unit supposed to be 80,000 may, instead, be only about 40,000 (see Section 3.2.). Smithies and Connell (S8) presented experimental support for the theory of stable polymers. The different bands obtained on starch gel electrophoresis kept their appearance and mobility also after being rerun. Depolymerization was achieved by a combination of urea and thioglycolate.

Atypical Hp phenotypes have, though very rarely, been reported (B8, G2, G3, G5, G6, S8). It must be questioned whether all of them are inherited variants (B2). A modified type of 2-1 (2-1 M) has repeatedly been observed. In this phenotype the concentration of the haptoglobins of low-molecular weight is higher and the concentration of haptoglobins of high-molecular weight is lower than in the conventional 2-1 type. Hp 2-1 M is relatively common in Africa (B8, G5), but not in Europe (H2).

Recently Smithies *et al.* (S10) presented biochemical results proving the existence of a total of six common Hp phenotypes instead of the accepted three. The family studies supported the hypothesis of 3 Hp alleles (Hp^{1F}, Hp^{1S}, Hp^2).* "The phenotypes corresponding to the genotypes Hp^{1F}/Hp^{1F}, Hp^{1F}/Hp^{1S} and Hp^{1S}/Hp^{1S} are indistinguishable (being Hp 1-1), as are those corresponding to the genotypes Hp^2/Hp^{1F} and Hp^2/Hp^{1S} (being Hp 2-1)." The constitutional difference could not be revealed until after reductive cleavage of the haptoglobins with mercaptoethanol in 8 *M* urea.

Anhaptoglobinemia is usually secondary to increased release of Hb into the circulation (A3, N2) (see below). According to Allison (A4), it is likely "that a small proportion of Caucasians and a higher proportion of Africans congenitally lack haptoglobins." He suggests that further

* Superscript letters F and S stand for "fast" and "slow."

family studies are necessary to reveal whether the anhaptoglobinemia is secondary to homozygosity for a suppressor gene at another locus (A3, G1, H3).

3. Haptoglobin and Its Hemoglobin Complexes

3.1. Isolation

A pure complex between Hp and Hb was isolated by van Royen (R5), who utilized the observation of Jayle and Gillard (J10) that the solubility of the HpHb complex in ammonium sulfate is higher than that of Hp. All attempts to recover Hp from its Hb complex have hitherto failed.

Pure Hp was first isolated from the urine of a patient with the nephrotic syndrome (J7). Urines from other patients with this diagnosis have never proved suitable as a source for preparation of Hp.

3.1.1. *Material*

The best sources of Hp preparations are sera from patients with advanced cancer and/or with severe infections without coexisting abnormal hemolysis, i.e., in subjects with high-electrophoretic α_2-values. Sera of the same Hp type may be pooled and stored in the frozen state with no loss of its HbBC. Sera containing Hb visible with the naked eye should not be added to the pools. Ascitic fluid from patients with infections or cancer, but without abdominal hemorrhage, is a convenient source.

3.1.2. *Methods available*

In recent years several methods for isolating Hp have been proposed and used with a varying degree of success (C2, H5, H6, L7, L8, S2, S11),[2] but in our opinion no ideal method is available for the preparation of stable, native Hp, at least not in more than trace amounts.

Jayle, Herman-Boussier, and Moretti have shown that different types of human Hp in amounts large enough for analysis can be prepared by fractional ammonium sulfate precipitation combined with a rather conventional, preparative electrophoresis in an acetate buffer of pH 5.8. Schultze and Heide (S2) utilize a more complicated procedure, in which preparative electrophoresis (acetate buffer, pH 4.4) is likewise the final step.

3.2. Properties

It is not difficult to obtain electrophoretically pure ($> 95\%$) Hp preparations, but the range of variation of the isolated compounds is,

[2] The complete technical details of the procedure are available on request.

as judged by the HbBC, too wide to be regarded as satisfactory. We feel that the degree of reproducibility of our results and of their agreement with those of Jayle's group are not sufficient to serve as evidence for or against the validity of their results (Table 1). We obtained, e.g., increasing nitrogen content in the order 1-1, 2-1, 2-2 in disagreement with Herman-Boussier. Schultze and Heide (S2) give a Biuret value of 81 for

TABLE 1
PERCENTILE COMPOSITION OF DIFFERENT HAPTOGLOBINS PREPARED BY HERMAN-BOUSSIER (H5)[a]

	Hp 1–1	Hp 2[b]
Nitrogen (Kjeldahl)	11.3	11.2
Protein (Biuret)	76.5 ± 2	74 ± 2
$E_{1cm}^{1\%}$ (278 mμ, pH 7)	11.9	12.1
Hexosamine	5.6	5.2
Galactose, mannose	7.7–10.4	10.5–12.7
Fucose	1	1
Neuraminic acid	6.3	5.0
Glucuronic acid	0	0
Tyrosine	5	5.9
Tryptophan	2.6	2.4

[a] All figures are expressed as per cent of dry weight except the absorbancy, *E*.
[b] Hp 2 is a mixture of 2-2 and 2-1.

Hp type 1-1 against Herman-Boussier's (H5) 76.5 ± 2%. We are therefore unable to recommend any preparative method as gentle and practical. In this case the discrepancies between physicochemical constants, unless ascribable to analytical errors, cannot be regarded as being of limited interest from a medical point of view, since they may indicate that one or more of the methods used causes denaturation of some of the protein. A rather sensitive indicator of any denaturation of the isolated compound is its HbBC per unit of protein. Like Herman-Boussier, when utilizing Connell and Smithie's method we obtained products with too low a HbBC indicating partial denaturation. We can confirm the observation of Herman-Boussier (H5) that if the pH of the acetate buffer is below 4.4, the HbBC of the Hp is rapidly lost.

As stressed by Herman-Boussier (H5), the Hp may rapidly lose its HbBC during the isolation procedure, even at neutral pH. She claims that her aqueous, nonsaline Hp solutions, especially of type 2-2, rapidly lose their HbBC which can, however, be preserved for months in the presence of $(NH_4)_2SO_4$ (0.4 *M*) or in $KHCO_3$ (0.02 *M*). This contrasts with our pure preparation, which can be dialyzed against distilled water without loss of HbBC. This difference in stability of the purified Hp is

ascribable to the principal difference in the isolation procedure used. One step in our method probably denatures a serum compound responsible for inactivation of Hp at low ionic strength. This step comprises precipitation with acetone in the cold and fractional elution with dilute cold acetone. This treatment protects the Hp product against later "spontaneous" inactivation. It has not yet been possible to ascertain the type of reaction resulting in loss of HbBC of Hp. Hp or serum may be heated up to 53°C for 30 minutes without any demonstrable loss of HbBC. Herman-Boussier *et al.* (H7) claim that Hp is more stable when prepared from plasma than when it is prepared from serum.

The sedimentation and diffusion constants (H7) of Hp 1-1 are 4.4 ± 0.1, and $4.7 \times 10^{-7} \pm 0.02$[3], respectively. With Herman-Boussier's partial specific volume (0.766), a molecular weight of $100{,}000 \pm 10{,}000$ is obtained. This final calculation gives a result which is higher than that based on the earlier observations by Jayle's team (J8). They emphasized that 1.10 mg Hp is saturated by one milliliter of *M*/80,000 Hb solution, i.e., containing 0.826 mg protein (molecular weight of Hb $4 \times 16{,}520$ is accepted). Linkage one to one will then give a molecular weight of 88,000 for Hp. Nobody has ever claimed that any polypeptide is released on reaction between Hp and Hb.

The literature contains no physicochemical studies of solutions containing molecules representing the separated, single Hp lines observable on starch gel electrophoresis when analyzing Hp 2-1 or 2-2. Schultze and Heide (S2) have reported data on solutions containing the whole Hp 2-1 and 2-2 spectrum of molecules in the ultracentrifuge. They have given the following sedimentation figures for Hp type 2-1: 4.5, 6, and 7 S in the ratios 34:47:19 and for type 2-2: 7 and 8 S in the ratio 79:21. It is too early to state anything definite about the exact size of the molecules in the different Hp lines obtained on starch gel electrophoresis. The simple hypothesis (A4, S8) that Hp 1-1 is the base unit in size and that the distance of the Hp-lines from the starting point varies largely with the number of polymers sounds plausible, but it has not yet been proved (A4) (cf. Section 2). According to this hypothesis, the 8th line of Hp types 2-2 would have a molecular weight of about 900,000, i.e., approximately the same as Schultze's α_2-macroglobulin (820,000) (S1), and they migrate together in starch gel (S6). The suggestion by Herman-Boussier *et al.* (H7) that the size of the polymer unit corresponds to half the molecular weight of Hp 1-1 may be

[3] We wish to thank Dr. A. Ehrenberg of Medicinska Nobelinstitutet, Stockholm, who has confirmed our findings.

correct despite the weakness of their experimental evidence. This would imply that the simplest Hp molecule, like Hb itself, is a dimer. The other haptoglobins would then be polymers of a higher order of magnitude, but the size does increase less rapidly than supposed by Allison.

After treatment of purified Hp of type 2-1 and 2-2 with a combination of urea and thioglycolate, Smithies and Connell (S8) found that these haptoglobins had been depolymerized. That the different Hp lines represent different degrees of polymerization is also supported by the findings of Kluthe and Isliker (K1).

The isoelectric points (i.p.) of different Hp types have been given as pH 4.20–4.25 for type 1-1, and as 4.10–4.20 for a mixture of 2-1 and 2-2. The last i.p. figures may be too low since the slight difference in electrophoretic mobility (Hp 1-1 faster) on free electrophoresis is attributed to the larger amount of acetylneuraminic acid in Hp 1-1 (5.3%) than in 2-2, and 2-1 (3.4 and 3.4%, respectively) (S2). It is therefore probable that Hp 2-2 has a slightly higher isoelectrophoretic point than Hp 1-1. We found the minimum solubility of HpHb complexes of type 2-2 to be higher at higher pH than that of 1-1, which would also suggest a difference in charge of about 0.3 pH units. On paper, in agar gel, and in moving boundary electrophoresis with a neutral phosphate or a slightly alkaline barbiturate buffer, all human types of haptoglobins migrate in the α_2 peak at approximately the same rate.

The mucoprotein nature of Hp was soon recognized by Jayle. The polysaccharide part comprised 22% of the molecule according to Cheftel *et al.* (C1) and 18.5 according to Schultze and Heide (S2).

Solubility curves for different types of Hp have been presented (H5, H7). The higher solubility of type 1-1 than that of the others is in conformity with its lower molecular weight. The irregularity of the solubility curves for Hp of 2-2 and 2-1 type reflects the molecular heterogeneity of these two proteins. It is known from fractionation experiments with ammonium sulfate as well as with ethanol that the slower Hp bands are enriched in those Hp fractions that are precipitated first. So far, we have not been able to separate any of the Hp bands completely from the others, except the 1-1 band in ascitic fluid of type 2-1.

The solubility of Hp in different acids has been given by Jayle and Moretti (J12). The relatively high solubility in 0.6 *M* perchloric acid explains why some of the haptoglobins are included in "Winzler's mucoproteins" (V2). Judging from our experience, on measurement of the mucoprotein by perchloric acid precipitation according to Winzler, the supernatant contains a relatively larger amount of Hp 1-1 than of Hp 2-2.

Within experimental error the various types of Hp give identical spectra and extinction coefficients (H5) in ultraviolet light.

Electrophoretic investigations have shown that haptoglobins link Hb and globin firmly over a wide pH range (at least 9 to 4.4). Van Royen (R5) proved the reaction between Hp and globin (L8, R4). In an attempt to test the stability of the HpHb and Hp-globin complexes in the pH range of physiological interest we performed the following experiments:

> The saturated HpHb complex of type 1-1 was formed in solution and mixed with a pure solution of globin. The proteins were then separated by starch gel electrophoresis with a neutral phosphate buffer. The procedure was afterward repeated, but with a solution of Hp-globin complex mixed with an Hb solution. The relative rates of migration of the proteins were: Hp > HpHb/2 ~ Hp globin/2 > HpHb ~ Hp-globin > Hb ~ globin. Comparison of slices treated with benzidine and with amido black, respectively, revealed the presence of globin or Hb in a given fraction. In none of the experiments did free Hp or any of the intermediate complexes (see below) appear as a result of dissociation. In the first experiment no signs of any exchange between the Hb in the HpHb complex and the globin were observed. A faint benzidine color was observed at the site of the Hp-globin complex in the gel in the second experiment. This may indicate either that a small amount of globin in the Hp-globin complex had been expelled by Hb, or that a slight exchange of hemin had occurred, which seems probable since part of the HbO_2 was converted into HbOH during the experiment.

At any rate these results prove that the degree and the rate of dissociation of the HpHb and Hp-globin complexes are extremely low. The results are also in agreement with findings made in the investigation of the exchange between free and Hp-bound Hb with labeled (Fe^{59}) dog Hb (N3).

According to Jayle and Moretti (J12), human Hp combines with Hb from different species (monkey, horse, cow, dog, rabbit, and mouse). No difference has been found between the spectra of free Hb and Hp-bound Hb (G8, J12, R5). Spectroscopically the HpHb complexes gave the same CO, OH, and CN compounds as Hb. In intravascular hemolysis, methemalbumin forms only after Hp has been saturated with Hb, i.e., when small amounts of free Hb begin to appear in the plasma (A6, B14, L4,

N8). It may therefore be concluded that $HpHbO_2$ is normally less readily converted to HpHbOH than Hb to HbOH, i.e., the heme linkage is more stable. Judging from the higher resistance of HpHb to oxidation by I_2 (R5) at slightly acid pH, the heme is probably more firmly bound in the HpHb complex than in free Hb. The HpHb complex does not undergo acid hydrolysis as readily as Hb (R5). The peroxidase activity of the HpHb complex with pH optimum about pH 4.2 also indicates that the linkage of heme to globin is altered after reaction of Hp with Hb. The peroxidase activity of the HpHb complexes varies from species to species and depends primarily on the structure of the globin part of the Hb. Horse Hb shows roughly twice the peroxidase activity of human Hb (R5). Dog Hb gave a Hp complex that was almost devoid of peroxidase activity (R5). It may then be expected that the structural differences between the various normal and abnormal human Hb's will be reflected in the specific peroxidase activity of some of their Hp complexes. This may be a further earmark of the different abnormal Hb's and therefore requires systematic investigation. From Nyman's (N5) comparison between peroxidase values and HbBC values of unselected sera, it can be inferred that the peroxidase activity per unit of bound Hb is independent of the Hp type. She has also presented other data supporting this statement.

The difference between the solubility of the HpHb complexes and that of the corresponding haptoglobins can be utilized for salting out fairly pure HpHb complexes of the different types. On prolonged dialysis against distilled water pure HpHb of type 2-2 will separate off as a viscous fluid gel.

Kluthe and Isliker (K1) have isolated the two homozygotic Hp types as Hb complexes by utilizing the solubility difference of free and bound Hp in 0.01 *M* zinc acetate. Their last step was chromatography on carboxymethyl cellulose.

The nature of the linkage between Hp and Hb is still obscure. According to Robert *et al.* (R2), it includes no S—S linkages. After blocking of the amino groups of Hb, the reaction with Hp was inhibited (R5). Such substances as protamine, heparin, and alginates interfere with the reaction between Hp and Hb [Bajic 1958, cited by (N5)]. It is evident from available centrifugal data that Jayle's early finding that one mole Hp (1-1) links one mole Hb is correct (B4, L8, S2, S6). The exact molecular size of the different polymers corresponding to the different Hp lines of the other Hp types observed on starch gel electrophoresis have not been determined (B4, C1, K1, L8, S2, S6). If simple polymers of almost identical Hp units are accepted, and if no

steric hindrance for Hb linkage is assumed to occur even in the biggest polymer, the quotient heme : protein will, on saturation with Hb, be the same for all polymers independent of size. If the two genetically controlled elementary polymer units differ measurably in composition, this quotient will vary from type to type, as will the ratios between the different polymers within type 2-1. Available data are insufficient to warrant any definite statement on this point (H5). The problem is of practical importance since it is desirable to report Hp concentration in absolute weight units.

If Hp type 1-1 is partly saturated with Hb, the starch gel electrophoretic pattern will include a band between that of Hp and that of HpHb. The corresponding complex was observed earlier (A6, L10) on paper, but then it is less sharply separated from HpHb. In view of the migration rate of the complex in gel and on paper, and of its relative intensity when stained with benzidine and amido black, one of us (L8) suggested that the complex consisted of 1 molecule of Hp and ½ molecule of Hb. Preliminary ultracentrifugal data failed to reveal the exact molecular weight. Herman-Boussier (H5) has confirmed these observations, but she and her co-workers (H7) suggest, on the basis of their optical studies of the complex, the principle composition ½ molecule HpHb, i.e., they believe that the bonds between the polypeptide chains within both the Hb and the Hp molecules are less strong than the bonds between Hb and Hp polypeptides. Herman-Boussier's hypothesis does not, however, explain the difference in charge between HpHb and the intermediate complex. According to both hypotheses, the Hb molecule is split on reaction with Hp. No attempts have been made to discover the geometry of the dissociation—does each half contain one α- and one β-chain or not? The globin part of the Hb molecule has hitherto been considered stable in neutral but not in acid solutions. Judging from the observations above, in the presence of Hp, the Hb molecule is probably easily dissociated in the physiological pH range. When the peroxidase activity of a pure Hp solution was tested by Jayle's activation method, it was found that the peroxidase activity increased linearly with the Hb added, until the Hp was completely saturated. This indicates that the specific peroxidase activity with regard to the heme bound is the same for the intermediate complex as for the fully saturated complex. This may indicate that no reassociation of the Hb parts has occurred, not even in the saturated complex, and that the peroxidase activity of Hb occurring after linkage to Hp may be explained as secondary to the dissociation of the Hb molecule.

In experiments in which increasing amounts of Hb are added to pure

Hp of types 2-1 and 2-2 it was observed (L8) that complexes intermediate with regard to charge and Hb content are also formed from these Hp's. The number of intermediate complexes has not been determined.

3.2.1. *Immunological Properties of Hp*

The precipitation line corresponding to Hp in the immunoelectrophoretic pattern was identified as early as 1954 by Burtin *et al.* (B14), who used a total antihuman serum and the same antiserum absorbed with Jayle's urinary Hp fraction. A specific antihaptoglobin serum is obtained by adsorbing a total antihuman serum of a high anti-Hp titer with a serum void of Hp, e.g., most cord sera. The identity or very close relationship of the dominating antigenic determinants of the different human Hp types has been demonstrated by agar gel diffusion and electrophoresis experiments (A7, B4, B10, F1, H8). Anti-Hp sera precipitate also the HpHb complexes (B10, W5) in gel, but it appears that no attempts have been made to ascertain the number of reactive sites on Hp and HpHb, respectively. The depolymerization of HpHb (type 2-2) obtained with cysteine has been shown with Ouchterlony's gel diffusion technique (K1).

The HpHb and the Hp-globin complexes can also be detected (R4) with rabbit antihemoglobin serum. The combination between Hp and anti-Hp is not influenced even if the neuraminic acid has been split off (L5, L6, P5, S3). That the antigenic structure of dog Hp is related to that of human Hp is evident from the fact that our potent antidog Hp serum cross-reacted with Hp in human sera and that our potent antihuman Hp serum cross-reacted with dog Hp.

4. Methods for Determination of Concentration and of Type

4.1. Quantitative Determination

Starting from the observations that HpHb has a much higher *peroxidase* activity at acid pH and is more resistant to I_2 than Hb, Jayle (J5) devised two similar methods for measuring Hp—the "activation" and the "saturation" methods—which are still in common use. The difference between the *electrophoretic mobility* of HpHb and of Hb was also later (L10) utilized to estimate the serum Hp concentration. The general agreement between results obtained by both methods was shown by Nyman (N5).

4.1.1 *Peroxidase Methods*

Jayle's activation method is technically fairly simple, but complicated from a kinetic point of view. Some of the reaction steps of the method have recently been carefully reinvestigated by Nyman (N5), who also

presented a detailed description of the method. We have used a slight modification of Jayle's method in our clinical work ever since ethyl hydroperoxide became available in nonexplosive solution. We think the method is recommendable for serial analysis, since it is rapid and relatively simple. Our simplification concerns mainly the setup. Instead of using a reaction temperature of 32°C we perform the analysis at room temperature. The influence of temperature on the reaction rate has been tested within the interval 26° to 20°C, and conversion factors are available. Our technical instructions are given below. Detailed information and discussions concerning the original method are available in Nyman's thesis (N5).

Connell and Smithies (C2) have proposed a photometric peroxidase method with guaiacol as substrate and H_2O_2 instead of ethyl hydroperoxide. According to Nyman (N7), the method is less specific than Jayle's method. In the clinically interesting low range of HbBC (0–30 mg/100 ml) the method is unreliable. A simplification has been presented by Owen *et al.* (O2). No report exists on its specificity, accuracy, or precision.

4.1.2. *Jayle's Activation Method (Modified) for HbBC Determination*

4.1.2.1. *Principle.* When serum is mixed with an Hb solution, the Hp is bound. The rate at which the mixture oxidizes I^- to I_2 in the presence of C_2H_5OOH is, under the conditions used, proportional to the HbBC (Hp content) of the serum. Hb added in excess is partially inactivated by addition of I_2. The amount of I_2 formed during the reaction is estimated by titration with sodium thiosulfate.

4.1.2.2. *Reagents.* 1. Horse Hb. One volume of red cells, washed 3 times with saline, is hemolyzed with 2 volumes of distilled water.

Ghosts are separated off by acidification with 1 *N* HCl to pH 5.8 and centrifugation. The solution is then neutralized with 1 *N* NaOH to pH 7.1 and the small amount of precipitate formed is centrifuged off. The solution is diluted until the concentration of the Hb is 1.6 g/100 ml. In the frozen state it can be kept for years without alteration. Before use it is diluted to about 80 mg of Hb/100 ml. This solution can be kept 2 weeks at +4° C.

2. Human Hb is prepared and stored in the same way as horse Hb.

3. 0.1 *M* Potassium iodide.

4. 0.1 *M* Acetate buffer pH 4.4.

5. 0.01 *N* Sodium thiosulfate solution is prepared daily by dilution of stock solution of known concentration.

6. 0.5% (w/v) Starch solution.

7. 2–3 *N* Ethyl hydroperoxide,[4] must be kept in the dark at +4° C. It is diluted until 0.11 *N*. This solution can be kept for a week in the dark at +4° C.

The normality of the stronger solution is checked once a month in the following way: 0.1 ml ethyl hydroperoxide solution +2 ml potassium iodide (20 g/100 ml) + 2 drops of glacial acetic acid are placed in a porcelain crucible, covered with a lid, and allowed to stand at room temperature for 4 hours. The amount of iodine released is then titrated with 0.05 *N* sodium thiosulfate.

8. 0.007 *N* Iodine solution.

4.1.2.3. *Equipment.* A 2-ml burette graduated in 0.02 ml and 25 ml glass beakers.

4.1.2.4. *Procedure.* Ten tests are done in series: 0.1 ml of horse Hb solution is pipetted into each beaker; 0.020 ml serum is added to nine of the beakers. The content of the remaining one is used as a blank. Three ml 0.1 *M* KI is added to each beaker. A mixture of 100 ml acetate buffer, 10 ml ethyl hydroperoxide solution and 10 ml iodine solution (8) is then prepared. (This mixture is only fit for use for 20 minutes.) At 60-second intervals 10 ml of this mixture is added to the beakers. Nine minutes and 15 seconds after the addition, 2 drops of starch solution are added, and titration with 0.01 *N* $Na_2S_2O_3$ is started after 9 minutes and 30 seconds. The titration should last, as closely as possible, 30 seconds. The temperature is measured in the reaction mixture.

4.1.2.5. *Calculation.* When horse Hb has been used: Titration value in milliliters (test—blank) × 96 × temperature factor (from tabulation) = HbBC in milligrams per 100 ml. When human Hb has been used: Titration value in milliliters (test—blank) × 185 × temperature factor.

Temp. ° C	Temp. factor	Temp. ° C	Temp. factor
+20	1.80	+24	1.57
+21	1.73	+25	1.52
+22	1.67	+26	1.47
+23	1.61	—	—

[4] Commercially available from AB Ferrosan, Malmö C. Sweden.

4.1.2.6. *Comments.* When the sera are hemolytic, human Hb is used instead of horse Hb. The blank, too, must then be run with human Hb.

When the volume (test subtracted by blank) of 0.01 *N* $Na_2S_2O_3$ consumed, is plotted against HbBC, the curve is slightly arched but in practice it may be regarded as straight until 1.2 ml when horse Hb is used and 0.6 ml when human Hb is used. When higher titration values are obtained, the titration must be repeated with diluted sera.

The reaction continues during the titration. This must therefore be finished close to the aforementioned time limit. If the titration is continued for, say, 30 seconds beyond the standard time, the values obtained will be about 5% too high.

The reaction velocity is dependent on temperature, which must therefore be measured. According to Nyman (N5) and judging from our own experience, the method is sensitive down to a HbBC of 5 mg/100 ml.

4.1.3. *Electrophoretic Methods*

Hb and HpHb can be separated by paper or gel electrophoresis. A time-consuming but safe variant is to run electrophoresis on a series of solutions containing a standard amount of serum but Hb in increasing concentration preferably in a neutral buffer (A6, L10, N5). The accuracy of the results depends on the magnitude of the differences between the concentration of Hb added. If precision is desired, it is useful to ascertain the approximate Hp value in an electrophoretic screening run and to rerun the sample with the addition of Hb in a series of concentrations in the neighborhood of the approximate value. High accuracy may be obtained, but the method is too time consuming for routine work. It is advisable to use Nyman's electrophoretic technique to standardize other HbBC methods. Her staining with malachite green can preferably be replaced by Owen's *o*-dianisidine staining (O4).

Electrophoretic separation of methemalbumin HpHb, and Hb after addition of excess amounts of Hb, staining with benzidine (L4) or *o*-dianisidine (J2), and finally scanning of the spots are other possibilities available for routine work. At low Hp concentration these methods do not give exact values since HBG is not taken into account, but the results are accurate enough for routine clinical work. Hommes (H10) eluates the HpHb spots and estimates the peroxidase activity. In this method HbBC is not taken into account.

A recently described method (D1) utilizes the difference between the protein concentration in the α_2 region before and after addition of Hb. This can only give the approximate Hp content of serum.

4.2. Determination of Type

Several modifications of Smithies' original Hp-typing technique (S5) have been proposed. It has generally been accepted as simplest and safest to identify the Hp by its saturated Hb complexes (HpHb), since these are more easily recognized and developed than Hp itself in starch gel and in immunoelectrophoresis. To convert all Hp to HpHb before the analysis, more Hb is added to serum than can be bound by the Hp.

4.2.1. *Starch Gel Electrophoresis*

Smithies' vertical starch gel electrophoresis (S7) separates the plasma proteins more distinctly than any other method. If the Hp concentration is normal, the Hp type can generally be recognized directly after the staining for proteins, but sensitive and more specific staining for heme groups, e.g., benzidine, *o*-dianisidine (O4), and malachite green (N5) are preferable. This technique consumes more hydrolyzed starch than the simpler original horizontal electrophoresis technique (S5).

4.2.1.1. *Limitations.* When the serum HbBC is low (below 20 mg/100 ml), the Hp type may be difficult to recognize, because the HpHb lines are faint. They may be so faint for types 2-2 or 2-1 that the minute amount of trailing Hb may mask the characteristic pattern of the type. Concerning type 1-1, minute amounts of HpHb complex are not readily recognized since the intense, but not sharply demarcated, Hb zone is near the faint HpHb line together with the HBG zone.

> The *Hb solutions* generally used are obtained by simple osmotic hemolysis of normal red cells followed by elimination of the ghosts. The molecular heterogeneity of such solutions of adult Hb is revealed by the starch-gel electrophoresis. The Hb line is therefore not quite distinct, which is a minor drawback when the solutions are used for Hp typing. To stabilize the Hb solutions, it is advisable to bubble CO through them before they are ampouled and stored in the frozen state.

4.2.1.2. *Modification.* A method for large scale typing (160 sera per 2 hours) in modified trays for starch gel has recently been described by Baitsch and Liebrich (B1). They utilize the power supply and the cooling system of the Pherograph designed by Wieland and Pfleiderer (W3) for high voltage electrophoresis of low-molecular substances. The

quality of the separation obtained is not apparent from their publications, but it seems to be good enough to recognize the Hp type. In questionable cases supplementary studies are made with conventional technique or such minor modifications as starch gel electrophoresis at pH 7 or below (L9). The separation of the plasma proteins is less distinct at this pH than at the conventional, more alkaline pH (> 8). However, below pH 7 the free Hb changes its direction of migration from the anode to the cathode, and thus moves in opposite direction to the HpHb complexes from the insertion. It is easier for the untrained analyst to decide the Hp type after benzidine staining if a neutral, instead of alkaline, buffer has been used. This technique is especially recommended for grouping of sera containing trace amounts of haptoglobins, when only one-tenth of the standard amount of Hb is preferably added to the serum. If further HbCO or fresh HbO_2 is used, no HBG will be developed; this prevents misinterpretation of HBG as Hp of type 1-1.

The following phosphate buffers are useful:

The starch is boiled with 0.007 *M* phosphate buffer, pH 7.5, the electrode vessels are filled with 0.03 *M* phosphate buffer of pH 7.0, which is also used for the bridges. Smithies' simple plastic trays (S5) for horizontal starch gel electrophoresis will give results clear enough for routine work.

4.2.1.3. *General Remarks.* A prerequisite for obtaining conventional patterns is that the Hb has been added to the serum in amounts sufficient to saturate the Hp. As a standard procedure the Hb concentration in serum is increased to 500 mg/100 ml serum before the electrophoretic run. However, sera from patients may contain Hp in a concentration above a HbBC of 500 mg/100 ml (highest value observed was 1400). In such cases the electrophoretic pattern will show supplementary abnormal HpHb lines, since also the intermediate complexes are recognized (see Section 3.2.). The pattern may then be misinterpreted as new Hp types (B5).

A theoretical disadvantage of the methods utilizing HpHb complexes for Hp typing is that the Hb reactive groups of Hp may be blocked by compounds bound more firmly than Hb. The pattern obtained will then simulate that of anhaptoglobinemia. No such cases have been reported by clinical workers studying sera from adults, but the finding, in umbilical cord sera, of an immunologically typical haptoglobin with too high an electrophoretic mobility and too low a peroxidase activity for a Hb complex, may be an example of blocked Hp (F1). If an anhaptoglobinemic serum contains HBG, it may be erroneously classified as type 1-1. No

misinterpretation will be possible if reference sera of Hp type 1-1 and HbCO (instead of HbO_2) are used in doubtful cases.

4.2.2. *Paper and Agar Gel Electrophoresis*

Budtz-Olsen (B13) has shown that Hp grouping is possible on vertical paper electrophoresis if Consden and Powell's (C3) borate-barbiturate buffer is used, since differences between the migration rate of the different Hp's is then great enough. It is, however, doubtful whether this is possible when the concentration of the Hp is low.

Hirschfeldt (H8) has suggested that the small differences in migration rate of the different HpHb complexes in agar gel at pH 8.6 with a Ca^{++}-containing barbiturate buffer can be used for ascertaining the Hp type. In our opinion the results obtained by this simplified procedure are less reliable than those obtained by the somewhat more complicated starch gel electrophoretic method.

4.2.3. *Immunological Methods*

Recently Aly *et al.* (A7) proved that Hp typing is possible by a micro-immunoelectrophoretic technique or by a simple agar gel diffusion test utilizing the precipitation lines obtained between an Hb-saturated serum and an antihuman serum. The hematin or heme-containing precipitation lines formed are developed by a benzidine-hydrogen peroxide staining procedure. As reference for judging the migration and the diffusion rate in the agar gel electrophoresis they utilize the HBG (β_{1B}) line, which also appears after such staining if an appropriate antiserum has been used.

In the simpler agar-diffusion technique the relative distances of the HpHb lines and the HBG line from the serum and antiserum wells are used as a basis for typing.

The position of the precipitation lines depends on, e.g., the diffusion rate of the different HpHb complexes, of the HBG and of the antibodies. The molecular size (the type) of the HpHb complex will therefore influence the result of the diffusion tests in agar. The technique seems to be simple and promising. So far, the data available on the sensitivity and reliability of the method is not sufficient to warrant recommendation of the procedure.

4.2.4. *Concluding Remarks*

The migration or diffusion rate of HpHb complexes or of Hp alone are of importance in several of the methods discussed above. It should be observed that the electrophoretic mobility of the Hp in aged sera may decrease if the sample has been contaminated with any of the common

sialidase-producing bacteria (L6, P5, S3). Such an enzymatic effect on the serum proteins may be suspected if the mobility of the α_1-fraction in the paper electrophoretic pattern is abnormally low, since the mobility of the α_1-glycoproteins is a sensitive indicator of the sialidase content of a serum.

5. Homeostasis of Haptoglobin in Health and in Disease

5.1. Normal Plasma Values

5.1.1. *Healthy Subjects*

The largest serum material (277 subjects) analyzed for both Hp type and HbBC is that of Nyman (N5). The mean HbBC, independent of Hp type, was 110 (S.D. 41) mg/100 ml, which is higher than Jayle and Boussier's figure of 75 (S.D. 31) mg/100 ml based on analysis of 1000 subjects (J8). No explanation can be offered for this discrepancy; it cannot be ascribed to the difference in the distribution of the Hp types in the two materials. Nyman's mean value for sera of type 1-1 was 136, for type 2-1 it was 108, and for type 2-2 it was 82 mg/100 ml. The differences between the mean values for each type were significant. The mean value for men (113) was significantly higher than for women (90). According to Jayle, in adults the value increases somewhat with age. Premature children are born anhaptoglobinemic (F2). In about 90% of cord blood samples and in samples collected during the first 14 days of life the Hp content is immeasurably small by conventional techniques (A6, G1, P1, T3). The Hp content then gradually rises (H9, W6) and at 4 months it reaches the mean level of healthy adults (H9).

