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Review Article

Some aspects of drug action: a comparison with intramolecular processes occurring in pharmaceutical and biochemical systems*

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Mechanism of enzyme action

Enzymes are biological catalysts and although the reactions they promote are well known, it is only as the result of intensive studies within limited classes in recent years that their mechanism of action has been revealed. Current theories regard the enzyme as a surface on which a substrate(s) is held by interatomic forces and subjected to bi-functional or polyfunctional catalytic processes such as those previously described.

The major part, or in some cases the whole, of the enzyme consists of a polypeptide chain of amino-acid units arranged in a definite conformation such as a helix and supported in such an arrangement by hydrogen bonds between adjacent parts of the chain. Somewhere along the chain is an area known as the active-site where the substrate is complexed and the catalytic processes occur. Defined in space this site may embrace one or more adjacent parts of the chain. The functional groups responsible for catalysis are present on those parts of the chain making up the active-site and are correctly orientated to perform their functions on the substrate. Hydrogen bonding, electrostatic interaction, van der Waals' forces or hydrophobic forces, enable the substrate to bind at the active site in the correct three dimensional arrangement for "intramolecular" catalysis to occur. The dimensional requirements of the substrate are probably defined by groups on the protein chain adjacent to the active site which perform a steric function.

Enzyme reactions may be differentiated from the normal catalytic processes occurring in chemical reactions such as hydrolysis or oxidation, by two features: substrate specificity and the speed of the reactions which occur in the pH range 2-10. These features become understandable if we consider that enzyme reactions proceed by an "intramolecular" mechanism involving a combination of the processes previously described where correct fit of a substrate at the enzyme surface will invoke the tremendous power of such reactions. Furthermore, hydrogen or hydroxyl ions present in the medium are only required to provide a suitable ionising medium for the catalytic functions and do not themselves participate in

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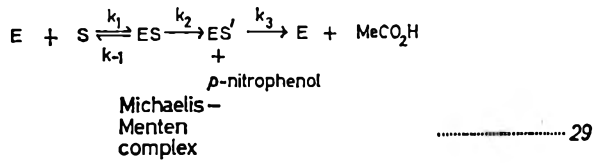
* The second part of a review on this topic. The first part appeared in the September, 1965 issue of this Journal.

the reactions. The mapping of the active-sites of enzymes is still in its infancy, but it seems reasonable to assume that all enzymes incorporate similar catalytic processes although additional refinements such as the presence of oxidation-reduction systems as co-enzymes are necessary to explain the mechanism of action of enzymes such as the dehydrogenases. The mechanism of enzyme action outlined above will now be illustrated in some detail for the hydrolytic enzymes, α-chymotrypsin and acetylcholinesterase.

α-CHYMOTRYPSIN

α-Chymotrypsin is an enzyme capable of hydrolysing amide and ester linkages in certain substrates and is found in pancreatic juice where it is stored in the form of a precursor, chymotrypsinogen. The enzyme consists of a polypeptide chain of amino-acid units only, and does not require a co-enzyme for its activity. Chymotrypsin has been isolated



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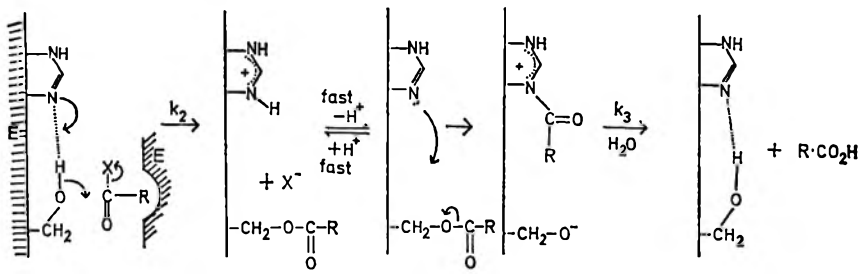
in crystalline form, and has a molecular weight of 23,000 (Rao & Kegeles, 1958). Inhibition studies using dyflos indicate that there is only one active site present in each molecule (Jansen, Nutting, Jang & Balls, 1949; Jansen, Nutting & Balls, 1949).

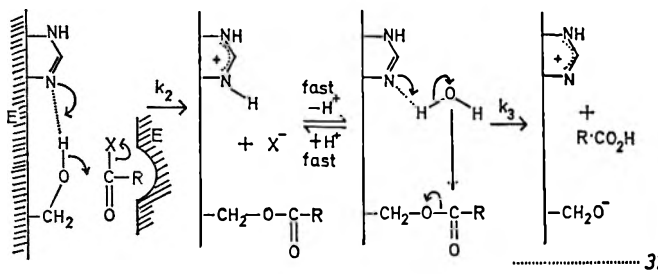
Chymotrypsin hydrolyses *p*-nitrophenyl acetate to acetic acid and *p*-nitrophenol and kinetic measurements indicate preliminary complexing between the substrate and enzyme in accordance with the Michaelis-Menten scheme for enzyme action. The complex then rapidly breaks down into an acyl-chymotrypsin (ES') with the release of *p*-nitrophenol, and the acyl-chymotrypsin is then slowly hydrolysed to acetic acid with regeneration of the enzyme (Hartley & Kilby, 1954) (eqn *29*, above).

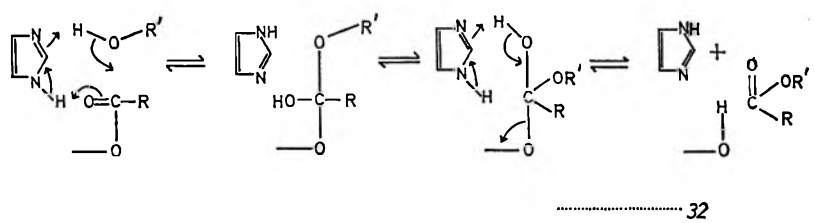
The acyl-chymotrypsin formed is stable in acid solution and has been isolated (Balls & Wood, 1956). The values for k_2 and k_3 for *p*-nitrophenyl acetate hydrolysis have been determined at various pH values and found to be pH dependent. Calculation shows that the catalytic groups involved in these steps have pK_a values of 6·7 (Gutfreund & Sturtevant, 1956) and 7·4 (Spencer & Sturtevant, 1959) respectively. Bender, Schonbaum & Zerner (1962b) have since shown that the same de-acylation rate (k_3) is obtained for five different esters of *trans*-cinnamic acid with chymotrypsin, indicative of the formation of a common intermediate *trans*-cinnamoyl α-chymotrypsin. These results may be interpreted as participation by an imidazole group (present in a histidine moiety) in the catalytic process since this has the required pK_a.

The presence of a second functional group necessary for the catalytic process was shown by Dixon, Dreyer & Neurath (1956), who found that the reaction of acetyl-α-chymotrypsin with hydroxylamine to give the hydroxamic acid at pH 5·5, was abolished by 8M urea. The reactivity of the

acyl-enzyme was restored when the concentration of urea was decreased by dilution. These results may be interpreted as an alteration by the urea solution of the conformation of the helix and thus the proximity of the functional groups on adjacent chains at the active site so that the deacetylation reaction is prevented. The second functional group involved in the catalysis is probably the hydroxyl group of a serine moiety since inhibition of chymotrypsin by dyflos followed by acid degradation gives serine phosphate (Schaffer, May & Summerson, 1953).







Various workers have attempted to show that in the acylated enzyme, the acyl group is attached either to imidazole or the serine hydroxyl, since this knowledge is essential for any proposal of mechanism of action of the enzyme. Dixon & Neurath (1957) concluded from a spectroscopic investigation that the acyl group initially resides on serine and the acyl-enzyme formed is stable at low pH values but at higher pH values it undergoes nucleophilic attack by the imidazole ring nitrogen atom to give

an acetylimidazole which is slowly hydrolysed to acetic acid with regeneration of the enzyme. Their scheme for the mechanism of action of chymotrypsin is shown in equation *30* (page 603). The imidazole nitrogen atom behaves as a general base catalyst in (i) and (ii) and then as a nucleophilic catalyst in (iii), (iv) and (v).

