PROGRESS IN MEDICINAL CHEMISTRY

Volume 4

G. P. Ellis &
G. B. West

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PREFACE

THE present volume of five reviews provides up-to-date information on a wide variety of topics. The first two chapters are short and contain material about the mechanism of action of drugs in general; the third attempts to provide the latest ideas on the combination of drugs with cells at the subcellular level; and each of the last two chapters is a more detailed account of a specific type of drug.

The first chapter is devoted to the study of experimental hypersensitivity reactions in an attempt to determine some of the more important factors involved in human allergy. The severity of the reaction appears to be controlled in part by the glucocorticoid hormones of the adrenal cortex and hence results from changes in the metabolism of carbohydrates. The review on the mechanisms of toxic action, however, shows how little understood are these effects, how important is the species of animal used, and how results in animals cannot be translated in many cases to man.

At the present state of knowledge, any discussion on drug-receptor interactions must be highly speculative. We know much about the structure of drugs but very little about the receptor material in the cell with which the drug combines. The future isolation of the receptor substance is now so important. The interaction between these materials is discussed in the third chapter of the present volume.

We have allocated one chapter of nearly 100 pages to the synthesis, assay and clinical importance of various polypeptides because it appears that major advances in this field of medicinal chemistry are not far away. The importance of insulin, oxytocin and vasopressin is well known but not so much stress has been laid on the polypeptides, angiotensin and bradykinin.

The final chapter on the chemical aspects of analgesic drugs is complementary to that on the testing and development of these compounds which appeared in Volume 2. Major advances in the metabolism of analgesic drugs and in the pharmacology of analgesic antagonists have provided some insight into their mechanism of action, and the structure-action relationships are discussed in the present volume.

Lastly, we are grateful to reviewers and others for their encouragement and suggestions. Reviews take many months to compile and further months to edit and print so that delay between completion and publication is inevitable. Our thanks are due to the staff of Butterworths and to the authors, societies, and publishers for permission to use illustrations and tables in this and previous volumes.

G. P. ELLIS
G. B. WEST

February, 1965

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A. H. Beckett, D.Sc., Ph.D., F.R.I.C., F.P.S., and A. F. Casy, B.Sc., Ph.D., F.R.I.C., F.P.S., *School of Pharmacy, Chelsea College of Science and Technology, London, S.W.*3

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EXPERIMENTAL HYPERSENSITIVITY REACTIONS

P. S. J. SPENCER and G. B. WEST

INTRODUCTION

A SUBSTANTIAL proportion of the human race each year suffers to a varying degree from asthma, hay fever, food allergies, drug allergies, contact dermatitis, and other unpleasant kinds of allergic manifestations. The undesirable exogenous substance (termed the antigen) reacts with some form of endogenous antagonistic substance (termed the antibody), and a train of events suffered by these unfortunate individuals is started. Very little is known as to how damage is then caused to the cells of the body, and in fact there are still no concise definitions as to the general chemical nature of either antigen or antibody.

In the earlier studies in this field, the specific changes in the response of the individual concerned immunity, or the immune state which remains after recovery from a bacterial or viral disease. A peculiar sort of protein belonging to the globulin fraction of blood and combining with the undesirable substance was generally found in the plasma of the immune organism. These so-called antibodies were then shown to possess protective and curative properties. Later, antibodies were found to be formed not only against pathogenic micro-organisms and their products of metabolism but also against a great variety of both proteins and polysaccharides. Simple chemical substances are now known to be able to form complex antigens and induce the production of specific antibodies, and hence it is impossible to state that such antibodies have a protective or any other useful function.

After primary contact with the antigen, the response of the individual to renewed contact is so altered that peculiar local and sometimes general reactions may result. It is this state of specifically altered reactivity which is generally termed allergy. The immediate allergic reaction involves antibodies in the circulation and may have a mechanism similar to that involved in the anaphylactic reaction in animals. In this reaction, the animal has received a small dose of the antigen two or three weeks before a fairly large dose of the same antigen is given intravenously. The second dose of antigen combines with the antibodies located mostly in the tissues and the animal may die from anaphylactic shock. Many patients who react badly to aspirin, for example, seem to have had a history of an allergic disease such as asthma, and so may well be placed in this category. On the other hand, the delayed allergic reaction does not appear to be the initial reaction of antigen and antibody, as antibodies cannot be found in the free state in the plasma or tissues of such individuals. This type of response may have a mechanism similar to that involved in the anaphylactoid reaction in animals, where the first dose of antigen or antagonistic substance produces a delayed

severe allergic-like response. Many individuals who have not had a history of an allergic disease but possess some unexplained hypersensitivity to drugs such as the barbiturates, sulphonamides or salicylates may be included in this category of delayed reactivity.

Although allergy is usually assigned to the subject of immunology, its reactions are distinctly pharmacological in nature. The responses are complex and the symptoms include eczema, oedema, gastro-intestinal upsets, urticaria and asthma. The human skin is highly sensitive to capillary poisons and therefore furnishes a useful index of allergic hypersensitivity. A number of drug idiosyncrasies show such a striking likeness to most of the characteristics of protein idiosyncrasies (for example, food poisoning) and to allergic reactions generally that an intimate connection between these suggests itself. All involve only small doses of the irritant and all may involve similar tissues of the host. Whatever else the allergic reaction may do to cells, histamine is released from them and this release is one of the more important consequences of cell stimulation or injury. Many but not all of the profound local and systemic signs and symptoms of allergic reactions can be traced to the actions of the released histamine. Although histamine produces vasodilatation and increases the capillary permeability, two vascular effects often occurring in allergy, this is not the sole chemical agent released. Other agents to be considered are 5-hydroxytryptamine (5-HT), the slow-reacting substances (S.R.S.), and various kinins (for example, bradykinin).

STUDIES ON HISTAMINE

Histamine (I) is a natural constituent of many plant and animal tissues. It was first synthesized in 1907 by Windaus and Vogt[1] who prepared it by a Curtius degradation of histidine (II). The discoverers were unaware of the powerful biological properties of their compound and histamine aroused little immediate interest. Three years later, Barger and Dale[2] showed that it was a constituent of ergot, and Ackermann[3] found that putrefactive bacteria form histamine when incubated with the amino acid. Dale and Laidlaw[4] studied its pharmacological actions and confirmed its presence in the intestinal mucosa of mammals. These authors were impressed by the similarity between the immediate symptoms of anaphylactic shock and those evoked by large doses of histamine, and even in 1911 suggested that histamine plays a role in anaphylaxis.

During the 1920's, histamine was shown to be released in sensitization reactions and then found to play a major role in the 'triple response' of the skin (Lewis[5]). Some years later, mainly during the World War II, came the discovery and development of the antihistamine drugs. The diverse pharmacological actions of histamine, coupled with its unknown physiological significance, had stimulated the search for effective blocking agents and it was abundantly clear that such antagonists would be valuable research tools and might even be useful in therapeutics. Today, the range of clinically-effective antihistamines is probably wider than that of any other group of drugs.

Another major advance came with the discovery of the well-known histamine liberators. These are substances capable of freeing histamine from

the tissues without causing gross tissue damage. In 1939, it was shown[6] that crude curare releases histamine from muscles of the dog, and ten years later MacIntosh and Paton[7] published their classical paper on chemical histamine liberators. More recent work has shown that the property of releasing histamine is common to many simple compounds. The knowledge gained by the use of histamine liberators in animals has been considerable, and this has been surveyed by Paton[8] in a comprehensive review of the mechanism of histamine release.

The most recent contribution of outstanding significance to the study of histamine has been the finding of Riley and West[9] that the bulk of the histamine in a number of tissues is located in mast cells. Earlier work[10] established that there is a correlation between the heparin content of a tissue and its mast cell population, and later work by Asboe-Hansen[11] showed that the hyaluronic acid content of many normal and pathological tissues also parallels their mast cell content. Thus three substances—histamine, heparin and hyaluronic acid—are closely linked with the physiology of these cells. They may contain other highly-active agents (for example, slow-reacting substances), and their function remains a matter for speculation.

When tissues are injured, mast cells disrupt and release their contents into the tissue fluids. It is remarkable that the highest concentrations of histamine and mast cells generally occur at surfaces where the organism is in contact with the outside world (that is, the skin, the lungs and the alimentary tract). Paton[12] suggested that perhaps histamine is in these locations to produce vasodilatation and so reduce the pathogenicity of invading bacteria. It is significant that many mast cells are found at perivascular sites and the histamine they release acts by increasing capillary permeability. This in turn floods the tissues with a protein-rich oedema fluid and so assists in the mobilization of the fixed mesenchymal tissue and in the removal of foreign matter[13].

The wide distribution of histamine in animal tissues led to speculation about its origin. The presence of an enzyme in mammalian tissues capable of decarboxylating histidine to form histamine was first shown by Werle[14] in 1936, and later studies, particularly by Waton[15], illustrate that the distribution of this enzyme varies widely from species to species. It now appears that most of the tissue histamine is not in equilibrium with the main metabolic stream, as it reaches the body from various sources. Animals which do not make their own histamine probably absorb it from the gut[16]. Later studies by Waton[17] have supported this hypothesis.

The fact that animal tissues inactivate histamine was presented for the first time by Dale and Laidlaw[4] in 1911, and later it was found that there are marked species differences in the distribution of the enzyme termed histaminase[18]. This enzyme catalyses the oxidative deamination of histamine to 4-imidazolylacetic acid (III) which then appears in the urine as 1-ribosyl-4-imidazolylacetic acid[19]. It has been known for some time that the urine of many mammalian species also contains some free histamine and some in a conjugated form having properties similar to those of acetylhistamine[20,21] (IV). Since estimates of conjugated histamine in the urine depend largely on the diet, Gaddum[16] suggested that estimates of free histamine in the

urine are most likely to be of value as an index of the amount of histamine released in the whole body. Another major metabolic pathway in some species is methylation of the imidazole ring nitrogen[22]. Dogs for example use methylation as the principal means of inactivating injected histamine, and cats and man methylate both oral and injected histamine. The product formed is 1-methylhistamine (V) which is then in some species deaminated to form 1-methyl-4-imidazolylacetic acid (VI).[23,24] It is of interest that female rats and mice excrete much more free histamine than do the males of these species.

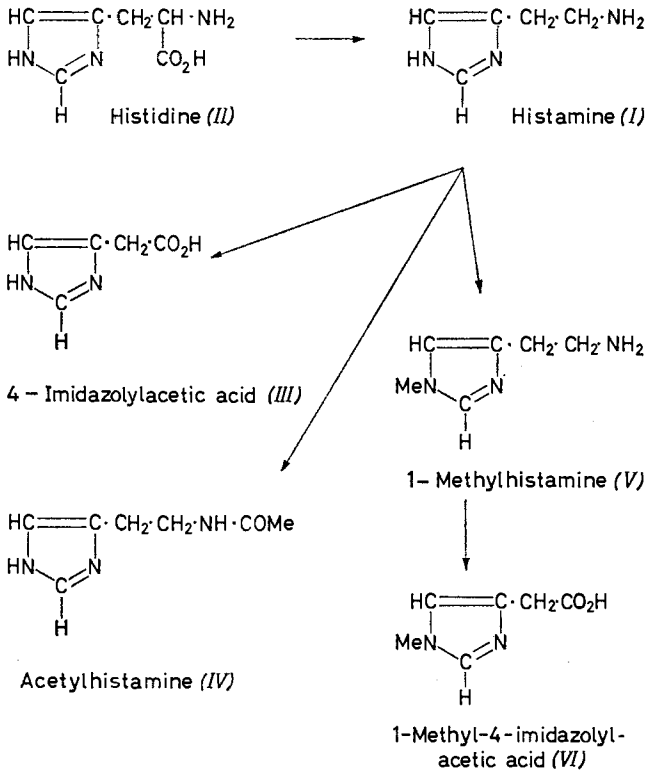


Figure 1.1. Principal pathways in the metabolism of histamine

STUDIES ON 5-HYDROXYTRYPTAMINE

The history of 5-HT covers no more than 15 years and the compound has been referred to under many names. These include vasoconstrictine, vasotonin, spatgift, thrombocytin, thrombotonin, enteramine and serotonin. The largest quantities of 5-HT are found in the gastro-intestinal tract where it is held chiefly in enterochromaffin cells in the mucosa. In the blood, 5-HT is contained mostly in platelets where it appears to be bound to adenosine triphosphate. The amounts of 5-HT in platelets of different species vary enormously, there being for example 15 times more in those of the rabbit than in human platelets. Considerable amounts are also to be found in the

spleen, and in the amygdala, hypothalamus and mid-brain areas of the central nervous system. The only other considerable source of 5-HT in mammals is in the mast cells of the rat and mouse. It is the mast cells which give the skin of these two species their high 5-HT content. Venoms and sting fluids such as those of the wasp, scorpion, toad, octopus, stinging nettle and cowhage also contain much 5-HT.

The biological synthesis of 5-HT was largely worked out[25] in 1957 and its course is indicated in *Figure 1.2.* Synthesis starts from tryptophan (VII)

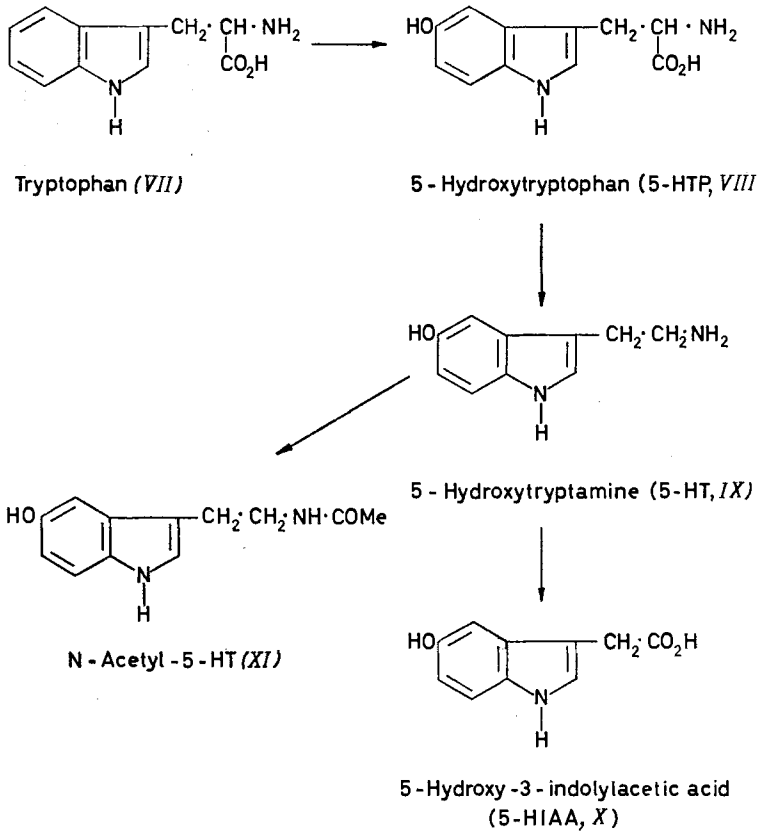


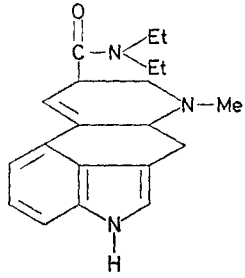
Figure 1.2. Principal pathways in the metabolism of 5-hydroxytryptamine

and this is hydroxylated into 5-hydroxytryptophan (VIII) although little is known of the sites in the body at which this stage occurs. The decarboxylating enzyme responsible for the formation of 5-HT (IX) from 5-HTP (VIII) is widely distributed and its activity in many tissues is considerable. Pyridoxal-5-phosphate is its co-enzyme since 5-HT synthesis is markedly depressed in pyridoxal deficiency. 5-HT is easily oxidized in the body to 5-hydroxy-3-indolylacetic acid (X) which is excreted in the urine. The other principal metabolite is N-acetyl-5-HT (XI) which may account for up to 25 per cent of the total.

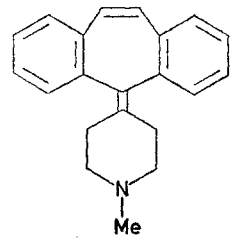
The changes in the circulation after the intravenous injection of 5-HT

into experimental animals are complex and have not been completely analysed. On the respiratory system, hyperpnoeic and apnoeic responses may both be seen. Generally, smooth muscle of mammalian origin is contracted by 5-HT. Evidence that 5-HT plays a physiological role in peristalsis was obtained when Bülbring and Crema[26] found that 5-HT is released from the intestinal wall into the lumen of the gut and that this release was related to the intra-luminal pressure both in the isolated preparation and in the living animal. When 5-HTP is injected into mammals, there is a rise in the 5-HT content of the brain, liver, heart and blood, and this is accompanied by tremors, ataxia, lachrymation and diarrhoea. Changes in hydroxyindole metabolism or in the concentration of 5-HT in the blood have been found in the malignant carcinoid syndrome, in some forms of mental deficiency, and in some blood diseases. Carcinoid tumours may develop anywhere along the gastro-intestinal tract and in such cases there is an increased excretion of both 5-HT and 5-HIAA in the urine. Phenylketonuria is an inherited condition due to a recessive gene and is associated with mental deficiency usually of a gross degree. There is a failure to oxidize phenylalanine to tyrosine which leads to a high blood phenylalanine concentration and to the formation and excretion in the urine of a number of products of phenylalanine metabolism normally not formed or only formed in small amounts. Of these, the ones found in largest amounts are phenylacetic, phenylpyruvic and phenyllactic acids.

Much work has been done using drugs with actions antagonistic to those of 5-HT and the subject has been reviewed[27]. At first it was considered that all antagonists must contain the indole structure (for example, lysergic acid diethylamide, XII) but recently compounds without such a structure have been shown to be potent antagonists (for example, cyproheptadine, XIII).



Lysergic acid diethylamide *(XII)*



Cyproheptadine *(XIII)*

The injection of reserpine into an animal is followed by a large increase in the excretion of 5-HIAA (X) in the urine and a loss of 5-HT (IX) from sites where it is normally found. This type of treatment has been extensively used in studying the role of 5-HT in allergic and hypersensitivity reactions. Humphrey and Jaques[28] were the first to observe in 1954 that 5-HT was released with histamine from rabbit platelets during the antigen-antibody reaction, and other workers have found that the urinary excretion of 5-HIAA

is increased after anaphylactic shock in rabbits[29]. In rats, however, anaphyaxis occurs even after the animals have been treated with reserpine to remove 5-HT from the brain, gastro-intestinal tract, platelets, spleen and skin, so there may be considerable species variation in the importance of 5-HT.

The functions of histamine and mast cells are closely related and the location of some 5-HT in the skin mast cells of the rat and mouse suggest that there may also be a function of 5-HT linked with that of mast cells and histamine. In these two species, 5-HT is many times more potent than histamine in increasing capillary permeability[30] and it is possible that at least in these two species 5-HT takes over the postulated defence role of histamine in other species[31].

STUDIES ON BRADYKININ

In 1949, Rocha e Silva, Beraldo and Rosenfeld[32] described the release of an active peptide from serum globulin by trypsin or snake venoms. They named the peptide bradykinin, because it caused a relatively slow contraction of the isolated guinea-pig ileum. These workers distinguished this substance from acetylcholine, histamine, adenosine and other active substances by a series of biological tests. For example, bradykinin contracts most isolated smooth muscle preparations such as the intestine of the guinea-pig, rabbit, cat and dog but relaxes the duodenum of the rat. It is also a powerful vasodilator and markedly increases capillary permeability. The isolated rat uterus, though one of the most sensitive test preparations *in vitro*, is practically unaffected by bradykinin *in vivo*[33]. Bradykinin produces bronchoconstriction in the guinea-pig but fails to contract isolated bronchial muscle of dog or man. It produces pain when applied to a blister base on human skin[34]. It is of interest that the bronchoconstrictor action of bradykinin in the guinea-pig is specifically suppressed by relatively small doses of acetylsalicylic acid, phenylbutazone and amidopyrine[35].

Bradykinin is a nonapeptide[36], although the early studies indicated that it was an octapeptide[37]. There are many other kinins which develop in plasma under a variety of conditions, but all seem to be derived from α_2-globulins. They are of considerable interest from the pharmacological point of view since their biological potencies are often as great as those of acetylcholine or adrenaline. The recent progress in the isolation and synthesis of these peptides however will undoubtedly accelerate the discovery of their significance in physiology and pathology. It is possible that bradykinin-like material is formed during the antigen-antibody reaction in some species and antagonists will also help in determining its role in pathology.

THE ANAPHYLACTOID REACTION

In the course of their experiments with egg-white, Parker and Parker[38] reported difficulty in separating the symptoms due to anaphylaxis from those due to the immediate toxicity of the protein. The rats reacted in a similar way after the initial dose and after the challenging injection of antigen, showing mild congestion of the lungs and intestines. Later, Selye[39] noticed a peculiar reaction in rats after the first intraperitoneal injection of fresh egg-white. This was characterized by oedema and hyperaemia especially of the face and extremities. A more thorough investigation of the

reaction[40] showed that the rat is naturally hypersensitive to egg-white and the symptoms attributed to anaphylaxis by previous workers might well have been due to this hypersensitivity state. However, even after large doses of egg-white, no fatalities were reported and the response was termed an anaphylactoid reaction.

Egg-white is not the only substance to elicit this reaction in the rat. A similar response is given with dextran, a high molecular weight polymer of glucose[41], and other examples are glycogen, globin, kaolin, yeast, hyaluronidase and bradykinin. The anaphylactoid reaction is readily elicited on intravenous, intraperitoneal or intrapleural injection of the agents. Small amounts of dextran injected subcutaneously also produce a marked local reaction in the paws and in distant shock organs. Selye[39] suggested that a combination occurred between dextran and the tissues of the shock organs and this was responsible for the anaphylactoid reaction in regions remote from the local injection site. After a single intraperitoneal injection of dextran or egg-white, the rat develops violent scratching especially of the face, and the pruritus soon becomes generalized. Vasodilatation and oedema of the snout and paws follow to reach a maximum about 90 minutes after injection. The symptoms then regress and by 6 hours nothing remains but patches of hyperaemia. The anaphylactoid reaction is now accepted as an acute inflammatory reaction elicited in the rat.

The role of histamine in the anaphylactoid reaction has been indicated by many workers[42–44], yet the symptoms observed are not reproduced in full by injections of histamine, and relatively large doses are necessary to elicit local reactions in the rat. In most areas of skin of the rat, Parratt and West[45] found that there is a relationship between the histamine content and mast cell count on the one hand and the 5-HT content on the other, and 5-HT was considered as another amine involved in the anaphylactoid reaction. Previously, Rowley and Benditt[46] reported that egg-white and dextran each released both histamine and 5-HT from rat skin. Furthermore, the intraperitoneal injection of egg-white or dextran was shown to produce the full anaphylactoid reaction even after the histamine content had been lowered to minute amounts. Depletion of tissue 5-HT, on the other hand, prevented the onset of the full reaction to these substances. Parratt and West[45] also detected small amounts of 5-HT in the oedema fluid but no histamine. Treatment with antagonists of 5-HT prevented the reaction whereas treatment with specific antihistamines such as mepyramine had no effect. Lastly, 5-HT is more than 100 times as effective as histamine in increasing capillary permeability in the rat[47]. However, whilst 5-HT may be more important than histamine in the anaphylactoid reaction, neither amine when injected alone fully reproduces the reaction and it is possible that other substances such as heparin[10], hyaluronic acid[11], bradykinin[37] and slow-reacting substances[48] are involved.

The anaphylactoid reaction in rats has recently been re-investigated by Harris and West[49], since these authors found that more than one-fifth of the rats secured from one colony failed to react to dextran and egg-white, no matter by what route or in what dose they were given. The skin of rats resistant to dextran and egg-white (the non-reactors) was not deficient in either histamine or 5-HT, and these rats were not less sensitive to injected

histamine and 5-HT than were the reactor rats. Although dextran released no histamine from the perfused hind quarters of the non-reactors, more powerful histamine liberators such as polymyxin B were effective. Procedures which inhibited the reaction in reactor rats included the production of alloxan diabetes and the pretreatment with glucose[50] or 2-deoxyglucose[51] but non-reactors were not diabetic and they had no glycosuria. Procedures which enhanced the reaction in reactor rats did not change the resistance of non-reactor rats. It was also noted that anaphylaxis was induced both in guinea-pigs using non-reactor serum as antigen and in non-reactor rats using horse serum as antigen. This result illustrates that rats resistant to egg-white and dextran produce antibodies to foreign protein and also that their serum acts as an antigen in a heterologous species. Consequently anaphylaxis may occur in a rat which does not show the anaphylactoid response. Guinea-pigs and rabbits are similar in this respect as the anaphylactoid reaction has never been found in these two species.

The combination of dextran or egg-white with a blood or tissue component necessary to effect the release of histamine and 5-HT may not occur in non-reactor rats. This component may be an enzyme, a metabolic product or an antibody. Since dextran reactivity is closely linked with carbohydrate transport as exemplified by the action of glucose and insulin and by the work of Beraldo, Dias da Silva and Lemos Fernandes[52] with sugars other than glucose, an abnormal metabolic intermediate may be formed. Alternatively, if an antigen-antibody reaction is involved in the production of the anaphylactoid response[53], then it is likely that this antibody to dextran is lacking in non-reactor rats. This non-reactivity has been found only in the Wistar strain of rat but it can be outbred into other strains. Recent work shows that this character is controlled by an autosomal recessive gene[54]. This important finding may have a counterpart in human allergy which is usually of an hereditary character. The mechanism by which the non-reactivity property in rats is brought about has not so far been established.

INFLUENCE OF THE ADRENAL GLAND

Dale and Richards[55] were the first workers to draw attention to the possibility of an antagonism between adrenaline and histamine and to suggest that a function of adrenaline is to maintain capillary tone against the depressant action of histamine and other products of cellular injury and metabolism. Later, Dale[56] showed that adrenalectomy in the cat renders it several times more sensitive to the effects of histamine. In the rat, adrenalectomy lowers the resistance to both histamine[57] and anaphylaxis[58]. Some years later, Perla and Gottesman[59] showed that this hypersensitivity in adrenalectomized rats is reversed by adrenaline or by adrenal cortical extracts. These results have been repeatedly confirmed and the protection has been found to be due to the gluco-corticoids and not to the mineralo-corticoids.

In a similar manner, adrenalectomy profoundly increases the intensity of the anaphylactoid reaction. This was first shown by Selye[39] and confirmed later by other workers[60–62]. Egg-white or dextran when injected into such rats elicits within minutes the severe symptoms of shock and vascular collapse, followed by death. Repeated large doses of cortisone protect adrenalectomized rats against the lethal effects of dextran[63], and often acute

pre-treatment with adrenaline is effective[61]. Treatment with ACTH for several days prior to challenge also inhibits the reaction in intact animals but is ineffective in adrenalectomized rats. Both the adrenal cortex and the adrenal medulla therefore secrete hormones which increase the resistance of the intact rat to dextran, egg-white, histamine and anaphylaxis.

In 1938, Rose and Browne[64] showed that injected histamine was less readily inactivated by rats after adrenalectomy, an effect which was counteracted by adrenal cortical extracts. The ability of the rat to inactivate exogenous histamine therefore appears to be influenced by changes in the level of adrenal cortical secretion. Endogenous histamine is similarly affected and after adrenalectomy the histamine content of various rat tissues is markedly raised[65–69]. Tissue levels of 5-HT are also raised after adrenalectomy[67], effects which are overcome by giving cortisone (but not deoxycorticosterone, a mineralo-corticoid). In 1961[70], a marked reduction in the skin histamine and 5-HT levels in the rat was reported after the administration of some new synthetic gluco-corticoids. Those compounds possessing the greatest gluco-corticoid activity were the most active whilst the mineralo-corticoids were inactive.

The formation and binding of new histamine in rat abdominal skin was shown by Schayer, Davis and Smiley[71] to be controlled by the adrenal cortex. Using radioactive histidine and the tracer techniques, these workers found that the rate of binding of new histamine is strongly inhibited by cortisone and increased after adrenalectomy. Telford and West[72] investigated the histidine decarboxylase activity of rat tissues and found that the liver has a far greater histamine-forming capacity than any other tissue, suggesting that this tissue in the rat supplies the histamine requirements of most of the body. Gluco-corticoids depressed the liver histidine decarboxylase activity in amounts sufficient to account for the depletion of histamine from the skin and lungs. Cass and Marshall[73] later confirmed the effects on the tissue histamine and 5-HT levels but attributed these changes to an action on the uptake and storage of these amines and not to a depression of synthesis. It is clear, however, that the adrenal cortical secretion, which is mainly corticosterone in the rat and thus gluco-corticoid in nature, exerts a functional control over the general metabolism of histamine and 5-HT, and this property may explain the therapeutic effect of gluco-corticoid hormones in allergic and inflammatory reactions in other species including man.

INFLUENCE OF THE THYROID GLAND

It is well known that thyroxine potentiates anaphylaxis in most animal species and thyroidectomy reduces the severity of the shock[74]. There is also considerable evidence to show that the thyroid hormones influence histamine metabolism in the rat. Gotzl and Dragstedt[75], for example, found that the removal of the thyroid gland decreased the histamine content of rat tissues whereas injections of thyroid extract increased them. A similar trend in skin histamine levels has been noticed by Feldberg and Loeser[76]. On the other hand, Arvy[77] showed that rats made hypothyroid by feeding with thiouracil showed a marked increase in the numbers of tissue mast cells and thus by implication the histamine content was raised. The literature is thus confusing on this point.

On the anaphylactoid reaction in rats, Leger and Masson[60] showed that the mild oedematous response after egg-white changed to a severe one resulting in death when the animals had received thyroxine. Thyroidectomy produced the opposite effects. More recently, Parratt and West[30] showed that, besides egg-white, doses of dextran, polymyxin B and compound 48/80 produce severe shock and death in thyroxine-treated rats. A pattern of events similar to that found in adrenalectomized animals is recorded, and microscopical examination of the submucosa of the jejunum revealed extensive haemorrhagic lesions. Oedema extended up to the villi where the epithelial cells showed desquamation, just as is seen in anaphylaxis in this species[78]. Parratt and West also found that rats under thyroxine treatment became much more sensitive to histamine and 5-HT, amines liberated in both anaphylaxis and the anaphylactoid reaction. The rats excreted more free histamine and the histaminase activity of the intestine was markedly reduced. Thus the greatly enhanced anaphylactoid reaction in thyroxine-treated rats is probably due to the marked increase in sensitivity to histamine and 5-HT (about 30-fold) and an impaired ability to inactivate these amines.

Later work by Spencer and West[79] showed that daily subcutaneous injections of either thyroxine or tri-iodothyronine greatly increased the severity of the anaphylactoid reaction in both male and female rats. Males proved to be more sensitive than females, whilst tri-iodothyronine was more potent in this respect than was thyroxine. The degree of oedema, as measured by a plethysmographic apparatus[80], did not increase although the speed of oedema formation was markedly accelerated in thyroxine-treated rats. A peak time of sensitivity occurred at 14 days in males and 17 days in females when treated with thyroxine sodium. Despite further treatment, the severity of the anaphylactoid reaction declined towards control levels, suggesting that some internal compensatory mechanism had become effective. The adrenal cortex and its secretions were thought to be involved.

Various aspects of histamine and 5-HT metabolism were therefore examined after making the rats hyperthyroid by injecting tri-iodothyronine. Marked increases in the sensitivity of the tissues to histamine and to 5-HT were found, as with thyroxine treatment, and the rate of removal of dextran-liberated histamine was partially reduced. Intestinal histaminase levels were somewhat lowered and tissue histamine contents were temporarily raised. The increase in the severity of the anaphylactoid reaction after tri-iodothyronine treatment thus appears to be mainly the result of the increased sensitivity of the tissues to the amines released by the anaphylactoid agents.

When hypothyroidism was induced in rats by thyroidectomy or by feeding antithyroid drugs (for example, methylthiouracil), the severity of the anaphylactoid reaction was reduced. This also was only temporary and within 14 days the sensitivity to dextran had returned to control levels. Again, as with hyperthyroidism, an internal compensatory mechanism was considered to be operating.

The mouse, like the rat, is resistant to the systemic effects of histamine and 5-HT, but this resistance is lowered by pre-treatment with *Haemophilus pertussis* vaccine[81,82] or by adrenalectomy[83]. Spencer and West[84] found that daily doses of thyroxine increased the sensitivity of mice to both histamine and 5-HT, coupled with increases in tissue amine levels and decreases in the

intestinal histaminase activity and in the rate at which histamine disappeared from the blood. Of greatest significance, however, was the temporary nature of the changes. As in the rat, alterations in amine metabolism reached a peak after which, despite continued treatment, the animals returned to their original sensitivity.

In both rats and mice, thyroxine and tri-iodothyronine produced marked adrenal hypertrophy which was confined to the cortex. In the rat, the increase was significant after some 14 days of treatment at a time when the severity of the anaphylactoid reaction was at a peak. Further treatment resulted in further adrenal enlargement and a reduction in the enhanced anaphylactoid sensitivity. To show that increased corticosteroid secretion was responsible for this compensation, hydrocortisone or prednisolone was given simultaneously with the thyroid hormone and there was no increase in sensitivity to dextran or to histamine. Adrenalectomy on the contrary increased the sensitivity of the rat to dextran and hypothyroidism did not modify this effect. That is, hypothyroid adrenalectomized rats were as sensitive to dextran as were euthyroid adrenalectomized animals. The induction of the hypothyroid state before or after adrenalectomy in mice similarly did not prevent the increase in histamine or 5-HT sensitivity found in adrenalectomized mice.

Telford and West[85] suggested that there is a relationship between the gluco-corticoid activity and the anti-anaphylactoid activity of adrenal steroids, since mineralo-corticoids are ineffective in this respect. Hydrocortisone and prednisolone, two potent gluco-corticoids, reduced the severity of the anaphylactoid reaction and masked the potentiating effect of thyroxine. Cortisone was less effective[79]. Thus cortical hypertrophy is an important aspect of the thyroid hormone action.

The view is no longer held that degradation to tri-iodothyronine is essential for the biological activity of thyroxine[86]. Nevertheless, glucocorticoids may alter the penetration of the thyroxine into cells, though this does not explain why the effects of tri-iodothyronine are reduced after chronic administration. It also offers no explanation for the initial compensatory release of adrenocorticotrophic hormone (ACTH) from the hypophysis or for the adrenocortical hypertrophy. The observation that hypothyroid-adrenalectomized rats are as sensitive to dextran as untreated adrenalectomized animals, together with the report[87] that rats are not protected from the histamine-sensitizing effects of adrenalectomy by thyroidectomy, suggests that the level of thyroid hormone is not directly responsible for the level of tissue sensitivity to histamine and 5-HT.

It is more logical therefore to suggest that the tissue levels of adrenocorticosteroids are more important than the level of the thyroid hormones in determining the sensitivity of rats to agents like dextran, and the effects produced by thyroxine and tri-iodothyronine may be mediated through an effect on the adrenocortical hormones. The effects of cortisone administration have already been shown[88] to be modified by the simultaneous induction of a hyperthyroid state, an increased utilization and more rapid destruction, inactivation and excretion of the steroid being suggested. This may be true, since an increased rate of cortisone removal occurs in hyperthyroid rats[89,90].

In the light of these results, the following hypothesis is put forward to

explain the mechanism whereby thyroxine and tri-iodothyroxine increase the sensitivity of rats to dextran, egg-white, histamine and 5-HT. Most important is the tissue level of corticosteroids since this determines the level of sensitivity. The daily administration of thyroid hormone results in an increased rate of steroid breakdown in the liver and a state of partial adrenocortical insufficiency is produced. This is the factor which is responsible for the increase in dextran, egg-white and amine sensitivities. However, the fall in plasma corticosteroid results in a compensatory release of ACTH from the pituitary gland; the adrenal responds by hyperactivity and hypertrophy. This compensatory activity increases until it overcomes the thyroxine or tri-iodothyronine effects; despite the increased ability of the liver to inactivate corticosteroids, the output of steroid is then sufficient to maintain normal levels of tissue steroid and the various effects on histamine and 5-HT metabolism are reversed. This hypothesis explains the following observed results: (1) the effects of thyroxine and tri-iodothyronine are transient; (2) the effects of thyroxine are inhibited by the simultaneous administration of gluco-corticoid; (3) hypothyroidism results in a depressed response due to a temporary accumulation of corticosteroid as the liver's steroid-reductase activity is reduced, but the decreased ACTH output and consequent reduction in steroid output (signified by adrenal atrophy) leads to a return to normal of tissue steroid level and sensitivity to dextran and egg-white; (4) adrenalectomized rats made hypothyroid show as high a level of sensitivity as do intreated adrenalectromized animals, due possibly to the lack of perpipheral corticosteroid; and (5) female rats, possessing larger adrenal glands than males, show greater hypertrophy during thyroxine treatment, and consequently are less sensitive to dextran.

INFLUENCE OF INSULIN

Gluco-corticoids and insulin exert opposite effects on carbohydrate metabolism, and this antagonism may therefore extend to some inflammatory processes such as the anaphylactoid reaction. In 1957, it was shown[91] that a single dose of insulin rendered rats more sensitive to the inflammation produced by injections of egg-white and dextran (the anaphylactoid reaction) Later in that year, other authors[92] showed that alloxan-diabetic rats did not react to dextran unless they had received previously a dose of insulin. It appeared that the blood sugar level was a factor determining the presence or absence of the anaphylactoid response. Other chemical histamine liberators were unaffected by the diabetic state or by insulin and released the amine. However, the subsequent injection of dextran or egg-white after treatment with chemical histamine liberators was ineffective.

One of the actions of insulin is to promote the entry of various sugars into tissue cells and it is likely also that it promotes the entry of large molecular substances such as dextran or ovomucoid, particularly if they contain sugar components. Thus insulin may speed up the access of dextran and ovomucoid to those mechanisms which are concerned in the anaphylactoid reaction. The latest work on this topic[93] shows that an active amine-releasing intermediate plays an important role in the anaphylactoid reaction. Dextran reactivity and insulin are so closely linked with carbohydrate transport that the target organs may be made more sensitive to the active

materials by virtue of a change in the permeability of the endothelial cells in the tissues as a result of hypoglycaemia. The insulin effect is not mediated through the nervous system since hypersensitivity to dextran occurs when the spinal nervous pathways are interrupted[94].

Goth[51] found that 2-deoxyglucose inhibited the anaphylactoid reaction in rats but did not affect the oedema reaction of synthetic chemical histamine liberators. The action of 2-deoxyglucose was prevented by pre-treatment with insulin, again pointing to the important role of glucose permeability in certain inflammatory states. Insulin was thought to diminish in several ways the amount of free glucose, leaving the transport mechanisms more free to carry the dextran across cell barriers in larger amounts to the sites of anaphylactoid inflammation. The formation of a histamine-releasing substance after the *in vitro* incubation of dextran and rat serum has also been shown[53] to be prevented by an excess of glucose, so dextran may combine with components of both serum and tissues. Further, the simultaneous injection of a large amount of glucose prevents the dextran reaction in the whole animal[95].

The course of anaphylaxis is also greatly modified by insulin. For example, Sanyal, Spencer and West[96] sensitized rats to egg-white and injected insulin a short time before challenge with the antigen. Anaphylactic shock was greatly increased. Formation of antibodies was not affected but the animals were rendered more sensitive to the antigen-antibody reaction or to the products of this reaction. The potentiating effect of insulin on the anaphylactoid reaction and on anaphylaxis may thus be similar to that of the thyroid hormone or of *Haemophilus pertussis* vaccine, or when the animal is adrenalectomized. Hypoglycaemia is a factor common to these different procedures and may be accelerating the absorption or distribution of egg-white or dextran.

DISCUSSION

Comparatively little is known about the influence of the endocrine glands on the course of allergic diseases in man although there have been many attempts to establish a relationship between their secretions and various hypersensitivity reactions. On the other hand, numerous experiments support the contention that an endocrine effect is involved in anaphylaxis in experimental animals. The anaphylactoid reaction in the rat closely resembles anaphylaxis in this species both in regard to severity and symptomatology. Although preliminary sensitization is not necessary, it may be that a combination between the challenging agent and some blood globulin occurs before the mediators of the reaction are released.

The severity of the anaphylactoid reaction after treating the rat with thyroxine has now been followed at various intervals and maximal sensitivity has been obtained after a characteristic length of treatment. When the dose of thyroxine is increased, the severity of the reaction occurs more quickly. Tri-iodothyronine is more active than thyroxine in this respect, just as it is in effects on metabolic activity. The tissues of the rat first become more sensitive to the amines released during the anaphylactoid reaction and later as thyroid treatment continues they become less sensitive. Thyroxine also increases the histamine and 5-HT sensitivity in the mouse and raises the

tissue levels of these amines. The effects are transient and disappear despite continued treatment with thyroxine. Adrenocortical hypertrophy may be the cause of this return to the control levels in both the rat and the mouse. It is postulated that the changes are brought about by enzyme effects in the liver, for in hyperthyroid animals this organ is capable of inactivating increased amounts of corticosteroids. Thus, for a few days until compensatory adrenocortical hypertrophy occurs, the animal is in a state of partial adrenocortical deficiency, and it is during this time that alterations in the metabolism of histamine and 5-HT occur. Two experiments in particular confirm these views. Firstly, small doses of gluco-corticoids prevent the effects of thyroxine on the anaphylactoid reaction. Secondly and of more importance, animals deficient in both adrenocortical hormones and thyroid hormones are as hypersensitive to dextran as animals deprived only of their adrenocortical hormones.

There is evidence that changes in thyroid activity in man are also accompanied by changes in the severity of certain allergic states. For example, Curschmann[97] reported that two patients who suffered intermittent Grave's disease invariably exhibited asthmatic attacks coincidentally with the more pronounced thyrotoxic symptoms. More recently, Waldbott[98] described how thyroidectomy of asthmatic patients with hyperplastic thyroids cured the asthma, and other workers have cured asthma by irradiating asthmatics with enlarged thyroids. Hyperthyroid patients also tend to suffer severe attacks of urticaria and alleviation of the thyroid disease generally brings about a distinct improvement in the allergic tendency. Thus in various types of allergy the condition becomes exaggerated when the patient develops a hyperthyroid state. There is of course no proof from these clinical observations that the hyperthyroid state is responsible for establishing the allergic reaction.

In guinea-pigs, it has been shown[99] that passive anaphylactic arthritis is potentiated by the prior administration of thyroxine or the thyroid-stimulating hormone of the pituitary. The antibody titre is similar in both situations so it must be an effect of the thyroid hormone on the shock organs, namely the capillaries. The effects of hyperthyroidism and of adrenalectomy on the sensitivity of rats and mice to histamine and 5-HT are probably brought about by a similar process. At the shock organs, it is possible that changes in carbohydrate metabolism occur both in experimental animals and in man. A very low incidence of asthma and allergic symptoms has already been reported[100] among diabetic patients and in fact hyperinsulinism may be necessary for the appearance of asthmatic attacks.

From this survey, the conclusion is reached that in laboratory animals and probably also in man, the severity of some hypersensitivity reactions is controlled by the gluco-corticoid hormones of the adrenal cortex. Changes in the carbohydrate metabolism appear to be one of the more important factors involved in allergy. In states of gluco-corticoid deficiency, there is an increase in the sensitivity of the blood capillaries and tissues to mediators of these hypersensitivity reactions. The thyroid hormones probably exert their potentiating effects by inducing a deficiency of gluco-corticoid secretion or utilization.

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2

MECHANISMS OF TOXIC ACTION

J. M. Barnes and G. E. Paget

INTRODUCTION

In the context of modern knowledge, the toxic action of a drug or of any other chemical with poisonous properties should ultimately be described in biochemical terms to explain the physiological disturbances which are observed during life, the pathological lesions revealed at necropsy and the subsequent microscopic examination of the tissues.

At the outset, it must be emphasized that anything approaching this ideal has been attained hitherto in very few studies of toxic compounds. Interesting facets of the whole picture are available in a few instances and some of these will be discussed in detail whether or not they refer specifically to the action of drugs. As this chapter is primarily concerned with mechanisms, no attempt will be made to list or describe all the known toxic actions of drugs. An attempt at selection has been made to illustrate both the general problem of understanding mechanisms of toxic action and the peculiar problems presented by the toxic action of some drugs.

BIOCHEMICAL BASIS OF SOME TOXIC ACTIONS

Arsenic has long been known to be poisonous and certain organic arsenicals apparently exert a selective toxic action on spirochetes and trypanosomes so that it is possible to use them effectively as drugs. However, the basis of the toxic action of arsenic was not understood until work on the chemical warfare agent lewisite by Peters provided an explanation[1]. Trivalent arsenic was shown to inhibit the oxidation of pyruvic and other keto acids and it was believed to do this by reacting irreversibly with —SH groups. The therapeutic action of dimercaptopropanol (BAL) by its superiority over other sulphydryl-containing molecules suggested that arsenic attached itself to two vicinal thiol groups[2]. This was ultimately shown to be the case by Gunsalus[3] who identified lipoic acid as the co-enzyme in α-keto-acid oxidation that was rendered inactive by the attachment of arsenic. The oxidation of pyruvate is an essential step in the oxidative pathways and its inhibition may produce local tissue injury or evidence of more general poisoning. The general action is illustrated by a decreased ability to remove pyruvate from the blood stream in the pyruvate tolerance test.

Many poisonous substances react readily with thiol groups but they do not necessarily produce the same type of injury as trivalent arsenic. An assumption that they are poisonous solely because of their ability to react with thiol groups present in abundance everywhere, cannot be made until the component with which they react has been positively identified as in the case of lipoic acid.

Fluoroacetates were developed as potential chemical warfare agents and

a study of their mode of action showed that they were treated initially like acetate by the enzymes in the tricarboxylic acid cycle but when fluorcitrate was formed this could not be attacked by aconitase. The metabolic cycle was blocked and citrate accumulated. A failure of this vital metabolic pathway leads to the death of the animal, though species vary greatly in their sensitivity to fluoroacetate and while some die after convulsions others apparently die from acute heart failure[4].

2,4-Dinitrophenol and the related 2,4-dinitro-6-methylphenol (2,4-dinitro-*o*-cresol, DNOC) were found 30 years ago to stimulate metabolism and had a short-lived vogue as slimming agents until a number of deaths and cases of cataract led to their being discarded. Their effect on metabolism was not understood until Loomis and Lipmann[5] showed some 12 years later that they uncouple oxidative phosphorylation. Normally in the course of consuming one atom of oxygen, mitochondria incorporate three atoms of phosphorus to form three moles of adenosine triphosphate (ATP) which is needed for so many of the reactions involved in tissue metabolism. Under the influence of dinitrophenols, the ratio P/O falls so that more oxygen has to be consumed in order to make the required amount of ATP. Animals poisoned by dinitrophenols die in their attempt to consume enough oxygen to maintain adequate levels of ATP in their tissues and the level of this substance only falls at the time of death[6]. Mitochondria taken from animals at the height of poisoning have been shown to contain the same concentration of dinitro-*o*-cresol as is present in mitochondria to which DNOC had been added *in vitro* so as to produce a serious degree of uncoupling[7]. Poisoning by dinitrophenol in the rat, if not rapidly fatal, is quickly and completely reversed as the dinitrophenol is either excreted or partially reduced to the inert aminonitrophenol[8]. While the general toxic effects of these drugs can readily be explained in biochemical terms, the basis of their action in producing cataract in some individuals who took them as drugs remains a mystery.

Another well-known drug—salicylate—also uncouples oxidative phosphorylation and in many ways its action on isolated preparations simulates that of the dinitrophenols[9]. However, there is no evidence that either the therapeutic action or the toxic effects of excessive doses are due to this biochemical disturbance.

The inhibition of a specific enzyme—acetylcholinesterase—is believed to be the basis of the toxic action of a whole group of organophosphorus compounds as well as the alkaloid eserine and the related synthetic product neostigmine. While there is no doubt that the inhibition of cholinesterase to a severe degree takes place in poisoned animals, there is no clear-cut explanation of just how this kills the animal. While there is good evidence that cholinesterase at the periphery is essential to the normal function of muscles such as those of respiration, the therapeutic value of atropine in cases of poisoning by cholinesterase inhibitors is undoubtedly due mainly to its central effects, presumably in counteracting the effects of excessive amounts of undestroyed acetylcholine. Although these organophosphorus compounds inhibit the action of an enzyme whose role in the functioning of the nervous system is apparently well understood, it cannot be said that the exact mechanism of the lethal action of these well-studied compounds is finally agreed[10].

INTERPRETATION OF BIOCHEMICAL DISTURBANCES

The difficulties of explaining the effects seen in the whole animal in terms of biochemical mechanisms studied *in vitro* in isolated tissues is illustrated in the case of triethyltin salts. These salts are extremely active inhibitors of oxidative phosphorylation when studied on mitochondria from either the liver or the brain[11]. The brain from poisoned animals if examined *in vitro* shows a diminished oxygen consumption but the liver from these animals does not, despite the fact that it contains much more triethyltin than the brain[12]. These animals show an increasing lethargy and weakness from toxic doses of triethyltin and develop brain oedema. Men poisoned by these compounds in a therapeutic misadventure showed similar reactions[13]. It seemed natural to assume that the poisonous effects of triethyltin on the brain might be due to the inhibitions of oxidative phosphorylation which would in turn produce a fall in the ATP levels of the brain. However, when the levels of ATP in the brain of poisoned animals were measured, they were found to be slightly raised[14]. The difficulties of interpreting the significance of biochemical disturbances studied in isolation in relation to the effects of the same toxic substances in the whole animal are thus illustrated.

RESEARCH INTO TOXIC MECHANISMS

Each of the studies outlined above represents a great deal of work carried out over a number of years in several different laboratories, largely those engaged in academic work. The state of knowledge of biochemistry and physiology is not yet sufficient to enable the significance of relatively isolated studies to be properly placed in the context of the behaviour of the whole animal. The incentive to study each of these problems largely stemmed from the belief in the minds of the investigators that such a study would help to throw light on fundamental processes in the whole animal. Chemicals synthesized as potential drugs and which prove to be toxic either before or after their introduction into therapeutics are of no value to their inventors. Rather than study the mode of action of such compounds the inventors naturally bend their energies towards the preparation of new compounds which they hope will be free from objection. It is perhaps therefore not surprising that there is relatively little to be written about the mechanisms of the toxic action of drugs. However, this problem is certainly important and the sort of knowledge that does exist on toxic mechanisms merits discussion if only to illustrate the gaps.

Much effort is devoted to discovering whether or not drugs have toxic effects which may offer a barrier to successful use. The sort of work that should be done to this end has recently been outlined[15,16]. Enough is known about the toxic effects of chemicals including drugs to say that predictions of the presence or absence of toxicity on analogy in chemical structure may offer some guide but can never be accepted by themselves as a basis for deciding whether or not a drug will be toxic. Any increase in our knowledge of the mechanisms of toxic action should make better predictions possible. This indeed is an argument for devoting more research towards the study of mechanisms of toxicity.

GENERAL ASPECTS OF DRUG TOXICITY

Some toxic effects will be the result of excessive dosing of a drug thereby extending its effect beyond its intended therapeutic action. These, however, should be predictable and readily recognized when they occur. Far more important are the toxic side-effects of drugs. These are often unsuspected and may indeed sometimes go unrecognized for long periods. Such side-effects may or may not have have been seen in the animals in which the toxicity of the drug was studied. They may be trivial or they may be fatal.

Toxic effects of drugs may be due to an excessive dosage or prolonged period of administration. They may only be seen in some individuals because they have an inborn sensitivity to that particular drug. Others may be unduly sensitive to the toxic side-effects of a drug because of concurrent disease with localized injury to special organs, particularly the liver or kidney. In other cases the toxic effects of one drug may become apparent because of the simultaneous administration of other drugs.

Some examples of the ways in which toxic materials may injure some of the most commonly affected organs are briefly described.

The Liver

The liver is perhaps the most frequent site of toxic action of drugs. This is not surprising since the liver will be subjected to a higher concentration of ingested material than any other organ, with the possible exception of the intestinal mucosa. It is, in addition, concerned with the metabolism and detoxication of various compounds. The liver also at first sight offers singularly favourable conditions for a variety of investigations of its structure and function. It is disappointing that, despite a considerable amount of work devoted to this subject, no toxic action on the liver is fully understood at any level of investigation. One of the difficulties leading to this situation is that only a small proportion of toxic actions of importance in man can be demonstrated in other species. Since the possibility of using other species to investigate a particular toxic action will determine the nature and extent of possible investigations, it is convenient to consider what is known of mechanisms in this field separately according to whether the action is peculiar to man or not.

Toxic actions apparently confined to man

Actions in this category can be further sub-divided into two fairly clearly defined clinical syndromes, and a third class of less well-defined conditions. An appreciable amount of investigation has been devoted to one of the clinical syndromes, cholestatic jaundice: the other well-defined syndrome which typically follows the administration of monoamine oxidase inhibitors has been less well studied. The third group of conditions is so various that it is not possible to consider any general mechanisms to explain their occurrence. However, it can be said as a generalization that the drugs responsible for any one of the toxic actions show no obvious relationship in either structure or pharmacological action. The incidence of the particular undesirable effect varies considerably from drug to drug[17]. The drugs thought to be associated with liver injury have been listed, but for present purposes it is sufficient to consider what is known of the best studied examples of each type of toxic action.

Cholestatic jaundice

Cholestatic jaundice is one of the more remarkable manifestations of drug toxicity. It is probably most commonly associated with use of the drug chlorpromazine, and it is estimated that about 1 per cent of patients receiving the drug will show this syndrome. Typically the patient begins to develop jaundice within a few weeks of commencing treatment. The jaundice may become very severe and persist for up to two years, even though the drug be stopped. Such patients are not as a rule severely ill and recovery usually occurs. When recovery has taken place, further attacks may or may not be provoked by further administration of the drug.

Pathological studies of biopsy material, operative exploration and biochemical studies of liver function, show that there is no organic obstruction of the intrahepatic or extrahepatic bile-ducts. There is no necrosis of liver cells and only a little cellular infiltration. Some bile plugs may be seen in the finest ramifications of the biliary tree[18]. At least two aspects of the toxic action are obscure. It is not clear either what lesions in the liver account for the clinical picture of failure of liver excretory function, or by what mechanism the drug produces these changes. As the condition cannot be produced in animals, studies even of the pathological mechanism are necessarily confined to human material and suffer from the limitations imposed by this approach.

Electron microscopic investigations of affected livers offer some hope that the first question might have been answered and attention has been focused on the bile canaliculi[17]. These structures are the finest branches of the biliary system and are bounded by liver cells on all sides. The surface of the liver cells adjacent to the bile canaliculus is folded to form finger-like projections called microvilli. Schaffner and Popper[18] believe that in cases of cholestatic jaundice the microvilli are oedematous and reduced in number although the rest of the liver cell appears relatively normal. Such a lesion would provide an attractive explanation for the clinical observations since it might be supposed to affect only the excretory function of the organ. Unfortunately, neither the observations themselves nor their interpretation is beyond question. Recent developments in the technique of specimen preparation for the electron microscope have shown that techniques such as those used by Schaffner and Popper may themselves give rise to considerable artefacts due to shrinkage. Furthermore, changes in the microcilli are found in a variety of conditions not associated with cholestatic jaundice and may in fact occur during the course of biliary obstruction from whatever cause[19].

A somewhat similar syndrome can be caused in human beings by androgenic and anabolic steroids. In man and experimental animals, changes similar to those found in chlorpromazine jaundice and involving the microvilli have been found[20]. The claim is made that such changes can be induced in animals by the steroid, norethandrolone. This observation should clearly be repeated, and possibly extended, using acceptable modern techniques of specimen preparation. Such investigations may clarify the morphological lesion responsible for the syndrome of cholestatic jaundice. Direct repetition of these experiments, using modern embedding agents for electron microscopy, has failed to reproduce the effects noted by the original

investigators. However, this failure may be due to strain differences in the animals used or to environmental factors such as nutrition[21].

If the pathological mechanism by which the jaundice arises is obscure, the mechanism by which the drug produces any liver change is more obscure. It is customary to consider cholestatic jaundice as a manifestation of sensitization. The only grounds for this assumption appear to be the low incidence of the condition. This low incidence may in fact be spurious since about 20 per cent of all patients receiving chlorpromazine show changes in liver function tests that do not progress in most cases to the point of frank jaundice but, even with continued therapy, gradually return to normal[22]. Patients in whom frank jaundice occurs do not necessarily show a recurrence of jaundice if chlorpromazine is given after the initial attack of jaundice has regressed. None of these features are characteristic of a phenomenon involving immunological mechanisms. Another major objection to classing this (or any other drug reaction) as a sensitization phenomenon is that this classification is of itself thought to represent an explanation of the phenomenon and therefore prevents the proper investigation of its cause. It is perhaps for this reason that adequate studies of the metabolism of chlorpromazine in patients susceptible to cholestatic jaundice and its comparison with metabolism in patients that do not show this reaction do not appear to have been carried out. The condition, however, shows many resemblances to other pharmacological situations where tolerance to a particular action of a drug may be established. In most of these cases and, it is suggested, in the case of chlorpromazine, the explanation is probably that metabolic adaptation to the drug has occurred. The cholestatic jaundice which follows steroid administration is clearly not due to sensitization since the majority of patients receiving a sufficient dose show changes in liver function tests which persist as long as the steroid is administered.

Jaundice after monoamine oxidase inhibitors

A small number of patients show liver injury after the administration of monoamine oxidase inhibitors. These patients differ from those showing the syndrome of cholestatic jaundice in that they are obviously ill and the condition has a high mortality rate. Pathologically, the changes are similar to those of acute viral hepatitis. It has been suggested that the drug does, in fact, activate a latent viral hepatitis[23]. This hypothesis satisfactorily explains the pathological findings. It cannot be proved until the virus of infective hepatitis can regularly be recovered from cases or some immunological index of infection has been established.

Drugs which are toxic to the liver in most species

A substantial class of chemicals will produce liver injury in the majority of species when a sufficient dose is used. Some drugs are included in this class but naturally the existence of this property tends to debar the use of the compound as a drug. The most completely studied substances acting on the liver are probably the chlorinated hydrocarbons, particularly chloroform and carbon tetrachloride. Both these substances have been used as drugs in human medicine but despite some continuing use of chloroform as an anaesthetic, their use is declining because of their toxic hazards.

The mechanism of their action is considered in more detail here than is warranted by their status as drugs, since studies of their action illustrate the numerous difficulties of such investigations. The pathological effects of carbon tetrachloride and chloroform on the liver are similar and are well known. Briefly, the administration of a sufficient amount of chloroform or carbon tetrachloride by any route, will, in the majority of mammalian species that have been examined, cause extensive necrosis and fatty changes in the liver and, less constantly, changes in other organs. This necrosis is manifest only a few hours after the administration of the poison and has a very characteristic distribution. As is well known, the conventional anatomical picture of the microscopic structure of the liver is of an organ composed of lobules consisting of cords of cells radially arranged around a central vein. At the periphery of such a lobule are found structures containing the bile-ducts, portal veins and hepatic arteries. In chloroform or carbon tetrachloride poisoning, the changes are closely related to this structure. Cells surrounding the central vein become necrotic, the next most peripheral cells show severe fatty change, whilst cells at the very periphery of the lobule are either normal or show only trivial cytoplasmic changes.

Work on this problem has been obscured by doubt as to whether the pathological changes are a direct consequence of the action of the substances on the liver cells, which later become necrotic, or whether this necrosis follows vascular changes produced either by a pharmacological action of the substance or by actions on cells which show only minimal changes and do not later become necrotic.

Early investigators were of the opinion that the centrilobular necrosis was a consequence of ischaemia, caused by swelling of cells at the periphery of the lobule. This work has been reviewed and the conclusion reached that the evidence for a vascular change occurring within the liver was weak, since many of the experiments on which the view was based were unrepeatable, and the evidence for a relatively undisturbed blood supply was strong[24]. Work since that time has done little to reverse this conclusion.

More recently it has been suggested by Brody, Calvert and Schneider[25] that the alteration in blood flow is neurogenic. This is a variation of the suggestion that alteration in blood flow arises within the liver itself. Nevertheless, the experiments of Brody and others demonstrate that the alteration of many variables may have an effect on the final result of carbon tetrachloride intoxication. Such changes cannot be interpreted as affecting the fundamental mechanism of the intoxication since many peripheral factors are concerned in the complex series of events before the liver cell is ever placed at risk. It can be shown that, for example, administration of beryllium salts inhibits chloroform necrosis. Almost certainly this is due to interference with reticuloendothelial function and has, therefore, little relevance to the fundamental problem of how the changes in liver cells are produced[26]. In all the arguments of the proponents of the vascular origin of necrosis in chloroform or carbon tetrachloride poisoning, it is assumed that hypoxia will produce such changes by itself. These arguments tend to be supported by reference to conditions such as shock or heart failure, in which hypoxia is but one aspect of a more fundamental physiological disturbance. In fact, uncomplicated hypoxia does not seem to produce liver necrosis unless a further factor is added, and careful

studies have shown only vacuolation commonly associated with anoxia and no evidence of centrilobular necrosis[27]. Centrilobular necrosis was encountered in the experiments where animals were subjected to hypoxia at the same time as ultra-violet irradiation of their liver surface[28]. It is significant that the necrosis was confined to the superficial lobules, that is, those lobules submitted to irradiation additional to deprivation of oxygen.