5.1.1.1. *Comment.* The anhaptoglobinemia frequently found at birth has been discussed by Fine *et al.* (F1), who conclude on the basis of immunological studies that Hp probably exists in cord blood, but has not got its "definitive structure." Their experimental observations and suggestions are interesting, but owing to lack of quantitative data their interpretation is debatable.

Nyman suggested that the minor sex difference may be ascribed to the influence of the sex hormones (B11, S12) on Hp homeostasis (see Section 5.4.).

The normal range of variation of each Hp type is remarkable. It is wider than what is usually found for substances of biological importance and is of the magnitude generally found for substances in transport. From the wide range of variation it is reasonable to assume that no such feedback mechanism exists and that the rate of Hp synthesis is influenced, directly or indirectly, by the plasma level.

The observed differences in the mean value of subjects belonging to the different Hp types are of some practical interest when deciding whether a subject is "normal" or not, e.g., when Hp values are used supplementary to the E.S.R. as a screening test in population studies. The reason for the difference between the HbBC of the various types remains to be explained. The mean value of HbBC for a given Hp type may be taken as its mean metabolic steady state. The differences found in the plasma level between the types may thus reflect differences in rate of Hp formation or in rate of elimination. The latter possibility is not improbable if we assume that one of the factors determining the normal catabolic rate of Hp is the amount of Hb or globin entering the plasma. We accept that the different Hp lines found on starch gel electrophoresis represent different polymers composed of two types of elementary Hp units and equivalent in HbBC. We know that once the HpHb complexes are formed, they are rapidly eliminated from plasma and catabolized (see Section 5.2.1.). It is then reasonable to assume, although it has not been proved experimentally, that the intermediate (incompletely saturated) HpHb complexes are also eliminated. This would imply that the higher the mean valency of the Hp molecules for Hb in plasma in persons with the same absolute Hp level, the greater will be the absolute amount of Hp lost during continuous inflow of small amounts of Hb to plasma. Not only will the Hp concentration of the plasma decrease, but the initial ratio—dictated by synthesis—between the different Hp polymers within a subject's plasma will change, resulting in an increasing overrepresentation of the simpler polymers until a new steady state is reached—the "normal" concentration pattern. The validity of this explanation for the different mean Hp values found within the different groups may be checked experimentally.

Jayle and Boussier (J8) have found that in view of the wide biological range of variation, the plasma Hp level in a given healthy subject is strikingly steady. No attempts have been made to ascertain whether the level is genetically controlled. This *individual* stability is less apparent in menstruating women owing to the rhythmic tissue destruction, proliferation, and estrogen output (B11). The *diurnal variation* (N5) does not seem to exceed the variation due to daily changes in water content of the plasma. During *pregnancy* the Hp level remains constant (N5, P1) which, in view of the substantial changes in output especially of cortisol and estrogens, is most remarkable. Experimental interference with homeostasis of these hormones markedly influences the Hp content of plasma in nonpregnant women (B11, V1). The concentrations of the plasma proteins ceruloplasmin and fibrinogen increases during pregnancy. The

concentrations of these two proteins vary in disease and are usually highly correlated with that of the haptoglobins (K3, N5).

5.2. Plasma Haptoglobin in Relation to Hemoglobinemia, Hemoglobinuria and Erythrocyte Turnover

When running routine paper electrophoresis of serum, many investigators have probably observed that the electrophoretic pattern in the α-β region is atypical in hemolytic sera. Tuttle (T3) and Wieme (W4) ascribed the phenomenon to interaction between α_2 proteins and hemoglobin. Wieme stated more precisely that the fraction appearing between α_2 and β was the HpHb complex. This was evidence of the high degree of stability of this complex at neutral pH and prompted us to study the interaction between Hp and Hb *in vivo* by injecting Hb intravenously (L10, L11). The injected Hb was immediately bound to available Hp. Free Hb did not appear until all plasma Hp had been saturated with Hb. No rapid inflow of Hp to the plasma occurred after the plasma Hp had been blocked by Hb. The whole HpHb complex was eliminated from the plasma without the appearance of any measurable amount of Hp during the following few hours. The Hp level gradually returned to the initial level during the next few days and seemed to be normalized within a week. No hemoglobin appeared in the urine until more Hb had been injected than could be bound by the Hp. These observations favored the hypothesis that the "renal threshold" for Hb depends primarily on the Hp concentration of the plasma and not, as was previously generally believed, on the capacity of the tubular renal cells to reabsorb Hb. The new hypothesis was based on the fact that a stable HpHb complex is immediately formed when Hb contacts Hp *in vivo* and that this complex, in contrast to free Hb, has too large a molecular size to pass into the glomerular filtrate. Allison and Rees (A6) proposed the same explanation for the renal threshold for Hb and showed that patients with paroxysmal nocturnal hemoglobinuria and cold hemoglobinuria usually have no demonstrable Hp in their plasma. Vanderveiken *et al.* (V1) proved that the renal Hb excretion in dogs varies with the concentration of free plasma Hb. A number of clinical and experimental observations (A6, L10) made after intravenous injection of Hb were also found to support our hypothesis, the essence of which can be traced back to a period before the time was ripe for plasma protein research. In this connection it might be convenient to refer to the early observation in 1916 by Sellard and Minot (S4) who studied the appearance of hemoglobinuria after intravenous injections of Hb. They found that hemoglobinuria could be provoked in patients with pernicious or hemolytic

anemia by injection of a smaller Hb dose than in healthy persons. They wrote: "We only wish to point out that there is a definite relationship between tolerance of hemoglobin and the blood destruction." This is in complete agreement with the finding that patients with hemolytic or pernicious anemias usually have no demonstrable Hp in plasma (A3, A6, B12, J6, L3, L10, N1, N2, N4, N5, N8, O1, P2), presumably because of an increased elimination rate of the Hp (secondary to the Hb complexing) without any compensatory increase of Hp synthesis.

It is apparent from the studies of Gydell *et al.* (N8), and of Brus and Lewis (B12) that anhaptoglobinemia is common in subjects with twice the normal turnover rate of the erythrocyte cell mass.

In the presence of infection, malignant diseases, and steroid therapy, haptoglobins may be demonstrable even if the rate of hemolysis is more than twice the normal rate (B12).

When human red cells were killed by storage in saline and reinjected intravenously, no increased consumption of Hp was observed (G9). Neither did the plasma Hp level change when the normal erythrocyte turnover was accelerated by injection of nicotinic acid (G10). Patients have also been observed who rapidly eliminated all red cells infused without consequent hemoglobinemia or decrease in the plasma Hp.

These partially contradictory observations give rise to the question whether the normal catabolism of Hp is related to the normal destruction of red cells or not. The Hp consumption secondary to erythrocyte destruction in a given person must reasonably depend upon the mechanism by which the red cells are destroyed. Neither in health nor in disease is the bulk of the red cell destruction due to intravascular rupture. A minor part of the cells may, however, rupture intravascularly, damaged cells may link Hp, and part of the Hb of the dying cells may be returned to plasma from the organs destroying red cells.

Garby and Noyes (G4) have measured the kinetics of the disappearance of labeled, small amounts, of Hp-bound Hb in plasma. If their data are pooled with the normal mean values presented by Hank *et al.* (H1) for plasma Hb (0.3 mg/100 ml plasma), it will be evident that about 0.5 g Hb enters and leaves plasma per day, i.e., a little less than 10% of the normal Hb catabolized is released into, or returned to, the circulation. These data—if correct—can also be used to calculate the approximate amount of Hp engaged in our normal erythrocyte (Hb) turnover. We must assume that the dominating Hp complex formed when Hb enters the circulation in minute amounts is one molecule Hp and ½ molecule Hb, since the saturation of Hp is very low. We accept that the molecular weight of Hp is 100,000. About 1.4 g of Hp will then be con-

sumed daily, secondary to the Hb transportation of 0.5 g. Assuming normal plasma Hp and normal plasma volume, the plasma Hp pool is about 4 g. An extracellular pool of the same size would imply a total pool of about 8 g Hp, i.e., a fraction of 0.18 of the total Hp body mass thus disappears in an average person daily on account of the normal erythrokinetics. Like other plasma proteins, Hp must also be lost by other pathways (intestinal tract) and true catabolism within the body must be supposed, so that the normal half-life of Hp must be shorter than 2 days. The plasma Hp level in a given person is fairly constant but varies widely from person to person. This may be due to *small* differences in Hp synthesis or to interindividual differences in the daily amount of Hb that enters the circulation (minor variation in red cell structure, in destruction mechanism, or in the organs destroying the aged cells). It may therefore be understandable that the normal Hp level varies widely and that anhaptoglobinemia often, but not regularly, develops at slightly increased red cell turnover provided that changes in the Hp level per se do not influence Hp synthesis.

From the comparison by Nyman *et al.* between plasma Hp level and different red cell destruction indices (half-life of Cr^{51}-tagged erythrocytes, endogenous CO formation, stercobilin (urobilin) excretion in feces) it is evident that anhaptoglobinemia is a very common, but not an invariable accompaniment of increased red cell turnover. The exceptions are possibly persons with increased Hp synthesis. Individual differences in efficiency to destroy red cells outside the circulation without Hb return may be reflected by the wide normal variation range of plasma Hp as well by the promptness with which anhaptoglobinemia develops on even slight increase in Hb turnover.

5.2.1. *Plasma Clearance of the Hemoglobin-haptoglobin Complex*

In humans the clearance rate of Hb is higher than that of HpHb (L4, L11). Murray *et al.* (M6) found this also to hold for rabbits and, by studying the elimination in nephrectomized animals, they also proved that the difference was not due to urinary loss of Hb. Analysis of the organs proved that the HpHb complex and Hb were assimilated mainly in the liver and were catabolized with an early reappearance of the iron as transferrin iron within 30 minutes. The free Hb accumulated also in the tubular cells of the kidneys. No data have been published suggesting that the spleen is of any appreciable importance in this respect. No typical exponential clearance of the HpHb complex from plasma was observed (L10, L11) in the first few experiments. Lathem and Worley (L4) found that HpHb disappeared at a simple exponential rate in 5

subjects and linearly in 5. The mean decrease of plasma Hb per hour was 15 mg/100 ml (L4). At very low plasma Hb concentration Garby and Noyes (G4) found that the elimination rate followed a simple exponential rate. The slow return (about a week) of Hp to normal after rapid elimination (L10) suggested that the extracellular pool was small. However, studies on Hp in dog lymph showed the same ratios between albumin and Hp in plasma and in lymph during health, and during increased Hp level after surgical trauma or turpentine abscesses (G7).

5.3. Hypo- and Anhaptoglobinemia

On analysis of the hospital records of 246 patients who had been found to be anhaptoglobinemic at least once, Nyman (N7) arranged the subjects in a few groups: anemia—202 cases; severe liver injury (without anemia)—28; infectious mononucleosis (without anemia or apparent liver injury)—11; and 5 cases without any of these signs or diagnosis. The anhaptoglobinemic sera were found among about 3000 hospitalized subjects. The finding of only 5 cases with unexplained anhaptoglobinemia —after incomplete ferroerythrokinetic study—indicates that Allison's postulated suppressing Hp gene is rare in the south of Sweden (A3, H3, J9), at least if the suppressing force is supposed to be strong enough to inhibit the appearance of recognizable amounts of Hp in plasma in disease.

In an attempt further to elucidate the anhaptoglobinemia in patients with anemia, Nyman (N7) studied the plasma Hp in 335 cases of anemia (Hb less than 11 g/100 ml) mainly from a department of internal medicine. The partition she found (Table 2) is instructive from a pathophysiological point of view. The frequency of subnormal Hp values found within the different diagnostic groups may fairly well reflect the hemolytic trait in the different types of anemia. Subnormal Hp possibly secondary to the increased red cell destruction is a common finding also in polycythemia.

Anhaptoglobinemia or subnormal Hp values, often found in acute and chronic liver disease, and in mononucleosis, may also be caused by an increased consumption and not by decreased synthesis. In both disorders there exists a tendency for the development of splenomegaly, i.e., a tendency to retarded splenic blood flow with slightly shortened survival time of the red cells as a consequence. If we do not presume a half-life of Hp in normals below one day, the main part of the Hp catabolism must be secondary to Hb release. Hence, subnormal Hp values will probably appear in conditions with no clinically observable increased hemolysis or slightly decreased Hp synthesis. The latter may be a con-

TABLE 2
HAPTOGLOBIN VALUES IN ANEMIA (N7)[a]

	HbBC (mg/100 ml)											
	0	5	30	60	90	120	150	180	210	240	270	>300
Pernicious anemia	53	4	1	—	—	—	—	1	—	—	—	—
Congenital hemolytic anemia	23	1	—	—	—	—	—	—	—	—	—	—
Acquired hemolytic anemia	29	4	1	1	—	—	—	—	—	—	—	—
Iron deficiency, bleeding anemia	1	—	—	7	14	7	4	—	—	—	—	—
Aplastic anemia	1	—	5	3	2	4	—	1	1	—	—	—
Lymphatic leukemia	8	7	3	1	6	1	5	1	3	—	—	—
Myeloic leukemia	3	2	—	2	2	4	2	5	1	2	—	4
Sideroblastic anemia	—	1	2	1	—	—	1	—	—	—	—	—
"Hypersplenic" anemia	14	4	—	—	—	—	—	1	—	—	—	—
Drug-induced anemia	6	2	2	2	1	2	2	1	—	1	—	—
Cancer	5	—	—	1	—	1	1	2	3	1	—	6
Sternberg's disease	1	1	—	—	—	1	—	—	—	1	—	3
Rheumatoid arthritis	—	—	2	—	—	—	—	1	1	1	—	6
Nephropathy with uremia	8	—	3	3	1	—	1	1	1	1	—	8

[a] The normal range is within the heavy vertical lines.

tributory factor to the frequent anhaptoglobinemia in severe acute liver damage or in advanced uncomplicated cirrhosis.

5.4. Hyperhaptoglobinemia

The plasma Hp level rises in disease, like the concentration of fibrinogen and orosomucoid (J8, N5), and this is most likely the result of increased synthesis. The degree of abnormality seems largely to follow the activity of the inflammatory reaction of the disease (infections, tumors, aseptic necrosis). Treatment with cortisol will normalize all three of the above-mentioned plasma proteins. We do not yet know the link between inflammatory reaction and the changed synthesis of Hp and the other plasma proteins. A deeper knowledge of Hp synthesis and the pathophysiology of the inflammatory reaction is necessary for proper utilization in clinical work of the individual Hp values found in diseased subjects.

After surgical trauma, myocardial infarction, or after administration of pyrogens (A9, J6, M5, N5) the Hp level will be pathologically elevated within one or two days. In patients with acute pneumonia receiving efficient chemotherapy the plasma Hp will return to normal values at a rate corresponding to an approximate half-life of 5.4 days of the excess Hp (N5). The figure is interesting with regard to the high correlation ($r = 0.89$) between the plasma Hp values and the electrophoretic α_1 fraction in acute infections (Hp—seromucoid, $r = 0.99$) (J8) and the recent finding of Weisman *et al.* (W1) that the half-life of orosomucoid was slightly more than 5 days, independent of the type of disease.

Abnormally high Hp values suggest that the patient is ill and ". . . that infection, rheumatic disease, malignant growth or aseptic necrosis, thrombosis and the like may be suspected. Repeated determinations of the Hp value in one and the same patient gives a good idea of the course of the disease" (N5). Kluthe and Müller (K3) have recently stressed the value of estimating plasma Hp in rheumatoid arthritis (L1) to obtain information on the activity of the disease. The plasma Hp response to cancer is so irregular (N5, R1) that Nyman (N5) claims: "Determination of the Hp level is thus of no value in the diagnosis of early or even advanced cancer,"—though this attitude is perhaps too negative. She also reinvestigated the findings by Jayle's group (J12, J13) concerning plasma Hp and liver disease, and could confirm that a high Hp level in icteric patients argues strongly for obstructive jaundice. Her experience, which has been confirmed by others (O3), is, however, that "the haptoglobin level is of limited value in the differential diagnosis between obstructive jaundice and hepato-cellular disease."

The plasma haptoglobins in *renal disease* (J11) attracted special interest since the first pure Hp preparation was isolated from a patient losing Hp with the urine (J7). The remarkably high concentration of electrophoretic α_2-globulins found in the nephrotic syndrome is due mainly to an increase of α_2-globulins ($S\alpha_2$) other than haptoglobins (J6). The correlation between the plasma Hp and fibrinogen values in most diseases is high, but the nephrotic syndrome is an exception to this rule, which is to be expected even if both are synthesized at the normal rates, since the Hp molecules, especially of type 1-1, are more readily lost in the urine than fibrinogen. Marnay (M2), however, claims in a preliminary communication on haptoglobins in urine from patients with the nephrotic syndrome that Hp 2-2 is never found, that Hp 2-1 appears in low concentration (mainly the rapidly migrating) and that Hp of type 1-1 sometimes occurs in high concentration. If the findings can be confirmed, such analyses will be of general importance in the future for the evaluation of the type of lesion responsible for the protein leakage in individual cases of proteinuria. In a preliminary communication, Kluthe and Meyer (K2) claimed that the Hp values are high in all types of nephritis and that in several cases of chronic pyelonephritis the Hp values are abnormal in spite of normal E.S.R. The finding is remarkable and needs confirmation since the electrophoretic α_2-values are usually normal in this type of nephritis.

If ACTH or cortisol is given to healthy subjects, it will cause a rise in the electrophoretic α_2-fraction, which is mainly secondary to an increased Hp level in plasma; this also holds for dogs (V1). In Cushing's disease slight hyperhaptoglobinemia is demonstrable (B3).

Administration of androgens (dose undefined) to women for short periods rapidly doubled the plasma Hp (S12). A corresponding decrease in Hp concentration appeared in women after the first week of treatment with diethylstilbestrol and ethynylestradiol (B11). Estriol produced no such effect. A significant increase of the endogenous CO formation during the same period suggested increased Hb catabolism. HbBC values up to 1000 mg/100 ml have been observed in castrated female patients with widespread mammary cancer and skeletal metastasis treated with massive doses of androgens. No other conditions are known in which the patients may feel well in spite of HbBC values above 500 mg/100 ml. One such patient with complicating empyema had HbBC values which reached such a high value as 1400 mg/100 ml suggesting an additive effect of cancer, androgens, and infection on the Hp synthesis (L12).

5.5. Concluding Remarks on Haptoglobin Metabolism and Homeostasis

The site of the main organ for Hp synthesis is still unknown. The remarkable increase of plasma Hp in inflammatory conditions does not seem to depend on a local release from the inflammatory focus (M5), since there the concentration is not higher than in the general circulation.

The prompt, regular plasma Hp increase following different types of trauma contrasts with the wide normal range of variation and suggests that the synthesis and/or the Hp mobilization (inflow) is not influenced by the Hp level. No deficiency symptoms have been observed in patients with life-long anhaptoglobinemia, e.g., congenital spherolytic anemia. However, no clinical or experimental finding has been presented suggesting the occurrence of individuals who never synthesize more than traces of Hp, i.e., true anhaptoglobinemics. On the contrary, patients with chronic anhaptoglobinemia may suddenly become hyperhaptoglobinemic during acute illness and thereby reveal the symptomatic nature of the anhaptoglobinemia. It is evident from the earlier discussion that the interpretation of the Hp level is somewhat more complicated than that of other plasma proteins, since no absolute relationship exists between the Hp level and the Hp synthesis, not even during the steady state. The elimination is composed of at least two independent variables: (*a*) the common catabolic processes for plasma proteins, and (*b*) the flow rate of Hb or globin into the intravascular space. The amount catabolized by system (*a*) varies with the plasma concentration, and the amount taken up by (*b*) is independent of the level and is normally fairly constant. Hp synthesis below or equivalent to the Hb-inflow rate will result in anhaptoglobinemia. The normal rate of synthesis is unknown, but the magnitude may be guessed from clinical observations. As a rule, anhaptoglobinemia does not seem to appear until the erythrocyte turnover is doubled, which would imply that the normal mean Hp pool (8 g in 70 kg) represents at least half the daily synthesis, if the amount of the plasma Hp loss per dying erythrocyte is accepted as constant. From the normal plasma Hb concentration and turnover (G4) the daily Hp loss owing to erythrocyte destruction was calculated as approximately 1.4 g. There is a discrepancy between the values obtained by the two methods of calculation, but the correct figure is probably of such a magnitude that elimination curves obtained with conventional turnover studies using tagged Hp will not fit a simple exponential function. The wide normal range of variation is also easier to understand in the light of this dual drainage of Hp. The maximum half-life of plasma

Hp calculated by Nyman as 5 days may represent the half-life as a result of the normal catabolism—type (*a*)—if we ignore the erythrocyte destruction. It is meaningless to speak of half-life values of Hp when discussing Hp turnover; absolute amount per unit of time and weight should, instead, be used.

One of the biological functions of Hp may be to prevent urinary loss of iron as Hb and to prevent blocking of the renal tubular cells by a heavy daily load of reabsorbed Hb. Whether this postulated function is important or not is not known, since no cases of true anhaptoglobinemia have ever been observed. Another biological function of Hp is to prevent the formation of methemalbumin.

Since inflammatory reactions are not accompanied by any remarkable change in the erythrocyte survival, it is difficult from a teleological point of view to understand the increased Hp synthesis or pool output during inflammatory reactions. It is possible that the main biological function of Hp has not yet been discovered. Suggestions of its importance in the immune defense have been proposed (S10), and it is apparently an essential part of what the virologists call the Francis inhibitor (S10).

6. Haptoglobin in Animals

No counterpart to the Hp polymorphism found in humans has been observed in animals. Most of the mammalian species studied—chimpanzee, baboon, monkey, horse, cow, pig, seal, rabbit, squirrel, arctic marmot rat, guinea pig, and mouse—have haptoglobins similar to human type 1-1. Sometimes double Hp bands have been found—monkey, marmot, seal, and mouse (A5, A8, B7, B9, M1, M5).

The Hp concentration in the conventional laboratory animals, rabbit (I1, B5), guinea pig, and rat (R3), is considerably lower than in humans, a difference possibly due to the shorter survival of red cells in these species than in humans.

We have prepared pure dog Hp by our method for human Hp type 1-1. Rabbit-antidog Hp serum was easily obtained. It was found to cross-react with human Hp. The Hp-level in dog plasma increases after injection of endotoxin or turpentine (G7), and, as in humans, it increases after surgical trauma (A9) and disappears after Hb injection (V1). In rats, guinea pigs, and rabbits the plasma Hp level rises markedly after induction of different types of inflammatory states (I1, M5, R3), but the absolute values never reach the high level seen in pathological conditions in man.

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MICROBIOLOGICAL ASSAY METHODS FOR VITAMINS[1]

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I. Introduction

At the time when an increasing number of clinical conditions were recognized as deficiencies of one or another chemical substances, these so-called vitamins were measured by their effect as supplement to a basal diet upon the growth and health of some species of laboratory animals. Vitamins are organic substances of natural origin, required in minute quantities, and their effects are not based on their caloric value, but exclusively on their catalytic nature. Elucidation of their structure and biosynthesis are exciting chapters of organic chemistry and their identification with the prosthetic groups of vital enzymes is a signal contribution to biochemistry.

The recognition of their structure permits the determination of vitamins by the tools of analytical chemistry, but while such methods are widely used in industrial production, the minute quantities in body fluids and tissues limit the purely chemical approach to a few members of this group present in relatively high concentration, e.g., vitamin C (K5). Microchemical methods are in use for the determination of thiamine, riboflavin, and some of the fat-soluble vitamins, based on the most sensitive colorimetric and, in particular, fluorometric techniques. Vitamin D, on the other hand, is determined by animal assay.

Vitamins, especially the water-soluble ones, provide the coenzymes for the most fundamental cellular reactions. Thus, their presence is required by vertebrates and invertebrates as well as by monocellular organisms, such as bacteria and other fungi and by protists. One finds in nature many microorganisms which need those vitamins that they are unable to produce at all or in sufficient quantities. Thus, one has a wide choice of

microbes for the assay of vitamins (S14). In the following review we shall discuss such microorganisms and the conditions under which they may be utilized for the assay of vitamins.

Nutritional deficiency diseases are relatively rare in the temperate zone. The etiology of numerous other clinical conditions involve vitamin deficiencies, due to faults in absorption, transfer, or utilization. Because of the central position of the vitamins as sources of coenzymes, such functional deficiencies are important in malabsorption, where the picture is often complicated by multiple deficiencies, in anemias where the defect is in general highly specific, and in many other diseases where the deficiency is secondary to other pathologic events, but nevertheless of grave consequences.

Microbiological assays are applicable when a microorganism responds to a metabolite for which physical and chemical determinations are neither sensitive nor specific enough. One must select a microorganism sensitive to the substance under assay; it should be cultivable with ease; the growth response should be easily measurable and the response should be specific; it should be non-pathogenic. Systems in which the vitamin only stimulates growth are impractical. The culture medium must not support any growth without the vitamin and permit full growth with it.

The growth of the microorganism, measured most simply by optical density (O.D.), should be linearly proportional to the concentration of vitamin over an appreciable range. A graph of O.D. as ordinate and concentration as abscissa should thus yield a straight line passing through origin; its slope should be such that it shows e.g., at double concentration double the O.D. If the log of the concentration rather than concentration itself is used as abscissa, the curve has the well-known sigmoid shape. The O.D. of the unknown is read on the standard curve and the abscissa of this point gives the vitamin concentration of the sample as tested. The vitamin content of the original sample is computed from this value. The titer of the vitamin, tested at three different concentrations, should yield values falling within a range of 10% one from another.

The standard curve will in some instances not follow a straight course or will have a "wrong" slope. If the slope is too steep, i.e., if the double concentration gives more than double a value of optical density, one will suspect the presence of an inhibitor or toxic factor, relatively more effective at lower concentrations, or of a stimulating factor relatively more effective at higher concentrations. In the case of strongly colored samples, optical compensation can be achieved in the reading of the

standard curve by insertion of a noninoculated sample at the proper dilution or of suitable color filters.

Microbiological assay should stress accuracy over precision. Standardization of an assay method should include comparisons with at least one other organism having a different nutritional pattern and specificity toward the compound being assayed. Such a comparison was made for cyanocobalamin (vitamin B_{12}) content of human blood and serum, using four microorganisms differing in their cobamide requirements and metabolism (B9).

A good basal growth medium is part of a microbiological assay; it must contain appropriate concentrations of all stimulating factors likely to be encountered. Many media prove good enough for purified materials, but are unsatisfactory for biological fluids such as blood, serum, and for cells and tissues. Basal media can be improved ad infinitum, but if diverse crude materials, supplied at levels approaching the tolerance of the organism, do not increase final growth more than 10%, the basal medium is satisfactory. Actually, stimulants in natural materials are nearly always diluted out, except when an assay approaches the limit of sensitivity for low-protency materials.

Reports on microbiological assays usually dwell upon apparatus, general technique, measurement of growth, choice of assay organism, etc., but since there are many reviews of these subjects (B26, G3, G4, H19), they need not be discussed here. We shall deal here with the development and use of microbiological assays for clinical purposes.

Before the introduction of the microbiological assay for B_{12} in human serum, deficiency could be diagnosed only by symptoms of hematological stimulation upon administration of liver extract or vitamin B_{12} preparations. A similar problem was experienced with folic acid. Because folic acid is metabolically related to vitamin B_{12}, microbiologic assays had to be devised for differential diagnosis. This has been solved not only for the vitamin B_{12}-folic acid pair, but also for thiamine, pantothenic acid, nicotinic acid, biotin, folinic acid, vitamin B_6 (pyridoxamine and pyridoxal), and unconjugated pteridines. These additions to the diagnostic arsenal have proved their value for assaying the titer of the respective vitamins in blood, serum, urine, and cerebrospinal fluid, and in tissues. The techniques and their value will be the main subject of this review.

2. Thiamine

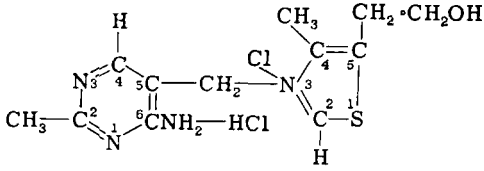
Thiamine catalyzes decarboxylations, as of pyruvic acid and transketolations in carbohydrate metabolism. Free thiamine is carried by the

plasma to the liver and kidneys, where it is phosphorylated to cocarboxylase (G20).

Straightforward thiamine deficiency in man, beri-beri, is characterized by accumulation of pyruvic and lactic acids in the blood and brain, and impairment of cardiovascular, nervous, and gastrointestinal function (D10, G17, P4, Y1). Neurological lesions characterize thiamine deficiency in growing rats (B40), guinea pigs (M6), mice (M13), chicks, and pigeons (B30). The effects of thiamine deficiency on the central nervous system of animals have been reviewed (D10).

Classic beri-beri, rarely seen in the United States and Europe, except in alcoholism (P4), is endemic in the Far East because of the prevalent diet of decorticated rice (F6). It occurs in two forms: wet beri-beri, characterized by edema and cardiovascular symptoms (G6), and dry beri-beri with peripheral neuritis, paralysis, and atrophy of the muscles. Conditions which may predispose to deficiency by increasing thiamine requirements are pregnancy (see section 2.4), and lactation, hyperthyroidism, malignant disease, febrile conditions, increased muscular activity, high carbohydrate diets, and parenteral administration of glucose solutions. A constant supply of thiamine is required for optimal nutrition because storage in the liver and elsewhere is limited. Thiamine is synthesized by bacteria in the intestinal tract of various animals, but this is not a dependable source for man.

Thiamine contains a pyrimidine moiety (A) and a thiazole moiety (B), joined by a methylene bridge as shown in (I). Microorganisms which



(I)

Thiamine.

require thiamine fall into five categories: they may require (1) intact thiamine, (2) the pyrimidine moiety, (3) the thiazole moiety, (4) either the pyrimidine or the thiazole moiety, and (5) both moieties. Man and other animals utilize only the intact thiamine. Hence, the assay organism must respond only to the intact vitamin in assessing thiamine status in animal materials. Thiamine is one of the few vitamins assayable chemically and chromatographically (A10, B38, K3), but this technique is often impractical in clinical situations. Compared with chemical

methods, microbiologic assay methods for thiamine are more sensitive, more specific, require less investment in equipment and less material for assay—important considerations when biologic samples are to be analyzed.

2.1. Microbiological Assay Methods

The microbiological methods based on lactobacilli and yeasts (B20–B22, E1) suffer from poor reproducibility, partly because non-chemically defined media are used; also lactobacilli are often stimulated nonspecifically by substances in biologic materials (D4, H19), since these stimulants are not adequately represented in published basal media. The particle-ingesting phytoflagellates, *Ochromonas malhamensis* and *Ochromonas danica* require intact thiamine; they do not respond to the thiazole or pyrimidine moieties alone as do the yeasts (H2, K9), nor can they combine the thiazole and pyrimidine moieties. The media are adequate for growth and are not appreciably stimulated by natural fluids such as blood. The useful range is 0.3–30 mμg/ml, which is adequately sensitive and wide. The method based on *O. malhamensis* has been very successful in our hands for assaying biologic fluids. An alternative method with *O. danica* will be reported soon; results agree with the *O. malhamensis* method. The method using *O. malhamensis* is described here.

2.2. Materials and Methods

Ochromonas malhamensis is obtainable from the American Type Culture Collection, or the Culture Collection of Algae and Protozoa, The Botany School, Downing Street, Cambridge, England. The basal medium is given in Table 1. Standard solutions of thiamine-HCl are made up in distilled water, volatile preservative added (H19) and stored at 4°; every month fresh standards are prepared. One milliliter of the sample to be assayed, e.g., blood, urine, CSF, is diluted with 2 ml of buffer solution. The buffer consists of 0.5 g of *trans*-aconitic acid in 100 ml of distilled water to which triethanolamine had been added to reach a pH of 4.5. The final pH of the solution should be between 4.5 and 5.0. The solution is then autoclaved for 30 minutes at 120°C. and cooled. For every milliliter of the original sample, 2 ml of an enzyme solution is added, prepared by dissolving 0.3 g of Diastase (Fisher Scientific Co., New York) in 100 ml of water. The original sample has now been diluted 1:5. Volatile preservative is added and the solution is incubated overnight at 37°. It is then autoclaved 10 minutes and centrifuged if necessary. The supernatant is diluted so that most measurements will fall on the straight-line portion of the standard curve. The final dilution should be between 1:25 and 1:100, provided the sample did not come from a patient

receiving vitamin therapy. The fluid is then assayed. The enzyme solution is also assayed to determine any thiamine contamination.

The inoculum for the assay is prepared by growing *Ochromonas* for 4–6 days in the basal medium supplemented with 10 mμg/ml of thiamine. The culture is then diluted 1:100 with basal medium; a drop of this dilution serves as an inoculum for each assay flask. There is no

TABLE 1

MEDIUM FOR THIAMINE ASSAY WITH *Ochromonas malhamensis*[a]

Compound	Concentration in final medium (mg/100 ml)
$(NH_4)_2H$ citrate	100
$CaCO_3$	15
K_3PO_4	30
$3MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$ ("basic" $MgCO_3$)	40
$MgSO_4 \cdot 7H_2O$	10
$KHCO_3$	30
Metals mix[b]	1.0 ml
Mo[as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$]	0.1
V(as $VOSO_4 \cdot 2H_2O$)	0.002
Sucrose	1200
L-Glutamic acid	300
L-Histidine·HCl	50
L-Arginine·HCl	40
DL-Methionine	80
DL-Asparagine·H_2O	100
Biotin	0.0005
Cyanocobalamin (vitamin B_{12})	0.0005
p-Aminobenzoic acid[c]	0.125
Acid-hydrolyzed casein (salt-free)	500

[a] pH 4.8–5.2 (adjusted with KOH).

[b] 1.0 ml contains: citric acid·H_2O, 3.0 mg; Zn (as $ZnSO_4 \cdot 7H_2O$), 4.0 mg; Mn (as $MnSO_4 \cdot H_2O$), 0.5 mg; Fe [as $Fe(SO_4)_2 \cdot 6H_2O$], 0.2 mg; Cu (as $CuSO_4 \cdot 5H_2O$), 0.1 mg; and Co (as $CoSO_4 \cdot 7H_2O$), 0.03 mg.