Spencer & Sturtevant (1959) re-examined the work of Dixon & Neurath and concluded that the observations made by these workers were due to changes occurring in the enzyme itself in being raised from pH 4·8 to pH 8·2. These changes result in light scattering as a consequence of protein precipitation occurring during the alteration in pH (Wootton & Hess, 1960). Spencer & Sturtevant consider that the imidazole functions as a general base for the acylation and de-acylation reactions, and their scheme is shown in equation *31*.

Bender & others (1962b), have detected spectrophotometrically *trans*-cinnamoyl-α-chymotrypsin formed during hydrolysis of *o*-nitrophenyl-cinnamate, indicating that the acyl-enzyme is an intermediate rather than an artifact. Furthermore, although the spectral changes noted did not differentiate between an acyl-imidazole or acyl-serine intermediate they were compatible with acylation on a serine hydroxyl which is in the environment of an aspartate carboxylate anion. Additional evidence is now available which, with these findings, identifies the group acylated as a serine hydroxyl group (see Bender & Kézdy, 1964, for review).

Bender, Schonbaum & Zerner (1962a) have recently shown that the pK_a values for acylation and de-acylation for each of three ester substrates studied are in reasonable agreement. This evidence, together with the similar effects of deuterium oxide, solvent composition and substrate structure on the overall catalytic and de-acylation rate constants, leads Bender (1962) to consider that the acylation and de-acylation steps are equivalent and carried out by the same catalytic functions, thus ruling out the mechanism proposed by Dixon & Neurath (1957) (eqn *30*, page 603) since the two steps are not equivalent. One of the rate-dependent steps involves proton transfer since, when the catalysis is carried out in deuterium oxide, the rate is decreased two to three-fold. This rules out proposed mechanisms which involve only a nucleophile (Westheimer, 1957; Rydon, 1958). The latest mechanism proposed by Bender & Kézdy (1964) is summarised in equation *32* (page 603) which embraces the equivalent steps of acylation (R′-OH = serine) and de-acylation (R′-OH = water). The imidazole functions as a general acid-general base catalyst through its two nitrogen atoms in a concerted process which gives a tetrahedral intermediate.

Some substrates of α-chymotrypsin give bell-shaped pH-rate profiles for the overall catalytic rate constant where the rate determining step is the acylation reaction, whereas a sigmoid profile is obtained when de-acylation is the rate determining step (Bender, Clement, Kézdy & Zerner, 1963; Bender, Clement, Kézdy & Heck, 1964). In addition to the imidazole group involved in acylation and de-acylation, it is proposed that there is present in acylation an acidic group ($pK_a \sim 9$) which is either absent or not concerned in the bond-changing processes of de-acylation.

These workers initially reached the same conclusion as Erlanger, Castleman & Cooper (1963) by regarding this acidic group as a serine hydroxyl in a "perturbed" state. However this acidic group has now been identified as the α-ammonium group of the *N*-terminal isoleucine residue (Labouesse, Oppenheimer & Hess, 1964; Oppenheimer, Labouesse, Carlsson & Hess, 1964). The function of this group is to induce conformational stabilisation of the active site in acylation, a function which is not necessary in de-acylation because of the covalent link of the acyl-enzyme. This implies that the conformation of the free enzyme is pH dependent and evidence has been presented to support this (Bender & Kézdy, 1964). The induced conformation is possibly due to an electrostatic bond between those parts of the enzyme surface at the active site carrying the ammonium group and a carboxylate ion.

In the proposed scheme *32*, the steric requirements between the imidazole molecule, the serine hydroxyl and the substrate are not fulfilled in a model. Recent evidence indicates that the two histidine residues in chymotrypsin are spatially very close, the actual sequence being His–Phe–Cys–S–S–Cys–His (Brown & Hartley, 1963; Hartley, 1964). It is suggested that conformation is important in the enzyme in order to ensure the close relationship between these two imidazole groups so that both participate in proton transfer in accordance with equation *32*, one acting as a general-base and the other as a general acid (Bender & Kézdy, 1964).

Bender, Kézdy & Gunter (1964), have analysed the kinetic factors responsible for the differences between the hydroxyl ion and α-chymotrypsin catalysed hydrolysis of *N*-acetyl-L-tryptophan amide. They were able to predict the enzymatic rate from a quantitative consideration of the intramolecular character of the general acid and general base catalysis by imidazole, together with a freezing of the substrate at the enzyme surface in a conformation where the ground state resembled the transition state for the reaction (i.e. energy barrier for reaction low). They concluded that the activity of the enzyme α-chymotrypsin could be discussed on a straightforward chemical basis where each factor involved was based on firm chemical analogy.

ACETYLCHOLINESTERASE

Two hydrolytic enzymes closely related to chymotrypsin are "pseudo"- and "true" (acetyl-) cholinesterase. Acetylcholinesterase hydrolyses acetylcholine (70) (page 607) faster than any other choline ester whereas "pseudo" cholinesterase hydrolyses other choline esters equally well. Furthermore, pseudocholinesterase shows saturation by excess substrate in accordance with the Michaelis-Menten scheme but not inhibition (Wilson, 1954), whereas acetylcholinesterase is inhibited by excess acetylcholine above a certain substrate concentration and the rate of hydrolysis falls off (Zeller & Bissegger, 1943).

Adjacent to the active site of acetylcholinesterase is a negatively charged site known as the anionic site where the quaternary nitrogen of the substrate is considered bound (Zeller & Bissegger, 1943). Evidence

for this view is provided from a study of the inhibition of the enzyme by tertiary bases and quaternary ammonium compounds which prevent the hydrolysis of acetylcholine. A tertiary base such as physostigmine (71) is far more effective as an inhibitor in acid solution where it exists in the protonated form than as the free base in alkaline solution, whereas quaternary ammonium compounds such as neostigmine (72) are equally effective over a wide pH range (Wilson & Bergmann, 1950a). The possibility that acetylcholinesterase has two anionic sites has been proposed; this would conveniently explain the inhibition noted with excess substrate (Bergmann, 1955).

The acetylcholine molecule with its three methyl groups attached to the cationic nitrogen is bound to the enzyme only as tightly as the corresponding dimethyl compound. However, with the former compound there is a marked decrease in entropy of the acetylcholine-enzyme complex (Wilson & Cabib, 1956) and this has been associated with the occurrence of a profound structural change in the enzyme-substrate complex (Belleau & Lacasse, 1964).

The kinetic scheme proposed for the hydrolysis of acetylcholine by acetylcholinesterase closely follows that proposed for chymotrypsin and other esterases, where an acyl-enzyme is initially formed which is decomposed by water to give products (eqn *33*, page 607) (Wilson, Bergmann & Nachmansohn, 1950).

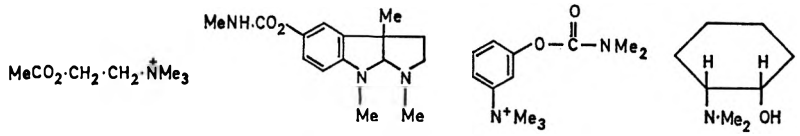
The acetylated function in the acyl enzyme is protected from hydrolysis by water as a result of excess substrate complexed at the anionic site. This accounts for the inhibiting effect of excess substrate (Wilson & Cabib, 1956; Krupka, 1963).

An examination of the rate-pH profiles obtained for the hydrolysis of a number of substrates by the enzyme shows that a basic and acidic group with pK_a 6·2–7·2 and pK_a 9·0–9·6 respectively, participate in the reaction at the active site (Wilson and Bergmann, 1950b; Bergmann, 1955; Bergmann, Segal, Shimoni and Wurzel, 1956; Krupka & Laidler, 1960). There is general agreement that the groups concerned are imidazole and the phenolic hydroxyl of tyrosine.

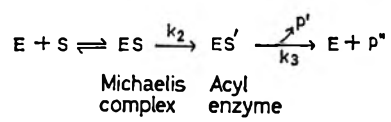
Phosphorylation of acetylcholinesterase leads to inactivation of the enzyme (see page 545) and this process proceeds in a manner analogous to substrate hydrolysis, except that the phosphorylated enzyme is stable to hydrolysis by water although it can be reactivated with stronger nucleophiles (Wilson, 1951). It seems very likely that phosphorylation (and by analogy, acylation) occurs on serine since inhibition of the enzyme with organophosphorus compounds containing ^{32}P followed by degradation gives serine ^{32}P-phosphoric acid (Schaffer, May & Summerson, 1954).