Among earliest co-ordinated studies of the fundamental changes in the liver cell are those of Judah and Rees, who showed that some twelve hours after exposure to carbon tetrachloride, enzymatic changes could be detected that indicated injury to the mitochondria, which were themselves abnormal when isolated from liver homogenates *in vitro*[29,30]. Later it was shown that certain antihistamines, notably promethazine, can protect the liver against necrosis which follows carbon tetrachloride administration[31]. This protection appears to involve the mitochondrial systems studied in the earlier work. The administration of promethazine does not afford a complete protection since it can be shown that some soluble liver enzymes reach the serum in the same amounts in protected animals as in unprotected controls[32]. It has been suggested that the protective action of promethazine and by implication therefore a part of the toxic action of carbon tetrachloride involves lipid films[3]. These studies clearly constitute a most important step in unravelling the changes in the liver cell not only in carbon tetrachloride intoxication but in many other situations as well. However, much evidence makes it clear that the phase of injury studied by these investigators is relatively late in the genesis of the lesion.

Such evidence stems from various sources. The flow of perfusion fluid through the isolated rat liver has been studied[34]. Chloroform was incorporated into the perfusion fluid at pharmacologically relevant concentrations. It was found that there is a considerable increase in perfusion flow during the first hour of the experiment, while at the same time, bile flow is progressively and greatly reduced. Incidentally, this work is relevant also to the controversy concerning the vascular origin of liver necrosis noted earlier. These results show, not only that severe injury occurs within the first hour of intoxication, but also that such injury occurs in the presence of an increased vascular flow.

Electron microscope observations confirm the very early onset of liver changes after intoxication with such agents and changes have been demonstrated in the endoplasmic reticulum occurring within the first few hours of intoxication[35,36]. Such changes may indeed be present within the first 30 minutes of exposure to chloroform[21]. The observable change in the endoplasmic reticulum is accompanied by a defect in protein synthesis[36], which is probably located in the ribosomes[37]. This evidence suggests that in the poisoned animals these particles are not aggregated normally into the larger aggregates necessary for complete function. The inhibition of protein synthesis and damage to the lipo-protein secretory mechanism may occur simultaneously and from this it has been deduced that carbon tetrachloride primarily attacks the endoplasmic reticulum[38].

It is therefore possible to begin to construct an outline of some of the important events involved in acute chlorinated hydrocarbon poisoning.

First, the poison must be absorbed since it may then have some pharmacological effects on the organism generally and may alter the ultimate manifestations of its action on the liver. Next, it must be transferred from the blood to the liver cell. Interference here may allow the poison to be excreted before dangerous concentrations are achieved in the liver cell. Once having entered the liver cell, it seems probable that the first site of attack is the rough-surfaced endoplasmic reticulum, causing defects in protein synthesis and possible changes in the 'enzyme profile' of the liver cell. At this stage some liver cell enzymes may leak into the blood. Probably as a consequence of enzymatic changes the lipo-protein secretory mechanism is destroyed and, at the same time, some conditions, as yet obscure, are set up which favour interference with the integrity of the larger cell organelles, particularly the mitochondria and the lysosomes. As a consequence of these changes, particularly in the lysosomes, cells that are sufficiently severely damaged undergo necrosis, while less severely damaged cells merely accumulate fat. A complete description of the mechanism of intoxication involves the integration and understanding of investigations deriving from pharmacology pathology and biochemistry and their techniques. It is not, therefore, surprising that no toxic action of a drug (or any other substance) on the liver is completely understood in a fundamental way.

The Kidney

The kidneys are the seat of a number of serious, progressive and largely incurable natural diseases affecting the vessels, the glomeruli or the tubules. Lesions in one sector may predominate though it is usual for all to be involved ultimately. By contrast with the liver, the functions of the kidneys are relatively circumscribed and lend themselves to detailed investigation, particularly as the end product of renal function is readily available in its totality if so desired.

The kidney in its excretory role plays an important part in the body's defence against the effects of drugs or toxic chemicals. Nevertheless, the mechanisms by which drugs or poisons may injure the kidney have not been so intensively studied as in the case of the liver and the reasons for this are by no means readily apparent. Rather than attempt a general survey of the toxic effect of drugs on the kidney, a few examples will be cited and a special discussion will centre on mercury and its compounds.

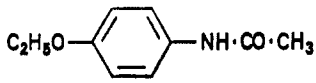
Drugs given in order to exert their therapeutic effects elsewhere in the body may damage the kidney in several ways. A simple example of one type of injury is the mechanical blocking of the renal tubular system by crystals of the drug or its metabolite. Many of the sulponamide type of drugs are acetylated by the liver and excreted in this form, but unfortunately in the case of some—for example, sulphapyridine and sulphathiazole—the acetylated compound is poorly soluble in water and may precipitate out unless the pH of the urine is on the alkaline side of 7·6. This mechanism is dependent on the metabolic fate of the drug after it had been absorbed and passed through the liver and the occurrence of the kidney injury can be explained in relatively simple chemical and physical terms.

Specific injury to the glomeruli by drugs is not common but there is some evidence that hydralazine causes an extensive fibrinoid necrosis and 'wire

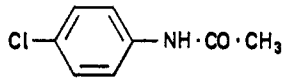
looping'[39]. This drug also causes the lupus erythematosus syndrome with lesions in the skin and changes in the circulating leucocytes but no connections between these two groups of toxic effects are apparent though a sensitizing effect is probable.

Lesions of the renal tubules are probably the commonest sequelae to the administration of drugs and poisons. The chemical may be secreted through the tubular cells into the urine or its toxic effects may be enhanced as water is reabsorbed from the glomerular filtrate.

In recent years in some European countries, a chronic tubular damage has been found in people addicted to large doses of phenacetin (I). This popular and widely distributed ingredient of analgesic tablets is known to be capable of causing severe methaemoglobinaemia and haemolysis in a few sensitive individuals but the part phenacetin plays in producing a chronic interstitial nephritis is less generally accepted. However, there appear to be groups of individuals, particularly in some industrial populations, who habitually take large doses of phenacetin to relieve headaches and other discomforts produced by their occupation. However, large doses of phenacetin in rats and mice do not readily produce evidence of interstitial nephritis.



(I)



(II)

The presence of some renal infection may also play a role in producing renal interstitial nephritis, as rabbits given the drug together with *Escherichia coli* intravenously, exhibit lesions. When phenacetin was given to a group of patients with pre-existing renal damage, the quantity of urinary sediment appeared to be increased. It has also been shown that an impurity, aceto-4-chloranilide (II) is much more injurious to the kidneys but this can only be present if the drug is synthesized by one particular route[40]. Nothing is known about the way in which either the drug or the impurity causes the renal lesions found in those taking large quantities.

However, in relation both to the physiology and some aspects of the pathology of the kidney, mercury and some of its compounds hold pride of place. Some compounds of mercury are well known for the damage they do to the kidney as well as to other organs, and the lesions produced by mercury salts are mainly in the tubules. Other mercurials are valuable for their diuretic action, particularly in cases of chronic cardiac failure. There seems to be general agreement that the diuretic action of mercurials is exerted in the renal tubules though the precise part of the tubule remains a matter of some disagreement. It seemed therefore that it might be profitable, in searching for mechanisms of toxic action, to try to discover what was known about the actions of these mercury compounds, since one group is almost entirely destructive in its effects while others exert a purely pharmacological action with little evidence of gross toxic action even in excessive doses.

The mercury compounds may conveniently be considered in three main groups.

(1) *Hg^{++} mercuric salts*, of which the best known is mercuric chloride, a general corrosive poison often taken for suicidal purposes. Those who die with this type of compound often show evidence of complete renal failure. Experimentally, small doses intravenously (3 mg/kg) kill animals in a few days after extensive kidney damage, particularly tubular necrosis. Given by other routes, mercuric chloride causes extensive damage especially to the intestinal tract. Despite its toxic action, mercuric chloride in smaller doses has a good diuretic action.

(2) *RHg^{+}*. Where R is simple alkyl group, for example, methyl or ethyl, the salts are notorious for the destructive though circumscribed lesions produced in the brains of experimental animals or of accidentally-poisoned man. They damage the kidney tubules but more attention has been paid to their effects on the nervous system because of their irreversible, and hence very serious, nature. These compounds have no diuretic action[41], appear to be relatively stable in the animal, and tend to reach and remain in the brain[42,43].

With R as a simple aryl group, usually phenyl or tolyl, a number of salts have been widely used as fungicides and bacteriostatic agents. Apart from blistering of the skin, they have not been responsible for occupational poisoning. They produce renal tubular lesions in animals but in smaller doses have no diuretic action[41]. They are comparatively unstable in the body and appear to break down readily to release inorganic mercury[44].

(3) *RHgX*. The mercurial diuretics may have X as a simple cation such as chloride but more usually it is theophylline. R is usually a substituted propyl group attached directly to the mercury, the middle carbon of the propyl group usually being a methoxy group and the terminal carbon large and often somewhat complex[45]. Thus the mercurial diuretics are a modification of the alkyl mercury compounds although they are free of adverse effects on the central nervous system of both man and animals.

Before discussing the mechanism of the toxic effects of mercuric salts, group (1), the mechanism of the diuretic action of compounds in group (3) is best considered. The reason for this can be made clear at the outset by pointing out that a view widely held is that the mercury diuretics exert their pharmacological effects because they release mercuric (Hg^{++}) ions within the renal tubular cells[41]. It is generally accepted that mercurial diuretics exert their pharmacological effects on the cells of the renal tubule and probably act mainly by preventing the reabsorption of sodium from the glomerular filtrate. Their effects upon other transport mechanisms, particularly potassium, remain obscure and uncertain. The action of the mercurial diuretics is much enhanced in acid urine, and while the conditions under which acid urine is produced might affect the distribution of mercury in the kidney, it does not influence its excretion. The secretion of acid urine might also involve mechanisms that are more susceptible to mercurial diuretics. The simplest explanation of the effect of acid urine on diuresis is that it hastens the release of mercury ions from the mercurial diuretic. All mercurials effective as diuretics are less stable in acid; mercury compounds stable in acid have no diuretic effect[45]. Whether or not mercuric ions are released to exert their diuretic action, the mercury itself is largely excreted conjugated with cysteine. It is not a loss of cysteine that brings about diuresis, for

non-diuretic mercurials also lead to an excretion of the mercury-cysteine complex. The fact that mercuric chloride is a relatively poor diuretic cannot be used against the hypothesis that it is the release of the mercuric ion that is responsible for the effects of the diuretic. Mercuric chloride is extremely reactive and after injection the concentration within the kidney builds up much more slowly than it does in the case of the diuretics[45].

The distribution of mercury in the kidneys of dogs and rats given diuretic doses showed that the greater part was located in the soluble (supernatant) fraction of kidney homogenates; there was some in the mitochondrial fraction but much less in the nuclear fraction[46]. The mitochondria isolated from kidneys grossly swollen by excessive doses of mercurial diuretics still functioned normally, and they continued to do so even when the supernatant containing most of the mercury was added to them[47]. The activity of five enzyme systems in the renal tubules, some of them thiol-dependent, have been studied histochemically but no disturbance of activity was noted until the dose of mercurial compound was great enough to produce tubular damage detectable by conventional histological methods[48].

The possible role of the reaction between mercury and thiol groups in the induction of diuresis must naturally be considered. Mercury reacts with thiol groups in the kidney for most of it rapidly appears in the urine as the mercury-cysteine complex. The administration of cysteine does not reverse the diuretic action of mercury but the dithiol—dimercaprol—does. *p*-Chloromercuribenzoic acid, on the other hand, which presumably reacts with thiol groups has no diuretic effect but also blocks the action of mercury diuretics[49]. It is therefore suggested that for diuresis the mercury must combine with two sites, only one of which is a thiol. That the mercury binds selectively is indicated by the fact that free thiol groups can be demonstrated in kidneys containing a fully effective dose of a diuretic. If the diuretics act by reason of the mercuric ion released from them, a clue to their action might be found by a search for biochemical mechanisms that would release the mercury. The evidence from renal function studies and histological and histochemical work is that the mercury is acting at several different places along the renal tubule[45].

Although the mercurial diuretics may exert some toxic actions in man, these are usually remote from the kidney. Some of the compounds are tolerated by rats in doses of over 50 mg/kg daily for a month after the animals have received smaller doses for longer periods. Even after 12 months no histological evidence of renal tubular damage was found[50].

Mercuric chloride or phenyl mercuric salts readily produce severe renal tubular damage if given repeatedly in such doses. The damage can be prevented or reduced in man and animals by dimercaprol[51] and also by the monothiol penicillamine[52]. The monothiols cysteine and glutathione do not modify the diuretic action of mercurial drugs[45], and their therapeutic value in mercury poisoning is not known.

Turning now to the toxic effects of mercuric ions, it has been shown that mercuric chloride injected into rabbits is predominantly located in the distal part of the proximal tubule[53]. Mercury is present in the kidney mitochondria but there are high concentrations in the microsome fraction[54]. The mitochondrial enzyme succinic dehydrogenase, which is thiol-dependent, has a

curiously patchy distribution in the renal tubule of the rat but its activity as judged by histochemical techniques is not disturbed until there are tubular lesions visible in conventional histological preparation and the mitochondria shows gross damage[55]. A promising line that may one day reveal in more detail how mercury salts damage kidney tubules has come from a study of the effects of diet on the susceptibility of animals to mercuric chloride. Twenty years ago it was found that dogs with low plasma protein were not affected by a dose of 3 mg/kg of this salt that killed normal dogs[56]. If rats are fed a diet predominantly of sucrose for 3 weeks they, too, will tolerate a dose of mercuric chloride that kills normal rats within 6 days[57]. Rats made nephrotic by injections of dimethylaminopurine are also resistant to mercuric chloride[58]. In neither case is the excretion of mercury more rapid in the resistant rats. In the sugar-fed rats, there is the same amount of mercury in the kidney as in normal rats given the same dose but a higher proportion of mercury in the microsome supernatant fraction of a kidney homogenate and less in the nuclei and mitochondria[59]. Most of the thiol groups, of which 25 per cent is glutathione, are found in the microsome supernatant fraction. The other thiol groups are protein bound. The molar ratio of Hg:SH is about 1:50. Kidney homogenates from normal and sugar-fed rat 24 hours after a dose of mercuric chloride, that would kill the normals, have been compared with that of the kidneys before mercury. There was a depression of the tricarboxylic acid cycle activity to zero in the normally fed rats but only by about 40 per cent in the sugar-fed rats. Succinic dehydrogenase and cytochrome oxidase activities were both depressed to a greater extent in the normal fed rats[60].

The precise nature of the action of mercury salts and mercurial diuretics is far from clear, and it is possible that the main toxic action of mercury ions is exerted on the nuclei and mitochondria of renal tubular cells, whereas the diuretic action takes place in the soluble fraction of the cells where mercuric ions are clearly much less noxious, possibly because they are harmlessly bound to thiol groups. It would be interesting to know more about the mode of excretion and diuretic action of mercuric chloride and mercurial diuretics in the sugar-fed insusceptible rats.

The Blood

Marrow and haematopoiesis

Cyanocobalamin (vitamin B_{12}) is a general haematopoietic principle which is only absorbed from the gut if so-called 'intrinsic factor' is present. It plays a role in the maturation of red cells, white cells and platelets.

Folic acid which occurs naturally in plants is also essential for haematopoiesis but the main source of this for man is probably that synthesized by the gut flora. The main function of folic acid is probably that of a coenzyme involved in the transfer of methyl groups in the production of thymine while cyanocobalamin may be involved in the synthesis of thymidine by means of uracil deoxyribose. (See[61] for further details.) Another factor in red cell production is erythropoietin—a mucoprotein secreted by the kidney and probably inactivated to some extent in the liver. It is present in plasma and its production is stimulated in certain types of anaemia; its role is unknown.

Pyridoxal phosphate is essential for the synthesis of porphyrins and haematopoiesis and this may be why anaemia is seen in cases of pyridoxine deficiency. Of these four substances known to be essential to blood formation, it is only folic acid and disturbances in its metabolism that can be incriminated in some of the toxic actions of drugs on bone marrow.

Analogues of folic acid such as Aminopterin have been synthesized with the aim of antagonizing folic acid and used in the treatment of leukaemias and other tumours. Excessive dosage will naturally interfere with haematopoiesis and cause marrow aplasia.

A deficiency of folic acid such as occurs in sprue gives rise to a macrocytic anaemia which is an indication in the cells of the peripheral blood that maturation of the red cells in the marrow is not satisfactory. The same type of anaemia occurs when there is a deficiency of cyanocobalamin. The commonest anaemia of the kind is pernicious anaemia associated with achlorhydria and a deficiency of intrinsic factor. The result is a failure to absorb cyanocobalamin in adequate amounts. The same type of anaemia is also sometimes found in association with malnutrition, kwashiorkor, malabsorption syndrome and other deficiencies.

In 1954 two cases of macrocytic anaemia were described in young women with normal gastric mucosa and one with free acid in the stomach. Their blood cyanocobalamin levels were normal. Both were epileptics receiving phenobarbitone and phenytoin sodium. One responded to cyanocobalamin, the other did not, but did respond to folic acid. The author[62] stated that since no cases of macrocytic anaemia had hitherto been described among the thousands of people who had taken phenobarbitone—'we have been wondering if the anticonvulsant drugs they have been receiving could have played a part'. This speculation proved to be correct and since then many cases of macrocytic anaemia have been described in patients receiving phenytoin sodium and related drugs with or without barbiturates. A survey of an epileptic colony showed that while some degree of macrocytosis was prevalent, frank anaemia was rare[63]. These subjects had normal blood cyanocobalamin levels, and ascorbic acid blood levels, and no evidence of liver dysfunction. Although phenytoin had been in use since 1938, the first cases of anaemia were only reported in 1954 and even now, though many cases have been described in the United Kingdom, very few reports have come from the U.S.A.

Some cases have been investigated in detail[64,65]. Typically they show free acid in the stomach, normal blood cyanocobalamin levels and good absorption of cyanocobalamin from the gut together with normal absorption of vitamin A and fat. Not only is the absorption of folic acid from the gut normal but also its excretion in the urine and rate of fall in the blood are as in normal people. Nevertheless, all cases respond to the administration of folic acid whether or not the drug is stopped. This had led to the hypothesis that the drugs act as direct competitors for folic acid in the marrow. Structurally the molecules of primidone and phenytoin bear some resemblance to a small part of the pteroylglutamic acid molecule, but so too does phenobarbitone[64]. However, it is usual for the doses of the hydantoin drugs to be 2–3 times as great as those of phenobarbitone. It is difficult to understand why relatively few of those taking the drug develop the anaemia but, those

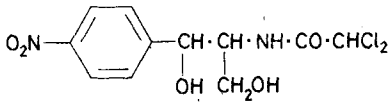
who have done so once and been cured may or may not relapse if the drug is again administered without the folic acid[66].

While some understanding of the toxic action of the hydantoin drugs has been gained from a careful study of affected patients, there is no experimental work which has been directed towards elucidating the enzyme system involved in the alleged competition between hydantoin and folic acid.

These same drugs can also bring about an unusual but reversible adenopathy which has some of the histological features of Hodgkin's disease and the malignant reticuloses. Unlike the cases of anaemia, the lymphadenopathy cases tend to develop soon after the administration of hydantoin, on an average within 4 months and sometimes within a week. In a review of 82 cases no mention is made of macrocytic anaemia nor is there any indication that its presence had been sought in these cases[67]. It is not reproducible in animals and whether or not the mechanism of this toxic effect of hydantoins has any connections at all with folic acid metabolism, remains quite unknown.

Drugs such as the nitrogen mustards that interfered with cell division affect many cells in the body but are particularly liable to damage the bone marrow and interfere with the production of red cells, leucocytes and platelets. Various degrees of marrow aplasia are to be expected when any of these drugs are given. However, there are a great many drugs now known that can occasionally cause agranulocytosis and aplastic anaemia both of which may prove fatal. Long lists of drugs that have been reported to have produced marrow aplasia will be found in text books of haematology. They vary widely in chemical structure and no obvious possible mechanism of action comes to mind on looking at their formulae. The important feature of them all is that in relatively few patients who take these drugs is the bone marrow adversely affected and in no case has it been possible to produce marrow damage in experimental animals. On the other hand, the well-known industrial solvent, benzene, will induce marrow damage regularly in a variety of animal species. However, men exposed to it in industry show a wider range in response and some apparently remain unaffected for years of work in an atmospheric concentration that has produced leucopenia or worse in their work-mates. Despite its well-known propensity for damaging the marrow and the fact that it can induce this experimentally in animals, there is nothing yet known about the mechanism of the toxic action of benzene. While it is believed that one or more of the metabolites may be responsible and these metabolites are well known[68,69], no evidence has been produced that they damage bone marrow.

The problem of sporadic marrow damage is worth examining further in the case of a relatively new drug which has gained a reputation for being able to produce agranulocytosis and aplastic anaemia.



(III)

Chloramphenicol (III) is a useful antibiotic with a number of almost

unique therapeutic virtues which make it indispensable in modern therapy. In the first extensive report on its use—mainly for cases of typhus in the Far East—there is a statement that, 'The presence of the nitrobenzene radical in the structure of chloramphenicol led to suspicion that the drug might be toxic to the haematopoietic system'. Careful investigation failed to disclose any evidence of this[70].

There is now no doubt that marrow damage can follow the administration of chloramphenicol but the incidence of such cases and the factors that may influence their appearance are not yet known or agreed upon. In a survey of 31 cases mainly of aplastic anaemia, 14 had had over 60 g of the drug while a recommended full course of treatment would have involved the administration of not more than 26 g[71] and for most patients the treatment went on for over 24 days. In a study of 15 cases it was found that the red cell system was affected first and while all the evidence appeared to support a direct toxic effect of chloramphenicol on the marrow, a few cases occurring after small doses suggested a hypersensitization[72]. This question of hypersensitivity in relation to marrow aplasia from drugs has been raised many times including studies on Pyramidon, the first drug to be recognized as being capable of producing occasional marrow damage[73,74]. There is relatively little outside evidence in support of hypersensitivity as other allergic manifestations are rare in these cases. Nor is there any evidence of this being an auto-immune type of damage as there is no haemolytic phase in these cases[75].

If the toxic effect of chloramphenicol is a direct one on the marrow and primarily one on red cell maturation, then the fact that the normal life of the red cell is 120 days means that many mild cases of marrow injury from chloramphenicol would escape notice[72]. This may explain why the administration of massive doses of chloramphenicol (6 g or more) to four patients with incurable cancer only produced anaemia in one case during the relatively short period of observation but there were small falls in reticulocytes in all cases[76]. The changes were reversible. Similarly changes have been seen in the marrow erythroblasts and pronormoblasts of patients receiving chloramphenicol, and these too were completely reversible[77–79]. Recent developments in the study of radioactive iron can throw light on the state of erythropoiesis. If the rate of disappearance of ^{59}Fe from the plasma is followed, it is shown to be slowed in cases where erythropoiesis is subnormal. While the administration of alkylating agents will always delay the removal of ^{59}Fe, in 15 patients receiving chloramphenicol only 5 showed any such delay and this was completely and rapidly reversed when the chloramphenicol was withdrawn[80]. However, when a group of 48 patients received an analogue of chloramphenicol—a methanesulphonate replacing the nitro in the para position—32 had a definite and 7 a borderline depression of erythropoiesis. Whereas of 49 receiving chloramphenicol (1 g twice daily for 28 days) only 1 had a definite and 3 a borderline depression[80]. Chloramphenicol given to neonatals is very toxic because the liver is unable to conjugate the molecule so that it can be excreted. There is no report of marrow damage in infants poisoned by chloramphenicol[82,83]. In adults there is much evidence consistent with the idea that chloramphenicol has a direct toxic effect on erythropoiesis, though there is much variation in the susceptibility of individuals

to the drug just as there appears to be in the case of benzene. Unfortunately, however, there is no evidence that chloramphenicol can produce marrow aplasia in experimental animals. An anaemia and fall in reticulocytes in ducks that had been given chloramphenicol was shown to be a sequel to the loss of appetite resulting from giving the drug[84,85]. Claims that dogs could be made anaemic by large doses of chloramphenicol were subject to critical examination and again it was found that the severe loss of appetite and inanition was responsible for the blood changes observed. Young dogs kept for 2 years on a daily dose twice that recommended for man remained healthy. Other laboratory animals were reported as being unaffected by chloramphenicol[86]. Monkeys were given large doses of chloramphenicol six times a week for 15 months and remained well; they included three animals, rendered anaemic beforehand by a vitamin B deficient diet, which recovered from their anaemia while receiving the chloramphenicol[87]. As experimental animals provide no means of studying the toxic action of chloramphenicol, it is worth looking at evidence of its toxicity on other systems. If leucocytes or bone marrow are incubated with folic acid, a substance is formed which stimulates the growth of *L. citrovorum* and reverses the inhibition of growth produced on this organism by chloramphenicol[88]. If rats are given a diet containing chloramphenicol, the xanthine oxidase levels in the liver fall to zero but the intestinal level of the enzyme is not affected. Folic acid metabolites are also known to inhibit xanthine oxidase. The hypothesis based on this evidence is that chloramphenicol in some way interferes with folic acid metabolism and removal of its metabolites[89]. This is at least consistent with the fact that the administration of folic acid is not effective in anaemia caused by chloramphenicol. A suggestion that chloramphenicol, by its supposed structural resemblance to phenylalanine, might interfere with the incorporation of this amino acid into bacteria as part of its bacteriocidal action has no basis either in theory or observation[90]. The incorporation of all amino acids is inhibited by chloramphenicol and its action against bacteria is more probably associated with the prevention of peptide bond formation[91]. Thus there is no biochemical basis upon which to begin to erect an hypothesis about the mechanism of the toxic action of chloramphenicol on bone marrow.

Recent work on the toxicity of soya bean meal from beans extracted with trichloroethylene offered some early promise of a readily available means of studying experimentally induced bone marrow aplasia. It has been shown that the toxic factor in the meal is probably dichlorovinylcysteine—a reaction product of cysteine and trichloroethylene. This can be synthesized and, like the toxic meal, readily produces aplastic anaemia in calves. Unfortunately it has no such effect when given to rats, mice, guinea-pigs, hamsters or dogs, nor did doses effective in the calf produce any evidence of marrow depression when given to patients[92].

Species differences in sensitivity to substances capable of damaging the bone marrow are perhaps not unexpected, though to radiation, radiomimetic drugs and benzene the response in all species tested is remarkedly consistent. There has been no extensive testing of drugs known to cause marrow aplasia in some patients on animals other than the common laboratory species. Perhaps the sensitivity of the calf to some of these drugs,

particularly the more notorious of these potentially injurious to the marrow, might be worth some examination. At present, however, it is not possible to go further than to suggest that for some reason the haematopoietic system of some people is unusually sensitive to a variety of drugs. It seems unlikely that all these drugs have a final common pathway whereby the biochemical injury is produced in the marrow. It seems more probable that some people have a haematopoietic system that runs with a smaller margin of safety than in the majority of people, and they are in some way unduly sensitive to anything that adds a strain on one or more haematopoietic mechanisms. It is perhaps worth noting that an acute deficiency of no known haematopoietic factors will produce such a dramatic injury as the aplastic anaemia following the administration of certain drugs.

It is tempting to suggest that these people have some inborn deficiency that renders their haematopoietic system unduly sensitive to any strain put upon it. With this thought in mind, it is appropriate to consider a condition affecting the circulating red cells of man which has been the subject of intensive study in recent years.

Circulating blood

For some years it had been known that the antimalarial drug primaquine may induce a haemolysis in some people—more commonly among negroes than white Americans. By transferring labelled red cells to and from sensitive and insensitive people, it was shown that it was the cells themselves that were sensitive to the effects of the drug[93]. Unlike most haemolytic anaemias, that due to primaquine is self-limiting because it is the older red cells that are more sensitive[94]. A number of drugs other than primaquine will produce this haemolysis as well as some natural products such as the bean *Vicia faba*[94,95]. In stained films, cells not destroyed by haemolysis may show the blue inclusions known as Heinz bodies and first described in men exposed to certain toxic chemicals in industry. An *in vitro* system was established for studying the effect of these drugs on red cells and it was found that oxygenation increased the damage done[96]. When the stroma of the cells from sensitive and insensitive people was examined in systems reducing glutathione and oxidizing glucose-6-phosphate, the cells of sensitive people were shown to be deficient in the enzyme glucose-6-phosphate dehydrogenase[97]. Apparently as a result of this defect, the cells of sensitive people when incubated with one of the toxic drugs show a sharp fall in the level of reduced glutathione. This does not occur in normal people and is a convenient test for this type of sensitivity[98]. Clearly the defect in the single enzyme results in a failure of the cells to maintain adequate levels of reduced glutathione and for this reason they are sensitive to the oxidative reaction products of the toxic drugs. The actual toxic entity is probably hydrogen peroxide which is normally prevented from attacking the haemoglobin or other cell structure by glutathione peroxidase[99]. The glucose-6-phosphate dehydrogenase maintains the equilibrium between the phosphopyridine nucleotide and its reduced form, and so regenerates supplies of reduced glutathione. As far as is known at present, this particular deficiency is peculiar to humans. Tests on over 128 monkeys, 22 cats and 160 rabbits revealed none with defective glucose-6-phosphate dehydrogenase systems in their red cells[100]. Fortunately this defect

can be readily studied *in vitro* on red cells removed from sensitive people and this probably explains the rapidity with which its nature was elucidated in the absence of suitable experimental animals.

Other inborn defects have been discovered recently to provide a basis for the differences in the reaction of individuals to drugs, for example, isoniazid and suxamethonium. The genetic basis for these variations has recently been reviewed[101,102]. The possibility that some similar mechanism has taken the sensitivity of some individuals to the toxic side effects of drugs must clearly not be overlooked, particularly where the toxic effect seems to be one that is peculiar to man.

CONCLUSIONS

The subjects discussed illustrate how little is understood about the mechanisms of the toxic action of drugs. The main difficulties arise in the analysis and interpretation of data obtained during the study of biochemical and physiological phenomena carried out in relative isolation.

What relations do the observations made in a Warburg flask or on a pithed animal have to events in the intact animal? They provide a useful starting point in any investigation of the nature of a toxic reaction but the glib assertion that a compound inhibits oxidative phosphorylation in liver mitochondria and that this observation provides an explanation of a toxic or pharmacological effect carries the problem no further forward at all. The important point is that only by many different approaches will a useful composite picture of the nature of a toxic reaction be obtained. There is no means of predicting how valuable an apparently rather bizarre experimental procedure such as the feeding of rats on sucrose might have in uncovering the mechanisms by which mercury ions poison kidney tubules.

Probably the most important consideration in the study of toxic reactions is that of species differences and in particular the place of man in relation to other species. The relationship between susceptibility and/or insusceptibility to the haemolytic effects of primaquine has been so clearly established as a gene-linked enzyme deficiency that it seems very probable that other toxic mechanisms peculiar to man may have a similar basis. In populations of laboratory animals the members tend to react very much alike, differing quantitatively rather than qualitatively in their responses to drugs and poisons. The most serious toxic responses to drugs seen in man are those that are not reproduced readily, if at all, in experimental animals. Drugs that do produce toxic effects in animals will be used with more circumspection in man so that their adverse effects will the more readily be noticed.

There is a great need for more research into the mechanisms of toxic action and from what has been said above, this is likely to be a fruitful starting point in gaining knowledge of physiological and biochemical processes in the whole animal. The toxicologist is never likely to be a popular member of a research team producing new drugs, and given an opportunity for research together with the necessary resources, better drugs may eventually emerge just from the knowledge gained in a thorough study of toxic mechanisms.

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3

DRUG RECEPTOR INTERACTIONS

E. W. GILL

INTRODUCTION

PHARMACOLOGICALLY active substances may be roughly divided into two groups: (1) those compounds whose biological activity can be correlated in terms of some general physical property and is only indirectly dependent on chemical structure, for example, volatile anaesthetics[1], (2) those compounds whose activity is dependent on chemical structure and where minor alterations in this structure can have a profound effect on activity. It is in the attempt to rationalize the behaviour of this latter class of compound that the idea of a 'receptor' is most frequently introduced. The concept of a receptor proves to be remarkably elusive, considering the central position it occupies in the theory of pharmacology, and any attempt at a rigorous definition is probably fruitless[2]. A receptor will be considered as the component of a cell with which a drug combines to initiate a response. The vagueness of this definition reflects our lack of precise knowledge about receptors. Langley first suggested the existence of a 'receptive substance' to explain the actions of curare and nicotine and this idea has been common currency for almost as long as pharmacology has existed as a distinct body of knowledge; it is the keystone of almost all discussions of structure–activity relationships, and of the quantitative description of the effects of drugs; yet, in so far as it is not yet possible to write a recognizable chemical structure for one receptor, it could be said that no more is known about receptors now than in the days of Langley. It is, however, not difficult to find reasons for this apparent lack of progress. The ability to differentiate sharply between different chemical substances is one of the most fundamental properties of living systems, and the central problem of pharmacology—why an organ responds in a certain way to a particular drug—is only one part of this larger question. Progress has therefore been dependent on parallel advances in the fields of biophysics, cytology, enzymology, and physical and macromolecular chemistry.

Fischer[3] coined the graphic image of the lock and key to explain enzyme specificity, and this idea, writ large as 'molecular complementarity' has offered a satisfying general explanation of the profound alterations in biological activity that can often follow minor alterations in the structure of a drug. Briefly, it is suggested that the receptor has a complementary structure to the drug and that, owing to the weakness of the forces binding the drug to the receptor, a very close fit between the two is needed for firm binding. Owing to the cooperative and complementary nature of these binding forces any deviation from the optimal structure, for example by the substitution of a bulky group for a small one, by rearranging the constituent groups of a molecule in space by inversion at an asymmetric centre, or by

altering the direction of a dipole within the molecule can cause a profound alteration in the stability of the drug-receptor complex. This explanation is probably basically correct, and indeed it is difficult to conceive of any other explanation for the great changes in biological activity that often follow inversion of an asymmetric centre in a molecule[4]. This review attempts to give more precision to these widely accepted general ideas and summarizes (1) the factors influencing the size and shape of drug molecules in solution, (2) the nature and properties of some of the components of cellular systems that may be responsible for the specific combination of drugs, (3) the forces that may bind drug molecules to these receptor substances, and (4) the possible effects of drug combination on the structure and properties of cellular components.

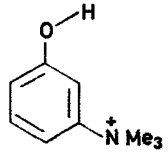
At the present state of knowledge any discussion of drug-receptor interactions must be highly speculative: something is known of the structure of the drug; practically nothing is known about the equally important second component of the reaction—the receptor. An indication of the kind of reaction that may be occurring can be made, and it may be asserted that the action of drugs on cellular systems is understandable in principle in terms of forces that are already known. Any more explicit statements concerning the receptor must await the completion of the formidable task of the isolation and complete structure determination of the receptor substance.

THE DRUG

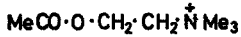
The receptor theory of drug action implies that the pharmacological properties of a compound are dependent not only on the nature and properties of the constituent groups within the molecule but also in the way in which these groups are distributed in space. This follows from the idea that the receptor is a discreet, spatially organized structure, and that maximum activation of the receptor only occurs when there is close apposition between the drug and the receptor. The necessity of considering the spatial arrangement of the groups in a molecule, its size and shape, has been emphasized recently by Ing[5].

The properties of the constituent groups found in drugs, considered in isolation, are available in text-books of organic chemistry. The way in which these constituent groups are arranged in space is, on the other hand, sometimes a more difficult and subtle problem. No great difficulty arises over the consideration of the shape of aromatic molecules: the rings are flat and rigid and their dimensions are well known; the spatial properties of the molecule are apparent from its structural chemical formula. Albert's work[6] on the antibacterial properties of acridine derivatives is a classic example of such an analysis. Similarly the steroids, with their complex perhydrophenanthrene ring system do not present a problem: the conformations of the different rings are locked and the precise shape of the molecule and the orientation of groups attached directly to the ring system can be determined directly from accurate models based on the known conformations of the steroids. The major uncertainty arises in those drug molecules, often with quite simple structures, which contain extended chains of singly bonded atoms. For example, whereas the spatial

distribution of groups in the ion (*I*) is apparent from its formula, that of ion (*II*) is not.



(*I*)



(*II*)

In recent years extensive studies on the physical properties of short flexible chains have given some insight into the way such chains arrange themselves in space. No previous extended analysis of the possible conformations of biologically active molecules seems to have been made, so the remainder of this first section is devoted to a brief review of the factors influencing the conformations of flexible molecules in solution and the effect of different chemical groups on this conformation.

Factors Influencing the Size and Shape of Drug Molecules in Solution

Many drug molecules contain as part of their structure open chains of atoms, for example polymethylene chains, often interruped by ester, ether, amino or other groups. The presence of these flexible units within a molecule greatly complicates any discussion of its size and shape. Early studies[7] ignored the possibility that several different conformations of simple open-chain molecules are possible and concentrated attention on the distances between different groups when the molecule was in the fully extended conformation. Schueler[8,9] later pointed out that the maximum possible distance between two groups in a flexible molecule has no great significance, and that owing to the possibility of rotation about the bonds forming the chain there was no unique conformation for the molecule; it was necessary therefore to consider a whole range of structures of differing probabilities. However, although rotation about bonds is possible in open-chain molecules such systems cannot be treated as a series of freely rotating rods restricted only by the valency angles. Due to interactions between non-bonded atoms in different conformations of the chain, energy barriers prevent free rotation and severely limit the number of possible conformations. The freely-rotating model serves to explain the properties of long chain polymers[10] but gives a completely false picture of the possible conformations of the comparatively short chains that occur in many drugs. The magnitude of these non-bonded interactions depends on the constituent atoms of the chain; it is also possible for other specific interactions for example, electrostatic forces and hydrogen bonding, to exert an influence on the conformational possibilities open to a chain. There is no general solution to the problem of the conformation of a flexible chain of atoms: different sequences must be treated separately.

(1) *Normal paraffin chains*

The simple polymethylene chain is a very common structural element in drugs and is also the system that has been most carefully studied. If

Figure 3.1 represents butane, then all possible rotational conformations can be obtained by rotating C_1 in a circle with the C_2–C_3 bond as axis. It has been shown[11,12] that the interaction energy E_ϕ between the two terminal methyl groups varies between 0 and 4·1 kcal, depending on the angle ϕ.

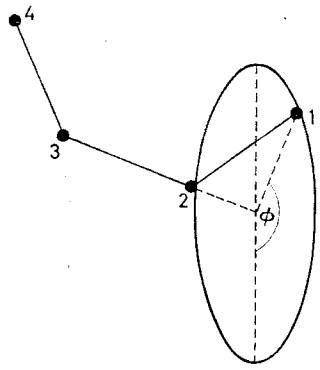


Figure 3.1.

The most stable conformation is the *trans*, with $\phi = 0$; the relative probability P_ϕ of any other conformation is given by

$$P_\phi = ke^{-\frac{E_\phi}{RT}}$$

where k is the normalizing constant, e the natural logarithmic base, R the gas constant, and T the absolute temperature. E_ϕ and P_ϕ are plotted against ϕ in *Figure 3.2*. It can be seen that, far from a series of freely rotating rods,

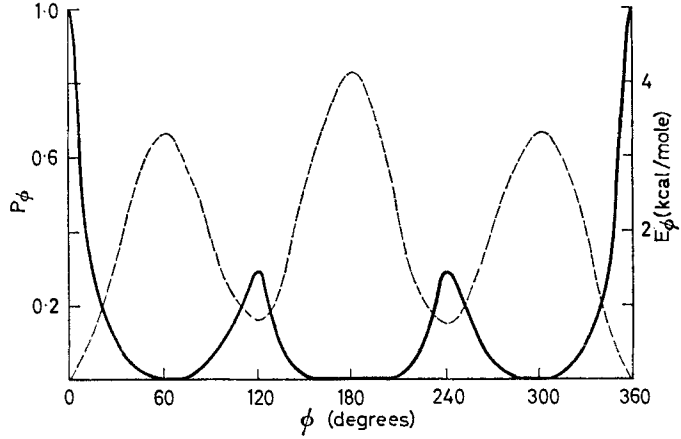


Figure 3.2. The non-bonded interaction energy E_ϕ (broken line) and relative probability P_ϕ (solid line) for all possible conformations of the carbon chain of butane.

butane must be considered as existing in three distinct conformations, the *trans* (*Figure 3.3a*) and the two skew (*Figure 3.3b* and *c*), with a limited amount of torsional oscillation about these positions. The relative probabilities of these three conformations (at 37°C) are 1·0, 0·272 and 0·272 respectively;

as the two skew conformations are equivalent, roughly two-thirds of the molecules in a sample of butane are, at any moment, in the fully extended form and one-third in the bent (skew) form. The planar *cis* form (*Figure 3.3d*) is a highly hindered conformation, with the maximum interaction energy of about 4·1 kcal/mole[11,12]. Ito[13] calculated a value as high as 6·1 kcal/mole

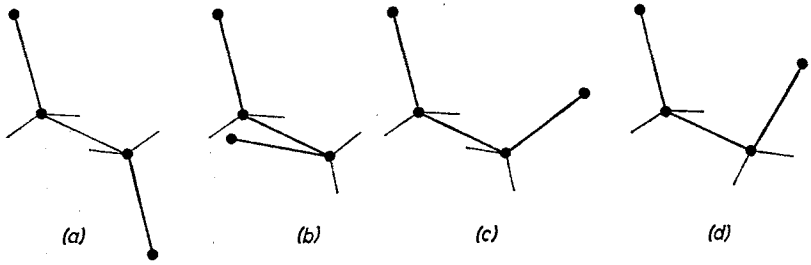
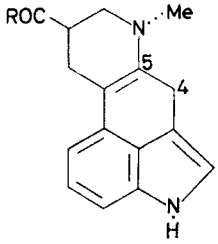


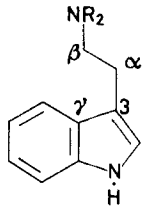
Figure 3.3. Possible conformations of butane. The hydrogen atoms of the terminal methyl groups have been omitted.

for this interaction energy; with the lower value, only about 1 in 1000 molecules would have this configuration at normal temperatures.

This does not mean, of course, that such energetically unfavourable configurations are irrelevant when interactions with a receptor are involved. If a very stable bond, with a free energy of formation greater than 4–6 kcals/mole can be formed when the molecule is in this strained configuration, then the molecule may be deformed into this configuration and the bond made. Such a molecule would, however, be less firmly bound than another molecule that adopted the same arrangement of reactive groups with less internal strain. Greenberg[14], in a study of the actions of tryptamine analogues on the *Venus mercenaria* heart, found that lysergic acid diethylamide *III*, R = NEt_2 produced a threshold effect at concentrations some 10^{-6}–10^{-7} lower than those required for simple tryptamine analogues. He argued that as in lysergic acid diethylamide C_4 and C_5 are fixed, by the geometry of the



(III)



(IV)

other rings, in the plane of the indole rings, it is necessary for the corresponding carbon atoms (C_α and C_β *IV*) in simple tryptamine analogues also to lie in the plane of the indole rings in order to combine with the receptor. This is an energetically unfavourable configuration, with C_α, C_β, C_3 and C_γ in a planar *cis* configuration. Assuming an energy barrier of 4–6·5 kcals one in 10^3–10^5 molecules would be present in this configuration. It is

then only necessary to assume that lysergic acid diethylamide was only about 100 times as active as a simple tryptamine derivative *in the planar configuration* to account for the considerable difference in activity between the two types of molecule.

As the carbon chain increases in length the number of bonds about which rotational isomers can form also increases and the total number of possible conformations increases rapidly. In the case of butane the most probable distance between the two terminal carbon atoms in the chain is the maximum distance, with the chain in the fully *trans* conformation. In all other simple paraffin chains the fully extended *trans* conformation is also that with the lowest energy. However, the fully extended conformation is unique, since the maximum separation of the two terminal atoms can only be obtained

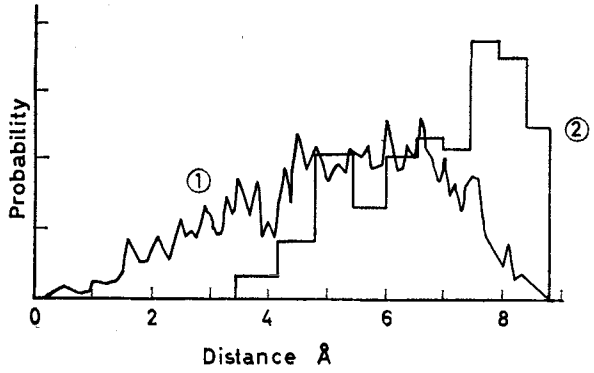


Figure 3.4. Probability distributions of the distance between the terminal carbon atoms of n-octane. Distribution 1 was obtained using a Monte Carlo method, with no restriction of rotation about carbon-carbon bonds. Distribution 2 was obtained when the effect of rotation barriers is taken into account.

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with one particular arrangement in space of the atoms in the intervening chain. The distance between the ends of the molecule decreases as the molecule adopts skewed conformations at some points along the chain. With chains of moderate length any given distance, shorter than the maximum, can be attained in several different ways with skewed conformations about different links in the chain. Although any one of these skewed conformations is energetically less favourable than the fully *trans* conformation, the greater statistical weight of the slightly bent conformations counterbalances the effect of a greater internal energy. As a result the most probable distance between the ends of paraffin chains longer than butane is rather less than the maximum possible. Very short distance between the ends of the molecule are precluded as these could only be attained in a limited number of conformations each involving high energy non-bonded interactions. As the number of possible rotational configurations about any one bond is reduced to only 3 of any significance, it is possible to calculate the effective length and the internal energy of all probable conformations of paraffin chains of up to 10 atoms, and hence calculate a length-probability distribution for each chain that allows for the effect of the rotation barrier[15]. *Figure3.4* shows the distribution calculated for a 7 link chain (octane) compared with the distribution calculated by a Monte Carlo method[16] for the same chain but ignoring the effect of internal rotation barriers. It can be seen that the probability distribution obtained when allowance is made for the rotation

barrier is rather erratic. The reason for this is that for a fixed angle between each succeeding bond, certain ranges of distances can only be obtained by forcing some segments of the chain into highly unfavourable conformations. The fully extended chain is still an important configuration but the most probable distance between the ends of the molecule is less than this, at about 80 per cent of the maximum. The freely-rotating model gives full effect to the greater statistical weight of the more folded configurations but ignores the greater internal energy of these forms: as a result the most probable distance between the ends is given as about 60 per cent of the maximum and far too great a weight is given to very short distances. In fact, conformations of carbon chains of moderate size with very short distances between the ends—pseudo cyclic structures—are unlikely; the bulk of the molecules have distances greater than 70 per cent of the maximum. As the carbon chain gets longer the most probable distance between the ends slowly decreases and the fully extended conformation diminishes in importance: nevertheless the more extended conformations continue to predominate.

These conclusions are confirmed by the observation, well known to organic chemists, that syntheses involving the closure of rings of about 10 atoms are very difficult to achieve[17]. The calculations referred to above apply to molecules that are free from intermolecular forces, a condition that applies in dilute solution, the state most relevant to the study of drug action. Mizushima[18] has studied the conformations of a series of hydrocarbons in the solid and liquid state using Raman spectra. He found that in the solid state all paraffins existed in the fully extended, all *trans* form, the molecules being held in this way by the lattice forces of the crystal. It is only when the molecules are in an extended rod-like conformation that they can be close-packed into an ordered lattice: such close packing would be impossible if the molecules adopted skewed conformations. In the liquid form, when the crystals have melted and the restrictions imposed by the necessity to fit into lattice removed, Raman bands due to folded forms were observed. With the shorter chains the band attributed to the fully extended form was still observed and it was concluded that the fully extended form was one of the possible conformations in the liquid state. However, with longer chains, for example cetane, $C_{16}H_{34}$, only bands due to folded forms were observed. These observations on liquid hydrocarbons are complicated by intermolecular interactions between the carbon chains and so do not give an accurate picture of an isolated chain, the condition most relevant to a drug in dilute solution, but they directly confirm the description based on considerations of restricted internal rotation. These observations also demonstrate the very important point that studies on the structure of flexible molecules in the solid state give no reliable information on their possible conformations in dilute solution.

The qualitative picture of a hydrocarbon chain that emerges therefore is not that of a flexible piece of string but rather one of a stiffly jointed system of rods occupying a range of conformations, for the most part near to the fully extended forms.

(2) *Branched paraffin chains*

The introduction of methyl groups along the chain has a disturbing effect on the distance-probability distribution of a paraffin chain. The 3 major

conformations of 2-methylbutane are shown in *Figure 3.5*. One *trans* (*Figure 3.5a*) and two skewed conformations (*Figure 3.5b* and *5c*) are possible but their relative energies are not as in butane. It can readily be shown* that the *trans* form (*Figure 3.5a*) and one of the skewed forms, (*Figure 3.5c*) are of equal energy, and that the second skewed form (*Figure 3.5b*) has an internal energy 800 cal above the other two conformations. At 37°C, the three conformations thus have relative probabilities of 1, 0·27 and 1: the two skewed forms therefore have a slightly greater combined probability than the *trans* form. The introduction of branching methyl groups into a paraffin

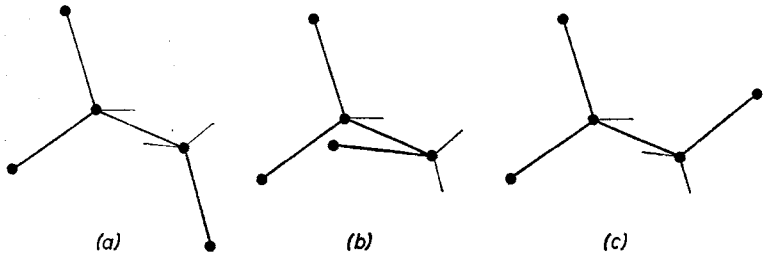
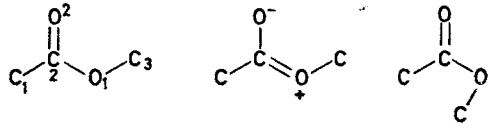


Figure 3.5. Possible conformations of 2-methylbutane. The hydrogen atoms of terminal methyl groups have been omitted.

chain therefore increases the probability of bent or skewed conformations and as a result the maximum in the distance—probability distribution moves to shorter distances.

(3) *Esters*

The introduction of an ester linkage into a paraffin chain produces an alteration in the pattern of possible conformations. Because of resonance between forms (*V*) and (*VI*) free rotation about the CO–O bond is severely restricted. An electron diffraction study[19] of a series of simple aliphatic



(*V*) (*VI*) (*VII*)

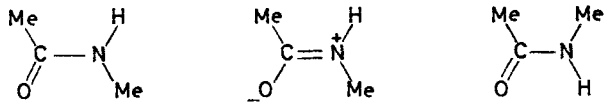
esters showed that of the two possible conformations (*V*) and (*VII*), the *cis* form (*V*) predominated, the *trans* form (*VII*) making little contribution. It was also found that, due possibly to steric interaction between C_3 and O_2,

* In the *trans* conformation in butane (*Figure 3.3a*) the terminal methyl group is interacting with 2 hydrogen atoms. In the skew positions (*Figure 3.3b* and *3c*), it is interacting with one methyl group and one hydrogen atom. Let the interaction energy between a methyl group and another methyl group be E_C and that between a methyl group and a hydrogen atom be E_H. The interaction energy in the skewed form of butane is 800 cal greater than in the trans form $\therefore E_C + E_H - 2E_H = E_C - E_H = 800$ cal. The interaction energy in the *trans* form (*Figure 3.5a*) of 2-methylbutane $= E_C + E_H$: for the skewed form (*Figure 3.6c*) this energy is also $= E_C + E_H$. In the conformation (*Figure 3.5b*) the energy $= 2E_C$. The difference between conformation (*Figure 3.5b*) and the two equivalent conformations (*Figure 3.5a* and *5c*) $= 2E_C - (E_C + E_H) = E_C - E_H = 800$ cal.

the carbonyl bond was tilted out of the plane of $C_2O_1C_3$, with an average angle of 25°, varying between the limits of 0–35°, suggesting two conformations skewed about the planar *cis* form (*V*). However, these results may mean that the carbonyl group executed torsional oscillations with an amplitude of $\pm 35°$ about the planar form. The qualitative conclusion is that an ester linkage in a chain tends to reduce the number of possible conformations, compared with a simple paraffin chain, and tends to favour conformations with the chain fully extended.

(4) *Amides*

The effect of a single amide linkage on chain conformation is similar to that of an ester linkage. Studies of a range of properties of *N*-methylacetamide (*VIII*) showed that due to resonance between forms (*VIII*) and (*IX*) the molecule was essentially planar and that the *trans* form (*VIII*), rather than



(VIII) *(IX)* *(X)*

the *cis* form (*X*) predominated[20]. Badger and Rubaclava[21] deduced from spectroscopic evidence that the *trans* form is at least 2 kcal/mole more stable than the *cis*, which means that at normal temperatures not more than 2 per cent is in the *cis* form. The amide link is more rigid than an ester linkage, and torsional oscillations about the C–N linkage are of much smaller amplitude. Corey and Pauling[22] estimated the deformation energy of this bond and only 1 per cent, at normal temperatures, probably has the carbonyl group deviating by more than 17° from the C–N–C plane. The amide linkage therefore, imposes a greater restraint than an ester linkage on the number of possible conformations and also tends to move the most probable distance between the ends of the chain, closer to the maximum.

(5) *Ethers*

In contrast to the ester and amide linkages, which tend to stiffen a chain compared to a corresponding polymethylene chain, an ether linkage

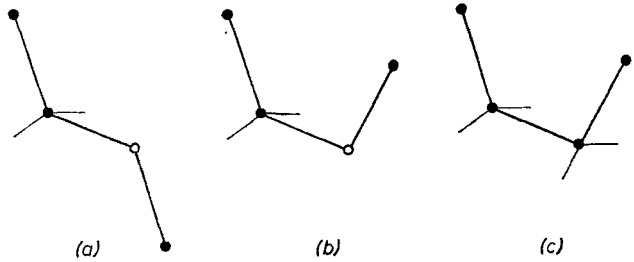


Figure 3.6 a and *b*. Possible conformations about an ether linkage.
c. Skew conformation of butane.

increases flexibility. Eucken and Franck[23] found a rotation barrier about the C–O bond (*Figure 3.6a*) of about 2·5 kcal/mole and distinct spectroscopic

evidence that significant numbers of molecules at normal temperatures were in *cis* conformations (*Figure 3.6b*). This greater freedom of rotation can be attributed to the absence of hydrogen atoms on the oxygen atom coming to repel the hydrogen atoms on the adjacent carbon atom when in the *cis* conformation (compare the *cis* butane conformation (*Figure 3.6c*)). An ether linkage introduced into a chain therefore increases the flexibility of the chain, widens the range of possible conformations and moves the maximum in the distance probability distribution to shorter distances.

(6) *Amines*

The amino group produces the least deviation from the conformation distribution of the simple paraffin chain. Aliphatic secondary and tertiary amines are strong bases and at physiological pH values they are almost completely protonated and in the charged tetracovalent form. The tetracovalent nitrogen atom is tetrahedral and, apart from minor variations in bond lengths, resembles a carbon atom in its geometry. Thus a secondary amine group in a chain closely resembles a methylene group (*Figure 3.7a*), compare *Figure 3.3a*, and an *N*-methyl tertiary amine (*Figure 3.7b*) resembles the corresponding branched polmethylene chain. Therefore, considering only steric influences, a chain containing a secondary amine group within it should have a length-probability distribution very similar to the corresponding polymethylene chain. An *N*-methyl group produces the same effect as a *C*-methyl group. However, a positively charged group within a chain interacts with other charged groups within the molecule, and this electrostatic effect, together with the possibility of hydrogen bonding (see below) may seriously alter the pattern of possible conformations.

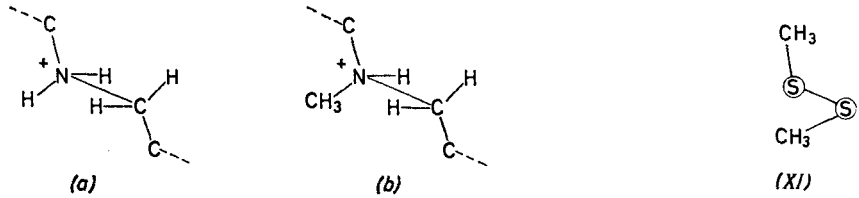


Figure 3.7. Trans conformations of *a.* secondary amino. *b. N*-methyl tertiary amino components of a carbon chain (compare *Figures 3.3a* and *3.5a*).

(7) *Disulphides*

The disulphide link has most remarkable conformational properties. A study of the structures of 2,3-dithiabutane (*XI*) and 3,4-dithiahexane shows that there is a very large energy barrier of 9·5 kcal/mole hindering free rotation about the S–S bond and which prevents the molecule adopting either a *cis* or *trans* planar conformation[24,25]. The two C–S bonds are therefore held at right angles to one another, and the high energy barrier severely restricts any torsional oscillations about the S–S axis. A disulphide linkage produces a right angled kink in the chain, effectively shortening the maximum possible distance between the ends and, as the two sulphur atoms and the two adjacent carbon atoms are fixed relative to one another, introduces a

rigid elements into the chain and reduces the number of possible conformations.

(8) *Other effects modifying the conformation of flexible chains*

The effects discussed so far have concerned local non-bonded interactions which are mainly a consequence of the space-filling properties of the elements making up the chain. The conformations that are possible for a flexible molecule may be further modified by other interactions between specific groups in the molecule. In the dilute aqueous solutions that are relevant to the problem of drug action, intermolecular interactions between different drug molecules may be ignored and the two most important intramolecular modifying influences to be considered are electrostatic forces and hydrogen bonding.

Electrostatic forces—The presence of more than one charged group within a flexible molecule affects its conformation: two charges of opposite sign attract one another and the two groups tend to come closer; like charges repel and the two groups tend to move away from one another. The polymethylene bis-quaternary group of ganglion-blocking and neuromuscular-blocking agents (*XII*) serve as an example where electrostatic effects have

$$Me_3\overset{+}{N}\cdot(CH_2)_n\cdot\overset{+}{N}Me_3$$

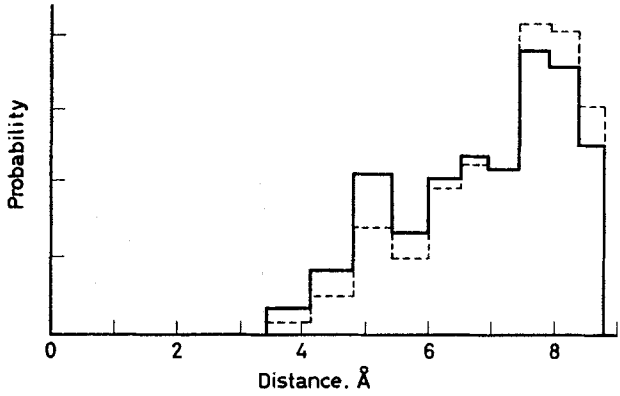
(*XII*)

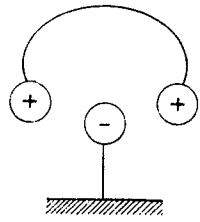
an effect on conformation. The effect of electrostatic forces on conformation in this case has been discussed in detail[15] and it has been shown that the mutual repulsion of the two terminal charges has the expected effect of making very short distances between the ends of the molecule less likely to occur than in an uncharged polymethylene chain. Consequently, there is an increase in the probability of the more extended conformations, although this effect is not overwhelming, and the general pattern of possible conformations is still largely determined by stereochemical interactions within the chain.

The major difficulty in calculating the magnitude of electrostatic effects within molecules lies in determining the effective dielectric constant of the medium separating the two charges. The molecule carrying the charge is embedded in a solvent of different composition and consequently the charges are separated by a medium of varying dielectric constant and are interacting over distances of the same order of magnitude as the dimensions of the molecules comprising the solvent. In the case of the polymethylene chain the problem may be simplified by assuming that the chain is bent out of the direct line between the two charges and that the paraffin chain does not seriously distort the spherical symmetry of the dielectric round the charges. It has been shown[26,27] that the dielectric constant of an aqueous solution is essentially constant at the bulk phase value to within 4 Å of a univalent ion. From this the electrostatic contribution to the conformational free energy of a flexible molecule can be calculated: it varies from 800 to 330 cal/mole as the distance between the ends of the chain varies from 5 to 12 Å. The electrostatic energy, therefore, is small compared to the magnitude of the energy barriers which determine the configuration of a carbon chain. The effect of the repulsion of the two terminal charges in hexamethonium (*XII*, $n = 6$) on the distance probability distribution is shown in *Figure 3.8.* At

distances less than 4 Å the effective dielectric constant would decrease and at such short distances electrostatic interaction would be considerable.

Cavillito and Grey[28] have criticized this treatment of the electrostatic effect and have suggested that ion-pair formation neutralizes the mutual





(XIII)

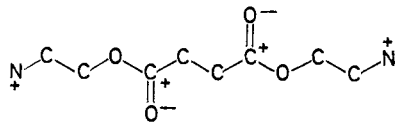
Figure 3.8. Effect of repulsion between the terminal charged atoms of $Me_3\overset{+}{N}\cdot(CH_2)_6\cdot\overset{+}{N}Me_3$. Solid line: length probability distribution of an uncharged seven link chain. Broken line: length probability distribution when corrected for charge repulsion.

repulsions of the two terminal charges and that a significant fraction of bis-quaternary molecules forms 'chelate-like' structures with a cellular anion (*XIII*).

Such a proposition grossly overestimates the importance of electrostatic effects on chain conformation. The formation of an ion pair between the quaternary group at one end of the chain and an anion converts the repulsion between two like charges into a less powerful interaction between a dipole and the remaining positive charge. It has been shown that the stronger repulsion between two like charges has little effect on the distance probability distribution: the ion-pair complex would have a distribution close to that of an uncharged chain. The energy barriers preventing heavily folded cyclic structures still dominate the configuration distribution and make the chelate-like conformations proposed by Cavallito and Grey unlikely.