[c] Added to prevent any growth inhibition produced by samples containing sulfa drugs (H17). Other antibiotics do not affect growth.

significant carry-over of thiamine. The tests are incubated at room temperature, not higher than 30°, for 4–6 days under constant illumination provided by five 40-watt "warm-white" fluorescent tubes at a 1.0 m distance. Growth is measured in optical density (O.D.) units with a Welch Densichron. If an optical density greater than 1.0 is reached, the cultures are diluted with water to read below O.D 1.0. The reading is then multiplied by the dilution factor. This procedure avoids reading

errors resulting from settling of the heavy suspensions and Rayleigh scattering error.

2.3. Normal Range

A typical assay curve (Fig. 1) illustrates the sensitivity and the usable growth range.

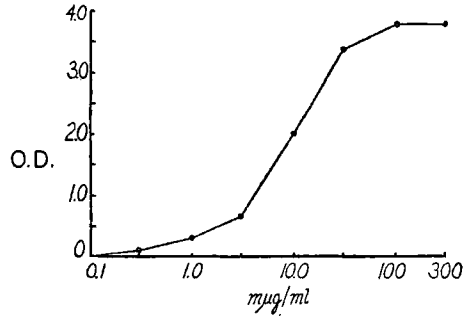


Fig. 1. Typical growth curve in thiamine assay with *Ochromonas malhamensis* [Baker *et al.* (B15)].

Extraction of thiamine from biologic fluids deserves comment. Acid extraction and autoclaving effect complete solution of the various forms of the vitamin. At the resulting pH of the extract and that of the basal medium used in the assay, pH 4.5–5.0, thiamine is stable. During the subsequent 37° incubation the enzyme, a phosphatase, hydrolyzes thiamine phosphate liberating free intact thiamine. After incubation, a 10-minute autoclaving is needed to stop enzymatic activity. With body fluids, enzymatic digestion is necessary, since *Ochromonas* does not produce an enzyme capable of splitting cocarboxylase (thiamine pyrophosphate). Enzymatic treatment of whole blood is important, since 90% of the thiamine in the red blood cells is present as thiamine pyrophosphate (J1). Trichloroacetic acid (TCA) precipitation of proteins (E1) was abondoned because it resulted in low values; some of the thiamine is retained with the TCA precipitate (M9). For example, a blood sample yielded 25 mμg/ml thiamine after enzymatic hydrolysis, but only 5 mμg/ml after TCA treatment. The addition of 50 mμg/ml thiamine gave as a result 75 and 56 mμg/ml, respectively; thus, the recovery of added thiamine was exactly 100%.

The normal values for thiamine in human blood vary from 25–80 mμg/ml (average of 27 cases), from 110–370 mμg/ml in urine (27 cases), and from 13–17 mμg/ml in cerebrospinal fluid (45 cases). These specimens were obtained from normal subjects, receiving no vitamin therapy and in the fasting state, to eliminate dietary influences. The

values shown for blood and cerebrospinal fluid are in agreement with those determined by manometric and chemical methods (D11, G16, S1).

The clinical significance of thiamine and its necessity for pyruvic acid oxidation has been discussed. Recent reports concerning the coenzyme function of thiamine in pentose (H13), tryptophan (D2), and lipoic acid metabolism (R6) have increased our knowledge of thiamine in metabolism and lend added interest to the role of thiamine in clinical problems. This method has also been used to assay thiamine in liver and brain.

2.4. Thiamine Deficiencies

We have determined thiamine levels of mother and infant at parturition (B11). Thiamine in the fetal circulation is 5 times higher than in the mother's. A similar situation exists in the guinea pig (B35).

In rats, thiamine deficiency results in low levels of neutral glycerides in the liver (A8). Vitamin studies, especially those involving hard to induce deficiency states, have been greatly facilitated by antivitamins. Some have been used as therapeutic agents, e.g., antifolics for leukemia, others to accelerate induction of specific vitamin deficiencies for metabolic studies. Administration of pyrithiamine provokes a deficiency closely resembling that induced by withholding thiamine (K6). Not much is known about natural antithiamines in food, however the literature on thiaminases is extensive. Active thiaminases were obtained from organ extracts of warm-blooded animals (rabbit or chick); the high thiaminase content of fish (K9) is held responsible for the thiamine deficiency in minks (Chvostek paralysis) (S18).

3. Pantothenic Acid

The nearly universal distribution of pantothenic acid in living cells suggests a vitamin function in cellular metabolism. Pantothenic acid deficiency reduces acetylations and it leads to a variety of pathological changes, e.g., in blood cholesterol partition. Deficiency notably affects the adrenal cortex, the nervous system, skin and hair; it also reduces longevity in mice (B27, L7, N4, P3). Deficiency influences the rate of oxidation of pyruvic acid, as expected from pantothenate as a component of coenzyme A. The adrenal cortex has an especially intense pantothenic metabolism: the adrenal cortex of the rat is high in coenzyme A. Pantothenate deficient rats have lesions of the adrenal cortex with hypertrophy, depletion of lipids, and finally hemorrhage and necrosis. Administration of the antivitamin, ω-methylpantothenic acid, to rats resulted in impaired production of corticosterone by the adrenal glands (A5). In the blood plasma of pantothenate deficient subjects, α-globulin is increased, γ-

globulin is decreased, ascorbic acid disappears, cholesterol is low, and red cells and hemoglobin are depressed; leucocytes remain normal. Carbohydrate metabolism is affected: glucose tolerance is decreased and all subjects have increased insulin sensitivity. Impaired adrenal function is seen in the decline in urinary 17-ketosteroids and the lack of eosinopenic response to corticotropin and diuresis after a load dose of water (H9)—quite a range of metabolic functions.

3.1. Assay Method

The assay for pantothenic acid in whole blood, serum, urine, and cerebrospinal fluid is described here. *Lactobacillus plantarum* ATCC No. 8014 (formerly *L. arabinosus*) is used for the assay. The basal medium for assay (Table 2) is made up in double strength; 2.5-ml portions of the medium are distributed into 10-ml borosilicate micro-Fernbach flasks provided with aluminum caps (H18). Solutions to be assayed are added and distilled water used to bring the volume to 5 ml. The techniques for maintenance and assay are the same as those for *L. casei* (B12). Full growth at 37° takes 3 days.

TABLE 2

Modified Basal Medium for *Lactobacillus plantarum*[a]

Components	Final medium (mg/100 ml)
Acid-hydrolyzed casein[b]	0.5 g
L-Cystine[c]	0.01 g
L-Tryptophan	0.01 g
Adenine[c]	0.5
Guanine[c]	0.5
Uracil[c]	0.5
Xanthine[c]	0.5
Salts mix I[d]	0.5 ml
Salts mix II[e]	0.5 ml
Glucose	2.0 g
Thiamine·HCl	0.1
Riboflavin	0.1
Nicotinic acid	0.1
Pyroxidine HCl	0.2
p-Aminobenzoic acid	0.01
Biotin	0.00025

[a] pH 6.6–6.8, adjusted with KOH or H_2SO_4.

[b] Nutritional Biochemicals Corp., Cleveland Ohio.

[c] Dissolved in KOH before addition.

[d] Mix I: 1 ml contains 100 mg each of KH_2PO_4 and K_2HPO_4.

[e] Mix II: 1 ml contains 40 mg $MgSO_4 \cdot 7H_2O$, 2 mg $MnSO_4 \cdot 4H_2O$, 2 mg NaCl, and 2 mg $FeSO_4 \cdot 7H_2O$.

A concentrated Ca-D-pantothenate solution (1 mg/ml) is prepared in distilled water and dilutions made as needed. Refrigerated solutions are stable for 6 months. Pantothenate is added at 5, 10, 20, 40, 60, 80, and 100 mμg/ml final concentrations; the control flask consists of basal medium alone for estimation of carry-over error—i.e., the pantothenate activity of the inoculum. The details of aseptic technique have been discussed elsewhere (H18, H19). Growth is measured in optical density units with a Welch Densichron, equipped with a red-sensitive probe to minimize blank readings due to the color of the medium.

3.2. Normal Range

3.2.1. *Pantothenate in Blood and Serum*

Citrated blood is diluted 1:10 with enzyme buffer solution, and preservative is added (H19). The buffer is prepared by dissolving 0.2 g of Clarase (Fisher Scientific Co., New York) in 100 ml citrate buffer (5 g potassium citrate monohydrate and 1 g citric acid monohydrate in 1000 ml distilled water, pH 5.6). The solution is incubated for 3 days at 37°. After incubation, it is autoclaved 15 minutes to stop enzymatic action and coagulate proteins. It is filtered, and 1.0, 1.5, and 2.0 ml of the supernatant is added to individual flasks and assayed. Control flasks are included to estimate pantothenic acid contamination of the enzyme.

3.2.2. *Pantothenate in Urine*

Urine is diluted 1:20 with citrate buffer without enxyme and autoclaved for 10 minutes to remove any protein; 0.5, 1.5, and 2.5 ml is used for assay.

3.2.3. *Pantothenate in Cerebrospinal Fluid*

The cerebrospinal fluid is treated as whole blood except that it is diluted 1:5 with enzyme buffer solution; 1.0, 1.5, and 2.5 ml of the supernatant is used for assay.

The range of pantothenic acid in normal blood and in cerebrospinal fluid is approximately the same (Table 3); in the urine it is higher. Ninety-nine

TABLE 3
Pantothenic Acid in Normal Subjects

No. of subjects	Fluid	Assay with *L. plantarum*	
		Range (μg/ml)	Average (μg/ml)
367	Blood	0.22–1.9	0.56
103	Cerebrospinal fluid	0.10–1.7	0.52
38	Urine	0.76–4.1	2.90

to 105% of added pantothenic acid was recovered; growth stimulation from the samples was negligible. Usable assay range with both organisms is the same; above 100 mμg/ml, the curve flattens.

The pantothenate in blood and cerebrospinal fluid is conjugated; without enzymatic hydrolysis no pantothenate activity is detected. In urine, pantothenate activity did not increase with enzymatic treatment.

3.3. Comments

Pantothenate in blood and tissues is bound (R9) and released by autolysis or hydrolysis. More vitamin could be released by use of an alkaline phosphatase and an enzyme from avian liver (L6). This method liberates pantothenate from coenzyme A in a variety of foods and tissues (N3, N4). A comparison of hydrolytic methods in blood suggested autolysis to be the most advantageous method (N3); in our hands, treatment with Clarase gave more reliable results as compared with autolysis, acid hydrolysis, treatment with Mylase P, or combination of Clarase and papain, or liver enzyme and alkaline phosphatase. In urine, pantothenic acid is unbound; our results show no increase with Clarase treatment. The vitamin has presumably a low threshold. Pantothenic acid shows the same concentration in blood and cerebrospinal fluid.

Lactobacillus plantarum is a reliable reagent for pantothenate in foods, tissues, and biologic fluids (M4, T5). It is also useful in studying the pantothenic acid content of tissues, in particular the liver. As with thiamine, the pantothenic acid levels in serum of the mother at term, although higher than in other individuals, are 5 times less than the fetus (B11).

ω-Methylpantothenic proved to be the most potent antagonist. Pantothenic acid antagonists have been studied in detail (B31); their clinical effects still need elucidation.

4. Nicotinic Acid and Amide

Nicotinic acid derivatives occur in biologic materials as the free acid, as nicotinamide, and in two coenzymatic forms: nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP). These coenzymes act in series with flavoprotein enzymes and, like them, are hydrogen acceptors or, when reduced, donors. Several plants and bacteria use a metabolic pathway for the formation of nicotinic acid that is different from the tryptophan pathway used by animals and man (B39).

Among mammals, symptoms of nicotinic acid deficiency have been

observed only in man and dog. In man, deficiency results in dermatitis, diarrhea, and delirium. As with other B-vitamin deficiencies, pellagra often accompanies poverty, chronic alcoholism, dietary fads, fever, hyperthyroidism, and pregnancy. Since B-vitamin deficiencies usually do not occur singly, some of the symptoms of pellagra may reflect deficiencies in other vitamins. Treatment of primary deficiency with nicotinic acid or nicotinamide produces a prompt response.

Recently nicotinic acid has been found to lower serum cholesterol in hypercholesteremia, and also in normal persons and rabbits (A3, P2). It was shown that the hypercholesteremia, induced by a 48-hour fast, could be completely corrected by giving the animals large doses of nicotinic acid during the fast. In contrast to nicotinic acid, nicotinamide does not lower the cholesterol level (M10). Several explanations are offered for the action of nicotinic acid: (1) it inhibits cholesterol biosynthesis, (2) it interferes with coenzyme A, and (3) it involves a hitherto unknown pharmacologic effect. The renewed clinical interest in nicotinic acid induced us to look for a more specific and sensitive assay for nicotinic acid (B7, M8).

4.1. Assay Method

The organism used is *Tetrahymena pyriformis* (variety I, mating type II). Assay technique and methods of maintaining *T. pyriformis* have been described (B4). Citrated blood is diluted 1:20 with buffer and autoclaved for 30 minutes at 118–121° to liberate the vitamin. The coagulum is centrifuged off, and the clear supernatant is assayed. To 2.5 ml of double strength basal medium (twice the concentration listed in Table 4a) are added 0.25 ml, 0.5 ml, and 1.0 ml of the supernatant. The medium is then brought to a final volume of 5.0 ml with distilled water, representing 1:400, 1:200, and 1:100 dilutions of the original blood. Urine is treated and assayed like blood (cf. Tables 4a and 4b).

Tetrahymena pyriformis utilizes nicotinic acid or nicotinamide; when they are added in the same concentration, the increment of growth is less than that predicted from the algebraic sums of the individual increments (Table 5).

Early investigators assumed that human erythrocytes could convert nicotinic acid, but not the amide, into NAD (H3, H8). There are later reports to the contrary, i.e., that nicotinamide, but not the acid, produced increased synthesis of NAD-active material (L3). To resolve these discrepancies, standards for assaying nicotinic acid activity were prepared by mixing equal weights of the acid and amide, because these

two compounds account for most of the activity of nicotinic acid and its derivatives in biologic materials. There are very few substances known to interfere with such activity (S12). A solution containing 1 mg/ml of nicotinic acid and 1 mg/ml of the amide is prepared by dissolving the pure compounds in distilled water. Dilutions are made with distilled water to yield 0.3, 1.0, 3.0, 10, 100, and 300 mμg/ml final concentrations in the assay medium. Solutions are kept at 4° with

TABLE 4a

BASAL MEDIUM FOR *Tetrahymena pyriformis*

Components[a]	Final medium (mg/100 ml)	Components	Final medium (mg/ml)
HEDTA[b]	10	Na_3 guanylate·$4H_2O$[e]	12
$MgCO_3$	60	Cytidylic acid[e]	3.0
KH_2PO_4	10	Uracil[e]	1.0
Metals mix[c]	10	Thymidine[e]	1.0
Citric acid·H_2O	40	Ca pantothenate	0.1
Na acetate·$3H_2O$	100	Na riboflavin·$5PO_4$	0.05
Glycerol	800	Thiamine·HCl	0.05
Diacetin	100	Pyridoxine·HCl	0.03
Glucose	150	Pyridoxal·HCl	0.03
Hycase[d]	1200	Pyridoxamine·2HCl	0.03
Glycine	10	Folic acid	0.01
L-Tryptophan	10	DL-Thioctic acid	0.001
Yeast adenylic acid (5'-adenylic-acid)[e]	2.5	Biotin	0.0002
		Hydrolyzed yeast nucleic acid[e]	40

[a] To the components of the medium is added distilled water to make 100 ml. The pH is adjusted to 6.6–6.8 with KOH or H_2SO_4.

[b] Hydroxyethylethylenediamine triacetic acid (Chel DM Acid, Geigy Pharmaceuticals, Ardsley, New York).

[c] See Table 4b.

[d] Acid-hydrolyzed salt-free casein (Sheffield Chemical, Norwich, New York).

[e] Dissolved in KOH before addition to basal medium.

added volatile preservative (H19); a control flask consisting of basal medium alone is maintained to allow estimation of carry-over error owing to the nicotinic activity of the maintenance medium. For inoculum 5 ml of a 24-hour culture, prepared as described (B4), are diluted with 10 ml of full strength basal medium. The flasks are inoculated with one drop of this suspension and incubated at 28–30° for 3 days. The final growth is measured in optical density units and the nicotinic acid concentration calculated. Additional technical details have been described (B7, B12).

TABLE 4b
METALS MIX[a]

Element	mg/100 ml	Salt	Gravimetric factor salt/element	g/1000 liters
Zn	0.79	$ZnSO_4 \cdot 7H_2O$	4.4	34.8
Mn	0.67	$MnSo_4 \cdot H_2O$	3.1	20.8
Fe	0.55	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	7.0	38.5
Co	0.074	$CoSO_4 \cdot 7H_2O$	4.8	3.55
Cu	0.022	$CuSO_4 \cdot 5H_2O$	4.0	0.88
Mo	0.034	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.8	0.61
V	0.004	$Na_3VO_4 \cdot 16H_2O$	9.2	0.37
B	0.016	H_3BO_3	5.7	0.91

[a] To make 1000 liters of metals mix, the salts in the quantities listed are combined. For each 100 ml of final medium, 10 mg of the metals mix are dissolved in 10 ml of water acidified with 4 mg sulfosalicylic acid. Further details for making metals mix in dry form have been described (H18, H19).

Hydrolyzed Yeast Nucleic Acid. Five grams of yeast nucleic acid are suspended in 50 ml of distilled water. KOH (10%) is added until the nucleic acid is dissolved and a final pH of 9.0 is attained. The mixture is then autoclaved at 118° C for 5 minutes and brought to pH 7.0 with HCl and a 125-ml volume with distilled water, making a 4.0% solution. Preservative is added, and the solution is kept at 4°.

TABLE 5
GROWTH EFFECTS OF NICOTINIC ACID AND DERIVATIVES ON *T. pyriformis*[a]

Control	Optical density at concentration (mμg/ml): 0.1	0.3	1.0	3.0	10	30	100	300	1000
Nicotinic acid	0.20	0.20	0.22	0.24	0.32	0.48	0.94	1.20	1.22
Nicotinamide	0.22	0.24	0.24	0.24	0.36	0.70	1.18	1.30	1.30
Nicotinic acid +[b] nicotinamide	0.26	0.30	0.30	0.32	0.48	0.88	1.22	1.34	1.34
Nicotinuric acid	0.20	0.24	0.24	0.24	0.24	0.30	0.36	0.40	0.40
Nicotinmethylamide	0.24	0.26	0.26	0.26	0.34	0.44	0.76	0.98	1.20
Nicotindimethylamide	0.22	0.22	0.24	0.24	0.24	0.26	0.26	0.40	0.44
Nicotinethylamide	0.22	0.22	0.22	0.22	0.24	0.26	0.48	0.70	0.90

[a] Growth measured in optical density units with a Welch Densichron.
[b] Equal concentration of each; total is twice the value given.

4.2. RANGE

The range of nicotinic acid activity in blood, for a group of 28 normal subjects is 3.9–9.6 μg/ml. Only a fraction of less than 1% (0.016–0.05 μg/ml) of this quantity was present in serum. The range in urine was 1.16–1.54 μg/ml. Ninety-seven to 101% of added combinations of nicotinic acid and nicotinamide was recovered when added to blood and urine. In blood, appreciable nicotinic activity is observed only after

30 minutes autoclaving, which deconjugates nicotinic acid-active material from protein; in urine it is free. Nevertheless, urine is autoclaved for at least 10 minutes before assay in order to precipitate proteins, when present, and salts. These precipitates interfere with measuring growth.

4.3. Comment

Use of *T. pyriformis* for measuring nicotinic acid activity permits a wider range and specificity than does *Lactobacillus plantarum*. In our hands the narrower range of *L. plantarum* was 3–30 mμg/ml as compared with 1–300 mμg/ml for *T. pyriformis*. Several naturally occurring nicotinic acid derivatives were tested with *T. pyriformis* (Table 5); only nicotinic acid and nicotinamide permitted full growth, nicotinmethylamide and ethylamide showed some activity, and compounds having other modifications of the carboxyl groups did not enhance growth. Trigonelline (the betaine of nicotinic acid), which has no animal activity, proved inert for *T. pyriformis*; the organism's requirement seems to parallel that of higher animals. Unfortunately, N^1-methylnicotinamide and its 6-keto derivatives, some of the principal excretion products in man, were not available. The cellular components of whole blood contribute the greatest nicotinic acid activity; (compare with whole blood, see Section 4.2). Such results are expected since red blood cells contain much NAD and NADP activity (B7, H3, H8). Among the excretory products of nicotinic acid in human urine is nicotinuric acid (nicotinoylglycine). *Tetrahymena pyriformis* does not respond to nicotinuric acid, whereas *L. plantarum* does (L3), another point of specificity favoring *T. pyriformis*; this superiority is based on the assumption that clinical status is reflected better by nicotinic acid activity than by nicotinic acid plus its catabolic derivatives.

The reason for the absence of an additive response to nicotinic acid plus nicotinamide, seen with whole blood and in recovery experiments, remains obscure. Nicotinic acid and nicotinamide seem to interfere with each other's utilization. There may exist a competition between nicotinic acid and nicotinamide for methylation; growth therefore would not reach an algebraic sum when the two are combined because of the limited number of methyl groups available for these compounds.

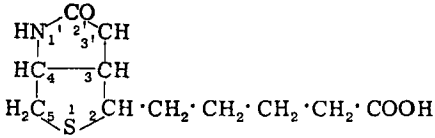
The biosynthesis and metabolism of nicotinic acid in disease has received little attention; metabolic studies deal mainly with normal animals and man (O1, R5). After a tryptophan load dose, the main catabolites in the urine are nicotinuric acid, N^1-methylnicotinamide, nicotinamide, quinolinic acid, kynurenine, 6-pyridone, anthranilic acid, and 3-hydroxyanthranilic acid. These excretory products were estimated

in normal man and in patients suffering from typhoid fever, cholera, small pox, cirrhosis of the liver, and infectious hepatitis. In all diseases studied, except in typhoid and small pox, urinary excretion of nicotinic acid and its metabolites was greatly diminished; tryptophan loading increased the excretion of nicotinic acid metabolites (B19). During pregnancy, conversion of tryptophan to nicotinic acid is more efficient (W6).

The antagonists of nicotinic acid are 6-aminonicotinamide and, less potent, 3-acetylpyridine and pyridine-3-sulfonic acid (H15, J4). Nicotinamide has also been reported to be effective in experimental cancer (S3). It is supposedly converted to nonphysiological nucleotide analogs of NAD and NADP because it becomes attached to available apodehydrogenase; the resulting enzyme cannot function in hydrogen and electron-transfer reactions essential to normal cellular metabolism (D7).

5. Biotin

Biotin is an optically active organic acid. The vitamin has the structure shown in (II). Only the dextrorotary form shows activity. Its chemistry and physiology have been extensively reviewed (T1).



(II)

Biotin (2′-keto-3,4-imidazolido-2-tetrahydro-thiophene-*n*-valeric acid).

A variety of biotin-requiring microorganisms have been used to assay biotin: *Saccharomyces cerevisiae* (H6), *Lactobacillus casei* (S2), *Lactobacillus arabinosus* (now *L. plantarum* ATCC No. 8014) (W14), *Micrococcus sodonensis* (A1), *Neurospora crassa* (H10), and *Rhizobium trifolii* (W7). None have been applied successfully for assaying biotin in biologic fluids. Because the flagellate *Ochromonas danica* had a specific and sensitive biotin requirement (A2), it was utilized as a reagent for biotin in blood, serum, urine, brain, and liver tissue (B3b).

5.1. Methods

Nutritional and cultural techniques for growing *Ochromonas danica* in light and darkness have been described (A2, H18). It was grown under constant illumination from five 40-watt "warm-white" fluorescent lights, ca. 1.0 m from the cultures. Transfers were made weekly in 10 ml of maintenance medium; 1 ml was used for transfer (Table 6).

TABLE 6
MAINTENANCE MEDIUM FOR *Ochromonas danica*[a]

Components		mg/100 ml
K_3 citrate·H_2O		25
Na acetate·$3H_2O$		50
$MgSO_4 \cdot 7H_2O$		10
Sucrose		200
Glycerol		100
Starch		400
Trypticase[b]		600
Yeast autolyzate[c]		200
DL-Lactate[d]		40
Liver L[e]		20
Distilled water	to	100 ml

[a] The pH is adjusted to 6.7–7.2 with KOH or H_2SO_4.
[b] Baltimore Biological Laboratory, Baltimore, Maryland.
[c] Albimi Laboratories, Inc., Brooklyn, New York.
[d] Added from a 40 mg/ml solution adjusted to pH 7.0 with KOH, before addition.
[e] Nutritional Biochemicals Co., Cleveland, Ohio.

The basal medium is made up in double strength (twice the amounts listed in Table 7); 2.5 ml of portions of the medium were distributed in 35-ml borosilicate micro-Fernbach flasks provided with glass caps (H18). Solutions to be assayed were added, and the final volume was brought to 5 ml with distilled water. They were then autoclaved for

TABLE 7
BASAL MEDIUM FOR *Ochromonas danica* IN BIOTIN ASSAY[a]

Components	Final medium (mg/100 ml)
Nitrilotriacetic acid[b]	20
KH_2PO_4	30
$MgCO_3$ (basic)	40
$CaCO_3$	5
Metals mix[c]	1
NH_4Cl	50
$MgSO_4 \cdot 7H_2O$	100
Thiamine·HCl	0.1
L-Glutamic acid	300
L-Histidine·HCl·H_2O	40
L-Arginine·HCl	40
Glucose	1000
Distilled water to	100 ml

[a] The pH is adjusted to 5.0 with KOH or H_2SO_4.
[b] Eastman Organic Chemicals, Rochester, New York.
[c] See Section 5.1 for details.

30 minutes at 118–121°C. For inoculation, 2 ml of a 5-day maintenance culture was diluted with 10 ml of distilled water; a drop of this solution served as an inoculum for each flask. There was no significant carry-over of biotin. Incubation was at room temperature (not higher than 32°C) for 3–5 days under light. Growth was expressed in optical units (O.D.) with a Welch Densichron equipped with a red-sensitive probe.

A concentrated standard solution of D-biotin, U.S.P. (1 mg/ml) was prepared in distilled water; a few drops of 1 *N* KOH were added to

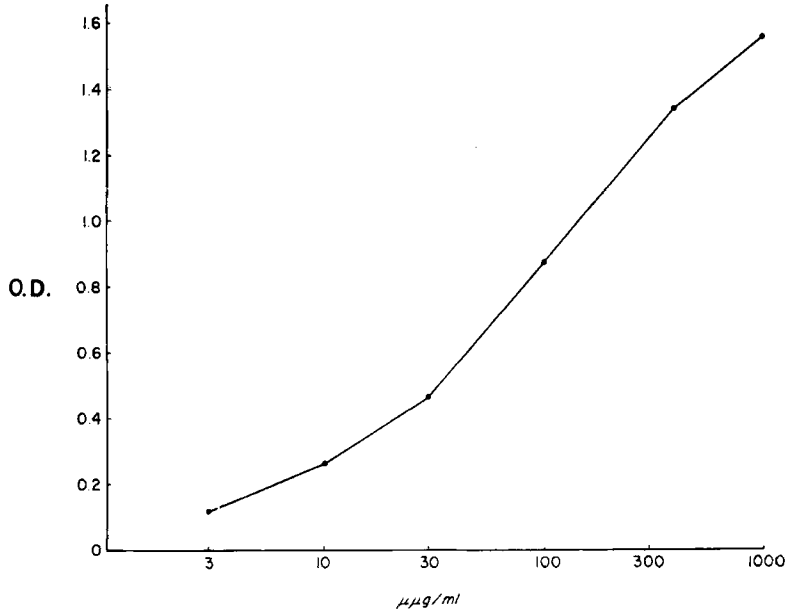


FIG. 2. Typical biotin curve for *Ochromonas danica* [Baker *et al.* (B3b)].

dissolve the crystals. Appropriate dilutions were made with distilled water from the concentrated standard. A standard biotin curve is prepared for assay by adding 3.0, 10, 30, 100, 300, and 1000 μμg/ml biotin to 2.5 ml of double strength basal medium. Solutions are then brought to a final volume of 5.0 ml with distilled water. Fresh standards must be prepared every month because biotin solutions slowly deteriorate. A control flask consisting of basal medium alone was always included for estimation of carry-over error, i.e., the biotin activity introduced by the inoculum. The basal medium and biotin standards were refrigerated at 4°C with volatile preservative added; additional technical details are described elsewhere (H19). A typical standard growth curve illus-

trates the sensitivity and the usable growth range for the biotin assay (Fig. 2).

The chemically-defined medium (Table 7) obviates the use of natural products such as casein hydrolyzate, beef extract, etc., which usually have appreciable biotin content. A "metals" supplement added as dry mix (B3b) to the basal medium provides: Fe, 0.1 mg [as $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$] Zn, 0.05 mg (as $ZnSO_4 \cdot 7H_2O$); Mn, 0.025 mg (as $MnSO_4 \cdot H_2O$); Cu, 0.004 mg (as $CuSO_4 \cdot 5H_2O$); Co, 0.005 mg (as $CoSO_4 \cdot 7H_2O$); B, 0.005 mg (as H_3BO_3); Mo, 0.0025 mg [as $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$]; V, 0.0005 mg (as $Na_3VO_4 \cdot 16H_2O$). Nitrilotriacetic acid is used as a nonmetabolizable chelating agent.

5.1.1. *Biotin in Blood*

Blood, obtained from an antecubital vein, was citrated and diluted 1:5 with enzyme buffer solution. The buffer is prepared by dissolving 19.2 g citric acid $\cdot H_2O$ in 100 ml of distilled water (Solution 1), and 71.7 g $Na_2HPO_4 \cdot 12H_20$ in 1000 ml of distilled water (Solution 2). Take 44 ml of Solution 1 and 56 ml of Solution 2 and dilute to 1000 ml with distilled water. The pH should be 5.5. The enzyme solution is prepared by suspending 1.0 g of crude papain in 100 ml buffer and homogenizing the suspension in a blendor. It is important to filter off the debris, since it proved to be a source of biotin contamination; the biotin in the debris is released by the enzyme during the incubation period preparatory to assay. The solution, with preservative added, was incubated overnight at 37°C to permit liberation of biotin from the biotin-protein complex. There is relatively little free biotin in blood. After incubation, the solution is autoclaved at 16 psi, 118–121°C for 10 minutes to stop enzymatic action and coagulate protein. The coagulum is centrifuged off and 0.5, 1.5, and 2.5 ml of the supernatant were added to individual flasks and assayed. This was equivalent to 1:50, 1:16, and 1:10 dilutions of blood. Control flasks of water and enzyme-buffer solution were included for blank determination. The enzyme solution prepared as described had insignificant biotin content.

5.1.2. *Biotin in Serum*

Blood was permitted to coagulate at room temperature. The coagulum was centrifuged off and the serum reserved for assay. Serum was assayed for biotin as described for blood.

5.1.3. *Biotin in Urine*

Urine was diluted 1:40 with buffer without enzyme. Biotin is free in urine, making enzymatic hydrolysis unnecessary. The solution was

autoclaved to remove any protein or salts; 0.25, 0.5, and 1.0 ml were used for assay. This represented a 1:800, 1:400, and 1:200 dilution of the original sample.

5.1.4. *Biotin in Liver Tissue*

Human liver obtained at autopsy was freed from coagulum and adhering tissue, sliced, washed 5 times, suspended in water, and homogenized. The tissue was lyophilized to dryness. This procedure standardized determinations since wet-weight values tend to fluctuate. Fifty milligrams of the dry powder were suspended in 100 ml water to which 1.0 g papain had been added (refer to Methods, Section 5.1, for preparation). Buffer is not necessary because the enzymatic liberation of biotin is independent of pH over the range from 3.5–7.5. The suspension was incubated overnight at 37°C; longer hydrolysis did not increase biotin yields. After incubation, the solution was autoclaved 10 minutes to stop enzymatic action and to precipitate the proteins. It was then further diluted 1:2 with distilled water. The debris was centrifuged off, and 0.5, 1.0, and 1.5 ml of the supernatant was used for assay. This represents 25, 50, and 75 μg of the original liver tissue per milliliter of final assay medium.

When assaying tissue, growth stimulation by unknown factors is always a drawback. In the assay of rat liver with *L. plantarum* such stimulation was noted; the drift was overcome by the addition of enzymatically hydrolyzed casein (F1). When assaying human liver, we also noted growth enhancement for *O. danica* by unknown factors; the 1:2 dilution, as described above, obviated any drift. Biotin recoveries ranged from 96 to 103%.

5.1.5. *Biotin in Brain*

Specimens for assay were prepared by suspending 500 mg of lyophilized brain in 50 ml of the buffer-papain mixture and hydrolyzing overnight at 37°C. The solution was then autoclaved for 10 minutes. This procedure yielded higher results than autolysis or prolonged enzymatic hydrolysis. After centrifuging off the debris, 0.5, 1.5, and 2.5 ml of the supernatant were used for assay. This represents 1, 3, and 5 mg of the original brain tissue per milliliter of final medium.

5.2. Range

In a group of 12 normal subjects, the values for serum ranged from 213–404 μμg/ml, those for blood slightly lower, 170–279 μμg/ml. The urine values, 6,260–32,700 μμg/ml, agree with those reported for "total"

biotin by the *Neurospora crassa* assay (W13), and also bear out our observations that biotin is in the free form in urine. Histologically normal lyophilized human liver tissue contains between 155 and 1140 μμg/mg biotin. Eight of the 10 livers assayed fell within the 230–660 μμg/mg range. Because biotin is conjugated in the liver, hydrolysis with papain is needed to free the biotin and make it assayable; heat denaturation frees only a small proportion of conjugated biotin.

The average value in the 10 histologically normal lyophilized brains assayed for biotin was 230 μμg/mg with a range of 210–240 μμg/mg. Values for biotin content of lyophilized rat brain were the same as those for human brain.