An early mechanistic scheme for the action of esterases including acetylcholinesterase proposed by Cunningham (1957) and later slightly modified for chymotrypsin by Dixon & Neurath (1957) (eqn *30*, page 603) assumes catalysis of the acylation and de-acylation steps by imidazole. An alternative scheme has been proposed by Krupka & Laidler (1960) who have included the acidic group at the active site (eqn *34*, page 607). The Michaelis complex has no ionisable groups present at the active site,

whereas the free enzyme and acyl-enzyme have the basic and acidic groups previously noted. This suggests that the substrate and enzyme in the complex are bound through these groups and that these groups are liber-



(70) (71) (72) (73)



Michaelis complex Acyl enzyme

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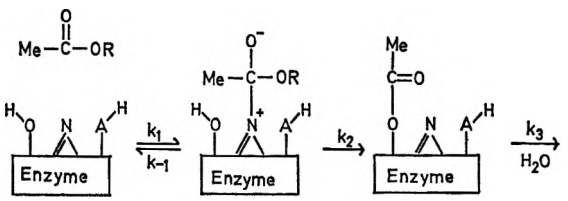

ENZYME + $MeCO_2H$

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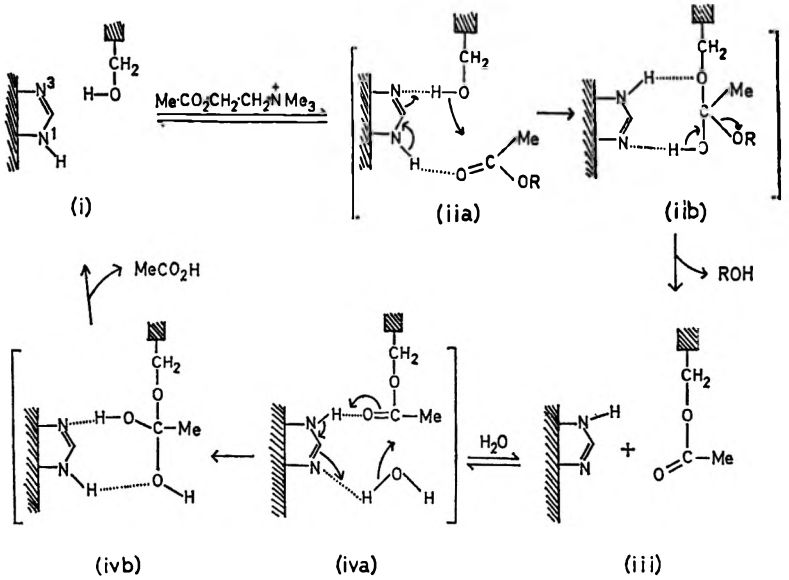

(i) (iia) (iib) (ivb) (iva) (iii)

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ated when transfer of the acyl function to serine occurs (Krupka & Laidler, 1960). Inhibitors of acetylcholinesterase may be divided into two classes; small molecules which block de-acylation by combining with the anionic site and acidic site, e.g. (73), and larger molecules such as

neostigmine (72) which are bound only at the anionic site and do not block de-acetylation (Krupka & Laidler, 1961). Krupka & Laidler have deduced from these facts that the anionic site is 5·0 Å and 2·5 Å from the basic (imidazole) and acidic sites respectively.

A recent scheme by Brestkin & Rozengart (1965) introduces the idea that acetylcholine participates in activation of the catalytic site and that the reactive form of serine does not exist in the molecule before interaction of the enzyme with the substrate (eqn *35*, page 607). Hydrogen-bonding between the carbonyl oxygen atom of the acetylcholine molecule, bound at the anionic site, and N–1 of the imidazole ring increases the basicity of N–3 which bonds with the serine hydroxyl (iia). This bonding increases the nucleophilic nature of the serine oxygen which attacks the acetylcholine carbonyl group with formation of a cyclic Michaelis complex (ii b). Electron rearrangement in (ii b) with expulsion of choline gives the acyl-enzyme (iii), which reacts with water through (iva, b) with regeneration of the enzyme and formation of acetic acid. The role of the acidic group at the active site is probably to transfer a proton from imidazole to either the choline or serine oxygen anions formed in (iib) and (ivb) respectively.

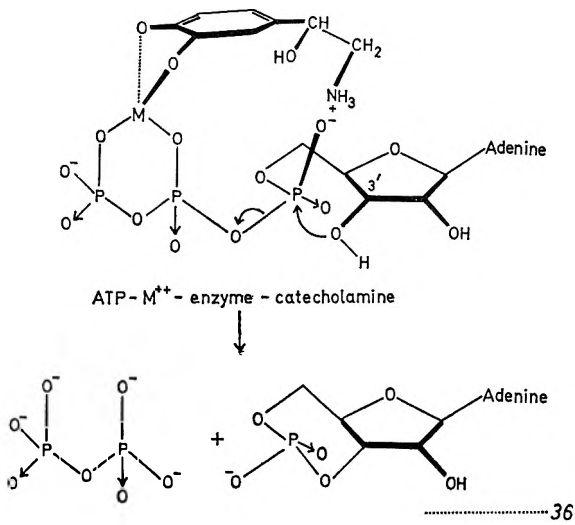
In conclusion it can be said that the proposed mechanisms for the action of α-chymotrypsin or acetylcholinesterase have not yet been universally accepted. However, from the foregoing discussion it is clear that the workers in this field consider that enzyme action can be explained in terms of the simple intramolecular processes which constitute the basis of this article.

Hormones

Whereas much is known about the general actions of hormones on organs *in vivo* and in tissues *in vitro*, very little information is available concerning the biochemical mechanisms which are influenced at cellular level. There has been some speculation about the nature of the receptors involved (Schwyzer, 1963). Some hormones may act upon the deoxyribonucleic acid (DNA) of chromosomes enabling genes to initiate the synthesis of ribonucleic acid (RNA), specific proteins and enzymes. Others may also interact with enzyme systems or with cell membranes (including membranes of subcellular structures).

Evidence has been forthcoming about the nature of the biochemical carrier of the phosphate group at the adrenergic receptor (Rall & Sutherland, 1959; Haynes, Sutherland & Rall, 1960). Adrenaline was shown to have a catalytic effect on the cyclisation of adenosine triphosphate (ATP) to adenosine-3′,5′-phosphate (3,′5′-AMP) which by activating phosphorylase increases the rate of glycogenolysis. The catecholamine is thought to interact with the system ATP-Mg^{++}–enzyme leading to 3′,5′-AMP as a result of (*a*) electrostatic attraction between the ammonium ion of the hormone and the phosphate ion, and (*b*) the formation of a co-ordination complex involving the phenolic groups of the hormone and the metal ion. The cyclisation process involves nucleophilic attack

by the 3′-OH group of the ribose moiety on the phosphorus, resulting in the ejection of pyrophosphate and 3′,5′-AMP, (eqn *36*, below) (Belleau, 1960). Under physiological conditions the oxygen atom on the phosphorus bears a negative charge which, as a result of interaction with the positively charged nitrogen atom of the hormone, becomes more effectively distributed. This increases the electrophilic nature of the



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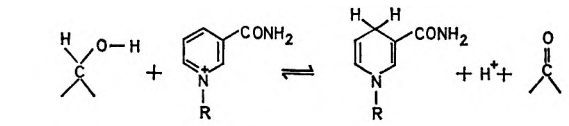
phosphorus reacting centre and consequently facilitates the process of 3′,5′-AMP formation and pyrophosphate ejection. Thus the hormone, in assuming the role of a general acid catalyst, supplements the activity occurring at this enzymatic site. [The blocking of the adrenergic receptor by dibenamine (554) can now be explained in terms of the esterification of the phosphate anion.]