Dipole-dipole interaction is a weaker form of electrostatic interaction. A common unit in flexible chains possessing a sizeable dipole moment is the carbonyl group, and this will serve as an example for discussion. The carbonyl group is polarized with the negative charge of the dipole on the oxygen atom. Dipoles that are aligned parallel to one another and with their negative poles pointing in the same direction will repel one another; a parallel arrangement with the dipoles pointing in opposite directions will tend to attract one another. Because the magnitude of the charges involved are less than those on a quaternary nitrogen atom, dipole-dipole interactions are weaker electrostatic interactions than those already discussed and are only significant when the two groups are very close to one another. Thus in succinylcholine (*XIV*), the alignment in opposite directions of the dipoles of the carbonyl groups in the ester linkage in the fully *trans* configuration

(*XIV*) probably makes a small addition to the stability of this sterically favoured conformation.



(*XIV*)

Hydrogen bonding—The hydrogen bond is an important mode of intermolecular and intramolecular interaction and is responsible for a wide range of phenomena, from the high boiling point of water to the crunch of a lump of sugar. The properties of this bond have been repeatedly reviewed[29,30] and an extensive discussion of this bond will not be given here. The properties of the hydrogen bond are briefly as follows.

(1) Hydrogen bonding is an interaction between a hydrogen atom linked to an electronegative element with another molecule or part of the same molecule also containing an electronegative element. We may refer to the group to which the hydrogen atom is most firmly bound as the donor, and the other electronegative group as the acceptor. In biological systems the most important donors are —OH and =NH; the thiol group SH is only a very feeble donor. The most important acceptors are oxygen (O=) and nitrogen (N=), with sulphur interacting weakly.

(2) Interaction occurs only over short distances: the separation between the two electronegative elements is in the range 2·5–2·7 Å. There is very little interaction at distances greater than 3·0 Å.

(3) The bond is directional: maximum interaction occurs when the hydrogen atom lies on the line joining the two electronegative elements, but some deviation from linearity is possible.

(4) The bond is quite stable, with an average heat of formation of about 5 kcal/mole. Very roughly the order of stability of hydrogen bonding between different elements is in the order OHO > OHN > NHN.

With a heat of formation of the order 5 kcal/mole it might be thought that the possibility of intramolecular hydrogen bond formation would have a profound effect on the conformation of flexible chains, tending to favour conformations where such a bond could form. There is, however, one important qualification that must be made when considering hydrogen bonding in the environment relevant to drug action, that is in dilute aqueous solution. Water is a very effective hydrogen bonding agent, acting both as a donor and as an acceptor, and most of its peculiar physical properties can be attributed to this fact. Therefore, in aqueous solution, any group in a molecule that is capable of forming a hydrogen bond, either as a donor or acceptor, will be bonded to water. In considering the possibility of hydrogen bonding between two different groups in an aqueous solution the relevant thermodynamic quantity is not the free energy of formation of a hydrogen bond *per sec* but the *difference* in free energy between donor/water, acceptor/water and the donor/acceptor, water/water hydrogen bonds (*equation 1*).

Equation 1

$$H_2O\cdots H{-}R + A\cdots\cdots H_2O \rightarrow A\cdots\cdots H{-}R + H_2O\cdots\cdots H_2O$$

Pauling and Pressman[31] in a study of antigen-antibody reactions estimated the difference in free energy of formation of a hydrogen bond between a hapten and antibody and hapten and water as only 400–700 cal/mole, a much lower order of magnitude. The geometrical requirements of the hydrogen bond, necessitating the alignment of the bonded groups, greatly restricts the rotational freedom of the intervening structures, resulting in a considerable loss of entropy. The low heat of formation of the hydrogen bond in water in general would hardly compensate for this entropy loss, consequently, it is unlikely that hydrogen bonding between different parts of a small molecule would have any great effect in aqueous solution. This point will be discussed further in the section on protein structure.

Summary

The general conclusion that can be drawn from this survey of the effect of different structural features on drug conformations is that alterations in the composition of a flexible chain of atoms, whilst keeping the number of atoms constant, produces effects over and above those due to the chemical reactivity of the added groups. Profound alterations can be made in the spatial configuration of the molecule, a property that is now considered to be of great importance in modifying drug activity. Different groups tend to stiffen a chain, or make it more flexible; they also tend to bend it or straighten it out. The introduction of two ester groupings into decamethonium (*XII*, n = 10) to give succinylcholine (*XIV*) does more than introduce two hydrolysable groups into the chain: the ester groups also alter the whole pattern of possible conformations, making the molecule more rigid and so causing it to adopt more extended conformations. On the other hand the bis-quaternary ether analogue[32] (*XV*) of pentamethonium (*XII*, n = 5) is expected to have a

$$(CH_3)_3\cdot\overset{+}{N}\cdot CH_2\cdot CH_2\cdot O\cdot CH_2\cdot CH_2\cdot\overset{+}{N}(CH_3)_3$$

(*XV*)

more flexible and compact size distribution than its polymethylene analogue. These conclusions serve to underline the common observation that the introduction of chemically inert groupings of the same general size into a molecule does not necessarily produce a compound with the same pharmacological activity.

The general considerations outlined above can now be applied to a consideration of the possible conformations of some molecules of pharmacological interest.

Acetylcholine

Despite the great importance of acetylcholine as a transmitter substance, and the part it has played in the development of pharmacology, there is still a great deal of uncertainty about its conformation in solution. The conformation of acetylcholine is dependent on the possibility of rotation about two

bonds: the C–C and the C–O bonds of the choline fragment. There are three possible conformations about each bond, giving a formal possibility of 9 conformations for the molecule as a whole—the fully *trans*, and four pairs of identical skewed forms (*Figure 3.9a–e*). The cyclic, doubly skewed form

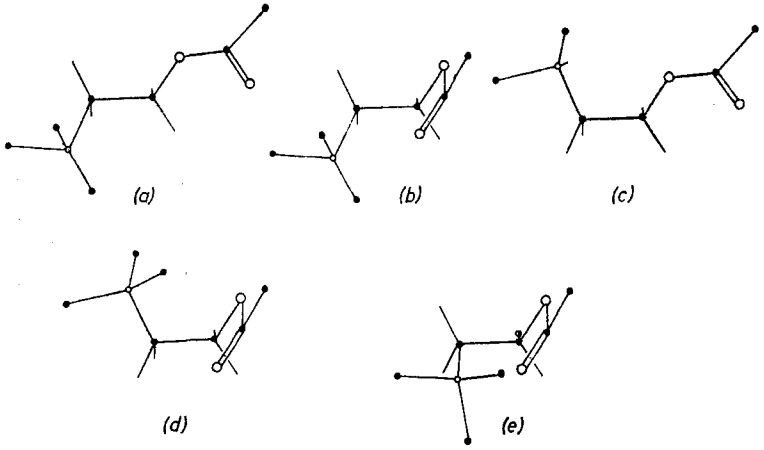


Figure 3.9. Possible conformations of acetylcholine. Solid circles = carbon; small open circle = nitrogen; large open circle = oxygen. Hydrogen atoms of the *N*-methyl and acetyl methyl groups have been omitted.

(*Figure 3.9e*) permits interaction between the carbonyl oxygen atom and the positively charged nitrogen atom, and several authors[28,33] have suggested that this is the most likely conformation of acetylcholine. However, calculations of interatomic distances show that in a cyclic conformation with minimum interaction energy within the chain there is a large overlap of the van der Waals radii of the carbonyl oxygen and the quaternary methyl groups, leading to very large steric compression. Slight deviations from this structure to increase the separation of the oxygen atom and the methyl groups are only possible at the expense of greatly increased intra-chain interactions. The electrostatic interactions between the ester dipole and the quaternary head could not compensate for the great steric compression in such a cyclic form and hence the cyclic conformation (*Figure 3.9e*) may be discarded from consideration.

The rotation barriers within the molecule are largely determined by the bulky trimethylammonium group. Precise values are not known, but reasonable estimates can be made. The trimethylammonium group, with a van der Waals radius of 3·4 Å from the central atom[34] is much larger than the terminal methyl group of butane. The interaction energy when it is in the skewed conformation (*Figure 3.9c*) is considerably greater than in the corresponding conformation in butane: a value of 2 kcal/mole is assumed. The carbonyl group of the ester linkage is also larger (van der Waals radius 2·6 Å) than a methyl group and a value of 1 kcal/mole is assumed for its skew conformation. Therefore, on the basis of intra-chain stereochemical interactions alone the relative probabilities of conformations (*Figure 3.9a–d*) will be 1:0·40:0·08:0·02.

However, an ester group is a dipolar unit, and the possible conformations of this ester will be affected by interactions with the quaternary ammonium head. There will therefore be an interaction between the positive charge of the quaternary nitrogen atom and the ester dipole depending on the orientation of the dipole and its distance from the nitrogen atom. In the fully *trans* form (*Figure 3.9a*) and in two of the skewed forms (*Figure 3.9c,d*) the ester dipole is aligned roughly at right angles to the line joining its centre to the nitrogen atom: dipole interaction is therefore negligible. In the remaining skewed form (*Figure 3.9b*) the dipole is directed towards the nitrogen atom and hence the dipole interaction will tend to increase the probability of the most favoured of the skewed forms. The precise value of the interaction energy depends on the value chosen for the effective dielectric constant between the charged groups. The distance between the two groups is about 4·0 Å which means that a value rather less than the bulk value must be chosen. A simple calculation then gives an interaction energy of 100–400 cal. When corrected for this dipole interaction the relative probabilities of the four forms (*Figure 3.9a–d*) of acetylcholine in solution become roughly 1 : 0·55 : 0·08 : 0·02.

Sörum[35] determined the crystal structure of acetylcholine bromide and found two configurations in the same crystal lattice namely *Figure 3.9a* and *3.9c*. Both Sörum and Kennard[36] suggested that this skewed form (*Figure 3.9c*) predominates in nonpolar media and this could explain the behaviour of acetylcholine at lipid water interfaces. However, it has been pointed out earlier that the conformations of molecules constrained within a crystal lattice are often very different from those adopted in solution, when the molecule is free from intermolecular interactions, and that evidence derived from crystal structures must be treated with reserve. In this particular case the acetylcholine molecule in the crystal lattice has to accommodate itself to the geometrical requirements of its anion, in this case bromide: it would be of interest to see whether the same skewed form is present in crystals containing other anions. Preliminary surveys of the crystal structures of acetylcholine chloride[37] and iodide[38] have been made but they were not carried through to a structure determination. There is no close similarity between the crystal structure of the bromide and that of either the chloride or iodide. The crystal structure of acetylcholine bromide is also unusual in that in several places adjacent molecules approach one another to distances less than normal van der Waals radii; lattice compression forces must be considerable (compare with reference[36]). In dilute solution, the anion has little effect on conformation.

The analysis of the conformation of acetylcholine in solution shows that the ester linkage makes the major contribution to the flexibility of the molecule and that the most important forms are the fully *trans* and the skewed form (*Figure 3.9b*). These conformations are mean positions, there being limited torsional oscillation of groups about these positions.

Adrenaline

The point of most interest in the conformation of adrenaline concerns rotational isomerism about the C–C of the ethyl side chain, although rotation about other bonds is also possible. However, owing to a lack of knowledge

of the rotation barriers in molecules with such large groups present, the discussion must be qualitative.

In order to minimize interaction between the two *ortho* carbon atoms of the benzene ring and the two substituents on the C_1 carbon atom the benzene ring usually lies with its plane bisecting the angle C_2–C_1–O. Three possible conformations about the C_1–C_2 bond are possible and these are shown in *Figure 3.10*. As a general rule, a group on one carbon atom will arrange itself so that it is closest to the two smallest groups on an adjacent carbon atom. Thus conformation *Figure 3.10a* will predominate, with the amino group

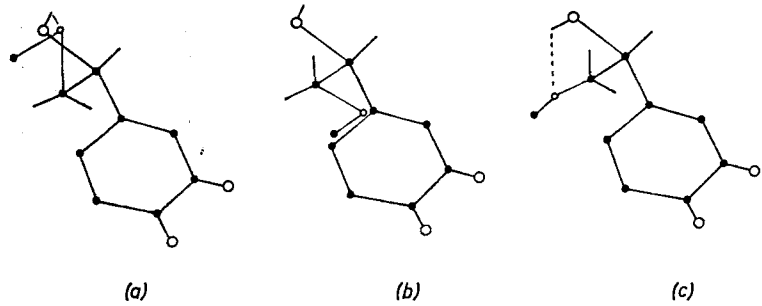


Figure 3.10. Possible conformations of adrenaline. Solid circles = carbon; small open circles = nitrogen; large open circles = oxygen. Hydrogen atoms of the *N*-methyl group and the aromatic ring have been omitted. In conformations *a* and *c* intramolecular hydrogen bonding is possible (broken line).

between a hydrogen atom and a hydroxyl group, and farthest away from the bulky benzene ring. Both the other conformations involve interactions with the benzene ring and are much less likely. On steric grounds alone, conformation *Figure 3.10b* with the amino group between the benzene ring and a hydrogen atom, would be preferred to conformation *Figure 3.10c* with the amino group between the benzene ring and the hydrolxyl group. However, there is the possibility of hydrogen bonding between the hydroxyl group and the amino group in conformations *Figure 3.10a* and *3.10c*, but not in *Figure 3.10b*. It has been pointed out that intramolecular hydrogen bonds in molecules in aqueous solution do not confer a great deal of stability but there is evidence that they exist and exert an effect[39]. In this particular case conditions are most favourable: the chain linking the two groups is very short and the number of alternative configurations is small, so that the entropy loss is slight. This marginal possibility of hydrogen bonding could counterbalance the unfavourable steric interactions of the amino and hydroxyl groups and consequently the order of stability for the three conformations is *Figure 3.10a* $\gg$ *10b* $\simeq$ *10c*.

THE RECEPTOR

The receptor is the all-important second component of the drug-cell interaction, and it represents the central problem of pharmacology, for it underlies nearly all present ideas concerning the mechanism whereby cells differentiate and respond to different chemical compounds. There are still uncertainties about the precise structures of the drugs applied to tissues:

this uncertainty pales into insignificance when the almost total ignorance concerning the structure of receptors is considered. A receptor is that component of a cell with which a drug combines to initiate a response. A discussion of the receptor must proceed forward tentatively from this.

The interior of a cell may be regarded as a specialized environment, carrying out its special functions by means of an interlocking matrix of enzymes, with their attendant substrates; intercourse between the intracellular and extracellular environment is regulated by the cell membrane. Drugs may act either intracellularly, in which case their effects are, for the most part, due to an interference in some way with enzymic activity, or at the membrane. Enzymes are proteins, so drug-enzyme interactions are a part of the general problem of small molecule-protein interactions (*vide infra*). For the purpose of this discussion a further, possibly artificial[2], restriction of the meaning of the word 'receptor' may be placed, and attention confined to drug actions at the cell membrane. A discussion of possible receptor substances then resolves itself into a consideration of the chemical constituents of cellular membranes, followed by a detailed discussion of the properties of those components that appear to be capable of conferring a high degree of specificity to the membrane in its interactions with small molecules.

The Chemical Nature of Cell Surfaces

The ultrastructure of cell membranes has been the subject of comprehensive reviews[40a,b], so it will suffice to present the main conclusions without recapitulating all the evidence. Unfortunately the most intensively studied structures have been the axonal and Schwann cell membranes; the membranes of most interest to the pharmacologist—the chemically excitable synapses—have not been so thoroughly studied at the very high levels of resolution needed to obtain information about molecular organization. However, the studies that have been made indicate that the general structural pattern found in the axonal and Schwann cell membranes is also found at excitable synapses, so this structure can be taken as a reasonable basis for discussion.

The common structural unit of the cell membrane is thought to be a bimolecular layer of oriented lipid molecules sandwiched between two non-lipid, probably protein, layers. Finean[41] suggested that the lipid layers consist of a cholesterol/phospholipid complex with the hydrocarbon chain of the phospholipid aligned against the steroid ring system, the polar ethanolamine phosphate 'head' being folded back to form a hydrogen bond with the hydroxyl group of cholesterol (*Figure 3.11*). The two monomolecular layers of lipid are then arranged with their polar heads facing outwards[40], the two layers being held together by van der Waals attraction between the two nonpolar 'tails'. Finean also suggested, on the basis of the changes in low angle x-ray diffraction patterns during drying, that the exact orientation of the lipid complex within the membrane depended on the interactions of the polar head with the protein outer layers, and that the lipid molecules may be arranged with their long axes either perpendicular or tilted with respect to the plane of the membrane, depending on the structure of the outer layers. The exact nature of the forces combining the lipid layers with

the outer layers of the membrane are not known. Most studies of lipo-protein complexes have been directed towards the complexes present in solution, for example the plasma lipo-proteins, and here the combining forces seem to be electrostatic, with some van der Waals attraction[42]. Chargaff[43] pointed out that covalent bonding between protein and many of the naturally

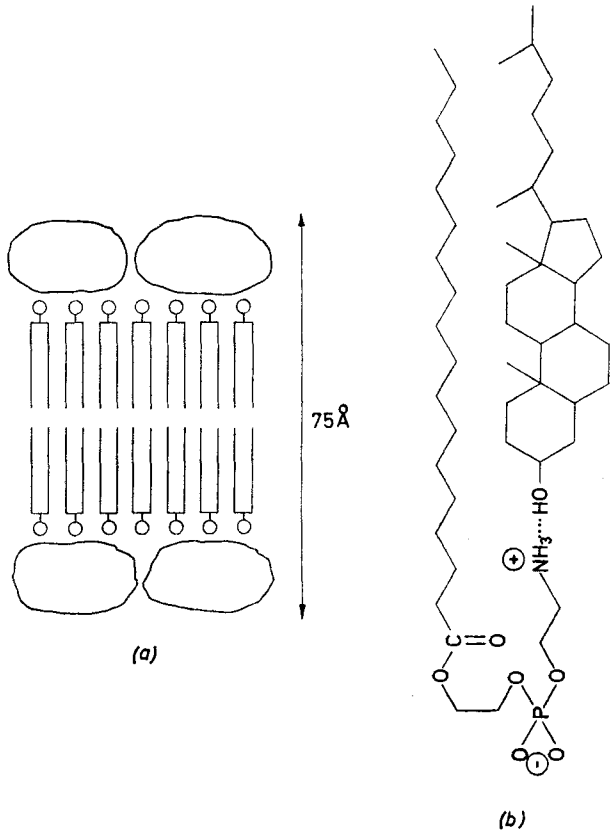


Figure 3.11a. Schematic representation of membrane structure, showing a bimolecular layer of lipid molecules with polar groups facing outwards, sandwiched between protein monolayers. *b.* Possible structure of a phospholipid-cholesterol complex.

occurring lipids, by ester and amide linkages is theoretically possible, but states that there is no evidence for such bond formation. The orientation of the lipid molecules proposed by Robertson implies that the layers are bonded primarily by electrostatic attraction between the negatively charged phosphate groups and the positively charged choline and ethanolamine groups of the lipid with corresponding positive and negative charges on ionized basic and acidic groups on the outer layers.

There is some uncertainty about the precise nature of the outer layers of the unit cell membrane. Early descriptions of the membrane[44,45] suggested that both layers were monolayers of protein. Robertson[40] however, suggested that the two non-lipid layers are different; the inner layer is protein and the outer layer may be a monolayer of polysaccharide. The Schwann cell membrane appears to be asymmetric, with the two faces differing in their staining properties, affinity for water and ability to cohere to one another.

Abood and Abul-Haj[46] presented histochemical evidence for the presence of a sulphated mucopolysaccharide in the gap between myelin and the neurolemma sheath, and Robertson therefore suggested that this substance may also be present in the outer membrane surface.

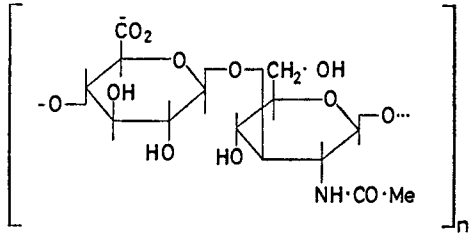
It should, however, be noted that it is not necessary to suppose that the two surfaces of the membrane are composed of different substances to account for the asymmetry of the unit membrane. The variation in the properties of different amino acid side chains makes it possible for two different proteins to exhibit widely differing properties, and hence the asymmetry of the membrane may be due to two layers of protein of different properties rather than to two basically different substances. The mucopolysaccharides are an important constituent of connective tissue and of bacterial cell membranes and it is obviously possible that they may form a constituent of excitable tissue membranes as well. However, it has been found[46,47] that hyaluronidase has no effect on axonal conduction, so the part played by such a polysaccharide in the function of the cell is not clear.

The triple-layered membrane, 75 Å thick, characteristic of Schwann cells has been found in many other types of cell[40], including the chemically excitable synapses. The asymmetry found in the Schwann cell membrane may also be found elsewhere. Couteaux[48] demonstrated the presence of cholinesterase on the surface of the muscle end-plate, and in ganglion cells, so it may be presumed that proteins form a part, at least, of the external surface of chemically excitable membranes.

If the primary action of many drugs is accepted as being exerted on the external surface of a cell membrane, and that the major chemical constituent of the outer layer of a cell membrane consists of a monolayer of protein, possibly together with some mucopolysaccharide, then it is necessary to consider in some detail the structure and properties of these macromolecules in order to get some insight into the possible structure of receptors.

Mucopolysaccharides

The mucopolysaccharides constitute a rather ill-defined group of substances of considerable importance: they form the amorphous 'ground substance'

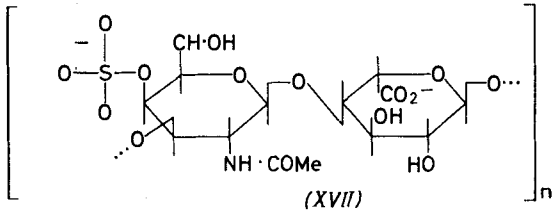


(XVI)

of connective tissue, the blood-group specificity substances, the protective mucous secretions of respiratory and digestive passages and are present in many types of nerve cell. They are nearly always found associated in some way with proteins[49,50]. The mucopolysaccharides differ from the typical

polysaccharides, such as starch and amylose, in that the carbohydrate units of the chains contain anionic groups such as the carboxyl group of glucuronic acid and sulphate ester groups, together with amino groups, which are, however, usually acetylated. Typical component sugars are D-glucosamine and D-galactosamine, D-glucuronic acid, L-iduronic acid, with N-acetyl and *O*-sulphate and *N*-sulphate substituents. The polymers are usually linear structures with an alternating sequence of two monosaccharide units. Thus hyaluronic acid, a non-sulphated mucin, consists of a chain of linked pairs of *N*-acetylglucosamine and glucuronic acid (*XVI*).

Chondroitin sulphate B, a sulphated polysaccharide consists of a chain of L-iduronic acid and sulphated *N*-acetylgalactosamine (*XVII*).



(*XVII*)

The mucopolysaccharides that have been isolated have very high molecular weights: values varying between 50,000 and $1{\cdot}4 \times 10^6$ have been quoted. It is sufficient for the present discussion simply to note that these are very large molecules consisting of chains of several hundred units at least. In solution these high molecular weight molecules have the configuration of a loosely tangled skein. There is no evidence yet as to whether there is any secondary coiling of the main chain into helical structures. Such helical structures have been suggested for some of the simpler polysaccharides, for example amylose[51,52], but the mutual repulsion of the negatively charged groups regularly spaced along the length of the chain make such secondary coiling unlikely in the mucopolysaccharides. The presence of several possible hydrogen-bonding sites in each unit of the chain, such as hydroxyl and amide groups, suggest that the molecule is strongly solvated in aqueous solution. Rogers[49] quoted 0·3 ml/g for the 'chemically bound water' of hyaluronic acid, which corresponds to about 4 water molecules/unit of the chain.

In their aqueous biological environment therefore, the mucopolysaccharides would be expected to behave as strongly solvated cation exchange resins, and the binding of inorganic ions[46,53,54] and cationic detergents such as cetylpyridinium bromide[55] has been demonstrated. Because of the presence of many closely-spaced negatively-charged groups along the molecule they would show a greater affinity for bis-quaternary than for monoquaternary salts. The distance between the negative charges on mucopolysaccharides varies somewhere between 5–15 Å depending on the type of structure and its folded configuration. Some degree of dependence of binding of bis-quaternary salts on molecular dimensions may also be expected. The whole surface of the macromolecule would be solvated with water molecules and the absence of any large hydrocarbon groups in the mucopolysaccharide suggests that molecules with large hydrocarbon groups would be feebly

bound. This is the pattern of behaviour demonstrated by Milhaud and colleagues[56] for a mucopolysaccharide isolated from the electric organ of *Electrophorus electricus*. These authors drew attention to a correlation between the degree of binding of bis-quaternary and tris-quaternary ammonium salts and their ability to inhibit the discharge of the electric organ. However, it would first be necessary to demonstrate a significant difference between the mucopolysaccharides of active tissue and those of connective tissue, in their pattern of combination with quaternary ammonium salts before accepting these substances as models for receptor behaviour. It is, however, beyond doubt that the mucopolysaccharides of connective and other tissues do play an important part in the binding of ionized molecules and must constitute an important component of the so-called 'sites of loss'.

Proteins

Two of the most important mediators of molecular specificity in biological systems—enzymes and antibodies—are proteins, and the idea that proteins may be an important component of another agent responsible for molecular specificity—the receptor—hardly needs elaboration. Many of the concepts applied to receptors have been derived from the study of enzyme proteins, and indeed in some cases the receptor has been identified with an enzyme[57]. It may also be taken as axiomatic that the properties of a cell membrane are dependent on the structure and properties of its component proteins: Finean[41] pointed out that the structures of the internal lipid component of the membrane and the outer protein layers are interdependent, and that alterations in the structure of one would alter the structure of the other. Any change in the structure of the proteins of the membrane may be expected to produce a profound effect on the properties of the membrane as a whole. Therefore in considering proteins as possible candidates for the role of receptor substance, information is needed on the structure and stability of proteins in their aqueous cellular environment; furthermore, in considering the possibility of drug-induced alterations in protein structure it will be necessary to consider the forces which maintain the structure of proteins and the way these forces are modified by drug combination.

Protein structure has been one of the central problems of biochemistry and an enormous literature on the subject has grown up: any extensive and critical review of the problem is out of the question here and all that can be presented is a dogmatic view of the current situation, with a citation of reviews by Leach[58] and Kauzman[59] as two out of many recent discussions.

The present view of protein structure is the result of the confluence of several independent lines of inquiry, using different techniques and different proteins. Much of the most important information concerning protein structure has been derived from x-ray analysis of proteins in the solid state—the insoluble fibrous proteins and crystalline amino acids, peptides and globular proteins.

One structure proposed for fibrous proteins is a linear, extended structure, the so-called β-conformation. In this the polypeptide chain is fully extended, with the peptide links in the *trans* configuration with the side chain groups alternating on either side of the main chain. If chains in this linear conformation are laid parallel to one another, running in opposite directions, then it

is possible for the carbonyl groups of one chain to hydrogen bond to the NH groups of the adjacent chain to produce an extended pleated sheet, with side chain groups in rows, alternating above and below the plane of the sheet (*Figure 3.12*). Such an anti-parallel pleated sheet has been proposed as the structure of silk fibroin[60].

This extended structure is a relatively open one and it was known that

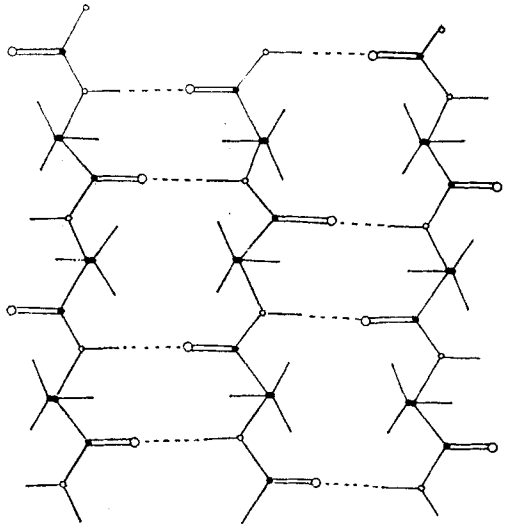


Figure 3.12. Extended polypeptide chains hydrogen bonded together to form a pleated sheet. Solid circles = carbon; small open circles = nitrogen; large open circles = oxygen. Hydrogen bonds are indicated by broken lines. The rows of β-carbon atoms run at right angles to the main polypeptide chains and alternate above and below the plane of the sheet.

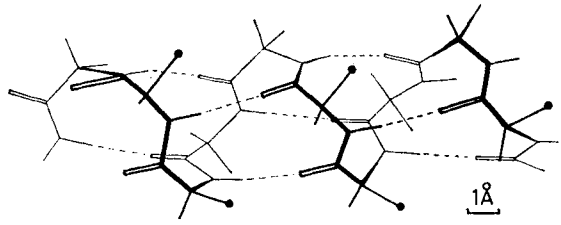


Figure 3.13. Orthogonal projection of an α-helix. Hydrogen bonds are shown as broken lines; solid circles indicate the positions of β-carbon atoms of amino acid side chains. Scale interval = 1 Å.

many fibrous proteins required a denser form of packing. Pauling, Corey and Bransom[61] therefore proposed a helical structure for the peptide backbone, in which the NH group of one peptide link was hydrogen bonded to the carbonyl group of the peptide link four residues along the chain, that is intra-chain hydrogen bonding, as distinct from the inter-chain bonding of the sheet structures (*Figure 3.13*). This α-helix (other helices are possible with more residues in each turn of the helix, for example the γ-helix with 5 residues/turn) has been widely accepted as the most stable form; evidence for its presence in many proteins has been presented[62]. The most recent contribution to our knowledge of the structure of proteins in the solid state is the x-ray analysis of crystalline myoglobin[63]. Myoglobin is a globular protein and it was found that the structure consisted of several lengths of α-helix connected by stretches of the polypeptide chain in the extended conformation, with the whole structure looped and folded back on itself to form a rough sphere.

It will be convenient at this point to introduce the nomenclature proposed by Linderstrøm Lang[64] and Bernal[65] to describe the hierarchy of protein structures. The primary structure of a protein is the amino acid sequence of the polypeptide chain: the secondary structure describes any local coiling or folding of the main chain into helices or similar structures: the tertiary structure describes the arrangement in space of the helical and non-helical segments of the whole molecule: the quaternary structure refers to the aggregation of individual macromolecules into groups, as for example, the aggregation of four myoglobin-like units to form the haemoglobin molecule.

The evidence described so far has referred to proteins in the solid state. The necessity for the individual protein molecules to pack into an ordered crystal imposes restraints on the molecule which are not present when it is in solution. Further study was needed therefore in order to establish whether some form of secondary and tertiary structure was present in proteins in solution. It is not immediately obvious that this should be so: even allowing for the rigidity of the peptide linkage there are still two bonds/residue about which rotation is possible and so a completely random tangled chain would be a possible structure for proteins in solution, as is found for many synthetic macromolecules. In fact, the evidence[58] suggests that there is some ordering of protein structures in solution. For example, it was found that when simple peptides of two or three residues are exposed to D_2O there is a rapid exchange of the peptide NH hydrogen, together with the hydrogens of any primary amino, carboxyl or hydroxyl groups present. For many proteins, however, for example insulin, β-lactoglobulin, ribonuclease, serum albumin, only a proportion of the total possible exchangeable hydrogen atoms were replaced rapidly, the remainder exchanging very slowly. Linderstrøm Lang[66] suggested that the slowly exchanging hydrogen atoms are the peptide bond NH hydrogen atoms which are firmly bound in the central helical core of the molecule, and which could only exchange if this central core was disrupted. Further support for the proposition that proteins in solution could have at least part of their peptide chain coiled into a helix has come from a study of the optical rotation dispersion of protein solutions, that is the variations of optical rotation with wavelength[67] and far ultra-violet absorption spectroscopy[68].

In general it may be said that, depending on the particular protein, between 15 and 50 per cent of the amino acid residues in native globular proteins in aqueous solution are in a coiled configuration.

It follows from this that proteins potentially may adopt many different 'structures'—that is, different conformations in space without any alteration in the covalently bonded polypeptide sequence. The range of possible structures extends from a completely coiled rod-like structure, through a partially disorganized structure containing segments of helix interspersed with open chain segments, the whole possibly folded back and intertwined into a loose sphere, to the random chain without any secondary structure, which, due to the possibility of rotation about bonds would adopt a randomly tangled conformation primarily restricted by chance interactions between widely separated amino acid residues in the chain. The evidence presented so far suggests that many proteins, whether in solution or in the crystalline form are in the intermediate, partially organized form. The potential

variability of the conformation of a protein, the possibility of a transition from one structure to another with a different degree of order is one of the most characteristic properties of proteins. These changes in structure can be brought about by slight changes in the environment. The phenomenon of protein denaturation has been recognized for a very long time. A drastic change in structure, leading to a completely disorganized structure may lead to the precipitation of protein from solution and may be irreversible. Such gross alterations in properties are easily recognized and are familiar: with the availability of more sensitive methods for measuring the overall size and shape of proteins in solution, for example viscosity, light scattering and optical rotation dispersion measurements, it has been found that completely reversible changes in protein structure are possible in solution by such comparatively mild procedures as slight alterations in pH or salt concentration.

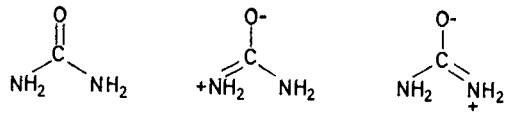
These conclusions are obviously relevant to any consideration of the behaviour of proteins when they are dispersed as a monolayer to form part of a cell membrane. In such a monolayer they are in a state intermediate between that of a crystal lattice and being in solution. As intermediate degrees of organization are possible in both these latter states it seems reasonable to suppose that proteins forming part of a cellular membrane could show a similar degree of molecular organization. It is apparent that any such reversible change in the structure of the constituent proteins of a membrane may cause a profound alteration in the properties of that membrane. However, before concluding that such structural alterations are the fundamental molecular mechanism for drug induced alterations in cell properties it is necessary to analyse in more detail the precise nature of the forces which maintain the structure of a protein, and in what way they would be influenced by combination with a drug.

The nature of the forces maintaining organized protein structures in aqueous solution

It must first be emphasized that for a macromolecule to exist in solution, free from the intermolecular interactions of a crystal lattice, in a partially organized form, rather than as a completely random chain, is itself quite a remarkable phenomenon. The unrestricted flexible molecule, free to rotate about bonds within the chain, may adopt an enormous number of possible configurations, and consequently its configurational entropy is very large: in an organized structure most, if not all, of this entropy is lost. Estimates of this configurational entropy vary between 3 and 7 e.u.[69,70] with a value of 4 e.u. per bond being most popular. If we consider a protein containing about 150 residues (for example myoglobin) then the flexibility of the random chain leads to a difference in free energy of 180 kcal/mole between the random chain and the completely inflexible organized protein. This entropy factor alone therefore overwhelmingly favours the random unfolded chain: the intramolecular interactions that lead to the formation of an ordered structure must result in a corresponding loss of free energy to compensate for the entropy loss. The nature of the forces responsible for the secondary and tertiary structures of proteins have been discussed by Lindestrøm Lang and Schellmann[71] and Kauzman[59]. There is general agreement concerning the types of forces but there is still a considerable divergence of view on the precise quantitative significance of these different forces.

Inter-peptide hydrogen bonding—Possible NH · · · O=C hydrogen bonding between different peptide links was considered by Pauling and Corey as one of the main factors leading to the formation of the α-helix in crystalline proteins. The heat of hydrogen bonding for O · · H–N bonding has been quoted as 6–8 kcal/mole[29] and as every peptide bond in the α-helix is engaged in a hydrogen bond, with the exception of the peptides at the ends of the helical segment, at first sight it appears that the α-helix is a very stable structure indeed, which would be very difficult to disrupt. However, the stability of the peptide-peptide hydrogen bond is greatly reduced in aqueous solution, because water is an extensively hydrogen-bonded substance which can act as either a hydrogen-bond donor or acceptor: it follows from this that any pair of groups within a molecule which can form an intramolecular hydrogen bond with one another are equally capable of forming separate hydrogen bonds with water. Thus the relevant reaction in an aqueous environment is one of hydrogen bond exchange rather than simple hydrogen bond formation (*equation 1, p.* 52).

Schellman[70] used thermodynamic data on the association of urea in water to derive an estimate for the free energy of formation of the peptide NH · · OC hydrogen bond, and obtained a value, at 25°C, of —0·3–0·6 kcal/mole. This is quite a low value, and Schellman concluded that the intra-peptide hydrogen bonds 'taken by themselves give a marginal stability to ordered structures which may be enhanced or disrupted by interactions of side chains'. Schellman's thermodynamic data was derived from a study of the association of urea in water, and although urea may be considered as an amide it would be expected to possess slightly different properties from a simple peptide (amide) linkage. Thus the C=O bond distance in urea is 1·26 Å, significantly longer than the C=O bond distance in simple amides and peptides, where an average value of 1·22 Å is found. The longer bond length in urea indicates a greater single bond character, estimated by Pauling[72] as 40 per cent, indicating considerable resonance contributions from forms (*XIX*) and (*XX*) out of the three possible simple canonical forms. The greater negative charge on the carbonyl oxygen of urea may lead to stronger hydrogen bonding in urea than that found in simple peptides.



(XVIII) *(XIX)* *(XX)*

Klotz and Franzen[73] used *N*-methylacetamide as a more realistic model of the peptide unit and investigated its association in a range of solvents, including water, using infra-red overtone spectra. They obtained a positive value for the free energy of formation of the peptide hydrogen bond of +3·1 kcal, which means that the equilibrium in *equation 1, p.* 52 lies well over towards the left, the non-bonded, hydrated form. They concluded roundly that 'for protein molecules in aqueous solution inter-peptide

hydrogen bonds cannot contribute significantly to macromolecular organization . . .'. Unfortunately these workers were not able to detect any association between urea molecules in aqueous solution by their infra-red technique: such assocation of urea molecules is clearly indicated by the vapour pressure measurements of urea solutions, and this conflict between the two different techniques still leaves a degree of uncertainty about the crucial values for the thermodynamic constants of the intra-peptide hydrogen bond. However, whatever the precise value, Schellman, and Klotz and Franzen are in agreement in saying that the intra-peptide hydrogen bonded system can make only a very small contribution, if any, to the stability of helical structures in an aqueous environment.

Having eliminated the main peptide backbone as a major stabilizing influence on protein structure, it follows that the important stabilizing influence must be the interactions of the amino acid side chains. Three different types of interaction have been suggested (1) hydrogen bonding between side groups, (2) hydrophobic bonding and van der Waals attraction, (3) electrostatic attraction; these will be considered in turn.

Intra-side-chain hydrogen bonding—Many of the side chains of protein molecules contain groups that are potentially capable of forming intra-chain hydrogen bonds; for example the hydroxyl groups of serine and tyrosine, the carboxyl groups of aspartic and glutamic acids; the amide groups of asparagine and glutamine, the amino groups of histidine, arginine and lysine. The most extensive quantitative treatment of hydrogen bonding between side chains is that due to Laskowski and Scheraga[74]. They calculated the stability constants for the various types of hydrogen bonded interaction possible, and from these stability constants they developed a theoretical treatment of the effect of intra-chain hydrogen bonding on the dissociation constants of acidic and basic groups, the binding of small molecules, denaturation, and the proteolysis and synthesis of peptide bonds[75,76].

There are several important consequences which follow from the formation of a hydrogen bond between two side chains on a protein. An isolated side chain, free from neighbouring interactions possesses a degree of rotational freedom. When this side chain forms a hydrogen bond it invariably loses nearly all its rotational freedom and is fixed in one particular conformation, in order to satisfy the geometrical requirements of the hydrogen bond. This loss in rotational freedom represents a loss of entropy, the extent of which depends on the length of the side chain, that is the number of bonds 'frozen in'. This loss in entropy, it was suggested, was counterbalanced by a fall in heat content due to a loss of torsional energy when the side chain is fixed in a particular conformation (amounting to 600 cal (at 25°C) per bond 'frozen') together with the heat of formation of the hydrogen bond. A rough value of 5 e.u. was assigned to the entropy associated with torsional oscillations about a single bond: hence for a tyrosine-glutamic acid hydrogen bond the total entropy loss was −20 to −25 e.u. Allowing −3·0 kcal for the loss in torsional energy and assigning a value of at least −3·0 kcal for the heat of formation of a single hydrogen bond, then the free energy of formation of a tyrosine-glutamic acid hydrogen bond would be approximately zero—that is about one-half of all such potential hydrogen bonds would be formed. For an aspartic acid-aspartic acid interaction two hydrogen bonds would be

formed and a similar calculation gives a free energy of formation of such a bond as −2·4 kcal. In general, values of the free energy of formation of side chain hydrogen bonds were calculated to range from 0 to −3 kcal. This would represent a considerable contribution to the overall stability of a helix, considering the large number of such interactions that are possible.

The only objection that can be raised to this treatment is to the value of at least −3·0 kcal assigned to the heat of formation of the hydrogen bond. In the discussion of the intra-peptide hydrogen bond it was pointed out that in an aqueous environment intramolecular hydrogen bonding was a process of hydrogen bond exchange, and that the relevant heat of formation was not that of a hydrogen bond between the two groups in isolation but the difference between such a hydrogen bond and the hydrogen bonds between the two groups and water. As most hydrogen bonds are approximately equal in strength, this difference is very small. Pauling and Pressman[31] estimated it as only 400 cal/mole: Klotz and Franzen[73] estimated it as zero. If we assign these lower values then a *positive* free energy of formation of 2·0–3·0 kcal is found, which is the value found by Klotz and Franzen for the free energy of association of peptide groups in water.

It is possible that, in the case of a heavily folded protein, the centre of the molecule is shielded from the external water by interacting side chains, and in such a water-free environment intra-chain hydrogen bonding is possible. However, these bonds would not stabilize the folded form relative to the extended random configuration for the difference in free energy of formation of the intra-chain hydrogen bond in the screened interior of the molecule and the free energy of formation of hydrogen bonding by the same groups with water molecules when in the extended configuration would be zero, or would favour the extended form, as solvation would not impose any restrictions on the rotational freedom of side groups. It must therefore be concluded that intra-side chain hydrogen bonding cannot have any significant effect on stabilizing organized protein structures in equilibrium with water.

Hydrophobic bonds—Many of the amino acids present in proteins have simple hydrocarbon side chains, without any polar groups—alanine, valine, leucine, *iso*leucine, phenylalanine, proline—and the presence of these non-solvated side chains has a profound effect on protein structure. Water is, of course, a most complex solvent; due to its ability to act as hydrogen bond donor or acceptor it is strongly associated, with individual water molecules sliding from one adjacent molecule to the next. When a hydrocarbon chain is introduced into water the water molecules immediately adjacent to the hydrocarbon chain cannot solvate it and therefore, in order to accommodate the hydrocarbon chain within the body of the solvent rearrange themselves into a quasi-crystalline structure, to form a cavity into which the hydrocarbon chain fits. The effect of a hydrocarbon chain, therefore, is to induce a degree of order into its neighbouring water molecules compared to the bulk phase, and it is the loss of entropy resulting from this localized order that is the cause of the poor solubility of hydrocarbons in water. This effect would also occur when a protein chain is in the extended conformation, with its hydrocarbon side chains isolated from one another: each side chain would be surrounded by a separate quasi-crystalline 'iceberg' of water molecules.

If the protein molecule folded in such a way that the hydrocarbon side chains were directed towards the interior of the molecule, where they could pack together and be shielded from solvent water molecules, there would be a great gain in entropy, relative to the extended form, due to the collapse of these quasi-crystalline aggregates of water molecules round the individual hydrocarbon side chains. This formation of a folded conformation which excludes nonpolar side chains from contact with water is called 'hydrophobic bonding'[59].

The model usually adopted for estimating the quantitative significance of hydrophobic bonding is as follows: the protein is assumed to fold so that the nonpolar residues are closely packed to form a non-aqueous hydrocarbon-like region in the centre of the protein sphere. The free energy change in moving a single hydrocarbon chain from this central core to the external aqueous environment is then calculated and this gives a measure of the stability of the folded form over the extended form of the protein. Kauzman[59] suggested a value of +3 to +5 kcal/side chain for this transfer: Tanford[77] finds values ranging from 0·7 to 2·9 kcal, depending on the nature of the side chain. Using a different model for the calculations, values of 0 to −1·5 kcal for the free energy of association of hydrocarbon side chains have been suggested[78]. Although other explanations for the large negative entropy of solution of hydrocarbons in water, which do not involve induced lattice formation in water have been offered[79], nevertheless the entropy effect exists and the semi-empirical calculations by Tanford show that the effect is important. Tanford showed from calculations based on the amino acid composition of the proteins, and on the assumption that all nonpolar side chains are shielded from solvent interaction, that 'the stability of the native conformations of myoglobin and β-lactoglobulin in water can be explained within the uncertainty inherent in the calculation, entirely on the basis of the hydrophobic interactions of the nonpolar parts of the molecule'.

Van der Waals forces are the weak electronic dispersion forces that act between all saturated molecules. The semi-empirical estimates of hydrophobic-bonding energies by Tanford, derived from the solubilities of amino acids, includes a component due to these interactions.

The model used for assessing the importance of hydrophobic bonding is admittedly an over-simplification. It is, for instance, unlikely that all the nonpolar side chains in a protein would be directed towards the interior of the protein, and be shielded from solvent interactions. In the only case where we have explicit information on this point, in the crystal structure of myoglobin, only 45 per cent of all the available nonpolar residues are directed towards the centre: however, this does include nearly all of the large aromatic residues, and furthermore, most of the polar residues are on the external surfaces of the molecule. The centre of the myoglobin molecule is a relatively closely packed area consisting essentially of hydrocarbon units, with only a few water molecules and so does resemble the model. However, although hydrophobic bonding must be an important element regulating protein structure, a consideration of the thermal stability of proteins shows that it cannot be the only factor responsible for ordered structures. The hydrophobic bond is endothermic[78] and hence is more stable at high temperatures than at low: if this were the only factor then proteins should be more stable at

higher temperatures, whereas, of course, the reverse is true. Some other process must also play an important part in controlling the configuration of native proteins.

Electrostatic interactions—Electrostatic interaction between negatively charged acidic side chains (aspartate and glutamate) and positively charged basic side chains (histidine, arginine, lysine) is a third possible force responsible for maintaining native protein structures. The quantitative evaluation of electrostatic interaction is a difficult and complicated calculation: an extended treatment of this problem is given by Tanford[80]. Whether or not electrostatic interaction can exert a stabilizing or destabilizing effect depends, for instance, on the precise geometrical distribution of the charges, different distributions in space producing different interaction energies. Tanford calculated the electrostatic energy for a simple model consisting of 6 carboxyl groups and 6 basic groups separated by distance of 6·5 Å and lying 1 Å below the surface of a sphere of radius 10 Å; the internal dielectric constant of the molecule was taken as 2, that for the external medium at 80. Calculations for 2 different spacial arrangements of the charged groups and at different values for the net charge on the molecule gave values varying from +7·5 kcal to −11·0 kcal. A value of −10 kcal was obtained for one model with a net charge of zero, that is equal numbers of positive and negative charges. Electrostatic interactions can therefore have an important but variable effect in different proteins.

Tanford's treatment considered the effect of electrostatic interactions on the structure of the molecule as a whole—on the tertiary structure. Electrostatic effects may also be important in stabilizing elements of the secondary structure of a protein. The effect of forming an α-helix is to bring groups that would be widely separated in the extended open chain form very close together. If the two groups thus brought into juxtaposition bore similar charges then the resultant electrostatic repulsion would reduce the stability of the helical form: conversely, if they were oppositely charged then this would stabilize the helix.

If we consider the α-helix, then side chains that are adjacent on successive turns of the helix (amino acids in a 1:4 or 1:5 relationship in the primary amino acid sequence) are separated by a distance of about 6·0 Å. When the helix is unfolded and the chain fully extended the distance between these groups increases to about 12 Å, resulting in a decrease in the electrostatic interaction.

Precise calculations of intramolecular interactions are complicated by uncertainties over the value to be assigned to the dielectric constant of the medium separating the charges. Tanford[80] in his model assumed a value of 2 for the dielectric constant of the interior of a protein, and placed his charges 1 Å below the surface. For side-chain interactions of polar groups directed towards the outer aqueous environment, and probably hydrated[63] a rather larger value is probably appropriate, although the screening effect of adjacent nonpolar groups would tend to keep the value low. Assuming a value of 20 as an upper limit, and ignoring the effects of distant charges within the molecule, then the change in electrostatic energy due to a helix-open chain transition is at least 2 kcal. If the charges are of opposite sign, then the helical form would be stabilized by this amount. Electrostatic

interactions would not restrict the rotation of side groups and hence would not lead to any entropy loss on this account.

An examination of the amino acid sequences of the helical and non-helical segments in myoglobin shows that in the helical segments acid-base pairing in a 1:4 or 1:5 relationship occurs 9 times, including a very favourable triplet interaction of two bases adjacent to one acid (H 9 lysine, H 12 glutamic acid, H 15 arginine); acid-base pairing occurs twice in non-helical regions, but it is possible that in these cases (and in some of the helical pairings also) the acid is present as an amide, in which case there would be no electrostatic interaction. Two cases of 1:4 base pairing, that is cases where there would be electrostatic repulsion between the groups, making the helical form unstable occurs in non-helical regions: no cases of base pairing occur in helical sequences. It could be estimated therefore that electrostatic interactions could contribute some 20 kcal/mole to the stability of the folded myoglobin structure.

Conclusions

The mucopolysaccharides are a closely related group of very polar macromolecules, carrying large numbers of negatively charged groups in close array, strongly hydrated, and loosely coiled in a random open skein: in the presumed absence of any secondary organization this loose open structure is probably quite stable. The proteins present a more complex picture. They can exist in a variety of different structures at different levels of organization: the large entropy factor favouring a completely random chain is largely, but not completely, counterbalanced by hydrophobic bonding in the folded native configuration. This hydrophobic bonding is supplemented by the summation of a large number of marginally weak interactions, such as hydrogen bonding within a helix and possibly between side chains, and by electrostatic interactions between charged groups. These weak interactions are dependent on the structure of the protein and are cooperative: the formation of a helix brings otherwise widely separated groups into close contact, allowing further interactions. Because of this cooperative nature of the stabilizing forces the stability of the protein as a whole is dependent on the stability of its component sections: a disturbance of part of the structure may lead to the collapse of the whole.

DRUG-RECEPTOR INTERACTIONS: THE FORCES

If it is accepted that the external surface of the cell membrane is a matrix of protein molecules, possibly interspersed with mucopolysaccharide molecules, and that the primary interaction between drug and cell is at this monolayer, then it is possible to analyse in detail the possible modes of drug receptor interaction. The first step is to identify the kind of chemical groupings that are present on the cellular surface and exposed to the extracellular environment. A mucopolysaccharide presents an array of negatively charged groups embedded in a matrix of the solvated hydroxyl groups of the monosaccharide units of the chain. A protein molecule would seem to afford a far wider range of potential combining groups. In myoglobin 55 per cent of the nonpolar side chains and 70 per cent of the polar side chains are outward facing. When a protein is part of a membrane, and assuming, for

the moment, that it retains some globular form of structure, then all these outward facing groups will not be exposed to the external environment. Some will be engaged in bonding the protein to its immediate neighbours and others to the polar groups of the underlying bimolecular lipid leaflet. Nevertheless it is still reasonable to suppose that a protein could expose to the extracellular environment combinations of any of the side groups that occur in proteins. Such groups are: nonpolar aliphatic carbon chains and aromatic rings; dipolar, and potentially hydrogen-bonding groups such as hydroxyl, amide, unionized carboxylic acid and amino groups; positively charged protonated bases; negatively charged dissociated carboxylic groups. A protein therefore presents a more complex and almost infinitely variable surface to the external environment. It is this variability, compared to all the other biologically important macromolecules, considered in conjunction with the great variety and selectivity of drug receptors that makes it most likely that our understanding of the receptor depends on a fuller understanding of the forces regulating protein structure.

Given this range of groups present on the cell surface, and given the almost infinite range of groupings possible in drug molecules, what are the most likely forces leading to drug-receptor interaction? These forces are essentially those already discussed in the consideration of protein structure: they can be briefly recapitulated with particular reference to the interaction of separate small molecules with a macromolecular array.

Covalent Bonding

The possibility of the formation of covalent bonds between drugs and receptors must be mentioned for the sake of formal completeness. Only a very small number of drugs, albeit quite important ones, have been shown to act in this way—the β-haloalkylamine antiadrenaline compounds and the fluorophosphate anticholinesterases, for example. By and large the actions of these compounds are explicable in terms of chemical theory and, although the details may be complex, their understanding does not seem to present any fundamental difficulties. Of much greater importance to the pharmacologist are the great majority of drugs which seem to form a very transient complex with the cell. Subsequent sections will be devoted to an analysis of the weaker and more readily reversible interactions that are possible between drugs and a tissue receptor.

Hydrophobic Bonding

The nonpolar sections of a drug molecule in aqueous solution will also be surrounded by quasi crystalline water lattice. Tanford[77] showed that there is a constant positive increment in free energy per methylene group when an amino acid is transferred from a nonpolar to a polar environment. He attributed this mainly to hydrophobic interaction with water, and we may suppose therefore that this hydrophobic effect will be larger the more extensive the nonpolar component of a drug's structure. If a receptor protein presents an extensive nonpolar interface then association of the drug with this interface would effectively remove part of the molecule from contact with the solvent, and the gain in entropy consequent on the dissolution of the 'iceberg' round the drug molecule would make an important contribution

to the free energy of drug binding. Scheraga and colleagues[78], using a different method from the semi-empirical approach of Tanford, calculated the free energy of hydrophobic bonding between individual side chains, a procedure relevant to a consideration of drug/protein side chain interactions, and obtained free energy changes ranging from 0 to −1·5 kcal for such hydrophobic bonding. Hydrophobic bonding must be an important component in the binding of many drug molecules. The binding of steroidal hormones to enzymes[81,82] has been attributed to hydrophobic bonding: Kauzman drew attention to the large positive entropy of binding of many anions to proteins[88–85] and also attributed this to hydrophobic bonding. It should, however, be noted that this interpretation of a large positive entropy of association as hydrophobic bonding is not the only possible one: the large positive entropy of binding of testosterone to serum albumin is attributed to a disturbance of the tertiary structure of the protein itself, rather than of associated water[86]. Thermodynamic measurements by themselves do not offer any means of distinguishing between these two alternatives.

van der Waals Forces

The van der Waals, or London dispersion forces are a second component of the interactions of hydrocarbon chains. All molecules exert a weak attractive force on one another when separated by distances greater than roughly 4 Å. These forces are weak and, for small molecules, are inversely proportional to the sixth power of the distance separating them. Thus for two isopropyl groups separated by a distance of 4·5 Å there would be an energy of interaction of approximately −0·45 kcal/mole. This may be compared with the value of −1·1 kcal/mole calculated[78] for the hydrophobic interaction of these two groups. For small groups the net fall in free energy is small, and because of its sixth power dependence on distance, requires very close conjunction of the interacting groups: at a distance of 6 Å the energy of interaction falls to 0·08 kcal.

The van der Waals force is probably much more significant for larger molecules, for example steroids and fatty acids[87]. Salem[88] showed that when the distance between two hydrocarbon chains is short compared with the length of the molecule (and given the limit imposed by the van der Waals radii on the distance between two molecules, this condition admits only long chain molecules) then it is found that the dispersion forces obey a fifth power law[89] rather than the normal sixth power law. This effect is significant when long chains can come into close conjunction: the energy of interaction of two molecules of stearic acid lying parallel and separated by a distance of 4·8 Å was estimated as −8 kcal/mole. A protein molecule with an extended array of, say, leucine molecules could interact with a long chain hydrocarbon in a similar way, and under these circumstances van der Waals interactions could make a significant contribution to drug binding.

It should also be noted that, amongst the group of biologically important macromolecules, the presence of large numbers of nonpolar hydrocarbon groups is a property peculiar to proteins. This fact, taken in conjunction with the repeated demonstration of the important function of the nonpolar parts of a molecule in determining biological activity, lends support to the idea that proteins must be an important constituent of receptors.

The methylene group has been considered in some detail as it is an important unit of molecular structure and indicates the magnitude of the van der Waals effect. Pauling and Pressman[31] discussed the variation in van der Waals interactions between haptenes and antibodies caused by different aromatic substituents. They derived the expression

$$\Delta W = \frac{4 \times 10^5}{r^6} (R_A - R_H) \text{ cal/mole}$$

where ΔW is the change in van der Waals energy produced by substituting a group A for hydrogen in a molecule, r = distance between molecule and protein, R_A and R_H the molar refractions of the group A and hydrogen respectively. Interaction is greater the greater the polarizability of the group: the substitution of an iodine atom for hydrogen would increase the van der Waals interaction at a distance of 6 Å by approximately 120 cal/mole: a fluorine substituent, which is slightly less polarizable than hydrogen would cause a very small decrease in interaction energy. This effect is of some interest when the enhanced activity of some of the fluorinated steroids is considered.

Electrostatic Interaction

In aqueous solution ion-pair formation between univalent ions is a comparatively minor phenomenon in salt solutions at low concentrations. Ion-pair formation will only occur when the energy of association between oppositely charged pairs of ions is sufficiently great to counterbalance the energy due to the random thermal motion of the separate ions. In aqueous solutions the effect is small for two reasons: (1) the high dielectric constant of the solvent reduces the electrostatic interaction between the ions, (2) the spheres of solvating water molecules round the ions prevent them from coming too close to one another and hence sets a further limit to their interaction[90]. However, it is well known that ionic association does occur between proteins and simple ions such as chloride[91], as well as with larger anions, and it is necessary to consider briefly how the presence of a macromolecule alters this simple situation.

Intramolecular electrostatic interactions in small molecules and in proteins has already been discussed briefly: the intermolecular interactions between macromolecules and small molecules presents another aspect of the same general problem. At the risk of gross oversimplification it can be treated along the following lines. The classical electrostatic energy of interaction between two charges q, separated by a distance r in a medium of dielectric constant D is given by

$$E_{el} = \frac{q^2}{D.r.}$$

Thus at a given distance of separation, and with constant charges, the magnitude of the interaction is dependent on the dielectric constant. When considering electrostatic interactions within small molecules the distortion of the dielectric medium between the charges by the intervening molecular structure is relatively small and as a first approximation the bulk phase value of the dielectric constant (80 for water) can be used. In a previous section it has been shown that a globular protein is a heavily folded sphere,

with polar water molecules largely excluded from its centre, a region consisting of closely-packed nonpolar groups. A protein may therefore be treated as a region of low dielectric constant embedded in a surrounding medium of high dielectric constant, and for charges interacting within the protein molecule a value of 2–5 for the dielectric constant has usually been assumed. In considering association between an ion in solution and a charged group within a protein, the presence of this extended region of low dielectric constant has a marked effect on the effective dielectric constant of the medium between the ions. Schellman[92] applied the methods of classical electrostatics to this problem of ionic interaction near an interface between regions of high and low dielectric constant. When applied to the experimentally determined value of the association constant of chloride ion with serum albumin a value of 28 for the effective dielectric constant was obtained: this is lower than the minimum possible value calculated from the simple electrostatic model, but considering the limitations of the model, this treatment was in qualitative agreement with experiment.

A different approach to this problem was adopted in a study of electrostatic effects on antigen-antibody interactions[93]. The association constants of small molecules with an antibody were evaluated by measuring the degree of inhibition by the hapten of the precipitation reaction between an antibody formed against an antigen which contained a diazo coupled phenyltrimethylammonium group. The contribution of electrostatic forces to hapten binding was estimated by comparing the affinity constants of 'H acid'-*p*-azophenyltrimethylammonium ion and 'H acid'-*p*-azo-t-butylbenzene for the antibody. These two molecules are almost identical in shape and size, only differing by the positive charge carried by the former. The difference in the free energy of binding of these two molecules, 1·5 kcal/mole was attributed to electrostatic interaction between the positive charge on the hapten and a single negative charge on the antibody. The effective dieletric constant between the two charges was evaluated by using the relationship derived by Schwarzenbach[94] from the variation in the two dissociation constants of dibasic acids. The application of a relationship derived from interactions within small molecules to the problem of protein interactions may be questioned, but it was found that the difference in free energy of association between the charged and uncharged molecule could be accounted for on the assumption that the positively charged ion approached to within 7 Å of a negative charge on the antibody, with an effective dielectric constant between them of 31. This distance of approach is reasonable, considering the dimensions of the trimethylammonium group and of a hydrated carboxyl group: the effective dielectric constant is close to that calculated by Schellman. This method[93] of calculating electrostatic interactions developed has since been used by Adams and Whittaker[95] and by Wilson and Bergman[96] to deduce the size of the negative charge present at the active site of the cholinesterases.

The presence of charged groups on naturally occurring macromolecules, and the experimental observations[93] on the magnitude of the electrostatic interaction between a charged molecule and a protein would suggest *a priori* that electrostatic interactions should play a significant part in drug cell interactions. This has been amply confirmed by experiment—the dominant

part played by the quaternary ammonium group in the large series of compounds related to acetylcholine is an obvious example. It is, however, rather curious that nearly all drug molecules that can bear a charge are cations: very few drugs are anions.

The electrostatic interactions between groups on the receptor and groups within a drug molecule bearing an integral number of electronic charges are quantitatively the most important electrostatic interactions we have to consider. Other interactions of a lower order of magnitude are, however, of some importance. These are, in decreasing order of magnitude ion-dipole, dipole-dipole and ion-induced dipole interactions.