5.3. Inhibitors

One unit of avidin obtained from egg white, combines with 1 μg biotin. Avidin is denatured and inactivated by heat. It is sterilized by passage through a bacteriological filter. One avidin unit, added aseptically to hydrolyzates of blood, serum, and urine, prepared as described here, prevents *O. danica* growth. Avidin forms complexes with biotin (F5) thus rendering it unavailable to the organism. Pimelic acid (up to 1.0 mg/ml), aspartic acid (up to 1.0 mg/ml), or Tween 80 (up to 3.0 mg/ml), alone or in combination, do not stimulate growth of *O. danica* in the absence of biotin. These compounds effectively replace biotin as an essential nutrient in the medium for some yeasts and lactobaccilli (A12, T1).

Dethiobiotin, the sulfur-free analog of biotin, competitively inhibits the growth of *O. danica*; the inhibition index is 10. Biocytin (ε-*N*-biotinyl-L-lysine) stoichiometrically replaced biotin for *O. danica*. Because *O. danica* is phagotrophic (A2), it can probably ingest low-molecular forms of biotin, e.g., biocytin. Other forms of biotin were not studied.

5.4. Comments

Biotin is a growth factor for many bacteria, protozoa, plants, and probably all higher animals. In the absence of biotin, oxalacetate decarboxylation, oxalosuccinate carboxylation, α-ketoglutarate decarboxylation, malate decarboxylation, acetoacetate synthesis, citrulline synthesis, and purine and pyrimidine syntheses, are greatly depressed or absent in cells (M11, T1). All of these reactions require either the removal or fixation of carbon dioxide. Together with coenzyme A, biotin participates in carboxylations such as those in fatty acid and sterol syntheses. "Active CO_2" is thought to be a carbonic acid derivative of biotin involved in these carboxylations (L10, W10). Biotin has also been involved in

several transaminations (R13) and in carbohydrate metabolism via the hexokinase system (W8).

The physiologic sequelae of biotin deficiency are almost unexplored. Severe skin lesions, especially seborrheic dermatitis and Leiner's disease (*Erythroderma desquamativum* or exfoliative dermatitis), were increased in young infants born of mothers on a restricted diet low in eggs, livers, and other biotin-rich foods. After biotin administration the lesions healed. There are claims that excess biotin produces a fatty liver characterized by heightened cholesterol content. Choline has no effect in the prevention of biotin-fatty livers (G2, M2). In mice with transplanted tumors, both the tumors and the blood levels of biotin are below normal (R8). More recent studies established a protection with avidin, the biotin-binding fraction of egg white, against tumor formation (K4). More data along these lines are still needed for confirmation.

Spontaneous biotin deficiency is unlikely to occur as a result of simple dietary restriction, since biotin has high potency, is widely distributed in foods, and is also synthesized in the intestine. The human requirement for biotin is not known, but subjects on a low-biotin diet recovered in 3–5 days, when 75–300 μg biotin was administered daily (S23, S24).

Biotin's biochemical role is becoming clearer. As mentioned, biotin has been implicated in CO_2-fixation. Good examples for this reaction in animals are (a) the combination of pyruvic acid and CO_2 to form oxaloacetic acid, or aspartic acid (B26, H19) to replace biotin, and (b) the growth stimulation of certain bacteria in the presence of biotin, and bicarbonate (L1).

Biotin is also involved in purine biosynthesis (M11): C^{14}-incorporation into adenine and guanine was considerably reduced in the biotin-deficient animal (M3). It plays a dual role in purine synthesis, through the ability to fix CO_2 and through the synthesis of aspartic acid. The conversion of 5-amino-4-imidazole ribotide to 5-amino-4-imidazole carboxamide ribotide requires biotin, aspartic acid, and bicarbonate. Aspartic acid and bicarbonate showed no effect on this conversion with biotin (M11). Completion of the pyrimidine ring in the purine molecule therefore depends on biotin. The status of biotin in diseases, showing disorder in purine anabolism and catabolism, has not yet been investigated. Biotin is also involved in lipogenesis via the conversion of acetate to fatty acids (W3), suggesting a CO_2-fixation step in fatty acid synthesis. A previous report (L10) demonstrated that this CO_2 fixation produces malonyl-CoA. This enzyme is an intermediate in fatty acid synthesis, e.g., malonyl-CoA and butyryl-CoA are intermediates in the biosynthesis of palmitic acid (S20). The possible relationship of biotin to the ability of the animal to esterify

and store fatty acid as well as cholesterol has been studied (S8). Although results are inconclusive, the subject is of interest, since cholesterol deposition in the arterial walls and the relationships of such a process to atherosclerosis are major topics of clinical and biological research. These and many other observations regarding biotin in animal and human nutrition and metabolism will undoubtedly foster more extensive investigations.

6. Inositol

Because the role of inositol in animal metabolism complies with the definition of vitamin, it is usually included in this class of substances, although the quantities and concentrations involved are of much higher magnitude.

TABLE 8

MEDIUM FOR INOSITOL ASSAY USING *Saccharomyces carlsbergensis*

Medium	mg/100 ml
Sugar and salts[a]	50 ml
Potassium and citrate buffer[b]	10 ml
Ammonium sulfate	750
Calcium pantothenate	0.5
Pyridoxine	0.05
Thiamine	0.05
Biotin	0.005
Casein (acid hydrolyzed)	1000

[a] 100 ml contains 20 g of dextrose C.P. (anhydrous), 220 mg of monopotassium phosphate, 170 mg of potassium chloride, 50 mg of calcium chloride ($CaCl_2 \cdot 2H_2O$), 50 mg of magnesium sulfate, 1 mg of ferric chloride, and 1 mg of manganese sulfate.

[b] 100 ml contains 10 g of potassium citrate ($K_3C_6H_5O_7 \cdot H_2O$) and 2 g of citric acid ($H_3C_6H_5O_7 \cdot H_2O$).

It may be determined by various chemical methods, but its analysis in body fluids lends itself to a microbiological assay method.

Among various methods reported, we wish to describe an assay technique with *Saccharomyces carlsbergensis* 4228. This yeast had been used by Atkin *et al.* (A11) for the determination of vitamin B_6. We have omitted inositol from the medium and have added an excess of pyridoxine (S19). The composition of the medium is given in Table 8. The method has an accuracy of $\pm$ 0.1 mμg/ml.

6.1. ASSAY METHOD

Since part of the inositol occurs in blood plasma in conjugated form, the sample must be hydrolyzed. This serves at the same time for deproteinization. Ten milliliters of oxalated plasma obtained in the fasting state are hydrolyzed with 100 ml of 18% HCl for 6 hours and the hydro-

lyzate filtered, concentrated, diluted with distilled water, treated with Norite, and the volume made up to 50 ml after the solution has been brought to pH = 5.5 with a few drops of alkali. Two, 3, and 4 ml samples of this hydrolyzate, corresponding to 0.4, 0.6, and 0.8 ml of plasma, are added to 5 ml of medium, made up to 9 ml with distilled water and inoculated with yeast. The nephelometric reading is performed in the Klett-Summerson Photoelectric Colorimeter with filter No. 66. A standard curve is established in the conventional manner.

6.2. Range

The normal range of inositol in plasma by this technique was 0.42–0.76 μg/ml. Higher values up to 1.9 μg/ml were found in individual patients. Destruction of some inositol during acid hydrolysis can not be excluded. The state of inositol in the plasma and the possible clinical significance of inositol need further investigation.

7. Vitamin B_6

Vitamin B_6 (pyridoxine, pyridoxal, pyridoxamine) like nicotinic acid is a pyridine derivative. Its phosphorylated form is the coenzyme in enzymes that decarboxylate amino acids, e.g., tyrosine, arginine, glycine, glutamic acid, and dihydroxyphenylalanine. Vitamin B_6 participates as coenzyme in various transaminations. It also functions in the conversion of tryptophan to nicotinic acid and amide. It is generally concerned with protein metabolism, e.g., the vitamin B_6 requirement is increased in rats during increased protein intake. Vitamin B_6 is also involved in the formation of unsaturated fatty acids.

Severe hypochromic microcytic anemia, responding only to vitamin B_6 and not to iron, a typical symptom of B_6 deficiency in many species of animals, is related to the dependence of porphyrin biosynthesis on vitamin B_6, preceding the δ-aminolevulinic acid stage, at the condensation of glycine with succinate to yield α-amino-β-ketoadipate, the immediate precursor of δ-aminolevulinic acid.

Epileptiform fits associated with degenerative changes in the myelin sheath of peripheral nerves and spinal cord occur in B_6-deficient animals. Lesions in the arteries, resembling those of human atherosclerosis, have been observed in B_6-deficient monkeys. Recently, a state of B_6 deficiency in human infants, characterized by loss of ability to convert tryptophan to nicotinic acid, by impaired growth, convulsions, and hypochromic anemia, has been described, following omission of vitamin B_6 from the diet.

Because of the immense literature which has accumulated on vitamin

B_6 action and effects, the reader is referred to the following articles for (a) metabolic effects (A6, C5, D1, L5, V1, W5), (b) vitamin B_6 in pregnancy (P1, W2), (c) vitamin B_6 in anemias (E3), hemosynthesis (R7), and blood diseases (G5), (d) endocrine function (W11, W12), neurophysiologic effects (M1, T6, T7), and chemistry (B34, S13).

7.1. Methods

Vitamin B_6 can be determined chemically (T4) by measuring the fluorescence of the cyanohydrin of pyridoxal, which is obtained by microbiological transamination of pyridoxamine with *Escherichia coli* mutants (D6), *Saccharomyces carlsbergensis* 4228 (M14), *Streptococcus fecalis* and *Lactobacillus casei* (S11). No assay has been used successfully for the detection of vitamin B_6 in biologic fluids. Perhaps the best indirect methods for determining the B_6 status in biological fluids are the transaminase methods, the xanthurenic acid method, and the method utilizing circulating leucocytes. The transaminase system is based on the dependence of pyridoxal-5-phosphate, as part of the alanine (SGPT) and aspartic (SGOT) transaminases in the serum. Both transaminases are important as indicators of hepatic and of cardiovascular disorders (W15). Deficiency of pyridoxine results in decreased plasma alanine and aspartic transaminases; the alanine enzyme appears to be more sensitive (A9). A disturbance of B_6 metabolism, through a derangement of tryptophan metabolism, gives rise to excretion of excess xanthurenic acid in the urine; this is used as an index of the B_6 status in man. There is disagreement about the validity of the xanthurenic acid titer as an index of B_6 deficiency; e.g., high xanthurenic acid levels in human urine were unrelated to significant metabolic changes (G1). Non-gravid women (M7) and women in the last trimester of pregnancy (T8) excreted tryptophan catabolites equivalent to 2 to 3 times the tryptophan intake; the absorption of vitamin B_6 was normal. On the assumption that xanthurenic acid is an abnormal metabolite, a tryptophan load test is used to assess departure from B_6 normality. Marked increases in xanthurenic acid excretion accompanied toxemias of pregnancy (W1); elderly subjects excreted about twice as much xanthurenic acid as young subjects in a 24-hour period after tryptophan loading; vitamin B_6 therapy corrected this effect in the elderly subjects (R4).

The leucocyte method estimates pyridoxal phosphate in isolated leucocytes; it is based on a coenzyme-catalyzed tyrosine decarboxylase system from S. *faecalis* (B32). Enough data are not yet on hand to evaluate this method. The determination of circulating or available vitamin B_6 should offer a more direct approach.

We have developed a direct assay for vitamin B_6 in blood, serum, urine, cerebrospinal fluid, and tissue, based on the ciliate, *Tetrahymena pyriformis*. The techniques are essentially those described for nicotinic acid (see Section 4.1), except that vitamin B_6 is omitted from the basal medium; both nicotinic acid and its amide are added each at 0.1 mg/100 ml of basal medium. The method for blood, serum, urine, cerebrospinal fluid, and tissues is given below.

7.2. Microbiological Assay

For every milliliter of citrated blood (Fig. 3) 1 ml of pH 6.1 phosphate buffer is added (see Folic Acid, Section 8.1). The mixture is autoclaved

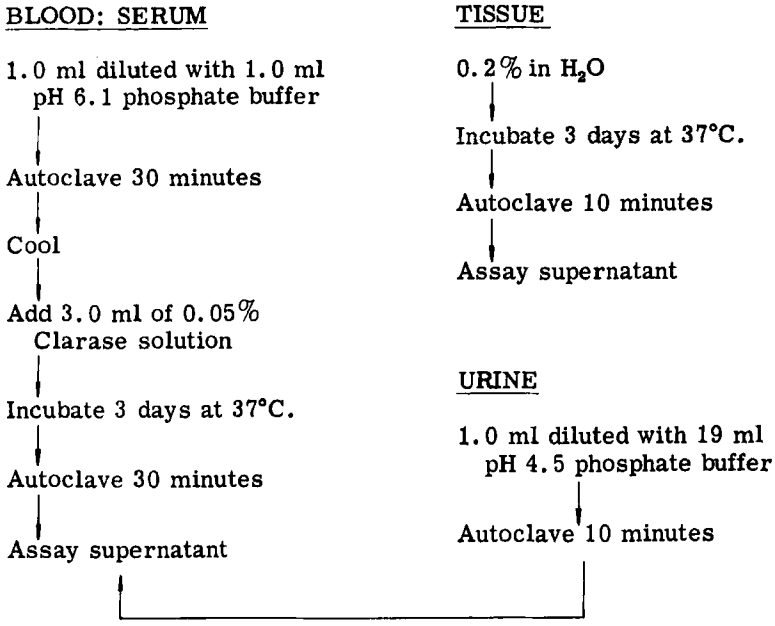


Fig. 3. Assay of vitamin B_6 in biologic fluids and tissues.

30 minutes at 118°–121°, 16–17 psi. The coagulum is homogenized and 3 ml of a 0.5 mg/ml solution of Clarase (Diastase—Fisher Scientific, New York) is added to the coagulated blood solution. The mixture is incubated for 3 days at 37° to allow the enzyme to liberate more of the bound vitamin. After this period of incubation, the mixture is autoclaved for 30 minutes. The coagulum is centrifuged off and the supernatant is assayed. To 2.5 ml of double-strength basal medium 0.5, 1.0, and 1.5 ml, of the supernatant is added. The medium is then brought to 5.0 ml with

distilled water. This represents 1:50, 1.25, and 1:16 dilutions of the original blood. Serum is treated like blood. Cerebrospinal fluid is assayed in higher concentration, e.g., 1:25, 1:16, and 1:10. No incubation is necessary for urine since vitamin B_6 occurs in unconjugated forms. The chief excretory product of B_6 catabolism, pyridoxic acid, the 4-carboxylic acid of vitamin B_6, is inert.

Liver or brain tissue is homogenized and lyophilized. The dried tissue is suspended in water (1 mg/ml), incubated 3 days at 37°, and autoclaved 10 minutes to coagulate protein. After removing the coagulum, the supernatant is assayed at 1, 1.5, and 2.0 ml, which represents 0.4, 0.6, and 0.8 mg/ml of basal medium (weight/volume) of dried tissue.

7.3. Range

Blood from normal subjects contains 14–60 mμg/ml, serum 25–75 mμg/ml, urine 100–200 mμg of B_6/ml of fluid; cerebrospinal fluid contains less than 1 mμg/ml. Normal human liver, obtained by biopsy, contains between 10–20 mμg of B_6/mg of dried tissue; rat brain between 3.5–5 mμg/mg. (cf. Table 9).

TABLE 9
Vitamin B_6 Levels

	Vitamin B_6 (mμg/ml)		Vitamin B_6 (mμg/mg)	
Fluids	Human	Tissues	Human	Rat
Blood	14– 60	Liver	4–12	8–13
Serum	16– 42	Brain	—	4–6
CSF	0– 0.75			
Urine	120–200			

The usable range for *T. pyriformis* is from 0.3–300 mμg/ml. The organism utilizes pyridoxal, pyridoxamine; pyridoxine, and pyridoxal-5-phosphate. Pyridoxamine + pyridoxal yielded the best growth; approximately 120 times more pyridoxine is required to yield the same growth as pyridoxamine (Fig. 4). As with nicotinic acid and its amide, when these compounds are added together in the same concentration, the increment of growth is less than the sum of the individual increments. Upon an intramuscular load dose of 100 mg of pyridoxine, peak vitamin B_6 levels are reached 2 hours after injection. The curves for 4 normal individuals are illustrated in Fig. 5.

The organism is competitively inhibited by isonicotinic acid hydrazide, but not by deoxypyridoxine. The deoxypyridoxine results confirm those observed by others (K2a). Recovery of vitamin B_6 added to biologic

fluids or tissues amounts to 92–98%. Our preliminary studies on patients with nontropical sprue and hyperthyroidism show them to have low-circulating levels of vitamin B_6. These results were found to be well correlated with the levels of xanthurenic acid and kynurenine excretion after a tryptophan load test.

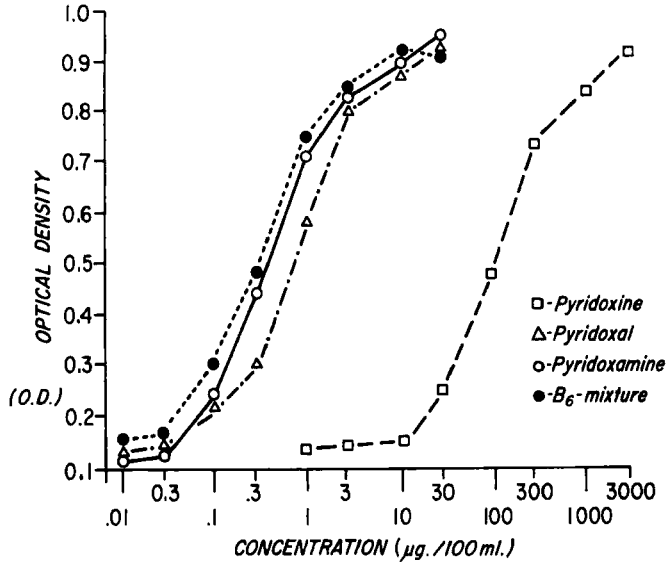


FIG. 4. Growth of *Tetrahymena pyriformis* with members of the vitamin B_6 group.

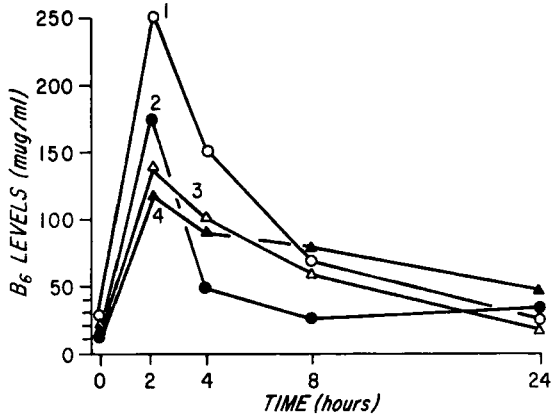


FIG. 5. Vitamin B_6-levels in blood upon intramuscular injection of 100 mg pyridoxine–HCl.

7.4. Comments

The microbiological assay is well suited for routine application. It is less time consuming and does not require the clinical supervision necessary in tryptophan-loading tests.

Deoxypyridoxine, pyramine (toxopyrimidine—the pyrimidine moiety of thiamine), and isonicotinic acid hydrazide are the most potent B_6 inhibitors. All can induce deficiency symptoms. There are several ways by which these inhibitors act. Some pyridoxine analogs act by being converted to coenzyme analogs competing with pyridoxal-5-phosphate for union with the specific apoenzyme, and by inhibiting the biosynthesis of pyridoxal-5-phosphate. Thus, tyrosine decarboxylase is inhibited competitively by 4-deoxypyridoxine-5-phosphate. Toxopyrimidine also acts as a competitive inhibitor for the reactivation of tyrosine apodecarboxylase. Isonicotinic acid hydrazide, used in recent years for blocking of pyridoxal-5-phosphate enzymes, combines with pyridoxal phosphate to form a hydrazone and thereby competitively inhibits tryptophanase, amino acid decarboxylases, and transaminases (B34). Mention should be made of the monoamine oxidase inhibitors. Monoamine oxidase is widespread in animal tissues; it is responsible for oxidative deamination of tyramine and adrenaline. In brain, monoamine oxidase activity is concentrated mostly in the central gray matter and hypothalamic region. Iproniazid may specifically inhibit enzymes that require pyridoxal phosphate as a cofactor. Monoamine oxidase inhibitors may affect the glutamic acid decarboxylase activity of the brain, a B_6-dependent system. The monoamine oxidase inhibitors may act as B_6 antagonists; further data are needed (R10, Z1).

8. Folic Acid

The chemistry, metabolism, and clinical importance of folic acid have been the subject of many excellent reviews (A7, G11, H14, H20, R1). Folic acid deficiency leads to a macrocytic anemia and leucopenia. These symptoms are due to inadequate synthesis of nucleic acid. The synthesis of purine bases and of thymine, required for nucleic acid synthesis, is impaired in folic acid deficiency. Detection of folic acid activity in biologic fluids and tissues is of the utmost importance; it distinguishes between the various anemias, e.g., those due to vitamin B_{12} or folic acid deficiency. Because morphology of the abnormal red cell does not help in diagnosing vitamin deficiency, one must rely on assay methods for differential diagnosis. Treatment of pernicious anemia with folic acid has led to subacute combined degeneration of the spinal cord despite

the hematological remission (R12); the ensuing neurologic damage is irreversible.

Chemical methods for folic acid detection are not useful for unfractionated biologic materials (H16). Reduction of folic acid in acid yields a methylpteridine and *N*-(*p*-aminobenzoyl)glutamic acid. The latter is estimated by a method for aromatic amines (B33). Another assay method of historical interest is the growth of chicks as a measure of the folic acid content of crude biological mixtures.

Nearly all microbiologic assays for folic acid activity have used *Streptococcus faecalis* and *Lactobacillus casei.* Earlier it appeared that these organisms could not detect folic acid deficiency in man (C2, L8); for example, in one study using S. *faecalis* there was no detectable activity in the fasting serum of humans (C3). Administration of a loading dose of folic acid with subsequent assay by S. *faecalis* (G9) has served as a workable means of determining folic acid deficiency (C6), a technique having definite drawbacks (G10).

An indirect method for detecting folic acid deficiency was based on the excretion of formimino-L-glutamic acid (FIGlu), an intermediate in the catabolism of histidine (B36, H7, S6). Although this method seems to be generally accepted, there have been reported instances of vitamin B_{12} deficiency in which FIGlu excretion was demonstrated and cases of folic acid deficiency in which FIGlu was not excreted (B37, F4). The method, therefore, is not dependable for detecting folic acid deficiency in man.

A microbiologic assay for folic acid activity in human serum was devised (B12). It agrees with the clinical folic acid status (W4).

8.1. Assay

The organism is *L. casei* ATCC No. 7469. The basal medium (J6) has been modified; it contains twice the concentration listed in Table 10a, and is stable when stored in an amber bottle in the cold. It is kept free from contamination with a volatile preservative. *L. casei* was maintained in medium given in Table 10b; 10 ml of this medium is dispensed into screw-capped tubes and autoclaved for 30 minutes at 118°, 16 psi. It should be transferred every 2 weeks; one drop of an 18-hour culture into 10 ml of maintenance medium suffices with subsequent 18-hour incubation at 37°. Cultures should be stored at 4°.

Blood is transferred to test tubes and permitted to clot at room temperature for approximately 3 hours. The clot is removed by centrifugation and the serum saved for assay. Only serum was used; it can be stored frozen with ascorbic acid added.

A concentrated pteroylglutamic acid (PGA) solution (1 mg/ml) is prepared with crystalline PGA dissolved in 20% (v/v) ethanol/water. The pH is adjusted with 0.1 *N* NaOH to pH 10–11 to dissolve the PGA, 0.06 *N* H_2SO_4 is then used to readjust the pH to 7.0. Appropriate dilutions

TABLE 10a
MEDIUM[a] FOR PGA ASSAY USING *Lactobacillus casei*

Medium	Concentration in final volume (mg/100 ml)
Hy-case "vitamin free"[b]	500
L-Tryptophan	10
Adenine	0.5
Guanine·HCl	0.5
Uracil	0.5
Xanthine	1.0
L-Asparagine·H_2O	30
L-Cysteine·HCl	25
Riboflavin	0.05
p-Aminobenzoic acid	0.1
Pyridoxine·HCl	0.2
Thiamine·HCl	0.02
Ca pantothenate	0.04
Nicotinic acid	0.04
Biotin	0.001
Glucose	2000
Tween 80[c]	0.005 ml
Glutathione (reduced)	0.25
Salt mix[d]	0.5 ml
Na acetate (anhydrous)	2000
K_2HPO_4	50
KH_2PO_4	50
$MnSO_4 \cdot H_2O$[e]	10

[a] pH 6.6–6.8 (adjusted with H_2SO_4 or KOH).

[b] Salt-free hydrochloric acid hydrolyzate of casein, Baltimore Biological Laboratory Inc., Baltimore, Maryland. These casein hydrolyzates can be substituted by Casamino acid (Difco), enzymatically hydrolyzed casein or acid-hydrolyzed casein.

[c] Atlas Powder Co., Wilmington, Delaware. The commercial preparation is diluted with ethanol to appropriate concentrations.

[d] 1.0 ml contains: $MgSO_4 \cdot 7H_2O$, 40 mg; NaCl, 2 mg; $MnSO_4 \cdot 4H_2O$, 2 mg; $FeSO_4 \cdot 7H_2O$, 2 mg.

[e] Added after pH adjustment.

are made with distilled water from the concentrated standard. Solutions are kept frozen and are thawed as needed; under these conditions PGA activity is stable for one month. A standard PGA curve is prepared for every assay by adding 5.0 ml of double strength basal medium (Table 10a),

into 10 ml micro-Fernbach flasks. PGA is added at 0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 mμg/ml final concentration; the control flask, consisting of basal medium alone, is included to estimate carry-over error, i.e., that error due to folic acid activity of the maintenance medium. The standard solutions in each flask are brought to a final volume of 10 ml with distilled water.

Serum samples are prepared for assay by a modification of a previous method: serum is used instead of whole blood (B18, T3). Serum is diluted 1:10 with buffer at pH 6.1 to which fresh 0.05% ascorbic acid has been added. To prepare buffer dissolve 27.8 g of $NaH_2PO_4 \cdot H_2O$ in 1000 ml distilled water (Solution 1); dissolve 71.7 g $Na_2HPO_4 \cdot 12\ H_2O$

TABLE 10b
MAINTENANCE MEDIUM[a] FOR *Lactobacillus casei*

Medium	Concentration in final medium (g/100 ml)
Yeast extract	0.75
Proteose peptone (Difco)	0.75
Glucose	1.0
KH_2PO_4	0.2
Tomato juice (filtered)[b]	10 ml
Tween 80	0.1 ml
L-Cysteine·HCl	0.1

[a] pH 6.8–7.0 (adjusted with KOH).
[b] pH adjusted to 7.0 with KOH before addition.

in 1000 ml distilled water (Solution 2). Take 212.5 ml of Solution 1 and add 37.5 ml of Solution 2; dilute to 1000 ml with distilled water. The pH should be 6.1 (D3). Some investigators (W4) use 0.1% ascorbic acid to make up for heat destruction of the ascorbate during autoclaving, however, if autoclaving is carried out in a double-walled autoclave operating on house steam, the additional ascorbate is not necessary for protecting the folic acid activity of serum. Gas and electrically operated autoclaves take longer to heat to appropriate temperature and pressure. Hence, the prolonged heating completely destroys the protective action of the ascorbate. The favorable effect of ascorbic acid has been reported (D3, T2). The serum buffer solution is autoclaved 10 minutes at 118°, 16 psi. The coagulated proteins are centrifuged off and the clear supernatant is assayed; 0.5 ml, 1.0 ml, and 1.5 ml of the supernatant are added to 5.0 ml of double strength basal medium and then brought to final volume, 10 ml, with distilled water. These represent 1:200, 1:100, and 1:67 dilutions of the original serum. The flasks are covered with alu-

minum caps, put into Pyrex utility trays, and autoclaved 30 minutes at 118°, 16 psi. After autoclaving, another tray is inverted to cover the sterile flasks.

The inoculum is prepared by transferring 1.0 ml of a fresh 18-hour *L. casei* culture grown at 37° in a tube of maintenance medium (Table 10b). The new culture is incubated 6–8 hours at 37°. This culture is then diluted 1:10 with full-strength basal medium; the flasks are inoculated with one drop of this suspension. The trays are sealed with masking tape and the flasks incubated 16-18 hours at 37°. Growth is measured with a Welch Densichron equipped with a red-sensitive probe to eliminate error due to the color of the medium. The standard growth densities of the PGA-standards are plotted on semilogarithmic paper and serum values calculated from the curve. Normal folic acid serum values range from 6.0–24 mμg/ml.

8.2. Results

Table 11 gives results with the above procedure. The vitamin B_{12} values are included to differentiate in megaloblastic anemias those due to

TABLE 11
Assay for PGA in Serum

Diagnosis	Number of subjects[a]	Ranges	
		B_{12} (μμg/ml)	Folic acid (mμg/ml)
Normal	10	200–660	6.0–24
Primary B_{12} deficiency (pernicious anemia)	9	15–110	9.0–23
Primary PGA deficiency (alcoholic cirrhosis with megaloblastic anemia)	8	200–875	1.0–4.4
B_{12} and PGA deficiency	8	32–125	1.2–4.8

[a] All subjects were untreated.

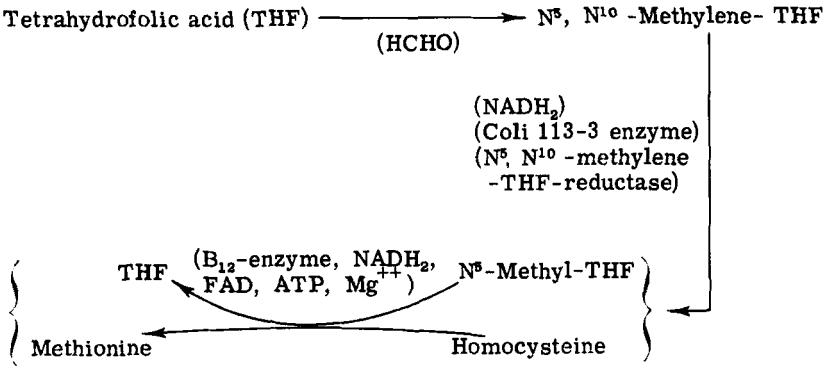
B_{12} from those due to folic acid deficiency. In all cases the folic acid determinations agreed with the clinical findings; more detailed results are given elsewhere (B12).

8.3. Comments

Because of the multiplicity of folic acid factors reported in whole blood (U1), the microbiologic assay for folic acid in whole blood and serum was regarded as valueless (C2, L8, W9); results based on S. *faecalis* methods (C2, C3) did not contradict this view. *Streptococcus faecalis* is inferior to *L. casei* in its utilization of the PGA polyglutamates

(H20, S7). In fact, the ability to utilize PGA polyglutamates may be responsible for the superiority of *L. casei* over *S. faecalis*.

Serum, rather than whole blood, was used for assay, since whole blood contains a multiplicity of PGA-active materials and anticoagulants affecting folic acid activity (T2, U1). There is evidence that red blood cells may also be overwhelmingly rich in PGA-by-passing factors for microorganisms (H20). Serum is apparently low in these by-passing substances but may be rich in polyglutamates or N^5-methyltetrahydrofolic acid, available to *L. casei* only (L2).



SCHEME 1: Derivatives of tetrahydrofolic acid.

For correlation with the serum folic acid, FIGlu determinations were carried out using two methods (L9, S6). Both proved insensitive, notably where no urinary FIGlu was excreted despite clinical proof of PGA deficiency.

A modification of the above serum folic assay method was recently described (W4). The investigators confirmed the validity of this technique as a practical means of differentiating patients with "folic acid" from "vitamin B_{12}" megaloblastic anemias. Another modification of this method was also described (C4). These investigators reduced the over-all sensitivity by high serum dilutions and thus made the assay, as they used it, valueless as a diagnostic tool.

The nature of folic acid activity in serum is still obscure despite many clues. Perhaps the best lead has been furnished by the isolation of the previously mentioned N^5-methyltetrahydrofolic acid. This is a newly isolated intermediate which is involved in the synthesis of methionine via the reaction in Scheme 1 (L2). This intermediate supports

the growth of *L. casei*, but not that of *Streptococcus faecalis* or *Pediococcus cerevisiae* (the organism used for the assay of folinic acid—See Section 8.4). Since the folic acid material in serum yields little folinic acid activity or *S. faecalis* activity, N^5-methyl THF (tetrahydrofolic acid) is probably the important folic acid derivative in serum. N^5-methyl THF may act as a metabolic trap for folic acid compounds. In a B_{12}-deficient system (e.g., pernicious anemia), N^5-methyl THF would decrease (A7). Possibly, in folic acid deficiency the lack of folic acid compounds would tend to decrease N^5-methyl THF and thereby reduce *L. casei* growth. There is also the probability that the naturally occurring prefolic acid A, which yields THF and formaldehyde on enzymatic oxidation is N^5-methyl THF (D8, D9, K2).

The *L. casei* method described here can also be used to determine absorption of folic acid. Five milligrams of folic acid is given by mouth; samples are obtained at 0, 2, 4, 6, and 8 hours. In cases of deficient folic acid absorption, normal peak levels are not obtained: in the malabsorption syndrome, a flat curve indicates no absorption. Normal peak levels are maintained during 2–4 hour intervals (B3).