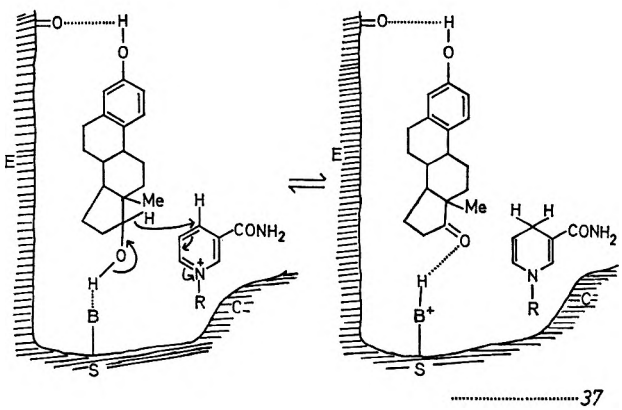
The formation of a complex between the catechol ring and the metal ion greatly increases the affinity of the hormone for the enzyme. Furthermore, molecular models reveal that chelation and ion pair formation can occur without the development of strain in the complex. As a means of obtaining further information about the β-adrenergic receptor site a series of tropolones biologically isosteric with the catechol system was shown to be effective as blocking agents (Belleau, 1963; Belleau & Burba, 1963). This clearly further implicates chelation as an important factor in the interaction of catecholamines with these receptors.

In attempting to correlate the structure and biological activity of 5-hydroxytryptamine (5-HT), Csötőstők, Per-enyi & Földes (1963) have found that the dissociation constant of the amino-group of 5-HT is 16 times greater than that of 4-HT which compares well with differences in their biological activities. Since ionisation is a prerequisite for the biological activity of 5-HT the possibility exists that this hormone, like adrenaline, becomes actively involved in the chemical changes taking place at an important receptor site. In this respect, the possibility

that 3′,5′-AMP acts as a mediator in the activity of 5-HT has already been contemplated (Haynes & others, 1960) to account for the stimulating effect of the hormone on the rate of glucose uptake by certain tissues. These authors contend that the adrenocorticotrophic hormone (ACTH) acts by controlling the phosphorylase level in the adrenal cortex. This suggestion follows from the discovery that ACTH causes an increase in the intracellular concentration of 3′,5′-AMP. Finally, as a result of these many observations, it is postulated that 3′,5′-AMP may function extensively as an agent of hormonal control.



VIII



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The occurrence of reduced and oxidised forms of many hormones in the body, e.g. hydrocortisone and cortisone, oestradiol and oestrone, suggests that this may be related to their physiological activity. Thus the action of oestradiol may be that of a catalyst involved in the transfer of hydrogen between reduced and oxidised forms of pyridine nucleotides. The net effect is the transfer of hydrogen from nicotinamide-adenine dinucleotide phosphate (NADP) to nicotinamide-adenine dinucleotide (NAD) (Talalay & Williams-Ashman, 1958).

$$NADPH_2 + NAD \rightleftharpoons NADP + NADH_2$$

Hagerman & Villee (1959) believe that oestradiol combines with the enzyme activating it, enabling it to effect a direct transfer of hydrogen between the NADP and NAD systems.

In addition to this transhydrogenation there are thought to be two oestradiol dehydrogenases catalysing reactions (1) and (2) respectively.

(1) NAD + oestradiol $\rightleftharpoons$ oestrone + NADH + H^+

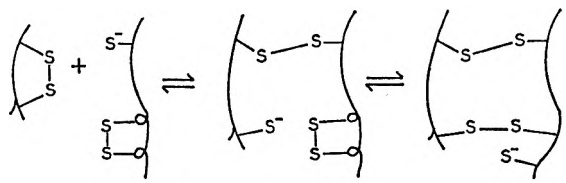
(2) NADP + oestradiol $\rightleftharpoons$ oestrone + NADPH + H^+

Laidler & Krupka (1961) have discussed reaction (1) in detail (see VIII, page 610) as an example of a molecular mechanism by which hydrogen transfer may occur in enzyme systems. This reaction is similar to the oxidation of lactic acid by NAD for which considerable kinetic information is available (*vide infra*). The rate-pH profile for the reaction shows a maximum, which suggests that a basic group –B and an acidic group –A–H play important roles. Studies involving isotope exchange (Loewus, Ofner, Fisher, Westheimer & Vennesland, 1953; Vennesland, 1955) suggest that this transfer of hydrogen is a direct one from substrate to coenzyme. A mechanism is proposed in which the rate-determining step for the enzyme-oestradiol-NAD system is visualised as in equation *37*. The non-polar oestradiol is probably attached to the enzyme surface by "hydrophobic bonds" arising as a result of the contact between non-polar groups leading to an increased degree of hydrogen bonding between solvent molecules. These "bonds" may also be supplemented by a hydrogen bond involving the oestradiol hydroxyl group. The transfer of hydrogen from the steroid to the NAD is facilitated by the active participation of group –B (enzyme site S; eqn *37*) as a general base catalyst. Oestradiol is converted to oestrone which now becomes attached by hydrogen bonding to the newly formed $-^{+}B-H$ group. It is possible that other acidic and basic groups on the enzyme surface are involved (i) in the binding of NAD to site C and (ii) in the activation of NAD to facilitate hydrogen transfer. This action of oestrogen on a specific enzyme system causes a marked increase in the cellular energy available for directing chemical reaction towards synthesis.

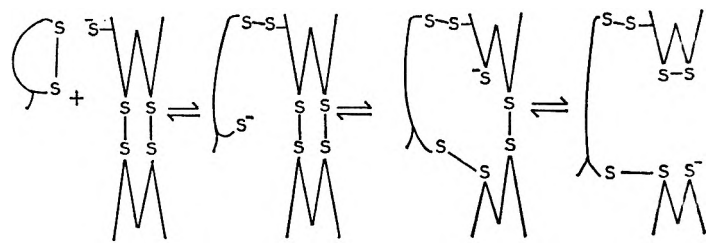
More recent studies using spectrophotometric and polarographic methods (Allison, Poever & Gough, 1962) have demonstrated the electron donor and acceptor capacity of several hormones including 5-HT, indoleacetic acid, prolactin and natural and synthetic oestrogens. Consequently, hydrogen bonding and the formation of charge transfer complexes could be involved in the activity of many hormones. Villee (1963) provides further evidence for this view by demonstrating that both corticosterone and progesterone increase the rate of reduction of nicotinamide adenine dinucleotide (NAD) by glutamic dehydrogenase.

It has been shown that insulin, ACTH and vasopressin all affect the permeability of cell membranes. A possible mechanism to account for this alteration in permeability has been suggested as a result of studies with vasopressin (Fong, Silver, Christman & Schwartz, 1960; Rasmussen, Schwartz, Schoessler & Hochster, 1960; Schwartz, Rasmussen, Schoessler, Silver & Fong, 1960). The increase in permeability to water of the isolated toad bladder was found to be related to the amount of tritium-labelled vasopressin bound to the bladder protein. Since a large proportion of the bound material could be liberated by incubation with thiol compounds it is suggested that the hormone combines with membrane protein by a thiol-disulphide interaction. This view is supported by the observed inhibition of hormone action when the pH of the solution falls below 7. Under these conditions the nucleophilic mercaptide ion becomes more highly protonated and less able to react with the hormone S–S

bridge. This SH, S–S interchange between hormone and receptor, which proceeds readily as a result of an initial electrostatically induced alignment of the relevant surfaces, could induce profound conformational changes in the protein of the diffusion barrier by breaking critical disulphide cross-linking (eqn *38*, below) with the formation of channels through which water, urea and Na^+ could flow. It is also possible that the hormone induces a separation of disulphide-linked fibrillar structures (eqn *39*, below) which could initiate a series or wave of SH, S–S interchanges as shown. Wave-like reactions of SH, S–S interchange



Hormone Receptor Hormone-Receptor Complex *38*



Hormone Receptor *39*

have also been implicated in a number of other important biological systems such as blood clotting and the changes involved during the mitotic cycle (Jensen, 1959). Disulphide and thiol groups occur widely in protein molecules and are potentially among the most reactive of protein functional groups. Normally, their reactivity is restricted by the conformation of the protein molecule. However, the control of many important physiological processes may depend on factors which establish conditions under which "intramolecular" nucleophilic interaction between protein SH and S–S groups can take place.