The energy of interaction, E_1 between a single fixed charge and a dipole is given by

$$E = \frac{Ne\mu \cos \phi}{D(r^2 - d^2)}$$

where N = Avogadro's number; e = electronic charge, μ = dipole moment; ϕ = angle between the line joining the fixed charge to the centre of the dipole and the direction of the dipole; D = effective dielectric constant of the separating medium; r = distance between the fixed charge and the centre of the dipole; d = length of the dipolar bond. This interaction can either strengthen or weaken drug association depending on the orientation of the dipole; if we consider a fixed negative charge on the receptor the drug-receptor complex will be stabilized if the positive end of the dipole is nearest the fixed charge, and destabilized if the dipole is reversed. It is also dependent on the orientation of the dipole (angle ϕ) and on the effective dielectric constant. Assuming a value of about 30 for the latter then ion-dipole interactions could contribute $\pm$500 cal/mole to a drug receptor interaction.

A permanent charge on one molecule can polarize the electrons of an adjacent, neutral molecule, inducing a dipole and leading to a net force of attraction, the magnitude of this interaction depending on the polarizability of the neutral molecule. Adams and Whittaker[95] estimated the magnitude of this effect in the interaction of a choline, a positively charged quaternary ammonium salt and plasma cholinesterase and obtained a value of about 100 cal/mole. This is probably an upper limit for this effect.

Dipole-dipole interaction could also affect the stability of a drug-receptor complex: the effect is a feeble one and highly dependent on the distance between, and orientation of, the two dipoles: values of $\pm$100 cal/mole are probably appropriate.

Hydrogen Bonding

This has been discussed during the consideration of intramolecular hydrogen bonding, and little more need be added. In general, any group capable of forming a hydrogen bond will be solvated by water: there will therefore only be a very small net change in free energy in exchanging a hydrogen bond with a water molecule for one with a protein constituent. It is unfortunate that at the moment we do not have any reliable thermodynamic data on this point: the small changes in heat content involved in these bond exchanges cannot be measured accurately and calculations of the entropy changes are fraught with uncertainty.

It can, however, probably be safely concluded that the presence of a potential hydrogen-bonding group may, in certain circumstances, be a destabilizing influence. As in the case of intramolecular hydrogen bonding when applied to protein molecules, it makes little difference whether a group is hydrogen bonded to another group within the molecule, or to a solvent molecule; the formation of the bond does not favour one state at the expense of the other. However, any intermediate state, in which a potential hydrogen-bonding group is neither solvated nor hydrogen bonded to a neighbour is very much less stable than either of these two hydrogen-bonded states. Therefore, when a drug molecule which contains a potential hydrogen-bonding group forms a complex with a receptor it must either retain its solvated water molecules, or be able to exchange them for another hydrogen-bonding group on the receptor. If the combinations of interacting groups in the receptor and drug molecule are such that neither of these conditions is satisfied then the drug-receptor complex will be destabilized by an amount of free energy equal to that lost in the formation of a hydrogen bond. Polyhydroxy compounds, such as carbohydrates are only very weakly bound to proteins, if at all[97–99]. Such molecules are very strongly solvated and it is probable that, in general, large solvated areas of the protein surface, free from hydrophobic nonpolar groups are not sufficiently numerous to allow extensive binding. Smaller molecules, with only one or two hydroxyl groups are more firmly bound[99].

DRUG-RECEPTOR INTERACTIONS: THE CONSEQUENCES

It seems that the concept of 'molecular dissection', that is, the attempt to determine the essential structural features of a complex molecule by the synthesis of analogues containing differing segments of the parent molecule, is an essentially false and misleading one. Substitution of a variety of inert non-reactive groupings into a molecule may have a profound effect on the spacial distribution of the groups within that molecule, as it must be considered as a three-dimensional entity. When any molecule possesses a structural component that confers a degree of flexibility, then the molecule must be considered as a consortium of different structures; this is not an infinite number, as the older conceptions of free rotation about single bonds would have it, but a restricted number, definable within limits. The effects of minor changes in the chemical structure on the relative importance of these different conformations must also be considered when relating chemical structure to biological activity, as well as their effects on the other, well known, modulators of biological activity, such as tissue distribution and metabolic disposal. Each member of a series of molecules must be treated as an individual case, and comparisons between different compounds must be based on an extensive analysis of its properties and not rely simply on the demonstrations of similarities of pattern in their formal structures.

The forces that lead to interaction between drugs and receptors are very weak, with the exception of the strictly limited number of cases where covalent bonds are formed. The analysis of these forces has shown that the correlation between the drug and the receptor must be good for the interaction energy to be great enough to overcome the random thermal motion of the drug and allow a reasonable duration of binding. The necessity for

multiple group interaction, the importance of the correct relative orientation of the different groups within the molecule, the consequences of the alterations in the bulk or shape of a molecule are all, in principle, understandable in the light of our present understanding of physical chemistry. It could, however, be said that all this was implicit in the terms of the old 'lock and key' hypothesis and that the present analysis only serves to make explicit what was already understood. This analysis has, however, implications beyond the demonstration that many of the phenomena revealed by the study of structure-action relationships are, in principle, explicable in terms of known forces.

Much more knowledge is needed about the chemical constitution of mammalian cell membranes. At the present state of knowledge it is probably hoping for too much to attempt the isolation of the receptor substance. An immediate requirement, which should be within the reach of present techniques, is to know whether there is any great difference in the composition of the chemically excitable areas and the chemically inexcitable areas of excitable cells. This would go a long way towards removing the present uncertainty concerning even the general chemical category of the possible receptor substance, let alone its detailed structure. In the face of this uncertainty and lack of definite information concerning cellular membranes any attempt at a basic chemical mechanism of drug action must require an extensive extrapolation from established facts. Such attempts are useful in that they may suggest experiments that are possible with presently available apparatus and techniques.

Reasons have been given above which make some form of protein the most likely candidate for the receptor. Many studies of structure action relationships have been carried out and many speculative attempts made to deduce something about the nature of receptors from them. Unfortunately too many of these have been content with a topographical description of the receptor, referring to planar areas, hills[100], crevices[101], slots and troughs. Such descriptions are unhelpful and sterile; they ignore the obvious fact that the membrane must be composed of chemical substances, consisting of atoms held together by bonds and separated by distances of the same order of magnitude as the dimensions of the drug molecule. At the present state of knowledge any discussion of possible receptor structures must be speculative, but there is no reason at all why the speculation should not be in terms of discrete and recognizable chemical structures. If a working hypothesis is accepted that the receptor is part of a protein, then several consequences follow from what is already known about the structure of proteins.

The discussion of protein structures in a preceding section concentrated on information gained from a study of proteins either in solution or in the crystalline state. Rather less is known about the structure of proteins in a membrane. It has been shown[102] that when enzymes are adsorbed onto lipid monolayers they can be partially or completely denatured, the extent of protein unfolding depending on the nature of the charged groups in the lipid layers, negatively charged groups particularly tending to unfold adsorbed proteins. The outer non-lipid layer of the membrane is about 25 Å thick. This is roughly the diameter of a globular protein: a layer of extended,

denatured protein would be much thinner. The outer layers may, therefore, consist either of several layers of protein in the form of extended sheets, related to the pleated sheet structure of silk fibroin, or of a single layer of globular protein, possibly resting on a single layer of protein in an extended form[103]. Even with these uncertainties over the detailed form of the membrane proteins, this knowledge of protein structures in general does impose some limitations on receptor structures.

The protein structure must be well defined: if an extended segment of the protein was in the random chain form, with its component parts executing normal thermal motions, then no particular array of groupings would ever exist for any reasonable length of time and no real degree of specificity towards an external molecule would be possible. Given some elements of secondary and tertiary structure, the receptor relies for its specificity on particular spatial combinations of amino acid side chains. The amino acids are finite in number and this goes some way towards defining possible chemical components of the topographical features that have figured, so largely in some receptor descriptions. Large planar areas can only be phenylalanine, tyrosine or tryptophan residues. The hydroxyl groups of serine and threonine are attached to the β-carbon atom of the amino acid side chain: this carbon atom would be fixed in space in any regular secondary structure such as an α-helix or bonded-extended chain, and therefore such hydroxyl groups would constitute well-defined hydrogen-bonding sites: the same applies to hydroxyproline. The imidazole ring of histidine would also be well-defined in space and could also function as a discrete hydrogen bond donor and acceptor: on the other hand, the terminal basic groups of arginine and lysine are at the ends of chains of 4 or 5 atoms, and, because of the limited free rotation about these intervening links, the groups may occur in a wide variety of positions and would not be expected to confer a high degree of specificity. Similarly, in their positively charged forms the histidine imidazole ring would be a more structurally specific group than either of the other basic amino acids. A similar argument applies for the anionic groups: a serine phosphate or aspartic acid carboxyl group is on short chains and so would be defined in space, within limits; the carboxyl group of glutamic acid, with its extra carbon atom in the chain could range over a wider area. None of these negatively charged groups would be fixed in space even though they formed part of a rigid α-helix; the probability of a range of distances separating two anionic groups has been deduced from a study of bis-quaternary ammonium compounds[15].

The formation of secondary protein structures also imposes limitations on the overall spacing of possible receptor groupings. The α-helix is a compact structure, with the β-carbon atoms of the amino acids separated by distances of about 5·5 Å from their nearest neighbours. The extended sheet form with its rows of side groups is rather more open: 4–5·5 Å between β-carbon atoms in one row and 7·0 Å between rows. In a linear-extended peptide chain groups are on alternate sides of the main stem separated by distances of 7·0 Å: in a complex tertiary structure short stretches of extended peptide chain may exist which are not long enough to permit extensive thermal motions of intermediate links and these could be folded in several ways. The existence of rotation barriers imposes limitations on the possible spatial

positions of groups attached to these β-carbon atoms (that is in all amino acids except glycine and alanine, and the prolines).

Most descriptions of receptor structures on macromolecules are divided between two extremes: they are either so schematic[104] that they convey

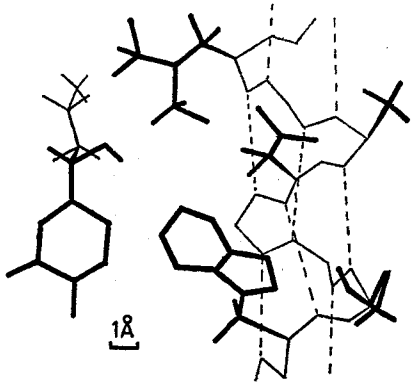


Figure 3.14. Orthogonal projection of an α-helix showing spacial arrangement of side-chain groupings on one 'face' of the helix. The side-chain groupings of trytophane, serine, aspartic acid and leucine are shown. A molecule of adrenaline, on the same scale, is also shown. Scale interval = 1 Å.

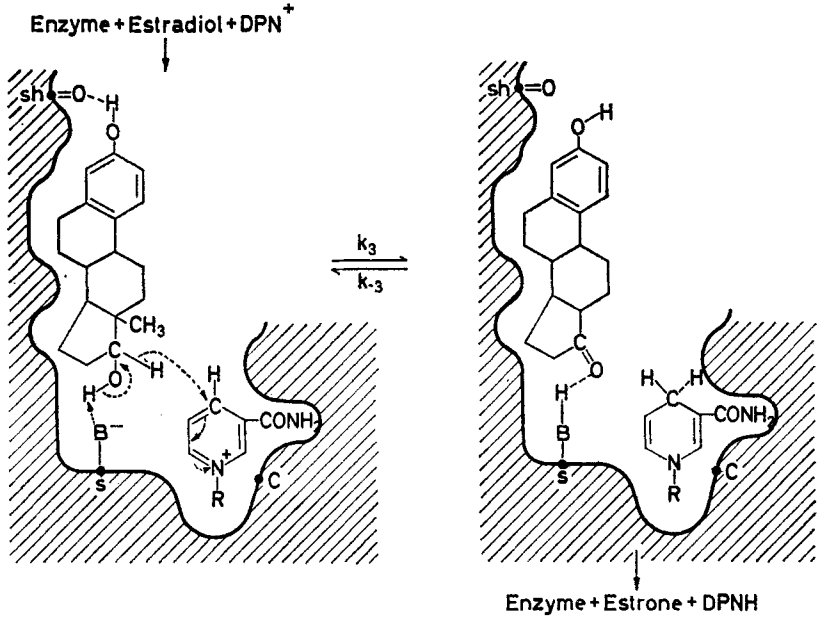


Figure 3.15. Representation of the estradiol/enzyme complex formed during the oxidation of estradiol by DPN. From Laidler and Krupka[81].

Reproduced from *Mechanism of Action of Steroid Hormones*, by courtesy of Pergamon Press

nothing concerning the chemical structure of the receptor, or they are represented as a structure that can faithfully follow the atomic contours of the combining molecule[81]. This latter view, springing directly from the lock and key simile, implies an unrealistic view of the structural elements that could possibly make up a receptor; such subtle changes in the surface of a receptor cannot be produced by a structure consisting of units of the same

size as the drug molecule itself. This important consequence of considering the receptor as a discrete chemical structure is demonstrated in *Figures 3.14* and *3.15*. Whilst, obviously, these highly schematic representations of receptors were not intended by their authors to be taken literally, nevertheless, this reluctance to consider receptors as discrete chemical structures has tended to add to the unreality of the concept.

A second consequence of the finite spacing of the groups projecting from a protein structure is that the number of side-chain groups that can be involved in a receptor pattern is very limited. If consideration is restricted to molecules up to the size of (+)-tubocurarine, then it is apparent, when the short range nature of most of the forces leading to drug-receptor interaction is considered, with the consequent necessity of close apposition of groups on receptor and drug, that for most drugs approximately six side-chain groupings at the most can possibly be involved in the drug-receptor complex. If small molecules such as acetylcholine or histamine are considered the number is even smaller. This is not, of course, to say that the remainder of the protein molecule is irrelevant: the particular orientation of the receptor groupings would be dependent on the orientation of adjacent segments within the same molecule and even in adjacent molecules. The fact that a membrane is probably a two-dimensional lattice of protein molecules each interacting with a neighbour implies that it may prove exceedingly difficult to isolate a receptor protein from its neighbours and still retain its active structure. The study of structure-action relationships is at best a very indirect way of studying receptors, and is beset with many difficulties of interpretation unconnected with the basic problem of the drug-receptor interaction. Nevertheless it is a technique that is potentially capable of yielding much valuable information about the receptor if the results are consistently interpreted in terms of a limited number of identifiable chemical structures. A concensus of such studies could then possibly give information on the sort of chemical groups most likely to occur in receptors.

So far attention has been concentrated on the formation of the drug receptor complex. It is also necessary to consider the immediate consequences of the formation of this complex. The response to a drug ultimately observed by the pharmacologist is the final consequence of a chain of events whose length and complexity is to some extent dependent on the degree of sophistication of the pharmacological recording technique. This chain is initiated by the formation of the drug-receptor complex and it is a legitimate question to ask what are the immediate consequences, in chemical terms, of the formation of such a complex? To take a particular example: from the study of the electrical properties of the motor end-plate membrane, the combination of acetylcholine with this membrane causes a transient increase in its permeability to all ions, this increase in permeability being recorded externally as a change in membrane potential. Why does the formation of a loose molecular complex between acetylcholine and a small segment of one of the proteins that constitute the membrane produce such an effect? Any satisfactory description of a receptor must eventually be able to answer questions of this sort.

At the present time any preliminary considerations of this question are hampered by uncertainties as to the precise part played by the drug receptor

complex in initiating the cell reaction. In the quantitative theories of Clark[105] and Gaddum[106] the assumption was made that the tissue response was proportional to the number of receptors occupied. This theory has been subsequently modified by Ariens[107] and Stephenson[108], who introduced the concept of 'efficacy', that is, different drug-receptor complexes could differ in the efficiency with which they initiated this response. However, the basic idea that the response is some function of the number of drug-receptor complexes was retained. Paton[109] suggested a different mode of analysis based on the assumption that excitation by a stimulant drug is proportional to the rate of drug-receptor combination, rather than to the proportion of receptors occupied by the drug. These two different theories have different implications when the detailed molecular mechanism of stimulant action is considered.

Several chemical mechanisms have been suggested to explain the depolarizing action of acetylcholine at the neuromuscular junction. Cavallito[110] suggested that as there is a large intracellular concentration of adenosine triphosphate (ATP) in muscle cells it is also a probable component of the cell membrane; he suggested that the polyphosphoric anion of ATP could be likened to a semi-conductor and that acetylcholine could combine with this anion and interfere in some way with its semi-conductor properties. Fatt and Katz[111] considered the possibility of an ion-exchange mechanism, the acetylcholine molecule displacing potassium ions to produce the change in membrane potential. They rejected this idea on the grounds that one molecule of acetylcholine was apparently capable of liberating very large numbers of potassium ions. Paton[109] extended this hypothesis and suggested that any one acetylcholine molecule could combine repeatedly with the membrane, being displaced each time by a potassium ion from the inside of the cell. In this way an amplification of the ion exchange would be observed. Whether the rates of dissociation of acetylcholine from the membrane are large enough to account for the very large ratios observed (at least 100 moles of potassium/mole of acetylcholine)[111,112] remains to be seen. Fatt and Katz[111] concluded that 'we must think in terms of some chemical breakdown of a local ion barrier which occurs as soon as acetylcholine combines with it'.

If the organized protein constituents of a membrane do play an important part in regulating ion flow across it then this suggestion may be re-phrased to read 'some transient rearrangement of a protein component of the membrane'. This suggestion is attractive in that it suggests a direct link between a protein functioning as receptor and a protein functioning as a membrane ion flux regulator. The analysis of the forces regulating protein structure reveals that secondary and tertiary protein structures are very unstable systems; they are held together by a large number of very feeble interactions, and the structure of the whole is dependent on the structural integrity of its sub-sections. The adsorption of a small molecule onto a protein could interfere with the interactions of a side chain and hence influence the stability of the protein as a whole. For example, it has been shown that electrostatic interaction betwen oppositely charged groups could be an important stabilizing influence. If acetylcholine were bound sufficiently firmly, by virtue of dipole interactions or hydrogen-bond interchange, so that an ion-pair

was formed between its positively charged quaternary ammonium head and an anionic group on the receptor, then any electrostatic interaction between the receptor anion and an adjacent positively charged protein group would be annulled. Hence the combination with acetylcholine then produces disorder in an organized segment of the receptor protein and, because of the integrated and co-operative nature of the protein structure, leads to a general alteration in the protein's tertiary structure with a consequent alteration in its ion regulating properties.

There is a great deal of evidence to suggest that the association of small molecules with proteins leads to structural changes. Karush[113] used the term 'conformational adaptability' to account for the variations in dye binding constants as increasing numbers of dye molecules were bound to a protein; he suggested that at least a portion of the entropy increase observed on dye binding arose from some structural alteration of the protein. Koshland[114,115] drew attention to some limitations in the simple lock and key hypothesis when applied to enzymes. He showed that there are many cases where different substrates react at different rates even when the enzyme is saturated with substrate, that is, when there are equal amounts of enzyme substrate complex. He introduced the idea of substrate-induced fit to explain these effects. He suggested that the enzyme substrate reaction was a two-stage process: the substrate combined with a limited number of groups on the enzyme, and on combination induced a change in the conformation of the enzyme so that the enzymically active site was only assembled after substrate combination. Different substrates, all equally bound initially could then differ in the efficiency with which they induced this enzyme transformation into an active form. A change in enzyme conformation accompanying the formation of an enzyme substrate complex has been demonstrated by optical rotation dispersion studies[116]. This explanation bears a striking resemblance to the concepts of affinity and efficacy introduced by Ariens and Stephenson, and provides a chemical interpretation for these two terms: affinity measures the ease of formation of the initial complex; efficacy measures the ability to induce the subsequent protein rearrangement. The difference between the enzyme and the receptor would then be that in the case of the enzyme the induced structural change in the protein led to a change in the substrate, in the case of the receptor the induced change would lead to a change in the properties of the matrix of proteins of which the receptor protein was a part. The idea of a rearrangement of a membrane protein produced by stimulant drug could also provide a physical interpretation of Paton's 'kinetic' model of drug action. Combination of a stimulant drug with the receptor induces a structural change: this structural change may alter the configuration of the groups which lead to the binding of the stimulant and therefore a necessary consequence of the protein rearrangement would be dissociation of the stimulant-receptor complex, one of the necessary conditions of Paton's analysis. Combination with a drug which did not lead to a rearrangement would result in a stable binding of the drug and a blocking action would be observed.

Association of a molecule with a protein need not necessarily lead to a disorganization of the protein: the drug-protein complex could stabilize the protein. Thus the simultaneous linking of two anionic groups by a

bis-quaternary molecule could prevent their separation in a protein rearrangement[15]. Charge-dipole interaction coupled with considerable hydrophobic bonding between large carbon groupings in the drug and similar nonpolar regions of the receptor is another possible mechanism for such a stabilization. The stabilization of proteins against many denaturation processes following the binding of fatty acid molecules has been demonstrated[117–119]. The correlation of drug stimulation with protein rearrangement and drug blocking action with protein stabilization would appear to be a not unreasonable inference from the known complexity and marginal stability of proteins. It should not be necessary to await the isolation of the receptor substance before this mechanism is subjected to experimental proof: Bergel[120] pointed out that such changes in conformation could probably be detected by a study of the optical properties of intact membranes during drug treatment.

At the present state of knowledge any survey of drug-receptor interactions can only serve to indicate the great gaps in knowledge and must of necessity be highly speculative and idiosyncratic. The great importance of the topic is the main justification for attempting such a discussion.

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4

POLYPEPTIDES OF MEDICINAL INTEREST

H. D. LAW

INTRODUCTION

PEPTIDE chemistry entered a new and fruitful phase in its development with the introduction of chromatography. This technique facilitated the elucidation of peptide structures and, directly and indirectly, stimulated the development of methods of peptide synthesis. As a result, many polypeptides of medicinal interest have been synthesized; those based on compounds of mammalian origin form the subject of this review.

Historically, the first compound to qualify for inclusion in a review of this type was oxytocin, the synthesis of which, by du Vigneaud and his colleagues in 1953, represented the first synthesis of a polypeptide hormone. Synthesis of polypeptides of powerful biological activity pointed the way to a more meaningful study of the structure–activity relationships of polypeptides. The preliminary communication reporting the synthesis of oxytocin[1] was concluded with the anticipatory words: 'What effect slight changes in the structure of a compound of such complexity might have on chemical, physical and biological properties must be investigated'.

As in other fields, it has been the hope that the investigation of structural analogues, as well as throwing light on the mode of action of the parent compounds, would result in the development of new compounds of clinical value. To a limited extent both objectives have been realized.

The compounds discussed in this review are oxytocin, vasopressin, corticotropin, the melanocyte-stimulating hormones, angiotensin, bradykinin, kallidin, insulin and glucagon. This list includes most pharmacologically-active polypeptides of medicinal interest which have been the subject of synthetic studies. It does not include the hypotensive polypeptide eledoisin[2–12], which is derived from a species of squid and which has been studied extensively in recent years, nor is consideration given to the large group of polypeptide antibiotics.

To facilitate discussion of specific peptides, the first part of the review is devoted to an outline of techniques in common use in peptide chemistry.

TECHNIQUES OF PEPTIDE CHEMISTRY

METHODS OF STRUCTURAL ELUCIDATION

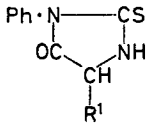
The methods which were employed to deduce the structures of the peptides discussed in this review are not new. However, although the strategy of sequence determination, particularly in its application to large molecules, has evolved to some extent, remarkably little change has taken place in the actual techniques. These were reviewed by Fraenkel-Conrat, Harris and Levy[13] in

1955 and the conclusions of these authors are still pertinent. Several recent reviews[14–16] have appeared and present comment is confined to stating the principal methods in common use.

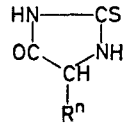
Long amino-acid residue sequences are deduced by integrating the sequences of smaller derived structures. This concept is now so familiar as to require no elaboration here. Recent trends favour the use of enzymes or specific chemical cleavage[17,18] for the fragmentation of the parent compound. In this way, fewer degradation products are obtained than, for example, by partial acid hydrolysis, and it is therefore easier to isolate them and to determine their orientation within the parent molecule. This approach is particularly favoured with large molecules, the stages of degradation being followed by quantitative amino-acid analysis. Partial hydrolysis under a variety of conditions is generally required for small peptides.

The identification of *N*-terminal residues is carried out most commonly by Sanger's technique, in which the peptide is reacted with 1-fluoro-2,4-dinitrobenzene (FDNB) and subsequently hydrolysed. The terminal residue is identified as its yellow 2,4-dinitrophenyl derivative. *N*-Terminal sequences may sometimes be deduced from the kinetics of enzymic degradation with leucine aminopeptidase. Otherwise, stepwise degradation from the *N*-terminal residue almost invariably employs Edman's method in which phenyl isothiocyanate is reacted with the peptide to form a phenylthiocarbamyl derivative, which is subsequently cyclized with cleavage of the terminal peptide bond, to give the corresponding phenylthiohydantoin derivative (*I*).

There are no chemical techniques of comparable value for the stepwise degradation of peptides from the *C*-terminal residue. Enzymic cleavage with carboxypeptidase is still the most satisfactory method. Hydrazinolysis is sometimes used for the identification of the *C*-terminal residue, which is released as the free amino acid; other residues are converted to hydrazides. The Schlack and Kumpf procedure is used occasionally and in theory, at least, is capable of stepwise application. In this procedure, an acyl peptide is reacted with acetic anhydride and ammonium thiocyanate to form the corresponding acyl-peptidyl-thiohydantoin derivative (*II*), from which the *C*-terminal residue in the form of the substituted thiohydantoin (*III*) is cleaved by mild alkaline hydrolysis.



(*I*)



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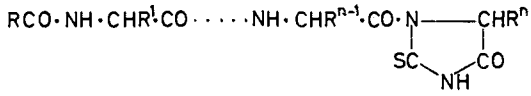
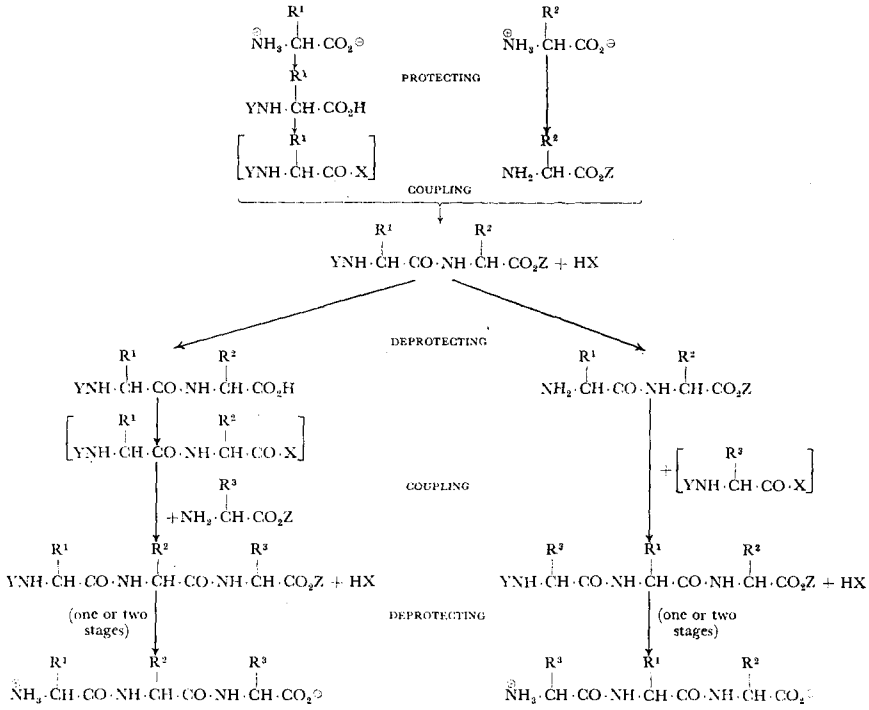


(*II*)

METHODS OF PEPTIDE SYNTHESIS

Several reviews dealing with methods of peptide synthesis have been published within the last few years[16,19–23] and the synthesis of peptides possessing

high biological activity has been described[24]. A small discussion group, the European Peptide Symposium, has met annually since 1958, principally to discuss methods of peptide synthesis, and the proceedings of these meetings have been published[25–30]. In view of the richness of this review material and because of the wide interest in the field engendered by recent spectacular successes in the synthesis of natural products, it is judged unnecessary to restate here the fundamental requirements of peptide synthesis. A comprehensive survey of available methods also will not be attempted. Such a project would be not only space consuming, but would tend to shift the emphasis of this review from where it properly lies, on the biological properties of peptides seen in relation to their chemical structure. Only the most commonly employed methods are outlined and attention is drawn to some recent developments which, in the opinion of the reviewer, will probably find wide application.



Schematic representation of the synthesis of a tripeptide
X = Carboxyl-activating group.
Y = Amino-protecting group.
Z = Carboxyl-protecting group.

For those unfamiliar with the field, the various stages of peptide synthesis may be brought into perspective by reference to the above synthesis of a hypothetical tripeptide. It is assumed that no side chain protection is necessary in this particular example, but when R^1, R^2 or R^3 in themselves carry reactive functions, further selective protecting groups are generally required.

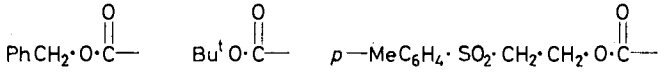
Protecting Groups

The use of protecting groups in organic chemistry has been reviewed by McOmie[31a].

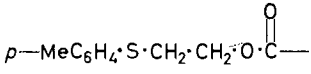
Amino-protecting groups

Amino-protecting groups have been discussed in detail by Boissonnas[31b] and only references which postdate this review are cited here.

The benzyloxycarbonyl group (*IV*) is still the most widely used α-amino-protecting group and isotope dilution studies with several amino acids have recently confirmed that no racemization occurs during its introduction[32]. It is generally removed by the action of hydrogen bromide in acetic acid solution, by catalytic hydrogenolysis, or by sodium-liquid ammonia reduction. Substituted benzyloxycarbonyl groups which are coloured or which show a different lability, have sometimes been used to advantage. The tertiary butyloxycarbonyl group (*V*) is widely used because of its extreme lability under conditions of mild acidity. Various other acid-labile alkoxycarbonyl groups, including the trityloxycarbonyl, 1,1-dimethylpropyloxycarbonyl and benzhydryloxycarbonyl groups, have been investigated, but seem to offer no advantages over the tertiary butyloxycarbonyl group[30a]. *p*-Tolylsulphonyl-ethyloxycarbonyl (*VI*) (and *p*-tolylthioethyloxycarbonyl (*VII*), from which it may be derived) seems a promising alkali-labile protecting group, but has not been intensively investigated[33].

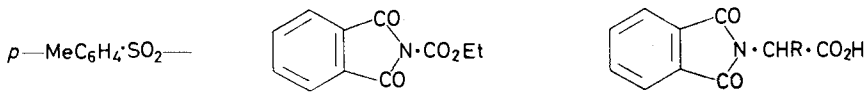


(IV) *(V)* *(VI)*



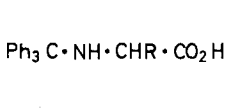
(VII)

The tosyl (*p*-toluenesulphonyl) protecting group (*VIII*) is still useful in peptide synthesis, although the coupling procedures which may be used with *N*-tosyl amino acids are somewhat limited[34–36]. Removal of the group with sodium-liquid ammonia proceeds by direct reduction of the S–N bond to form toluene sulphinate[30b,37]. *N*-Ethoxycarbonylphthalimide (*IX*) is now used for the preparation of optically-pure phthaloyl amino acids (*X*). More work on the synthesis of these compounds via phthalamic acid derivatives has been described[38]. The use of the phthaloyl protecting group has been facilitated by the observation that its removal, by reaction with hydrazine, takes place even in weakly acidic solution, but activated phthaloylamino acids have been shown to racemize more readily, in the presence of triethyl-amine, than their benzyloxycarbonyl counterparts[39].



(VIII) *(IX)* *(X)*

N-Tritylamino acids (*XI*) find occasional application, although their use is limited both as regards methods of preparation and methods of coupling. Similar acid-labile protecting groups which do not suffer from these disadvantages are the *o*-nitrophenylthio (*XII*)[29a,30c,40,41], 2,4,5-trichlorophenylthio (*XIII*)[30d] and, to a lesser extent, the tritylthio (*XIV*)[29a,40] groups. Use of the *o*-nitrophenylthio group, in particular, seems most promising.



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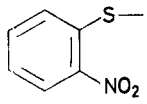

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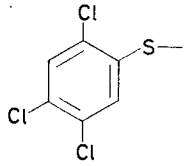
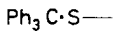


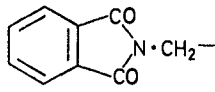
(*XIII*)



(*XIV*)

Carboxyl-protecting groups

Methyl and ethyl esters find frequent application in peptide synthesis and improved methods for their preparation have been described. Saponification sometimes leads to undesirable side reactions and occasionally enzymic hydrolysis of methyl esters has proved useful[42]. The use of benzyl esters and tertiary butyl esters, which may be removed by hydrogenolysis and under mildly acidic conditions respectively, avoids these difficulties. Amino acid benzyl esters are now readily available. Procedures which have been recently described include their preparation from dibenzyl sulphite[43] and from the dibenzylacetal of *N*,*N*-dimethylformamide[44,45]. Tertiary butyl esters of amino acids are prepared by a variety of methods: an improved procedure for the acid catalysed alkylation of benzyloxycarbonyl amino acids with



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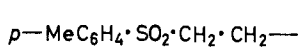


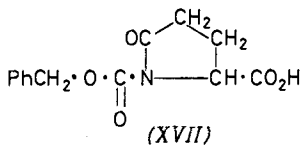
(*XVI*)

isobutylene has been described[46]. Other derivatives which may be cleaved under mildly acidic conditions and which may find general application are the benzhydryl[30c,47,48], *p*-methoxybenzyl and phthalimidomethyl (*XV*)[49] esters. *p*-Toluenesulphonylethyl (*XVI*)[33] esters may be cleaved under mildly alkaline conditions. Protecting groups which may be removed under unusual and specific conditions may also be useful in certain instances: *β*-*N*-phenylhydrazide[50], removable by oxidation with manganese dioxide in aqueous acetic acid solution, is one such group.

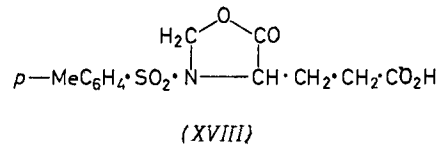
Side-chain protection

Basic side chains—The ω-guanidino group in the arginine side chain has been commonly protected by protonation or by nitration. Protonation is usually satisfactory in the synthesis of larger peptides, but frequently causes difficulties with smaller peptides. Nitroarginine derivatives are usually satisfactory from this point of view, but side reactions occur during the removal of the nitro group by hydrogenolysis. The group is not removed by sodium-liquid ammonia[51]. Investigations with model compounds[30e] suggest that the best conditions for its removal are hydrogenolysis in methanolic solution at 40°C in the presence of Raney nickel catalyst and 2·5 equivalents of acetic acid. Tritylation and benzyloxy-carbonylation of the guanidino group have also been studied. Tosylation[52–54] is probably the best method available at present for its protection. The ε-amino group of lysine has been protected, in general, like α-amino groups; the histidine imidazole ring has usually remained unprotected. The N^a-benzyloxycarbonyl group may be removed selectively from N^a,N^{im}-dibenzyloxycarbonylhistidine[55].

Acidic side chains—Carboxyl groups in side chains are generally protected as esters[29b,56–60], but their protection is not always necessary[61]. In the case



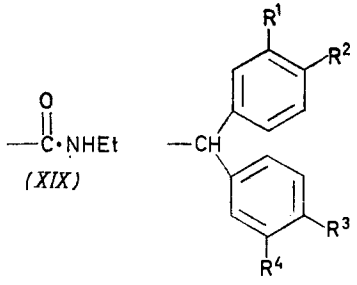
(XVII)



(XVIII)

of glutamic acid, the possibility of forming cyclic compounds also exists. Tosyl-pyrrolidone carboxylic acid has been frequently used in this role: derivatives of *N*-benzyloxycarbonyl-2-pyrrolidone-5-carboxylic acid (*XVII*)[62,63] and β-[3-tosyl-5-oxo-oxazolidinyl-(4)-]propionic acid (*XVIII*)[64] have recently been described. The β-*N*-phenylhydrazide group has been used[50] to protect the γ-carboxyl group of glutamic acid.

Neutral side chains—Troublesome side reactions which involve alcohol functions sometimes occur, both during the coupling reactions and during the removal of *N*-protecting groups. Alcohol functions are perhaps best protected as the benzyl ethers[30f], but tertiary butyl[65,66] ethers have also been used. The benzyl group, removed by sodium-liquid ammonia reduction, is still



(XIX)

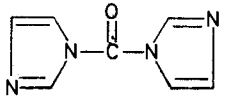
(XX)

(*a*) $R^1 = R^2 = R^3 = R^4 = H$
(*b*) $R^1 = R^4 = H$; $R^2 = R^3 = OMe$
(*c*) $R^1 = R^2 = R^3 = R^4 = OMe$

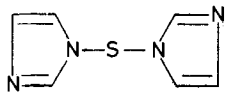
widely used for thiol protection. *S*-Acyl derivatives, especially *S*-benzoyl, *S*-acetyl and *S*-benzyloxycarbonyl compounds, have recently been used in peptide synthesis[29c,67]. Like the *S*-(*N*-ethylcarbamoyl) derivative (*XIX*)[30d], these protecting groups are removed under mildly alkaline conditions. *S*-Trityl protection has been reinvestigaged and this group may be cleaved by treatment with silver nitrate-pyridine or by the action of hydrogen bromide in acetic acid solution. It is well suited for selective cleavage in the presence of *S*-benzhydryl derivatives (*XX*a)[29c,68]. The benzhydryl group is cleaved by the action of hot trifluroacetic acid; 4,4′-dimethoxybenzhydryl (*XX*b) and 3,3′,4,4′-tetramethoxybenzhydryl (*XX*c) groups behave similarly and in addition are chromogenic, which facilitates their detection[30g].

Coupling methods

A large number of methods are known for the formation of the amide bond in peptide synthesis[16,19–24], but only five are in common use. These involve the use of azides, mixed anhydrides, acylimidazoles, active esters and di-imides. The problem of racemization during the formation of the peptide bond is constantly under review and the azide method is the only one which has not been faulted[22,30h,69]. Unfortunately, the azide method is subject to several side reactions[70,71] and the formation of the initial hydrazides sometimes gives trouble. Mixed anhydrides formed with isobutyl chloroformate and ethyl chloroformate still find wide application. The use of pivaloyl mixed anhydrides has extended mixed anhydride coupling to tosylamino acids[35] and to sterically-hindered compounds[72] and recently a pivaloyl mixed anhydride was used, in an important instance, to activate a long chain *C*-terminal glycine peptide[73]. Acylimidazoles, formed by the reaction of the carboxyl component with *N*,*N*′-carbonyldi-imidazole (*XXI*) or *N*,*N*′-thiodi-imidazole (*XXII*), react smoothly with suitably protected amino components, forming the desired peptide bond and imidazole. The presence of imidazole[74–78] accelerates alkyl and active ester couplings and bifunctional compounds containing weakly acidic and weakly basic groups have also been used to increase yields in this type of synthesis[30i,79].



(XXI)

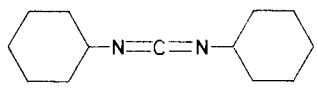


(XXII)

Active ester couplings have probably received the widest attention in recent years. *p*-Nitrophenyl esters[80] are most commonly used and new methods for their preparation have been described[81–83]. One very useful attribute of the *p*-nitrophenyl ester method is that it may often be used with amino-acid base salts in aqueous solutions; a limitation is that the formation of the active ester from the free carboxyl group of a peptide is always accompanied by varying degrees of racemization. From a survey of a wide number of active esters it was concluded[84] that, from some points of view, 2,4,5-trichlorophenyl esters are better than *p*-nitrophenyl esters. The chlorinated phenol is less expensive than *p*-nitrophenol and, in the event of its incomplete removal

after the coupling stage, is not reduced to troublesome coloured byproducts during subsequent hydrogenolyses. Probably a more formidable opponent for the *p*-nitrophenyl ester method employs *N*-hydroxysuccinimide esters[85], which react, if anything, in slightly better yields than *p*-nitrophenyl esters and have an overwhelming advantage in that the byproduct, *N*-hydroxysuccinimide, is water soluble.

N,N'-Dicyclohexylcarbodi-imide (*XXIII*) is still the most widely used di-imide in peptide synthesis, although in isolated instances valuable use is made of di-imides which give water soluble ureas as byproducts in the coupling reaction. The mechanism of the di-imide reaction has been the subject of several recent studies[86–88] and an *O*-acylisourea derivative similar to the postulated active intermediate (*XXIV*) has been isolated[88]. It is possible that formation of a symmetrical anhydride of the carboxyl component is involved in the coupling. This is reminiscent of the formation of benzyloxycarbonylglutamic anhydride in the reaction of benzyloxycarbonylglutamic acid with *N,N'*-dicyclohexylcarbodi-imide[62,89]. Clearly related, both to active ester and to di-imide couplings, is the use of reagent K, *N*-ethyl-5-phenylisoxazolium-3'-sulphonate (*XXV*)[90,91], which probably forms the active ester (*XXVI*). The method has been used on several occasions and is particularly useful because the byproduct of the coupling is water soluble.



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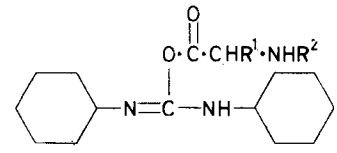

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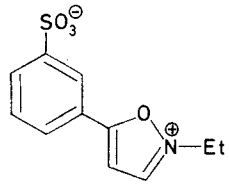
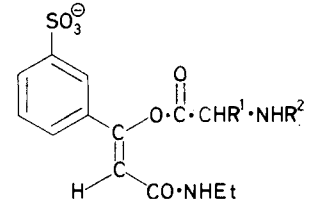


(*XXV*)



(*XXVI*)

Peptide synthesis by the established pattern of operations, coupling, 'deprotecting', coupling, 'deprotecting', is a laborious and wasteful procedure and many attempts have been made to synthesize peptides without isolating the intermediates. A recent promising and original example was described by Merrifield[92a]. The *C*-terminal residue was esterified and rendered insoluble by reaction with a chloromethylated styrene-divinylbenzene polymer. *N*-Benzyloxycarbonyl amino acids were subsequently added in a stepwise manner, by the use of *N,N'*-dicyclohexylcarbodi-imide, to the immobilized *C*-terminal residue, removing the benzyloxycarbonyl group at each stage by

the action of hydrogen bromide in acetic acid. The peptide was finally liberated from the resin by saponification*.

POLYPEPTIDES

METHOD OF PRESENTATION

In this section, amino-acid residue sequences are denoted in accordance with the suggestions of the Committee on Nomenclature which reported at the Fifth European Peptide Symposium[29]. As is now customary, the residue positions in any given peptide have been numbered beginning with the amino terminal position. In the naming of analogues, the noun form of an amino acid has been used, prefixed by the number of the residue position, to indicate the replacement of one amino-acid residue by another. The adjectival form of the amino acid has been used to indicate acyl substitution. By these conventions, promulgated by du Vigneaud,

Arg·Pro·Pro·Gly·Phe·Ser·Pro·Phe·Arg is bradykinin;
Lys·Arg·Pro·Pro·Gly·Phe·Ser·Pro·Phe·Arg is 1-lysylbradykinin;
Lys·Pro·Pro·Gly·Phe·Ser·Pro·Phe·Arg is 1-lysine bradykinin.

Intermediates used in syntheses of specific peptides are tabulated according to the sequences involved; protecting groups and methods of coupling are discussed in the text. It should thus be possible, by tracing the reference numbers from the tables of analogues to the tables of intermediates, to deduce at least the general pattern adopted for the synthesis of any given analogue. Where several analogues are covered by one reference, superscript letters have sometimes been added to assist cross reference between the tables. Every effort has been made to make the tables comprehensive, covering work published to the end of 1963.

In many papers quoting biological properties it is not stated whether these refer to anhydrous or to hydrated materials, to free bases or to salts, and, in general, no account is taken of these differences in this review. The discrepancies arising in this way are usually not large since the molecular weights involved are in excess of 1000. However, there is much to be said for reporting biological properties in terms of activity per unit weight of anhydrous free base, or in terms of activity per micromole; the activity of an analogue is sometimes adequately represented as a fraction of the activity of the parent compound.

The discussion of structure–activity correlations in this review may seem occasionally to be biased towards considerations of the significance of the contributions of individual amino-acid residues to biological activity. Any such bias arises from the way in which most structure–activity studies have been formulated (to determine the effect on biological activity of replacing one amino-acid residue by another). This is also a convenient way to summarize the principal facts which any theory concerned with mechanisms of activity must correlate. However, all residues must be considered in relation to their environment. When one residue cannot be replaced without loss of

* A recent communication by Merrifield[92b] reports the synthesis, by similar means, of bradykinin in eight days starting from tertiary butyloxycarbonyl amino acids (overall yield 32 per cent).

activity, it has become customary to say that 'this residue is essential' for the manifestation of activity. In the absence of other evidence, this is not to imply that this particular residue participates directly in producing the biological effect. Substitution of one amino-acid residue by another might lead to far reaching changes, for example, in the conformation of the molecule or in its metabolism in the body. The same considerations apply to the removal of specific functional groups. On the other hand, when one residue can be replaced by another without loss of activity, the minimum deduction is that the residue is not uniquely essential for activity; when a functional group can be removed without loss of activity, clearly, it is not directly implicated in producing the biological effect.

In general, the peptides reviewed here show a high degree of specificity, both in relation to the changes which can be made to the molecules without loss of biological activity and in their pharmacological properties. Whilst one cannot disregard the possibility that similar biological properties may be exhibited by polypeptides of radically different structures,[93] the significance of this possibility seems to diminish as research progresses.

OXYTOCIN AND VASOPRESSIN

Introduction

The ability of pituitary extracts to raise blood pressure (pressor effect) in mammals was discovered by Oliver and Schäfer[94] in 1895 and it was soon demonstrated that this property was a feature of the posterior lobe of the gland[95]. Reports of further biological properties of posterior pituitary extracts followed, the principal ones being an oxytocic effect (ability to cause uterine contractions)[96]; a milk ejecting property[97]; and an antidiuretic effect (ability to cause an inhibition of urine secretion)[98].

In 1928, oxytocin, the agent responsible for the oxytocic effect, was almost completely separated from vasopressin, which is responsible for the antidiuretic effect[99]. In the following twenty years the chemistry of these substances was studied extensively, especially by du Vigneaud and colleagues, but appreciable further purification was not achieved until Craig developed his technique of counter-current distribution. The application of this method by du Vigneaud and colleagues resulted in the purification of oxytocin[100] and of vasopressin[101] to the point at which detailed structural studies were undertaken.

Structural Elucidation

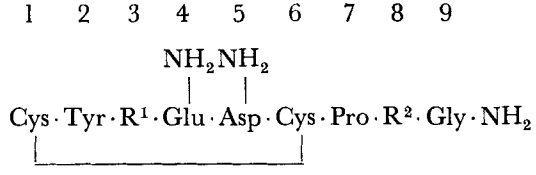
An acid hydrolysate of oxytocin was shown by chromatographic analysis to contain eight amino acids in equimolar proportions, together with three moles of ammonia[102,103]. One of the amino acids was cystine and since neither performic acid oxidation[104] nor Raney-nickel desulphurization[105] of oxytocin resulted in cleavage of the molecule, it was deduced that a cyclic disulphide structure was present.

The sequence of the amino-acid residues in the molecule was investigated by the use of FDNB[106], and by Edman degradation of the performic acid-oxidized material[107]. Partial acid hydrolysis[107] and specific chemical cleavage

with bromine water[108,109], a reaction which at that time could not be rationalized[17], were utilized for the production of smaller peptides. The terminal carboxyl group was thought to be present as an amide because no *C*-terminal residue could be demonstrated by the Schlack and Kumpf procedure[107] and because the molecule was resistant to carboxypeptidase[110]. The other two moles of ammonia were accounted for by assuming that the aspartic and glutamic acid molecules produced on hydrolysis were derived from glutaminyl and asparaginyl residues.

By these means du Vigneaud, Ressler and Trippett[107] were able to deduce the structure of oxytocin (*XXVII*a). The same structure was derived independently by Tuppy and Michl by the use of performic acid oxidation followed by acidic and enzymic hydrolysis and by extensive use of the FDNB method[111,112].

Subsequent work has established the essential similarity between oxytocin and the vasopressins. Arginine vasopressin (*XXVII*b)[113,114] occurs widely in mammals, lysine vasopressin (*XXVII*c)[113,115] occurs in the pig and hippopotamus.

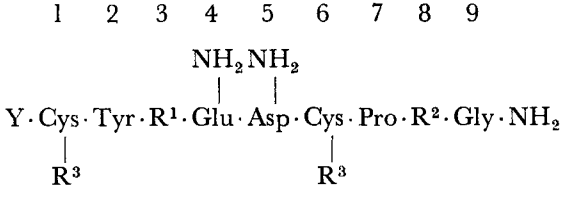


(*a*) R^1 = Ile, R^2 = Leu.
(*b*) R^1 = Phe, R^2 = Arg.
(*c*) R^1 = Phe, R^2 = Lys.

(*XXVII*) Oxytocin and Vasopressin

Synthesis

Most syntheses of oxytocin and all syntheses of the vasopressins have proceeded via an *S,S'*-dibenzylnonapeptide intermediate (*XXVIII*a) and the final stages of these syntheses have involved removal of the benzyl groups by



(*a*) R^3 = —$CH_2C_6H_5$
(*b*) R^3 = —$C(C_6H_5)_3$
(*c*) R^3 = —CONHEt

Y = amino protecting group

(*XXVIII*) Protected Nonapeptide Intermediates used in the Synthesis of Oxytocin and Vasopressin

sodium-liquid ammonia reduction and aerial oxidation of the resulting sulphydryl compound to the disulphide form. The feasibility of this route having been demonstrated[116,117], it was subsequently used in the first synthesis of oxytocin[1,118]. This work and the structural elucidation of oxytocin has been reviewed by du Vigneaud[119]. *S*-Benzylcysteine derivatives have been used in all of the reported syntheses of oxytocin and vasopressin analogues.

Oxytocin has also been synthesized by the use of *S*-trityl[120] (*XXVIII*b) and *S*-carbamoyl[30d] (*XXVIII*c) derivatives. An improved technique[121] for the conversion of the sulphydryl to the disulphide form involves the use of 1,2-di-iodoethane.

$$R—S^{\ominus} + ICH_2CH_2I \rightarrow R—SI + CH_2 = CH_2 + I^{\ominus}$$
$$R—SI + R—S^{\ominus} \rightarrow R—SS—R + I^{\ominus}$$

In the synthesis of some oxytocin analogues, ferricyanide oxidation has proved advantageous[122].

The preparation of the protected nonapeptide in the oxytocin series has been based generally on one of the following three patterns:

Method A[123].

$$1—2 + 3—5 \rightarrow 1—5(+6—9) \rightarrow 1—9$$

Method B[124,125].

$$\left.\begin{matrix} 4—6 + 7—9 \\ 4—5 + 6—9 \end{matrix}\right\} \rightarrow 4—9(+1—3) \rightarrow 1—9$$

Method C[126].

$$8—9(+7) \rightarrow 7—9(+6) \rightarrow 6—9(+5) \rightarrow \text{etc.} \rightarrow 2—9(+1) \rightarrow 1—9$$

In procedures A and B, benzyloxycarbonyl and tosyl groups have been used for protection of the terminal amino function; coupling methods, particularly in the more recent adaptations of these procedures, have involved the use of mixed anhydrides, azides and *N,N'*-dicyclohexylcarbodi-imide. The terminal carboxyl group has been blocked in the form of the ethyl ester, which has been converted to the amide by ammonolysis, usually at the tripeptide (7–9) or tetrapeptide (6–9) stage. In other procedures, glycine amide has been used from the outset. Glutaminyl peptides have been formed both by the ammonolysis of tosyl-pyrrolidone derivatives and directly, from benzyloxycarbonylglutamine.

Method C, which has influenced considerably the preparation of intermediates in methods A and B, has found wide application in peptide synthesis. The strategy of this method[80] is to start with the carboxyl protected *C*-terminal residue and to add the other residues in a stepwise manner in the form of their *N*-benzyloxycarbonyl *p*-nitrophenyl esters. Hydrogen bromide in acetic acid removes the benzyloxycarbonyl group after each coupling. By this approach, the possibilities of racemization are minimized and, since excess *p*-nitrophenyl esters can be readily removed, high yields can be obtained at the coupling stages. In several examples of approach B, the tripeptide 1–3 has been coupled to the hexapeptide 4–9 by a *p*-nitrophenyl

ester method, although, on one recent occasion, appreciable racemization of the residue in position 3 occurred[30j].

In view of the close resemblance between the structures, it is not surprising that synthetic routes in the vasopressin and oxytocin series are similar. Satisfactory methods analogous to procedures A[127], B[128,129] and C[130] have been described. The ε-amino group of the lysine residue has been universally blocked by the tosyl group in syntheses of lysine vasopressin: in syntheses of arginine vasopressin, the guanidino group has generally been blocked by protonation[131–136], but use of the recently introduced ω-tosylarginine derivatives[52–54] is preferable[128,129].

The intermediates used in the synthesis of oxytocin, vasopressin and their analogues are collected in *Tables 4.1* and *4.2*.

Assay Procedures

Assay procedures for oxytocin and vasopressin have been reviewed by Thorpe[202a]. The vasopressin assay is usually based on: (1) the increase in arterial blood pressure in rats (pithed or anaesthetized with urethane)[203,204], and (2) the reduction in the flow of urine from the cannulated bladder of rats (anaesthetized with ethanol and given a water load)[205–207]. Oxytocin is also active in these tests but is far less active than vasopressin. The oxytocin assay is usually based on: (1) the contraction of a segment of uterine horn (guinea-pig or rat), suspended in a suitable salt solution at a constant temperature, which results from the addition of oxytocin to the solution[208–213], (2) the increase in milk ejection-pressure which results from contractions of the myoepithelial tissue of the lactating mammary gland (rabbits and guinea-pigs) after intravenous injection of oxytocin[214–217], and (3) the decrease in the arterial blood pressure of fowl (anaesthetized with barbitone) which is produced by intravenous injections of oxytocin[206,218–220]. Vasopressin, to a lesser degree, is also active in these tests.

The bladder of the toad, *Bufo marinus*, is relatively impermeable to water and urea under resting conditions but the permeability increases markedly in the presence of vasopressin or oxytocin. An assay technique based on this water transport was developed by Bentley[221] and has been studied extensively[222–224].

Oxytocin and its analogues stimulate, to varying degrees, the active transport of sodium ions through the skin of the frog, *Rana esculenta*, and an assay method for this so-called natriferic activity, measuring the increase in short-circuited current, has been applied to a number of compounds by Morel[225a].

Following the description of neurohypophysial and hypothalamic extracts which possessed high corticotropin-releasing properties and an amino-acid content very similar to that of lysine-vasopressin with, in addition, serine and histidine[226], several analogues of vasopressin have been assayed for their ability to bring about the release of corticotropin[138]. Lysine-vasopressin itself possesses an appreciable corticotropin-releasing activity *in vivo*[227]. Mangili, Martini and Pecile, in a recent study[225b], find the ratio of corticotropin release: pressor activity to be 3·3 for arginine-vasopressin (defining corticotropin release as 1000 for this compound) and 3·25 for lysine-vasopressin. 1-Histidylseryl-8-lysine vasopressin on this scale has a ratio of 4·0.

Table 4.1. Intermediate fragments used in the synthesis of oxytocin and analogues

Sequences	References	
	Oxytocin	*Analogues*
(—3) to 2		137
(—2) to (—1)		138
(—1) to 2		137, 139
1–2	1, 118, **123**, 124, **140**, **141a**, 142, 143	**139**, 142, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156
1–3	124, **141a**, **141b**, 143, 157 158	30j, 128, **139**, **141b**, 144, 146, 147, 149, 150, 153, 154, 159, 160, 161, 162, 163, 164, 165, 166
1–4	**141a**	
1–5	123, 142	142, 148, 151, 152, 167, 168
1–6		169
1–9		122, 138, 170
2–3		145, 163
2–9	126	122, 163, 171, 172, 173, 174a, 175
3–5	1, 118, 123, **140**, 176	148, 151, 152, 169, 177
3–6		169
3–9	1, 118, 125, 126, **140**	122, 137, **139**, 155, 163, 172, 174a, 175
4–5	125, **178**	156, 160
4–6	124, 125	144, 153, 161, 162
4–8		153
4–9	124, 125, 126, 143, 158	30j, 128, **139**, 144, 145, 146, 147, 149, 150, 153, 154, 156, 158, 159, 160, 161, 162, 163, 164, 165, 166, 172, 174a, 175
5–6	124, 125	144, 161
5–9	126	30j, 164, 166, 174a, 175
6–9	1, 118, 123, 125, 126, **140**, 142, 143, **179**, **180**	142, 148, 151, 152, 156, 160, 164, 166, 167, 168, 174a, 175, 177
7–8	142	142, 153
7–9	124, 125, 126, 142, 143, **179**, **180**, **181**	142, 144, 153, 156, 161, 162, 168, 174a
8–9	124, 126, **179**, **180**	153, 162, 174a

References in heavy type are concerned with the preparation of intermediates.

Table 4.2. Intermediate fragments used in syntheses of the vasopressins and analogues

Sequences	*References*		*Sequences*	*References*	
	Arginine and lysine-vasopressin	*Analogues*		*Arginine and lysine-vasopressin*	*Analogues*
(—3) to (—1)		182	3–9	130, 135, 184	174a, 183, 197
			4–5		200b
(—2) to (—1)		138, 182	4–6	128, 129, **134**, 189	158, 190, 192, 193
(—1) to 2		183	4–9	128, 129, 130, **134**	128, 136, 149, 150, 158, 174a, 182, 188, 190, 191, 193, 200a
1–2	127, 128, 131, 135, 184, 185, 186	128, 149, 150, 187, 188, 200b	5–9	130	174a, 193
1–3	128, 129, **134**, 189	128, 136, 149, 150, 158, 182, 188, 190, 191, 192, 193, 194	6–7	131, **133, 201**	
			6–9	127, 130, 131, 132, **133**, 184, 185, 186, **201**	174a, 187, 193, 194, 196, 200b
1–5	127, 131, 132, 185, 186, **195**	196			
1–6	**134**, 189	192	7–8	128, 189	128, 190
2–5	132, **195**		7–9	127, 128, 129, 130, **133**, 185, 189, **201**	128, 158, 174a, 190, 192, 193, 196
2–9	130	174a, 197, 198			
3–5	127, 131, 132, 184, 185, 186, **195, 199**	187	8–9	127, 128, 129, 130, 131, **133, 201**	128, 158, 174a, 193, 196

References in heavy type are concerned with the preparation of intermediates.

The standards employed in the assay of oxytocin and vasopressin are related to the International Standard 'Pituitary (Posterior Lobe) Powder' which is defined as containing 2 units of activity per mg.

Structure–Activity Correlations

From the study of analogues it is now possible to assess the significance for biological activity of various structural features of the oxytocin and vasopressin molecules, but the mechanism of action of these hormones at a molecular level is still largely unknown. Several analogues with clinical indications have been produced.

Analogues which had been rigorously purified and for which adequate pharmacological data was available were reviewed by Boissonnas and colleagues, 1961[228]. These authors very properly emphasized the importance of assaying each analogue by several different tests. As one might expect, a modification to the structure of the parent hormone frequently affects its activity in different tests to different degrees. The literature up to about the same period has also been reviewed by Sawyer[229] and by Hofmann[230].

Presented in *Tables 4.3* and *4.4* is biological data on all analogues of oxytocin and vasopressin published to date. Any single analogue of oxytocin is also an analogue of vasopressin, and compounds which, on the basis of their biological properties and structures, are obviously intermediate between oxytocin and vasopressin have been produced. To this extent, it is illogical to deal with oxytocin and vasopressin separately. However, in the following tables, analogues which structurally most resemble oxytocin have been collected under one heading and vasopressin analogues under another. It is hoped that this facilitates comparison between the activities of any one analogue and those of its nearest parent compound. An almost infinite number of cross-references between the compounds can be made and a corresponding number of tables would be required to bring them all into juxtaposition.