8.4. Folinic Acid

We have developed an assay for folinic acid (citrovorum factor) which will soon be applied to serum assay; it has proved its usefulness in tissue analysis. The organism used is *Pediococcus cerevisiae* (formerly *Leuconostoc citrovorum*) ATCC No. 8081. It is maintained like *L. casei*. The basal medium has been described (J6). It has been modified by substitution of 1.0% salt-free acid-hydrolyzed casein for the specially prepared acid-hydrolyzed casein recommended, and by inclusion of 0.5 mg% thymidine (G22). For assay, 25 mg of lyophilized liver or brain is suspended in 25 ml, pH 6.1 phosphate buffer (see Section 8.1) to which 12.5 mg of ascorbic acid has been added. This pH allows maximum release of activity of folic and folinic acids. The suspension is incubated at 37° overnight, and then autoclaved to coagulate proteins. The coagulum is centrifuged off and the supernatant diluted 1:10 with water and 1, 2, and 4 ml of the supernatant are asayed for folic acid and folinic acid; this represents 10, 20, and 40 μg of liver per milliliter of basal medium. Folinic acid standards are prepared by dissolving synthetic calcium DL-leucovorin in water and using it at the same strength as the folic acid standards. The potency of the DL-leucovorin is assumed to be one-half that of the natural form. The natural folinic acid activity of liver, when synthetic folinic acid is used as a standard, is determined by dividing the folic

acid value obtained by 2. Since 1 mg of calcium leucovorin stimulates *L. casei* to the extent of 0.5–0.55 mg of folic acid, the natural folic acid content of liver is determined by the following formula: FAA = F —(NCF/2), where FAA equals folic acid activity (PGA), F equals total folic acid activity obtained by the *L. casei* method, and NCF is the folinic acid value obtained with *P. cerevisiae* and leucovorin as standard. On this basis dried normal human liver contains approximately twice as much folic acid activity as folinic acid activity. For rat liver a similar ratio was observed. In human liver, rat brain, and rat liver, fluctuation of the folic-folinic ratio occurs during anesthesia; the liver is particularly affected by ether (D5). From the results with human liver mentioned above, it seems that some of the folic acid is stored in the liver as folinic acid, or at least in some assayable reduced form of folic acid. Different ratios have been reported for mammalian liver (G8, S22).

8.5. Radioactive Folic Acid

Recently, tritiated folic acid became available, making possible a nonmicrobiological method for studying the metabolism of folic acid. It obviates the toxic effects of folic acid antagonists on microbial assay organisms. This technique was used to follow the uptake, metabolism, and excretory products of folic acid (A4, J2, J3).

8.6. Unconjugated Pteridines

A review on folic acid should not ignore the metabolism of unconjugated pteridines. This subject was reviewed (H20), but unconjugated pteridines with an aliphatic substituent at carbon 6 or carbon 7 have yet to find their place in mammalian metabolism. Using the trypanosomid flagellate, *Crithidia fasciculata* (N2), the content of unconjugated pteridines in human blood and serum was assayed (B8). The blood serum was treated as for folic acid, except that ascorbic acid was omitted from the buffer so as to destroy purposely the folic acid activity to a level below the range that could stimulate the growth of the organism. Whole blood contains between 27–70 mμg/mg, and serum between 11–43 mμg/ml of unconjugated pteridines. Exceptionally high levels were observed in sera from patients with gout, although folic acid activity was in the normal range (B8). Possibly, in patients with gout, not enough xanthine oxidase remains to metabolize the pteridines sufficiently; pteridines are substrates for mammalian xanthine oxidase (B29).

The function of pteridines in the metabolism of some microorganisms was studied. They were found to influence the production of vitamin B_{12}

(N1). This line of inquiry is likely to become more active if the unconjugated pteridine, biopterin, proves to have the proper microbiological activity and so can serve as standard.

8.7. Antifolics

The antimetabolites of folic acid are the only antivitamins that have been put to therapeutic use, notably in leukemias and chorioepithelioma (M5). They differ greatly in structure, but were designed to be closely related to substances normally utilized by the cell for metabolism and growth (K8). "Antifolics" interfere with the synthesis or structure of the nucleic acids, preventing the proliferation of many types of normal and neoplastic cells. The 4-amino analogs of folic acid, aminopterin, interferes with the reduction of folic acid to its coenzyme form and to the formation of its formyl derivative, folinic acid. It renders single carbon fragments unavailable for the synthesis of purines, pyrimidines, and amino acids. The most widely used antifolic preparation is 4-amino-N^{10}-methylfolic acid (amethopterin). Resistance to treatment is the usual sequel of prolonged amethopterin therapy (H4). The assay of folic acid activity in serum aids in determining efficacy of antifolic therapy (H12).

9. Vitamin B_{12}

The recent review on vitamin B_{12} in this series (G18) provides an excellent survey. In current research emphasis is placed on the biosynthesis and the coenzyme forms of vitamin B_{12} (R2). Similarity between biosynthesis of porphyrin and vitamin B_{12} was shown by the incorporation of δ -aminolevulic acid into the vitamin by a microorganism (S4). The 1-amino-2-propanol moiety of vitamin B_{12} may be formed by decarboxylation of threonine (K10). Nothing is yet known about the nature of the precursors of the dimethylbenzimidazole portion of vitamin B_{12}.

Knowledge of the coenzyme forms of vitamin B_{12} has increased steadily. The first coenzyme of B_{12} isolated from bacteria had similarities to pseudovitamin B_{12}; it contained adenylic acid instead of 5,6-dimethylbenzimidazole, but differed in lacking cyanide and having an extra molecule of adenine which was assumed to be bound to the cobalt atom by the coordination site, often occupied by cyanide (B24). This coenzyme, adenylcobamide, was completely inactive for *Ochromonas malhamensis*, but active for *Escherichia coli* 113-3.

Ochromonas malhamensis responds to vitamin B_{12}, but not to pseudovitamin B_{12}; *E. coli* responds to both forms. Later other coenzymes were isolated (B25). One contained benzimidazole (BC), the other

5,6-dimethylbenzimidazole (DBC) in place of the 1-ribose-bound adenine moiety (B23) (see formulas III and IV). The DBC coenzyme differed from vitamin B_{12} by having adenine instead of CN; exposure to light transforms DBC to hydroxycobalamin by release of the adenine-containing moiety. Both BC and DBC coenzymes were active for *O. malhamensis* and *E. coli.*

Vitamin B_{12} catalyzes incorporation of single carbon units, e.g., the S-linked methyl of methionine and the C-methyl of thymine; it also participates in the redox reactions leading to deoxyribose (S10). The many techniques for measuring vitamin B_{12} activity fall into 2 classes: (a) microbiological, and (b) radioactive.

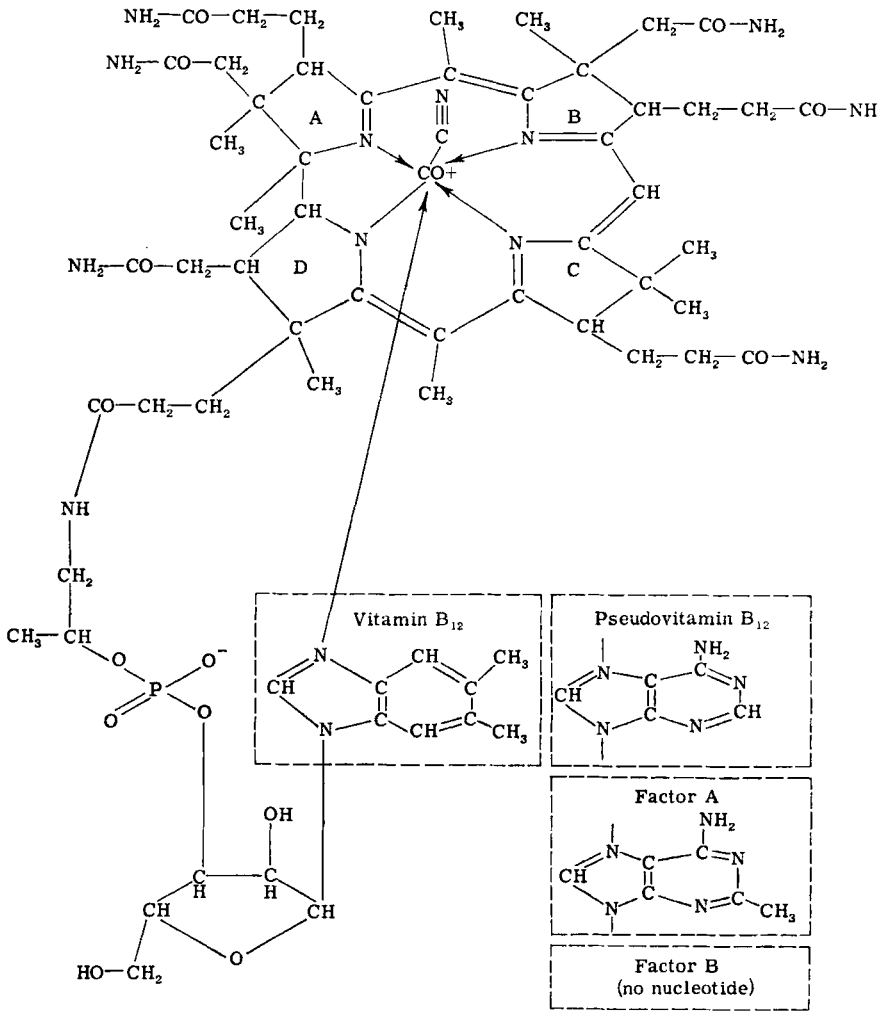
9.1. Tracer Techniques

The availability of Co^{57}, Co^{58}, Co^{60} has permitted the wide application of tracer techniques. The pros and cons of the radioactive techniques have been discussed (G18). They are more expensive and less applicable to routine use than the microbiological ones. The application of radioactive techniques requires the close cooperation of patient and analyst at all times. The Schilling Test (C1) for example, requires that the patient must abstain from food for 12 hours and from water for 4 hours before the test. After the radioactive oral dose, the patient must wait 90–120 minutes for an intramuscular dose of nonlabeled vitamin B_{12} (G19). In the B_{12}-absorption feces test, the feces must be diligently collected by the patient after a radioactive load dose (H1). In the hepatic-uptake test (G14, G15), the radioactivity after an oral dose is measured with a surface scintillation counter over the liver after the radioactivity has disappeared from the intestine, either spontaneously or after purgation. Indeed, in these tracer tests, the patient's and the tester's patience is critical for success. One must also take into account the residual radioactivity remaining with the patient after these tests, 72 days, 270 days, and 5.3 years being the half-time values of Co^{58}, Co^{57}, and Co^{60}, respectively (G18).

9.2. Microbiological Assay

Microbiological assays for vitamin B_{12} are diverse; most are simple, but require some experience and confidence. In our hands they have proved uniformily reliable for evaluating the vitamin B_{12} status of patients and are extremely useful research tools. Perhaps too much emphasis has been placed upon "space, good and meticulously cleaned equipment, and trained personnel" (G18). The B_{12} assays to be discussed here have been developed as practical methods. Four organisms have

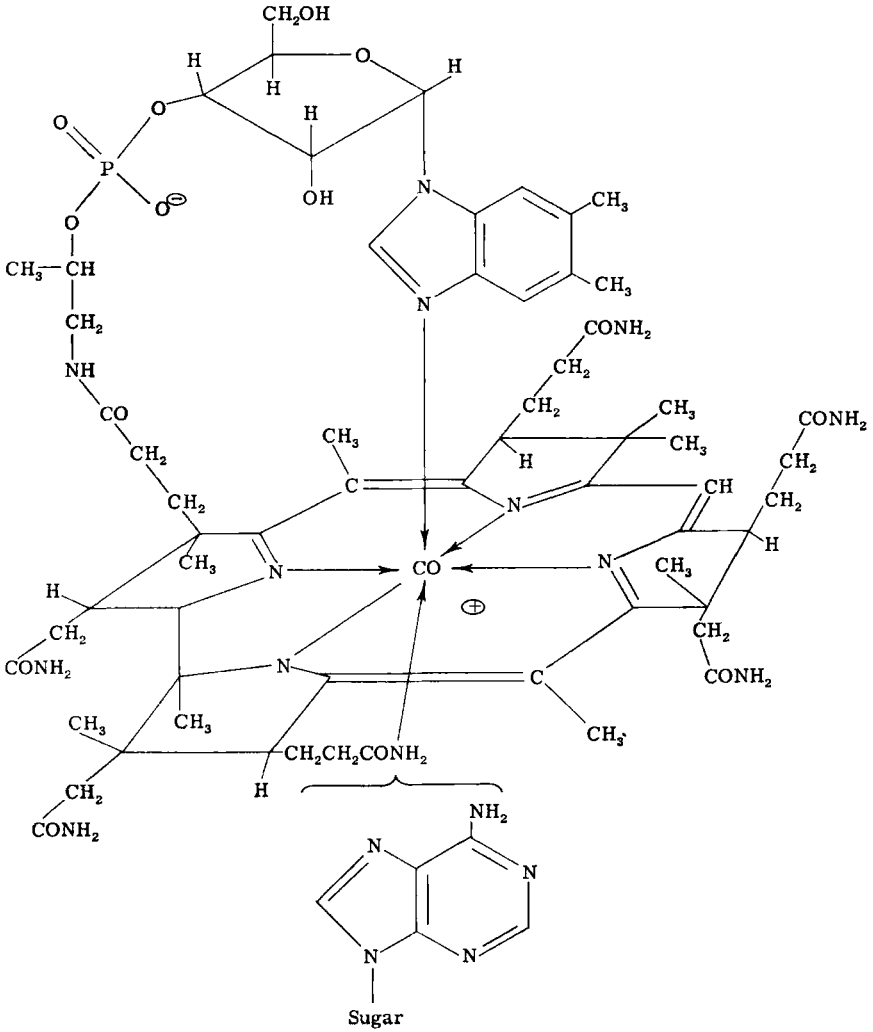
been used in the routine assay for vitamin B_{12}: (a) *Escherichia coli* 113-3 (G21), (b) *Lactobacillus leichmannii* (ATCC 4797, 7830 and NCIB 7884) (R11), (c) *Euglena gracilis* (Z, t, and bacillaris) (H17, H21), and (d) *Ochromonas malhamensis* (B17, F2, F3, K1). The latter two are protozoa with the advantage of sensitivity and specificity (H5, H11, H17). The basal medium and the maintenance medium for each



(III)

Vitamin B_{12} and Congeners.

of these organisms are listed in Tables 12a, 12b, and 13. The vitamin B_{12} assay medium used for *O. malhamensis* is the same as that for thiamine (see Thiamine, Section 2.1) except that (a) 0.1 mg/100 ml of thiamine is used in place of vitamin B_{12}, (b) 0.2 mg/100 ml of NaCN and (c) 500 mg/100 ml of salt-free acid hydrolyzed casein are added. The organism is transferred every week; one drop into 10 ml of main-



(IV)

Vitamin B_{12} Coenzyme.

tenance medium suffices. The *Euglena* B_{12} basal medium is given in Table 13; the maintenance medium in Table 12b. The *E. coli* 113-3 B_{12} basal medium is given in Table 14. It is maintained by streaking onto nutrient agar, (Difco) incubating overnight at 37°, and monthly transfer. Baltimore Biologic Laboratories, Baltimore, Maryland, has available a vitamin B_{12} assay mix for use with *L. leichmannii* ATCC 7830. The pH

TABLE 12a
Ochromonas malhamensis MAINTENANCE MEDIUM[a]

Constituents	Concentration (g/100 ml)
Trypticase[b]	0.2
Yeast autolyzate[c]	0.2
Sucrose	1.0
"1:20 Liver"[d]	0.01
Glycerol (w/v)	0.5

[a] pH adjusted to 5.0 (use KOH or H_2SO_4).
[b] Baltimore Biologic Laboratories, Baltimore, Maryland.
[c] Albimi Laboratories, Brooklyn, New York.
[d] Nutritional Biochemicals Co., Cleveland, Ohio.

TABLE 12b
Euglena gracilis Z STRAIN MAINTENANCE MEDIUM[a]

Constituents	Concentration (g/100 ml)
KH_2PO_4	0.002
K_3 citrate·H_2O	0.004
$MgSO_4 \cdot 7H_2O$	0.0025
Fe	0.0001
Trypticase[b]	0.06
Yeast autolyzate[b]	0.04
Thiamine·HCl	0.04 mg
Cyanocobalamin (Vitamin B_{12})	0.05 μg
Na acetate·$3H_2O$	0.04

[a] pH 6.2–6.6 (use KOH or H_2SO_4).
[b] See Table 12a.

should be adjusted to 7.0. If prepared as per directions, it suffices for B_{12} assay of biological fluids. The maintenance medium is the same as that used for *L. casei* (see Folic Acid, Section 8.1). It is transferred every week; the addition of one drop into 10 ml of maintenance medium is followed by overnight incubation at 37°. The bacteria are stored at 4° after incubation. The protozoa are grown and maintained under five 40-watt "warm white" fluorescent tubes at a distance of approximately 1.0 m at room temperature (between 26–32°). Standard curves of vitamin

B_{12} are prepared from a triturate of vitamin B_{12} and mannitol (Nutritional Biochemicals Co., Cleveland, Ohio). Double-strength basal medium (twice the value listed in the tables) is prepared and dispensed in 2.5-ml quantities into 10-ml micro-Fernbach flasks (Kimble Glass

TABLE 13
Euglena B_{12} ASSAY MEDIUM[a]

Constituent	Concentration (g/100 ml)
KH_2PO_4	0.04
$MgSO_4 \cdot 7H_2O$	0.05
L-Glutamic acid	0.3
$CaCO_3$	0.01
Sucrose	2.0
DL-Aspartic acid	0.2
Glycine	0.3
Metals mix[b]	0.6 ml
Mo[as $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$]	0.2 mg
V (as $VOSO_4 \cdot 2H_2O$)	0.01
NH_4HCO_3	0.1
Thiamine·HCl	0.075 mg
DL-Malic acid	0.2
NaCN	0.2 mg
p-Aminobenzoic acid	0.125 mg

[a] pH 3.4–3.6.

[b] 1.0 ml contains: ethylenediamine tetraacetic acid, 2.5 mg; Zn (as $ZnSo_4 \cdot 7H_2O$), 2.5 mg; Fe (as $FeSO_4 \cdot 7H_2O$), 1.0 mg; Mn (as $MnSO_4 \cdot H_2O$), 0.5 mg; Cu (as $CuSO_4 \cdot 5H_2O$), 0.1 mg; Co (as $CoSO_4 \cdot 7H_2O$), 0.05 mg; B (as H_3BO_3), 0.02 mg.

TABLE 14
BASAL MEDIUM FOR *E. coli* 113-3[a]

Constituent	Concentration (g/100 ml)
KH_2PO_4	0.6
K_2HPO_4	1.4
Na_3 citrate·$2H_2O$	0.1
$MgSO_4 \cdot 7H_2O$	0.02
$(NH_4)_2SO_4$	0.2
L-Asparagine·H_2O	0.8
L-Arginine·HCl	0.02
L-Glutamic acid	0.02
Glycine	0.02
L-Histidine·HCl	0.02
L-Proline	0.02
L-Tryptophan	0.02
Sorbitol	1.0

[a] pH 6.8–7.0

Co.) 0.3, 1.0, 3.0, 10, 30, 100, 300, and 100 μμg/ml of vitamin B_{12} is added to the double-strength medium. Distilled water is added to make the final volume 5 ml. For *L. leichmannii* 5 ml of double-strength basal medium is added and the volume is made up to 10 ml.

Blood of normal subjects was obtained from an antecubital vein, diluted 1:5 with pH 4.5 buffer,[2] and autoclaved 30 minutes to convert bound cobalamin into its microbiologically active form; serum was treated like blood. This procedure allowed estimation of total vitamin B_{12}. For the subsequent inoculation of specimens: (a) *E. coli* as a loopful from nutrient agar suspended in 25 ml of medium, (b) *L. leichmannii*, an 18-hour culture diluted 1:10 in basal medium, (c) *E. gracilis*, strain Z, and (d) *O. malhamensis* are inoculated directly from a 5-day culture grown in liquid maintenance medium. One drop into a culture flask served as inoculum. The bacteria required 18-hours for full growth; protozoa, 4–5 days.

9.3. Comparison of Assay Organisms

Figure 6 summarizes the results obtained with the 4 assay organisms for serum and whole blood. As may be seen, the values for *O. malhamensis* are lowest; serum values range from 135 to 643 μμg/ml, and those for blood from 115 to 450 μμg/ml. The *E. gracilis* values are

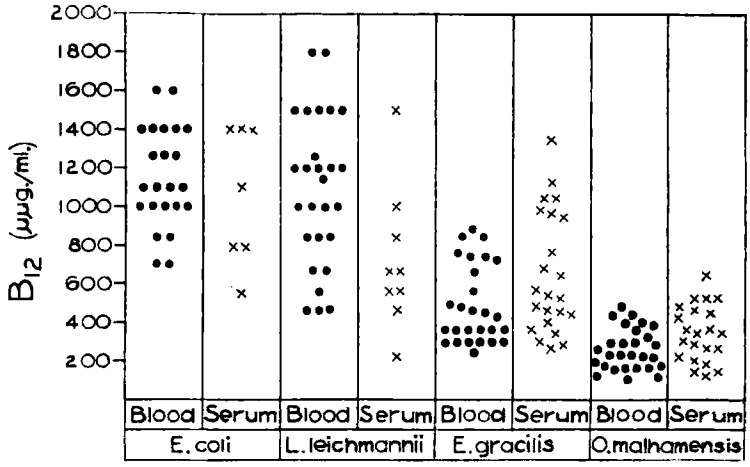


Fig. 6. Vitamin B_{12} in whole blood and serum [Baker *et al.* (B9)].

[2] One-half gram of *trans*-aconitic acid per 100 ml of distilled water, to which KOH had been added to reach pH 4.5. Fifty milligrams of sodium metabisulfite had also been added to 100 ml of buffer for stabilization of vitamin B_{12} analogs.

the next lowest; serum values range from 285 to 1335, those for blood from 305 to 875 μμg/ml. With *L. leichmannii,* serum values range from 215 to 1500 μμg/ml, those for whole blood from 465 to 1500 μμg/ml. With *E. coli* 113-3, serum ranges are 565 to 1400 μμg/ml, whole blood values 700 to 1600 μμg/ml.

9.4. Blood and Serum

In Table 15, 8 cases are listed in which complete B_{12} analyses were carried out with the 4 assay organisms. With *O. malhamensis* the serum values were equal to or higher than those for whole blood; about half

TABLE 15
Comparison of Vitamin B_{12} in Normal Blood and Serum

				B_{12} (μμg/ml)							
				E. coli		*L. leichmannii*		*Euglena*		*O. malhamensis*	
Subject	Sex	Hematocrit[a]	Hb (g)	B	S	B	S	B	S	B	S
J.R.	F	40	12.8	700	835	565	465	305	285	115	185
N.C.	M	39	17.7	700	1000	665	215	375	370	140	170
A.W.	M	43	15.8	835	565	1000	565	335	440	205	270
M.R.	F	41	14.8	835	1100	1200	565	350	560	205	205
M.K.	F	39	13.4	1265	835	835	665	450	435	250	340
I.P.	F	39	13.1	1000	1400	1500	665	735	635	280	425
W.N.	M	45	18.8	1400	1400	1500	835	560	975	315	405
M.G.	F	42	14.8	1400	1400	1000	1500	750	675	340	465

[a] Normal for females, 42; normal for males, 45.
B = blood; S = serum.

the cases showed the reverse on assay with *E. gracilis.* This reverse relationship, with one exception, held for all results with the *L. leichmannii* assay; with *E. coli* 113-3, the results were high in both serum and whole blood. They resembled the *O. malhamensis* results as the serum values were generally higher than those for whole blood.

Although B_{12} can be assayed biologically in mice, chicks, and rats, especially with the use of radioactive cobalt, the microbiologic method of assay is preferred because it is economical and sensitive. One serious drawback of bacterial B_{12} assay procedures is the lack of specificity and sensitivity. The extreme sensitivity (1×10^{-12} g) and relative freedom of stimulation in blood, serum, and urine make protozoa the choice assay tools (B17, F3, H19). The most specific is *O. malhamensis* (B17, F3); *Euglena* is known to be stimulated by pseudo-B_{12} (F2). Serum and blood from normal subjects have a growth-promoting effect on *Euglena* (M12) above that seen with *Ochromonas.* The reason for such

stimulation is obscure, since no known pseudoforms of B_{12} have been found in biologic fluids. Increased growth in the bacterial assay, which lacks specificity in comparison to animals and to protozoa, is probably due to nonspecific stimulants present in blood and serum such as amino acids, nucleosides, or different forms of B_{12}.

9.5. Assay in Tissue

Liver tissue B_{12} is assayed by suspending 50 mg of lyophilized human liver in 100 ml of aconitate buffer to which 100 mg sodium metabisulfite is added. The suspension is autoclaved for 30 minutes at 16 psi to liberate the bound forms of the vitamin. Aliquots are diluted 1:10 after autoclaving to reduce metabisulfite toxicity on microorganism growth. Each milliliter now contains 50 μg of liver (w/v); 1.0, 1.5, and 2.0 ml of this hydrolyzate is added. After dilution to a volume of 5 ml, this represents an assay of 10, 15, and 20 μg of liver per milliliter of final solution. Normal liver contains between 2–14 mμg of vitamin B_{12}/mg liver powder.

9.6. Comments

Low serum and blood B_{12} is not only found in pernicious anemia, but also in cases of intestinal malabsorption, total gastrectomy, partial gastrectomy, gastroenterostomy, small intestinal loops and diverticula, megaloblastic anemia of pregnancy and puerperium, and the anemias associated with the administration of anticonvulsant drugs (G12). Maternal B_{12} levels are usually lowered during pregnancy; the fetal levels are approximately twice as high as the maternal levels (B2, B18). Plasma levels closely reflect the B_{12} supply of the tissues, even though the blood cells from patients with pernicious anemia often contain more than half as much B_{12} as normal cells (B16, S17). B_{12} in normal cerebrospinal fluid ranges from 0–30 μμg/ml (S15); during exacerbation of multiple sclerosis and in other neurologic disorders the levels in this fluid are high (S16).

The ability of the liver to act as a depot for vitamin B_{12} (B28, G13) enables us to use this vitamin as an index of proper hepatic function. Hepatic disorders lead to an increased B_{12}-binding in the serum (J5, R3), suggesting that the blood assumes a greater role in the conservation of B_{12}. We have reported that patients with liver disease excreted invariably less than 10 μg of B_{12}, 8 hours after a 50-μg intramuscular load dose of the vitamin. In contrast, normal subjects excreted 24–40 μg, i.e., 50–80% of the vitamin in the same test (B14). These results were correlated with various chemical determinations indicative of hepatic disorders (B1). In Table 16 the clinical diagnosis and the various liver-

function tests are compared with the excretion of vitamin B_{12}. The material in Table 16 is organized according to the results of the cephalin-flocculation test. The total series comprises 22 patients with hepatic disorders and 16 control patients with other diseases. The control patients were included in order to establish the degree of specificity of the test for liver disorders. This study led to the conclusion that the B_{12}-load test is in general indicative of parenchymous disease of the liver. It runs fairly parallel with other liver function tests, but appears to be more sensitive during early stages and during recovery.

In hyperthyroid subjects, the whole blood values before and after a load dose of B_{12} were lower than in the normal and myxedematous groups, suggesting that B_{12} turnover and demand is appreciably greater in hyperthyroidism and lower in myxedema (Z2). Recently it was shown that B_{12} was the only vitamin to overcome the antagonistic action of various thyro-active compounds on the growth of the B_{12} requirer *O. malhamensis* (B10). These findings suggested that *O. malhamensis*, like animals (G7) could be used as an indicator of thyro-active substances and thyroid antagonists.

An alkali-stable factor from human blood was shown to by-pass the B_{12} requirement of *O. malhamensis* (B13). Chromatographic studies show this factor not to be cyano- or hydroxycobalamin nor the co-enzyme forms of vitamin B_{12}. It is released from the liver into the circulation by vitamin B_{12}. Normally this factor does not exist in the circulation (L4). Its microbial activity, unlike that of other alkali-stable factors described (S5), does not depend on reducing conditions. Recovery experiments show vitamin B_{12} and folic acid to be destroyed in hydrolysis under such alkaline conditions. This factor was also extracted from human and beef liver and from thermophilic microorganisms growing at 55°C (B13). Treatment of patients with pernicious anemia in relapse shows that crude extracts of this factor permit a complete reversal of megaloblastosis with hematocrit levels returning to normal (e.g., from 19 to 39) within 2 weeks after treatment with a total of less than 1.0 μg of B_{12}-like activity as assayed with *O. malhamensis*. The B_{12} levels in the serum of these patients remained low throughout the hematological reversals observed. The initial B_{12} levels were, e.g., 28 μμg/ml. After correction of megaloblastosis and the return of the hemoglobin levels to normal, the B_{12} titer was 4 μμg/ml; the liver levels obtained by punch biopsy showed a decreased rather than increased level of vitamin B_{12} remission. The absorption of this factor is not dependent on intrinsic factor, since oral doses are as effective as those given intramuscularly (B13).

TABLE 16

CORRELATION OF VITAMIN B_{12} EXCRETION AND LIVER FUNCTION TESTS 8 HOURS AFTER A 60 μg LOAD DOSE

No.	Patient	Age	Sex	Diagnosis	Total bilirubin mg/100 ml)	Serum alkaline phos-phatase (K.A.U.)	A/G (%)	Urinary excretion of B_{12} (μg)
				Cephalin flocculation negative				
1	B.F.	61	F	Diabetes	—	7.0	1.4/3.1	30
2	E.L.	43	F	Congest. heart failure	2.4	6.1	3.8/2.9	12
3	J.V.	20	F	Psoriasis	—	5.8	4.6/3.3	11.6
4	A.T.	60	M	Ulcer	—	6.0	3.4/3.2	11
5	A.W.	59	M	Polycythemia	0.29	6.1	3.9/2.3	9
6	S.J.	50	F	Hypertension	—	6.3	3.6/3.2	9
7	C.L.	30	F	Rheum. heart disease	0.7	6.5	4.0/4.0	0.8
8	F.T.	24	F	Schistosomiasis	0.6	7.4	3.4/3.2	28
9	P.M.	52	M	Cirrhosis	0.9	26	3.6/3.3	25
10	C.C.	14	M	Homologous serum hepatitis (recovering)	2.0	14.1	4.3/3.7	13.4
11	T.R.	40	F	Schistosomiasis	0.6	6.4	4.3/3.9	9.8
12	T.C.	50	M	Hepatitis (recovering)	0.2	6.0	3.4/3.3	4.2
				Cephalin flocculation +				
13	P.L.	45	M	Duodenal ulcer	0.5	6.5	4.0/2.9	22.1
14	P.D.	43	F	Thrombophlebitis	0.3	5.9	3.4/4.0	13.2
15	E.D.	57	F	Rheum. heart disease	0.7	6.6	4.2/3.8	4.9
16	A.V.	66	M	Congest. heart failure	—	6.8	3.5/3.7	2.4
				Cephalin flocculation ++				
17	M.G.	44	F	Congest. heart failure	0.68	12.4	4.3/4.0	10.6
18	V.T.	35	F	Sarcoid	—	9.5	3.2/5.4	3.2
19	P.S.	23	M	Infectious hepatitis	2.9	17.7	3.0/3.3	6.4

TABLE 16 (*continued*)

No.	Patient	Age	Sex	Diagnosis	Total bilirubin (mg/100 ml)	Serum alkaline phosphatase (K.A.U.)	A/G (%)	Urinary excretion of B_{12} (μg)
20	J.V.	18	M	Infectious hepatitis	1.3	12.5	4.4/4.2	6.0
21	A.A.	15	M	Homologous serum hepatitis	1.6	10	4.2/3.3	5.2
22	T.C.	50	M	Hepatitis	2.0	9.0	3.7/3.6	4.3
23	P.S.	23	M	Hepatitis	3.3	15.2	4.1/3.5	1.4
24	L.S.	72	F	Cirrhosis	0.9	14.0	2.9/4.5	0.8
25	J.P.	30	M	Cirrhosis	3.2	36	2.7/4.1	0.1
				Cephalin flocculation +++				
26	M.M.	49	F	Unknown liver disease	1.3	11	3.2/4.4	7.5
27	J.F.	38	M	Cirrhosis	0.9	8.2	3.0/4.5	3.4
28	J.A.	57	F	Cirrhosis	0.8	7.1	2.6/3.2	2.5
29	F.M.	36	M	Homologous serum hepatitis (recovering)	20	14	3.4/4.5	2.2
30	F.M.	36	M	Homologous serum hepatitis	42	18.5	4.3/4.9	0.3
				Cephalin flocculation ++++				
31	T.N.	53	F	Cirrhosis	28	13.4	3.1/4.4	3.8
				Cephalin flocculation not recorded				
32	W.M.	—	M	Pneumonia (recovering)	—	—	—	24
33	S.R.	39	M	Empyema	—	—	—	12
34	G.S.	59	F	Emphysema	—	—	4.2/3.3	10.4
35	M.S.	70	F	Hepatitis (recovering)	1.2	13.7	2.4/4.7	9.0
36	A.R.	—	M	Acute yellow atrophy	—	—	—	8.8
37	E.P.	—	M	Cirrhosis	—	—	—	7.5
38	J.I.	—	M	Cirrhosis	—	—	—	4.9

Studies on competitive inhibition of B_{12} activity show that certain substituted benzimidazoles, purines, and pteridines have weak inhibitory effects in bacterial systems (S10a). Substituted amides of B_{12} (S9) inhibited the growth-promoting effects of vitamin B_{12} in protozoic and bacterial systems (B5, B6). The anilide of B_{12} shows a definite inhibitory effect on maturation and proliferation of blood-forming cells of the chick embryo; an opposite effect is induced by vitamin B_{12} (E2).

10. Conclusions

The establishment of quantitative methods for the determination of vitamins in body fluids and tissues by microbiological assay techniques should stimulate the search for the significance of vitamins in disease, not only in nutritional deficiency, but in the much wider field of all metabolic disturbances. Functional vitamin deficiencies are produced by malabsorption, by inhibitors of the vitamin function through products of the body, and particularly through drugs and other toxic substances. Vitamin deficiencies may be relative deficiencies whenever an individual's metabolism is deranged so as to require enhanced quantities of a given vitamin to cure or to counteract certain symptoms as, e.g., in Darier's disease (keratosis follicularis) (P2a).

There is, moreover, the field of hypervitaminoses, which has been explored for the fat-soluble vitamins, but hardly touched in the water-soluble vitamins. The production of combined system disease by folic acid therapy of pernicious anemia belongs to this group, but many more instances wait to be recognized. The indiscriminate use of polyvitamin preparations by poorly informed clinicians is bound to mask such states and to delay their discovery. Also, the use of flushing doses of vitamins in diagnostic tests may cause acute hypervitaminoses.

Like other constituents of blood or serum, the deviation of their titer from the normal range may mean a number of things; reduced intake or absorption, increased utilization, increased demand, increased excretion will all cause a decrease of titer, their opposites an increase.