An attempt has been made to interpret the activity of insulin in terms of such an interaction involving S–S linkages of the peptide chain and SH groups of a cellular receptor (Cadenas, Kaji, Park & Rasmussen, 1961). This interpretation has, however, been questioned by Carlin & Hechter (1962) who were unable to demonstrate any inhibition of insulin activity after exposing the receptor tissue to thiol blocking agents.

It has often been stated that the overall effects of a hormone may well be due to its simultaneous action on a number of enzyme systems when the balance between the rates of reactions would be an important factor. Nevertheless, the exploration at cellular level of hormonal action may

continue to reveal common fundamental mechanisms underlying multiple biological activities. Recently published findings (Tata, Ernster, Lindberg, Arrhenius, Pederson & Hedman, 1963) related to possible biochemical mechanisms underlying the activity of thyroid hormone lend further support to this view.

Drug action

The fact that most biologically active substances are so constituted as to be capable of ionisation leads one to reflect on the role of such elements as nitrogen, oxygen and sulphur in drugs. This immediately brings to mind well established concepts such as those related to the penetration of membranes and the formation of drug-receptor complexes. When coupled with more recent information emerging from studies on the mechanism of enzyme and hormonal actions (*vida infra*) such reflections readily evolve into speculations which may suggest fresh concepts from which a further insight into the action of drugs at cellular level may be gained. It is the purpose of this section to examine these concepts in the light of those which are already familiar.

A large number of drugs contain a nitrogen atom whose "lone pair" of electrons can play a significant role in the biological activity of the molecule. Thus this "lone pair" may bond covalently with a hydrogen ion to form a salt in accordance with the equilibrium,

$$R_3N + H^+ \underset{}{\overset{k}{\rightleftharpoons}} R_3N^+H$$

and the ratio of ion to neutral molecule at physiological pH will depend on the ionisation constant. It is thought that many substances penetrate cell membranes as neutral molecules to exert their biological activity within the cell as ions.

A drug produces its biological response as the result of an interaction with a functional or organised group of atoms referred to as a receptor site. In order to facilitate such interaction the drug molecule must be correctly aligned at the site. To achieve this alignment, binding forces are invoked by the presence of groups on both drug and receptor surfaces which are complimentary to one another. In this respect, ionic "bonds," hydrogen bonds, ion-dipole and dipole-dipole interactions and van der Waals' forces all play their part. Ionisation at physiological pH occurs in aliphatic amino-, carboxyl, thiol and sulphonamido-groups so that potential ionic "bonds" are frequently found in drugs containing nitrogen, oxygen or sulphur. Each of these elements possesses at least one "lone pair" of electrons and is capable of forming hydrogen bonds. Differences in electronegativities between carbon and oxygen or nitrogen lead to dipoles which are able to form weak bonds with regions of low or high electron density, such as ions or other dipoles. Such dipolar functions as carbonyl, amide and ether are often located in drugs. Since, in most cases, it is essential for a drug to dissociate from the receptor site when the concentration in the extracellular fluid decreases, irreversible covalent bonds are undesirable.

On attaining a good "fit" to the receptor the drug may act either by initiating a response or by decreasing the activity normally associated with the site. In the latter instance, the drug blocks access to the site by the molecule responsible for producing a biological response. The view is firmly held that this effect explains the mode of action of drugs such as sulphonamides, antihistamines and neuromuscular, ganglionic and adrenergic blocking agents.

We would support a thesis which recognises that some drugs may fulfil an active role in biochemical mechanisms proceeding at or near a receptor site. It thus becomes possible to envisage a system whereby a drug, suitably ionised at physiological pH and correctly orientated at a receptor site, could influence a biochemical reaction occurring at a closely situated enzymatic site. A clinical condition could conceivably originate from a depletion of the required catalytic species at an important biochemical site of activity. A drug which alleviates this condition might possibly owe its effect to, say, a cationic group which as a result of general acid catalysis supplements the abnormally low activity at this site. Drugs which simulate the actions of adrenergic hormones may well behave in this manner.

Such speculations as these might tentatively be extended to include several types of drugs where the activity could depend upon the presence of nitrogen, oxygen or sulphur in anionic forms. Thus barbiturates, being ionised (N^-) at physiological pH, may influence an important enzymatic reaction as a result of nucleophilic reaction. The mechanism by which central nervous system depressants uncouple oxidative phosphorylation may well involve such considerations. Further conjectures could possibly include other substances such as salicylates, phenols and thyroxine which are also known to uncouple oxidative phosphorylation.

The pronounced activity of thiol groups in hormones has previously been cited. This group representing the tautomeric forms of thioamides, is found in several classes of synthetic biologically active compounds, e.g. dithiocarbamates, thiouracil. In its ionisable form it may enable some of these drugs to act in a manner previously envisaged.

To continue with these speculations may not be appropriate or even desirable at this present stage in our understanding of fundamental mechanisms occurring at cellular level. However, our survey of the extremely powerful intramolecular mechanisms which occur at enzyme surfaces as a direct consequence of the close proximity and correct orientation of groups, leads us to suggest that our hypothesis as outlined in this section, may well be within the bounds of possibility.

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Research Papers

Influence of the length of the stimulus period and frequency of sympathetic stimulation on the response of the guinea-pig isolated vas deferens to bretylium, guanethidine and amphetamine

M. D. DAY

The height of contraction of guinea-pig isolated vas deferens preparations in response to pre- or postganglionic sympathetic nerve stimulation at various stimulus frequencies was shown to vary with varying length of stimulation period. Short stimulation periods, as used by some workers, produced suboptimal contractions especially at the lower frequencies. It is suggested that variations in the length of the stimulation period could account for some of the contradictory results reported by various workers using this preparation. Thus the effect of low concentrations of dexamphetamine was qualitatively changed as the length of the stimulation period was increased; with short stimulation periods dexamphetamine impaired the responses to sympathetic stimulation but enhanced them if stimulation was continued until contractions had reached the maximum height obtainable. Higher concentrations of dexamphetamine impaired the contractions, the effect being most marked against the higher stimulus frequencies. Using stimulation to maximal effect a qualitative difference in the blocking actions of bretylium and guanethidine was demonstrated. Bretylium reduced the responses to the higher stimulus frequencies to a greater extent than it reduced the responses to the lower frequencies. Guanethidine reduced the responses to all frequencies so that there was a parallel shift of the frequency/response curve to the right. The possible significance of these findings is discussed.

THE isolated sympathetically-innervated vas deferens preparation of the guinea-pig was first described by Huković (1961) and has since been widely used to elucidate sympathetic nervous mechanisms. The published pharmacological work on this preparation contains several conflicting reports concerning the action of various drugs. Some of these differences may be explained on the basis of the recent convincing evidence that most fibres in the hypogastric nerve supplying the vas deferens synapse at or near the organ (Bentley & Sabine, 1963; Birmingham & Wilson, 1963; Kuriyama, 1963; Ohlin & Stromblad, 1963).

Other contradictory results cannot be explained in this way. Edge (1964) showed that low concentrations of amphetamine potentiated the contractions of the vas deferens in response to hypogastric nerve stimulation, the effect being most marked at low frequencies of stimulation. Higher amphetamine concentrations inhibited the responses to sympathetic nerve stimulation with the higher frequencies being most affected. Using the same preparation and drug, Morrison & Parkes (1964) obtained the opposite effects. They found that low concentrations of amphetamine reduced responses to all frequencies of stimulation except the highest, which were potentiated; higher concentrations reduced responses to all frequencies of stimulation, the effect being most marked against the lower frequencies. Furthermore, Morrison & Parkes (1964) were unable

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to confirm the observations of Green and others, made on different preparations, that the sympathetic nerve blocking actions of bretylium and guanethidine could be differentiated by their blocking efficacy at different stimulation frequencies (Boura & Green, 1962; Green & Robson, 1964).

In view of the increasing use of the vas deferens preparation and of the drugs bretylium and guanethidine in the investigation of the sympathetic postganglionic mechanism, an attempt has been made to resolve these conflicting reports.

Experimental

Methods

Guinea-pigs weighing 400–500 g were killed by a blow on the head and vasa deferentia were removed and set up in 75 ml organ baths containing Tyrode solution gassed with air and maintained at 32°. Longitudinal contractions of the preparations were recorded kymographically by means of an isotonic frontal writing lever. The preparations were stimulated electrically either through the hypogastric nerve ("preganglionic") as described by Huković (1961) or by means of parallel electrodes ("postganglionic") as described by Birmingham & Wilson (1963).