Uterotonic, avian depressor, milk let-down, rat pressor and antidiuretic effects

General correlations—Dibenzyl derivatives of all oxytocin-vasopressin analogues prepared to date have been 'inactive'. The reduced form of oxytocin has also been found inactive under anaerobic conditions[248] and it is generally thought that the disulphide bridge is essential for biological activity. Incontrovertible proof of this is not to be found in the analogues so far studied, since simple modifications of the disulphide (for example conversion to a dialkyl derivative or dithiol) must create profound conformational changes in the parent molecule. An ideal compound to resolve the difficulty would have a space separation between the α-carbon atoms of residues 1 and 6 approximately equal to the separation of the α-carbon atoms in cystine. The importance, for oxytocic activity, of the size of the 20 atom disulphide ring is evinced by the low activities of 1-hemihomocysteine-oxytocin (compound 10)[152], homo 2:3 tyrosine-oxytocin (compound 19)[145,156], homo 2:3 isoleucine oxytocin (compound 20)[235], des-isoleucine-oxytocin (compound 21)[236], 4-isoglutamine oxytocin (compound 35)[160,238] and 5-isoasparagine oxytocin (compound 40)[161]. Evidence is accumulating to show that the disulphide

Table 4.3. Analogues of oxytocin

Compound number	*Residues as in oxytocin unless marked to the contrary* 1 2 3 4 5 6 7 8 9	*Rat uterus IU/mg*	*Avian depressor IU/mg*	*Milk let-down IU/mg*	*Rat pressor IU/mg*	*Anti-diuretic IU/mg*	*Water transport (% arginine vasotocin) 225d‖*	*Natriferic units/mg 225a‖*	*References*
		450 ± 30	450 ± 30	450 ± 30(Rb)	5 ± 1	5 ± 1(Rt)	4	450	228
1†		486 ± 5	507 ± 15	410 ± 16	3·1 ± 0·1	2·7 ± 0·2			231, 232
2	DCys	0·25*	0·1*	0·38*(G)		0·0033*(Rt)	0·016 0·014	24	137
3	MeCys	0·25*	0·025*	0·98*(G)	(<0·001)	0·0039*(Rt)	0·027	9	137
4	Sar·Cys	2·3*	0·85*	1·3*(G)	(<0·001)	0·027*(Rt)	0·00030	2·7	137
5§	Gly·Cys	0·33*	(0·7)*	anti(G)	0	0·11*(Rt)	0·00003		137, 170
6	Leu·Cys	23*	(3·0)*	anti(G)		0·27*(Rt)			137
7	Leu·Gly·Gly·Cys	0·2*		anti(G)	0	0·034*(Rt)		2·1	137
8	His·Ser·Cys				0·27 ± 0·03				138
9	Ser·His·Cys				0·04 ± 0·02				138
10	homoCys	0·75	~0 anti		~0		0·015		152
11	desaminoCys	684 ± 32	733 ± 23	400 ± 8	1·1 ± 0·1	14·9 ± 2·1	0·45	198	171, 173, 231
12§	acetylCys	1·7 ± 0·2	anti (5000:1)	2·1 ± 0·2(Rb)					233
13§	Phe	30	60	60					148
		31·5 ± 2·4	63·2 ± 9	141 ± 21(Rb)	0·4	0·5(Rt)			147, 234
		25*	50*				0·098	36	155
14§	Me | Tyr	anti	8*	10*(G)	tachy	0·01*(Rt)		8·6	155
		~5	~5		anti		0·064		151
15§	MeTyr	1·2 ± 0·4 (anti)	0·32 ± 0·05 (anti)	3·1 ± 0·7(Rb) (anti)		~0·001(Rt)			163
16	DTyr	34 ± 3		19	6·1	0·01			30k

	1 2 3 4 5 6 7 8 9							
17	Leu	0·44*	1·2*	1·7*(G)		0·02*(Rt)	0·00064	8·2	155
18§	Ser	<0·01	<0·01	<0·01(Rb)	<0·01	<0·01(Rt)			150
19§	Homo‡ 2:3 Tyr	anti 500:1	anti 500:1	~0·5(Rb)					145, 156
20§	Homo‡ 2:3 Ile	~0·1	~0·05	~0·2(Rb)		<0·01(Rt)			235
21	—	'INACTIVE'							236
22	ale	24* 27	19* 24	125*(G) —	—* ~0·18	0·046*(Rt) —	0·25	8·1	154
23§	Val	51*	38* 39·5	67*(G)	~0·035*	0·55*(Rt)	0·093	1	154, 159
		59 ± 8	57 ± 4	207 ± 14(Rb)	~0·2	~0·8(Rt)			144, 162
24§	Leu	4·4* 5·0	10* 7·1	35*(G) —	~0·03* —	1·0*(Rt) —	0·19	17·1	154, 159
		45 ± 7	42 ± 1	101 ± 13(Rb)	~5	~5(Rt)			144, 162
25	NorLeu	5·1* 14	14* 25	25*(G)	~0·19*	0·46*(Rt)	0·0095	61	154
26	NorVal	6·3* 7·2	10* 14	85*(G) —	~0·14*	5·25*(Rt)	0·038	2·8	154
27§	Tyr	0·1 ± 0·03	~0·03	1·5 ± 0·3(Rb)	~0·01				149
28§	Try	0·04 ± 0·01	~0·1	0·10±0·06(Rb)	<0·01				150
29§	Phe	~20	~30	~60(Rb)	~3	~30(Rt)			144, 167, 237
30	Ser	195 ± 30	230 ± 20	255 ± 45(Rb)	<0·1	0·06±0·01(Rt)			166
31	Ala	36 ± 6	65 ± 3	240 ± 55(Rb)	<0·01	<0·01(Rt)			166
32	Asp(NH_2)	108 ± 29	202 ± 15	300±128(Rb)	0·13 ± 0·03	0·044 ± 0·005(Rt)		18	164, 166
33	descarboxamido-Glu(NH_2)	75 ± 2	108 ± 5		~0·1	0·2–0·3			175
34	Glu	1·6	0·5						30k
35§	IsoGlu(NH_2)	<0·01	<0·05		anti 500:1				160, 238
36	Glu(NH_2)	1± 0·2	0·8 ± 0·03	7·3 ± 1·1(Rb)	0·01			2·6	164

Table 4.3. Analogues of oxytocin (*continued*)

Compound number	*Residues as in oxytocin unless marked to the contrary* 1 2 3 4 5 6 7 8 9	*Rat uterus IU/mg*	*Avian depressor IU/mg*	*Milk let-down IU/mg*	*Rat pressor IU/mg*	*Anti-diuretic IU/mg*	*Water transport (% arginine vasotocin) 225d‖*	*Natriferic units/mg 225a‖*	*References*
37	descarboxamido-Asp(NH_2)	0·2–0·3	0·2–0·3		(<0·01)	(<0·002)			175
38	Ser	0·7 ± 0·2	0·2 ± 0·01	4·1 ± 0·8(Rb)	<0·1	<0·01(Rt)			166
39	Ala	<0·05	<0·1	<0·05(Rb)	<0·01	<0·01(Rt)			166
40§	IsoAsp(NH_2)	<0·01	<0·05		<0·01				161
41§	—	3·8 ± 0·9	anti 1000:1	1·6 ± 0·2	<0·01	<0·01		18	153
42	—	4·5 ± 1	anti 1000:1	~3	anti 1000:1	0·07 ± 0·008		660	153
43§	Ile	289 ± 21	(498) ± 37 489	328 ± 21	6 ± 1	1·1 ± 0·1		507	162, 239
44§	Val	200 ± 15	280 ± 17	310 ± 20	9 ± 1	0·9 ± 0·1		610	162
45	DLeu	~20	20	⩾50	0 anti	low			168
46§	Arg	75	150		125				146
		(155) ± 15	285 ± 40	~210(Rb)	245 ± 15	250 ± 35(Rt)		1140	240
		115 ± 15 90 ± 12	285 ± 40 224 ± 32	~212 ~165	245 ± 15 193 ± 12	250 ± 35 197 ± 28	100		241
47§	Lys	78 ± 10	210 ± 3 190	180 ± 25(Rb)	130 ± 13 130	24 ± 3(Rt)	25	940	158, 172, 240, 191
48	Orn	42 ± 5	90 ± 3	95 ± 6	103 ± 10				158
49	Cit	~100%	~50%		low		6·6		174a, 174b
50§	NH_2	4·2 ± 0·7	anti 10,000:1	~3	anti 10,000:1	~0·02		1110	153
51	Sar				low**	low**	2·5		142

	1	2	3	4	5	6	7	8	9							
52								NH_2	—	~3·3	<0·03	~1·1	<0·01		0·23	169
53				Ser				Ile		150 ± 12 130	320 ± 15	300 ± 15	0·06 ± 0·01	0·18 ± 0·03		165, 242, 243
54§		Phe						Lys		1 ± 0·1	~8	12 ± 3	32 ± 6	1·0 ± 0·1		244
55		Phe						Arg		2 ± 0·4	~10	~10(Rb)	125 ± 15	109 ± 13(Rt)		240
56				NH_2 NH_2 | | Asp Glu						0·4 ± 0·08	0·3 ± 0·01		0·01			164
57	desaminoCys	DTyr								30 ± 1		14	1·0	0·01		30k
58§		Phe	Phe							3·3 ± 0·3	5·4 ± 0·9	34·6 ± 9·0(Rb)	~0·9	5·7 ± 0·6(Rt)		149
59§		Phe	Tyr							<0·01	<0·01		<0·01	~0·001(Rt)		149
60§		Ser	His							<0·01	~0·03					150
61§		His	Phe							<0·01	<0·01		<0·01			150
62§		Ser						Lys		<0·01	<0·01	~0·01(Rb)	~0·1	~0·06		150
63	desaminoCys	Phe								~25	61 ± 2		~0·04	~0·35		122, 171
64		homo 2:3 Tyr						Lys								177

For footnotes see end of *Table 4.4*

Table 4.4. Analogues of vasopressin

Compound number	*Residues as in vasopressin unless marked to the contrary* 1 2 3 4 5 6 7 8 9	*Rat uterus IU/mg*	*Avian depressor IU/mg*	*Milk let-down IU/mg*	*Rat pressor IU/mg*	*Anti-diuretic IU/mg*	*Water transport (% arginine vasotocin) 225d‖*	*Natriferic units/mg 225a‖*	*References*
65	Lys	5 ± 0·5	40 ± 5	60 ± 10(Rb)	270 ± 20	~250(Rt)	0·40	5·4	191
		4·8 ± 0·3	48 ± 2	31 ± 2	243 ± 3	203 ± 7			245
		16 ± 4	57 ± 6	64 ± 8(Rb)	380 ± 38	429 ± 42(Rt)			240
66	Arg				400 (430 dry)		1·3		129
					380 (415 dry)				129, 246
67	rn	10 ± 2	21 ± 1	~50	360 ± 26				158
68	Cit	At least = vasopressin			10–12·5%	10–12·5%	0·16		174a
69§	His	~1·5	~4·6		~1·5				190
70	Form Lys	13 ± 2	41 ± 1	~75(Rb)	32 ± 2	10 ± 1(Rt)			193
71	Gly·Cys Lys				15	40–10 dependent on dose			183, 225c
72§	His·Ser·Cys Lys				11 ± 1·3				138
73§	Ser·His·Cys Lys				2·0 ± 0·3				138
74	Acetyl·Ser·Tyr·Cys Lys								182
75	Acetyl·Ser·Tyr·Ser·Cys Lys								182
76	DesaminoCys Lys	12 ± 0·5	61 ± 2	32 ± 2	126 ± 2	301 ± 11			245
77	Acetyl·Cys Lys	low	anti	~0·1	~0 anti	~0·001			198
78	Acetyl·Cys Arg				~0 anti				136

	1	2	3	4	5	6	7	8	9							
79§			Phe					Lys		~0·3 ~0·06 <0·1	~0·15 ~1·4 0·5	~2·5(Rb) 3·0 ± 0·7	55 ± 7 79 ± 12 50–60	20 ± 2(Rt) 29 ± 3	0·0080	1·9	149, 240 197
80			Phe					Arg		~0·2	<1	3 ± 0·4(Rb)	122 ± 13	~350(Rt)			240
81			Me | Tyr					Lys					2·5Rt, 76Rb				183, 188, 225c, 247
82			Et | Tyr					Lys									225c
83			homo 2:3 Tyr					Lys									187
84§			His					Lys		<0·01	<0·01	<0·01	<0·01				150
85§			Tyr					Lys		~0·01	~0·1	~0·2(Rb)	1·6 ± 0·2	0·18±0·08(Rt)			149, 194
86§			Ser					Lys		<0·01	<0·01	~0·04(Rb)	<0·01	~0·02			150
87§			Try					Lys		<0·01	~0·08	<0·01(Rb)	~0·07				150
88				Asp(NH_2)				Lys		3 ± 0·4	22 ± 1	18 ± 3(Rb)	90% 54 ± 12	600–800(Rt) 24 ± 3(Rt)			192, 225c 193
89				Ala				Lys		0·5 ± 0·05	2·2 ± 0·1	~2(Rb)	1·6 ± 0·3	30 ± 0·3			193
90				Ser				Lys		0·9 ± 0·2	10 ± 0·7	7 ± 4(Rb)	3·3 ± 0·5	69 ± 11			193
91					Ser			Lys		<0·05	<0·1	0·06±0·01(Rb)	<0·1	0·04 ± 0·004			193
92							Val	Lys					0·7 ± 0·1	0·2 ± 0·03			193
93								Lys	Sar	~0	<0·01		0·4 — 0·5				196
94§			Phe	Tyr				Lys		<0·01	<0·01		0·14 ± 0·01	0·013 ± 0·002			149
95§			Ser	His				Lys		<0·01	<0·02		<0·02				150
96§			His	Ser				Lys		<0·01		<0·01(Rb)					150
97		Gly	·Cys	Me | Tyr				Lys					anti	1·5			183, 225c
98			Phe					Leu		3·3 ± 0·3	5·4 ± 0·9	24·6 ± 9·0	~0·9	5·7 ± 0·6			149

Table 4.4. Analogues of vasopressin (*continued*)

Compound number	*Residues as in vasopressin unless marked to the contrary* 1 2 3 4 5 6 7 8 9	*Rat uterus IU/mg*	*Avian depressor IU/mg*	*Milk let-down IU/mg*	*Rat pressor IU/mg*	*Anti-diuretic IU/mg*	*Water transport (% arginine vasotocin) 225d‖*	*Natriferic units/mg 225a‖*	*References*
99	homo 2:3 Tyr Leu								200a
100	homo 2:3 Tyr3Tyr Lys								200b

• I.U. per micromole
** Antiduretic activity of this compound is approximately ten times greater than its rat pressor activity.
† du Vigneaud and colleagues have also synthesized a tritiated oxytocin (trityl-leucine oxytocin; 130 microCuries/mg) with an oxytocic activity = 500 I.U. per milligram[232]
‡ Higher homologue—indicated by the term 'homo' preceding the new amino-acid residue(s). The numerals refer to the amino-acid residues between which the new one(s) has been inserted
G Guinea-pig
Rt Rat
Rb Rabbit
§ Compounds thus marked were tabulated by Boissonnas and colleagues[228]
‖ See references 225a and 225d respectively

bridge plays an essential role in the action of vasopressin on kidney tissues[249a].*

Recent research by Jaquenoud and Boissonnas[153] has carried a stage further the work of Ressler who found that the disulphide ring of oxytocin (compound 52) possessed biological activity of a very low order[169]. When any one of the three side chain residues is omitted a great diminution in the defined activities results and the compounds acquire inhibitory properties† in the avian depressor (compounds 41, 42 and 50) and rat pressor (compounds 42 and 50) assays.

One might conclude from the above that for an analogue of oxytocin to possess appreciable activity in the five tests under consideration, a primary backbone structure resembling that of the parent molecule is probably necessary.

Functional groups—Three functions are present in oxytocin which would normally be regarded as chemically reactive. These are the terminal amino, the tyrosine phenolic and the disulphide groups. The significance of the disulphide group has already been discussed. In the vasopressins a fourth reactive site must be considered—the basic side chain of residue 8. The significance of all these side chains has been investigated with appropriate analogues.

The terminal amino group. The most striking biological activities listed in the tables are probably those associated with desamino-oxytocin‡ (compound 11)[171,173,231]. This substance is appreciably more active than oxytocin in the rat uterus, avian depressor and antidiuretic tests. It has less pressor activity than oxytocin and approximately the same milk let-down effect. Desamino-oxytocin was the first compound to show a decrease in pressor combined with an increase in antidiuretic properties. Desamino-8-lysine vasopressin (compound 76)[245] is more active than 8-lysine vasopressin (compound 65) in the antidiuretic and less active in the pressor tests. It has approximately the same activity as the parent hormone in the milk let-down and is more active in the uterus and avian depressor assays. The synthesis and behaviour of desamino-8-arginine vasopressin have yet to be reported. Only two other oxytocin-vasopressin analogues, 8-isoleucine oxytocin (compound 43)[162, 239] and 4-asparagine 8-lysine vasopressin (compound 88)[192,225c] have been reported to exhibit higher activities than the parent hormones. In another laboratory[193] 4-asparagine 8-lysine vasopressin was found to be very much less active. Comparisons revealed that the discrepancies were due to differences in assay technique and between the reference substances used.

It seems evident from the above results that the terminal amino functions of oxytocin and vasopressin are not essential for manifestation of the biological activities under discussion. The *in vivo* responses of the desamino compounds are of the same duration as those of oxytocin and shorter than those elicited

* It has recently been shown that the disulphide bridge is not essential to the pressor activity of vasopressin[249b].

† Inhibition is observed thus: constant responses are obtained to a given dose of the reference standard and, subsequent to a dose of inhibitor, the responses to the given dose of the reference standard are reduced.

‡ As originally suggested by du Vigneaud, the prefix 'des' will be used throughout in place of 'de' which can be confused in certain contexts with 'D'.

by the USP standard[231]. Otherwise it might be thought that the increased responses were related to resistance to enzymic attack. Leucine aminopeptidase will deactivate oxytocin very slowly[250] whereas desamino-oxytocin is not deactivated[251]. Pregnancy serum destroys oxytocin rapidly but is completely without effect on desamino-oxytocin[252]. On the other hand, desamino-oxytocin is more rapidly attacked than oxytocin by chymotrypsin[251].

The results with desamino-oxytocin and desamino-vasopressin underline the limitations of using blocking groups to determine functions essential for biological activity. Thus 1-acetyloxytocin (compound 12)[228,233], 1-acetyl 8-lysine vasopressin (compound 77)[198] and 1-acetyl 8-arginine vasopressin (compound 78)[136] have been synthesized and found to possess biological activities of a very low order. This confirms earlier work in which vasopressin was treated with acetic anhydride[253].

When the terminal amino group is acylated by an amino acid or peptide, rather low activities are produced but the compounds sometimes have a prolonged action. 1-Glycyl oxytocin (compound 5)[137,170], 1-leucyl oxytocin (compound 6)[137] and 1-leucyl-glycyl-glycyl oxytocin (compound 7)[137] give protracted effects resulting in tachyphylaxis and inhibition of response to the reference standard in the milk let-down assay. It is possible that these compounds are resistant to attack by oxytocinase and that a slow degradation to oxytocin itself is responsible for the observed activity. Large doses of oxytocin cause tachyphylaxis and subsequent inhibition of response to further oxytocin[137,225e]. Protracted activity has also been observed with 1-aminoacyl-vasopressin analogues (compounds 71, 97)[183,225c].

The tyrosine phenolic group. 2-Phenylalanine oxytocin (compound 13)[147,148,155,234], 2-phenylalanine 8-lysine vasopressin (compound 79)[149,197,240] and 2-phenylalanine 8-arginine vasopressin (compound 80)[240], molecules in which the tyrosine phenolic group is absent, all retain a considerable amount of activity, qualitatively similar to that of the parent molecules. This suggests that the phenolic group augments the activity in some way but is not essential for activity. Studies by Rudinger and Krejčí[254] with rat uterus have shown that, in terms of the hormone–receptor hypothesis[255], 2-phenylalanine oxytocin has a high affinity for the receptor and a low intrinsic activity. 2-Leucine oxytocin (compound 17) has the same intrinsic activity as 2-phenylalanine oxytocin but a lower affinity[155,254]. Hence, the side chain of the residue in position 2 is binding as well as functional.

2-*O*-Methyltyrosine oxytocin, the methyl ether of oxytocin, (compound 14) is inhibitory in the rat uterus[155] and pressor assays[151]. In this case the analogue has a high affinity for the receptor and low or no intrinsic activity[254]. The importance of the ionic composition of the organ bath[210] shows up very clearly with this compound. When the concentration of calcium ions is high, 2-*O*-methyltyrosine oxytocin can induce contractions of rat uterus. The dose–response curve approaches that of oxytocin more and more as the calcium ion content is increased. A somewhat similar effect is related to the concentration of magnesium ions [225f,256,257].

Side chain in position 8. The basicity of the side chain in position 8 clearly influences the biological properties of the vasopressins. Arginine vasopressin (compound 66) is more active in all tests, especially pressor and antidiuretic assays, than lysine-vasopressin (compound 65). When a residue with a basic

side chain is present in this position and isoleucine is in position 3, the compounds are intermediate, both in structure and biological activity, between oxytocin and the vasopressins and are referred to as vasotocins. Arginine[146,240,241], lysine[158,172,191,240] and ornithine[158] vasotocin (compounds 46–48) are clearly intermediate in this sense, citrulline vasotocin (compound 49)[174] is more like oxytocin. Similarly, 8-ornithine vasopressin (compound 67)[158] is very similar to arginine vasopressin, whereas 8-citrulline vasopressin (compound 68)[174] has a much lower activity. When a neutral residue is in the 8 position in the vasopressin series, as in 8-leucine vasopressin, called oxypressin, (compound 29)[144,167,237] and 8-N^{ϵ}-formyl-lysine vasopressin (compound 70)[193], oxytocin-like activities are only slightly affected whereas vasopressin-like properties are considerably reduced. Somewhat surprisingly, in view of the tendencies apparent in the above series of compounds, 8-histidine vasopressin (compound 69)[190] has a very low degree of activity on rat uterus, avian depressor and rat pressor tests.

It may be concluded from these studies that the basicity and spacing of the amino function in the side chain of residue 8 is particularly important for the manifestation of vasopressin-like properties[225g]. When the basicity is reduced, pressor activity[190] and antidiuretic activity[258] are considerably reduced.

The terminal amino group has a similar relationship to the pressor properties of the molecule. In both desamino oxytocin (compound 11)[171,173,231] and desamino lysine vasopressin (compound 76)[245] the pressor activity is less than that of the parent compound. Antidiuretic activity, on the other hand, is greater. Carbamoylation of the terminal amino group of oxytocin causes a marked diminution in biological activity[259]. Carbamoyl-oxytocin inhibits the response of the rat uterus to oxytocin *in vitro*, but not *in vivo*. The pressor response (*in vivo*) is inhibited.

It is interesting to note that arginine-vasotocin seems to be the natural hormone in all cold-blooded vertebrates except the elasmobranches[260a] and there is a suggestion that it might occur in bovine pineal stalks[225h]. Its occurrence was first demonstrated in 1959[261,262], so that this is an example of a hormone which was synthesized (1958)[146], before its presence in nature was suspected.

Potential functional groups—There are three primary amide groups in the oxytocin-vasopressin structure and, although amides would not normally be regarded, chemically, as very reactive, there is always the possibility that biochemically, they might represent incipient carboxyl functions.

Terminal carboxamide group. Synthetic evidence for the significance of the terminal carboxamide group is not available. Natural oxytocin on mild acid hydrolysis gave one equivalent of ammonia and a biologically inert product in which the presence of *C*-terminal glycine could be demonstrated by the Schlack and Kumpf procedure[106]. This evidence seems to indicate that the presence of the terminal amide function is essential for activity. It should be borne in mind, however, that the presence of a carboxyl group in this position might prohibit biological activity and that the only real measure of function would be the biological activity of 9-descarboxamido oxytocin.

Glutamine side chain in position 4. In the oxytocin series, the problem of the significance of the γ-carboxamido group in this position has been resolved

conclusively by a study of 4-descarboxamido oxytocin (compound 33)[175]. This compound retains appreciable oxytocic properties indicating that the γ-carboxamido group is not essential for oxytocic acitivity. Other analogues, for example 4-serine oxytocin (compound 30)[166], 4-alanine oxytocin (compound 31)[166] and 4-asparagine oxytocin (compound 32)[166,164] also possess a relatively high order of oxytocic activity. Changes in the 4 position of lysine-vasopressin seem to reduce the vasopressin-like properties more severely. This is seen with 4-alanine lysine vasopressin (compound 89)[193] and 4-serine lysine vasopressin (compound 90)[193]. However, all of these results substantiate the claim that the γ-carboxamido group is not essential for biological activity. In contrast, synthetic 4-glutamic acid oxytocin (compound 34)[30k] is rather inactive and this must be attributed to the presence of the γ-carboxyl group.

In view of the apparent variability of the residue in position 4 in the oxytocin series, it is interesting to note that 4-serine 8-isoleucine oxytocin, ichthyotocin, (compound 53)[165,242,243] has been isolated from the neurohypophysis of teleosts[225i,263,264].

Asparagine side chain in position 5. The definitive test of the significance of the β-carboxamido group in this position has been made by a study of the biological properties of 5-descarboxamido oxytocin (compound 37)[175]. This substance is almost inactive which suggests that the β-carboxamido group plays an essential role in the manifestation of the biological activity of oxytocin. Other compounds modified in this position, for example, 5-glutamine oxytocin (compound 36)[164], 5-serine oxytocin (compound 38)[166] and 5-alanine oxytocin (compound 39)[166] are also inactive and the inactivity of 4-asparagine, 5-glutamine oxytocin (compound 56)[164] presumably is also due to the change in position 5. These results throw some doubt on the value of the inactivity of 5-isoasparagine oxytocin as evidence for the biological importance of the size of the disulphide ring. In the vasopressin series, 5-serine lysine vasopressin (compound 91)[193] is similarly almost inactive.

Position 3. Oxytocin and vasopressin are distinguished by the nature of the residues in positions 3 and 8. By the insertion of suitable residues at these positions, it has been seen already that compounds of an intermediate type of biological activity may be produced. Other variations in the 8-position with retention of biological activity have been discussed and it is not surprising that changes may similarly be made in the 3-position to produce biologically-active compounds. 3-Alloisoleucine oxytocin (compound 22)[154], 3-valine oxytocin (compound 23)[144,154,159,162], 3-leucine oxytocin (compound 24)[144,154,159,162], 3-norleucine oxytocin (compound 25)[154] and 3-norvaline oxytocin (compound 26)[154] are examples. These compounds still retain a considerable degree of biological activity, but are appreciably less active than the parent hormones. It is calculated that 3-alloisoleucine oxytocin, 3-valine oxytocin and 3-leucine oxytocin have about the same intrinsic activity as oxytocin but a decreased affinity for the receptor in the rat uterus test[154]. The rat uterus, avian depressor and antidiuretic activities of 3-alloisoleucine oxytocin might be accounted for by the presence of oxytocin arising from the known isoleucine contamination of the original alloisoleucine. The high milk let-down activity cannot be dismissed in this way however.

Water transport effect

Activity in the toad bladder assay is relatively specific to analogues of oxytocin and vasopressin; other peptides are inactive. The results in *Tables 4.3* and *4.4* are expressed as percentage activities of arginine vasotocin, the natural hormone in amphibians and, although in many cases very low percentages are recorded, these still represent considerable activity. All reduced analogues tested were found to be inactive in this assay, otherwise all of the analogues tested did possess some activity[225d].

The mode of action of vasopressin in this system has been studied using tritiated arginine vasopressin[265]. After intravenous injection into rats, tritiated arginine vasopressin becomes associated with a kidney protein. The radioactivity is lost if the protein is treated with thiols[265]. In the toad bladder assay similar results are obtained: labelled hormone becomes attached to the bladder tissue and may be detached by the action of thiols, particularly L-cysteine. The disappearance of physiological activity is paralleled by the disappearance of *S*-bound radioactivity[222,223,225j]. Two ionizable groups, $pK' = 7{\cdot}1$ and $pK' = 7{\cdot}8$, are important in determining the hormonal response. That with $pK' = 7{\cdot}8$ is associated with the receptor and could be a thiol function. This work indicates that thiol-disulphide interchange is probably the reaction which initiates the sequence of events leading to increase in permeability.

Natriferic effect

The natriferic activities of several oxytocin-vasopressin analogues, as measured by Morel[225a], are recorded in *Tables 4.3* and *4.4*. Compounds of high activity result from the replacement of the 8-leucine residue with any one of several amino-acid residues. Otherwise, changes in natriferic activity tend to parallel changes in rat uterus and avian depressor properties except in position 4 where substitutions for the glutaminyl residue caused decreases in natriferic activity more marked than the attenuation of oxytocic properties. Once more, arginine-vasotocin is the most active compound; no competitive inhibition by the less active analogues was reported. The three analogues, 2-phenylalanine oxytocin, 2-*O*-methyl tyrosine oxytocin and 2-leucine oxytocin differ from oxytocin, in this test, in their affinity for the substrate; they exhibit the same intrinsic activities as the parent hormone[225k].

Posterior Pituitary Hormones in Man

Oxytocin, vasopressin and several analogues of these parent structures have actual and potential clinical applications. In general, these are related to physiological, or probable physiological, functions of the hormones and it therefore seems logical to outline briefly what is known of the origins, functions and fate of these hormones in man, before considering clinical aspects. (For a more detailed account see the review by Sawyer[229].)

The concentration and distribution of oxytocin in the pituitary gland, hypophysial stalk and hypothalamus have been measured in man[266] and in other species[267]. It is now generally agreed that these hormones are synthesized in the hypothalamic region and pass down the hypophysial stalk to the pituitary. Within the hypothalamo–hypophysial region, the hormones

form a complex (the van Dyke protein) with a protein, neurophysin, in a rather loose association which probably involves the terminal amino groups of the hormones[268].

Physiological function is attributed to the antidiuretic effect of the vasopressins; the pressor effect is only noticeable at much higher concentrations of the hormone and is not likely to play a physiological role. Oxytocin is implicated physiologically in lactation; proof of its physiological role in the induction of labour is more tenuous, but high serum oxytocin concentrations may be demonstrated during the second stage.

The release of vasopressin from the posterior pituitary is controlled by an osmo-regulatory mechanism. Oxytocin can also be released by the injection of hypertonic saline into the carotid artery but in this case non-physiological osmotic pressures are required. Evidence indicates that the release of oxytocin is entirely under nervous control[260b]. Stimulation of the nipples in lactating woman and even preparation for breast feeding brings about oxytocin release. Labour has been induced by stimulation of the nipples.

Oxytocin and vasopressin in the general circulation are almost certainly in association with some kind of transport protein. This might form part of a mechanism to take the hormones to the target organs without prior destruction or dissipation[225p]. The hormones in non-pregnant women are primarily deactivated in the kidneys and liver[260c].

In pregnant women and apes, but not in any other genera investigated, the serum contains an enzyme, serum oxytocinase, a glycoprotein which is probably produced by the placenta and which rapidly destroys both oxytocin and vasopressin[225l,260d]. The concentration of oxytocinase in the serum increases rapidly as the pregnancy proceeds and attempts have been made to use oxytocinase concentration as a diagnostic aid for pregnancy[269,270]. Injections of oxytocin cause transient rises in the oxytocinase concentration of serum and measurements of this effect can be used to calculate the date of parturition or the amount of enzymic block which must be overcome to induce labour with exogenous oxytocin[225m].

Tuppy and Nesvadba[271] have shown that oxytocin is cleaved by this enzyme at the cysteinyl-tyrosine peptide bond. Cysteinyl-di-β-naphthylamide is cleaved by the enzyme to give rise to chromogenic β-naphthylamide and techniques based on this activity have proved very convenient research tools[260d]. Beránková, Rychlík and Šorm have shown that inhibition of serum oxytocinase is fairly specific whereas the generally-occurring tissue oxytocinase was inhibited by all cysteine compounds tested[272]. The most potent inhibitors of serum oxytocinase were desthio-oxytocin and *S,S'*-dibenzyl oxytocin[273].

The inactivation of oxytocin and vasopressin by the tissue enzyme is thought to proceed in two steps, the first involving fission of the disulphide bond and the second, cleavage of the cysteinyl-tyrosine bond[225n,225o].

Studies of the biological inactivation of vasopressin and oxytocin have led to the synthesis of analogues which one might expect to be resistant to enzymic degradation. 2-*N*-Methyltyrosine oxytocin (compound 15)[163] is an example. In this molecule the cysteinyl-tyrosine peptide bond is modified and therefore might not be susceptible to attack by serum or tissue oxytocinase. The compound is relatively inactive and is even slightly inhibitory towards

oxytocin in several tests. 9-Sarcosine 8-lysine vasopressin (compound 93)[196] might be expected to be resistant to trypsin attack, but this analogue too is virtually inactive.

1-D-Cysteine oxytocin (compound 2)[137], 1-*N*-methylcysteine oxytocin (compound 3)[137] and 1-sarcosyl-oxytocin (compound 4)[137] might be expected to be resistant to amino peptidases like tissue oxytocinase; these analogues too have very low activity.

A third group of compounds, 1-glycyl oxytocin (compound 5)[137,170], 1-leucyl oxytocin (compound 6)[137], 1-leucylglycylglycyl oxytocin (compound 7)[137] and 1-glycyl 8-lysine vasopressin (compound 71)[183,225c] have diminished activities but their effects are considerably protracted. This may be related to the enzymic release of the parent hormones rather than to resistance to enzymic attack.

Clinical Applications

Oxytocin preparations are widely used with or without amniotomy to induce and stimulate labour[274]. The classical approach has involved intravenous infusion, preferably at a rate of 1–2 mU per minute, which is well within the physiological range[260e], but other approaches have been investigated[275]. Recently sub-lingual application has been shown to be extremely effective[276,277]. It is interesting to note that in non-pregnant women infusion of oxytocin at rates of up to 128 mU per minute has no significant effects on the uterus.

For the stimulation of milk let-down[278], oxytocin may be given sublingually or in the form of an intranasal spray. Intravenous and intramuscular application have also been used.

Vasopressin finds its main medicinal use in the control of diabetes insipidus[279,280], but highly purified arginine-vasopressin has also been used to great advantage to produce uterine blanching by direct injection into the uterus during gynaecological surgery[281].

Synthetic analogues of posterior pituitary hormones are likely to find clinical application only if they possess a more selective response than the parent compounds, effects modified in some qualitative way, or, more speculatively, new and unique properties not possessed by the parent compounds. Because of the extremely high activity per unit weight of the parent structures, it is unlikely that substantial increases in activity alone will be sufficient to commend an analogue for clinical use.

2-Phenylalanine-8-lysine vasopressin (compound 79), due to its selective pressor effect, is used clinically in the treatment of shock, for producing local ischaemia during surgery and as an additive for local analgesics[282,283].

The activities of certain other compounds in *Tables 4.3* and *4.4* suggest possible clinical applications: 4-asparagine oxytocin (compound 32)[164,166] is clearly a more selective oxytocic agent than oxytocin itself. Whereas the analogue's oxytocic activity is one-fifth to three-fifths, its vasopressin activity is only one-hundredth to one-fiftieth that of oxytocin. 3-Alloisoleucine oxytocin (compound 22)[154] is very selective for milk let-down. The ratio of milk let-down to uterus activity is 5:1 for this compound as opposed to 1:1 for oxytocin. In the vasopressin series, desamino 8-lysine vasopressin (compound 76)[245] has a more favourable antidiuretic to pressor ratio (2·4) than

arginine-vasopressin (0·8). In general, the pressor effect of vasopressin does not complicate control of diuresis, but in specific instances, this greater selectivity might be advantageous. In the control of diabetes insipidus the significantly prolonged antidiuretic response of 1-glycyl-8-lysine vasopressin (compound 71)[183,225c] might make this compound useful. The unknown 1-glycyl 8-arginine vasopressin might also have a prolonged effect coupled with a higher antidiuretic response than the lysine compound.

CORTICOTROPIN AND THE MELANOCYTE-STIMULATING HORMONES

Introduction

A functional relationship between the pituitary gland and the adrenal cortex was discovered in 1926 by Smith[284,285] during his classical studies on anterior pituitary function. In hypophysectomized animals the adrenal cortex atrophies, but this may be avoided by regular injections of anterior pituitary extracts[286,287,288]. The substance which brings about this adrenal response is called the adrenocorticotropic hormone (ACTH) or corticotropin.

A decade before the observations relating to corticotropin, it was discovered that hypophysectomy of tadpoles leads to a reduction in the degree of pigmentation of the skin[289,290]. This effect too can be countered by the use of pituitary extracts, but in this instance the intermediate lobe of the gland has the greatest activity, although the anterior lobe is not completely inactive. The compounds responsible for these effects are termed the melanocyte-stimulating hormones (MSH's), melanotropins, or intermedins. They occur in primates and in many other species as well as in the amphibia.

It was not until 1942 that a protein preparation possessing specific corticotropic properties was isolated (from sheep and pig pituitary extracts)[291,292]. Li found that active polypeptides could be cleaved enzymically from the corticotropin protein by the action of pepsin[293] and subsequently, Astwood and colleagues separated the active components from the protein by adsorption on oxycellulose without prior peptic digestion[294]. By the use of ion exchange chromatography[295–299], zone electrophoresis[296,297] and counter-current distribution[295–297,299–302], pure corticotropins have now been prepared from oxycellulose concentrates.

Oxycellulose has also proved useful for the adsorption of melanotropins from acetic acid extracts of acetone-dried pituitary powder and, once again, final purification has been largely dependent on zone electrophoresis[303–305], counter-current distribution[304–310] and ion exchange chromatography[311,312].

Structural Elucidation

A first step of general value in structural studies on the corticotropins and melanocyte-stimulating hormones has been enzymic hydrolysis: chymotrypsin[300,302,306,309,310,312–320], trypsin[300,306,309,310,312–321] and, less commonly, pepsin[300,302,313,314,321] have been used to cleave the parent compounds into smaller peptides. These and the parent molecules have been studied by a variety of techniques including the Edman reaction[300,302,310,312–316,319],

the Edman reaction applied to paper strips[309,310,317,319,320,322], carboxypeptidase degradation [300,302,309,310,312,313,315–317,320,323], FDNB-labelling[302,309,310,319,320] and, in one case, to identify an acetyl group, hydrazinolysis[317].

Complete sequences have been deduced for corticotropins isolated from sheep, pig, beef and human pituitary glands (*XXIX*). Each of these molecules consists of a single chain of 39 amino-acid residues with free terminal amino and carboxyl groups. Slight differences in the sequences and types of amino-acid residue occur in the region of residues 25 through 32. The significance of other, apparently homogenous, fractions which possess corticotropic activity[301] has not been completely determined[324].

Two types of melanocyte-stimulating hormone, designated α and β, have been shown to occur in pig, beef, horse, monkey and human pituitary glands. α-MSH (*XXX*) is identical in each of the first four species, a 13 residue straight-chain peptide possessing an acetylated terminal amino group and a *C*-terminal primary amide. The human hormone has not been isolated in sufficient quantities to permit structural elucidation[312]. β-MSH (*XXXI*) from pig, beef, horse and monkey pituitaries is an 18 residue straight-chain peptide possessing free terminal amino and carboxyl groups. Limited species variations are found in the types of residues in the chain. In the hormone from human sources the peptide chain consists of 22 residues.

A comprehensive review by Li[325,326] covers details of the isolation of most of these compounds and early structural studies.

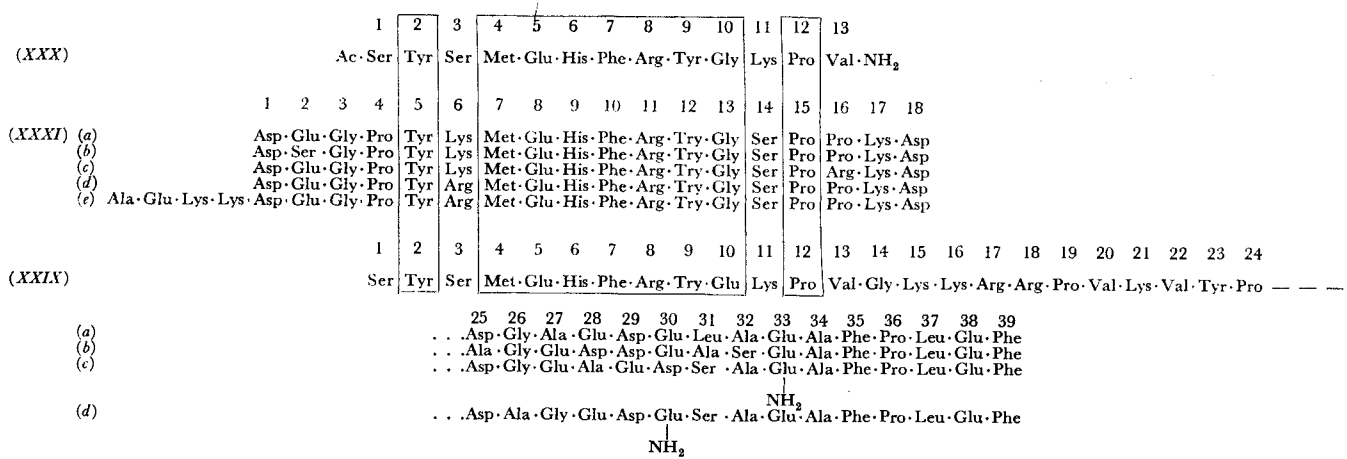
Synthesis

Most of the reported synthetic work related to corticotropin and the melanocyte-stimulating hormones has been carried out by three research groups. The individual contributions of these groups were reviewed to mid-1961 by Schwyzer[332], Hofmann and Yajima[333], and Li[334].

It was known from the work[313] on natural pig corticotropin that the molecule could be considerably degraded from the *C*-terminal residue without loss of corticotropic activity. Enzymic followed by mild acidic hydrolysis indicated that 15 residues could be removed in this way. On the other hand, it was shown by White[335] that the first two residues at the amino end of the corticotropin molecule were essential for corticotropic activity.

In the first place therefore, synthetic work was directed to the synthesis of fragments of corticotropin related to the 1–24 sequence. This part of the molecule overlaps α-MSH which is the *N*-acetyl 1–13 residue amide. Considerable interest was attached to determining the minimum chain length which would show corticotropic activity: α-MSH shows none.

One stumbling block to the synthesis of compounds of this series has been their non-specific degradation under conditions used for the removal of classical side-chain protecting groups. Guttmann and Boissonnas[336] observed this when they synthesized α-MSH, using sodium in liquid ammonia or catalytic hydrogenolysis for final cleavage. Similar difficulties in the use of sodium in liquid ammonia were experienced[337] in an attempted synthesis of β-MSH and in an attempt to synthesize 1–13 corticotropin amide[338] (*see*,



(*XXX*) (*a*) Pig[307,317,327], (*b*) beef[327,328], (*c*) horse[306] and (*d*) monkey[312] α-MSH

(*XXXI*) (*a*) Pig[318,319], (*b*) beef[305,329], (*c*) horse[309,310], (*d*) monkey[312] and (*e*) human[320] β-MSH

(*XXIX*) (*a*) Pig[300,302,313], (*b*) sheep [296,314,330,331], (*c*) beef[315,316] and (*d*) human corticotropin[298,321]

however, the synthesis of 1–19 corticotropin[339]). On the other hand, corticotropin is relatively stable in acid solution and this has been exploited synthetically.

Acetylation has been used to protect the terminal serine amino group and formylation to protect the N^{ε}-amino group of lysine derivatives[333,340–343]. The protecting groups can be removed, without prohibitive hydrolysis of peptide bonds, by the action of 0·5N hydrochloric acid at boiling water bath temperature[338]. The tertiary butyloxycarbonyl group has been used to protect the terminal serine amino group and N^{ε}-lysine amino group[73,332,344–350]. Reaction with anhydrous trifluoroacetic acid, to cleave tertiary butyloxycarbonyl groups and tertiary butyl esters, has proved an ideal final stage in these syntheses (this work is reviewed in some detail in reference 348). In their later work, Hofmann and colleagues[351] have used tertiary butyloxycarbonyl protection, together with carboxyl groups in the form of amides. It is not clear at present whether trifluoroacetic acid converts glutaminyl residues in these molecules into glutamyl residues or not.

All of the large fragments related to the 1–24 sequence of corticotropin which have been synthesized to date have been formed by the combination of smaller peptide sub-units. When peptide derivatives are activated for coupling to other peptides, there is considerable danger that racemization of the *C*-terminal amino-acid residue will occur. For this reason the larger sub-units have generally been chosen to end in *C*-terminal glycine or proline residues. These occur in positions 10, 12, 14, 19 and 24 and derivatives of many fragments which terminate at these positions have been reported (*see Table 4.5*).

The principal methods of coupling used in syntheses of fragments related to the 1–24 sequence have involved azides, *N,N'*-dicyclohexylcarbodi-imide, mixed anhydrides, *p*-nitrophenyl esters and, more rarely, Woodward's reagent K[355,356]. Some examples of side reactions[343,345,350,357], particularly with the use of *N,N'*-dicyclohexylcarbodi-imide, have been reported, and, with couplings involving *C*-terminal residues other than glycine and proline, various degrees of racemization[344,345,347].

Recently, the total synthesis of pig corticotropin was announced by Schwyzer and Sieber[73]. In this work, carboxyl groups were protected as tertiary butyl esters and amino groups as tertiary butyloxycarbonyl derivatives. The entire 25–39 sequence was built up from phenylalanine tertiary butyl ester in a stepwise manner by the addition of benzyloxycarbonyl amino acid *p*-nitrophenyl esters. This fragment was coupled to a 17–24 derivative by a mixed anhydride procedure using pivaloyl chloride. Further fragments were subsequently added by an azide procedure (11–16) and by the use of *N,N'*-dicyclohexylcarbodi-imide (1–10). Counter-current distribution was used to purify protected intermediates. The final 1–39 residue peptide was obtained homogeneous without further purification by reaction of the protected peptide with trifluoroacetic acid.

The protecting groups used by the Swiss workers in their recent work are clearly satisfactory for ease of removal. Further evidence of this is provided by the recent syntheses by these workers of α-MSH[349] and β-MSH[350] in which the final removal of protecting groups with trifluoroacetic acid proceded quantitively and in 84–90 per cent yields respectively. Tertiary butyl

Table 4.5. Intermediates used in the synthesis of peptides related to α-MSH and corticotropin

Sequences	*References*	*Sequences*	*References*	*Sequences*	*References*	*Sequences*	*References*	*Sequences*	*References*
1–2	352, 358, 361, 366, 368, 376	3–10	363, 373, 377	7–10	52, 344, 355, 369, 373, 382, 384, 389, 391, 393	12–14	337, 339, 385	18–20	342
		4–5	358, 360, 361, 363, 366, 376			12–15	374, 388	18–21	353
1–3	30f, 337, 360, 361, 366, 373, 386, 391	4–6	373, 382	8–9	352, 360, 367	12–16	353	18–22	374, 388
		4–8	377	8–10	52, 339, 355, 369, 375, 380, 381, 393	12–17	374, 388	18–23	390
1–4	337, 339, 344, 349, 352, 355, 361, 369, 383, 386, 391	4–10	337, 373, 382, 391	9–10	52, 355, 365, 369, 370, 373, 375, 381, 384, 389, 393	12–22	28d, 374, 388	19–20	342, 353, 360, 372, 387
		5–6	344, 373, 380, 382, 384, 391			13–14	337, 378, 385		
1–5	336, 338, 343, 351, 358, 360, 361, 366, 368, 371, 376, 379	5–7	352, 362, 380	10–11	353, 367	14–15	353, 360, 387	19–21	353, 372
		5–8	362, 384	10–13	360, 367, 383, 387	14–16	353, 360, 387	19–23	390
1–7	352	5–10	339, 344, 349, 355, 369, 370, 373, 380, 384, 391, 393	10–16	343, 353	14–20	360, 387	19–24	372
1–8	377			10–20	360, 387	15–16	337, 344, 345, 346, 348, 355, 378, 390, 394	20–21	344, 347, 357, 374, 388, 394
1–9	28a, 352			10–21	28b, 353	15–17	355	20–22	374, 388
1–10	73, 337, 338, 340, 341, 342, 346, 348, 349, 351, 355, 369, 373, 391	5–19	339	11–13	349, 368, 377, 378	15–19	337, 339, 344, 345, 348, (356), 392	20–24	344, 347, 348, 357, 394
		6–7	339, 352, 360, 362, 367, 375, 381	11–14	337, 339, 345, 346, 348, 355, 378, 385, 390, 394	16–17	359, 374, 388	21–24	347
		6–8	362, 365, 370, 375, 384, 389			16–19	339	22–23	347, 354, 357, 372
1–13	338, 349, 368, 371, 379	6–9	360, 367	11–16	73, 340, 342, 346, 390, 394	16–22	374, 388	22–24	344, 347, 357, 372 394
1–16	343, 346	6–10	52, 338, 339, 343, 351, 355, 363, 365, 368, 369, 373, 375, 377, 381, 384, 389, 393	11–17	355	17–18	359, 360, 387	22–28	28c, 354
1–17	355			11–19	337, 339, 344, 345, 348	17–19	337, 339, 344, 345	23–24	394
1–19	337, 339			11–20	342, 351		348, (356), 394	24–25	354
1–20	342, 351, 360			11–23	340, 341, 390	17–20	342, 360, 387	24–28	354
1–23	340, 341			11–24	344, 348, 394	17–21	353	26–28	354
1–39	73	6–13	336, 338, 367, 368, 371, 377, 378, 379	11–39	73	17–23	340, 390	26–30	28e
2–3	30f, 360, 366, 386			12–13	339, 349, 353, 367 374, 377, 388	17–24	73, 394	27–28	354
3–4	339, 352, 361	6–16	343			17–39	73	33–39	364
3–5	358, 361, 363, 368, 376	6–20	360			18–19	54, 337, 339, 344, 345, 348, (356), 374, 388		
		7–8	362, 373, 384, 389						

For fragments 11–16, 17–24, 25–39, 26–39, 27–39 . . . 38–39[73]

The following fragments related to bovine β-MSH have been utilized: 1–3, 1–7, 1–18, 2–3, 4–5, 4–6, 4–7, 8–13, 8–18, 10–13, 14–18, 15–18, 16–18, 17–18[350,395]

and tertiary butyloxycarbonyl will probably be the protecting groups of choice in future syntheses of corticotropin and other large peptides.

Assay Procedures

The melanocyte-stimulating activity of a preparation is usually measured in terms of the degree of skin darkening it will produce in amphibia. Both *in vitro* and *in vivo* methods are used (for a review *see* Landgrebe and Waring[202b]).

Shizume, Lerner and Fitzpatrick[396] described a technique in which the skin of *Rana pipiens* is immersed in a solution of the preparation under test whilst changes in the pigmentation of the skin are measured by a photoelectric reflection device. These workers use a standard prepared from a lyophilized aqueous extract of posterior pituitary powder, and define it as containing one unit of melanocyte-stimulating activity per 0·04 μg. The scale of activity (SLF scale) based on this standard is widely used. Alternatively, Landgrebe and Waring[397] used the international posterior lobe powder itself as the standard and have defined one unit as the amount of activity possessed by 0·5 mg. Li[326] determined that one of these units is equivalent to 10,000 units on the SLF scale.

Since corticotropin possesses melanocyte-stimulating activity, the SLF procedure is frequently used routinely to assay corticotropin. Specific corticotropin assays are usually based on (1) depletion of adrenal ascorbic acid in hypophysectomized rats[398,399]; (2) steroidogenesis, either *in vitro*[400,401] or *in vivo*[402,403], as measured by a variety of methods; and (3) the increase in weight of the adrenal glands which results from chronic administration. They are related to the activity of an international standard preparation of oxycellulose-purified porcine pituitary. Corticotropin assay has been reviewed by Fisher[202c].

Structure–Activity Correlations

The variety and degree of biological activity exhibited by peptides related to corticotropin and the melanocyte-stimulating hormones have been reviewed by Hofmann[404,405] and a general presentation only will be attempted here. In the main, the information summarized in *Table 4.6* has been published since the last review[405], although some of this information, unpublished or gleaned from preliminary communications, was reported at that time.

Melanocyte-stimulating activity

The existence of a common sequence of amino-acid residues, 4–10, in α-MSH, β-MSH and the corticotropins, all of which possess melanocyte-stimulating activity, prompted suggestions that the activity was in some way associated with this part of the molecule. Synthetic studies seem to confirm this speculation. The heptapeptide 4–10 possesses considerable melanocyte-stimulating activity[409] whereas the *N*-terminal octapeptide 1–8[377], and the N^{ε}-formyl-lysine derivative of the *C*-terminal heptapeptide amide, 7–13[410], are inactive. The inactivity of the heptapeptide is not due to the presence of the ε-amino protecting group, as is evinced by the existence of many active

Table 4.6. Biological properties of compounds related to corticotropin

Compound number	*Structure (residues as in pig β-corticotropin unless marked to the contrary)*	*Corticotropic activity*		*Lipolytic activity*	*Melanocyte-stimulating activity units/gm*	*References*
		Adrenal ascorbic acid depletion units/mg	*Steroidogenesis units/mg*			
1	Natural β-corticotropin	80–100	94·5 ± 10·6		$1{\cdot}7 \times 10^8$	301, 328, 406
2	Natural α-MSH				$1–1{\cdot}5 \times 10^{10}$	328
3	Natural β-MSH (pig)				$3{\cdot}8–5 \times 10^9$	328, 407
4	Natural β-MSH (beef)				$1{\cdot}2–2 \times 10^9$	328, 407
5	Natural β-MSH (monkey)				2×10^9	312
6	Natural β-MSH (horse)				$\sim 2 \times 10^9$	309
7	Natural β-MSH (human)				$3{\cdot}3 \times 10^9$	407
8	Acetyl 1–13 amide (≡ α-MSH)				$1{\cdot}2–4 \times 10^{10}$	349
9	Benzoyl 1–13 amide				$4–8 \times 10^9$	349
10	Synthetic β-MSH (beef)				$1{\cdot}2–4 \times 10^9$	350
11	1–16	<0·1	<0·1		$3{\cdot}7 \times 10^8$	343
12	1–16	1	5–10			346
13	1–17		6	≡ACTH	≡ACTH	355
14	1–19	s.c. 74 $\left(\frac{\text{s.c.}}{\text{i.v.}} = 2\right)$ i.v. 35	40		$1{\cdot}4 \times 10^7$	339
15	1–20 amide	110 ± 18·0 $\left(\frac{\text{s.c.}}{\text{i.v.}} = 0{\cdot}3\right)$	83 ± 7		$1{\cdot}1 \times 10^8$	342
16	4-α-aminobutyric acid 1–20 amide	s.c. 21·6 ± 3·3–32·4 ± 3·0 i.v. 31·2 ± 4·2–48·4 ± 10·4 $\left(\frac{\text{s.c.}}{\text{i.v.}} = 0{\cdot}7\right)$	30–40% ACTH		$1{\cdot}6 \times 10^7$	351
17	1–23	s.c. 38·5 ± 4·0–43·6 ± 5·0 i.v. 103 ± 10·4–116 ± 21 $\left(\frac{\text{s.c.}}{\text{i.v.}} = 0{\cdot}3\right)$	91 ± 12·6		$2{\cdot}0 \times 10^8$	341
18	1–24	s.c. 106 ± 14 (225 ± 50 after heat)	110		$1–2 \times 10^8$	348
19	1–39 (≡ pig β-corticotropin)	s.c. 140	110			73, 408
20	1–10 + 15–19		1	≡ corticotropin	$\sim\frac{1}{100}$ corticotropin	356
21	1–10			$\sim\frac{1}{10}$ corticotropin	$\sim\frac{1}{10}$ corticotropin	356
22	15–19			0	0	356

sequences protected in this position, one of which is the N^{ϵ}-tosyl-lysine derivative of the *C*-terminal octapeptide amide, 6–13[368,377,404]. This suggests, both that the histidine residue in position 6 plays some special role in melanocyte-stimulating activity, and that the heptapeptide sequence, 4–10, does not have to be present in its entirety for activity to exist. In fact, the pentapeptide, 6–10[52,365,368], is the smallest fragment so far reported to be active, but how far this fragment can be shortened from the *C*-terminus without total loss of activity has not been established. Presumably, at least the tryptophan residue in position 9 will be necessary for activity, since the *N*-terminal decapeptide of the α-MSH sequence, 1–10[338,404], in contrast to the octapeptide, 1–8, possesses considerable activity.

Changes can be made at positions within the pentapeptide sequence without total loss of activity. For example, an ornithine[375] or a nitroarginine[378,404] residue can be incorporated in position 8 instead of arginine and the peptides are still appreciably active. Much more surprising is the finding that D-arginine may be included in position 8 of the pentapeptide[410], or D-phenylalanine in position 7[381], or D-phenylalanine in position 7 together with ornithine in position 8[375], without loss of potency. The D-phenylalanine analogue is 10 times more active than the all-L compound.

This unusual feature of MSH activity is undoubtedly related to the prolongation of activity observed after melanocyte-stimulating compounds have been heated with sodium hydroxide[411]. Lee and Buettner-Janusch[412], have shown that the 7-phenylalanine and the 8-arginine residues in α-MSH are racemized by this treatment. It is noteworthy that the racemic compounds are completely resistant to attack by chymotrypsin or by trypsin at these positions. The melanocyte-stimulating activity of corticotropin and of several other peptides is enhanced as well as prolonged by heating with sodium hydroxide and this is probably dependent on the presence of a free *N*-terminal serine residue[413].

The tridecapeptide sequence of α-MSH seems to be the optimum chain length for melanocyte-stimulating activity and when the chain length is increased beyond this point, melanocyte-stimulating activity diminishes, although the activity of corticotropin itself is still considerable. In general, acetylated compounds are more active than those with free terminal amino groups.

Corticotropic activity

Corticotropic activity is associated with the presence of a free terminal amino group and selective acetylation of this group causes a marked diminution in this type of activity[414a,414b]. The smallest fragment to exhibit corticotropic activity is the 1–13, tridecapeptide amide[338,404,415] corresponding to the free amino form of α-MSH, so that in a sense, this compound marks the transition point into the corticotropin series.

The tetracosapeptide (compound 18)[348], the tricosapeptide (compound 17)[341] and the eicosapeptide amide (compound 16)[351] have activity in the standard assays equal to that of natural corticotropin. Smaller fragments (compounds 14–11) have reduced activities.

Corticotropin A[300,302], which is isolated from pig pituitary glands by a process incorporating oxycellulose adsorption and which is presumably the

same as pig β-corticotropin[313], is approximately three times more active by subcutaneous than by intravenous application (s.c./i.v. = 3). On the other hand, for corticotropin B, which is separated from the corticotropin protein by peptic digestion[293], the same ratio can be as low as 0·25[405]. The ratio is 0·3 for the synthetic eicosapeptide amide and for the tricosapeptide amide discussed above, whereas the ratio for the nonadecapeptide (compound 14), surprising by comparison, has a value approximating to 2. The difference between the synthetic materials and corticotropin A is a genuine biological distinction and might be attributed to differences in rates of absorption, inactivation, transportation and so on.

The importance of individual residues for corticotropic activity has only been ascertained in a few cases. Inactivation of corticotropin by acylation (*see above*) or by the action of periodate[416] is interpreted to indicate the importance of the *N*-terminal serine residue. Further evidence is provided by the deactivation of corticotropin by leucine aminopeptidase which parallels the rate of removal of the *N*-terminal serine residue[417].

Although corticotropic activity is reversibly abolished by the oxidation of corticotropin with hydrogen peroxide or other oxidizing agents, a change which probably involves only the conversion of the 4-methionine residue to the sulphoxide, the methionine residue in the 4 position is not essential for corticotropic activity. This is shown by the high activity of the 4-α-amino-butyric acid analogue of the eicosapeptide amide (compound 16)[351]. A similar conclusion has been reached with respect to the melanocyte-stimulating activity of α-MSH[333,405] which is reduced, but not completely abolished, by oxidation.

Some or all of the lysine ε-amino groups in positions 11, 15 and 16 are probably necessary for potent corticotropic activity since the 5-glutamine, tetraformyl tricosapeptide, 1–23, has only 0·5–0·7 IU/mg ascorbic acid depleting activity[341]. The lysine ε-amino group in position 21 is clearly not essential for activity in view of the potency of the eicosapeptide, 1–20.

Twenty-five biological properties of corticotropin were listed by Li[334] and extra-adrenal activities have recently been reviewed by Engel[418]. The structure-activity relationships described above have only been concerned with the relatively few properties widely exploited in assay procedures. One of the activities of corticotropin which has not been considered is its ability to release nonesterified fatty acids from rat epididymal adipose tissue (lipolytic activity). It is interesting to note that different structural features are required for this activity than for corticotropic (*see* compound 13)[355] or melanocyte-stimulating (*see* compound 20)[356] activities.

Clinical Applications

Intense clinical interest in adrenocorticotropic hormone may be said to date from the discovery[419] in 1949, that it and cortisone were equally effective in the treatment of rheumatoid arthritis. At the present time, corticotropin is used in the treatment of a wide range of disorders including rheumatoid arthritis, rheumatic fever, secondary adrenal hypofunction, severe bronchial asthma, hay fever, and acute inflammatory diseases of the skin.

Corticotropin from natural sources contains polypeptide impurities which sometimes give rise to severe allergic reactions and curtail its use. It was to be

anticipated that synthetic peptides with corticotropic activity would not suffer from this disadvantage and such has proved to be the case. The effects in man of the synthetic nonadecapeptide[334,420], tricosapeptide[333,405,421] and tetracosapeptide[422–425] have been measured. Neither the tetracosapeptide nor synthetic 1–39 β-corticotropin (pig), for example, give allergic reactions in patients sensitized with natural corticotropin; nor do they react with serum antibodies from sensitized patients[408]. It is to be anticipated that non-antigenic preparations of this type will find wide application.

ANGIOTENSIN*

Introduction

Two excellent reviews on angiotensin, by Schwyzer and Turrian[426b] and by Page and Bumpus[427], appeared in 1960 and 1961 respectively and no attempt will be made to cover the large volume of literature concerning angiotensin which antedates these reviews. In particular, work prior to the structural elucidation and synthesis of angiotensin will be mentioned only in passing.

Angiotensin (angiotonin, hypertensin) is produced by the action of renin, an enzyme which occurs in kidney tissue, on an α_2-globulin fraction of blood[428–431]. The compound formed by renin is a decapeptide, angiotensin I. This compound is converted to an octapeptide, angiotensin II, by an enzyme or group of enzymes, widely occurring in the body and referred to as 'converting-enzyme'[432,433]. Whereas angiotensin I is only slightly active *in vitro*, angiotensin II is equally active *in vitro* and *in vivo*[433–435]. It has the ability to contract smooth muscle and is the most potent pressor substance known.