A vitamin is often the etiological center of a disease as vitamin B_{12} and folic acid in macrocytic anemias. Here, because of the obvious implications for diagnosis and therapy the determination of the "nucleogenic" vitamins, B_{12} and folic acid, is imperative in the routine of clinical hematology.

Where the connection between a vitamin and a disease is less transparent, a wide field remains open for the discovery of meaningful correlations between vitamin content of body fluids and tissues and physiologic or pathologic events.

To single out the case of a physiological state of general importance, the vitamin equilibrium between a pregnant woman and fetus and its disturbances, have been studied by us (B11, B18), and open new aspects for the management of pregnancy, especially in regions where preventative shot-in-the-dark polyvitamin therapy is financially impossible. In the pathological field, the significance of vitamin B_6 in toxemia of pregnancy deserves further study.

The so-called normal range of blood and serum vitamin levels is always derived from observations on healthy young subjects. How about a comparison with healthy old subjects, whose percentage in the population is steadily increasing? Much may be learned about the cause of the decrease of physiological function and of the increased susceptibility to organic disease in old age, if the role of vitamins as parameter of these alterations were investigated with a view to preventive theory.

A glance at vitamins in clinical medicine opens a wide panorama with challenging aspects in hepatic conditions, in oxalosis and calculus disease, in obscure, but widely spread neurological diseases, and in many others; astute clinical observations, combined with knowledge of the function and mechanism of vitamin action, will bring vitamin analysis into the picture as a useful tool.

Special mention must be accorded to iatrogenic effects, where the usefulness of novel synthetic drugs is impaired by untoward side effects of obscure etiology. In some, if not many of them, these side effects may find their explanation in the inhibitory action of the drug upon a vitamin, as in the case of primidone vs. folic acid (B3a). These relationships appear to be fortuitous until the structural chemical kinship of drug and vitamin is recognized.

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DEHYDROGENASES: GLUCOSE-6-PHOSPHATE DEHYDROGENASE, 6-PHOSPHOGLUCONATE DEHYDROGENASE, GLUTATHIONE REDUCTASE, METHEMOGLOBIN REDUCTASE, POLYOL DEHYDROGENASES

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I. Introduction

1.1. Catalytic Functions and Types of Reactions Catalyzed by Dehydrogenases

Dehydrogenases catalyze the hydrogen transfer with pyridine nucleotides as coenzymes. Most of them are involved in the process of biological oxidation and belong to the group that has been named "key pathway" enzymes (B24). Others belong to the group of "special metabolic enzymes." Warburg *et al.* (W6) discovered the general and important principle of dehydrogenation reactions in living material, and the stoichiometric and reversible connection of substrate reduction with the conversion of pyridinium salts to dihydropyridines.

There exist numerous different apodehydrogenases in human, animal, and plant tissues, most of which catalyze oxidation-reduction reactions involving alcohols and the related aldehydes or ketones, and reactions involving aldehydes (or their derivatives) and the corresponding carboxylic acids (or their derivatives). Examples of the first group are LDH, MDH, SDH, and the alcohol dehydrogenases of liver and yeast. Representatives of the second group are G-6-PDH and D-glyceraldehyde-3-phosphate dehydrogenase. "Special" dehydrogenases catalyze the reduction of disulfide bonds or the reduction of trivalent iron.

Hence the enzyme proteins, i.e., the apodehydrogenases, contribute more toward substrate specificity than the coenzymes, the prosthetic groups. Indeed, there are only 2 pyridine nucleotides, NAD and NADP, that participate, according to present knowledge, in the hydrogenation (in their reduced form $NADH_2$ and $NADPH_2$) or dehydrogenation of about 100 different substrates. So the coenzyme action is quite less specific than the function of the protein moieties, the limitation of their action being invoked by their combination with the apodehydrogenase (K3, W1). Many pyridine nucleotide-linked processes are relatively specific for NAD, others are specific for the NADP system. Another group of

enzymes will act with both nucleotides, although a difference in reaction velocity can usually be observed. The apparent sharp specificity of G-6-PDH toward G-6-P is the exception rather than the rule.

1.2. Mechanism of Hydrogen Transfer by Pyridine Nucleotide Coenzymes

The mechanism by which dehydrogenation reactions occur has been clarified by Vennesland *et al.* (F1, V2) in 1953. In experiments with isotopes using deuterium as an indicator it has been found (F1) that the H-atoms localized in the hydrogenated reaction product are the same as those derived from the hydrogen donor, thus excluding an electron transfer. In this case, they would not be the same since they derive from the hydrogen ions of the water-containing medium. The

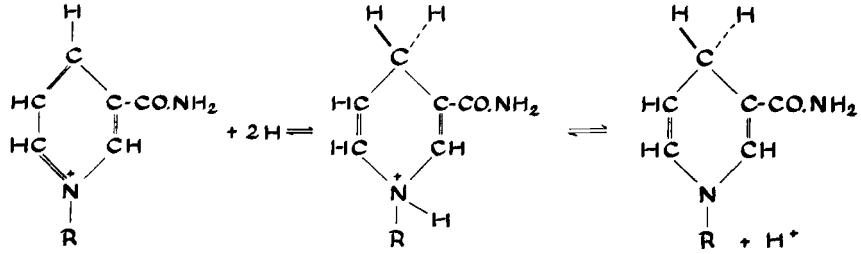

Fig. 1. General scheme of the hydrogen transfer as catalyzed by dehydrogenase reactions. R represents the residue of the pyridine nucleotide molecule.

first experiments were carried out with labeled ethanol (CH_3CD_2OH), NAD, and alcohol dehydrogenase. Furthermore, it was demonstrated that during dehydrogenase reactions a 1,4-reduction of the nicotinamide ring of the coenzyme will take place (L9). From the reduced coenzyme labeled with deuterium in the 4-position approximately the theoretical amount of deuterium will be transferred to pyruvate yielding lactate by LDH. But, no deuterium at all is transferred from either the 2- or the 6-position.

The enzymatic reduction is represented in Fig. 1; these reactions are exclusively 1,4-reductions.

It is not the purpose of this article to review all dehydrogenases that are known at present and not even all of those that have been described in human tissues and body fluids. A few of them have been selected that are of importance for diagnostic, prognostic or, as therapy controlling tools or, that are known as etiologic factors of pathological states.

1.3. General Considerations in the Determination of Enzyme Activities and Measurement of the Maximal Initial Reaction Velocity

Numerous methods based on different analytical principles have been offered for the measurement of dehydrogenase reactions. Most reliable and simple are spectrophotometric procedures ("optical test") founded on the assay of one of the participating coenzymes (or substrates).

The absorption of $NADH_2$ and $NADPH_2$ shows a maximum at 340 mμ. Therefore this wavelength is often used for measurements of dehydrogenase reactions. The extinction coefficient is 6.22×10^6 cm²/mole, i.e., 0.01 μmole = 6.63 μg NAD/ml will give an extinction of log $(I_0/I) = 0.0622$ at 340 mμ (1-cm light path) after reduction of the coenzyme (H14). Using a photometer the filters of which isolate the Hg-line at 366 mμ, the extinction coefficient is nearly half of that at 340 mμ (3.3×10^6 cm²/mole) (B5). Therefore the values of measurements also are about 50% of those at 340 mμ. Since NAD and NADP show practically no light absorption in the region 300–400 mμ, numerous dehydrogenase reactions, the coenzyme of which is NAD or NADP, can be determined by measuring the increase or decrease of the extinction at 340 or 366 mμ. Furthermore, other types of enzyme reactions may be determined in this way if the reaction product can be measured by an NAD (or NADP) dependent reaction, i.e., by the use of the appropriate dehydrogenase as an "indicator enzyme."

The function of an enzyme is the catalysis of a precisely known chemical reaction, and the enzyme activity can be measured as an absolute reaction rate, the indicator of the amount of enzyme present under carefully standardized conditions. The catalytic activity is a function of pH, ionic strength of the medium, temperature, time, and substrate-, coenzyme- and enzyme-concentrations, and other factors which may even be unknown (T2, W9). Unknown effectors of activating or inhibiting character may influence the reaction rate. This must be given special consideration when multiprotein solutions like crude homogenates or body fluids and undialyzed enzyme solutions (serum) are used for the assay procedure. When serum is used for the estimation of a dehydrogenase the fact must be considered that other dehydrogenases besides the interesting enzyme are present and will react with the corresponding serum substrate when an excess of coenzyme is added. To exclude errors by secondary reactions the test system is completed with the exception of substrate which is added after these reactions have come to an end. The slope of the straight line in Fig. 2 demonstrates the linear proportion between time and enzyme activity; the

progress curve is linear, i.e., the reaction is of zero order. But, while this simple relation may be observed sometimes, enzyme progress curves are often nonlinear, and the reaction rate will decrease with time no matter what initial substrate concentration, coenzyme concentration, etc., are chosen. Instability of the enzyme, formation of inhibitory products, back reactions of the products when equilibrium is approached, destruction of cofactors, and decrease of substrate concentration are the most common reasons.

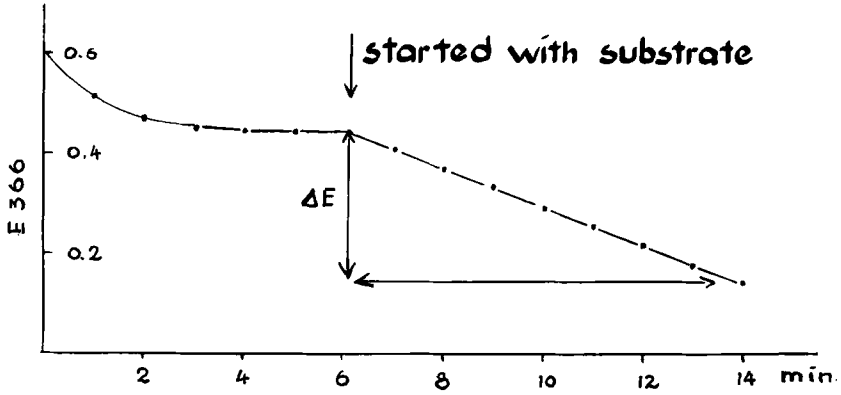

FIG. 2. Start of the enzymatic reaction by addition of substrate.

It is therefore a fundamental principle in the estimation of every enzyme that the rates measured should be maximal initial reaction velocities, tangents to progress curves at zero time. They really should represent the amount of enzyme protein in the reaction mixture.

Probably the most important variable to consider in defining "optimal conditions" or "standard conditions" is the initial substrate concentration. Most enzymes show a hyperbolic curve as relation between initial reaction velocity and substrate concentration, well known now as the Michaelis-Menten curve. With increasing substrate concentration (S) the velocity (v) rises asymptotically to a maximum value (V) (Fig. 3), according to the expression:

$$v = \frac{V_s}{K_m + S} \tag{1}$$

K_m is the Michaelis constant. In some cases such as hydrolases or lactic dehydrogenases (T2), the velocity may fall again with higher substrate concentrations, so that there is an optimum substrate concentration which approximates the theoretical value V, the maximal velocity, following the theory of Michaelis and Menten

$$(E) + (S) \rightleftharpoons (ES) \rightleftharpoons E + P \qquad (2)$$

where (E) is the enzyme concentration, (S) the substrate concentration, (ES) the activated enzyme-substrate complex, and P the reaction product. The initial concentration of S must be high enough to saturate the enzyme with substrate. At this point (*a*) in Fig. 3, E = ES, and the curve of the reaction will be linear, i.e., of zero order until the concentration of S has fallen to point *b*. Beyond point *b* an increasing curvature will appear. Between *a* and *b* the reaction rate will be *V* for any point of the time progress curve. Therefore with a standard method the *final* concentration should not be lower than that corresponding to point *b*. However, it must be mentioned that these considerations are only applicable under simple conditions in an uncomplicated system

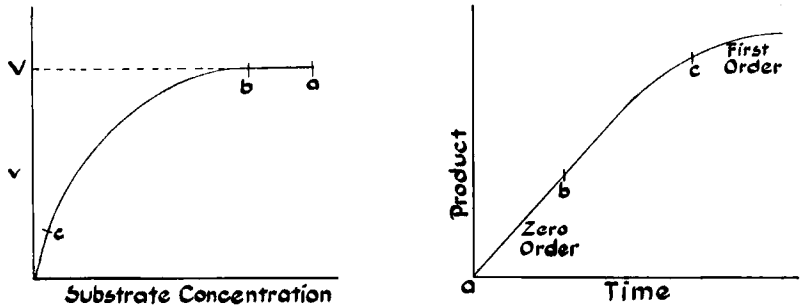

FIG. 3. Relation between substrate concentration and reaction velocity (Michaelis-Menten curve), and reaction velocity and time.

if the enzyme is stable during the time of incubation and if there is no appreciable back reaction or product inhibition.

The methods referred to in this paper have been found to be sufficiently reliable by many investigators and by the authors.

1.4. Units and Correlations of Enzyme Activities

In clinical chemistry the activity of an enzyme should be expressed as units and must be related to the amount of material used as enzyme source. Many authors define the unit of enzyme activity as that amount of enzyme in a given volume or weight unit of material which causes a certain change of absorbance [$\Delta \log (I_0/I)$] at 340 or 366 mμ per time unit under defined, but varying conditions, e.g., $\Delta A_s = 1.000$ or 0.001 per minute at 25°C. In European literature activities are often given as "Bücher-Einheiten" (B5) where the unit is defined as the amount in 1.00 ml medium causing the change of A_s of 0.1 per 100 seconds at 366 mμ and 25°C.

Other authors (e.g., G10, W18) define the unit as micromoles $\times 10^{-3}$ $NADPH_2$ (or $NADH_2$) formed per 1.00 ml enzyme solution diluted by the indicated test volume per hour, or as the quantity of enzyme in the test system reducing 0.01 μmole NADP (or NAD) per minute at a given temperature. Furthermore, it has been suggested that the unit be defined as the number of micromoles of substrate converted per milliliter of serum per one hour at room temperature.

This corresponds to the recommendations given in 1959 by a joint committee of the Clinical Chemistry Commission of IUPAC (International Union of Pure and Applied Chemistry) and the Enzyme Commission of IUB (International Union of Biochemistry). Thus, one unit of enzyme activity should be defined as that amount of enzyme which catalyzes the conversion of one micromole of substrate per minute under defined conditions (W9).

Since the enzyme solution used for the assay consists of different media (e.g., blood, serum, urine, cerebrospinal fluid, tissue homogenate, saliva), the activity must be related to different volumetric or gravimetric units.

For the determination in body fluids the unit may be related to one milliliter of material, or, where inconvenient numbers would arise by this way, to 1000 ml of the respective fluid (= milliunits, mU).

Enzyme activities of tissues are usually related to the following measurable quantities: fresh or wet weight, dry weight, fat-free dry weight, nitrogen (N), protein-N, noncollagen protein, and cellularity. In enzyme determinations in erythrocytes the units of activity can be calculated per gram hemoglobin or per the number of RBC (R9).

The variability of these correlation systems necessitates the use of an exact definition in expression of units. In the present paper all activity units will be defined as recommended by IUPAC, i.e., one unit equals the substrate conversion in micromoles per minute at 25°C per milliliter enzyme solution unless indicated otherwise. The referred data given in the literature have been recalculated for these units wherever possible.

2. Glucose-6-phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase

2.1. Introduction

The phosphorylated glucose (G-6-P) occupies a key position in carbohydrate metabolism and can be utilized by 4 main pathways: (a) by glucose release following enzymatic dephosphorylation; (b) by

glycogenogenesis after conversion to glucose-1-phosphate by phosphoglucomutase; (c) by glycolysis via the Embden-Meyerhof scheme; and (d) by the direct oxidative pathway (synonyms: hexose monophosphate shunt, pentose phosphate cycle, 6-phosphogluconate shunt, etc.).

The direct oxidative pathway involves 2 dehydrogenase systems that are of clinical interest because of the inborn errors, lacks and deficiencies of the enzymes involved. This hereditary trait, often race linked, leads to metabolic disorders accompanied by innocuous as well as by dangerous and severe symptoms.

Recent investigations have reduced some disease states from an "idiosyncrasy" of unknown etiology to a well-defined metabolic disorder due to enzymatic deficiencies with appropriate therapeutic conclusions.

2.2. The Pentose Phosphate Pathway Reactions

In 1931 Warburg and Christian (W5) detected an enzyme catalyzing the reduction of NADP by G-6-P, which he called "Zwischenferment":

$$\text{G-6-P} + \text{NADP}^+ \rightleftharpoons \text{6-PG} + \text{NADPH} + \text{H}^+ \qquad (3)$$

This enzyme is designated now as glucose-6-phosphate dehydrogenase. Its isolation from yeast in a pure crystalline state has been recently reported (N4). The first step of the reaction in Eq. (3) is the conversion of G-6-P into 6-phosphogluconolactone (C8). This was confirmed (H17) by the reversal of reaction (3). The immediate reaction product 6-phosphogluconolactone has a half period, as to its conversion to 6-PG by splitting the lactone ring, of 1.5 minutes at pH 7.4 (H17). A lactonase system controls this splitting function, its equilibrium being in favor of the final product (B20). In the course of the direct oxidative path, 6-PG is oxidized

$$\text{6-PG} + \text{NADP}^+ \rightleftharpoons \text{3 Ketogluconic acid-6-phosphate} + \text{NADPH} + \text{H}^+ \qquad (4)$$

under control of 6-phosphogluconate dehydrogenase. The reaction according to Eq. 4 is followed by decarboxylation in carbon-1 position, yielding ribulose-5-phosphate and CO_2 (H19). Since no direct evidence is available for the intermediate formation of 3 ketogluconic acid-6-phosphate (H19), the reaction of 6-PGDH may be summarized as:

$$\text{6-PG} + \text{NADP}^+ \rightleftharpoons \text{Ru-5-P} + \text{NADPH} + \text{H}^+ + \text{CO}_2 \qquad (5)$$

(H16, H19, M5, M12, S12, and others). The reversibility of reaction (5) has been proved (H16).

The resulting Ru-5-P [Eq. (5)] is in enzymatic equilibrium as well with R-5-P (H19) by ribose isomerase [Eq. (6)], as with xylulose-5-phosphate (B21, B22, M5) by an epimerase [Eq. (7)]:

$$\text{Ru-5-P} \rightleftharpoons \text{R-5-P} \quad (6)$$

$$\text{Ru-5-P} \rightleftharpoons \text{Xu-5-P} \quad (7)$$

Figure 4 summarizes the direct oxidative pathway with its relations to (a) the gycolytic route of G-6-P utilization, (b) the reduction of pyridine nucleotides, and (c) the influences on some reactions depending on reduced pyridine nucleotides.

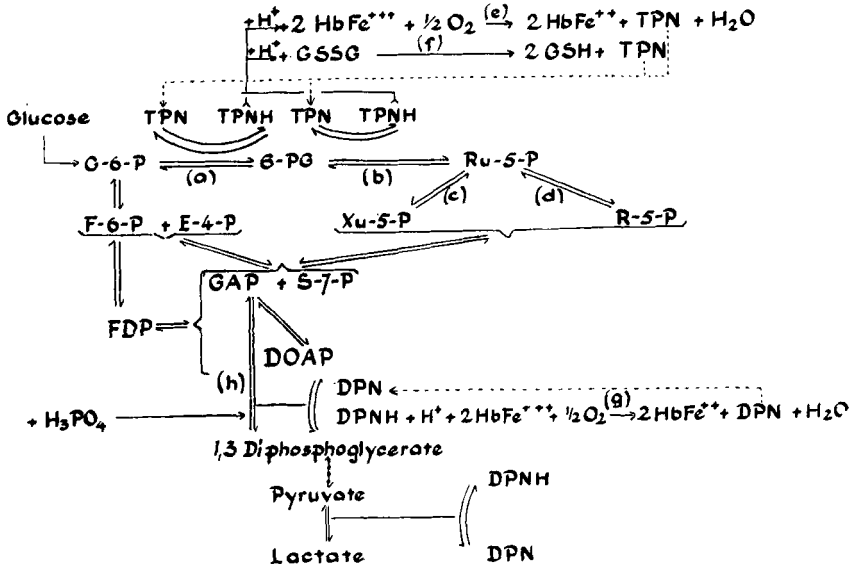

FIG. 4. The pentose phosphate cycle with its relations to glycolysis, reduction, and oxidation of nicotinamide adenine nucleotides and some reactions depending on $NADPH_2$ or $NADH_2$. (a) = G-6-PDH; (b) = 6-PGDH; (c) = pentose epimerase; (d) = ribose isomerase; (e) = $NADPH_2$ MHbR; (f) = GSSGR; (g) = $NADH_2$ MHbR; (h) = D-glyceraldehyde-3-phosphate dehydrogenase (Warburg's "oxydierendes Gärungsferment").

The literature concerning the pentose phosphate cycle has been reviewed (D5, D10, M5) and, especially the literature for erythrocytes (A7).

Since only less than 10% of G-6-P is channeled into the pentose phosphate cycle (under physiological conditions this percentage varies depending on the different tissues), the question must be discussed, what is the importance of this shunt. With regard to the resulting compounds Eqs. [(3), (5), (6), (7)] one mole $NADPH_2$ appears twice. Furthermore, pentose phosphates are furnished for biosynthesis of nucleotides, nucleic acids, and fatty acids (D5, D6, D10, H13, M5).

The conclusion that the significance of the pentose phosphate shunt may be in keeping NADP in its reduced state and furnishing pentose phosphates for biosynthesis, rather than G-6-P utilization, is closely confirmed by the fact that individuals deficient in or lacking G-6-PDH activity suffer from a number of metabolic disorders due to lack of $NADPH_2$ generation and nucleotide depletion.

2.3. Distribution and Coenzyme Specificities of G-6-PDH and 6-PGDH

Both G-6-PDH and 6-PGDH have been found in tissues of plants, microorganisms, and animals, suggesting the widespread importance of the oxidative pathway of glucose metabolism.

G-6-PDH and 6-PGDH were isolated from yeast (G9, H15, H19, K19, N1, N4), from *Neurospora crassa* (R3), from *Escherichia coli* (M12), from *Aspergillus flavus-oryzae* (H10), and from *Aspergillus niger* (J2).

The enzymes have been demonstrated to be present in varying amounts in almost all mammalian tissues and in blood (B4, D4, D7, D8, G8, G10, G11, H13, S12, S14, W5, W18). The highest activities are attributed to erythrocytes and to the other cellular components of blood. Recently, G-6-PDH and 6-PGDH activities in saliva have been described (R5).

The enzyme proteins were found only in the soluble fraction of disintegrated liver (G10). However, measurable activities of both the enzymes have also been described for isolated cell nuclei (S14, S15).

Some normal values of activity are given in Table 1. The comparability of the values is problematic, even when recalculated in international units (cf. Section 1.4), because of the different assay methods and relations of the units (for references cf. Table 1).

Generally, both G-6-PDH and 6-PGDH are regarded as being NADP specific (B4, C2, D7, D8, H13, H15, H16, N2, R12, W5, W6, W18). Nevertheless, the interaction of NAD in the reactions of Eq. (3) and (5) has been reported (C2, H10, L5), especially if high concentrations of NAD were used as a hydrogen acceptor (N2). The role that NAD plays in these reactions remains unexplained, but it may be of less importance under steady state conditions.

2.4. Activation and Inhibition of G-6-PDH and 6-PGDH

Studies on inhibiting and activating agents of enzymes are important for understanding the mechanisms and alterations in metabolic disorders.

In Table 2 is given a synopsis of known inhibiting and activating compounds for G-6-PDH and 6-PGDH.

The inhibition of G-6-PDH and 6-PGDH by heavy metal ions and its reversibility by adding EDTA demonstrates the requirement of active sulfhydryl groups (G10, R6). This finding is confirmed by the activating action of cysteine (N2, W18). However, an inhibition of

TABLE 1
NORMAL VALUES OF G-6-PDH AND 6-PGDH ACTIVITIES OF HUMAN SERUM, ERYTHROCYTES, AND RAT LIVER[a]

Material	G-6-PDH	6-PGDH	Remarks on assay method	References
Human serum (mU/ml/min)	0.75	—	pH 7.6; EDTA added	(B15)
Human serum (mU/ml/min)	5.60	—	pH 7.6; EDTA added	(L1)
Human serum (mU/ml/min)	5.42	—	pH 7.6; Mg^{++} added	(K6)
Human serum (mU/ml/min)	13.10	—	pH 7.6; Mg^{++} added	(K7)
Human serum (mU/ml/min)				
(a) Males	—	2.28 ± 0.6[b]	pH 7.5; cysteine and Mg^{++} added	(W18)
(b) Females	—	2.28 ± 1.0[b]		
(c) Term pregnancy	—	2.97		
Human erythrocytes (mU/4 × 10^9RBC)	2289.00	—	pH 7.6; EDTA added digitonin hemolyzed	(B15)
Rat liver homogenate (mU/g tissues wet weight)	4.6	5.90	pH 7.6; 2-substrate method	(G10)

[a] The units given in the literature were recalculated for international units (cf. Section 1.4).

[b] Standard deviation.

G-6-PDH activity in the presence of EDTA + Mg^{++} ions has been reported (M8), suggesting the probable formation of an inhibitory EDTA-Mg-substrate complex. Nevertheless, addition of EDTA may be important in the estimation of serum activities, since the levels of heavy metals (e.g., in hepatitis) may be elevated to inhibiting amounts, thus resulting in false values. Cu^{++} and Zn^{++} ions have been found to inhibit exclusively 6-PGDH activity without affecting G-6-PDH activity (G10).

TABLE 2
INHIBITION AND ACTIVATION OF G-6-PDH AND 6-PGDH ACTIVITIES

Inhibitors	Type of inhibition[a]	Inhibition of G-6-PDH	Inhibition of 6-PGDH[b]	References
Primaquine and related comps.	Noncomp.	+	+	(D4, and many others)
Nitrofurantoin	Noncomp.	+	+	(D4, K4)
Tolbutamide	Noncomp.	+	+	(D4)
Menadione sodium bisulfate	Noncomp.	+	+ Comp.	(D4)
Sulfonamides	Comp.	+	+	(A5, D4)
Phenothiazine derivatives	Comp.	+	+	(C4)
Nicotinamide	Comp.	+	+	(A4, M8)
p-Chloromercuri-benzoate	Noncomp.	+	+	(D4)
β-Naphthol	Noncomp.	+	+	(D4)
Acetylphenyl-hydrazine	Noncomp.	+	+ Comp.	(D4)
Dehydroiso-androsterone	Noncomp.	+	?	(M8)
Galactose-1-phosphate	?	+	0	(L2)
D-Glucosamine-6-phosphate	Comp.	+	?	(G9)
ATP	Comp.	0	+	(G10)
Norit	by absorption of coenzyme	+	+	(C3)
Heavy metal ions	SH-inhibition	+[c]	+	(G9, G10, H18, R6)
Phosphate ions	Comp.	+	+	(G9, T1)
EDTA + Mg++	EDTA-Mg-substrate complex?	+	?	(M8)

Activators		G-6-PDH	6-PGDH	
Cysteine	—	+	+	(N2, W18)
Mg++, Ca++, Mn++	—	+	+	(G9, G10, H18)

[a] Comp. = competitive; Noncomp. = noncompetitive.
[b] Type of inhibition indicated if different from that of G-6-PDH.
[c] Except Cu++ and Zn++ ions.

The inhibition of both enzymes by primaquine and related compounds (8-aminoquinoline derivatives) possesses remarkable clinical implications to be discussed later (cf. Section 2.8.3).

Galactose-1-phosphate has been found to inhibit G-6-PDH but not 6-PGDH (L2). This finding is important in galactosemia in which galactose-1-phosphate accumulates because of lack of p-galactose uridyl transferase in tissues and plasma.

ATP inhibits only 6-PGDH and not G-6-PDH (G10). As previously reported for other enzymes, phenothiazine derivatives inhibit both G-6-PDH and 6-PGDH competitive with NADP since NADP protects G-6-PDH depending on its concentration (C4). Neither inhibiting nor activating action has been observed in phenylbutazone treatment (F2).

2.5. Hormonal Influences on G-6-PDH and 6-PGDH Activities

Before a possible hormonal control of the activities can be discussed it must be stated, that, if there is a real controlling function, the enzyme activities involved will not be altered alone, but the whole metabolic system will be influenced. The change of activity of one enzyme or an enzyme system does not necessarily indicate the metabolic situation in the living cell, for the assay method will be performed under optimal conditions including pH, coenzyme concentration, and concentration of the substrate in the test system. Furthermore, a series of other regulating factors may play a role under steady state conditions. Nevertheless, sufficient background data must be accumulated (W10) before attempting an interpretation. Investigations were performed of changes in G-6-PDH and 6-PGDH activities of various tissues after hypophysectomy in rats (R13, W10). In the primary target organs (adrenals and gonads) G-6-PDH remained unchanged, calculated as activity per unit wet weight of tissue. In the secondary sex glands a decreasing activity of this enzyme was found. The corresponding results for 6-PGDH show a decreasing activity in the adrenals and no appreciable change in the secondary sex glands. The activities of both the enzymes in liver, heart, and brain tissues were found not to be remarkably changed after hypophysectomy (R13). However, liver tissue activity after removal of the pituitary gland is significantly decreased in starving animals (W10) (cf. Fig. 5).

Reduced G-6-PDH activity entails in intact rats a longer starvation period, thus suggesting an influence of pituitary function on maintenance of normal G-6-PDH level, or on the utilization of G-6-P by the oxidative pathway.

Histochemical studies of the distribution of G-6-PDH activity in

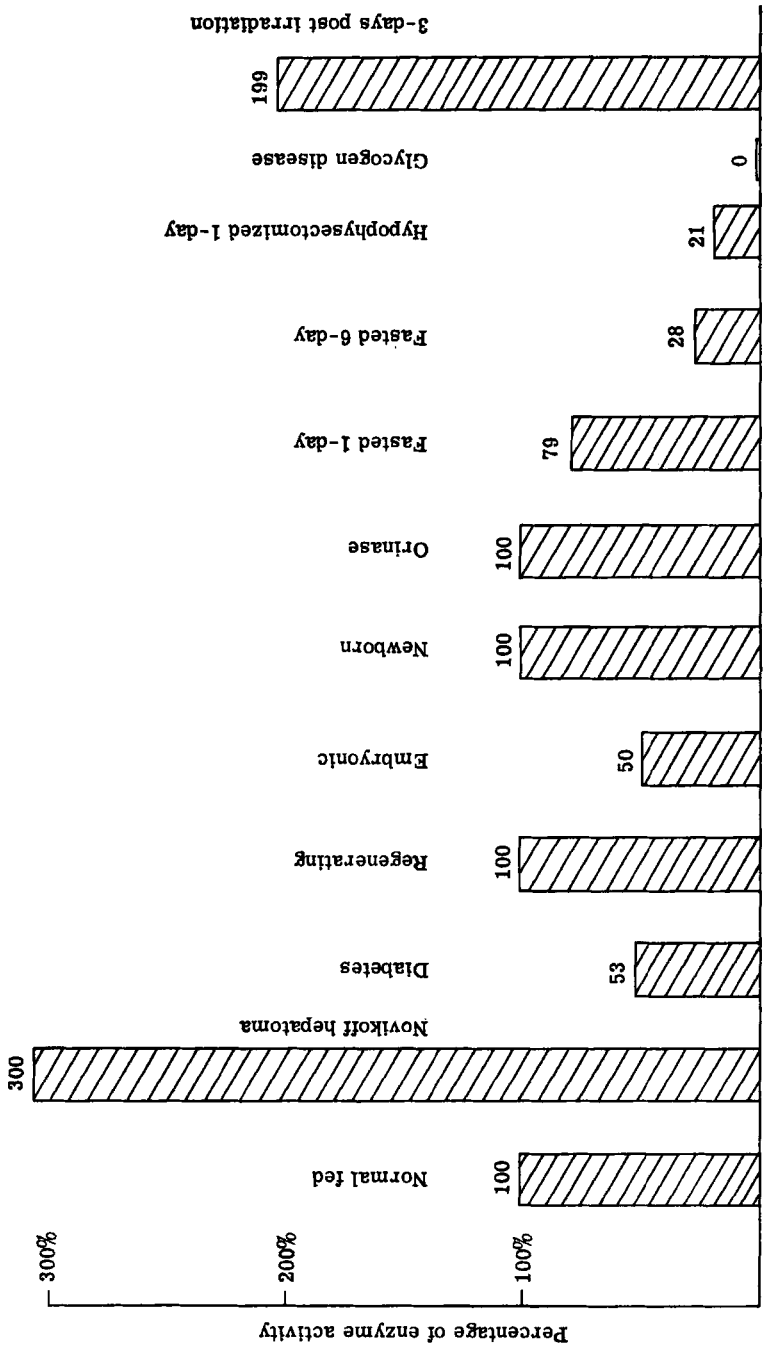

FIG. 5. G-6-PDH activity in liver cell homogenates of rats under physiological and pathological conditions. Activity in livers of normal fed animals = 100%. Cf. (W10).

adrenal cortex of normal and hypophysectomized rats exposed to cold stress resulted in the finding that activities did not vary in distribution or intensity in response to stress (C5). Since stress is normally followed by the ACTH-stimulated production of cortical hormones, the lack of depletion of glycogen and lipid stores in the adrenal cortex in hypophysectomized rats exposed to cold stress may suggest a mechanism of ACTH influence on steroid synthesis involving the action of enzymes of the oxidative pathway: ACTH stimulates glycogenolysis, thus providing a substrate for G-6-PDH and setting into motion the first step of the pentose phosphate cycle. By this, $NADPH_2$ (and energy) is provided for steroid synthesis resulting in the utilization of the cortical lipid stores. By histochemical techniques, the failure of lipid and glycogen store utilization following stress in hypophysectomized rats was proved (C5). If this theory is true, even in part, it is an example of the importance of substrate concentration for enzyme activity under steady state conditions.

Adrenalectomy causes a decrease of G-6-PDH activity in liver homogenates of rats. This can be restored to normal levels by administration of cortisone or corticosterone while hydrocortisone and progesterone dosages resulted in a partial restoration of activity (W17).