Preganglionic stimulation was applied by threading the nerve through bipolar platinum electrodes of the type described by Burn & Rand (1960) and postganglionic stimulation by passing the whole preparation through an electrode consisting of two platinum rings embedded in epoxy resin. In some experiments both pre- and post-ganglionic stimulation were applied in the same preparation.

Electrical stimulation was supplied from Palmer electronic stimulators delivering rectangular pulses of 2 msec pulse width and supramaximal strength. Stimulation was applied for periods of 2 or 5 sec repeated at intervals of 1 to 3 min, or (as in most experiments) continuously until a maximal response was obtained. In the latter instance 5 min periods were allowed from the end of one stimulation period before the start of the next. This method of stimulation produced more consistent results than were obtained with short fixed periods of stimulation.

Drugs

The following drugs were used: (+)-amphetamine (dexamphetamine), (−)-amphetamine, (±)-amphetamine, as the sulphates; concentrations in the text are expressed in terms of the base. Concentrations of guanethidine sulphate and bretylium tosylate (*p*-toluene sulphonate) refer to the salts.

Results

Effect of duration and frequency of stimulation on responses of the isolated vas deferens

Postganglionic stimulation. In the initial experiments preparations were stimulated via parallel electrodes. This produced results corresponding closely to those expected after postganglionic stimulation

(Birmingham & Wilson, 1963), and largely precluded the possibility of ganglionic effects.

The lowest frequency of stimulation which regularly evoked a recordable contraction was 2 pulses/sec. The size of the contractions increased with increasing frequency in a linear fashion up to 20 pulses/sec; stimulation at 50 pulses/sec produced responses which were usually little different

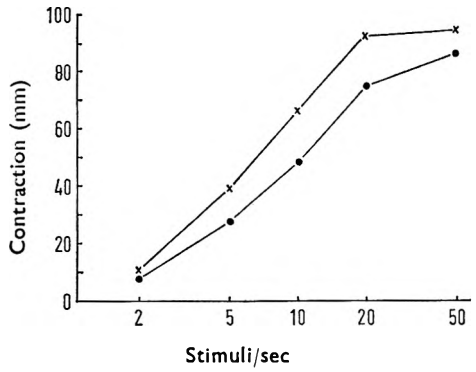

FIG. 1. Mean height of responses (in mm) from 17 preparations of guinea-pig isolated vas deferens stimulated postganglionically at various frequencies to maximal effect (— × —) and for 5 sec at each frequency (— ● —).

from those obtained at 20 pulses/sec. The size of the contraction at any one frequency was to some extent dependent on the duration of the stimulation period. Thus, stimulation for periods of 5 sec produced responses that were consistently smaller than those obtained in the same preparations at the same stimulation frequencies in which stimulation was continued until a maximal effect was obtained. The frequency/response curves obtained were nevertheless roughly parallel (Fig. 1).

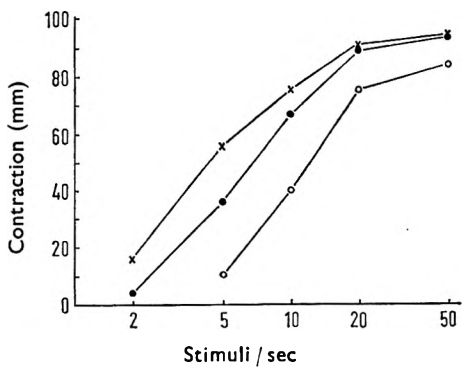

FIG. 2. Mean height of responses (in mm) from 6 vas deferens preparations stimulated preganglionically at various frequencies for 2 sec (— ○ —), 5 sec (— ● —) and to maximal effect (— × —).

Preganglionic stimulation. In 6 preparations frequency/response data were obtained over the frequency range 2 to 50 pulses/sec by stimulating the hypogastric nerve in each preparation for periods of 2 and 5 sec, and

to a maximal effect, at each frequency. As with postganglionic stimulation a 5 sec stimulus period was insufficient to produce a maximal effect at any frequency of stimulation. The difference was more apparent with low rates of stimulation and was even more marked when 2 sec stimulation periods were used. The mean responses from these experiments are plotted in Fig. 2.

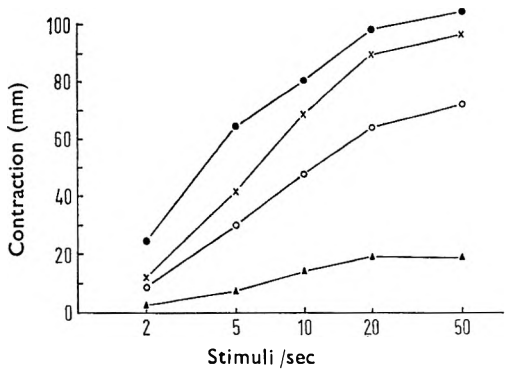

FIG. 3. Effect of dexamphetamine on the mean contraction heights (in mm) from 6 vas deferens preparations stimulated postganglionically to maximal effect at various frequencies; control observations (—×—), and in the presence of 1 μg/ml (—●—), 30 μg/ml (—○—) and 100 μg/ml (—▲—) of dexamphetamine.

Previous workers using this preparation have stimulated the nerve for periods of 2 to 5 sec (Huković, 1961; Morrison & Parkes, 1964; Large, 1965) or for a fixed number of pulses at each frequency (Burn & Weetman, 1963; Edge, 1964). The results plotted in Fig. 2 suggest the possibility that qualitative differences in drug actions obtained by various workers in this preparation may be a consequence of variations in methods of nervous stimulation.

ACTION OF DEXAMPHETAMINE ON THE VAS DEFERENS

Postganglionic stimulation. Dexamphetamine (1–10 μg/ml) caused a potentiation of the responses to all frequencies of sympathetic stimulation whether this was applied for 5 sec periods or continued until a maximal response was obtained. In both instances the enhancement was most marked at low frequencies of stimulation and was greater in the concentration range 1–3 than at 10 μg/ml. In concentrations above 10 μg/ml, dexamphetamine impaired sympathetic responses. Fig. 3 shows graphically the potentiation produced by a low concentration of dexamphetamine (1 μg/ml) and the impairment produced by higher concentrations (30 and 100 μg/ml) on supramaximally stimulated preparations.

The potentiation of responses with the low concentration of dexamphetamine are in accord with the results of Edge (1964) but differ from those obtained by Morrison & Parkes (1964) who reported that this concentration of amphetamine reduced low frequency stimulation (6 to 16 pulses/sec) but potentiated high (20 and 24 pulses/sec).

Preganglionic stimulation. Both Edge (1964) and Morrison & Parkes (1964) used preganglionic stimulation in most of their experiments so it is unlikely that a ganglionic action of amphetamine could account for the disparity in their results. However, Morrison & Parkes (1964) stimulated

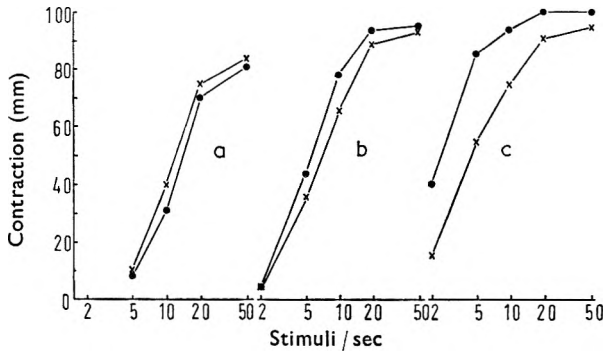

FIG. 4. Modification of the action of dexamphetamine on mean responses from 6 vas deferens preparations caused by changing the duration of the stimulation period. Preganglionic stimulation at various frequencies; control observations (—×—), and in the presence of 1 μg/ml of dexamphetamine (—●—). In 'a' with 2 sec stimulation periods the responses are slightly reduced after dexamphetamine whilst in 'b' with 5 sec periods the responses are slightly enhanced. In 'c' the stimulation was continued until a maximal response was obtained at each frequency, and dexamphetamine caused a potentiation of the responses which was most marked at the lower frequencies.

for fixed periods of 2 to 5 sec at each frequency and thus gave increasing numbers of stimuli with increased frequency whilst Edge (1964) stimulated with 100 pulses at each frequency. To determine whether the divergent results obtained by these workers were explicable on the basis of the differing duration of their stimulation periods, 6 preparations were stimulated over the frequency range 2 to 50 pulses/sec for periods of 2 and 5 sec, and to a maximal effect, both before and in the presence of dexamphetamine (1 μg/ml). The mean results from these experiments are plotted in Fig. 4 and show that both a qualitative and a quantitative change in the action of dexamphetamine occurs with increase in stimulation period.