The decapeptide and the octapeptide produced from horse serum were isolated[436,437] by the use of counter-current distribution. Peart[438] isolated the decapeptide from bovine serum by partition chromatography on Hyflo; the octapeptide from this species has not been isolated.

Structural Elucidation

The sequence of the equine decapeptide (*XXXIIa*) was deduced by the use of carboxypeptidase, chymotrypsin, FDNB-labelling and Edman degradation[439,440]. Conversion of the decapeptide to the octapeptide (*XXXIII*) involves the cleavage of the dipeptide, histidyleucine, from the *C*-terminal end of the molecule[439]. It is interesting to note that the *in vivo* pressor activity of the decapeptide after incubation with carboxypeptidase is extremely low when the *C*-terminal leucine residue has been removed. Presumably, 'converting enzyme' is unable to convert the des-leucine compound to the octapeptide.

Leucine aminopeptidase, chymotrypsin, carboxypeptidase, FDNB-labelling and Edman degradation were used to deduce the structure of the bovine decapeptide (*XXXIIb*)[441,442]. The only difference between the decapeptides from the two species is in position 5 which is occupied by an isoleucine residue in equine angiotensin and by a valine residue in bovine angiotensin. The two

* After this section was completed the proceedings of an important symposium on angiotensin and related compounds became available[426a].

compounds are generally referred to as isoleucyl[5]-angiotensin I and valyl[5]-angiotensin I respectively, but for consistency within this review, they will be termed 5-isoleucine angiotensin I and 5-valine angiotensin I. Porcine angiotensin is probably identical with the 5-isoleucine variety[443].

Short periods of tryptic hydrolysis result in the cleavage of a tetradecapeptide from the angiotensin precursor, angiotensinogen. The tetradecapeptide, which is hydrolysed by renin to angiotensin I and a tetrapeptide, has been shown by Edman degradation to possess structure *XXXIV*. The existence of a serine residue in the *C*-terminal position of this molecule has prompted the suggestion[444] that an ester linkage is involved in the attachment of the tetradecapeptide fragment to the rest of the angiotensinogen molecule. This could account for the remarkable ease with which the bond is disrupted since it is known that trypsin catalyses ester hydrolysis more rapidly than amide hydrolysis.

(*XXXIV*) Asp·Arg·Val·Tyr·R·His·Pro·Phe·His·Leu·Leu·Val·Tyr·Ser
↓ renin
(*XXXII*) Asp·Arg·Val·Tyr·R·His·Pro·Phe·His·Leu
↓ 'converting enzyme'
(*XXXIII*) Asp·Arg·Val·Tyr·R·His·Pro·Phe
1 2 3 4 5 6 7 8 9 10 11 12 13 14

(*XXXII*) (*a*) Horse angiotensin I, R = Ile
(*b*) Cattle angiotensin I, R = Val
(*XXXIII*) Angiotensin II
(*XXXIV*) Tetradecapeptide released by the action of trypsin on angiotensin precursor

Synthesis

The decapeptide renin substrate[445], 5-valine angiotensin I[446–448], 5-valine angiotensin II[449], 5-isoleucine angiotensin II[443,450–453] and many analogues of the angiotensins have been synthesized.

In most of the synthetic work in this series the benzyloxycarbonyl group has been used for protection of amino functions, but *p*-chlorobenzyloxycarbonyl[455], tertiary butyloxycarbonyl[452] and trityl[454] derivatives have also been employed. The *C*-terminal carboxyl group has frequently been blocked as the methyl ester, but this leads to possible rearrangements during the final saponification or acid hydrolysis. A better technique is to use benzyl[455,456,457a] or *p*-nitrobenzyl[448,451] esters, removable by hydrogenolysis. In view of the usual resistance of *p*-nitrobenzyl esters to the action of hydrogen bromide in acetic acid, the partial removal of the ester group when benzyloxycarbonyl valyl-histidyl-prolyl-D-phenylalanyl-histidyl-leucine *p*-nitrobenzyl ester was exposed to this reagent[448] is noteworthy.

Side-chain protection in these syntheses has been restricted mainly to the β-carboxyl group of the aspartic-acid residue and the ω-guanidino group of the arginine residue. An attempted synthesis of 5-isoleucine angiotensin II using N^{im}-benzylhistidine derivatives failed because the benzyl protecting group could not be removed from the imidazole ring, but not all of the possible cleavage reactions were investigated[458].

The β-carboxyl group of the aspartic acid residue has been protected as

its methyl ester[443], benzyl ester[445,448,451,458–461] or amide[446,449,450,452,462,463]. Use of the amide protecting group seemed satisfactory until recently, particularly since in all cases in which l-aspartyl and l-asparaginyl angiotensin analogues have been compared, their biological activities have been almost identical. However, it has now been shown[464] that hydrolysis of the amide group in l-asparagine 5-valine angiotensin II results in a partial rearrangement of the terminal aspartic-acid residue through an imide structure. A β-aspartyl derivative is formed as well as aspartic acid, heptapeptide and the required α-aspartyl compound.

With one exception, in which a salt was used[448], the ω-guanidino group of arginine has been protected by nitration during angiotensin syntheses and its subsequent liberation by hydrogenolysis has generally been satisfactory.

Coupling procedures have involved azides, *N,N'*-dicyclohexylcarbodiimide, mixed anhydrides and more rarely, cyclohexyl-morpholinylethylcarbodi-imide, *p*-nitrophenyl esters, diethyl chlorophosphite and *o*-phenylene chlorophosphite. One of the early routes to 5-isoleucine angiotensin II has been reinvestigated to advantage recently to determine the value of *N,N'*-carbonyl di-imidazole as a coupling agent[452].

In this series, no completely stepwise synthesis of a free octapeptide or decapeptide and only one such synthesis of a protected octapeptide has been reported. All other syntheses have involved the coupling together of carboxyl activated peptide units (*see Table 4.7*), with the attendant risks of racemization, and racemization of the valine residue in position 3[483] and of the tyrosine residue in position 4[465] has been observed. Side reactions during the formation of the valyl-tyrosyl peptide bond have also been reported[450,452,483].

Table 4.7. Intermediate fragments used in the synthesis of the angiotensins and analogues

Sequence	*References*	*Sequence*	*References*
1–2	443, 445, 446, 449, 450, 452, 455, 457b, 459, 462	3–14	445
		4–5	483
1–3	459	4–8	458
1–4	448, 450, 452	5–6	443, 446, 448, 449, 450, 452, 454–457b
2–3	483		
2–4	448, 454, 456, 483	5–7	448
2–5	483	5–8	445, 449–452, 454–458, 460–463, 467, 468
2–8	451, 454, 456, 457a, 458, 460, 461, 466		
		5–10	448
3–4	443, 445, 446, 448–452, 454–457b, 460–463, 468, 483	6–7	445, 448
		6–8	445, 451, 456, 458, 460, 467
3–5	446, 483	7–8	443, 446, 449–452, 454–458, 460, 467
3–6	443, 446, 449, 459		
3–8	443, 445, 449, 451, 455, 457a, 457b, 458, 461–463	7–10	446
		8–10	448
3–10	446	9–10	446, 448

9–11, 9–14, 10–11, 12–14, 13–14 . . . all reference 445

Assay Procedures

Both *in vitro* procedures, utilizing amongst others, rat uterus[469] and guinea pig ileum preparations and *in vivo* procedures, measuring the pressor response of intact animals[470], are used to assay angiotensin[427]. Isolated rat colon has recently been reported to show a high degree of specificity for angiotensin[471].

In the main, three standards have been used in assay procedures. According to Bumpus, Schwartz and Page[435], one of their units was approximately the same as 0·18 Goldblatt units[436] and 1·0 Kuether and Haney units[472a]. Bumpus and colleagues calculated that 6 of the units of oxytocic activity used in their assay of angiotensin on rat uterus preparations, are equal to 1 international unit of oxytocic activity (posterior pituitary powder)[470].

For structure–activity studies, results are best expressed as percentages of the activity of pure synthetic angiotensin.

Structure–Activity Correlations

Analogues of angiotensin which have been described in the literature are listed in *Table 4.8.** Several previous discussions of structure–activity relationships have appeared[426,427,473–475].

In contrast to the corticotropin molecule, the amino extremity of the angiotensin molecule seems less important for biological activity than the carboxyl end. Removal of the *C*-terminal phenylalanine residue to give the heptapeptide, 1–7 (compound 25)[463,473], its replacement with alanine as in 8-alanine 5-isoleucine angiotensin II (compound 28)[470], or conversion of the terminal carboxyl group to an amide derivative (compound 26)[473,479], results in considerable loss of activity. Similarly, replacement of the penultimate proline residue by an alanine residue as in 7-alanine angiotensin II (compound 24)[460], or photoxidation of the imidazole ring of the histidine residue in position 6, gives relatively inactive compounds. On the other hand, the desamino angiotensins (compounds 5 and 6)[466,476,477], the des-aspartic acid angiotensins (compounds 14 and 15)[301,457a,463,470,473], 1-asparagine 2-ω-nitroarginine angiotensin II (compound 16)[473,479], and 1-asparagine 3-leucine angiotensin II (compound 19)[473] are all appreciably active. The high activity of 1-asparagine angiotensin II and the low *in vitro* activity of angiotensin I were mentioned earlier.

The importance of the tyrosine phenolic group is brought out by 4-phenylalanine angiotensin II (compound 20)[462,473] and by 4-alanine angiotensin II (compound 21)[461]. Both of these compounds are considerably less active than angiotensin. 4-(*O*-Methyltyrosine) 1-asparagine 5-valine angiotensin II also has a low order of pressor activity. It has no inhibitory action towards angiotensin in rat pressor tests[480,481].

Angiotensin II is deactivated reversibly by solutions of urea, guanidine or arginine hydrochloride and it has been postulated that the conformation of angiotensin is critical to its biological activity. Optical rotatory dispersion measurements indicate an increase in the degree of order of the peptides in dilute acetic acid in the series: tetrapeptide, 5–8, hexapeptide, 3–8, octapeptide, 1–8. By the same criteria there is a reduction in the degree of order when urea is added to solutions of the octapeptide. The ultra-violet absorption peak at 275 mμ does not change with this treatment so that the phenolic group is unlikely to be involved in hydrogen bonding. The ionization potential of the phenolic group suggests that it does not interact with ionic side chains.

* An isostere of bovine angiotensin II in which the α carbon atom of the valine residue in position 3 is replaced by a nitrogen atom has also been described. In the isolated rat uterus and in rat pressor tests this compound possesses 1/100th and 1/200th respectively of the activity of 1-asparagine-5-valine angiotensin II[472b].

On the basis of this evidence, Smeby and colleagues[482] proposed a strained α-helix structure for the 5-isoleucine angiotensin II molecule. It is not possible to construct a perfect helix because of the rigidity of the proline residue in the penultimate position in the chain. The slight imperfection in the helix tends to bring the aromatic ring of the terminal phenylalanine residue, which is known to be important for activity, into proximity with the tyrosine side chain. The imidazole ring is also in this region, whereas the side chains of the apparently non-essential aspartic acid and arginine residues are on the other side of the model. The low order of activity of 7-alanine 5-isoleucine angiotensin II, which could presumably form a completely helical structure, has already been pointed out.

In support of this theory, the hexapeptide, 3–8, acylated at its terminal amino group with poly-*O*-acetylserine, which might be expected to stabilize a helical conformation (compound 51)[466] has a pressor activity ten times greater than that of the hexapeptide[466]. 1-(Poly-*O*-acetylseryl)-5-isoleucine angiotensin II (compound 8)[466], on the other hand, is less active than angiotensin II.

It has been reported that the pressor[484] and smooth muscle stimulating[485] activities of angiotensin are considerably enhanced when the solution is at pH $\geqslant$ 11. That this is probably associated with a conformational change in the polypeptide molecule is shown by the development of a new peak in its absorption spectrum at 250 mμ when the pH is 10 or higher. Since the enhancement of activity is reversible, it is unlikely that a rearrangement of the primary structure of the peptide is responsible.*

Only one analogue of angiotensin which possesses an appreciably enhanced pressor activity has been reported. This is the compound in which the aspartic acid residue in the 1 position is linked through its β-carboxyl group to the arginine residue in position 2 (compound 11)[464,476,477]. This analogue, formed by rearrangement of 1-asparagine angiotensin II, probably owes its enhanced activity, in part at least, to its resistance to enzymic decomposition. Angiotensin is rapidly deactivated *in vivo*[487–490] and this is due mainly to an aminopeptidase type of decomposition[477]. As well as the β-aspartyl compound, 1-(α)-D-aspartic acid 5-valine angiotensin II (compound 12), 1-(β)-D-aspartic acid 5-valine angiotensin II (compound 13) and desamino 5-valine angiotensin II (compound 6) all have prolonged activity compared to angiotensin II and this is attributed to their comparative resistance to aminopeptidase destruction[476,477]. In view of this it is perhaps surprising that prolonged activity has not been recorded for the 1-(poly-*O*-acetylseryl) analogues[466].

It is interesting to note that Paiva and Paiva[491], treated 1-asparagine-5-valine angiotensin II with acid and recorded an initial transient increase in activity. Early suggestions were that hydrolysis of the amide[427] or formation of the heptapeptide 2–8[491], might account for the increased activity, but the work described above suggests that an α → β aspartyl rearrangement might have occurred.

* Measurements of optical rotatory dispersion and of the kinetics of hydrogen-deuterium exchange for 1-asparagine 5-valine angiotensin II have led Paiva, Paiva and Scheraga[486] to conclude recently that the molecule in aqueous solution exists in a random conformation. This important finding does not exclude the possibility that angiotensin might adopt a specific conformation when it reacts with the 'receptor'.

Table 4.8. Analogues of angiotensin

Compound number	*Residues as in angiotensin unless marked to the contrary* 1	2	3	4	5	6	7	8	*Relative pressor activity** *a*	*b*	*c*	*d*	*References*
1					Val					*100*			449
2					Ile				100	100			30p, 443, 450–453, 457a, 473
3	$Asp(NH_2)$				Val					100	100	100	449, 455, 473
4	$Asp(NH_2)$				Ile					100	85	52	450, 452
5	DesaminoAsp				Ile				60				466
6	DesaminoAsp				Val					f			476, 477
7	NO_2benzoylAsp(NH_2)				Val				g				478
8	Poly(*O*-acetyl)serylAsp				Ile				41				466
9	Gly				Val					50	42		30p, 457a, 457b, 462, 473
10	Arg				Ile				14·7				427, 451, 470
11	βAsp				Val					150–200[f]			464, 476, 477
12	D-Asp				Val					f			476, 477
13	βD-Asp				Val					f			476, 477
14	—				Val					50			463, 473
15	—				Ile				21·6		15		30p, 457a, 457b, 470
16	$Asp(NH_2)$	$Arg(NO_2)$			Val					50		25	473, 479
17	Asp(R)	†Orn			Val				25	20		8·9[h]	462, 473
18	$Asp(NH_2)$	D-Arg			Ile						3		30p, 457a, 457b
19	$Asp(NH_2)$		Leu		Val					100			473
20				Phe	Val					10		4·9[h]	462, 473
21				Ala	Ile				3				461

	1	2	3	4	5	6	7	8					
22		Asp(R)†		homo4:5′Tyr						1%		0·33[h]	463, 473
23		Asp(NH_2)		Leu						25			473
24				Ile			Ala		1				460
25				Val			—			0·05			463, 473
26		Asp(NH_2)		Val			$PheNH_2$			3			473, 479
27		Asp(NH_2)		Val			$PheOCH_3$			10			449, 473
28				Ile			Ala		0				470
29		Asp(R)†		Val			Phe(Br)‡			50			473
30				Val			PheHisLeu			110			446, 448
31				Val			DPheHisLeu			1			448
32		Asp(R)†		Val			$PheHisLeuNH_2$			50–100			473
33		Asp(R)†		Val			PheProPhe			0·2			473
34	Asp(NH_2)	Lys		Leu						<1			473
35	—	D-Arg		Ile							33		30p, 457a, 457b
36	—	Lys		Ile							3		30p, 457a, 457b
37	—	D-Lys		Ile							23		30p, 457a, 457b
38	—	Harg		Ile							2		30p, 457a, 457b
39	—	D-Harg		Ile							11		30p, 457a, 457b
40	—	Hly		Ile							2		30p, 457a, 457b
41	—	D-Hly		Ile							7		30p, 457a, 457b
42	—	Orn		Ile							(2·7)[e]		30p, 457b
43	—	Cit		Ile							0·5		30p, 457b
44	—			Val			DPhe			0·2			473
45	———			Val						<0·1			473
46	———			Ile					1–2·3		0·3		466, 470
47	———			Ala	Ile				**0**				470
48	———			Phe	Ile				0				470
49	———			Tal§	Ile				i				427
50	———			Phe(F)‖	Ile				0				427
51	Poly(*O*-acetyl)seryl———				Ile				10·8				466
52	————				Ile				0				470
53	————				Ile				0				470

Table 4.8. Analogues of angiotensin (*continued*)

* Biological Data:

(*a*) Mainly after Page and Bumpus and colleagues and related to 5-isoleucine angiotensin II
(*b*) Mainly after Schwyzer and colleagues and related to 1-asparagine 5-valine angiotensin II
(*c*) After Havinga, Schattenkerk, Heymans, Visser and Th. Kerling[30p,457] and related to 1-asparagine 5-valine angiotensin II (CIBA)
(*d*) After Paiva and Paiva[492] and related to 1-asparagine 5-valine angiotensin II
(*e*) Related to 1-asparagine 5-isoleucine angiotensin II (CIBA): On the same scale, compound 4 = 83, 18 = 3, 36 = 2·3 and 37 = 17·5
(*f*) = 'prolonged activity'
(*g*) = 'active'
(*h*) = results refer to 1-asparagine derivative
(*i*) = 'very low activity'
† R = OH or NH_2
‡ Phe(Br) = *p*-bromophenylalanine
§ Tal = -2-thienylalanine
‖ Phe(F) = *p*-fluorophenylalanine

Possible Pathological and Physiological Roles of Angiotensin

The structure–activity relationships of angiotensin discussed in the last section were concerned almost entirely with its pressor and smooth muscle-stimulating properties. It has been known for some time that angiotensin also has an action on renal function, generally described as an antidiuretic effect[426a,427]. Sodium transport in isolated renal tissue is inhibited by angiotensin[493]. Recently, an accumulation of evidence has indicated that in several species angiotensin also brings about release of aldosterone from the adrenal cortex[494a,495,496,497]. Pretreatment of rabbits with aldosterone reduces the pressor effect of subsequent injections of l-asparagine, 5-valine angiotensin II[498]. Whether these biological activities are equally modified in angiotensin analogues or whether, as one might suspect, at least a partial separation of these properties is possible, has yet to be determined.

It has not been demonstrated directly that angiotensin is produced *in vivo* and its physiological role, if any, is still unrecognized. Although interest in hypertension provided the main impetus for the research which culminated in definition of the renin-angiotensin system, angiotensin has not been unequivocally implicated in hypertension.

Helmer[494b], and Judson and Helmer[499] used an aortic strip preparation which reacts to angiotensin II, but not to renin, angiotensin I or renin substrate, to measure the renin concentration of dialysed plasma. All evidence indicates that the enzyme measured is renin and that angiotensin II is directly responsible for the measured activity. The highest renin concentrations were found in the plasma of patients with accelerated hypertension or renal vascular occlusive disease and, in the latter state, renin estimations proved valuable to determine which kidney was implicated. However, the plasma of some patients with high pressures was inactive. Laragh[494a] reported that in malignant hypertension, in which renal damage is prominent, aldosterone secretion is always increased. Angiotensin infusion has a similar effect on aldosterone levels in normal human subjects.

Unfortunately, none of the analogues of angiotensin which have been synthesized have had an inhibitory action towards angiotensin. A compound with this type of activity might settle the question of angiotensin involvement in hypertension and might have valuable clinical applications.

Goldblatt and colleagues[500], 30 years ago, described a technique for producing chronic experimental hypertension in animals by constricting the renal artery. This hypertension has not been shown to be directly related to chronic hypertension in human beings, although the technique has been invaluable experimentally. Chronic experimental hypertension in the dog can be treated effectively by immunologically-produced antirenin[501], but this preparation is inactive towards human renin. The angiotensin molecule is too small to be antigenic. An anti-angiotensin preparation has been produced by Deodhar[478,502] by the use of bovine γ-globulin-azo-benzoyl-l-asparagine, 5-valine angiotensin II. The antibody preparation was not as active as the antirenin preparation and an incubation period of 75 minutes was necessary to produce maximal antiangiotensin response. 5-Valine angiotensin II was inactivated to the extent of 56·7 per cent, l-asparagine 5-valine angiotensin II, 30·7 per cent and 5-isoleucine angiotensin II, 51·5

per cent, by this preparation. The antigen antibody complex was soluble which might account for the incomplete inactivation.

Clinical Applications

Synthetic l-asparagine, 5-valine angiotensin II finds clinical application as a pressor agent[494c] in the treatment of cases of severe shock[494d] and in the correction of hypotensive states during anaesthesia[503]. Single intravenous injections may be used in emergency situations, but due to the rapid deactivation of angiotensin in the body, intravenous infusion is necessary for the maintenance of blood pressure. From this point of view, it is conceivable that the analogues of angiotensin with prolonged action, for example l-(β)-L-aspartic acid, 5-valine angiotensin II, might be used to advantage. Intradermal injection of angiotensin has been suggested as a diagnostic test for latent, labile or prehypertensive states[494c].

BRADYKININ AND KALLIDIN

Introduction

In 1925, Frey[504] showed that an enzyme present in urine was a potent vasodilator and later, when the enzyme was found to occur in comparatively large quantities in the pancreas, it was named 'kallikrein'[505]. It is clear that kallikreins from different sources are not all identical. For example, the kallikrein derived from human plasma, but not the kallikreins from human and porcine pancreas or human and canine urine, is inhibited by soya bean trypsin inhibitor[506a]. Kallikreins act, in general, by releasing highly active polypeptides from α_2-globulin fractions of plasma, as was shown in 1948 by Werle and Berek[507] who coined the name 'kallidin' for the particular peptide with which they were concerned. It has since been shown that there is more than one kallidin[506a,508,509,510a,510b].

Whilst Werle and Berek were studying kallidin, Rocha e Silva, Beraldo and Rosenfeld[511] were investigating the actions of trypsin and snake venom on plasma. They found that a substance, which they called 'bradykinin', was released from the pseudoglobulin fraction of the plasma. Bradykinin is a potent vasodilator which possesses strong and characteristically slow, smooth muscle stimulating activity. Some ten years later, trypsin-bradykinin was isolated by Elliott, Horton and Lewis[512] who also deduced its structure[506b,513,514], assisted by the synthetic work of Boissonnus and his colleagues[506c,515a,515b,516,517]. Trypsin-bradykinin, snake venom-bradykinin[518] and one of the kallidins, kallidin I, have proved to be identical[506a,509]: kallidin II is very closely related to them[506a,509]. Compounds of this type,

Plasma Kinin Formation and Degradation

Inactive precursors (kallikreinogen, plasminogen[506e])
$+\downarrow$ activator
kinin-forming enzymes (kallikrein, plasmin)
$+\downarrow$ α_2-globulin fraction
kinins (bradykinin, kallidin)
$+\downarrow$ kininase
inactive degradation products

vasodilator smooth muscle stimulating polypeptides, are referred to as plasma kinins[519].*

Blood itself contains enzymic systems capable of releasing kinins and these systems may be activated in various ways[506d], for example, by contact with glass[520,521], by dilution[522] or by heating with dilute hydrochloric acid[523]. Also present in blood and tissues is an enzyme or group of enzymes, referred to as 'kininase', which rapidly deactivates kinins. The interrelationships between these various substances have been discussed by Lewis[524] and are summarized diagrammatically on the previous page.

Isolation and Structural Studies

At present, little is known about the substrate upon which the kinin-forming enzymes act except that it is an α_2-globulin. Pharmacological evidence indicates that the substrates which give rise to bradykinin and to kallidin II are probably identical and distinct from the precursor of angiotensin which is also an α_2-globulin[525–527]. The relative amounts of bradykinin and kallidin II which are formed are dependent on the source of the kallikrein[506a,b]. Species differences might also exist between the kinin precursors.

Kallikrein has been prepared in relatively crude form for clinical use for many years. Recently, by the application of new methods which involve ion exchange chromatography on DEAE-cellulose and resins, gel filtration and starch gel electrophoresis, several highly purified samples of kallikreins have been prepared, including those from hog[506f,506g,506h,528,529] and human[506h] pancreas, hog[506f], human[506f] and horse urine[506i,530], hog sub-maxillary gland[506f] and hog plasma[506f]. Structural studies have been limited to molecular weight determinations and to investigations of the 'active centres'. The molecular weight of human urinary kallikrein is 40,000 and of human and hog pancreatic kallikrein, 32,000[506h]. Kallikreins are deactivated by incubation with di-isopropylfluorophosphate[531], from which it is inferred that the kallikrein molecule, like many other esterolytic and proteolytic enzymes, has a serine residue in its 'active centre'. The esterolytic activity of the kallikreins is at a maximum at pH 8·5 which suggests that the analogy with other enzymes of this type might extend even further, and that a histidine residue might be involved in the 'active centre'[506f]. This contention finds further support in the photochemical deactivation of pancreatic kallikrein[506f,532].

The presence of kininase in plasma is a complicating factor in the isolation of kinins. Frequently, the plasma has been treated with acid to deactivate the kininase[533,534] prior to formation of the kinins. This facilitates their isolation but introduces complications of its own, since acid treatment is sufficient to activate a kinin-forming system within the plasma itself. As Elliott has pointed out, this means that subsequent incubation with an enzyme from another source may produce a pattern of kinins not typical of that enzyme[506b]. On the other hand, kallidin II and bradykinin are said to be deactivated at different rates by kininase so that, even if the kinins can be isolated, unless kininase activity is completely destroyed, false patterns may result[506j].

* Kinins also occur in wasp and hornet venoms[506v]. In botany the word 'kinin' has a different connotation.

Chromatography with volatile buffers on carboxymethylcellulose has been used to isolate kinins from several sources. This method was introduced by Elliott, Lewis and Horton[512,535] for the isolation of bradykinin, approximately 3·5 mg of which was obtained from 90 l. of ox blood. The sequence of amino-acid residues in bradykinin was deduced from evidence provided by hydrolysis with chymotrypsin and by Edman degradation[513,514]. These studies were hampered by the partial decomposition of proline during acid hydrolysis which led initially to the postulation of an incorrect amino-acid analysis. A similar destruction of proline has since been observed both with bradykinin from another source[536] and with a synthetic analogue[537]. Bradykinin is a nonapeptide (*XXXVa*).

Pierce and Webster[509], by similar methods, were able to isolate two kinins from human plasma which had been incubated with human urinary kallikrein. One of these kinins, kallidin I, subsequently named kallidin-9 because of its nonapeptide structure[506k], was identical with bradykinin. The other kinin, kallidin II, contained the same amino acids as bradykinin and in addition, one lysine residue. Edman degradation showed that the lysine was *N*-terminal and that the structure of kallidin II was l-lysyl-bradykinin (*XXXVb*). It was later renamed kallidin-10, on account of its decapeptide structure. The same two kinins, but in different proportions, were obtained by Werle, Trautschold and Leysath[506f,510a] from ox plasma treated with porcine submaxillary kallikrein and subsequently, both kinins have been reported from several other sources[506k].

Recently, Elliott, Lewis and Smyth have reported the isolation of a third kinin (*XXXVc*), from plasma activated by acid[538].

R·Arg·Pro·Pro·Gly·Phe·Ser·Pro·Phe·Arg
1 2 3 4 5 6 7 8 9

(*XXXV*)
(*a*) Bradykinin, R = H
(*b*) Kallidin-10, R = Lys
(*c*) A third kinin obtained from plasma activated by acid, R = Met·Lys

Synthesis

Due to the error in the original amino-acid analysis, it was first thought that bradykinin was an octapeptide. This structure was soon shown by synthesis to be incorrect[516,539,540]. From a study of the chymotryptic hydrolysis of this compound, Boissonnas and colleagues[506c] were able to deduce a probable structure for bradykinin which, by synthesis and by further degradation of the natural product, was later confirmed[517,541–543]. Other syntheses of bradykinin[544,545] as well as syntheses of kallidin-10[30m,546–548] and of the new methionyl-lysyl-kinin[548] have since been described. Many synthetic analogues of bradykinin have been reported.

In all cases, benzyloxycarbonyl protection of amino functions has been used.* The terminal carboxyl group has been blocked as its methyl[537,545,549–552], benzyl[539] and *p*-nitrobenzyl[553–555] esters. More recently, with *p*-nitrophenyl ester couplings it has been blocked by salt formation[544]. The arginine

* The tertiary butyloxycarbonyl group has been used recently in a new type of synthesis (*see* reference 92b and page 94).

ω-guanidino group has been protected as its nitro[539,545–547,549,551–555], tosyl[537,544] and di-benzyloxycarbonyl[545] derivatives; the serine β-hydroxyl group has only occasionally been blocked, for example as the acetate[550,551] and as the benzyl ether[550]. Coupling procedures have relied mainly on *N,N'*-dicyclohexylcarbodi-imide[537,539,544,553,554] and *p*-nitrophenyl esters[544–547,549,552]; the serine carboxyl group has generally been coupled by an azide procedure. Reagent K[549,551] and mixed anhydride couplings have found occasional applications[549,551].

No synthetic difficulties peculiar to the bradykinin structure have been reported. In one instance, the attempted saponification of an ester led to hydantoin formation from a terminal benzyloxycarbonyl group[549], but this difficulty has been encountered in other instances in which a glycine residue has been adjacent to the *N*-terminal residue. Acetylation of the serine hydroxyl function during peptide synthesis has been reported[545,556].

Intermediates used in the synthesis of bradykinin and its analogues are listed in *Table 4.9.*

Table 4.9. Intermediate fragments used in the synthesis of bradykinin and analogues*

Sequences	*References*	*Sequences*	*References*†
(−2)-(−1)	30m, 548(B,y)	3–9	30m, 506w, 506m, 537, 545, 547, 548(A,a–f,m,r–t,v–x), 549, 550, 552, 558(a,b)
(−1)–2	30m, 548(A,v–x)		
1–2	30m, 506w, 517, 537, 547, 548(a–f,m,r,t), 549, 554, 555, 558(a)	4–9	545, 558(b)
		5–6	506w, 506m, 517, 537, 539, 544, 545, 549, 552, 554, 558(b)
1–4	30m, 517, 539, 544, 548(g–l,n–r,u)	5–7	506w, 549
1–5	506w, 549	5–9	30m, 506w, 506m, 517, 544, 545, 548(g–l,n–r,u), 550, 552, 555, 558(a,b)
1–9	30m, 548(B,y)		
2–3	539, 544		
2–4	539, 544	6–9	506w, 545, 549, 550, 558(a)
2–9	506w, 506m, 545, 550, 552, 557, 558(b)	7–9	506w, 506m, 517, 537, 544, 545, 549, 550, 552, 558(a,b)
3–4	506w, 517, 537, 549, 554, 558(a)	8–9	506w, 506m, 517, 537, 544, 545, 549, 550, 552, 554, 555
3–6	537		

* Retrobradykinin, an analogue in which the sequence of amino-acid residues is completely reversed, has been synthesized by the use of the following intermediates: 1–3, 4–6, 5–6, 7–8, 7–9[506w,551,553], 1–2, 1–6[553], 2–3, 4–9[506w,551].

† Several of the individual references in this table refer to more than one analogue of bradykinin and in these instances, superscripts have been added (e.g. 548(B,y) to relate the payments to the particular analogues concerned (*Table 4.10*).

Assay Procedures

Samples of pure bradykinin from two sources have been reported to possess 12,000–12,500 units/mg[559,560] and 5,000 units/mg[518], respectively, of bradykinin-like activity when compared with standard preparations. This discrepancy is undoubtedly due to variations in the standard preparations and, for structure–activity comparisons, activity is best presented in terms of the pure synthetic material.

Bradykinin brings about vasodilatation, smooth muscle stimulation, increase in capillary permeability, accumulation and migration of leucocytes and pain production[506n,561]. The first two effects are readily measured and have been widely used to assay bradykinin-like activity.

An idea of the potency of bradykinin as a vasodilator may be obtained from the fact that it is about as active on a weight basis in the cat hind limb as the very much smaller molecule, acetylcholine[506n]. For assay purposes, vasodilatation has been measured *in vitro*, for example in the coronary vessels of the isolated cat's heart, and *in vivo*, measuring vasodepressor activity in intact animals. The latter reponse is dependent to a considerable degree on the species used. For example, the vasodepressor activity of kallidin-10, expressed as a percentage of the activity of bradykinin, is 60 ± 12 per cent in the cat, 190 ± 40 per cent in the rabbit and about 200 per cent in the rat[506c,546].

Smooth muscle contraction similarly depends on the source and type of muscle. Kallidin-10 has 60 per cent of the activity of bradykinin on the isolated rat uterus; for guinea-pig ileum, the figure is 33 ± 8 per cent[506c]. Bradykinin causes contraction of isolated guinea-pig bronchial muscle and, when given intravenously in the intact animal, increases the resistance of the lungs to inflation[506o,515c]. This important effect[559,562] provides the basis of a fairly specific test for bradykinin-like activity, because the kinins, but not other substances active in this way, are antagonized by aspirin, amidopyrine and phenylbutazone.

Bradykinin, applied to an exposed blister base or injected intra-arterially[563–566], produces pain. The pain produced by intra-arterial injection, but not the pain produced in a blister base, is also antagonized by aspirin and by *N*-acetyl-*p*-aminophenol. Bradykinin may be distinguished from kinins produced by the action of glass on plasma, by the rate of onset of tachyphylaxis in the blister base test[506p].

The increased capillary permeability which bradykinin brings about may be measured by the diffusion of a dye from the capillaries[567] in the vicinity of an intradermal bradykinin injection[515d]. Histamine has a somewhat similar activity but there are qualitative differences in the response[568]. Bradykinin is about 15 times more active than histamine in this test.

Intradermal injection of bradykinin also causes migration of leucocytes to the area[524,569]. This may readily be seen by microscopy but is difficult to measure.

Structure–Activity Correlations

The analogues of bradykinin which have been reported to date, along with available biological data, are collected in *Table 4.10.*

Activity is at a maximum in the nonapeptides, bradykinin (compound 1), 3-alanine bradykinin (compound 14)[30m,548], 6-glycine bradykinin (compound 19)[30m,506w,548,549] and 8-*p*-fluorophenylalanine bradykinin (compound 30)[552]. The decapeptide, kallidin-10 (compound 9)[30m,506c,506m,546–548] is in general slightly less active but is of the same order of activity as bradykinin. 1-Methionyllysyl-bradykinin is reported to possess about a quarter of the activity of bradykinin on various smooth muscle preparations; in enhancing capillary permeability, the two compounds are equi-active in the guinea-pig, whereas, in rabbits[538], the endecapeptide is twice as active as bradykinin. Kallidin-10, 1-methionyllysyl-bradykinin (compound 57)[548] and 1-lysyl, 9-lysine bradykinin (compound 56)[548] are all more potent vasodepressor agents than bradykinin. All of the reported octapeptides (compounds 2, 12,

26, 33, 39, 40) and heptapeptides (compounds 42, 43, 44) are very much less active than bradykinin in all tests.

The arginine residues in positions 1 and 9 are important for activity as is shown by compounds 3, 4, 5, 6, 7, 8, 34, 36, 37, 38, 45, 46, 47, 50, 51, in which one or both of the residues in these positions is not arginine. All of these compounds have reduced activities even when the arginine residues have been replaced by other basic amino acids. Nonetheless, an appropriate chain length, terminated by arginine residues (compound 4)[506w,549] is not sufficient to confer bradykinin-like properties on a molecule.

Apart from the arginine residues, the only chemically reactive side chain in the bradykinin molecule is that of the serine residue in position 6. It is clearly seen from the high order of activity of 6-glycine bradykinin (compound 19)[30m,506w,548,549] and of 1-lysyl, 6-glycine bradykinin, that is, 7-glycine kallidin-10 (compound 49)[506w,549], that the serine side chain is not essential for activity. On the other hand, compounds with bulkier side chains at this position (compounds 23, 24, 25)[506m,558] tend to have diminished activity; a change in the sterochemistry at this position (compound 22)[506m,558] has the same effect to a less marked degree.

The importance of the aromatic ring in position 8 is demonstrated by the relative inactivity of 8-alanine bradykinin (compound 32)[30m,548]. Other analogues involving changes at this position, but in which the aromatic ring is retained, are still appreciably active, even when inversion at the α-carbon atom is involved (compounds 29 and 31)[552]. The other aromatic side chain in the bradykinin molecule, at position 5, is also of considerable importance for biological activity. Replacement of the phenyl group by a hydrogen atom (compound 17)[30m,548], or even *p*-hydroxylation, as in 5-tyrosine bradykinin (compound 18)[30m,548], leads to loss of activity. The results of these and of other changes, for example, replacing proline in position 2 (compound 13)[30m,548], glycine in position 4 (compound 16)[30m,548] or proline in position 7, could perhaps be most easily accounted for in steric terms. It would be interesting to know whether sarcosine could replace the proline residues in position 3 and 7. Arginylheptaglycylarginine is too simple an 'abbreviation' for the bradykinin molecule, but on the basis of evidence presently available, the minimum structure to possess appreciable bradykinin-like activity could still be fairly simple, for example

$$\begin{matrix} & \text{Pro}\cdot\text{Ala}\cdot & & & \text{Pro}\cdot & \\ \text{Arg}\cdot & & \text{Gly}\cdot\text{Phe}\cdot\text{Gly}\cdot & & & \text{Phe}\cdot\text{Arg} \\ & \text{Sar}\cdot\text{Gly}\cdot & & & \text{Sar}\cdot & \end{matrix}$$

There is some evidence[506q,570] that *in vivo* deactivation of bradykinin might involve carboxypeptidase-like hydrolysis with removal of the *C*-terminal arginine residue. Chymotrypsin is known to cleave the same bond rapidly[506b]. It would not be unreasonable to think that the analogues in which the residue in position 8 was of the D-configuration might be resistant to this type of inactivation, but prolonged activity has not been reported for either of them. On the other hand, 1-methionyl-lysyl-bradykinin is reported to be less readily inactivated than bradykinin by the plasma kininase of man, dog and guinea-pig[538]. It is difficult to see why acylation of the *N*-terminal

Table 4.10. Analogues of bradykinin

Compound number	1	2	3	4	5	6	7	8	9	Rat uterus in vitro	Guinea-pig ileum	Guinea-pig broncho-constriction*	Vasodepressor activity: Rat	Vasodepressor activity: Guinea-pig	Vasodepressor activity: Dog	Vasodepressor activity: Rabbit	Coronary vessels of cat heart in vitro	Capillary permeability	References†‡
1	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg	1	1	1(+)	1	1	1	1	1	1	506m, 557
2	—											$<\frac{1}{2,000}$		$\frac{1}{80}$	$\frac{1}{50}$, $\frac{3}{100}-\frac{1}{500}$				506m, 557
3	Orn											$\frac{1}{1,000}$		$\frac{1}{50}$	$\frac{1}{100}$, $\frac{1}{100}-\frac{1}{200}$				506m, 557
4	Lys									$\frac{1}{500}$	$\frac{1}{150}-\frac{1}{300}$	$\frac{1}{62}$		$\frac{1}{10}$	$\frac{1}{30}$, $\frac{1}{25}-\frac{1}{50}$	$\frac{1}{250}$			506m, 557; 30m, 548(c)
										$\frac{1}{400}$			$\leqslant\frac{1}{100}$				$\leqslant\frac{1}{100}$	$\frac{1}{1,000}$	506w, 550
5	Cit											$<\frac{1}{2,000}$		$\frac{1}{200}$, $\frac{1}{250}$					506m, 557
6	GluOH											$<\frac{1}{2,000}$		$\frac{1}{300}$					557
7	Ala									$<\frac{1}{1,500}$	$<\frac{1}{1,500}$					$\sim\frac{1}{1,000}$			30m, 548(a)
8	Gly										$<\frac{1}{5,000}$					$>\frac{1}{1,000}$			30m, 548(b)
9	Lys·Arg									$\frac{6}{10}$		$\frac{1}{3}$	2	$\frac{2}{3}$					506c, 506m, 546, 547
										$\frac{1}{10}$	1					2			30m, 548(A)
10	Phe·Arg									1	$\frac{1}{2}$					$\frac{1}{5}$			548(v)
11	Orn·Arg									$\frac{1}{10}$	$\frac{1}{3}$					$\frac{1}{5}-\frac{1}{10}$			548(w)
12		—									$\frac{1}{100}$								516, 555
13		Ala								$\frac{1}{300}$	$\frac{1}{150}$					$\frac{1}{200}$			30m, 548(d)

	1	2	3	4	5	6	7	8	9						
14			Ala							1	1	$<\frac{1}{2,000}$	$\frac{1}{2,000}$	1	30m, 548[f]
15			Pip												506m
16				Ala						$<\frac{1}{1,500}$	$<\frac{1}{1,500}$			$\frac{1}{300}$	30m, 548[g]
17					Ala					$\frac{1}{1,000}$	$<\frac{1}{1,500}$			$\sim\frac{1}{1,000}$	30m, 548[h]
18					Tyr					$\frac{1}{500}$	$\frac{1}{150}$			$\frac{1}{300}$	30m, 548[i]
															506w, 549
19						Gly				$\frac{1}{10}$	$\frac{1}{3}-1$			$\frac{1}{2}-1$	30m, 548[k]
20						Ala				$\frac{1}{10}$	$\frac{1}{5}$			$\frac{1}{10}$	30m, 548[j]
21						Sar				$<\frac{1}{1,500}$	$\frac{1}{1,500}$			$\frac{1}{1,000}$	30m, 548[l]
22						DSer						$\frac{1}{40}(+)$	$\frac{1}{2\cdot5}$		506m, 558[b]
23						Carbamoyl Ser						$<\frac{1}{500}(+)$	$\frac{1}{75}$	$\frac{1}{10}-\frac{1}{100}$	558[a]
24						Thr						$<\frac{1}{500}(-)$	1		558[b]
25						$Asp(NH_2)$						0	$\frac{1}{1,000}$		506m
26							—				$<\frac{1}{2,500}$				516, 554
27							Gly			$\frac{1}{100}$					506w, 549
28							Ala			$\frac{1}{80}$	$\frac{1}{100}$			$\frac{1}{1,000}$	30m, 548[n]
29								DPhe				$\frac{1}{20}\left(\frac{1}{60}\right)$ (+)	$\frac{1}{4}$	$\frac{1}{5}$	552
30								F Phe				1·4(+)	1·5	1·5	552
31								F DPhe				$\frac{1}{100}(+)$	$\frac{1}{3}$		552
32								Ala		$\sim\frac{1}{1,500}$	$\sim\frac{1}{1,000}$			$\sim\frac{1}{1,000}$	30m, 548[o]
33									—			$<\frac{1}{2,000}$	$<\frac{1}{2,000}$		506m
34									Gly	$<\frac{1}{1,500}$	$<\frac{1}{1,500}$			$\sim\frac{1}{1,000}$	30m, 548[q]

Table 4.10. Analogues of bradykinin (*continued*)

Compound number	1	2	3	4	5	6	7	8	9	Rat uterus in vitro	Guinea-pig ileum	Guinea-pig bronchoconstriction	Vasodepressor activity: Rat	Vasodepressor activity: Guinea-pig	Vasodepressor activity: Dog	Vasodepressor activity: Rabbit	Coronary vessels of cat heart in vitro	Capillary permeability	References†‡
35									Ala	$\frac{1}{500}$	$< \frac{1}{800}$					$< \frac{1}{1,000}$			30m, 548(p)
36									Lys	$\frac{1}{500}$	$\frac{1}{5}$					$\frac{1}{10} - \frac{1}{30}$			30m, 548(r)
37									Cit	$\frac{1}{400}$		$\frac{1}{1,000}$	$\leqslant \frac{1}{100}$	$\frac{1}{20}$			$\frac{1}{10}$	$\frac{1}{10} - \frac{1}{100}$	506w, 550 506m
38									His			$\frac{1}{200}$		$\frac{1}{20}$					506m
39			Gly	Pro			—				$< \frac{1}{2,500}$								516, 555
40				Phe	Gly		—				$< \frac{1}{2,500}$								516, 555
41		Gly	Gly	Gly	Gly	Gly	Gly	Gly		0									506w, 549
42		—									$< \frac{1}{2,500}$								516, 555
43	—								—			$< \frac{1}{2,000}$		$< \frac{1}{2,000}$					506m
44	Z								—			$< \frac{1}{2,000}$		$\frac{1}{400}$					506m
45	Dab								Dab	Inactive									537
46	Cit								Cit	$< \frac{1}{5,000}$			$< \frac{1}{100}$					$\leqslant \frac{1}{100}$	506w, 550
47	Lys·Lys											$\frac{1}{500}$		$\frac{1}{20}$	$\frac{1}{25} - \frac{1}{50}$				506m
48	Lys·Arg							F Phe				$\frac{1}{3}$		$\frac{2}{3}$	$\frac{1}{2} - \frac{1}{4}$				506m
49	Lys·Arg					Gly				$\frac{1}{4} - 1$									506w, 549
50	Lys								Lys	$< \frac{1}{1,500}$	$< \frac{1}{1,500}$					$\frac{1}{500}$			30m, 548(s)
51	Lys								BOC Ly :	$< \frac{1}{1,500}$	$< \frac{1}{1,500}$					$< \frac{1}{1,000}$			30m, 548(t)
52		Phe		Ser		Gly		Pro		Inactive at 80γ/ml	Inactive								506w, 551 553

	1	2	3	4	5	6	7	8	9									
53							Phe	Arg	Pro	$<\frac{1}{300}$	$\frac{1}{150}$					$<\frac{1}{1,000}$		30m, 548[u]
54	Pro	Arg								$\sim\frac{1}{1,500}$	$\sim\frac{1}{500}$					$\sim\frac{1}{1,500}$		548[e]
55				Ser		Gly					$\frac{1}{100}$					$\frac{1}{100}$		548[m]
56	Lys·Arg								Lys	$\frac{1}{500}$	$\frac{1}{100}$					1·5		548[x]
57	Met·Lys·Arg									$\frac{1}{3}$	$\frac{1}{3}$					2–3		548[B]
58	BOC | BOC·Met·Lys·Arg									$\frac{1}{150}$	$<\frac{1}{150}$					$\frac{1}{300}$		548[y]

* (+) indicates that this response was inhibited by aspirin
† Superior letters relate the References to *Table 4.9*
‡ The ratios quoted in references 30m and 548 refer to the activities of the following hydrated salts:

Compound number	1	4	7	8	9	10	11	13	14	16	17	18	19	20	21	28	32	34	35	36	50	51	53	54	55	56	57	58
Moles of acetic acid	2	1·5	0·75	3	2·5	1·5	3	1·5	1·5	1·5	2	2	2	1·5	1·5	2	0·5	0·5	0·5	2·5	2	1	1·5	1·5	1·5	2·5	2	1 (HCl)
Moles of water	6	6	5	7	6	5	5	1	3	10	5	4·5	4	5	5	5	5	3	3·5	5	3	3	4	5	4	6	6	3

residue should protect the *C*-terminal peptide bond from a carboxypeptidase-type of attack. Possibly new light will be thrown on the action of plasma kininase as a result of these observations.

Pathological and Physiological Roles of Kinins

Bradykinin has the ability to elicit all of the signs associated with the early stages of inflammation: vasodilatation, increased capillary permeability, accumulation and migration of leucocytes, pain. Although there is no direct evidence to show that bradykinin is involved in the inflammatory response, circumstantial evidence is compelling[506n]. Proteolytic enzymes are known to be activated during tissue injury and damaged tissue generally has a mildly acid pH. It is not known if the second effect is sufficient to activate the acid-sensitive kinin-producing system, but Edery and Lewis[571] have shown that it is sufficient to inhibit kininase activity. These workers have also drained lymph from areas of damaged tissue and shown it to possess enhanced kinin-forming abilities[572]. Chapman and colleagues[506r] detected bradykinin-like activity in subcutaneous fluid taken from painful areas of subjects suffering headaches of the migraine type[506p]. On the other hand, Horton has shown that the pain which bradykinin causes when applied topically to exposed blister bases is so subject to tachyphylaxis that it could not account for sustained severe pain. Of course, it is possible that pain receptors in inflamed tissue are different or react in a different manner to those in the blister base. Alternatively, bradykinin or similar compounds might react on pain receptors in inflamed tissue to modify their response to other agents.

The action of bradykinin in the guinea-pig, where it brings about bronchoconstriction which is antagonized by phenyl-butazone, amidopyrine and aspirin, has stimulated speculation that bradykinin might be involved in asthma[506o] and substances which antagonize bradykinin are sometimes effective in the treatment of asthma. Furthermore, bradykinin applied in an aerosol reduces the vital capacity of chronic asthmatics. Here again, the evidence is all circumstantial, but suggestive.

It is striking that the action of proteolytic enzymes on plasma proteins gives rise to a potent pressor substance, angiotensin, and to potent depressor substances, the kinins, and at first sight, it may seem that here is the making of a haemodynamic equilibrium. Rocha e Silva[506s,515e] investigated this possibility and concluded that no competitive antagonism occurs between angiotensin and bradykinin. A physiological antagonism is still possible, but whereas angiotensin acts predominantly by modifying the calibres of large vessels, bradykinin probably acts more particularly on the smaller vessels.

The occurrence of kallikrein in many glandular secretions led Hilton and Lewis[573] to investigate the possibility that bradykinin was responsible for functional vasodilatation in glandular tissue. With salivary glands, they were able to show that a kallikrein-like enzyme was released from the secretory cells into the interstitial space where it acted on plasma proteins to release kinin-like polypeptides. Under normal physiological conditions, the activity was destroyed or transported away from the gland mainly by the lymph. A similar role for bradykinin has been demonstrated in the glandular tissue of the tongue[574], in the sweat glands of the forearm[575] and in the pancreas[506t] and it seems well authenticated that bradykinin is physiologically implicated

in the functional vasodilatation of these glands. Other glands, however, possess no kinin-releasing system[576].

Clinical Applications

The kinins have at present no direct clinical application. Compounds with a prolonged hypotensive activity and compounds antagonistic to bradykinin might be useful, but no peptides with these properties have yet been produced.

Kallikrein preparations[577] are used for their vasodilator properties, for example, in the treatment of claudication, Raynaud's disease and circulatory diseases of the eyeground. An inhibitor[578] of kallikrein and of other proteolytic enzymes is extracted from parotid glands and from lymph nodes of cattle and is used to treat acute pancreatitis and other diseases associated with increased proteolytic activity. The clinical uses of kallikrein and of kallikrein inhibitors have been discussed by Forell[506u].

INSULIN AND GLUCAGON

Introduction

Insulin was first extracted successfully by Banting and Best[579]. It is secreted by the β-cells of the islets of Langerhans. Glucagon, first isolated by Staub, Sinn and Behrens[580], is secreted by the α-cells. These hormones are hypoglycaemic and hyperglycaemic agents respectively. The role of insulin in the control of diabetes mellitus and in the aetiology of this disease have formed the subjects of a recent symposium[581] and of several reviews[582,583,584,585]. Glucagon has only limited therapeutic applications—it may be used, for example, to counter insulin-produced hypoglycaemia[581a]—and has received much less attention.

Structural Elucidation

Insulin

The amino-acid residue sequence of beef insulin was determined by Sanger[581b]. This work represented the first complete elucidation of the primary structure of a protein and the methods which were employed have been of far reaching significance in subsequent structural studies on other proteins.

FDNB studies on the intact protein showed that glycine and phenylalanine residues occupied *N*-terminal positions in the molecule and it was postulated that two types of peptide chain were present[586]. Performic acid oxidation was used to cleave the disulphide bonds which were assumed to hold the chains together and it was then possible to separate the *N*-terminal glycine material, the A chain, from the *N*-terminal phenylalanine material, the B chain[587]. The sequence of residues in the B chain was determined by examining the products of partial hydrolysis produced by the use of acid, pepsin, trypsin and chymotrypsin[588]. FDNB and deamination techniques were used to identify *N*-terminal residues. The structure of the A chain was deduced in a similar manner[589].

Electrophoretic studies on the products of enzymic hydrolysis enabled the amide groups in the molecule to be located[590]. Fragments in which the cystine residues were intact were obtained both from enzymic hydrolysis

and by the use of acidic hydrolysis in the presence of thiols, which were found to inhibit disulphide interchange. It was thus possible to determine the position of the disulphide bonds[591], and this information, together with the knowledge that the molecular weight was 6,000[592], made it possible to write the primary structure of the beef insulin molecule (*XXXVI*).

Since this time, the amino-acid sequences in insulins derived from pig[593], sheep[593], sperm whale[594,595], sei whale[595], horse[594], man[596], rabbit[597], rat[597], and dog[597] have been determined. In these cases the structures are very similar to that of beef insulin. The differences are mainly restricted to the 8–10 sequence of the A chain, the portion of the molecule involved in the interchain disulphide ring (*see Table 4.11*).

Commercial beef-pork insulin is generally antigenic in man[598] and the antibodies produced also react with horse, sheep, human[599], dog, monkey, rabbit and whale[600] insulins. Some antisera can distinguish insulins from different species, beef and sheep insulins generally reacting more strongly than pig and horse insulins, and these differences have been related to similarities between the amino-acid residue sequences and to possible differences in secondary structure[599].

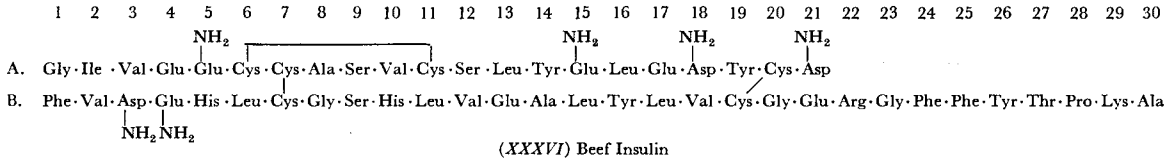
Wilson and Dixon[601] compared the reactions, towards antibovine insulin serum produced by horses and guinea-pigs, of insulins from seven species of bony fish and concluded that four of the insulins behaved like bovine insulin, but that the other three behaved quite differently. One of the three, cod insulin, isolated by Moloney and Wilson[602], was subjected to structural investigations and was found to differ to a marked degree from other insulins in its amino-acid content. *N*-Terminal residues were shown to be glycine and serine[601]. Kotaki[603] has studied insulin from bonito and has shown that it also differs considerably from mammalian insulins. A partial structure was derived for the A chain of bonito insulin II (*XXXVII*).

When cod A and B chains are separated and combined with ox B or ox A chains respectively, one of the resultant hybrid insulins (ox A cod B) resembles ox insulin immunologically, whereas the other (cod A ox B) resembles cod insulin[604]. This might indicate that the immunological specificity of insulin is determined primarily by its A chain. On the other hand 67 per cent (14 in 21) of the residues in the cod A and beef A chains are different, whereas only 30 per cent (9 in 30) are different in the B chains.

Berson and Yalow[605] found that some antisera will distinguish readily between whale and pig insulins, although the residue sequences in these molecules are identical. If the assumption is made that the amino-acid residues in these two species are in the same configurations, it must follow that the antisera are detecting differences in secondary and tertiary structures, and Berson and Yalow make the interesting suggestion that perhaps the folding, as well as the sequence of the residues is determined by the genetic apparatus.

Glucagon

The beef insulin molecule contains no tryptophan or methionine residues; if it were otherwise, structural elucidation would have been even more difficult. Glucagon contains both of these amino acids but consists of only one chain (*XXXVIII*). Chymotrypsin[606], trypsin[607], subtilisin[608] and acid[609] were

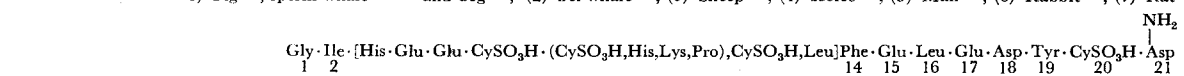


(*XXXVI*) Beef Insulin

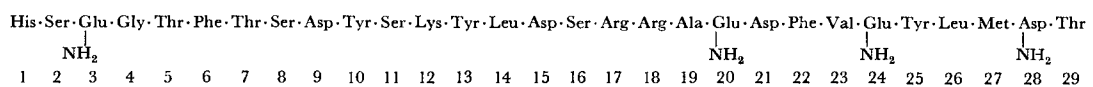
Table 4.11

	A *chain*				B *chain*		
	4	8	9	10	3	29	30
1		Thr	Ser	Ile			
2		Ala	Ser	Thr			
3		Ala	Gly	Val			
4		Thr	Gly	Ile			
5		Thr	Ser	Ile			Thr
6		Thr	Ser	Ile			Ser
7	Asp	Thr	Ser	Ile	Lys		Ser
8	Asp	Thr	Ser	Ile	Lys	Met	Ser

Differences between the residue sequence of beef insulin and insulin from:
1) Pig[593], sperm whale[594,595] and dog[597]; (2) Sei whale[595]; (3) Sheep[593]; (4) Horse[594]; (5) Man[596]; (6) Rabbit[597]; (7) Rat[597]; (8) Rat[597]



(*XXXVII*) Partial structure of the A chain of bonito insulin[603]



(*XXXVIII*) Glucagon

used to fragment the molecule, FDNB-labelling, hydrazinolysis and carboxypeptidase, for end group determinations[606–610].

Synthetic Approaches

Insulin

From the point of view of synthesis, the insulin molecule presents difficulties not encountered with the other compounds discussed in this review. The molecule is larger by twelve residues than that of corticotropin, but size alone is not the source of the problem. There are in the insulin molecule, six disulphide bonds. An unequivocal synthesis of this molecule would therefore necessitate the use of three different sulphur protecting groups: these would be capable of selective removal, two of them, at least, without the disruption of preformed disulphide bonds.

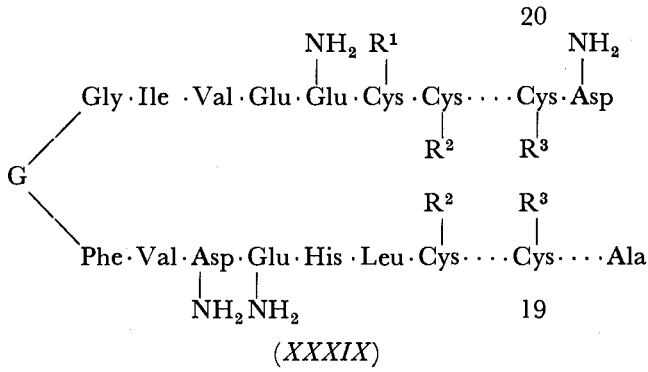
Several ingenious methods for thiol protection have been devised[29c,67,68] and these have facilitated the synthesis of a derivative of the A 6–12 sequence, containing an intact 6–11 disulphide bridge[30n,67].

Such selective protection of the thiol groups does not mean *per se* that the required disulphide bonds will be formed on their removal. Although unsymmetrical open-chain cystine compounds have been prepared[611–613], it is not at all certain that comparable methods could be used in the synthesis of more complex molecules. Unsymmetrical cystinyl compounds tend to be unstable and to rearrange to symmetrical compounds[614–617], so that formation of the first insulin interchain disulphide bond might give rise, either directly or after rearrangement, to two types of molecule: these would consist respectively of two A chains connected by a disulphide bridge and two B chains connected by a disulphide bridge.

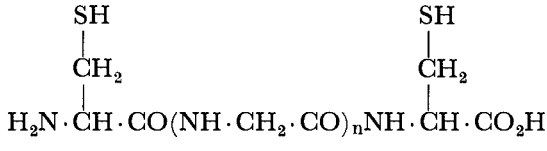
It has been suggested by Zervas[28f] that this problem might be avoided if the terminal amino groups of the A and B chains could be interconnected via a polyvalent amino protecting group (as in *XXXIX*), for example by the *p*-nitrobenzyl-phosphoryl, *p*-bromobenzyl-phosphoryl or *N*-benzyloxycarbonyl-L-glutamyl groups. In this event, formation of the first disulphide bond would give rise to a cyclic structure, for which, by analogy with the ring systems of oxytocin and vasopressin, some degree of stability might be predicted. The amino protecting group could be removed after the formation of the second interchain disulphide bridge.

This route is dependent on the practicability of forming the desired cyclic compound when the first disulphide bond is completed. Various degrees of polymerization might occur as well as, or instead of, the desired reaction. In oxidation studies with cysteinyl glycyl cysteine peptides (*XL*), only low yields of cyclic disulphide monomer with dipeptides to pentapeptides ($n = 0$–3)[618,619] were obtained whereas the hexapeptides to octapeptides ($n = 4$–6)[620] gave cyclic monomer almost exclusively. Similar results, including studies on the nonapeptide ($n = 7$) which behaves like the octapeptide, have been reported[621].

The proportion of monomer to polymer obtained in these reactions depends on the configuration of the reactant thiol compound. If the configuration of compound *XXXIX* is other than random, it is still possible that the desired cyclic disulphide will be unobtainable. However, in the model compounds, the effects of (1) increasing the number of residues which separate the



(*XXXIX*)



(*XL*)

cysteines and (2) varying the reaction conditions, for example, by the exclusion of metal ions[620], give reason to hope that this will not be the case. It is conceivable that the conformational preference for an insulin-like structure, as opposed to a polymer, might be greater if the interchain disulphide bridge distal to the *N*-terminal residues were formed first, or even if both interchain disulphide bridges were formed simultaneously. In the latter case, interconnection of the terminal amino groups, or an independent chain-link of another type, would be essential, because an anti-parallel alignment of the chains would otherwise be possible[618].