At the end of pregnancy, especially between parturition and weaning, elevated activities of G-6-PDH and 6-PGDH could be observed in liver and mammary gland tissues. Adrenalectomy at parturition or in the lactation period depresses these increased activities in mammary glands and in livers (G11). The hepatic activities are slightly increased for the immediate postoperative period (W16). However, adrenalectomy and substitution of cortical hormones were reported as having no influence on the rate of glucose metabolism via the pentose phosphate cycle in liver tissue of rats. Using histochemical methods, only gluconogenesis and decreased lipogenesis could be observed (A10).

An investigation of G-6-PDH and 6-PGDH activities of mammary cancer tissues in rats showed a remarkable decrease following administration of dehydrotestosterone. Ovariectomy also resulted in a depression of enzyme activities of these tissues, which could be reversed by administration of 17β-estradiol, an effect depending on dosage (R8).

A correlation between the activity of G-6-PDH and extractable renin in rat kidneys has been reported, suggesting that the macula densa cells and also the juxtaglomerular apparatus are parts of a system related to the formation of renin (H6).

With respect to the problem of hormonal influences on enzymes of the pentose phosphate cycle, investigations of the enzyme levels in

diabetes are important. Several authors found decreased G-6-PDH and 6-PGDH activities in liver homogenates of animals with alloxan diabetes (G12, G13, G14, W10). These results are confirmed by the finding that insulin treatment reverses the decreased activities of G-6-PDH (G14). Crystalline insulin has no *in vitro* effect on either G-6-PDH or 6-PGDH. However, effects of insulin on pathways of glucose utilization, other than Embden-Meyerhof, in mammary gland tissues of lactating rats have been reported. Increased activity of the pentose phosphate shunt has been found (A1).

Elevated G-6-PDH activity in hyperthyroidism has been found in erythrocytes (P1) and in liver cell homogenates (G13, G14), but pure, crystalline DL-thyroxine had no *in vitro* effect on the enzyme action (G13). A slight reduction of elevated hepatic levels in hyperthyroidism following thiouracil treatment was not significant (G13). Administration of growth hormone to rats caused no significant rise of liver cell activity, except perhaps in relation to seasonal variations, the G-6-PDH activities of liver cells being appreciably higher in summer than in winter (G13).

The mechanisms of all these alterations in G-6-PDH and 6-PGDH activities are as yet unknown and will have to be clarified. A special question is whether or not we can consider them as real hormonal control or as a simple coincidence conditioning alterations in equilibria of enzyme reactions involved.

2.6. Alterations of G-6-PDH and 6-PGDH Levels under Physiological Conditions

Since metabolism is not static, but rather an equilibrium including cell aging and propagating processes, the alterations of G-6-PDH and 6-PGDH levels are of interest from these aspects. In this respect, the larger standard deviation of 6-PGDH activity in human serum (cf. Table 1) of females in comparison with males becomes remarkable. At term pregnancy the serum level of 6-PGDH rises to about 130% of the normal (W18). In tissues of mammary glands of lactating rats, G-6-PDH and 6-PGDH levels are increased 60- and 20-fold, respectively (G11). The significance of these findings has been confirmed by the fact that the lactating glands do not augment in weight if correction for milk content is applied.

Decreasing G-6-PDH activity in aging erythrocytes has been described (L4, L8). This finding becomes important in hereditary G-6-PDH deficiency, explaining the self-limiting effect of drug-induced hemolysis (B9, B10, B11).

Investigations of changes in G-6-PDH activity of liver cells in starving rats resulted in the finding of reduced activity (A3) depending on the duration of the starvation period (W10).

The G-6-PDH activity of erythrocytes of human newborns was found to be approximately 100% higher than in healthy children and adults, although the red cells were GSH unstable (B1) (cf. Section 3.4).

2.7. Assay Methods

The determination of G-6-PDH and 6-PGDH activities is possible by 3 ways: (a) by measuring the O_2 uptake when the respective reaction product is oxidized, (b) by following spectrophotometrically the appearance of reduced NADP in the reaction mixture at wavelengths of 340 or 366 mμ, or (c) by observing the decolorization of a reducible dye such as methylene blue and brillant cresyl blue.

The manometric method using the Warburg apparatus is highly accurate, but complicated, and demands more time than the simple spectrophotometrical assay, its accuracy being equal.

Recently a colorimetric method for estimation of erythrocytic G-6-PDH was described (E1). This procedure is based upon the interaction of phenazine methosulfate as electron carrier between $NADPH_2$ formed in the reaction and dichloroindophenol, the rate of the reduction of the latter compound being followed at 620 mμ.

The observance of decolorization of a reducible dye may be suitable for a rapid screening test, but not for final examinations. Since the estimation of G-6-PDH activity may be necessary in laboratories where no photometer is available, both methods (b) and (c) mentioned above will be described.

2.7.1. *Spectrophotometric Methods for Estimation of G-6-PDH and 6-PGDH*

The method described (K19) and used by many investigators is based upon following the increase of $NADPH_2$ absorbance at 340 mμ in a glycylglycine buffer medium, pH 7.5, including enzyme, substrate (G-6-P or 6-PG sodium salt, respectively), NADP and $MgCl_2$ as activator. An analogous method for 6-PGDH has been reported (H18).

More recently the G-6-PDH method has been adapted especially for serum and hemolyzates (B15).

Reagents: triethanolamine buffer, pH 7.6, $5 \times 10^{-2}\,M$ containing EDTA $5 \times 10^{-3}\,M$, NADP solution $1 \times 10^{-2}\,M$ and G-6-P sodium $4.1 \times 10^{-2}\,M$; for hemolyzing the erythrocytes, the red blood cells of 0.2 ml blood are centrifuged off and washed 3 times with 0.9% sodium

chloride solution before being suspended in 0.5 ml cold saturated digitonin solution. After 15 minutes hemolysis at +4°C the stromata are centrifuged off (for 10 minutes at 3000 r.p.m.) and 0.1 ml of the supernatant is used for the reaction. The reaction mixture in 10-mm cuvettes consists of (values for hemolyzates in parentheses):

1.85 (2.75) ml buffer-EDTA
1.00 (0.10) ml enzyme, serum, or hemolyzate
0.10 (0.10) ml NADP solution

and, after a 5-minute equilibration period,

0.05 (0.05) ml G-6-P·Na.

3.00 (3.00) ml final volume.

Readings are taken at one-minute intervals at 340 or 366 mμ. The change in absorbance should not exceed 0.03 per minute, otherwise the enzyme must be diluted (e.g., with buffer-EDTA solution).

2.7.2. *Rapid Screening Test*

For a quick decision, if G-6-PDH activity can be expected in a normal pattern, a screening test has been described (G7). The procedure is performed as follows: 0.04 ml blood is mixed with 1.5 ml cold water for hemolysis; then 0.4 ml of tris buffer (pH 8.5, 0.74 *M*), 0.5 ml brillant cresyl blue (1×10^{-3} *M*), 0.4 ml H_2O, 0.1 ml NADP (0.1 mg), and 0.1 ml G-6-P solution (5 μmoles) are added. After thorough mixing and sealing the mixture by the addition of about 1.0 ml mineral oil, the samples are incubated at 37°C and observed for decolorization. It should be noted whether or not decolorization occurs within 100 minutes of incubation (= normal).

All determinations of G-6-PDH activity should be made as soon as possible after the withdrawal of the blood sample. After standing for 24 hours, the activity was reported to have been reduced to 50% of the original (K7); storage conditions were not indicated.

2.8. Clinical Implications of G-6-PDH and 6-PGDH Activities

2.8.1. *Introduction*

For clinical chemistry the most important question is whether or not changes in enzyme activity will accompany defined disease states, the materials being obtainable by ways practicable in clinical medicine. The enzyme levels of G-6-PDH and 6-PGDH are assayed in serum, blood (hemolyzates), and liver homogenates yielded by biopsy. In the latter case it is necessary most of all to take account of the probable differences between the assay conditions and the steady state. Furthermore, the reference system is of decisive importance (e.g., cellularity

per unit weight). In case of determinations in hemolyzates, activities may be related to the number of RBC per milliliter, or to grams hemoglobin, or to the red cell volume. If both the number of RBC and the red cell volume are known, the activity may also be referred to the mean enzyme content of one red cell, if disturbances by other corpuscular blood elements are excluded.

The discussion of the clinical implications of G-6-PDH and 6-PGDH will be divided into two sections: change of activities caused by diseases, and disorders caused by deficiency of enzymes.

2.8.2. *Alterations of G-6-PDH and 6-PGDH Activities Due to Disease States*

The levels of G-6-PDH in serum have been investigated in many patients suffering from a multiplicity of disorders (K6), without finding a significant change except in myocardial infarction (K7). In these cases, G-6-PDH activities reach their maxima later than other enzymes reported to increase following this event. The highest values were found about the sixth day after infarction (K6, K7).

Hepatic diseases do not change the serum levels of G-6-PDH (B15, K6, L1). In malignant occlusion jaundice, elevated levels of G-6-PDH have been reported (L1).

Increased 6-PGDH activity has been described in plasma of individuals suffering from anemia, in which the elevation in megaloblastic forms was noticeably higher than in blood-loss anemia (H1, H2). This may be related to the higher content of enzymes in megalocytes. The preformed reticulocytes flushed in peripheral circulation in cases of blood loss contain less G-6-PDH than newly generated ones (K1). The alterations of enzyme activities in red blood cells are of more interest in clinical chemistry for their higher content of enzymes involved. Most of these changes will be discussed in the next section. The decreasing activities of G-6-PDH and 6-PGDH of aging erythrocytes has been mentioned above. Increased activities in blood-loss anemia can thus be explained (H2, K1) and are confirmed by the observation of increased values in pernicious anemia (B15). In cases of Werlhof's disease, increased activities of G-6-PDH and 6-PGDH were reported, which returned to normal values after splenectomy (P2). In hepatic coma the G-6-PDH activity in hemolyzates rises about 30%, normal values being found in hepatitis and obstructive jaundice (nonmalignant) (B13). In hyperthyroidism a significant increase of G-6-PDH activity of erythrocytes was observed (P1). However, in liver tissues no change of G-6-PDH activities in thyrotoxicosis could be found (N3).

By a special technique the augmentation of pentose phosphate cycle activity in red cells of obese patients was shown by Šonka *et al.* (S16), increasing the glucose utilization by the oxidative pathway from 7.56 to 12.25%.

In leucocytes of individuals suffering from myelocytic and lymphatic leukemia a significant decrease of G-6-PDH and 6-PGDH has been described (B3, B4, G4), the activity of the latter being noticeably more reduced than that of the former.

Since G-6-PDH has been shown to be present in platelets (L6, R7), its activity has been studied in thrombocytopenia and leukemia (W22). Activity was found normal in the former and decreased in the latter disorder.

The changes in G-6-PDH and 6-PGDH activities of tissues (especially liver cells) have been extensively investigated. For the above-mentioned reasons the evaluation of the results obtained seems problematic. A remarkable increase in liver cell activity has been described in acute hepatitis (S3, S4). The increase in liver cell activity in chronic hepatosis (S5) is not as great as that for acute hepatitis. In cases of hyperbilirubinemia following hepatitis, G-6-PDH activity was also found to be elevated (K2). Decreased activities have been shown in liver cells of children suffering from glycogen storage disease (B16). Investigations of liver cell activities of both the enzymes in hepatocellular disorders by Verme *et al.* (V4), although showing elevated values which could not be statistically assured, were interpreted by the authors to be due, at least in part, to exogenic infiltration of the tissue by blood cells; thus, the difficulty of evaluation again becomes evident. An increase of G-6-PDH and 6-PGDH in liver cells of rats following total body irradiation has been reported (W10).

Whether finding of increased G-6-PDH activity in isolated glomeruli and kidneys of nephrotic rats will become important from the clinical chemistry point of view is questionable (D12).

A series of investigations were performed in search of alterations in enzyme activities of the hexose monophosphate shunt in tissues of various tumors (H13, K17, R8), and an increase of the activities of enzymes involved was found.

2.8.3. *Metabolic Disorders Due to Lack or Deficiency of G-6-PDH*

2.8.3.1. *General Views.* In 1926 Cordes[1] reported instances of hemolysis during malaria treatment with pamaquine (Plasmochin, an 8-amino-

[1] Original paper not available to authors: 15th Annual Report, United Fruit Company (Med. Dept.), pp. 66-71 (1926).

quinoline derivative) in Negroes. The observation of incident hemolytic attacks was confirmed by further studies of the same author (C7).

Since that time many investigations have been made to elucidate the mechanism of this drug-induced hemolysis [recently reviewed by Beutler (B7)]. The hemolytic symptoms were recognized to be hereditary and race linked. They were found to be due to lack of G-6-PDH activity in erythrocytes and other cellular blood compounds, and the mechanisms of the consequently appearing metabolic failures were emphasized.

2.8.3.2. *Distribution of Primaquine-Sensitive Individuals in the Various Population Groups.* Primaquine sensitivity was found in Negroes (B7, B19, C3, C7, G6, G7, G18, H21, M7), Caucasians (G18, L3, M7, W3), and certain Jewish tribes (S21, S23). The phenomenon could be detected in 4.6% of an unselected population of 305 North Americans. In healthy Negroes the sensitivity was more common (7.2%) than in Caucasians (1.3%) (G18). In the Jewish population, the defect was found in 20% of the non-Ashkenazic Jews originating from Iraq and Persia, in 5% from Yemen and Turkey, and in 2% from North Africa. Also 3% of the Arabs in Israel showed drug sensitivity.

In the Mediterranean population G-6-PDH deficiency has been described in relation to favism (S22, Z2, and others). Furthermore, hereditary enzyme deficiency was found in Malayans (W8) and Chinese (V1) (in the latter in 2.2% of 225 healthy individuals).

This distribution suggests a race linkage of the trait. Further studies on families and relatives of affected subjects revealed the deficiency to be related to a dominant gene which is sex linked and sex modified, since the full symptoms of hemolytic crisis occur only in males (G19, L3, M7, S21, S22, S24).

2.8.3.3. *Mechanism of Primaquine Sensitivity.* After the symptoms of drug administration and of sensitivity to the ingestion of fava beans [acute hemolysis, consequent drop of hemoglobin, hemoglobinuria, and even methemoglobinuria (H21)] had been recognized, investigations were performed on the localization of this defect. First, erythrocytes were found to be uniquely susceptible to the hemolytic reaction (D3). Further studies showed a close correlation between G-6-PDH deficiency and drug-induced hemolysis or favism (e.g., B11, C3, D1, D4, D9, G6, G15, G16, G18, G19, H21, K4, K16, L3, L5, M6, P5, S21, S22, W2, W3, Z2).

In sensitive individuals G-6-PDH activity of platelets was also found to be deficient (R7, W22). A reduced activity of saliva has been reported (R5). The results of these investigations led to the suggestion

that G-6-PDH activity is one of the regulating factors for maintenance of red blood cell integrity. At this point, the mechanism of drug action must be discussed. Primaquine and its derivatives were shown to inhibit G-6-PDH *in vitro* (D4). *In vivo*, the drug is transformed into intermediates interacting in the $NADPH_2$ or $NADH_2$ glutathione reductase system and is capable of depleting the cell of $NADPH_2$ and GSH (K5, L5). The importance of this system derives from the close connection with GSH level and with the function of the pentose phosphate cycle. Therefore it will be discussed in relation with GSSGR in Section 3.5.2 of this paper. The above suggestion is confirmed by the finding that in drug-induced hemolysis NADP and NAD levels in the cell are increased while those of $NADPH_2$ and $NADH_2$ are reduced (K4, W3). (See Section I of the Addendum, page 279.)

The observance of a "resistant" phase following recovery from acute hemolysis caused by drugs, in which a further destruction of erythrocytes occurs only if increased doses are given (A8, K5), supports the evidence of the mechanism described. This self-limiting effect of drug-induced hemolysis is due to the higher G-6-PDH content of the younger erythrocyte population, thus generating more $NADPH_2$ for GSSG reduction which is supposed to be the integrity-maintaining principle of erythrocytes. It has been found that erythrocytes of G-6-PDH defective individuals have a remarkably reduced life span (mean value of half life for normals, 66 days; for deficient erythrocytes, 48.2 days) (B19).

Recent investigations have shown the mechanism of hemolysis due to G-6-PDH deficiency to be more complex. It has been found that "lacking" G-6-PDH activity could be restored when erythrocytes of defective individuals were incubated with the stromata of normal red cells. It could be excluded that the postulated activator was eluted G-6-PDH of the stromata, and evidence is given for the enzymatic nature of the activating reaction which can be inhibited by heavy metal ions and *p*-chloromercuribenzoate. Preliminary experiments characterized this activator as relatively heat stable, susceptible to freezing, and inactivated by acetone (G17, R6, R11). This would lead to the suggestion that the defect in primaquine sensitivity consists of the lack of the activator, the enzyme G-6-PDH being present in inactive form. This has to be clarified by further investigations. In any event, a key position of G-6-PDH activity for metabolism and maintenance of integrity of the erythrocyte is evident.

2.8.3.4. *Other Disorders Discussed in Relation to Hereditary G-6-PDH Deficiency.* Severe hemolytic jaundice in newborns without incompatibility either in classic blood groups or in the rhesus system, but

exhibiting G-6-PDH deficiency has been reported (W8). However, deficient G-6-PDH activity could not be confirmed in all cases of congenital nonspherocytic hemolytic anemia (V5, Z1), but a striking parallelism between G-6-PDH activity and glutathione stability indicates the interaction of the G-6-PDH–GSSGR system in this disorder (B19, L5, P4).

Examinations of the color sense in G-6-PDH deficient individuals showed a fairly close linkage between the gene loci determining color blindness and enzyme deficiency (A2).

Thalassemia has been discussed in relation to G-6-PDH deficiency (M4) because of the finding that favism was often found among the relatives of subjects suffering from thalassemia. Since both conditions are common in Sardinia and surrounding countries, the relation may be fortuitous; this suggestion corresponds to the reported results (S24, V1).

3. Glutathione Reductase

3.1. Introduction

When in the past 10 years the mechanism of drug-induced hemolysis was studied, the implication of an enzyme system in the integrity-maintaining principle of the red cell became evident as did the probability of its interacting in the processes of maturation and senescence (B8, S1).

This system regulates the level of reduced glutathione and was first described by Kohman and Sanborn in 1937 (K18). Glutathione was first isolated from yeast in 1921 by Hopkins (F4) and recognized to be a tripeptide: L-glutamyl-L-cysteinylglycine. Glutathione exists *in vivo* in its reduced (GSH) and oxidized (GSSG) forms; 2 molecules GSH may oxidize to one molecule disulfide glutathione.

3.2. Properties, Distribution and Coenzyme Specificity of GSSGR

GSSGR catalyzes the reaction:

$$GSSG + NADPH + H^+ \rightleftharpoons 2\,GSH + NADP^+ \qquad (8)$$

The reversibility of reaction (8) has not been observed as yet (H20, M3, R4) and may be attributed to other systems (M2), such as assumed by Racker (R2), the oxidation of GSH to GSSG being coupled to the reduction of homocystine under control of a transhydrogenase.

GSSGR has been found in plant tissues (C6, K18, M3), in *Escherichia coli* (A12), in yeast (R1), in various animal tissues (R1, R4), and in human blood and serum (H20, M1).

GSSGR reacts with both $NADPH_2$ and $NADH_2$ (B25, H20, M3, R1).

However, other investigators found GSSGR sharply $NADPH_2$-specific (A12, C6, K6, R4). These contradictory results can be explained by the finding in this laboratory of 2 different pH optima in the $NADH_2$- and $NADPH_2$-linked reactions, the former being at pH 6.2, the latter at pH 6.5 (H20). If determinations are performed, e.g., at pH 7.6, it may be possible that no reaction with $NADH_2$ occurs.

Furthermore, the affinity of GSSGR for $NADH_2$ as coenzyme is lower than that for $NADPH_2$ (A12, H20, R1). The quotient of GSSGR activities in $NADPH_2$- and $NADH_2$-linked reactions was shown consistently to be 1.8 in human sera under different physiological and pathological conditions (H20), according to the following ratio:

$$\frac{\text{Initial maximal reaction velocity with } NADPH_2}{\text{Initial maximal reaction velocity with } NADH_2} = 1.8 \qquad (9)$$

The ratio was not significantly altered in various disease states such as pernicious anemia, tumor metastasis in liver, or porphyrinuria, ranging from 1.8 to 2.1 (H20).

The fact that GSSGR reacts with both the coenzymes may be implicated in transhydrogenation reactions between $NADH_2$ and $NADPH_2$, since these 2 coenzymes have been found to be decreased in hereditary lack of G-6-PDH (L5, W2). In Table 3 the normal mean values are summarized.

TABLE 3
NORMAL MEAN VALUES OF GSSGR ACTIVITIES OF HUMAN SERUM, RECALCULATED FOR INTERNATIONAL UNITS

GSSGH activity (mU)	Remarks on method used	References
13	pH 6.5 $NADPH_2$ phosphate buffer	(H20)
60	pH 7.4 $NADPH_2$ phosphate buffer	(K6)
62	Not indicated	(L1)

GSSGR activity was found to be inhibited by *p*-chloromercuribenzoate (A12), this inhibition being reversible by cysteine. An inhibition was also observed for tris buffer (H20, M1). In the $NADH_2$-linked reaction of GSSGR, sodium or potassium chlorides showed strong inhibiting action, although this could not be observed in the $NADPH_2$-dependent reaction (R1). Similarly, an inhibition by heparin could be shown for the $NADH_2$-linked reaction, less for the $NADPH_2$-linked GSSGR reaction (H20). An activation of only the $NADH_2$-linked reaction by phosphates has been described (R1), these findings being confirmed by the

observation that tris buffer exerts a stronger inhibition in $NADH_2$-linked reaction when phosphate ions are present (H20). Nitrofurantoin has been reported to inhibit GSSGR in concentrations reached by normal therapeutic dosages, this effect being reversed by dialysis (B26). However, no inhibition of the enzyme, either by nitrofurantoin ($1 \times 10^{-4} M$), or by aureomycin, chloromycetin, penicillin, and potassium cyanide could be found by Asnis (A12).

3.3. Assay Method

The determination of GSSGR activity is based upon Eq. (8) and can be followed spectrophotometrically by the decrease of $NADPH_2$ absorbance at 340 or 366 mμ. For the above-mentioned reasons phosphate buffer may be preferred. The typical reaction mixture contains in a final volume of 3.0 ml (H20): 1.6 to 2.4 ml phosphate buffer as described by Sörensen (0.076 *M*, pH 6.5); 0.2 ml $NADPH_2$ (0.4 μmole per test volume); 0.2 ml GSSG solution ($1.5 \times 10^{-3} M$ and equilibrated to pH 6.3–6.4 with NaOH); and 0.2–0.8 ml of serum. The blank contains water instead of substrate solution. Readings are taken at 340 or 366 mμ at room temperature at one-minute intervals, the light path being 1.0 cm. The decrease in absorbance should not exceed 0.03 per minute.

It is advisable to perform the assay of GSSGR within 6 hours after the blood has been drawn, although the activity was not found to be decreased when serum was kept frozen for one week—or for the same time in a refrigerator at +4°C (H20, M1).

3.4. The GSH Stability Test

In cases of primaquine sensitivity, erythrocytes were shown to be deficient in G-6-PDH activity and thus there is not sufficient $NADPH_2$ for the reduction of GSSG by its reductase system. In such individuals lowered GSH levels in erythrocytes were found (B6, B8, B11, S8, S24, and others). Furthermore, it can be shown that in these cases, when erythrocytes are incubated with acetylphenylhydrazine (B6, G18), the maintenance of even a lowered GSH level is not possible. The GSH stability test is based on this fact; it measures virtually the G-6-PDH activity and not that of GSSGR.

The GSH level in erythrocytes is determined by a nitroprusside method (G20) slightly modified (B6). Another blood sample is incubated with acetylphenylhydrazine, and after this, another determination of GSH is carried out. Normal erythrocytes show no or little change of the GSH level after incubation with acetylphenylhydrazine, while those of "unstable" ones exhibit a decrease in the GSH level averaging

44 mg GSH/100 ml of RBC (B6), as shown in Fig. 6. The GSH content of normal erythrocytes is 66.3 mg GSH/100 ml RBC (mean value).

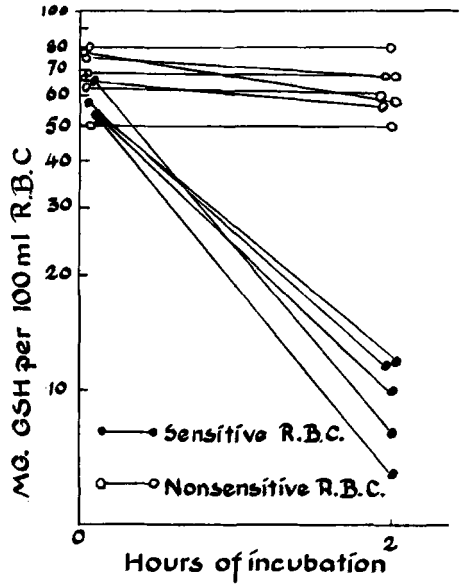

FIG. 6. "GSH stability" of sensitive and nonsensitive red blood cells. Reproduced by courtesy of *The Journal of Laboratory and Clinical Medicine* (B6).

3.5. CLINICAL SIGNIFICANCES OF GSSGR ACTIVITY

3.5.1. *Alterations of GSSGR in Various Disease States*

The determination of GSSGR activity has not as yet reached the widespread use of other enzyme tests in clinical medicine, although increased GSSGR activity of serum was reported in hepatic diseases including metastases in liver (D2, H20, K6, L1, M1, W11). The activity peak in homologous serum icterus and the return to normal values equals the development in SGOT and SGPT (M1). Increased serum GSSGR activity was described for malignant occlusive jaundice (L1).

Furthermore, elevated serum levels were found in pernicious and megaloblastic anemia, porphyrinuria (H20, W11), in myelocytic leukemia, hyperfunction of the adrenal cortex (and after administration of adrenal cortical hormones), and in patients suffering from pulmonary tuberculosis receiving isoniazid (K6, W11). In cerebrospinal fluid, GSSGR activity was reported to be increased in individuals with acute bacterial meningitis; likewise in serous effusions containing malignant

cells. In the latter cases the serum levels were not necessarily elevated (M1).

It seems to be important that in myocardial infarction no increase of GSSGR activity could be found (K6, L1), thus representing the possibility for differential diagnosis in connection with other enzyme tests.

3.5.2. *Implication of GSSGR in Hemolysis and Cell Aging*

In contrast to the finding of G-6-PDH deficiency in primaquine-sensitive individuals, an increased GSSGR activity has been demonstrated in hemolyzates of affected subjects (C3, S8). On the other hand, the GSH level in such erythrocytes is reduced (cf. Section 2.8.3.3). When the drug is given, the GSH level drops even more and, after the acute hemolysis, it rises but not to normal levels (S8). This connection between G-6-PDH and GSSGR is confirmed by the observation that, as GSH disappears an increase in GSSG occurs (B10), proving (a) that the lowered GSH level is due to its oxidation, and (b) that nonsensitive RBCs are able to protect their GSH level by the G-6-PDH-GSSGR system, since in the absence of glucose no GSH generation occurs. The above-mentioned elevation of GSSGR activity in primaquine-sensitive erythrocytes suggests that the increase compensates at least partially for the deficient enzyme, allowing it to work at a higher rate and increasing the availability of GSH (S8).

This hypothesis is confirmed by the finding that the younger RBC population even in "sensitive" cases contains more (but not normal) G-6-PDH activity, thus being responsible for the self-limiting effect in drug-induced hemolysis. A further confirmation was obtained by labeling erythrocytes of sensitive subjects with Fe^{59} and infusing them into healthy volunteers. Thus, the action of primaquine on erythrocytes of a narrow range of age could be followed. These cells were not affected by the drug between 8 and 21 days of cell age, while in the range of 63 to 76 days the cells were rapidly destroyed (B8, B9). With regard to the decreasing activity of G-6-PDH in the course of cell aging we can decide that (a) G-6-PDH activity is rate limiting for GSSGR activity in red cells by forming $NADPH_2$, and (b) the susceptibility to drug-induced hemolysis is a function of cell age.

The following mechanism of drug-induced hemolysis is suggested by Beutler (B8): Primaquine administration is followed by reversible alteration in the hemoglobin molecule, now reacting with GSH and oxidizing it to GSSG while the altered hemoglobin molecule is restored, provided that G-6-PDH activity is sufficient, and thereby the function

of the hexose monophosphate oxidative pathway. If it is deficient, GSSG will consequently accumulate, simultaneously exhausting the supply of GSH. By this, the altered hemoglobin molecule will denature with formation of Heinz bodies and the cell will be destroyed. This would be in accordance with the finding of elevated serum GSSGR levels in cases of "hemoglobin S-S disease" (W11).

Perhaps this may be considered in relation to the suggestion of Kellermeyer *et al.* (K5) that the drugs involved are transformed *in vivo* to redox intermediates. Furthermore, the reducing capacity of RBC was shown to be a function of GSH content. Reduction of this capacity by intravenous infusion of sodium thiosulfate solution reflects changes in the intracellular oxidation-reduction system of glutathione, the oxidized form being favored (C1, S9).

The results of all these investigations support the evident importance of the GSSGR system for the integrity-maintaining principle of RBC including the processes of maturation.

4. Methemoglobin Reductase

4.1. Introduction

In 1928 Barron and Harrop reported the stimulation of oxygen uptake of erythrocytes by methylene blue (B2). Two years later, Warburg and his co-workers pointed out, that methemoglobinemic red cells are able to reduce their methemoglobin to hemoglobin in the presence of glucose, glucose monophosphate, hexose diphosphate, or lactic acid (W4, W7). An enzyme system has been postulated that catalyzes the reduction of MHb. Based upon these findings and conceptions, methemoglobin reductase has been detected and recognized to be a nicotinamide adenosine nucleotide-linked enzyme involved in glucose metabolism by both the glycolytic and oxidative pathways (B14, D11, G5, G21, H22, H24, K8, K9, K10, K11, K12, K14). For the connection with glucose metabolism, cf. Fig. 4.

4.2. General Properties of MHbR

The enzyme MHbR catalyzes the following reactions:

$$2\,HbFe^{+++} + NADPH^{+} + H^{+} + \tfrac{1}{2}\,O_2 \rightleftharpoons 2\,HbFe^{++} + NADP + H_2O \quad (10)$$

$$2\,HbFe^{+++} + NADH^{+} + H^{+} + \tfrac{1}{2}\,O_2 \rightleftharpoons 2\,HbFe^{++} + NAD + H_2O \quad (11)$$

The reactions in Eqs. (10) and (11) indicate the links with glucose metabolism, the former to the direct oxidative pathway, the latter to the glycolytic route. The essential link in the reduction of MHb is the generation of reduced pyridine nucleotides (B14, R12, S10). The

electron carrier systems involved will be discussed in the next section. The importance of pyridine nucleotides for the activity of MHbR is confirmed by the finding that a reduction of MHb in hemolyzates occurs only if nicotinamide is present during lysis (G21), thus protecting NADP and NAD from enzymatic hydrolysis. Of course, the reduction will start on the readdition of either $NADPH_2$ or $NADH_2$. Likewise, the inhibition of MHb reduction observed after the addition of iodoacetate or fluoride (D11) can be explained. The reduction of MHb has been found to be promoted by several nucleosides such as adenosine, xanthosine, guanosine, inosine, 2,6-diaminopurine riboside, deoxyguanosine, deoxyadenosine, deoxyinosine, and by the pentoses, ribose and deoxyribose (J1), thus suggesting that all compounds which are actively metabolized by intact erythrocytes by pathways leading to the reduction of NADP or NAD will enhance the reduction of MHb.

MHbR has been purified from erythrocytes of many species including man (H21, K8, K11, K12, K14) and from brewers' yeast (A6). The 1000-fold purified preparation obtained by Kiese (K14) has been shown, by means of chromatographical studies, to contain FAD. Thus the enzyme has been identified as a flavoprotein. However, the same author could not find any further activating effect of FAD or FMN on active preparations, nor a reactivation of the enzyme previously inactivated by dialysis (K14).

On the other hand, highly purified preparations (180-fold) obtained by Huennekens and his co-workers (H22) have been shown to be a hemoprotein with a molecular weight of approximately 185,000. With regard to these different results it is interesting that in RBC of individuals suffering from hereditary methemoglobinemia a complete lack of NAD diaphorase has been reported (S10, S11); this would indicate the importance of an enzyme which contains FAD. The reasons for the discrepancies between the preparations obtained by two teams of investigators are not understood as yet. Perhaps they are implicated in the electron transport mechanisms or in the nature of a certain cofactor which is to be discussed now.

4.3. The Significance of Methylene Blue in the MHbR Reaction

The stimulating action of methylene blue and other reducible dyes on MHb reduction has long been established. However, this function is not constant, and may even be absent (H22, H23). In beef erythrocytes, and in MHbR prepared from yeast, no stimulating effect of methylene blue could be observed (A6, H22). When beef blood enzyme was boiled, a substance was obtained which activates the human MHbR without

requirement of methylene blue (H22). Furthermore, during purification of the enzyme the influence of added methylene blue increases. Nevertheless, the purified preparations from beef and human erythrocytes show very similar properties.

These findings lead to the conclusion that the reduction of MHb by its reductase requires a natural cofactor, which is abolished during the purification procedure and can be replaced by methylene blue (G5, H22, H23, K8, K14). Since methylene blue and the other effective dyes are redox intermediates, it is obvious that the postulated cofactor interacts in the electron transport sequence of the MHbR reaction (H23). This is confirmed by the finding that oxygen and cytochrome c serve as well as terminal electron acceptor as does MHb (H22, H23, K14). Nevertheless, it had been possible to separate a cytochrome c reductase from MHbR in yeast extracts (A6).

It was shown that trivalent iron was able to provide the electronic linkage and to produce a 4-fold stimulation of MHbR activity in the absence of any dye (H23). Furthermore, it has been found that the addition of methylene blue to crude enzyme preparations was necessary for reducing MHb but not for using oxygen as a terminal electron acceptor (H23). The enhancement of MHb reduction in methemoglobinemia by ascorbic acid (e.g., B14) also indicates the implication of reduction-oxidation mechanisms in MHbR activity.