With 2 sec stimulation periods the responses were slightly but uniformly depressed after dexamphetamine (Fig. 4a), whilst they were slightly enhanced if the stimulation period was extended to 5 sec (Fig. 4b). When the nerve trunk was stimulated until a maximal response was obtained the action of dexamphetamine was to produce a much greater potentiation of responses which was particularly marked at the lower frequencies (Fig. 4c). This latter effect was like that seen with this concentration of dexamphetamine on preparations subjected to supramaximal postganglionic stimulation (Fig. 3).

ANTISYMPATHETIC ACTION OF AMPHETAMINE ON THE VAS DEFERENS

As shown in Fig. 3 dexamphetamine in a concentration of 30 μg/ml or above caused a considerable impairment of the responses to sympathetic

nerve stimulation. Morrison & Parkes (1964) obtained a similar result with this concentration although they did not state which optical isomer of amphetamine they used in their experiments. Using (±)-amphetamine, Edge (1964) reported that concentrations up to 100 μg/ml potentiated the responses whilst 500 μg/ml were necessary to inhibit sympathetic responses. These divergent results suggest the possibility that there might be a marked difference between the blocking efficacy of the (+)- and (−)- form of amphetamine. Accordingly, experiments were made in which the effect of (+)-, (−)- and (±)-amphetamine was compared on the maximal responses to sympathetic nerve stimulation over the frequency range 2 to 50 pulses/sec, using both pre- and post-ganglionic stimulation. In these experiments it was found that both optical isomers and the racemic mixture produced identical results. Thus, each substance potentiated the responses to sympathetic stimulation in concentrations up to 10 μg/ml the effect being most marked at the low frequencies, whilst higher concentrations (30–100 μg/ml) caused impairment which was most marked at the higher stimulation frequencies.

COMPARISON OF THE BLOCKING ACTIONS OF BRETYLIUM AND GUANETHIDINE ON THE VAS DEFERENS

Boura & Green (1962) showed in the cat nictitating membrane preparation that the blocking actions of bretylium and guanethidine could be distinguished because guanethidine caused a preferential block of low frequency stimulation while bretylium preferentially blocked the high frequency. Morrison & Parkes (1964) were unable to confirm this observation in the vas deferens preparation; in their experiments both drugs produced a uniform degree of block at all stimulus frequencies. Since a qualitative difference between the blocking actions of bretylium and guanethidine may provide a clue to their precise mode of action at the sympathetic nerve ending, it was decided to re-examine this phenomenon on this preparation.

Postganglionic stimulation. In these experiments stimulation was applied until a maximal contraction was attained at each frequency over the range 2 to 50 pulses/sec. The effects of bretylium (3 μg/ml) and guanethidine (1 μg/ml) were compared in 12 preparations taken from 6 guinea-pigs; the two drugs being compared on preparations from the same animal in each experiment. Control frequency/response data were obtained initially and again after each drug had been left in the bath for periods of 30–45 min or until a virtually steady state of blockade had been attained. The mean results from these experiments are shown in Fig. 5; a clear qualitative difference was observed between the blocking actions of bretylium and guanethidine over the frequency range 2 to 20 pulses/sec. Bretylium had a relatively greater effect against the higher frequencies and thus flattened the frequency-response curve whilst guanethidine decreased the response at all frequencies causing a parallel shift of the frequency/response curve to the right. At 50 pulses/sec this difference was not so apparent, both drugs causing a greater degree of block than at 20 pulses/sec.

Preganglionic stimulation. Morrison & Parkes (1964) used preganglionic stimulation applied for short periods in their experiments and were unable to show a qualitative difference between the blocking actions of bretylium and guanethidine. In the present investigation their experiments were repeated using preganglionic stimulation but applying stimulation at each frequency until a maximal response was obtained. The results obtained were virtually the same as those shown in Fig. 5 for postganglionic stimulation.

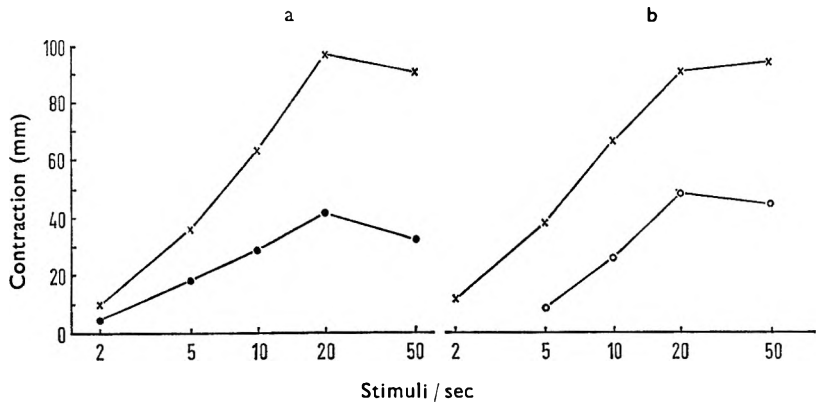

FIG. 5. Differential blocking action of bretylium and guanethidine on mean responses (in mm) from 12 vas deferens preparations taken from 6 guinea-pigs. Contractions in response to postganglionic sympathetic nerve stimulation at various frequencies and continued to maximal effect. In 'a', control responses from 6 preparations (—×—) were impaired most at the higher frequencies after 3 μg/ml of bretylium (—●—). In 'b' the contralateral preparations from 'a' were used and after 1 μg/ml guanethidine (—○—) the control responses (—×—) were preferentially blocked at the lower frequencies.

Reversal of guanethidine and bretylium blockade by dexamphetamine. As shown by previous workers (Day & Rand, 1963; Morrison & Parkes, 1964) dexamphetamine in low concentrations reversed the blocking action of bretylium and guanethidine on the vas. In the present experiments this restoration was found to occur over the full frequency range (2 to 50 pulses/sec) with both bretylium and guanethidine. This is similar to the behaviour of the cat nictitating membrane preparation (Day & Rand, 1963).

Discussion

The results described in this paper indicate that in the isolated vas deferens preparation both qualitative and quantitative differences in the action of a single drug can be obtained by varying the length of the period of electrical stimulation to the sympathetic nerves. Thus, using periods of stimulation of up to 5 sec as described by some workers (Hukovič, 1961; Morrison & Parkes, 1964) the contractions of the preparation are incomplete at all stimulus frequencies. Moreover, if frequency response data are obtained in this way the total numbers of stimuli applied at each frequency differ and consequently the lower frequencies will be more completely submaximal than the higher ones,

causing a steepening of the frequency/response curve. In the present experiments (Fig. 2), with 2 sec stimulus periods, the actual number of stimuli delivered varied between 4 (at 2 pulses/sec) and 100 (at 50 pulses/sec). It would be expected that 100 pulses at any frequency might produce a more nearly maximal response than would 4 pulses. That this was so is shown in Fig. 1; with stimulation periods of 2 or 5 sec the responses to the lower frequencies are smaller than those to the higher frequencies, when compared with the maximal responses obtainable at each frequency with prolonged stimulation.

Another disadvantage of stimulating this preparation for a fixed arbitrary time, irrespective of whether contraction is complete or not, is that drugs which affect the speed of contraction of the preparation may produce qualitatively different effects according to the length of the stimulation period. This is apparently the case with dexamphetamine; Morrison & Parkes (1964) stimulated for 2 to 5 sec and reported that amphetamine depressed the responses to all frequencies of sympathetic stimulation except the highest. In the present experiments a blocking action of low concentrations of dexamphetamine was similarly demonstrated (Fig. 4a) with 2 sec stimulus periods, but this was converted to a slight enhancement if stimulation was extended to 5 sec, and to a marked enhancement if stimulation was applied to maximal effect. The most likely explanation of this phenomenon is that amphetamine slows the rate of contraction of the vas in response to sympathetic stimulation whilst potentiating the actual extent of the contraction. Thus, it seems likely that differing durations of stimulation period could be an important factor in explaining the divergent results obtained by other workers using this preparation.