A compound which should prove useful for investigations of the final combination of the A and B chains has recently been reported by Meienhofer and Brinkhof[622]. This compound, produced by sulphitolytic cleavage of the disulphide bonds of natural insulin, a reaction which takes place preferentially with the interchain bonds, is the 6,11-disulphide-7,20-bisthiosulphate derivative of the A chain.

Several groups of workers[604,623–625] have shown that the separated A and B chains of insulin, which in themselves are inactive, may be recombined to give low yields (~1–2 per cent) of insulin. In anticipation of similar low yield final steps, several large fragments of the A and B chains have been synthesized with the sulphydryl groups protected non-selectively as benzyl derivatives. The philosophy of the synthesis of a natural product which entails a final stage of such dubious merit is clearly different to that involved in most classical natural product chemistry; nor is such a method readily adaptable to structure-activity studies. However, if activity of approximately the right order, even though it be only a few per cent, is obtained in these syntheses, it might be sufficient, in addition to ionophoretic and immunological evidence, to indicate that a synthetic insulin has been produced.

A partial synthesis of insulin by this approach has already been reported

by Katsoyannis, Tometsko and Fukuda[626a] who coupled synthetic sheep A chain to natural ox B chain with a recovery of 0·5–1·2 per cent of insulin activity. The activity could be destroyed by the use of anti-ox insulin serum. Synthesis of the B chain[627–633,648] are nearing completion.*

In syntheses of intermediates of both the A and B chains of insulin, terminal amino functions have been blocked almost exclusively by the benzyloxycarbonyl group. Exceptions are the use of the *p*-nitrobenzyloxycarbonyl[626a,634] and trityl[29c,67,68,635] groups. The *C*-terminal residue, in A chain intermediates, has been protected as its methyl or ethyl[636–641], benzyl[635,638,642a] or, *p*-nitrobenzyl[626a,642b,643] ester. In B chain intermediates, methyl or ethyl esters[627,629,630,632,633,644–649] have been used predominently and, more rarely, benzyl esters[650,651], primary amides[628] and salts[631,634]. Simple alkyl esters have generally proved satisfactory in these syntheses, but occasional difficulties have arisen, as in the saponification of benzyloxycarbonyl-threonyl-proline methyl ester (B 27–28), during which some hydrolysis of the peptide bond occurs[649].

Side-chain protection in the A chain has been concerned primarily with the cysteine residues in positions 6, 7, 11 and 20. Work involving the selective protection of the thiol groups at positions 6 and 11, with trityl groups, and in position 7, with a diphenylmethyl group, has already been mentioned[30n,67]. Apart from this example, the cysteine side chains have generally been blocked non-selectively as the benzylthioethers[626a,635,636,638,640–642a,652], although attempts at selective protection have been made using the benzylthiomethyl or benzyloxy-carbonyl groups at positions 6 and 11[639], the *p*-nitrobenzyl group at positions 7 and 20[639,642b,643,653,654] and the tetrahydropyranyl group at position 7[642a]. The glutamic acid residue in position 4 has been blocked as the γ-methyl[637] or γ-tertiary butyl ester[626a,636], that in position 17 as the γ-methyl[641] or γ-benzyl[626a,642b,643] ester.

The cysteine residues in the B chain have been protected only as the benzylthioether derivatives[628,630,633,645,646,648]. Glutamic-acid residues occur at positions 13 and 21 where they have been protected as γ-methyl[629,647], γ-ethyl[632,644] and γ-benzyl[628,630,631,645,648,650] esters. Pyrrolidone formation is a complicating factor in the hydrogenolysis of *N*-α-benzyloxycarbonyl γ-methylglutamyl peptides[647].

Additional side protection is required in the B chain intermediates because of the presence of basic amino-acid residues. The histidine residues at positions 5 and 10 have been protected as the benzylimidazole derivatives[632,633]. Tosylation[629,631,648] and nitration[627,645,647,650] have been used to block the arginine guanidino group (position 22). Tribenzyloxycarbonyl-arginine has been incorporated as an *N*-terminal residue at this position but removal of the *N*-α-benzyloxycarbonyl group proved impracticable[629]. Generally, the lysine side chain at position 29 has been blocked with the tosyl group[627,629,631,646–649] and on one occasion, the *p*-nitrobenzyloxycarbonyl group[634] was used for this purpose. Unlike the *N*-α-benzyloxycarbonyl group the *p*-nitrobenzyloxycarbonyl group in the N^{ε}-position was fairly resistant to the action of hydrogen bromide in acetic acid, so that some degree of selective protection was achieved.

* Since this section was completed the details of a total synthesis of insulin have become available[626b].

Synthetic intermediates of insulin are listed in *Tables 4.12* and *4.13*.

Table 4.12. Insulin A chain: residue sequences of synthetic intermediates

Sequences	*References*	*Sequences*	*References*
1–3	637	8–9	29c, 67, 68, 636, 642a
1–4	636	8–11	638, 642a, 652
1–5	637	8–12	29c, 67, 68
1–9	626a, 636	10–11	638, 639, 642a
1–21	626a	10–12	29c, 67, 68, 626a
2–3	637	11–12	29c, 67, 68, 626a
2–4	636	11–13	639
2–5	637	12–13	639
3–4	636	12–16	642b
3–5	637	12–21	642b
4–5	637	13–14	635, 641
5–9	636	13–16	626a, 635, 641, 642b
6–7	29c, 67, 68, 638, 642a	13–21	626a, 641
6–8	626a	14–16	642b
6–9	29c, 67, 68, 626a, 636	15–16	635, 641, 642b
6–11	638	17–18	641
6–12	29c, 67, 68	17–21	626a, 641, 642b, 643
7–8	639	18–19	635, 641
7–9	636, 639, 652	18–21	626a, 641, 643
7–10	639	19–20	641
7–10 pig and whale	640	19–21	641, 643
7–11	638, 652	20–21	626a, 635, 641, 643

Table 4.13. Insulin B chain: residue sequences of synthetic intermediates

Sequences	*References*	*Sequences*	*References*
1–8	633	17–19	628
2–8	633	17–20	645, 648
3–8	633	17–21	645
4–8	633	17–22	645
5–8	633	18–19	628
6–8	633	18–20	630, 646, 648
9–10	644	19–20	630, 645, 648
9–13	632, 644, 658	20–23	650
10–13	632	21–22	645, 647
11–12	628, 644	21–23	631, 650
11–13	632, 644	21–30	629, 631, 647, 648, 660
11–19	628	22–23	627, 631, 650
12–13	632	22–30	629
13–17	630	23–24	634, 646, 649, 651
13–19	628	23–25	651
13–20	630, 648	23–26	634, 646, 651
14–15	659	23–27	649
14–17	630	23–29	634
14–19	628	23–30	629, 646, 649
14–20	630, 648	24–30	627, 629, 631
14–30	648	25–26	634, 646, 649
15–17	630	25–27	649
15–19	628	25–30	627, 631
15–20	648	26–30	627, 631
16–17	630	27–29	634
16–18	659	27–30	627, 631, 646
16–19	628	28–29	634
16–20	648	28–30	627, 631, 646, 649
17–18	645	29–30	627, 646, 649

For the stepwise synthesis of units of both the A and B chains of insulin, *p*-nitrophenyl ester couplings have been widely used[627,628,630,632,633,636,642b,643,648] whilst *N,N'*-dicyclohexylcarbodi-imide, azides, and, more rarely, mixed anhydrides and reagent K[648] have been used for coupling peptides. Counter-current distribution and column chromatography have been used in purification procedures, but considerable difficulty is experienced in attempts to purify large peptides related to the B chain because of their insolubility.

Glucagon

Syntheses of several fragments of the glucagon sequence have been described[30f,655–657].

Biological Activity and Methods of Assay

The mode of action of insulin has been discussed by Tuppy[661]. Insulin has an effect of carbohydrate[581c], fat[581d] and protein[581e,662] metabolism and is assayed by techniques dependent on these activities. The principal bioassays are concerned with the production of hypoglycaemia in rabbits and convulsions in mice, with glucose and C^{14} amino-acid uptake by rat diaphragm segments, and with changes in metabolism of the rat epididymal fat pad. These methods have been reviewed by Smith[202d] and by Stewart[581f]. Methods for the estimation of glucagon have been reviewed by Bromer and Behrens[202e].

Structure–Activity Correlations

Some structure–activity correlations have been deduced for the insulin structure both by comparing the structures of insulins derived from different species and by studying the biological effects of chemical modification of natural insulins.

Despite the considerable differences in structure between mammalian insulins and those derived from cod and bonito, the biological activities of these compounds are the same. So far as the A chain is concerned, close similarities between bovine and bonito insulin are confined to the *N*-terminal dipeptide, the positions of the cysteine half residues in the chain and the *C*-terminal heptapeptide[603]. These features are constant in all insulins yet studied. The B chain of insulin shows species variations which involve both *N*-terminal and *C*-terminal residues as well as residues within the sequence.

Most commercial crystalline samples of insulin contain two active components, one possessing 6 and one 5 primary amide groups[663–667]. Desamido-insulin probably contains 85–90 per cent of insulin in which the β-amide group of the A chain *C*-terminal asparagine residue has been removed during the isolation procedure[667]. The rest of the material contains moieties in which the amide group has been lost from other residues. Acid treatment of insulin has been shown to involve the loss of amide groups progressively from 6 amide centres, but preferentially from the *C*-terminal asparagine residue[668].

Although the β-amide group of the *C*-terminal asparagine residue may be removed, studies with carboxypeptidase digestion confirm the impression, obtained from species comparisons, that the residue in this position is necessary for insulin-like activity. Whereas desalanine-desamido-insulin[667], in

which the B chain *C*-terminal alanine residue has been removed, has 60 per cent of the activity of insulin in the mouse convulsion test, desalanine-desasparagine-desamido-insulin[669], in which the A chain *C*-terminal asparagine residue is also removed, has less than 5 per cent.

Trypsin reacts with insulin to cleave alanine and a heptapeptide from the *C*-terminal sequence of the B chain. The remaining molecule, desoctapeptide-insulin[670,671], is virtually devoid of insulin-like activity. This evidence suggests that a site essential for biological activity is to be found in the B23–29 sequence of the insulin molecule.

Sequences near the amino end of the chains are not of such great significance. Smith, Hill and Borman[672] have shown that insulin degraded at least to the A6 and B7 residues by digestion with leucine amino peptidase retains 44 per cent of the activity of the parent insulin. Whether the partial loss of activity is due to some cleavage of one of the cysteinyl (A6 or B7) residues or whether it is associated with the removal of any particular residue, is not known. That the terminal amino groups of insulin are not essential for activity is confirmed by experiments in which they are converted to carbamoyl[673,674] or guanidino[675] derivatives without loss of activity.

Clinical Applications

Insulin has several clinical uses, but its most important application, by far, is in the control of diabetes mellitus. This is too large a topic to be considered in any detail in this review, but some disadvantages of insulin therapy will be discussed in the light of recent structural studies. The principal disadvantages of insulin therapy are: (1) that administration is by injection; (2) that insulin is antigenic; (3) that insulin is destroyed rapidly in the circulation.

Administration

Insulin, in the treatment of diabetes mellitus, is administered by injection once or twice daily. A preparation which was active by mouth would have obvious advantages and many attempts have been made to achieve this[676]. Although insulin is destroyed by the enzymes of the gastrointestinal tract, there is evidence that, provided such inactivation can be prevented, some absorption can occur[677,678]. However, such absorption is small and variable in amount. These difficulties may be overcome by the synthesis of an active insulin molecule of reduced size (to increase rate of absorption) and modified chemically (to render it resistant to chemical attack). From the work discussed above, there is reason to hope that this possibility may be realizable.

Immunology

Insulin-binding antibodies are generally detectable in patients who have been treated with commercial insulin preparations, but are never observed in patients who have not received insulin therapy. Local and generalized allergic reactions to insulin can usually be controlled by the use of highly purified insulin or by treatment with antihistamines, but, much more serious in certain patients, is the production of insulin-binding antibodies associated with the development of insulin resistance[581g].

The probable importance of the secondary structure of the molecule to the antigenicity of insulin[600] makes attempts to produce non-antigenic material

more difficult. Desalanine-pork-insulin, for example, which has the same residue sequence as human insulin except for the absence of the *C*-terminal residue of the B chain, is bound as well as pork-insulin by antisera of patients immunized with pork-insulin[679].

Although the insulin molecule can be reduced in size without loss of activity, it is unlikely that it will be possible to reduce it to a size where it is no longer antigenic. On the other hand, with a knowledge of the antigenic sites of the molecule, it might be possible to abolish the antigenic reaction. This possibility is dependent on the fact, clearly established by species comparisons, that the antigenic groups in the insulin molecule are different than the groups responsible for its antidiabetic activity.

Longevity of action

Insulin is deactivated in the circulation, principally in the liver, but also in kidney, muscle and other tissues[581h]. The exact mechanism of inactivation is not known but there is considerable evidence for the presence of disulphide-cleaving and exopeptidase systems in the liver. Ultimately, long acting insulin preparations might be produced by the incorporation of specific inhibitors of these enzyme systems, or by modification of the insulin molecule at the points of enzymic attack. The difficulties inherent in the use of the available insulin preparations have been discussed by Oakley[581a].

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5

ANALGESICS AND THEIR ANTAGONISTS: BIOCHEMICAL ASPECTS AND STRUCTURE–ACTIVITY RELATIONSHIPS

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In Volume 2 of this series, the present authors reviewed the pharmacological testing procedures available for the identification and assay of analgesics and described the development of the various classes of analgesic drugs. The present review is intended to be complementary to the earlier work and comprises the following additional aspects of analgesics: (*a*) estimation of analgesics in biological media; (*b*) absorption, distribution and excretion of analgesics; (*c*) metabolism of analgesics; (*d*) antagonists of analgesics; (*e*) relation of morphine-type analgesics to adrenergic and cholinergic mechanisms and other biochemical processes; and (*f*) structure–activity relationships.

ESTIMATION OF ANALGESICS IN BIOLOGICAL MEDIA

The two most commonly applied procedures for the determination of analgesics in biological media are the indicator-dye and tracer methods. The former depends on the fact that many organic bases combine with dyes such as methyl orange to form complexes that are readily soluble in organic solvents and sparingly soluble in water[1]. All organic bases are first separated from biological material by extraction with benzene (or ethylene dichloride) at an alkaline pH. The organic phase is then shaken with aqueous methyl orange at pH 5 when the dye dissolves in the organic solvent through complex formation in an amount proportional to the base concentration. The benzene solution is shaken with aqueous mineral acid to decompose the complex, and the dye—now in the aqueous phase—is determined photometrically. Since any amine which forms a solvent-soluble dye complex is measured together with the administered compound, it is essential to assess the specificity of the procedure. This is done by determining the distribution of the base in the organic solvent–water system over a range of pH values and comparing results with those obtained for the base to be analysed. The dye method is highly specific when used in conjunction with counter-current distribution[2]. Extraneous bases are normally metabolites which, being more water-soluble than the original drug, may often be removed by washing the total base extract with a suitable buffer prior to reaction with dye.

In methods based on tracer techniques, drugs labelled with radio-active isotopes are used; for example, N–$^{14}CH_3$ labelled pethidine and morphine have been made from the corresponding norcompound and ^{14}C-formaldehyde-formic acid[3,4], and 2–^{14}C methadone from methadone cyanide and $CH_3{}^{14}CH_2$ magnesium bromide[5]. The separation of unaltered drug from radioactive metabolites is facilitated by diluting tissue extracts with known

amounts of non-radioactive drug that acts as carrier during purification (reverse isotope dilution method). By this process, N–$^{14}CH_3$ morphine has been recovered from biological media as the crystalline dinitrophenyl derivative (its purity was established by its powder X-ray diffraction pattern) and has been detected in plasma at concentrations as low as 0·028 μg/ml[6].

ABSORPTION, DISTRIBUTION AND EXCRETION OF ANALGESICS

Morphine and other analgesics, administered parenterally as aqueous solutions of their salts, rapidly disappear from the injection site and their pharmacological or chemical effects are detected a few minutes after injection; peak effects may not occur until after one hour[7]. Passage of the drug from the injection site to the blood probably occurs chiefly by physical processes, bases penetrating tissue as lipoid soluble undissociated molecules. Absorption rates are thus dependent upon dissociation constants and partition coefficients of the bases. Basic drugs (pK_a'8 and above) are almost completely ionized in acid conditions and are thus poorly absorbed from the stomach. Irregularities in the absorption of analgesics after oral, in contrast to parenteral, administration is thus probably due to the fact that their rates of absorption are not appreciable until they reach the lower intestine, where a greater proportion of lipid-soluble un-ionized molecules exists.

The distribution of analgesics within the body follows the same pattern as that found for other basic drugs; the bases rapidly leave the blood and concentrate in parenchymatous and reticulo-endothelial tissue. This behaviour contrasts markedly with that of acids which have a limited ability to penetrate cells, tending to be distributed extracellularly, and is attributed to the significant amounts of un-ionized base that exist at physiological pH (7·2) enabling uptake by lipoid material. Thus the pK_a' values of most analgesics lie within the range 7·8–8·9, corresponding to 2–14 per cent of un-ionized drug at pH 7·2[8]. Studies on the distribution of analgesics, summarized by Way and Adler[7], show that they accumulate in organs such as the lung, spleen, kidneys, liver, adrenals and thyroid gland. The appreciable but comparatively lower concentrations found in the brain and C.N.S. indicate a significant blood–brain barrier to analgesics (see *Table 5.1* for results on pethidine distribution[9]). Correlations between concentrations in the C.N.S. and analgesic effect (tail reaction time to thermal stimulus) have been reported[10].

The respiratory depression commonly seen in infants delivered from mothers who have been given analgesics indicates that such drugs may pass the placental barrier[11]. Direct evidence of passage has been obtained with pethidine which was detected in the urine of newborn infants[9].

Distribution studies of morphine and other analgesics within the C.N.S. have been made. Radio-autographs of brain sections taken from rats injected with labelled morphine show concentrations of radio-activity in the vascular choroid plexus and ventricles rather than in the neural elements of the brain[10]. Such measurements, however, are still relatively crude in terms of molecular drug concentrations at receptor sites.

Table 5.1. Distribution of pethidine in rats after intraperitoneal administration[9] (mg/kg fresh tissue)

Dose schedule	*Blood*	*Lung*	*Liver*	*Kidney*	*Spleen*	*Brain*	*Heart*	*Muscle*
0 hr 50 mg/kg 1 hr 50 mg/kg 2 hr sacrificed	3	11	34	46	58	17	10	7
0 hr 50 mg/kg 1 hr 50 mg/kg 3 hr sacrificed	2 3 4 5 <2	20 28 20 14 19	16 19 14 11 14	110 57 43 22 20	21 27 60 16 29	10 8 23 14 12	6 15 8 7 10	5 4 8 4 4
0 hr 50 mg/kg 1 hr 50 mg/kg 5 hr sacrificed	<2 <2	<2 <2	<2 <2	<2 <2	3 <2	<2 <2	<2 <2	<2 <2

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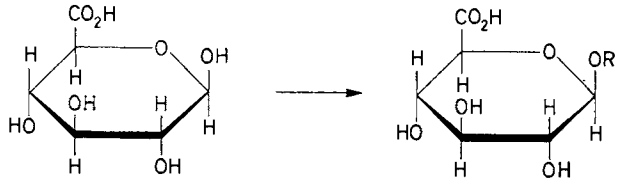
The rate of excretion of analgesics in the urine is most rapid soon after drug administration when plasma levels are at or near peak values. Tissue localization results in a rapid fall in analgesic plasma concentrations and excretion becomes very slow. Generally 5–10 per cent of the administered dose appears unchanged in the urine after 24 hours. Analgesics are chiefly excreted in the urine as more soluble metabolized products. Disposal of analgesics from the intestinal tract is minor; the small amounts of morphine and methadone found in faeces appear to be a result of biliary excretion[7].

METABOLISM OF ANALGESICS

The action of analgesics is chiefly limited by their biotransformations within the body. The main processes involved, namely conjugation, *N*- and *O*-demethylation and hydrolysis, are, with some exceptions, detoxification mechanisms since the metabolic products are usually pharmacologically less active and more easily excreted than the parent compounds.

Conjugation

The amounts of drug recovered from the urine of animals which have received morphine is considerably increased if the urine is subjected to acid hydrolysis prior to extraction. Such experiments establish the presence of 'bound' morphine. Conjugation is a well-known metabolic pathway and commonly involves combination of hydroxyl-containing molecules with



(I) *(II)*

glucuronic acid (*I*) to give glucuronides (*II*)[12]. Proof that 'bound' morphine is a glucuronide is now available. Urine from a dog infused with morphine,

when chromatographed and sprayed with an alkaloid reagent, produced two spots, one of which corresponded with morphine. The other was eluted, hydrolysed and rechromatographed. Treatment with the alkaloid reagent revealed one spot corresponding with morphine while a sugar reagent produced spots corresponding with glucuronic acid and glucuronolactone[13]. 'Bound' morphine that had the correct elemental analysis of morphine monoglucuronide dihydrate was also isolated from dog bile[14]. A negative test for free phenolic hydroxyl showed that this group was involved in the conjugate link. The conjugate was pharmacologically inert in dogs and mice, and failed to alleviate the severity of the abstinence syndrome in addicted monkeys during withdrawal of the drug[14]. Crystalline 'bound' morphine with infra-red absorption similar to that of morphine monoglucuronide dihydrate from dog urine was isolated from the urine of human addicts by Fugimoto and Way[15]. The phenolic hydroxyl was masked (ultra-violet absorption evidence) and the conjugate had two pK_a values which showed it to be a zwitterion (the conjugate linkage is β since hydrolysis may be effected using β-glucuronidase).

Some workers consider that more than one form of 'bound' morphine exists. Thompson and Gross[16], for example, differentiated 'bound' morphine from dog urine into two fractions, one more easily hydrolysed than the other, while Woods[14] isolated a second amorphous form of 'bound' morphine from dog urine which he considered was a diconjugate. However, chromatographic evidence and the fact that the crystalline conjugate may be obtained from the amorphous form indicate that only one form of 'bound' morphine is excreted in the urine[15].

Codeine[17], levorphanol (Dromoran)[18] and nalorphine[19,20] are conjugated *in vivo* and *in vitro*; bound forms have not been isolated but are probably glucuronides. Thus Axelrod and Inscoe[21] noted the disappearance of these substrates when incubated with liver microsomes and uridine diphosphate (UDP)-glucuronic acid. The same drugs, given to guinea pigs, were isolated from urine after its treatment with β-glucuronidase. Results with codeine showed that the secondary 6-hydroxyl group may serve as the site of conjugation when the phenolic hydroxyl is blocked.

Pethidine itself cannot be conjugated in any manner so far recognized but the presence in human urine of unidentified 'bound' forms of its metabolites, meperidinic and normeperidinic acid have been established[22]. Pethidine and norpethidine amounts increase after urine is esterified; these levels increase even more if the urine is hydrolysed prior to esterification. Acetylation of the primary aromatic amino group of anileridine has been established in various species, the conjugate being excreted as such and as the de-esterified form[23].

Enzymes located in liver microsomes are responsible for glucuronide formation, this process being closely allied to carbohydrate metabolism. Dutton and Storey[24] found that *o*-aminophenol is conjugated with glucuronic acid by an enzyme in liver microsomes that transfers glucuronic acid from uridine diphosphate-glucuronic acid to the phenolic acceptor. The formation of UDP-glucuronic acid is catalysed by an enzyme that oxidizes UDP-glucose and requires diphosphopyridine nucleotide (DPN^+) as the hydrogen acceptor.

N-Dealkylation

A metabolic route involving the *N*-methyl group of pethidine was established by the detection of $^{14}CO_2$ in the expired air of rats given N–$^{14}CH_3$ labelled pethidine[2] and by the identification (by counter-current distribution studies) of norpethidine in the rat's urine. In man, the same metabolic pathway has been proved by the isolation of norpethidine hydrochloride from human urine[22,25]. Plotnikoff, Way and Elliott[22] found that demethylated products (norpethidine, normeperidinic acid and its conjugate) in human urine accounted for approximately 30 per cent of the administered dose of pethidine, a result which emphasizes the importance of this metabolic pathway in man. These results have been confirmed by *in vitro* studies, norpethidine and formaldehyde being obtained after the incubation of pethidine with rat liver and rat brain preparations[26–28].

In contrast to studies on pethidine, evidence for the *in vivo* *N*-demethylation of morphine has proved more difficult to obtain and, until recently, rested mainly upon the detection of expired $^{14}CO_2$ after administration of N–$^{14}CH_3$ labelled drug[29,30]. In man, pulmonary excretion of $^{14}CO_2$ ranged from $3\frac{1}{2}$ to 6 per cent of the injected dose of N–$^{14}CH_3$ morphine in 24 hours, most of the recovered radioactivity being found in the urine.

Discrepancies between the amounts of expired $^{14}CO_2$ and nor-compound recovered are possibly due to uptake of the detached methyl group into the general body carbon pool from which radioactive carbon is slowly released by catabolic processes[17]. Transmethylation processes in which a $^{14}CH_3$ group is displaced by an endogenous methyl group may further invalidate expired $^{14}CO_2$ as a measure of *N*-demethylation. In 1961, however, normorphine was identified as a biotransformation product of morphine in the rat. Misra, Mulé and Woods[31,32], using tracer techniques, have demonstrated the *in vivo* formation of normorphine in the rat as a metabolite of tritium nuclear-labelled morphine. Urine from rats administered with tritium-labelled drug was autoclaved and the bases extracted after the addition of unlabelled normorphine as carrier. Chromatographic procedures showed radioactivity to be associated with two spots, with R_f values corresponding to tritium-labelled morphine and unlabelled normorphine respectively.

Milthers[33,34] has recently reported the quantitative recovery by paper chromatography of normorphine from the urine and faeces of morphine-treated rats. The total amount of normorphine excreted was 8–9 per cent of the dose of morphine given. Working with rats deprived of all abdominal organs except the urogenital organs, she obtained results indicating that *in vivo* transformation of morphine (and nalorphine) into normorphine may occur in the brain[35]. Normorphine amounted to 1 and 5 per cent of total morphine found in blood and brain, respectively.

In vitro evidence of the biotransformation of morphine to normorphine is also available. Under aerobic conditions $^{14}CO_2$ was released from rat liver slices incubated with N–$^{14}CH_3$ morphine as substrate[29]. Axelrod[26,27] obtained formaldehyde, the immediate precursor of carbon dioxide, when various analgesics, including morphine, were incubated with liver microsome preparations and identified normorphine as a metabolite by paper chromatography.

Evidence of *N*-demethylation in the brain was obtained by Elison and Elliott[36] who detected $^{14}CO_2$ when analgesics labelled with ^{14}C were incubated with brain slices of the rat. Brain tissue was much less active than liver but exhibited a qualitatively similar behaviour (e.g. pethidine and codeine were demethylated to a larger extent than morphine, while male tissue was more effective than female), a result suggesting that the demethylating enzymes from the two sources are similar.

Evidence for the *N*-demethylation of levorphanol and dextrorphan is similar to that relating to morphine. In monkeys 20 per cent of the dose of *N*–$^{14}CH_3$ labelled levorphanol was accounted for as expired $^{14}CO_2$, while in rats and dogs the values were 5 and 1 per cent, respectively[37]. Norderivatives have not been isolated[38] although free and bound 3-hydroxymorphinan have been detected in urine after its administration to dogs. Formaldehyde was isolated when levorphanol or dextrorphan were incubated with rat or mouse liver preparations[27,39] but none was obtained when dog liver was used.

Evidence for the *in vivo* *N*-demethylation of analogues in which the 3-hydroxyl group is masked has also been obtained. After administration of *N*–$^{14}CH_3$ labelled codeine to man, $^{14}CO_2$ was detected in expired air and norcodeine (largely conjugated) isolated from urine in amounts equivalent to 10 per cent of the dose[17]. Similarly 3-hydroxymorphinan has been identified in dog urine after administration of 3-methoxy-*N*-methylmorphinan[40].

In *in vitro* studies using rat or mouse liver preparations, substrates with masked phenolic hydroxyl groups or lacking phenolic groups are demethylated more rapidly than free phenols[27] (see *Table 5.2*). With the 3-methoxy analogues of morphine and levorphanol, these results suggest simultaneous *O*- and *N*-demethylations. Takemori and Mannering[39], however, found that when a single compound possessed both *N*- and *O*-methyl groups, the amount of demethylation occurring was greater than the sum of that obtained with its two analogues bearing only the *N*-methyl or *O*-methyl group. Furthermore, the more rapid demethylation of 3-ethoxymorphine (Dionine) over that of morphine (the aldehyde assay is specific for formaldehyde) and the isolation of norcodeine and morphine in the ratio 6 : 1 after codeine demethylation, shows that increased rates are due primarily to *N*- rather than *O*-demethylation. The rate of demethylation of *N*-methylmorphinan, which lacks a phenolic hydroxyl, is similar to that of codeine. Thus the free

Table 5.2. In vitro demethylation of analgesics by rat liver microsomes[27]

Substrate	*Relative activity*
Morphine	100
Codeine	195
(−)-3-Hydroxy-*N*-methylmorphinan	75
(−)-3-Methoxy-*N*-methylmorphinan	280
(+)-3-Hydroxy-*N*-methylmorphinan	25
(+)-3-Methoxy-*N*-methylmorphinan	160
(−)-Methadone	130
(+)-Methadone	60
Pethidine	165

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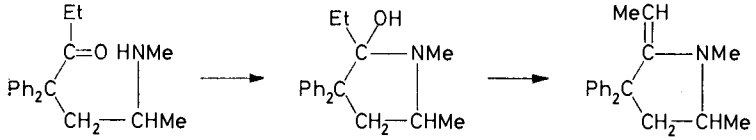
phenolic hydroxyl group of both morphine and 3-hydroxymorphinan retards *N*-demethylation. It may be significant that these *in vitro* results correlate with the established importance of *N*-demethylation as an *in vivo* metabolic route for analgesics that either lack phenyl hydroxyl groups (for example, pethidine) or have etherified phenolic groups (for example, codeine), and with the evidence that indicates the same pathway to play a less important role in the metabolism of phenolic analgesics such as morphine and levorphanol.

Metabolic *N*-demethylation of methadone occurs since incubation of (—)-methadone with rat liver preparations results in the formation of formaldehyde at a rate only somewhat less than that obtained with pethidine as substrate[27] (see *Table 5.2*). An unidentified basic metabolite was found in rat bile[41] and was shown to comprise most of the original molecule by tracer studies using methadone labelled at position 2 adjacent to the carbonyl group[10]. Chemical attempts to prepare nor-compounds that should result from demethylation of methadone and related substances have failed[8,42] since the nor-compound (*III*) may undergo further reaction giving the pyrrolidine (*IV*) or (*V*). Pohland[43] has synthesized the pyrrolidine (*V*) which has a very similar infra-red spectrum to that of the metabolite of methadone isolated from rat bile.



(III) *(IV)* *(V)*

Evidence for the metabolic *N*-demethylation of propoxyphene[44] comprises the *in vivo* and *in vitro* liberation of $^{14}CO_2$ in the rat after administration (or use as substrate) of labelled drug and the isolation from human urine of a crystalline dinitrophenyl compound presumed, from analytical and infra-red absorption evidence, to derive from de-*N*-methylpropoxyphene.

Few reports are available on the dealkylation of analgesics which bear groups other than methyl upon the basic centre. Nalorphine, which inhibits the *N*-demethylation of analgesics by liver preparations (see p. 178), is itself dealkylated in the same system, as shown by the identification of normorphine in the residual substrates[45]; its rate of dealkylation is more than twice that of morphine. As already noted, Milthers[35] isolated normorphine from the brain of rats given nalorphine. Mannering and Schanker[46] demonstrated the *in vitro* and *in vivo* biotransformation of levallorphan into 3-hydroxymorphinan. Evidence that the 2-*p*-aminophenethyl nitrogen substituent of anileridine is cleaved during metabolism is provided by the isolation from rat urine of a fraction that is either identical or very similar to *p*-acetylaminophenylacetic acid[23].

Schaumann[47] has proposed methylation, in contrast to demethylation, as a metabolic pathway for methadone. Using paper chromatographic methods he established the *in vitro* conversion of methadone into a methyl quaternary compound by liver slices. Further, Clouet[48] has demonstrated

that the nor-derivatives of morphine and codeine are *N*-methylated *in vitro* by liver and brain tissue preparations using *S*-adenosylmethionine as a methyl donor, and *in vivo* in brain with methionine-(^{14}C-methyl). An enzyme, isolated from rabbit lung, that transfers a methyl group from *S*-adenosylmethionine to the amino group of serotonin has also been found capable (in lesser degree) of methylating normorphine, norcodeine and norpethidine[49].

Enzyme Systems for N-Demethylation

Axelrod[27] studied an enzyme system in liver preparations of various species that was responsible for the demethylation of morphine-type analgesics; that from rat liver required oxygen and TPN^+ and operated most effectively at a pH of 7·0–7·5 using phosphate buffers (for mouse liver, the optimal pH is 7·9–8·1[39]). Rat liver was separated into nuclear, mitochondrial, microsomal and soluble fractions by differential centrifugation and each fraction examined for its ability to demethylate narcotics. Negligible activity was observed in each cellular fraction alone. However, when the microsomal and soluble fractions were combined, extensive demethylation took place. The low activity of whole homogenate was traced to the presence of thermolabile inhibitory factors in the nuclei and mitochondria. Microsomal preparations from kidney, brain, muscle and spleen had no demethylating activity. The importance of the soluble fraction appears to be partly due to a reducing factor since reduced TPNH was found to be half as effective as the soluble fraction and TPN^+ combined. Marked species differences occur in the demethylating activities of liver preparations. The ability of rabbit liver to demethylate *laevo*-methadone and pethidine was greater than that of rat liver; with morphine as substrate, the reverse was true. The results suggest the presence of more than one demethylating enzyme for narcotic drugs. In mice, activity differences extend to strains, some having high and some negligible activity. Marked sex differences in demethylating ability were found in the rat, liver preparations from males being more effective than those from females (a result confirmed by other workers[50]). In males, enzyme activity was depressed after administration of estradiol while females, treated with testosterone, showed increases in activity. The kinetics of demethylation have also been studied; the initial rate is rapid and linear, but after 15–20 minutes the rate decreases and reaction ceases after 2 hours[39]. The enzyme system demethylates morphine, morphinan, pethidine and methadone type analgesics. The retarding effect of a phenolic hydroxyl group has already been discussed. Axelrod[27] noted that *laevo*-isomers of 3-hydroxy-*N*-methylmorphinan, 3-methoxy-*N*-methylmorphinan and methadone were demethylated by rat liver 2–3 times more readily than the corresponding dextro enantiomorphs (see *Table 5.2*). Takemori and Mannering[39] found that mouse and rat liver preparations showed less substrate stereospecificity; although the demethylation of (—)-3-methoxy-*N*-methylmorphinan was markedly greater than that of the dextro isomer, levorphanol and dextrorphan were demethylated at equal rates. A recent report[50] has confirmed Axelrod's findings in this respect. Axelrod and Cochin[45] found that the demethylation of morphine and other analgesics by rat liver preparations was inhibited by nalorphine in a degree proportional to the latter's

concentration. Nalorphine itself underwent dealkylation in this system but its inhibitory effect was shown to be non-competitive, indicating that the enzymes concerned were different. Morphine demethylation by rabbit liver preparations was not inhibited by nalorphine. Inhibition of both the mouse and liver demethylation of *laevo*- and *dextro*-isomers of morphinan type analgesics was caused in equal degree by (—)- and (+)-3-hydroxy-*N*-allylmorphinan[39].

Axelrod[26] has drawn attention to certain features common both to the enzymes in rat liver which *N*-demethylate analgesics and to the receptor sites proposed for these drugs; they interact with the same substrate, they show in some cases similar degrees of stereospecificity, and they have actions that are inhibited by nalorphine. Further, chronic administration of morphine to rats resulted in a profound reduction in the ability of liver enzyme preparations from these animals to *N*-dealkylate morphine, nalorphine, hydromorphone (Dilaudid) and pethidine (*N*-demethylation of cocaine was unaffected)[26]. These animals showed a diminution of analgesic response to a test dose of morphine. Nalorphine, administered daily with morphine, partially reversed the effect produced by morphine. Both nalorphine and normorphine shared with morphine the property of depressing both enzymatic demethylation and analgesic response, but to lesser degrees. Chronic administration of levorphanol was also very effective in depressing the rat liver *N*-demethylation of several morphine- and morphinan-type analgesics while dextrorphan was relatively ineffective[39]. The observed depression was not reversed by simultaneous administration of nalorphine or levallorphan. Recent data[51], however, indicates that morphine may be a non-specific inhibitor of drug metabolism by virtue of its prevention of ACTH release.

Deuteration of the *N*-methyl group of morphine causes a reduction in both analgesic potency and rate of *in vitro* demethylation[50], a finding in accord with, but not proving, a relationship between *N*-demethylation and analgesic action (see Way and Adler[7] for a criticism of Axelrod's proposal that the liver *N*-demethylating system serves as a model for the study of analgesic receptor sites).

Many other substrates are known to be *N*-dealkylated by the TPNH-oxygen-liver microsome system[52] but the precise mechanism of the reaction has not been established. Similarly located enzymes that effect *O*-dealkylation[53], hydroxylation, deamination and side chain oxidation also require oxygen and TPNH[52]. These requirements at first suggested that the various oxidative pathways concerned were carried out by transfer of hydrogen from substrates, activated by dehydrogenase systems, with TPN acting as a hydrogen acceptor. The finding that reduced TPN or a reduced TPN generating system was actually required[27] showed that an unusual oxidative mechanism was involved in which the pyridine nucleotide is already in a reduced form and therefore unable to accept hydrogen. Gillette, Brodie and La Du[54] demonstrated the presence of an oxidase (TPNH oxidase) in liver microsomes which catalysed the oxidation of TPNH to TPN even in the absence of substrate. A product of this oxidation was found to have hydrogen peroxide-like properties and this material may be implicated in the various metabolic pathways for drugs mentioned above. All these enzymes are

inhibited by diethylaminoethyl 2,2-diphenylpentanoate (SKF 525-A)[52]. The

$$C_3H_7 \cdot CPh_2 \cdot CO_2(CH_2)_2NEt_2$$
SKF 525-A

oxidative sequence leading to demethylation may be formulated in two ways: (1) direct substitution of a hydroxyl group for an alkyl hydrogen with subsequent hydrolysis[55]; or (2) oxidation of the tertiary amino group to

$$R_2NMe \rightarrow R_2N \cdot CH_2 \cdot OH \rightarrow R_2NH + HCHO$$

an amine oxide followed by rearrangement to a carbinol and subsequent hydrolysis[56,57]. Tertiary amine *N*-oxides are known to undergo the latter

$$R_2NMe \rightarrow \underset{\underset{O}{\downarrow}}{R_2NMe} \rightarrow R_2N \cdot CH_2 \cdot OH \rightarrow R_2NH + HCHO$$

sequence under mild conditions (pH 5–7, 38°C) when catalysed by a ferric ion-tartrate complex[58].

O-Dealkylation

O-Demethylation is well established as a metabolic pathway for codeine. Pulmonary excretion of $^{14}CO_2$ occurred both in the rat and in man receiving O–$^{14}CH_3$ labelled drug. Morphine was isolated from human urine as the dinitrophenyl ether identified by its X-ray diffraction pattern[17]; its release has also been noted in the monkey[59], dog[60] and rat[61–63]. Codeine is also converted to morphine by liver preparations derived from several species[39,53,63] and it has been suggested that the pharmacological activity of codeine is due to morphine released from the parent drug during metabolism. Way and Adler[7] consider this to be unlikely since, among other reasons, the pharmacological effects of large doses of codeine in man are much less than those which result from the amounts of morphine known to be released metabolically. Recent evidence, however, indicates that the pharmacological effects of codeine are achieved only after release and distribution of its demethylated metabolites. Adler[64] measured the analgesic properties of codeine, norcodeine and morphine in the mouse after intraventricular injection (thereby achieving a closer approximation to intrinsic potency than is possible by the usual routes of administration) and found the potency of codeine to be much less than that found after normal administration of the drug. Further, Jóhannesson and Schou[65] found that the concentrations of morphine in the brain of rats given either codeine or morphine in doses resulting in equal degrees of analgesia were similar.

Both *O*- and *N*-dealkylated products have been detected by paper chromatographic procedures in dog urine after administration of dextromethorphan[40], while evidence of the *in vitro* *O*-dealkylation of 3-methoxy and 3-methoxy-*N*-methylmorphinan is available[39].

Hydrolysis

Hydrolysis is an important metabolic pathway in all analgesics that are esters and may be considered a detoxification process in cases where the significant products are acids, since these are rapidly excreted in the urine.

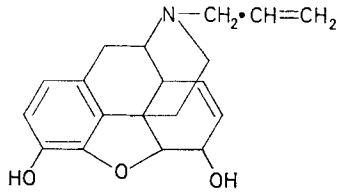
Bernheim and Bernheim[66] incubated pethidine with tissue preparations and sodium bicarbonate and used the volume of carbon dioxide released as a measure of the degree of hydrolysis. The liver was found to be the chief site for hydrolysis and the responsible enzymes were shown to differ from known tropine esterases, cholinesterases and esterases which hydrolyse aliphatic esters. Blood levels of pethidine in rats were increased after partial hepatectomy[67]. The presence of meperidinic acid in rat and human urine was established by the demonstration of increases in pethidine levels after esterification[22]. Similarly, normeperidinic acid was shown to be present in human urine[22]. These acids also occur in bound forms. No normeperidinic acid was detected in the urine of human subjects given meperidinic acid[25]; from this and other evidence Way and Adler[7] conclude that normeperidinic acid derives mainly from the hydrolysis of norpethidine and that meperidinic acid is not *N*-demethylated to any significant degree in the body. The acid derived from anileridine together with its *N*-acetyl conjugate has been reported in guinea pig, human and rat urine, esters and acids being separated by extraction procedures[23]. Hydrolytic products resulting from the hydrolysis of ethoheptazine have also been reported in the urine of several species[68].

Complete hydrolysis of heroin gives morphine while partial hydrolysis gives 6-monoacetylmorphine (6-MAM); the 3-acetyl group is particularly susceptible to hydrolysis in the presence of acid or alkali whereas the 6-acetyl group is relatively stable[69]. Wright[70,71], using manometric methods, found that various tissues from the rabbit and the rat could deacetylate heroin at both the 3- and 6-positions but that 6-MAM caused evolution of carbon dioxide much more slowly than did heroin. Until recently, morphine has been the only hydrolysis product isolated after heroin metabolism under both *in vivo* and *in vitro* conditions. Oberst[72], from the results of work on addicts, concluded that heroin was completely hydrolysed to morphine in the body. However, Way and his colleagues[73], using analytical techniques that minimized hydrolysis of metabolites during their estimation, established the conversion of heroin to both 6-MAM and morphine. These substances were identified by paper chromatographic methods in extracts of various tissue homogenates incubated with heroin. Deacetylation of heroin to 6-MAM and morphine was found to occur most rapidly and to the greatest degree in the liver. 6-MAM was further characterized by counter-current distribution of an extract of whole mouse homogenate incubated with heroin; from the same extract a crystalline substance was isolated that gave an infra-red spectrum identical to that of synthetic 6-MAM. Absorption of heroin and its subsequent disappearance from the animal body were both found to be very rapid. The heroin disappearance was accompanied by a rapid appearance of 6-MAM in the brain, followed shortly after by morphine. It was concluded that the pharmacologic effects of heroin are mediated primarily by 6-MAM and morphine.

ANTAGONISTS OF ANALGESICS

In 1943 Unna[74] reported the pharmacology of *N*-allylnormorphine (*VI*, nalorphine, Nalline, Lethidrone), an analogue of morphine in which the

N-methyl group of the latter drug is replaced by an allyl group. Dogs remained alert and showed no signs of depression after subcutaneous doses of 10–20 mg/kg. Morphine (5–10 mg/kg) given 20–60 minutes later failed to



(VI)

produce its usual effects (i.e. vomiting, drowsiness and muscular uncoordination). Dogs intoxicated with morphine were promptly aroused by a subsequent injection of nalorphine and regained their normal behaviour completely after 15 minutes. Similar results were obtained with cats. Nalorphine produced no Straub tail effect in mice and was effective in counteracting morphine poisoning in the same species. In mice, the threshold for pain perception in abdominal skin after electric shocks was not raised significantly by nalorphine. Morphine was ineffective in raising the pain threshold in mice previously treated with nalorphine; when given first, its analgesic effect was rapidly abolished after administration of nalorphine. Although the respiratory rate of rabbits was not significantly affected after intravenous doses of up to 20 mg/kg of nalorphine, 50 mg/kg caused death by respiratory failure. However, after nalorphine, morphine failed to depress respiration even when given intravenously in large doses (20 mg/kg); the respiratory rate, markedly depressed when morphine was given first, was rapidly restored to normal after subsequent injection of nalorphine.

The remarkable antagonism of the depressant actions of morphine upon pain perception and respiration observed by Unna is now well known and has been confirmed in many other animal species and in man (see the review by Woods[75]). Nalorphine antagonizes a wide spectrum of morphine effects including the excitation of cats, antidiuresis in rats, the hyperglycaemic response in rabbits and intestinal and biliary spasm in man[75,76].

In man, nalorphine is an effective antagonist against respiratory depression caused by an accidental overdose of morphine and other narcotics[77] and it finds important clinical application in this respect. It is also used to antagonize the moderate to severe respiratory depression which may develop during the course of analgesia[78]. Eckenhoff and Oech[79], in a recent analysis of the effects of narcotics and their antagonists upon respiration in man, point out that therapeutic doses of narcotics lead to a diminution in alveolar ventilation, which is primarily a result of a decrease in tidal exchange. Narcotic overdose, in contrast, results in characteristic slow, deep respirations. In both cases the carbon dioxide content of end expiratory air increases. Intravenous administration of nalorphine (the usual dose is 5 or 10 mg) to patients deeply narcotized and with marked respiratory depression results in a prompt rise in respiratory rate and minute volume, while the end expiratory carbon dioxide content decreases to normal. Nalorphine may

also be used to counteract the depression of newborn infants' respiration by narcotics given to the mother. Nalorphine, given either to the mother a few minutes before delivery or injected directly into the infant's umbilical cord, significantly reduces the infant's respiratory depression[79]. When nalorphine is given to patients with only mild respiratory depression, this condition may be increased rather than reduced[80]. Patients did not respond to nalorphine after a single therapeutic dose of morphine unless a 'priming' dose of morphine was given first[81,82]. Fraser[78], from work with addicts, found that nalorphine only restores normal respiration after a dose of morphine sufficient to induce severe respiratory depression. Thus, the effectiveness of nalorphine in restoring respiration to normal is directly related to the degree of depression present. The specificity of nalorphine action in this respect is illustrated by its inability to reverse the respiratory depression produced by non-narcotic drugs such as barbiturates, ether or cyclopropane[75].

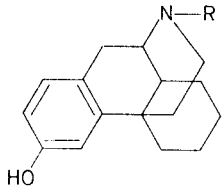
Administration of nalorphine to morphine-tolerant animals and to human addicts precipitates an acute abstinence syndrome. Use is made of this property in the diagnosis of addiction to morphine-type drugs. In subjects who have been taking as much as 120 mg of morphine or its equivalent for 15 days or more, subcutaneous injection of 3 mg of nalorphine produces abstinence symptoms, including profuse perspiration, pupillary dilation, hyperpnoea, gooseflesh, nausea, vomiting and defaecation, which appear within 20 minutes[78]. In the 'allyl' test for the addictive liabilities of drugs, use of nalorphine enables assessment to be made after relatively short withdrawal periods[83]. Attempts to produce addiction to nalorphine itself in former addicts have failed. Withdrawal of the drug after 28 days administration produced no symptoms of an abstinence syndrome[84]. Schrappe[85], however, has reported the incidence of abstinence syndromes, mild in degree and of short duration, after nalorphine administration to patients without previous narcotic experience.

Apart from an early report of its effect in rats[86] the analgesic properties of nalorphine in animals have been shown to be weak. In man, however, its potency was similar to that of morphine in alleviating post-operative pain[87,88]. It produced the same subjective side-effects as morphine but, in addition, caused unpleasant sensations (described as feelings of unreality) which were more pronounced when morphine had been given previously or when the patient was in an anxious state prior to operation. The respiratory depressant action of nalorphine shows the same species variation; in lower animals it is insignificant while in man its effect is similar in degree to that of morphine[75]. In normal subjects, Lasagna and Beecher[87] found that the minute volume response to 5 per cent carbon dioxide was reduced by 25 per cent after 10 mg of morphine and by 30 per cent after 5 mg of nalorphine. The response returned to normal more rapidly after the latter drug. As with morphine, available data indicates that nalorphine diminishes alveolar ventilation by a reduction in minute volume rather than by a change in respiratory rate[79].

In addition to its effect upon morphine, nalorphine also effectively antagonizes other potent analgesics[75], such as morphine derivatives (e.g. diacetylmorphine, metopon, hydromorphone), morphinans (e.g. levorphanol), 4-phenylpiperidines (e.g. pethidine, anileridine[89], alphaprodine),

diphenylpropylamines (e.g. methadone, isomethadone, phenadoxone) and thiambutenes[90,91]. Indeed, the antagonism of analgesia by nalorphine is accepted as one of the criteria by which a morphine-like analgesic may be differentiated from other central nervous depressants such as tranquillizers.

Levallorphan (*VII*, R=$\cdot CH_2 \cdot CH{=}CH_2$; Lorfan) related to levorphanol as nalorphine is to morphine, is also an analgesic antagonist[92]. Benson,



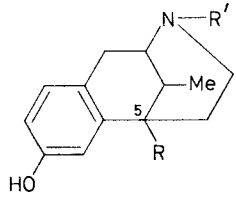
(VII)

O'Gara and Van Winkle[93] found that it effectively antagonized the respiratory depression and analgesia induced in rats by racemorphan, levorphanol, codeine and alphaprodine. The *dextro* isomer, related to analgesically inactive dextrorphan, lacked antagonistic properties. Levallorphan also antagonizes the actions of methadone, morphine and pethidine in rats[94]. There are several reports of its use in man to counteract respiratory depression produced by analgesics. Eckenhoff, Hoffmann and Funderburg[95] found that levallorphan and nalorphine were equally effective in this respect after morphine or levorphanol administration, but further work has shown levallorphan to be the more potent antagonist (the usual dose to counteract respiratory depression due to narcotic overdosage is 1 or 2 mg given intravenously)[79]. In rats also, levallorphan was more active than nalorphine in restoring depressed respiration to normal[94]. The same antagonists were equal in their action in suppressing the hyperglycaemic response of dogs to levorphanol[96]. In common with nalorphine, levallorphan has itself been shown to be a respiratory depressant[79]. (−)-3-Hydroxy-*N*-propargylmorphinan (*VII*, R = $CH_2 \cdot C{\equiv}CH$) is reported to be a potent antagonist to alphaprodine or morphine in the dog and to alphaprodine in man[97]. Given simultaneously with alphaprodine at 1/75th or 1/100th the dose of the latter, it counteracted the respiratory depression produced by the narcotic alone. The propargyl compound was as effective an analgesic as nalorphine in patients with post-operative pain[98]. *N*-Allyl and *N*-propargyl derivatives of noroxymorphone have also been reported as morphine antagonists[99,100]. 1-Allyl-4-phenyl-4-ethoxycarbonylpiperidine, the *N*-allyl analogue of pethidine, was effective in combating the respiratory depression produced by methadone, alphaprodine and pethidine, but only when used at high dose levels. It has no effect upon the analgesia produced by these drugs[94].

The most recently reported analgesic antagonists are benzomorphan derivatives related to the potent analgesic phenazocine [*VIII*, R = Me R′ = $(CH_2)_2Ph$].

In animals the *N*-allyl analogue (*VIII*, R = Me, R′ = $CH_2 \cdot CH{=}CH_2$) is an antagonist of phenazocine, morphine and pethidine[101] and is assessed,

together with the 5-ethyl analogue (*VIII*, R = Et, R′ = $-CH_2 \cdot CH{=}CH_2$) as being three times as effective as nalorphine in reversing pethidine-induced



(VIII)

analgesia[102]. Both derivatives relieved post-operative pain in man (5 mg/kg≡10 mg/kg morphine) but caused psychic and other side effects similar to those seen with nalorphine[102].

The possibility of selective reversal of the respiratory depressant and other undesirable side effects of narcotics, but with concomitant retention of their analgesic properties, has prompted the study of the effects of analgesic–antagonist mixtures. The effect of such mixtures upon respiration appears to depend, as does that of the antagonists themselves, upon the previous narcotic experience of the individuals concerned. Thus the respiration of normal healthy volunteers was depressed equally by morphine and by nalorphine–morphine mixtures in a 1:5 and 1:3 ratio[87]. Similar results were obtained with levorphanol and levallorphan–levorphanol mixtures[103,104]. However, in patients already taking analgesics, mixtures (with some exceptions) led to no more than slight respiratory depression. Ratios of analgesic to antagonist were as follows: morphine to nalorphine 3:1; levorphanol to levallorphan 10:1; alphaprodine to levallorphan 50:1; pethidine to levallorphan 100:1[79]. In nearly all clinical cases reported, patients had been given a narcotic either for pre-anaesthetic medication or for chronic pain. Respiratory depression was also effectively blocked in patients receiving analgesics after prior administration of an antagonist. Hamilton and Cullen[105] injected levallorphan (2·5 and 5·0 mg) in patients prior to operation and anaesthetized with nitrous oxide. A slight diminution in respiratory rate was noted but subsequent injection of levorphanol, pethidine or morphine did not produce the degrees of respiratory depression that were anticipated. These results are analogous to previous reports of the failure of morphine to depress the respiration of nalorphine-treated rabbits[74]. Experiments with mixtures of phenazocine and its *N*-allyl analogue are promising with respect to the development of a non-addicting analgesic combination. Mixtures that retained the analgesic activity of phenazocine in animals precipitated an abstinence syndrome, rather than supported physical dependence, in addicted monkeys[101].

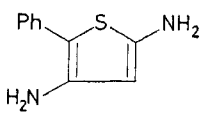
Several studies of the structural requirements for antagonist properties in animals have been made[106–108]. In morphine-type compounds, the substituent upon the basic centre is decisive in determining whether the derivative has analgesic or anti-analgesic properties. High anti-analgesic activity is obtained when this substituent is a straight 3-carbon chain, as with the allyl and *n*-propyl groups (the cyclopropylmethyl group confers an even

higher activity—see below). The high anti-analgesic properties of 3-hydroxy-*N*-propargylmorphinan is consistent with this observation. Further increases in chain length result in compounds that are inactive or only weakly active as antagonists while analgesic properties re-appear. In man, *N*-3,3-dimethylallyl analogues or levorphanol and phenazocine lack the antagonist properties of the unsubstituted *N*-allyl compounds and are potent analgesics[98,102]. The benzomorphan derivative (*VIII*, R = Me, R′ = $CH_2 \cdot CH{=}CMe_2$), termed pentazocine, is claimed to represent the first clinically useful potent analgesic with minimal addiction liability (see p. 212)[109,110]. Modifications of other functional groups in the morphine molecule affect the degree but not the nature of the compound's activity. Thus the anti-analgesic activity of nalorphine is reduced by 60 per cent if the phenolic group is methylated, while the corresponding *N*-*n*-propyl derivative loses all its anti-morphine activity. Activity is retained, although at a reduced level, after acetylation of the 3- and 6-hydroxy groups. This result contrasts with the enhanced analgesic activity of diacetylmorphine over that of morphine. However, other derivatives more potent than morphine, such as dihydromorphine, desoxymorphine and dihydromorphinone, have *N*-allyl and *N*-propyl counterparts with antimorphine activities greater (with one exception) than those of respective normorphine derivatives. Similarly levorphanol, a more potent analgesic than morphine, gives an antagonist, levallorphan, that is more potent than nalorphine, while the *N*-allyl analogue of the much weaker analgesic, pethidine, is inactive, or only weakly active as an antagonist. It appears therefore that marked anti-analgesic properties in *N*-allyl, *N*-propyl or *N*-propargyl derivatives are only to be expected when these substances are related to compounds that are themselves potent analgesics. The close similarity in properties between alkenes and cyclopropyl derivatives has prompted the testing of *N*-cyclopropylmethyl derivatives as antagonists[111]. Such analogues of morphine, levorphanol and phenazocine do, in fact, antagonize pethidine-induced analgesia in mice and are more active than the corresponding *N*-allyl compounds. Antagonistic properties are retained in *N*-cyclobutyl and *N*-cyclopentyl derivatives of the benzomorphan (*VIII*, R = Me) but are much reduced in the *N*-cyclohexyl member[108]. (−)-3-Hydroxy-*N*-cyclopropylmethylmorphinan has been shown to be a potent analgesic in man[111].

The relative absorption rates after subcutaneous injection of morphine and nalorphine and the relative tissue distributions of the same drugs have been studied[19,75,112]; also the C.N.S. distribution of tritium-labelled nalorphine has been reported[113]. In rats, the rate of absorption of nalorphine is considerably more rapid than that of morphine. Four hours after subcutaneous administration of 150 mg of nalorphine, the percentage of the dose recovered at the site of injection in each animal was as follows: nalorphine 0·3, 0·2 and 4·2; morphine 16, 17 and 15. Tissue distribution in both dogs and rats of both free and conjugated drug was generally lower for nalorphine than for morphine; in both cases highest concentrations were found in gall bladder bile. In dog brain tissue, however, the concentration of nalorphine 90 minutes after subcutaneous injection, was almost four times that of morphine. In rats, brain concentrations of nalorphine were ten times those of morphine 15 minutes after administration of equal doses[35]. Significant levels of

morphine persisted in the brain for 16 hours after injection but the amounts of nalorphine present rapidly decreased and after 4 hours were less than those of morphine. Almost all of the free nalorphine appeared in the urine 3–4 hours after injection. These results are in accord with the observed rapid effect and short duration of action of nalorphine. The dog conjugates nalorphine more rapidly and more completely than it can morphine, although *in vitro* studies have shown that dog liver slices conjugate the two drugs at approximately equal rates[20]. Reference has already been made to the more rapid rate of dealkylation of nalorphine by liver microsomal preparations compared with that of morphine[45]. Levallorphan is similarly metabolized to 3-hydroxymorphinan which is excreted mainly as a conjugate. In addition, an unidentified oxidized metabolite has been isolated[46].

Several reports of the clinical use of amiphenazole (*IX*, Daptazole) and



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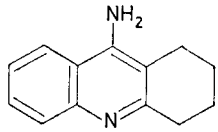


(X)

tetrahydroaminacrin (*X*) in morphine therapy have been published[114–118]. Their use derives from the observation of Shaw and Bentley[119,120] that a dog completely narcotized with morphine is restored to normal within a few minutes by an intravenous injection of amiphenazole. These compounds do not affect morphine-induced analgesia and their action in counteracting certain side effects of morphine, such as respiratory depression and narcosis, is probably analeptic rather than antagonistic. Both are respiratory stimulants. Combinations of these substances and morphine were used in the treatment of the intractable pain of terminal carcinoma when it was possible to give high doses of morphine (up to 100 mg four times a day) without patients becoming narcotized. Papadopoulos and Keats[121] advocate treatment of mild narcotic-induced depression with a non-specific analeptic and treatment of severe narcotic depression with a specific narcotic antagonist.

RELATION OF MORPHINE-TYPE ANALGESICS TO ADRENERGIC AND CHOLINERGIC MECHANISMS AND OTHER BIOCHEMICAL PROCESSES

It is well established that morphine causes a release of adrenaline; the adrenaline-depleting action of morphine upon the adrenal gland of cats and dogs, first reported by Elliott[122], has been confirmed by other workers (see Gunne[123] for summary). Gunne, in a continuation of earlier work, found that acute administration of morphine produced an activation of sympathetic part of the nervous system, leading to a depletion of brain and adrenal stores of catecholamines in rats and cats while chronic administration of the same drug induced an increased rate of resynthesis of catecholamines. It has been suggested that the analgesia produced by morphine-type

analgesics is mediated, wholly or in part, through a release of adrenaline from the adrenal medulla (see references cited by Millar and his colleagues[124]). Large doses of adrenaline have been reported to increase the pain threshold for a heat stimulus in humans[125] and to elevate the reaction threshold for electrical stimulation of the tooth in dogs[126]. Other sympathomimetic agents have the same effect in dogs and cats and potentiate the effects of morphine and pethidine in man[127,128]. In dogs and cats the rise in pain reaction threshold after morphine, pethidine and methadone was decreased after adrenalectomy[127,129]. Further, Watts[130] found the degrees of hyperglycaemia (related to adrenaline release) produced by *dextro* and *laevo* methadone to parallel their analgesic potencies in dogs.

However, evidence contrary to the implication of adrenaline in the mediation of analgesia is also available. Adrenal inactivation did not decrease tail reaction time to thermal stimulus in rats treated with morphine, while near lethal doses of adrenaline and noradrenaline were required to raise the pain threshold in mice[124]. The tetraethylammonium ion (TEA), which prevents the release of adrenaline and blocks morphine hyperglycaemia, did not reverse the effect of morphine upon pain thresholds in rats, mice and dogs. In the rat, near lethal doses of morphine (10–30 times the ED_{50} for analgesia) were required to affect adrenal medullary release as measured by the hyperglycaemic response. No appreciable depletion of adrenaline or noradrenaline in rat adrenal glands was found after administration of 20 mg/kg of morphine, a dose which failed to produce hyperglycaemia[131]. Finally, various sympatholytic agents did not reverse the rise in pain threshold after morphine in rats and methadone in dogs[125,132].