The differences between the enzyme preparations with respect to the requirement of added methylene blue are in good agreement with the finding of varying amounts of MHb in the normal blood of several species (H7) and variable, but species-linked abilities to reduce MHb (K9, K11, S17). Furthermore, an age variation in MHb reduction of rabbit erythrocytes has been observed (S17).

Nevertheless, the nature and function of this cofactor will have to be clarified by further investigations. It is possible that this problem is involved in the way by which the energy for reduction is provided, whether $NADH_2$ or $NADPH_2$ is oxidized by the action of MHbR (cf. G5, R12, S10, S11, S17). This may be implicated in the results showing the enhancement of MHb reduction by aliphatic and aromatic aldehydes effected by a NAD-linked aldehyde dehydrogenase (M9, M10).

4.4. Assay Methods

The assay methods reported in the literature vary with respect to the use of methylene blue or another dye, and to the coenzyme used, depending on enzyme source, etc.

Since the MHbR system has not been fully characterized as yet, the

evaluation of the results becomes difficult as does the standardization of units. In principle the estimation of MHbR activity is possible by manometric, spectrophotometric, and colorimetric methods. Futhermore, either oxygen or MHb may be used as terminal electron acceptors.

The manometric methods described (e.g., L7, W3) are based on the uptake of either carbon monoxide or oxygen, the former using MHb as electron acceptor in the presence of toluidine blue, the latter using the dye as the terminal acceptor in a system involving the following reactions:

$$\text{G-6-P} + \text{NADP}^+ \rightleftharpoons \text{6-PG} + \text{NADPH} + \text{H}^+ \quad (12)$$

$$\text{Toluidine blue} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{Leucotoluidine blue} + \text{NADP}^+ \quad (13)$$

$$\text{Leucotoluidine blue} + \tfrac{1}{2}\,\text{O}_2 \rightleftharpoons \text{Toluidine blue} + \text{H}_2\text{O} \quad (14)$$

Reaction (12) is under control of G-6-PDH, reaction (13) is catalyzed by MHbR and provides the limiting rate, while reaction (14) is nonenzymatic and indicates the measurable oxygen uptake. The blank in this method contains no dye and the unit of "MHbR system" is defined as the increase of O_2 uptake of one micromole per hour after the addition of an excess of toluidine blue (L7). The determination of MHbR activity in hemolyzates demands the further addition of G-6-P, G-6-PDH, and NADP to the system (W3), the enzyme activity being thus assayed independently from the physiological function of G-6-PDH system.

The spectrophotometric methods make use of either the different absorption spectra of MHb and Hb, or of the decrease of $NADPH_2$ absorbance at 340 or 366 mμ. MHb has an absorption peak at 630 mμ while oxyhemoglobin at 576 mμ. By this, the absorbance of oxyhemoglobin appearing or the decrease at 630 mμ may be a measure for MHbR activity (H23).

The estimation of MHbR activity by following the disappearance of $NADPH_2$ absorbance is not possible if any hemoglobin is present in the assay system. However, after denaturation by purification steps the activity can be measured in this way, described as follows (H22): 0.15 μmole $NADPH_2$ plus 100 μmoles phosphate buffer (tris or Veronal buffers may be used without change of results), plus 0.2 ml of enzyme solution and H_2O to a final volume of 3.0 ml. After a stable value of A_s has been obtained, the reaction can be started by the addition of 0.01 ml of a 0.1% methylene blue solution. In the blank $NADPH_2$ is omitted.

A further assay method using a G-6-P, G-6-PDH, NADP, MHb, MHbR system in which MHb was prepared from human hemolyzates as

cyanide-MHb and following the change of absorbance at 560 mμ has been described for yeast enzyme preparations (A6).

Finally, the methemoglobin reduction test may be briefly mentioned. The test has been developed for the detection of primaquine sensitivity and depends on the function of the G-6-PDH system. Its principle consists in the oxidation of Hb to MHb by sodium nitrite and the subsequent enzymatic reduction to Hb in presence of methylene blue. The activity of this system can be followed easily by observation of alterations in color after an incubation period or by means of MHb determinations before and after this period (B18).

4.5. Clinical Implications of MHbR Activity

4.5.1. *Introduction*

Methemoglobin reductase is responsible for keeping the hemoglobin molecule in its functional state, thus ensuring sufficient oxygen transport. Since hemoglobin reacts with oxygen *in vivo*, forming either oxyhemoglobin or methemoglobin (B14, H23), the presence of MHb can be expected in normal blood. This has been proved by many investigators as reported in an extensive review by Bodansky (B14). The normal mean values of MHb content in healthy human subjects range between 0.0 and 2.4% of the total blood pigment (cf. B14).

Methemoglobinemia arises from poisoning with MHb-forming substances and from the hereditary deficiency of an enzyme system which either provides reduced pyridine nucleotides for MHb reduction or is involved itself in the MHb reduction mechanism (e.g., electron transport system). (See Section II of the Addendum, page 280.)

4.5.2. *Methemoglobin-Forming Compounds*

The MHb-producing substances must be classified into 3 groups:

(a) compounds which directly cause the conversion of Hb to MHb,
(b) compounds that are converted *in vivo* into effective derivatives by the action of microorganisms, and
(c) compounds which are metabolized with formation of active substances.

In Table 4 some examples of these compounds are given from the review of Bodansky (B14). With respect to their effectiveness these compounds depend on the mode of ingestion, absorption and, through the latter, on their concentration in blood. Thus, most of the investigators expressed the MHb-forming capacity of any compound as the average molecular ratio of MHb formed to its dosage (B14). But, the variability

of the MHb concentrations effected by a stated dose scarcely permits one to average this molecular ratio, since the ability to reduce MHb is not only variable from one individual to another, but is also species-dependent.

TABLE 4
METHEMOGLOBIN-FORMING COMPOUNDS AND THEIR MODE OF ACTION

Compounds	Group[a]	Remarks
Nitrites	(a)	—
Nitrates	(b)	By reduction to nitrites in intestinal tract
Chlorates	(a)	—
Bivalent copper	(a)	—
Hydrogen peroxide	(a)(c)	—
Ferric tartrate	(a)	—
Quinones	(a)	—
Methylene blue	(a)	And other reducible dyes
Aromatic amino and nitro compounds	(c)	—
Hydroxylamines	(c)	—
p-Nitro-*o*-toluidine	(a)	—
Sulfonamides	(c)?	Or by inhibiting catalase, hydrogen peroxide accumulating
TNT	(a)	—

[a] Cf. Section 4.5.2.

The way in which aromatic amino compounds lead to the formation of MHb is of some interest in regard to the role played by the first reaction of the pentose phosphate cycle in this reaction system. It has been stated (L5) that nitrosobenzene effects within one hour the conversion of Hb to MHb to the extent of 80% of total pigment according to the following reactions:

$$\text{G-6-P} + \text{NADP}+ \rightleftharpoons \text{6-PG} + \text{NADPH} + \text{H}+ \quad (15)$$

$$\text{NADPH} + \text{H}+ + \text{C}_6\text{H}_5\text{-NO} \rightleftharpoons \text{NADP}+ + \text{C}_6\text{H}_5\text{-NHOH} \quad (16)$$

$$\text{HbFe}++ + \text{C}_6\text{H}_5\text{-NHOH} + \tfrac{1}{2}\,\text{O}_2 \rightleftharpoons \text{HbFe}+++ + \text{C}_6\text{H}_5\text{-NO} + \text{H}_2\text{O} \quad (17)$$

The reaction in Eq. (15) is under the control of G-6-PDH, that in Eq. (16) is catalyzed by MHbR, while that in reaction (17) is non-enzymatic. It is remarkable that nitrosobenzene is reduced enzymatically to phenylhydroxylamine which oxidizes Hb to MHb and is, by this, reoxidized to nitrosobenzene (L5); the latter substance acts catalytically and not stoichiometrically when forming MHb from Hb by the fact of its regeneration during reaction (17).

If this concept is right, a decreased MHb formation by nitrosobenzene must be postulated in subjects with lack of G-6-PDH activity. In fact, a deficient MHb formation has been demonstrated in such a case (L5, W3). Thus, reaction (15) has been proved to be rate limiting for reactions (16) and (17).

4.5.3. *Methemoglobin Reductase and Methemoglobinemia*

It is evident that methemoglobinemia results if MHbR activity is lacking, although quite normal values of MHb reduction in red cells of individuals suffering from methemoglobinemia have been found on the addition of G-6-PDH (and toluidine blue) to the G-6-P–$NADPH_2$–MHbR system (G5, K13, W3). On the other hand, normal MHb values have been found in the blood of patients lacking in G-6-PDH (W3), thus suggesting (a) that MHb reduction by the $NADH_2$-linked system is sufficient, and (b) that the failure in methemoglobinemia may be related rather to a defect in the electron transport mechanism than to deficient MHbR (G5, H23, J1, R12, S10, S11). However, deficient MHb reduction was found to be associated frequently, but not in all cases, with deficient G-6-PDH activity (R12).

Furthermore, an increased oxidation rate of Hb—the reduction capacity remaining normal—will result in methemoglobinemia (H23, K13, K20). By this, two essentially different forms of methemoglobinemia can occur: (a) deficiency in MHb reducing system and (b) acceleration of Hb oxidation. The question, whether or not the latter form of methemoglobinemia is identical with the so-called hemoglobin-M disease must be clarified by futher investigations. As yet, abnormal spectra of Hb as well as of MHb have been reported in such cases (H23, K13, K20, P3).

4.5.4. *Significance of Methylene Blue Treatment in Methemoglobinemia*

Methylene blue and other reducible dyes were shown to enhance the activity of $NADPH_2$-linked MHbR (K8, K9). This is confirmed by the finding that intravenous injections of methylene blue in methemoglobinemic patients result in a striking decrease of MHb levels (e.g., B14, K9, K10). This seems to be paradoxical, since methylene blue is capable of reacting with Hb with formation of MHb, but the dye reacts much more effectively as an artificial electron carrier in the $NADPH_2$–MHbR system (B14). It has been stated (K10) that methemoglobin reduction is associated with the formation of pyruvate in equivalent amounts, but that in reactions accelerated by reducible dyes no correlation between pyruvate formation and MHb reduction could be found.

This supports the evidence that only the $NADPH_2$-linked MHbR activity is involved in the interaction of methylene blue.

4.6. Concluding Remarks

All investigations made on the reduction mechanism of methemoglobin reveal that MHbR activity must be considered as a system of enzymes operating with pyridine nucleotides as coenzymes and depending on the presence of electron transport intermediates, which have not been decisively characterized as yet, particularly not under physiological or steady state conditions. The existence of two separately working enzyme systems, implicated in different stages of glucose metabolism and requiring either $NADH_2$ or $NADPH_2$ gives evidence for the importance of MHb reduction in the living organism. The fact that even in methemoglobinemic cells reducing activity can be induced may be considered as a safety factor for vital functions.

5. Sorbitol (Polyol) Dehydrogenase

5.1. Introduction

In searching for further enzymatic tests useful in clinical chemistry, and in allowing maximal differentiation and evaluation of the organs involved in the metabolic disorder, sorbitol dehydrogenase has been discussed in recent years. From a biochemical point of view, sorbitol represents only one of a number of substrates implicated by enzyme systems requiring pyridine nucleotides which control the equilibria between ketoses (or aldoses) and the corresponding polyhydric alcohols. Furthermore, the polyol dehydrogenases are involved in the conversion of glucose to fructose in carbohydrate metabolism.

In clinical chemistry however, these systems have not been differentiated as yet. Since the oxidation of an alcohol corresponds to the reduction of a ketose or an aldose, the designations "ketose reductase" and "aldose reductase," respectively, were suggested (H4, W14). In this paper however, the enzyme or enzyme system will be named polyol or sorbitol dehydrogenase (SDH), although the latter expression does not characterize exactly the enzyme's function in a general biochemical sense. But sorbitol or fructose have been commonly used as substrates in clinical chemical investigations.

5.2. General Properties and Distribution of Polyol Dehydrogenases

In 1951 Blakley (B12) described SDH activity in the soluble fraction of disintegrated rat liver. The author found small activities in suspensions of mitochondria and in the supernatant fluid of the latter, but

the activity was restored upon combining both the fractions. Therefore a requirement of cytochrome c for polyol dehydrogenase activity of the insoluble fraction was suggested. The enzyme in the soluble fraction was found to catalyze the following reaction:

$$\text{Sorbitol} + \text{NAD}^+ \rightleftharpoons \text{D-Fructose} + \text{NADH} + \text{H}^+ \qquad (18)$$

As we have found, the oxidation of sorbitol is favored kinetically at pH 9.6 (cf. Fig. 7), and at neutral reaction the equilibrium shifts to the left side of Eq. (18). The optimal concentrations of substrate and co-

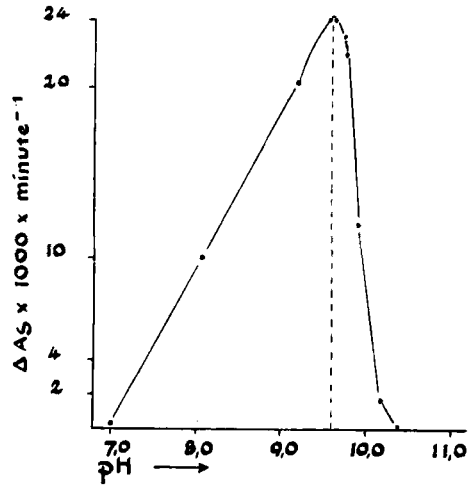

FIG. 7. pH curve of the SDH reaction using sorbitol as substrate. Tris-HCl buffer, pure tris solution and tris-NaOH mixture were used.

enzyme correspond exactly to those given in our SDH assay method (cf. Section 5.5). Studies of SDH activity in various buffer systems showed the preference of tris. As extracting medium for rat liver homogenates, distilled water was found superior to tris buffer and 1.22% potassium chloride solution.

In homogenates of rat liver, SDH activity remained unchanged when stored at $\pm 0°$ or $-18°$C for a period of 48 hours, while it had been decreased to 27% of the initial activity when exposed for 26 hours to $+22°$ or $+37°$C. However, a remarkable reduction of SDH activity in serum under various storage conditions has been reported in comparison with LDH and MDH (S20) as shown in Table 5.

Further studies on liver polyol dehydrogenases (e.g., H11, M11, T3, W14, W19) revealed the existence of a number of enzymes catalyzing reactions which can be written as follows:

$$\text{Acyclic polyol} + \text{NAD}^+ \rightleftharpoons \text{Ketose} + \text{NADH} + \text{H}^+ \quad (19)$$

or

$$\text{Acyclic polyol} + \text{NADP}^+ \rightleftharpoons \text{Ketose} + \text{NADPH} + \text{H}^+ \quad (20)$$

Reactions of the type in Eq. (20) were found when a method specially adapted to extract the enzyme from ruptured liver cell mitochondria had been used (H11).

Enzymes involved in the general reactions in Eqs. (19) and (20) have been observed in liver (B12, H11, M11, S2, T3, V3, W12, W13, W14, W19), in some accessory sexual organs (H3, K15, W14, W15), in kidneys (B12, S2), in ram spermatozoa (K15), and in some micro-

TABLE 5

STABILITY OF SERUM DEHYDROGENASES STORED UNDER VARIOUS CONDITIONS[a]

	Hours after storage									
	6		12			24		48		
Enzyme	+20°	+4°	+20°	+4°	−20°	+20°	+4°	+20°	+4°	−20°
SDH	7	6	14	12	5	31	22	58	38	22
LDH	8	8	21	16	16	28	27	36	38	28
MDH	1	1	11	6	1	17	12	21	13	2

[a] Values given in per cent decrease from initial activities determined 3 hours after withdrawal of the blood sample.

organisms (e.g., A9, C9, F3, M11, S13). Furthermore, activities especially of the type in Eq. (18) have been described in the following tissues: mucous membrane and muscular tissue of the stomach, lymph gland, pancreas, cerebrum (cortex and medullary substance), cerebellar hemisphere and lung (S2). No activity could be detected in skeletal and heart muscle, in uterus, adipose tissue, and erythrocytes (S2).

Thus far, the reactions in Eqs. (19) and (20) were considered, but in reaction (20) the ketose can be substituted by aldose according to:

$$\text{Polyol} + \text{NADP} \rightleftharpoons \text{Aldose} + \text{NADPH}^+ + \text{H}^+ \quad (21)$$

in which a number of aldehydes can replace the aldose (H3, H4). The enzyme was reported to be present in placenta and seminal vesicles of sheep (H5). While this enzyme could not be detected in placenta of rabbit, the liver, kidney, and cerebrum of various animals contain an analogous enzymatic system, but its activity toward glucose and certain pentoses is absent (H4).

The above-mentioned system is assumed to be involved in the conversion of glucose into fructose (H3, H5) according to the following reactions:

$$\text{Glucose} + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{Sorbitol} + \text{NADP}^+ \quad (22)$$

$$\text{Sorbitol} + \text{NAD}^+ \rightleftharpoons \text{Fructose} + \text{NADH} + \text{H}^+ \quad (23)$$

or summarized:

$$\text{Glucose} + \text{NADPH}_2 + \text{NAD} \rightleftharpoons \text{Fructose} + \text{NADP} + \text{NADH}_2 \quad (24)$$

The enzyme system catalyzing the reactions of Eqs. (21) and (22) has not yet been sufficiently purified for studying its substrate specificity. However, a number of aldoses and aldehydes serving as substrates in reaction (21) have been described (H4).

5.3. Substrate and Coenzyme Specificities

Different preparative procedures have been shown to yield protein fractions which are able to catalyze different types of reactions with respect to their requirement of either NAD or NADP as coenzymes [cf. Eqs. (19), (20), and (21)]. In sera of mice poisoned by carbon tetrachloride we found polyol dehydrogenases catalyzing the oxidation of the following polyols: (a) with NAD: sorbitol, ribitol, mannitol; (b) with NADP: sorbitol, ribitol. Erythritol and *myo*-inositol were not attacked at all. Figures 8 and 9 show the results of these determinations performed at pH 9.6. In the NAD system sorbitol and ribitol are oxidized at exactly the same rate, while in the NADP system ribitol does not reach the rate of sorbitol. The ratio NAD:NADP for sorbitol is calculated to be 4.20 and for ribitol 5.50. Mannitol is oxidized at 23% of the rate of sorbitol.

Soluble fractions of liver homogenates are reported to have no activity against mannitol (M11). The fact that we were able to find some oxidation of this alcohol confirms the suggestion that polyol dehydrogenases are present in the soluble fraction as well as bound to the mitochondria, since administration of carbon tetrachloride is believed to be related to the disruption of mitochondrial structures (W20). However, no mannitol oxidation has been found in preparations of mitochondrial enzyme of guinea pig livers (H11). These discrepancies may be involved in the different assay methods and enzyme sources, since a relatively labile mannitol dehydrogenase has been described in extracts of certain microorganisms grown in media with added sorbitol, exhibiting the requirement of NAD and assayed at pH 8.0 (A9, S13).

The reactivity of xylitol in the polyol dehydrogenase reactions has been extensively studied for its relations to pentosuria. Xylitol was found to be oxidized in two ways: (a) to L-xylulose by a highly specific NADP-requiring dehydrogenase and (b) to D-xylulose by a NAD-linked enzyme with lesser substrate specificity:

$$\text{Xylitol} + \text{NADP}^+ \rightleftharpoons \text{L-Xylulose} + \text{NADPH} + \text{H}^+ \quad (25)$$

and

$$\text{Xylitol} + \text{NAD}^+ \rightleftharpoons \text{D-Xylulose} + \text{NADH} + \text{H}^+ \quad (26)$$

Both the enzymes were prepared by a special technique from the insoluble portion of guinea pig liver mitochondria, and they are quite specific with respect to the requirement of pyridine nucleotide (H9, H11). However, dehydrogenases catalyzing reaction (25) with NAD as coenzyme have been reported (M11, S13, T3), thus confirming the importance of the source of the enzyme and the purification procedure employed.

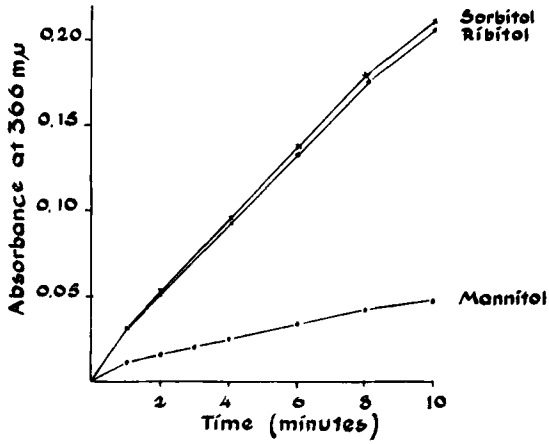

FIG. 8. NAD polyol dehydrogenase in sera of CCl_4-poisoned mice: sorbitol, ribitol, and mannitol oxidation (pH 9.6; polyols as substrates).

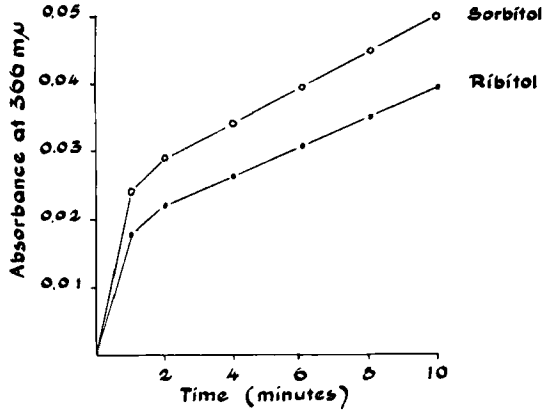

FIG. 9. NADP polyol dehydrogenase in sera of CCl_4-poisoned mice: sorbitol and ribitol oxidation (pH 9.6; polyols as substrates).

The evidence that polyol dehydrogenases can be considered as a group of different enzymes is supported by the finding that all reactions exhibit a specific stereochemical pattern (e.g., M11, S13, V3).

5.4. Metabolic Significance of Polyol Dehydrogenases

The role that polyol dehydrogenases play in metabolism has scarcely been elucidated. Approaches were made by investigating the metabolic fate of C^{14}-labeled sorbitol after oral and parenteral administration. It has been found that in normal animals $C^{14}O_2$ was expired following the administration of labeled sorbitol, while eviscerated, liverless subjects showed no $C^{14}O_2$ expiration under these conditions, thus confirming

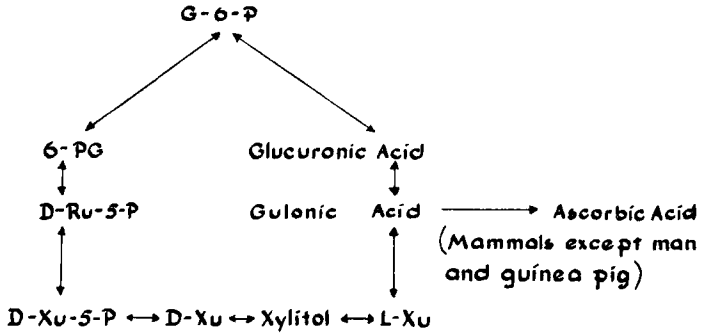

Fig. 10. The connection between the pentose phosphate cycle and the C-6 oxidation pathway.

the importance of liver enzymes for sorbitol metabolism (e.g., W12, W13). The probable route by which sorbitol is metabolized *in vivo* is its conversion to glucose by reaction (22).

Further evidence for the function of polyol dehydrogenases under physiological conditions was given by experiments on the mechanism of formation of seminal and fetal fructose (H3). It has been found that glucose labeled with C^{14} in the carbon-1 position was converted to fructose-1-C^{14} via reactions (22) and (23). The first step proceeds in the seminal vesicles or placenta, the second is effected by liver enzymes. These results were confirmed by the discovery of sorbitol in the glands as well as in the fetal blood of the ewe (H3). The over-all reaction in Eq. (24) could be inhibited by EDTA through inactivation of liver polyol dehydrogenases. Furthermore, polyol dehydrogenases catalyzing reaction (26) can be considered as a link between the pentose phosphate cycle and the formation of L-xylulose by the C-6 oxidation pathway of glucose metabolism (A11, H8, H11, H13). Figure 10 reflects the

probable reaction scheme. A specific xylulose kinase catalyzing the phosphorylation of D-xylulose by ATP to yield xylulose-5-phosphate has been described (S18). The suggestion that L-xylulose is a normal metabolic intermediate is confirmed by the observation of traces of L-xylulose in urine of healthy humans (H8, H14), and by the finding of increased L-xylulose excretion upon ingestion of D-glucuronolactone by pentosuric individuals, which has been proved by using C^{14}-labeled glucuronolactone and following the expiration of $C^{14}O_2$ (H8).

5.5. Assay Methods

The estimation of polyol dehydrogenase activities is principally carried out by manometric and spectrophotometric methods. In the former, the O_2 uptake by oxidation of substrate is measured, in the latter the reduction state of the appropriate pyridine nucleotide is determined by following the absorbance at 340 or 366 mμ. The spectrophotometric method may be preferred for the possibility it offers of following the reaction in both directions, i.e., with ketose and reduced pyridine nucleotide to the corresponding polyol, or with the alcohol and NAD (or NADP) to the respective ketose. In clinical chemistry, only the determination of NAD-linked sorbitol dehydrogenase has become usual. In our laboratories SDH activities are estimated using sorbitol as substrate and consequently following the increasing $NADH_2$ absorbance at 340 or 366 mμ. The pH optimum for the reaction in this direction was found to be pH 9.6 (cf. Fig. 7). The typical test mixture contains in cells of $d = 10$ mm in a final volume of 3.0 ml: 4×10^{-4} *M* tris-HCl buffer (1.0 ml of a 0.4 *M* solution), 3×10^{-6} *M* NAD (0.5 ml of a 0.006 *M* solution), 1.5×10^{-4} *M* sorbitol (1.0 ml of a 0.15 *M* solution) and 0.5 ml serum or homogenate in appropriate dilution, so that the change in A_s per minute does not exceed 0.02. The reaction is started by the addition of substrate, the blank containing water instead of substrate. For almost all clinical purposes the test can be performed at room temperature, although for kinetic studies it may be advisable to keep the reaction mixture constantly at 25°C by a thermostat. Under the conditions described curves were obtained which are strictly linear between the third and the eighth minute (cf. Figs. 8 and 9). An attempt to spare the blanks by preincubating the test cell for 10 to 20 minutes before the addition of substrate remained unsuccessful, since—especially in the estimation of human serum activities—no stable blank values could be obtained.

The determination of SDH activity by following the reverse reaction at pH 7.4 has been described by many investigators (e.g., G2, H12,

S6, S7, W14). An example of this kind of assay may be the following composition used in the "test combinations" now commercially available[2] and according to the work of Gerlach (G2): 1.75 ml triethanolamine-sodium carbonate buffer, pH 7.4, 0.2 M; 0.05 ml $NADH_2$, 0.015 *M*; 0.2 ml D-fructose, 3.33 *M*; 1.0 ml serum. The reaction is started by the addition of fructose solution after a preincubation period of one hour at 24°C or room temperature. After starting the reaction the decreasing $NADH_2$ absorbance will be followed at 340 or 366 mμ, the light path being 10 mm. No blanks are used in this method.

5.6. Clinical Implications of Polyol Dehydrogenases

5.6.1. *Serum SDH Activity in Disease States*

In blood or serum of healthy humans no SDH activity can be found. Since the polyol dehydrogenases are localized preponderantly in the liver cells, the appearance of SDH activity in serum should be attributed to liver cell damage. SDH has been designated as being highly liver specific (G2, G3, L1, S19, W21). In agreement with this conception SDH activity has been found in sera of patients suffering from hepatitis (B17, D2, G2, G3, J3, L1, S7, S19, W20, W21). Little SDH activity has been reported in obstructive jaundice, cirrhosis, nephritis, pancreatitis, and malignant tumors (L1, W21). Activity has also been found in the sera of some cases of myocardial infarction (G3, W21), thus posing the problem of its origin in these cases. It has been suggested (G3) that some acute disorders would lead to an increased permeability of damaged but surviving cells, thus releasing enzymes even from organs which had not been attacked directly. But, the observation of SDH activity in serum following myocardial infarction was connected in almost all cases with a protracted hemodynamic collapse (G3), thus leading to the supposition that the latter event directly caused hepatocellular damage. This would be in good agreement with the findings recently described (B23) that hypoxia caused by poisoning with carbon monoxide or low pressure will lead to the elevation or appearance of a number of serum enzymes due to the interruption of the electron transport sequence in biological oxidation, even if in this investigation SDH had not been particularly examined.

However, the high values of SDH activity in acute hepatitis make possible the differentiation of this disease from others (W21). The high level is found to be reduced within 2 weeks, thus representing convalescence.

[2] C. F. Boehringer & Soehne G.m.b.H., Mannheim, Germany, Article No. TC-N 15960.

Preliminary investigations on serum SDH activities in patients with carcinoma of the cervix uteri revealed a possible relation to malignant growth (R10). However, the increase of serum activity in cases of patients afflicted with carcinoma is much smaller than in cases of hepatitis.

5.6.2. *The Role of Polyol Dehydrogenases in Essential Congenital Pentosuria*

Pentosuria was classified as an inborn error of metabolism in 1908 by Garrod (G1). The harmless metabolic condition has been described almost exclusively in individuals of Jewish origin.

In pentosuria L-xylulose is excreted in urine, the daily amounts varying from 1 to 4 g (H8). The urinary excretion has to be distinguished from alimentary pentosuria in which other pentoses such as arabinose, xylose, or—to a lesser extent—D-ribose are found in the urine. However, traces of L-xylulose have been found in the urine of healthy individuals (cf. Section 5.4).

The excretion of L-xylulose in the amounts reported above suggests the accumulation of this compound in metabolism, which can be easily explained by the deficiency of NADP-linked xylitol dehydrogenase [cf. Eq. (8) and Fig. 10]. This suggestion is confirmed by the observation that the excretion of L-xylulose increases upon administration of D-glucuronolactone to pentosuric individuals, and proved by the finding that after uniformly C^{14}-labeled D-glucuronolactone had been given intravenously, only one-sixth as much of the isotope was recovered in the expired CO_2 as compared to normal subjects similarly treated (H8). This indicates the interruption of the linkage between the C-6 oxidation pathway and the pentose phosphate cycle at the stage of L-xylulose conversion to xylitol, since radioactive L-xylulose was found to be excreted under the conditions described.

ADDENDUM

I. *Cf. Section 2.8.3.3.*

After the preparation of this article, an extensive review of primaquine sensitivity was published [A. R. Tarlov, G. J. Brewer, P. E. Carson, and A. S. Alving, Primaquine sensitivity. *A.M.A. Arch. Internal. Med.* **109**, 209-234 (1962)]. The susceptibility to drug-induced hemolytic incidences was shown to be inherited by a gene of partial dominance carried on the X-chromosome. The mutant gene was reported to parallel the occurrence of *Plasmodium falciparum malariae.* The erythrocytes of affected individuals are not only deficient in G-6-PDH but also in GSH, thus suggesting their inability to regenerate $NADPH_2$. When the drug is given to deficient individuals, the partially degraded drug acts as an oxidant and oxidizes, e.g., HbFe++ and GSH to a larger extent than can be regenerated by the

function of $NADPH_2$-generating processes such as the conversion of G-6-P to 6-PG and then to Ru-5-P by G-6-PDH and 6-PGDH activities, respectively, known to be deficient in these cases. Thus the oxidized hemoglobin is denatured at the time of formation of Heinz bodies; intravasal hemolysis follows. This is in accordance with the work of Beutler (B8).

II. *Cf. Section 4.5.1.*

Recently an investigation of a variant of hereditary methemoglobinemia was published [P. L. Townes and M. Morrison, Investigation of the defect in a variant of hereditary methemoglobinemia. *Blood* **19**, 60-74 (1962)]. In this variant, methemoglobinemia arises through deficient glutathione synthesis and is not due to the lack or deficiency of either MHbR or the function of $NADH_2$- or $NADPH_2$-generating processes.

List of Abbreviations

In accordance with the recommendation of the Commission of the International Union of Biochemistry [R. H. S. Thompson, Classification and nomenclature of enzymes and coenzymes, *Nature* **193**, 1227 (1962)] the terms NAD and NADP have been used instead of DPN and TPN, except in Fig. 4. The generic term "nicotinamide nucleotides" is used with the same significance as pyridine nucleotides.

ACTH	Adrenocorticotropic hormone
A_s	Absorbance
ATP	Adenosine triphosphate
DOAP	Dioxyacetone phosphate
EDTA	Ethylenediaminetetraacetic acid
E-4-P	Erythrose-4-phosphate
FAD	Flavin adenine dinucleotide
FDP	Fructose-1,6-diphosphate
FMN	Flavin mononucleotide
F-6-P	Fructose-6-phosphate
GAP	D-Glyceraldehyde-3-phosphate
G-6-P	Glucose-6-phosphate
G-6-PDH	Glucose-6-phosphate dehydrogenase
GSH	Reduced glutathione
GSSG	Disulfide glutathione
GSSGR	Glutathione reductase
Hb	Hemoglobin
HbFe++	Hemoglobin
HbFe+++	Hemiglobin, methemoglobin
LDH	Lactic dehydrogenase
MDH	Malic dehydrogenase
MHb	Methemoglobin, hemiglobin
MHbR	Methemoglobin reductase
NAD	Nicotinamide adenine dinucleotide
$NADH_2$	Reduced NAD
NADP	Nicotinamide adenine dinucleotide phosphate
$NADPH_2$	Reduced NADP
6-PG	6-Phosphogluconic acid
RBC	Red blood cells

R-5-P	Ribose-5-phosphate
Ru-5-P	Ribulose-5-phosphate
SDH	Sorbitol dehydrogenase
SGOT	Serum glutamic oxalacetic acid transaminase
SGPT	Serum glutamic pyruvic acid transaminase
S-7-P	Sedoheptulose-7-phosphate
Tris	Tris(hydroxymethyl)aminomethane
Xu	Xylulose
Xu-5-P	Xylulose-5-phosphate

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