The inhibition of parasympathetic functions by morphine would be anticipated as a corollary to its possible promotion of adrenergic activity. Its paralysing effect upon the intestine and inhibition of the peristaltic reflex is well known. Other morphine-like analgesics have similar actions in this respect and some correlation is found between potencies relating to analgesia and inhibition of intestinal contractions[133,134]. As with analgesia, stereospecificity is also evident in the latter effect. Thus, while levorphanol is highly effective in inhibiting the peristaltic reflex, dextrorphan is inactive[135]. Paton[136] was unable to demonstrate conclusively the antagonism of nalorphine against morphine on the cholinergic nerves of guinea-pig ileum, nalorphine itself having a depressant effect on the intestines.

Another parasympathetic function affected by morphine is the vagal slowing of the heart. In the rat and rabbit, stimulation of the vagus produced much less slowing of the heart after morphine than before; a distinct delay occurred in the onset of such slowing as did occur. Nalorphine reversed, in part, these effects of morphine. In the guinea-pig, morphine did not reduce cardiac slowing while its effect in the cat was relatively small[137].

The mode of action of morphine in these respects is considered to be due to the inhibition of the release of acetylcholine at cholinergic synapses rather than inhibition of its synthesis[135,136,138]. Morphine has only a slight effect on the response of isolated guinea-pig ileum to acetylcholine and carbachol[139].

There is evidence, also, that morphine inhibits the release of chemical

transmitters from adrenergic synapses. Interference by morphine with the release of noradrenaline from the adrenergic nerve endings of the cat nictitating membrane has been reported by Trendelenburg[140]. This effect was not due to the action of morphine on the smooth muscle of this membrane since direct action of adrenaline and noradrenaline was not reduced. The rapid relaxation of isolated guinea-pig jejunum (contracted by previous administration of histamine) brought about by stimulation of the adrenergic nerves was inhibited by morphine, while the depressant action of noradrenaline on histamine contractions was not significantly affected by morphine[141]. Nalorphine reduced the effect of adrenergic stimulation only slightly and partially antagonized the effect of morphine with the latter drug present in the higher concentration. The minimum concentration of morphine that inhibited the adrenergic nerves was about the same as required to reduce the effect of coaxial stimulation on the guinea-pig ileum and these results indicate that morphine interferes with a step common to both cholinergic and adrenergic nerves[136].

In contrast to the action of morphine in inhibiting the release of acetylcholine from cholinergic nerve endings, it potentiates acetylcholine itself by reducing the activity of cholinesterases. Bernheim and Bernheim[142] found that very small concentrations of morphine markedly inhibited brain cholinesterase, as measured by the per cent increase in hydrolysis time of acetylcholine. Morphine, codeine, hydromorphone and desomorphine all inhibited brain and serum cholinesterase from man, dog or rabbit[143]. Similar actions have been reported for methadone[144] and pethidine[145]. Physostigmine potentiated the *in vivo* action of morphine on dog intestine and cat blood pressure[146] while prostigmine potentiated the analgesic effect of morphine upon the pain response of the cat to pressure[147]. Similar potentiation by neostigmine of the effect of racemorphan on rabbit intestine has been reported[148]. These results may be explained if morphine acts as a cholinergic agent since its actions should be increased by other similarly acting drugs. However, poor correlation between either analgesia or the effect on intestinal tone and the pattern of cholinesterase inhibition has been noted for a series of analgesics.[148] Further, in the morphinan series the analgesic antagonist, levallorphan, had the greatest anticholinesterase activity while Jóhannesson and Milters[149] report the activity of nalorphine to be greater than that of morphine in the same respect.

Analgesics have been shown to inhibit the oxygen uptake of brain tissue[150–155]. However, in general, the concentrations of analgesics which have been required to produce a significant inhibition have been far in excess of the concentrations which have been shown to reach the brain in the intact animal, and so the results are of little practical significance. Other workers[156,157] have shown that the oxidation of glucose, succinate, ascorbate and lactate by brain tissue is inhibited by analgesics. By reason of this inhibition of oxidation processes, Wang and Bain[158] have investigated the sensitivity to morphine of the various steps in the cytochrome system.

It is evident, from data presented in this section, that efforts to associate the analgesic properties of morphine with its effects upon functional systems within the body have so far been unsuccessful.

STRUCTURE–ACTIVITY RELATIONSHIPS

Introduction

In earlier summaries[159,160] of structural features common to potent analgesics, stress has been laid upon the following: (1) a tertiary basic function in which at least one of the groups linked to nitrogen is relatively small; (2) a central carbon atom, none of its valency bonds being linked to hydrogen; (3) a phenyl group or a group isosteric with phenyl connected to the central carbon atom; and (4) a two-carbon chain separating the central carbon from nitrogen for maximum activity.

During the past ten years, however, many potent analgesics have been described which possess structural features deviating from the above requirements (notably with respect to the permitted size of the basic group), and the necessity for a re-evaluation of structure–activity relationships in analgesics has been pointed out by Eddy[160]. In 1952 Beckett[161] emphasized the importance of stereospecificity in analgesics and speculated that the minimum requirement for activity is a hydrophobic group (or collection of groups) containing a basic centre with an overall optimum spatial arrangement. Subsequent stereochemical investigations of analgesics have advanced knowledge of the possible nature of the analgesic receptor site and are outlined in this section.

In assessing structure–action relationships, some attempt must be made to differentiate relationships reflecting the intrinsic activity of a compound (i.e. its power of inducing a pharmacological response at the site of action) from those which arise as a result of the effect of structure upon absorption, distribution, metabolism and storage of the drug (i.e. factors involved in the transportation of a drug to its locus of action).

The quantitative results of analgesic testing, in common with those of many other pharmacological assays, are subject to wide degrees of variation. Thus the type of test applied, the species and strain of animal used, the route of administration, and the period allowed to elapse between time of drug administration and time of measurement of analgesic response, all have a profound effect upon the estimate of the analgesic potency recorded. Ideally, structure–activity relationships should be drawn from results obtained in a standardized test carried out in one laboratory. However, comparisons often need to be made between compounds whose potencies have been assessed under differing conditions and in such cases only general trends may justifiably be noted.

Stereochemical Aspects

Many members of the various classes of potent analgesics are asymmetric and their stereochemical selectivity is well known, analgesic activity and other morphine-like properties largely residing in one member of each enantiomorphic pair. Examples of this phenomenon reported up to 1959 have been reviewed[162,163]. Further examples continue to be described and it is now almost standard practice to examine the activities of the enantiomorphic forms of all new asymmetric analgesics. Some more recent data in this respect are given in *Table 5.3.*

In terms of drug-receptor interactions, the significance of stereospecificity

Table 5.3. Recent examples of stereospecificity in analgesics

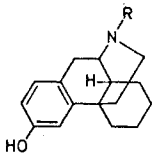
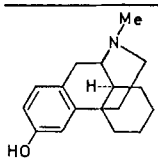
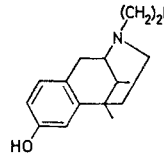
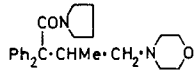
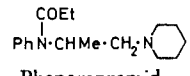
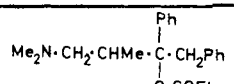
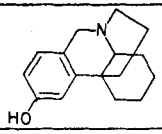
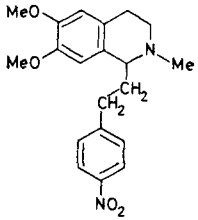
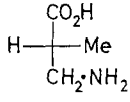
Structure		*Isomer*	*Analgesic activity (ED_{50} mg/kg in mice unless otherwise stated)*	*Reference*
 3-Hydroxy-*N*-Aralkyl Morphinan	*R* $(CH_2)_2Ph$	(−) (+)	0·11 100	164
		(−) (+)	0·01 100	
		(−) (+)	0·02 100	
		(−) (+)	0·02 100	
		(−) (+)	0·07 100	
 3-Hydroxy-*N*-methyl Isomorphinan		(−) (+)	8–10 times as active as morphine in rats Inactive	165
 Phenazocine		(−) (+)	0·11 7·6	166
N-Me analogue		(−) (+)	1·7 Inactive	166
 Moramide		(+) (−)	0·12 85	167
 Phenampromid		(−) (+)	9 (AD_{50} in rat) 36	168
 Diampromid		(+) (−)	3·6 (AD_{50} in rat) 11·7	169

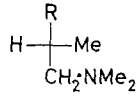
Table 5.3. Recent examples of stereospecificity in analgesics (*continued*)

 Propoxyphene	α-(±) α-(+)	25·4 7·5	160
$PhCH_2 \cdot CHPh \cdot NMe_2$	(−) (+)	0·5 to 0·33 as active as morphine Inactive	170
 3-Hydroxy-9-aza-*N*-morphinan	(−) (+)	4·28 Inactive	171
	(−) (+)	0·46 36	172

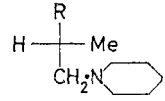
in drug action only becomes established when the more active isomers of enantiomorphic pairs are shown to possess identical configurations. Several such studies have now been reported. The more active isomers of certain methadone and thiambutene-type compounds have been shown to possess identical configurations related to D-(−)-alanine (see *Table 5.4*). Configurational identity also obtains among the more active isomers of analgesics containing the structural entity $>N \cdot CH_2 \cdot CHMe—$. Thus (−)-isomethadone[175], (+)-propoxyphene[176,177] and (−)-phenampromid[178] are all related to R-(−)-α-methyl-β-alanine (see below).



R-(-)-α-methyl-β-alanine



(-)-isomethadone
(R=CPh$_2$·COEt)

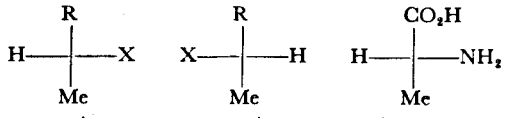


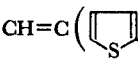
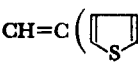
(-)-phenampromid
[R=N(COEt)Ph]

(+)-propoxyphene
[R=C(O·COEt)Ph·CH$_2$Ph]

Stereoselective adsorbents have been applied to assigning configuration to morphine-type analgesics. The method involves the preparation of an adsorbent in the presence of a reference configurational molecule which is then removed from the surface of the adsorbent; molecules with the same configuration as the reference are adsorbed more strongly than those of dissimilar stereochemistry. Data so derived indicate morphine, levorphanol

Table 5.4. Activities in mice and configuration of methadone and thiambutene analgesics[173,174]



R	X	*Isomer*	*Configuration*	*Activity**
$CH_2 \cdot CPh_2 \cdot COEt$ (Methadone)	NMe_2	(−) (+)	D L	180 10
$CH_2 \cdot CPh_2 \cdot SO_2Et$	NMe_2	(−) (+)	D L	180 10
$CH_2 \cdot CPh_2 \cdot COEt$ (Phenadoxone)		(−) (+)	D L	195 5
	NMe_2	(−) (+)	L D	30 170
	NEt_2	(−) (+)	L D	50 120

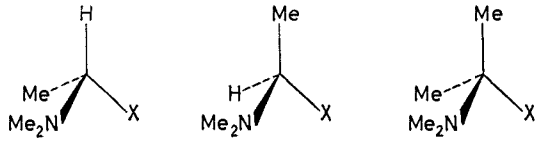
* (±)-methadone = 100.

and (−)-phenazocine to have identical configurations[179]. (−)-*N*,*N*-Dimethyl-1,2-diphenylethylamine (see *Table 5.3*) has the same configuration as morphine in respect of the analogous C_9 asymmetric centre of the latter[170]. Portoghese's report[180] that the more active isomers of diampromid and its *N*-benzyl analogue (which contain asymmetric centres similar to that present in methadone) are related to L-(+) rather than D-(−)-alanine, is the one result contrary to the general finding of configurational identity among analgesically active enantiomorphs (see p. 204).

Stereospecificity is also exhibited by the analgesic antagonist 3-hydroxy-*N*-allylnormorphinan, only the *laevo* isomer (levallorphan) being effective in this respect[92]. Levallorphan has the same configuration as levorphanol (nalorphine and morphine are related in the same sense).

In 1954 the present authors developed a hypothesis concerning the nature of the analgesic receptor site on the basis of stereochemical evidence and structural features common to analgesics and their antagonists known at that time[181]. They considered the stereospecificity of analgesics and the results of configurational studies to be indicative of a 'three point' association between drug molecule and receptor site. Three structural features of an analgesic molecule orientated in a specific manner, complementary to that of the surface features of the receptor, were felt to be implicated in the drug-receptor interaction. A basic group and a flat aromatic ring structure (both features are common to all known analgesics) were postulated as two of the 'active areas' of the molecule while the third was held to be a hydrocarbon

entity. Only one member of an enantiomorphic pair may present the three features in the correct orientation for association at the receptor site. It is probably more accurate to regard the drug-receptor association as being 'two point' in nature (the basic group and the aromatic structure being the two essential active binding sites) with the third group either augmenting binding to a secondary degree in the more active enantiomorph while seriously impeding it in the less active isomer. Evidence in support of the last aspect has been derived from a study of '6-methylmethadone' (6-dimethylamino-6-methyl-4,4-diphenylheptan-3-one)[181,182]. This compound (*XIII*), although possessing the same configurational arrangement of functions as is present in the potent (−)-enantiomorph of methadone (*XI*), is nevertheless devoid of analgesic activity—a result which may be interpreted in terms of the second methyl group positioned as in the inactive (+)-isomer of methadone (*XII*) preventing effective drug-receptor association. On the other hand, removal of both methyl groups gives an analgesic



(XI) (−)-methadone

(XII) (+)-methadone

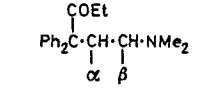
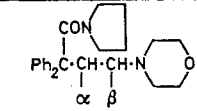
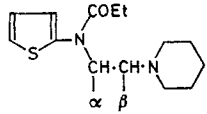
(XIII) 6-methylmethadone

X = $CH_2 \cdot CPh_2 \cdot COEt$

(normethadone) of much reduced potency[183]. This and other examples (see *Table 5.5*) establish the hydrocarbon entity as playing a positive rather than a purely passive role in the association of active molecules at the receptor site.

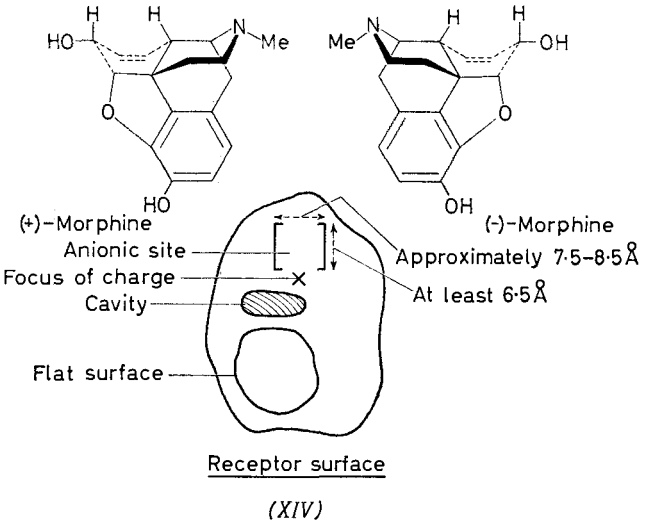
Some intimation of the delineation of the active sites on the receptor was gained by consideration of the actual shape of rigid analgesic molecules

Table 5.5. Influence of methyl groups on analgesic activity[167,183,184]

Structure	α	β	ED_{50} mg/Kg *in mice**
	H	Me	1·6
	Me	H	2·5
	H	H	2·5
	Me	H	1·25
	H	H	13·6
	H	Me	32
	Me	H	17·4
	H	H	150

* Hot plate test.

since their 'active' features should be complementary to corresponding features upon the receptor surface. Using (−)-morphine as the model, the diagrammatic representation of the receptor surface shown below may be derived. It is to be noted that the arbitrarily chosen morphine configuration



Diagrammatic representation of the three dimensional arrangement of morphine and the analgesic receptor site. The diagrams represent the lower surface of the drug and the upper surface of the receptor, i.e. complementary surfaces in front of, behind, and in the plane of the paper are represented by —, ····, and — respectively.

(XIV)

(Reproduced by courtesy of the Editor of *J. Pharm. Lond.*)

used as basis for the original receptor diagram (advanced in 1954)[181] proved, in the light of subsequent work, to represent (+)- rather than (−)-morphine and the diagram was amended accordingly[185].

The three essential sites described were:

(1) a flat portion which allows binding with the aromatic ring of the analgesic drug through van der Waals' type forces;

(2) an anionic site which associates with the positively charged basic centre of the drug molecule (in all cases studied, the pK_a' values of analgesics lie within the range 7·8–8·9 corresponding to about 86–96 per cent ionization at physiological pH)[10]; and

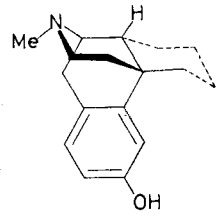
(3) a cavity suitably orientated with sites (1) and (2) to accommodate the projecting $-\overset{15}{C}H_2-\overset{16}{C}H_2-$ portion of the piperidine ring D which lies in front of the plane containing the aromatic ring and the basic centre.

It was considered that association of drug donor groups with sites (1) and (2) represented the primary site of analgesic action whereas correct alignment of a projecting hydrocarbon residue with the cavity in one enantiomorph enhances the drug-receptor contact and consequently the analgesic activity; in the opposite enantiomorph, the projecting group would impair the drug-receptor contact.

The antagonism of a wide range of analgesics by nalorphine and levallorphan provides convincing evidence that all types of analgesics (in the sense defined earlier)[186] act at a common receptor. Hence the receptor surface (*XIV*), formulated on the basis of the morphine molecule, must be capable of accommodating other structural types. This was originally shown to be the case in a number of instances[181]; these examples are reviewed here

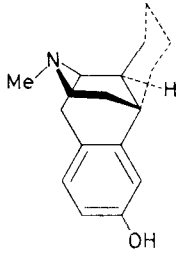
together with a consideration of important analgesics developed over the past ten years.

Levorphanol (*XV*) must fit the receptor (*XIV*) since its molecular shape closely resembles that of morphine (the steric identity of corresponding ring junctures in morphine and morphinan derivatives has been established[187]) while inactive dextrorphan has the incorrect configuration for drug-receptor association.



(XV)

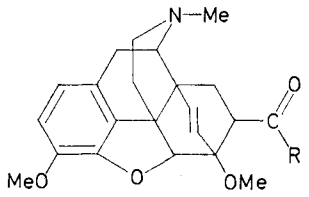
The validity of the proposed receptor surface receives support from the fact that the change from a morphinan (*XV*) to an isomorphinan (*XVI*) does not result in a loss in potency[165]. Isomorphinan has a *trans* juncture between rings B and C and differs in shape from morphinan (and morphine) in respect of the disposition of ring C. In both molecules, however, this ring is directed *away* from the receptor surface (see diagrams *XV* and *XVI*), and hence its stereochemistry should have little influence upon drug-receptor association. The numerous structural variations that may be made upon ring C in morphine with retention or augmentation of analgesic properties support the view that ring C is not implicated at the receptor surface[186]. It is remarkable that the stereospecificity of the analgesic receptor is such that it may differentiate enantiomorphic pairs (e.g. dextrorphan and levorphanol) and yet fail to discriminate between diastereoisomers (e.g. levorphanol and isolevorphanol).



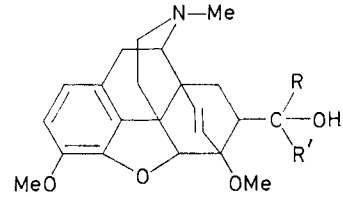
(XVI)

Bentley and Hardy[188] have recently described a series of highly potent analgesics obtained by Diels Alder reactions between thebaine (the diene) and vinyl methyl ketone (and other dienophiles). The resultant ketonic adducts (*XVII*) had activities comparable to those of morphine but derived alcohols (especially tertiary alcohols) were much more potent, several being one thousand or more times as active as morphine in mice and rats[189,190].

Structurally these alcohols may be regarded as derivatives of morphine in which the 6 and 14 carbon atoms of ring C are bridged by a bimethylene chain substituted by an alcoholic function. This bicyclic feature lies largely

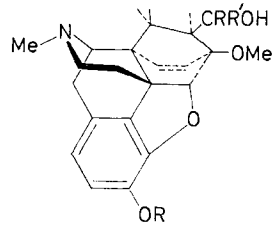


(XVII)



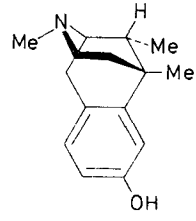
(XVIII)

behind the plane containing the nitrogen atom and the aromatic ring A; thus the 6,14 endoetheno derivative (*XIX*) would be expected to fit the proposed receptor. Evidence that the C-7 alcohol group may provide an additional binding site is provided by the fact that the two diastereoisomeric alcohols (*XVIII* R = Me and R′ = *n*-Pr) differ markedly in potency[189].



(XIX)

Benzomorphan derivatives (*XX*, e.g. methazocine) would be expected to fit the proposed surface; apart from ring C (which is reduced to alkyl fragments at C_5 and C_9) other structural and stereochemical features of morphinan are retained in the benzomorphan molecule[191]. Active diastereoisomers, related to benzomorphan as isomorphinan is to morphinan, are also known[192].

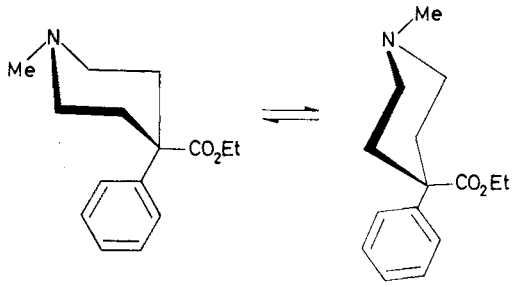


(XX)

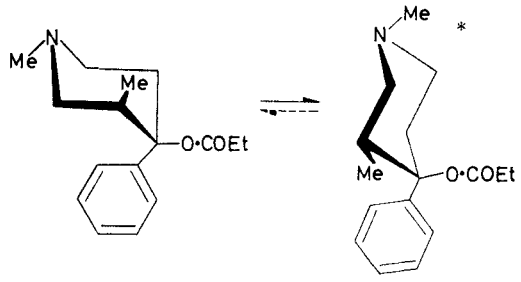
With less rigid molecules it is necessary first to decide whether particular conformations may be appropriate for drug-receptor association and secondly to consider the likelihood of the molecule adopting such conformations.

The analgesic activities of 4-phenylpiperidine derivatives indicate this

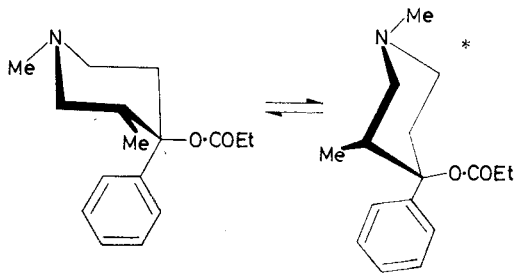
structural entity to be the essential feature of the analgesics so far discussed. The rigid nature of these polycyclic molecules requires the phenyl group to be linked axially to the 4-position of the nitrogen ring; in simple 4-phenylpiperidines, however, an equatorial phenyl conformation is more likely. Models reveal that both axial and equatorial conformations would be



Pethidine *(XXI)*



Alphaprodine *(XXII)*



Betaprodine *(XXIII)*

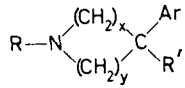
* Established as the conformation in the crystalline state[193,194]; in solution, however, the oxygen function may be enlarged by solvation, a factor making the axial phenyl conformations more favoured.

expected to fit the receptor surface although the less favoured axial phenyl conformer should fit the better (see *XXI–XXIII*). Derivatives in which axial phenyl conformations are more favoured are, indeed, more potent analgesics than stereoisomers in which this is not the case, e.g. alpha- and betaprodine[195].

In general, analgesic properties are retained (although in reduced degree) in seven-ring analogues of active piperidine derivatives but are absent or

weak in five-membered congeners (see *Table 5.6*). These results indicate the relative orientation of, and the distance between, the basic centre and the aromatic group to be optimal in a six-membered ring. Although the relationship of these two functions must be modified when an additional methylene group is included in the nitrogen ring, the molecule is free to

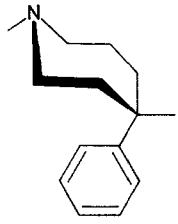
Table 5.6. Analgesic activities of some azacycloalkane derivatives (in rats unless otherwise indicated)



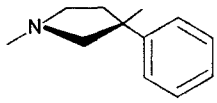
R	R′	Ar	*Analgesic activity (pethidine = 1)*			*Reference*
			5-series $x = 2$; $y = 1$	*6-series* $x = y = 2$	*7-series* $x = 3$; $y = 2$	
Me	CO_2Et	Ph	Inactive	1	0·3	159, 196
Me	COEt	*m*-OH-C_6H_4	—	10	0·7	159, 197
Me	SO_2Et	Ph	—	1*	0·3	159, 198
$(CH_2)_2Ph$	O·COMe	Ph	Inactive*	5·7*	2·5*	199
$(CH_2)_2Ph$	O·COEt	Ph	Inactive*	17*	7*	199

* In mice.

adopt a wide range of conformations[200] some of which may satisfy (in part) requirements for drug association at the receptor. In contrast, the distance between the nitrogen atom and the aromatic function in pyrrolidine derivatives must, of necessity, be less than that obtaining in piperidine analogues; furthermore, the orientation of the two features is restricted to narrower limits through the more rigid nature of the five-membered ring (see *XXI* and *XXII*).



(XXIV)

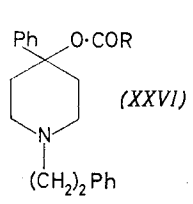


(XXV)

The basic centre–aryl group relationship obtaining in 4-phenylpiperidines is also radically disturbed in 4-benzyl analogues, replacement of phenyl by benzyl in the active piperidine derivatives (*XXVI*) resulting in a sharp fall in potency[201]. In view of these results, the high potency of Fentanyl (*XXVIII*) (claimed to be 400 times as potent as morphine in rats[202]) is unexpected since four atoms separate the basic function from the phenyl group in both this compound and the benzyl derivatives (*XXVII*). This anomaly, however,

may be the result of Fentanyl being related to diampromid and other basic anilides (see p. 201) rather than to pethidine.

Acyclic 1,1-diarylpropylamine analgesics possess the structural features of 4-phenylpiperidines (i.e. a basic centre and an aromatic ring linked by three



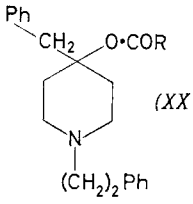
(XXVI)

Activity

R = Me, 6·3

R = Et, 3·5

Morphine 1·0



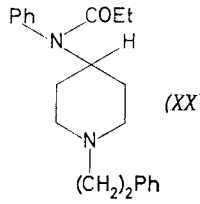
(XXVII)

Activity

R = Me, < 0·3

R = Et, < 0·3

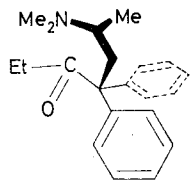
Morphine 1·0



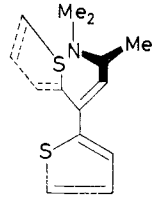
(XXVIII)

Fentanyl

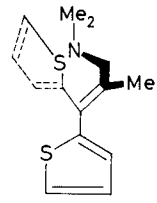
carbon atoms) and models show that molecular conformations exist in which these features are correctly aligned for association at the receptor surface. In such conformations, shown below for methadone (*XXIX*) and dimethylthiambutene (*XXX*), the basic centre lies approximately in the same plane as one of the aromatic rings. Rotation of the ring out of this plane increases steric interactions with the second aromatic ring (in the conformations shown the two rings are at their maximum distance apart) and a function of the additional ring may, in fact, be to aid in holding the 'active' conformations. Evidence from dissociation constants (indicative of N–C_{CO} and N–S interactions respectively) has also been advanced in support of the



(XXIX)

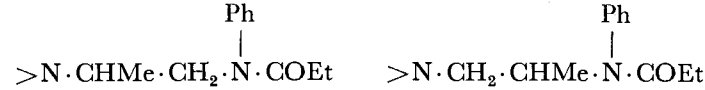


(XXX)



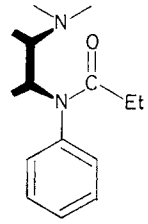
(XXXI)

favoured nature of these conformations[8,203]. Methyl group displacement from C_3 to C_2 in dimethylthiambutenes results in a greater loss in activity than does the comparable change in methadone[204]. A methyl group in the 2- position of a 1,1-diarylprop-1-ene has a fixed disposition since it forms part of the alkene link (see *XXXI*); a 3-methyl substituent is less restricted and may be capable of adopting conformations more conducive to drug-receptor association. In 1,1-diphenylpropylamines the freedom of movement of methyl groups at C_2 and C_3 are comparable.



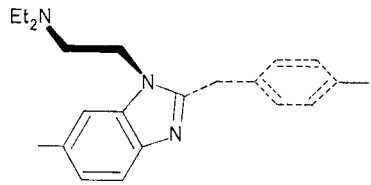
(XXXII)

Analgesically active basic anilides[205] (*XXXII*), in which the quaternary carbon atom and one aryl group of the diarylpropylamines is replaced by a nitrogen atom, may also adopt conformations suitable for fit at the receptor surface (see *XXXIII*). Since the second, buttressing, aryl group is absent, other factors (e.g. hydrogen bonding between amide carbonyl and protonated nitrogen and/or the rigidity imparted to the molecule as a result of the partial sp^2 character of the $N–C_{CO}$ link) may be important in determining the more favourable conformations.



(XXXIII)

Analgesics based upon 2-benzylbenzimidazole[206] similarly possess the essential features for fit at the proposed receptor site. Of the two aryl entities in the molecule, the benzimidazole benzene ring is the more likely feature to be associated at the receptor surface since it is linked through nitrogen (analogous to the quaternary carbon of other analgesics) to a 2-aminoethyl side chain. Models reveal that these features may be disposed suitably for fit at the receptor site (see *XXXIV*). The area of planarity in the molecule is greater than that in the other analgesics so far discussed and this factor may be related to the extremely potent nature of certain benzimidazole derivatives.



(XXXIV)

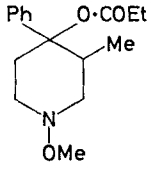
Role of the Basic Group in Analgesics

The basic group influences both the fit of the drug molecule at the receptor site (i.e. its *affinity* for the site) and the nature of the effect mediated upon drug-receptor association (i.e. the *intrinsic activity* of the drug molecule).

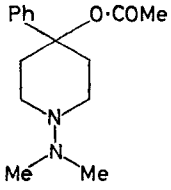
The basic group governs affinity in respect of both its degree of ionization and its steric dimensions. The basic strength of the drug molecule should be such that the molecule is largely ionized as a cation at physiological pH to allow of association with the anionic site of the receptor. The dissociation constants of many analgesics have been reported and none lie outside the

range 7·8–8·9, corresponding to about 86–96 per cent ionization as cation at *p*H 7·2[8]. On the other hand, the existence of small amounts of un-ionized base may be important in respect of the drug reaching its site of action. To do this, the drug molecule must pass various membranes that are lipid in character and hence relatively impervious to ionic species[207]. The pH in the immediate environment of the receptor site may be lower than the generally accepted figure of 7·2 (certain enzymes behave as though acting in environments some two pH units lower than the bulk[208]). Hence protonation of the basic centre of analgesic molecules near the receptor surface may well be complete.

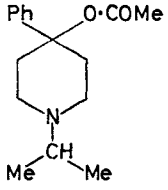
Two examples of compounds whose low analgesic potency may be attributed to their weakly basic nature are the *N*-methoxypiperidine[209] (*XXXV*) (an analogue of the potent analgesic alphaprodine) and the hydrazine derivative (*XXXVI*) [c.f. the activity of the *N*-isopropyl analogue (*XXXVII*)][210].



(XXXV)
Inactive in rats



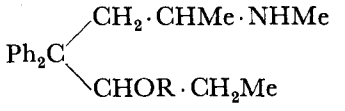
(XXXVI)
ED_{50} 40mg/kg
(in mice)



(XXXVII)
ED_{50} 15mg/kg
(in mice)

Size of Basic Group

Most analgesics possess tertiary basic groups. The exceptions are normorphine and the normethadol derivatives (*XXXVIII*)[160]. The influence of basic group size upon activity is most simply studied in analgesics possessing the 4-phenylpiperidine entity, since in such cases only one group attached to



R = H or COMe

(XXXVIII)

nitrogen may be altered with retention of the essential molecular skeleton. Until relatively recently, it was thought that replacement of *N*-methyl by a larger group would result in a fall in activity. Now, however, many potent analgesics are known that bear larger *N*-substituents[186]. The effects of replacing *N*-methyl by other groups may be summarized as follows:

(1) lower alkyl (Et, n-Pr, isoPr, n-Bu, isoBu)—activity falls sharply[107,164,211];

(2) higher alkyl groups (n-C_6H_{13} to n-C_9H_{19})—activity rises to above the *N*-methyl level, α-substitution reducing activity[212];

(3) benzyl—activity falls sharply[164,213,214];

(4) 2-arylethyl—activity rises, in many cases to much above the *N*-methyl level; 2-arylpropyl and 2-arylbutyl derivatives are also active and chain branching gives inactive or weakly active compounds[186];

(5) more complex groups—many other *N*-substituents confer high activities, e.g. $(CH_2)_2NHPh$, $(CH_2)_2COPh$, $(CH_2)_2O \cdot CH_2$—  ; in all cases an alkyl chain of at least two carbon atoms is directly linked to nitrogen[186].

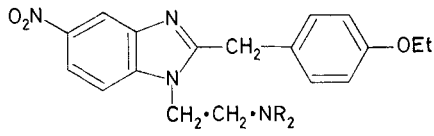
These results may be interpreted in terms of distribution and fit at the anionic site of the receptor (the degrees of dissociation of these derivatives would not be expected to differ markedly from those of *N*-methyl analogues). The presence of long alkyl chains or aralkyl substituents in the molecule probably increases lipid solubility of the drug and hence would be expected to facilitate passage across the many lipid barriers that the drug has to pass before it reaches its site of action. The wide range of functions present in *N*-substituents conferring high activities (e.g. NO_2, NH_2, $NH \cdot COMe$, CO, OH, OMe) is strongly indicative of the operation of a structurally non-specific effect (such as influence upon lipid solubility) rather than one dependent upon steric or electronic influences. *N*-(2-Benzoylethyl) norpethidines[186], being Mannich bases, would be expected to undergo *in vivo* dealkylation more readily than *N*-alkyl analogues and their high activities may possibly be attributed to this factor (see p. 207).

The sharp fall in activity following replacement of *N*-methyl by lower alkyl groups suggests that the anionic site can only accommodate a small bulk in the *immediate vicinity* of its locus of charge. The low activities of *N*-benzyl analogues and the disadvantageous effect of α-substitution in higher alkyl and phenalkyl derivatives support this view (see p. 207 for consideration of the NCD_3 group). In areas further removed from the focus of charge, spatial limitations appear to be less and additional bonding sites, especially for aryl groups as present in *N*-arylethyl substituents, may be available.

In acyclic analgesics, alterations in the basic function usually affect two of the groups linked to nitrogen. In methadone and related compounds, dimethylamino is the group which gives optimum activity. Diethylamino analogues are markedly less active while five and six membered alicyclic groups (pyrrolidino, piperidino and morpholino) yield compounds comparable in activity to dimethylamino derivatives[159]. Activity is lost again when the basic function is azacycloheptyl or azacyclo-octyl[203,215]. These results appear to reflect the steric limitations of the anionic site, the more compact medium-sized alicyclic basic groups fitting the site better than the flexible diethylamino function of greater van der Waals' radius. Methyl substituents in positions 2 and 3 of the piperidino ring increase the width of the basic group and activity falls, whereas a 4-methyl substituent, which on drug-receptor association should lie in a region of the anionic site where steric limitations are less, does not adversely affect activity[203]. In studies of normethadone and thiambutene type compounds, the optimum width of the basic group near the charged centre has been calculated to be 7·5–8·5 Å[203]. Surprisingly, in highly potent benzimidazole derivatives, the diethylamino member (*XXXIX*, R = Et) is fifty times as active as the dimethylamino compound (*XXXIX*, R = Me) in mice[216].

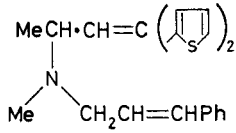
Replacement of one of the dimethylaminomethyl groups by an aralkyl residue increases the effective width of the basic function and on this account

would be expected to lead to a fall in potency. In the few examples available the results are inconsistent. Thus *N*-methyl-*N*-cinnamylthiabutene (*XL*) is

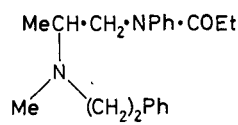


(XXXIX)

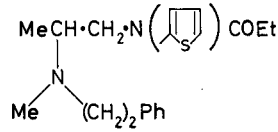
inactive[213] (the cinnamyl group confers high activities upon pethidine-type analgesics[214]) whereas diampromid (*XLI*) (containing an *N*-methyl-*N*-phenethyl group) is a potent analgesic[205] as is also the thienyl analogue (*XLII*)[184]. Even the *N*-benzyl-*N*-methyl basic anilidide related to diampromid has an activity greater than that of pethidine[169], *N*-benzyl derivatives of alicyclic analgesics invariably being of low potency or inactive.



(XL)



(XLI)



(XLII)

It may be of significance that basic anilides containing arylalkylmethylamino functions do not conform to the configurational requirements established for methadone and related compounds (see p. 193). Such inversion of configurational specificity also occurs in the methadols where the configuration of the oxygen function rather than that of the asymmetric carbon linked to nitrogen appears to be the prime factor governing activity. (See *Table 5.7.*) Portoghese and Larson[180] have suggested that the relative

Table 5.7. Analgesic activities of methadols and acetylmethadols in mice[217]
$Me_2N \cdot CHMe \cdot CH_2 \cdot CPh_2 \cdot CHOR \cdot Et$

Form	*Configuration**	*Methadols* (R=H) ED_{50} mg/kg	*Acetylmethadols* ED_{50} (R=COMe) mg/kg
L-(+)-methadone → α-(−) (25·7)†	L(L)	3·5	1·8
L-(+)-methadone → β-(+) (25·7)†	L(D)	63·7	4·1
D-(−)-methadone → α-(+) (0·8)†	D(D)	24·7	0·3
D-(−)-methadone → β-(−) (0·8)†	D(L)	7·6	0·4

* Bracketed capitals denote the arbitrarily chosen configuration of the oxygen function; the more active methadols have identical configurations at this centre.
† ED_{50} mg/kg.

orientation of oxygen function and basic group in basic anilides (which differs from that obtaining in methadone) may likewise influence the stereochemical specificity of the receptor site. Contrary to this interpretation, however, is the fact that acetylation of methadols restores the generally established configurational relationships (the more active acetylmethadols

are those derived from D-(−)-methadone) which also holds for corresponding nor-derivatives (the nor-analogue of α-(+)-acetylmethadol related to D-(−)-methadone, is almost nine times as active as the corresponding secondary amine related to L-(+)-methadone)[160]. Further, the configurations of the more active isomers of phenampromid (which contains the piperidino group, a basic function also encountered in 1,1-diphenylpropylamines) and isomethadone are identical (see p. 192) even though the dispositions of basic group to oxygen function differ in the two compounds.

Intrinsic Activity

Analgesic antagonists are characterized by their ability to abolish or reduce the analgesic and respiratory depressant effects of analgesics in laboratory animals. Such compounds given alone to animals behave like analgesics in respect of their effect on respiration but their analgesic potency is low. In man both the respiratory depressant and analgesic effects of antagonists are comparable to the analgesics to which they are related. The respiratory depression produced in man by a conventional analgesic is alleviated or prevented by an antagonist, analgesic effects being retained (see pp. 181–183).

This summary emphasizes the essential similarity in nature of analgesics and their antagonist and points to their associating with the same receptor site. The following observations support this view:

(1) morphine and nalorphine have the same configuration;

(2) levorphanol becomes an analgesic antagonist upon replacing the *N*-methyl group by *N*-allyl, whereas the corresponding change in the analgesically inactive dextrorphan fails to yield an antagonist[92];

(3) methylation of the phenolic hydroxyl group reduces analgesic potency in morphine and antagonistic properties in nalorphine (see pp. 185, 211);

(4) antagonists based upon morphine or morphinan antagonize not only their parent molecules but also many other active compounds (see p. 183).

The nature of the effect mediated by the association of a molecule with the analgesic receptor site, i.e. its intrinsic activity, is directly determined by the structure of the basic group present in the molecule. Antagonistic properties are specifically associated with a three carbon chain *N*-substituent and are best developed in *N*-allyl derivatives (see p. 185) (the closely related cyclopropylmethyl group also confers high activity in this respect).

Mode of Action of Analgesics and Antagonists

In 1956 a mechanism for the possible mode of action of analgesics and their antagonists was advanced based on the concept of agonist–antagonist competition for a common receptor site[218]. The interaction of the drug molecule (A) with the receptor (S) was represented as follows:

$$A + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} \underset{\text{complex}}{AS}$$

where K_1 = rate constant for association and K_2 = rate constant for dissociation.

The formation of the complex AS, while not of itself mediating a biological

effect, was regarded as triggering a sequence of reactions (given below) that induced an analgesic response

$$A + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} AS \xrightarrow{K_3} XS + Y \rightarrow \overset{\text{analgesic response}}{\overset{\uparrow}{X}} + S + Y$$

adsorption reaction desorption

X represents the compound which causes analgesic action or an essential intermediate in a further sequence of reactions which produces the biological effect. The desorption of X from the receptor S regenerates the latter for further combination with the drug. The degree of analgesia produced was considered to depend on the rate of formation of X, i.e. on the rate constant K_3. In the case of antagonists, it was presumed that the value of K_3 was much less than values obtaining in analgesics, resulting in X being formed only slowly and in quantities insufficient to produce a significant analgesic response. However, species variations in these constants would be expected; in animals nalorphine may be dealkylated at the analgesic receptor site more slowly than analgesic molecules, whereas in man the values of dealkylation of conventional analgesics and nalorphine may be similar.

The degree of inhibition of an analgesic by an antagonist depends upon (*a*) their relative concentrations in the phase in contact with the receptor, and (*b*) the change in free energy upon formation of the drug-receptor complex. Both aspects favour *N*-allyl derivatives. After administration of equal doses, concentrations of nalorphine in both dog and rat brain tissue were higher than those of morphine (see p. 186) while the replacement of *N*-methyl by *N*-allyl should increase drug-receptor attraction by increasing the non-bonded attractive forces between drug and receptor. This additional bonding represents a free energy change of about 2·5 kcal per molecule in the formation of the drug-receptor complex[219] and it has been calculated that a difference of this order would result in the two complexes being formed in equal amounts even if the concentration of analgesic were 40 times that of the antagonist[218]. Support for the proposed competitive nature of the antagonistic action of nalorphine has been provided by Cox and Weinstock[220] who found that the relationship between dose ratios and doses of nalorphine for analgesic activity in mice was linear in the case of morphine, methadone and M183 (the 3-*O*-acetyl analogue of (*XVIII*, R = Me, R′ = *n*-C_3H_7), see p. 197); analgesia induced by pethidine was only partially antagonized by nalorphine. Almost identical pA_2 values (indicative of competitive antagonism[221]) were calculated from this data. Similar results were obtained in a study of the antagonism by nalorphine of the lenticular opacity produced in mice by analgesics.

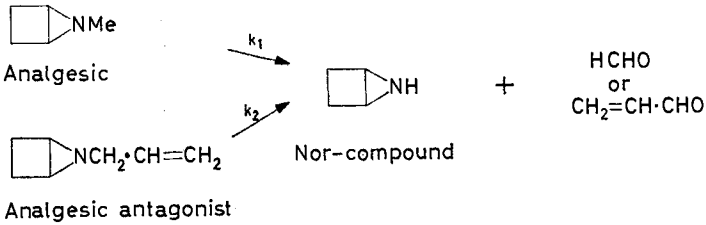
In a further development of the receptor site theory, conjecture was also made regarding the nature of the reaction immediately following formation of the drug-receptor complex[218]. The basic centre was considered to be the most likely site of reaction for the reasons listed below:

(1) Analgesic potency is extremely sensitive to structural changes in this group. A striking illustration of this fact is the significant fall in potency brought about in morphine by replacing *N*-methyl by *N*-trideutereomethyl[50], an effect more likely to be a consequence of chemical factors (reflecting

differences in the respective vibrational frequencies of C–D and C–H bonds) than steric since the effective kinetic size of the C–D bond is essentially similar to that of the C–H bond[222].

(2) The nature of the biological response appears to be governed by the structure of the basic centre, analgesics and their antagonists differing in this respect alone.

From a consideration of biochemical pathways involving the basic centre, oxidative *N*-dealkylation, resulting in the formation of a nor-compound at the receptor site, was proposed as the one most likely to represent the primary reaction.



When this postulate was first advanced, oxidative dealkylation, although a well established pathway in liver microsomes, was not known to occur in the C.N.S. Recently, however, Milthers[35] has obtained evidence of the transformation of both morphine and nalorphine into normorphine in the brain of hepatectomized rats, while Elison and Elliott[36] have demonstrated the *in vitro* *N*-demethylation of morphine, codeine and pethidine by rat brain slices. The demethylation of pethidine by brain tissue of rats has also been demonstrated by Herken, Neubert and Timmler[28].

These results are held to be complementary to those obtained from the study of liver biotransformations in demonstrating the common incidence of *N*-dealkylation in the body but are not advanced as direct evidence for occurrence of the same process at the analgesic receptor site. Dealkylation in the last respect is considered to take place at the *molecular level*, actual amounts of nor-products produced (assuming they undergo no further change) being so small as to render their detection by presently available techniques extremely unlikely. Nor is it felt that the drug-receptor dealkylation process is of necessity akin to that brought about by liver or brain enzymes. There is no consistent relationship between rates at which substrates are dealkylated in the liver and their analgesic potencies; codeine and pethidine (weaker than morphine) are more readily dealkylated than morphine by liver (and brain) preparations[27] while *N*-trideuteronormorphine (also weaker than morphine) undergoes this reaction less readily[50]. Nalorphine is more readily dealkylated than morphine by liver preparations[27], *in vivo* evidence indicating the reverse to obtain in the brain[35]. It is fundamental to the dealkylation hypothesis that a positive correlation exists between analgesic potency and dealkylation rates at the receptor site. Furthermore, as a result of the stereospecific nature of the receptor, only molecules of the correct configuration may undergo dealkylation. However, in further demonstration of the divergence between liver dealkylating enzymes and the system postulated to be implicated in the mediation of analgesia, the stereospecificity

of liver enzyme dealkylation of analgesics has been shown to be of an order far lower than that of the response of the analgesic receptor.[27,39,50]. Indeed, in view of the marked species, strain and even sex differences known to occur in the demethylating systems of liver preparations themselves (see p. 178), it is clear that the behaviour of known enzymes may only serve as an approximate guide to that of the system postulated as operating at the analgesic receptor site.

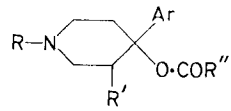
The question of the analgesic potency of normorphine requires examination. If normorphine is, indeed, an essential intermediate for the mediation of analgesia it would be expected to have a potency at least of the same order as that of morphine if administered correctly. This is, in fact, the case in mice provided the compound is administered by an intracisternal route, i.e. directly into the C.N.S.[218,223]. Its low activity by other routes[186] was originally accounted for by postulating the nor-compound to have a reduced ability to gain access to receptor sites (through decreased lipid solubility etc.)[218]. In accord with this view, Jóhannesson and Milthers[224] found morphine in the brain in higher amounts than normorphine after subcutaneous and intraperitoneal injection of equal doses in rats. When given intravenously, both drugs were found at the same brain concentration (by this route Orahovats and Lehman[225] found the analgesic potency of normorphine equal to that of morphine in dogs). However, when doses of morphine and normorphine of equal analgesic potency were given intraperitoneally to rats (morphine was found to be six times as potent as normorphine by this route) the concentration of normorphine in the brain was 4–5 times higher than that of morphine[224]. These results, however, relate to concentrations in total brain tissue and cannot be taken as indicative of relative concentrations in the vicinity of the receptor site where similar amounts of the two drugs are to be anticipated if their potencies are alike. It is probable that additional lipid membranes intervene between a drug and its locus of action even after penetration of the blood–brain barrier has been achieved. Hence, if normorphine and morphine are to reach the active site in comparable amounts the gross brain concentration of the former must be higher than that of the latter drug to produce an equianalgesic response.

It must be emphasized that the presence of the nor-compound *upon the receptor site itself* is regarded as the essential feature of the mechanism advanced for the mediation of analgesia. The nor-compound may either be the product of dealkylation following association between receptor site and a tertiary amine, or, if present in the surrounding media, be adsorbed directly onto the receptor surface. It may well be that dealkylation of a tertiary amine represents a more effective manner for producing the desired location of nor-compound as a result of the *N*-alkyl precursor being more readily adsorbed at the site. The antagonism of normorphine by nallorphine[223,226] is in accord with this view since the action of the antagonist has been interpreted in the light of its being particularly well absorbed upon the receptor site. Similarly, the apparent anomaly of normorphine being found in rat brain after administration of nalorphine (which lacks analgesic properties in this animal) is resolved once allowance is made for the nalorphine, also present, more readily gaining access to, and being preferentially adsorbed by, the receptor sites[224].

Aromatic Features of Analgesics

The most common aromatic entity present in analgesics is the phenyl group. Several reports have been made of the effects of variations in this group upon activity, and the results provide information relevant to the steric and electronic requirements of the flat area of the proposed analgesic receptor site. Much of the data relates to reversed esters of pethidine as a result of the versatile nature of 4-aryl-4-piperidinol syntheses. Gross increases in size of aryl group, as in naphthyl derivatives[201], leads to inactive compounds, while even 4-tolyl analogues are less active than the parent compounds in almost all cases (see *Table 5.8*). There is no consistent relationship between

Table 5.8. Effect of substitution in the 4-phenyl group of reversed esters of pethidine



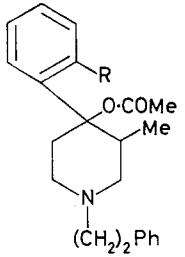
R	R′	R″	*Analgesic activity* (*morphine* = 1)				*Reference*
			Ar = Ph	Ar = *o*-MeC_6H_4	Ar = *m*-MeC_6H_4	Ar = *p*-MeC_6H_4	
Me	Me	Me	—	0·75	<0·2	0·3	227
Me	Me	Et	2	0·85	0·5	1·5	227
$(CH_2)_2Ph$	Me	Me	3·85	1·3	1·8	0·9	228
$(CH_2)_2Ph$	Me	Et	4·3	2·6	0·4	0·2	228
$(CH_2)_2Ph$	H	Me	6·3	1·2	—	0·5	229
$(CH_2)_2Ph$	H	Et	3·5	3·4	4·7	<0·3	229

			Ar = 2,3-$Me_2C_6H_3$	Ar = 2,4-$Me_2C_6H_3$	Ar = 2,5-$Me_2C_6H_3$	
$(CH_2)_2Ph$	H	Me	0·3	1	2·2	229
$(CH_2)_2Ph$	H	Et	—	0·5	<0·3	

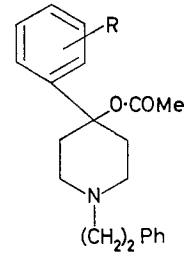
potency and position of substitution and only general trends may be discerned. Thus *para* substitution usually results in the greatest, and *ortho* in the least, fall in activity. In *o*-tolyl derivatives the adverse effect of aryl group enlargement upon drug-receptor contact may be offset by the shielding action that ortho groups have upon the ester function (see p. 212); this effect may also account for the high degrees to which activity is retained in *o*-methoxy derivatives (see formulae on page 210).

Isosteric replacement of phenyl, i.e. replacement of phenyl by other aromatic groups of essentially the same size (e.g. furyl, pyridyl and thienyl), in analgesics is generally disadvantageous. Thus the 2-thienyl analogues of pethidine[231] and ethoheptazine[232], the 2-furyl, 2-pyridyl and 2-thienyl

analogues of reversed esters of pethidine[201,233] and the 2-thienyl analogue of methadone[159] are all much weaker than the parent phenyl compounds. These results have been interpreted in terms of the bulky heteroatom

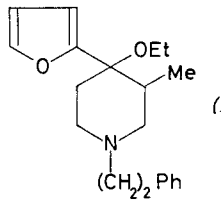


Activity[228]
R = H, 3·9
R = OMe, 4·0
Morphine, 1·0



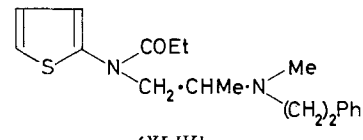
Activity[230]
R = H, 5·7
R = *o*-OMe, 3·0
R = *m*-OMe, 0·5
Pethidine, 1·0

(probably solvated) interfering with the fit of the aryl group at the flat portion of the receptor site[201]. However, the high activities of certain 2-furyl and 2-thienyl isosteres (see *XLIII* and *XLIV*) suggests that adequate association between these aryl groups and the receptor is, in fact, possible and that low activity, where it occurs, is due to other factors (e.g. the facilitation of ester hydrolysis in reversed esters of pethidine, discussed in the next section). A special case of isosteric replacement is that of the thiambutenes (*XLVa*);



(XLIII)

1·2–2·5 times as active as morphine in mice[233]



(XLIV)

As active as morphine in mice[184]

here the diphenyl analogue (*XLVb*) has negligible activity[234]. Electronic interactions considered of importance in maintaining the 'active' conformations of 3-amino-1,1-diarylbutenes (see p. 200) are absent in the diphenyl derivatives[203] (*XLVb*).

$R_2N{\cdot}CHMe{\cdot}CH{=}CAr_2$ *(XLV)* Ar = (a),  (b), Ph

The importance of π-electrons in analgesic drug-receptor association has yet to be established. A significant function for such electrons is not necessarily contra-indicated by the inactivity of certain reversed esters of pethidine in which phenyl is replaced by non-aromatic groups capable of providing π-electrons (e.g. —C≡CH and —C≡N) since the extent of π-cloud formation in the compounds tested may have been inadequate[235].

Oxygen Functions

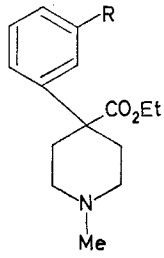
The importance of oxygen-containing functions in analgesics may be summarized as follows.

Analgesics with rigid skeletons

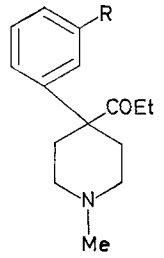
A free phenolic group is an important feature of analgesics based on morphine, morphinan and benzomorphan, its removal or masking by alkylation resulting in sharp falls in potency. Non-aromatic oxygen functions (e.g. the 4,5 ether bridge and the C- secondary hydroxyl of morphine) are not essential although they may enhance the potency of active compounds (as does, for example, a C_{14} hydroxyl group)[186].

Analgesics with a Non-rigid Skeleton

In contrast to the above, the presence of a phenolic group is not a prerequisite for high potency in analgesics of this class although such groups may be advantageous (see below).



Activity[159]
R=H, 1·0
R=OH, 1·5



Activity[159]
R=H, 0·5
R=OH, 10·0
Pethidine, 1·0

On the other hand, non-aromatic oxygenated functions are essential.

4-Phenylpiperidines

A wide range of structures is permitted for the oxygen function at C_4 comprising —CO_2R (as in pethidine), —O·COR (as in reversed esters of pethidine), —COR (as in ketobemidone) and —OR (as in 4-(2-furyl) piperidines)[233]. Functions of optimal size are shown below, sharp falls in potency occurring if these groups are made smaller or enlarged[159,236]. Weak activities result when oxygen is linked to acidic hydrogen, as in the

O ‖ —C·OEt	O ‖ —CEt	O ‖ —O·CMe	O ‖ —O·CEt	—OEt
Ethoxycarbonyl	Propionyl	Acetoxy	Propionoxy	Ethoxy

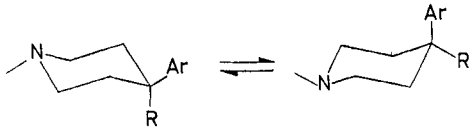
acid derived from pethidine and in 4-piperidinols[159,228].

Diphenylpropylamines

Potent members possess an oxygen function linked to carbon bearing an aryl group. Permitted structures are ketonic carbonyl (as in methadone), secondary hydroxy (free and acetylated as in methadol and derivatives), amidocarbonyl (as in dextromoramide), acetoxy and propionoxy (as in propoxyphene and related compounds)[186]. The thiambutenes (*XLVa*) provide rare examples of potent analgesics that entirely lack an oxygen group.

Some conjecture may be made regarding the role of oxygen functions in analgesics. In respect of distribution and metabolism, a phenolic group would be expected to militate against activity since it will increase the polarity of the molecule (and hence decrease lipid solubility) and will serve as a site at

which metabolic conjugation may readily occur (on the other hand metabolic *N*-demethylation is retarded by a free phenolic group, see p. 178). From the enhanced activities of phenolic derivatives relative to those of parent compounds it may be concluded that the phenolic group is directly implicated at the analgesic receptor site where it most probably serves as an additional binding site for drug-receptor association. This view is supported by the similar role assigned to phenolic groups in the uptake of catecholamines at adrenergic receptors[237]. Non-aromatic oxygen functions may also constitute supplementary binding sites, but in addition may have an important influence upon the conformation of the molecule either by generation of electronic interactions of the type $N–C_{CO}$, proposed for methadone[8], or through bulk effects. The last aspect may be important, for example, in 4-phenylpiperidines where the position of equilibrium of the system shown below will be critically influenced by the size of the oxygen function (*R*).



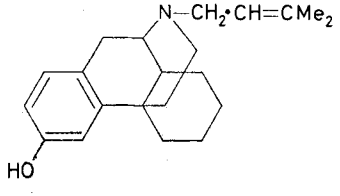
Metabolic transformations may convert analgesics which are esters into acidic-hydrogen containing species. Since the latter are inactive (either intrinsically or through failure to reach the locus of action) certain structure–action relationships in these esters may reflect interference or otherwise with metabolic hydrolyses. Thus the introduction of an ortho methyl or methoxyl group into 4-phenylpiperidines will significantly increase steric factors near the C_4 carbon atom and the generally higher potencies of such derivatives over *meta* and *para* substituted analogues (see *Table 5.7*) may be attributed, in part, to possibly slower rates of enzymatic hydrolysis in the former compounds. Conversely, reversed esters, in which structural features known to facilitate ester hydrolysis (e.g. 2-furyl and 2-thienyl groups) are present, would be expected to undergo *in vivo* hydrolysis more readily than 4-phenyl counterparts and to have reduced activities on this account[233,238]. In the same respect, propionyl and ethoxy groups may be more suitable than ester functions in 4-phenylpiperidines since the former groups are probably less prone to enzyme attack.

Addendum on Pentazocine

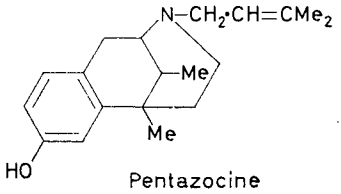
The observation that nalorphine is a potent, non-addicting analgesic in man has prompted the clinical evaluation of other morphine antagonists and related compounds in the hope of finding a potent analgesic that lacks the undesirable psychotomimetic side-effects (e.g. hallucination and disorientation) of nalorphine. In an earlier series, *N*-(3,3-dimethylallyl)-3-hydroxymorphinan (*XLVI*) proved the most promising[98]. It was found to be a potent analgesic (equipotent with morphine and nalorphine against post-operative pain) which lacked the psychotomimetic effects of nalorphine and was only half as depressant on respiration as morphine. However, it gave evidence in both monkey and human addicts of being a drug of addiction.

This work has been followed by an examination of a related series of

benzomorphan derivatives and particularly promising results have been reported with 2′-hydroxy-5,9-dimethyl-2-(3,3-dimethylallyl)-6,7-benzomorphan (*XLVII*, Win 20,228; pentazocine)[108]. Keats and Telford[109] found that this compound has an analgesic activity comparable with that of morphine against post-operative pain; the degree of respiratory depression was similar but was not antagonized by nalorphine. Its other side-actions



(XLVI)



Pentazocine

(XLVII)

were similar to those of morphine and included sedation, dizziness, nausea and vomiting. The major significance of this study with pentazocine was the discovery that the compound was free from psychic side-effects and had a low addiction liability in human addicts (its degree of addictiveness approximates to that of dextropropoxyphene)[110]. A recent clinical evaluation[239] confirms earlier work.

The pharmacology of pentazocine and related benzomorphan derivatives has been reported by Harris and Pierson[240]. Pentazocine did not give a positive response in the tail-flick and hot plate tests when administered in non-toxic doses to mice and rats. Unlike nalorphine (likewise a potent analgesic in man yet inactive in the hot plate test), pentazocine only feebly antagonized the effects of morphine on the tail-flick response. Most animal tests, as a guide to the clinical effectiveness of analgesics, are thus inapplicable to non-antagonists such as pentazocine and to potent antagonists such as nalorphine and *N*-propargyl-3-hydroxymorphinan[98]. Recently, however, phenylquinone-induced writhing in mice has been claimed to be a sensitive indicator of the analgesic activity of nalorphine, cyclazocine and pentazocine[241].

Despite intensive research resulting in chemical, biochemical and pharmacological advances in the analgesic field, the design of an analgesic drug devoid of side-effects and of known mechanism of action, is yet to be achieved. Time alone will tell whether signposts directing us towards this goal are already to hand amongst the vast amount of information now available, or whether the goal itself, by its very nature, is beyond our reach[241].

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