In the present experiments using dexamphetamine, bretylium or guanethidine the effects of these drugs were unchanged whether pre- or post-ganglionic stimulation was used. However, the presence of ganglionic synapses along the hypogastric nerve trunk is another factor which may account for disparity between the results of different workers using other drugs, as shown by Birmingham & Wilson (1963).

Using maximal stimulation it was shown that dexamphetamine in concentrations of 1 to 10 μg/ml potentiates the effects of sympathetic nerve stimulation the effect being most marked on the lower frequencies, confirming the observation of Edge (1964). However, doses of 30 μg/ml and above depressed the responses to sympathetic nerve stimulation the effect being most marked at the higher frequencies. This latter observation is not in complete agreement with the findings of Edge (1964) who reported that concentrations up to 100 μg/ml of amphetamine potentiated responses whilst 500 μg/ml were necessary to cause a blocking action. The reason for the difference between the blocking potency of amphetamine in the present experiments and those of Edge (1964) is not apparent. It is not due to differing potencies of the optical isomers of amphetamine since in these experiments both optical isomers and the racemic mixture produced indistinguishable effects.

Edge showed that high concentrations of amphetamine produced an

anti-adrenaline effect in addition to blocking the contractions to sympathetic nerve stimulation. However, the fact that high frequencies of stimulation are more impaired than low frequencies suggests some other mechanism perhaps in addition to an anti-adrenaline effect. Day & Rand (1963) postulated that the blocking action of dexamphetamine might be due to a weak adrenergic neurone blocking effect and this is compatible with the recent report that dexamphetamine has a very marked affinity for the noradrenaline uptake site (Iverson, 1964). Concentrations of dexamphetamine below those necessary to impair responses to sympathetic nerve stimulation oppose the blocking action of both bretylium and guanethidine.

The observations of Green and his colleagues (Boura & Green, 1962; Green & Robson, 1964) on the relative blocking efficiency of bretylium and guanethidine against high and low frequencies of sympathetic nerve stimulation have provided what may be an important clue to the precise mode of action of these drugs and perhaps to the phenomenon of tolerance to their effects sometimes met in clinical practice. By using prolonged stimulation to produce a maximal contraction at each stimulation frequency this differential blocking effect has been demonstrated in the vas deferens preparation which may therefore provide a convenient tool for the further examination of this interesting phenomenon.

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Urinary excretion kinetics of amphetamine in man

A. H. BECKETT AND M. ROWLAND

The urinary excretion of amphetamine has been examined in 11 male subjects after oral administration of (+)- and (−)- amphetamine sulphate. The excretion of unchanged drug was shown to be dependent upon urinary pH. Excretion of amphetamine, when the urine was maintained alkaline or acid, was measured in seven subjects. The implications of these results, especially in the evaluation of dosage forms, are discussed.

AMPHETAMINE is excreted in the urine of man substantially unchanged, the values of recovered drug varying from 12–100% (Richter, 1938; Beyer & Skinner, 1940; Jacobsen & Gad, 1940; Keller & Ellenbogen, 1952; Chapman, Shenoy & Campbell, 1959; Alles & Wisegarver, 1961; Cartoni & de Stefano, 1963). The various analytical techniques used may account for the spread of the recoveries although the changes in the urinary excretion of amphetamine with pH of the urine in the various trials may have been a contributing factor. Repeated use of amphetamine seemed to have little effect on amount of the dose recovered in the urine (Jacobsen & Gad, 1940; Harris, Searle & Ivy, 1947).

The present paper extends a preliminary communication (Beckett & Rowland, 1964a) which examined the influence of urinary pH on the excretion of unchanged amphetamine in man and also considered the kinetic significance of the results. Some of the clinical aspects have been discussed by Beckett, Rowland & Turner (1965).

Experimental

ORAL ADMINISTRATION OF AMPHETAMINE AND COLLECTION OF URINE

General method. Male subjects, 23–33 years, were used; no other drug (including alcohol) was taken for a day before and during the trials. Breakfast of tea or coffee and toast was taken at 8.0 a.m., urine voided just before 8.30 a.m. and the stated dose of (+)- or (−)-amphetamine sulphate then given orally in aqueous solution (50–100 ml). The urine was collected and measured every 2 hr for 16 hr and then at 24, 28, 32, 36, 40 and 48 hr. In most cases the pH was determined immediately, but never later than 12 hr after collection. If urination was not at the above times, the exact time was noted. In some trials, hourly samples were collected during the first day of the trial. The drug was occasionally administered in the morning other than at 8.30 a.m., but always 30 min after breakfast. The urine of seven subjects, who were not given the drug, was also examined.

Alkaline urine trials. The general method was used but an alkaline urine was induced and maintained alkaline by sodium bicarbonate (1 g/20 ml water orally). A typical regimen was a 3 g sodium bicarbonate dose 1 hr

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before drug administration, 2·5 g at the time of taking the drug and then every 2 hr for 14 hr. Urine was collected every 2hr for 16 hr after the drug had been taken. Trials were not begun until the urine was alkaline. A 40–48 hr sample of urine was collected in some instances.

Acid urine trials. The general method was used but an acid urine was induced and maintained acidic by ammonium chloride (0·5 g enteric coated tablets*). A typical regimen was 4 g ammonium chloride taken 2 hr before and 3 g taken 1 hr before the amphetamine and then 1 g hourly throughout the trial. Generally amphetamine was not taken until the pH was 5·3 or less. After amphetamine administration, urine was collected hourly for 16 hr and occasionally a 24 hr sample was taken. In several subjects urine was collected every 15 min for the first $2\frac{1}{2}$ hr.

INTRAVENOUS INJECTION OF AMPHETAMINE

Collection of urine. Approximately 13 mg (+)-amphetamine sulphate was given intravenously to three subjects with ammonium chloride-induced acid urine. After administration of the drug, urine was collected every 15 min for $2\frac{1}{2}$ hr and then hourly for 14 hr.

AMPHETAMINE IN OTHER BIOLOGICAL FLUIDS

Bile. (+)-Amphetamine sulphate, 10 mg, was given orally to a cholecystectomy patient with a bile duct fistula. The bile was collected 2, 4, 8 and 24 hr after drug administration.

Gastric contents. After an intravenous injection of approximately 10 mg (+)-amphetamine sulphate to two subjects, gastric contents were withdrawn continually by Ryle's tube, starting 10 min before and stopping 40 min after the injection. Volumes and pH values of the samples were noted.

Plasma amphetamine. Blood was collected from a subject (M.D.) taking 30 mg (+)-amphetamine sulphate daily. A narcoleptic patient (E.H.) taking 70 mg (+)-amphetamine sulphate daily was given an intravenous injection of 15 mg (+)-amphetamine sulphate and blood collected $\frac{1}{2}$ and $2\frac{1}{2}$ min after injection.

Clinical effects. Any subjective effects experienced after administration of the drug were noted.

DETERMINATION OF AMPHETAMINE

Amphetamine, in urine, was determined by the gas-liquid chromatographic method described by Beckett & Rowland (1965a). Some of the results were obtained by the method of Beckett & Rowland (1964b). In some instances amphetamine was further identified as its acetone derivative (Beckett & Rowland, 1965a). Amphetamine (2 μg/ml) was added to acid and alkaline urine, stored at 4°, and the amphetamine content determined daily for 4 days.

Amphetamine in gastric juice, plasma and bile was determined using the method of Beckett & Rowland (1965a). Known amounts of drug

* The ammonium chloride tablets caused acute diarrhoea on some occasions.

were also added to samples of the three fluids to check recoveries. These fluids, to which no drug had been added, were also analysed.

CALCULATION OF TOTAL AMPHETAMINE EXCRETED

The amount of amphetamine excreted at infinite time was calculated to determine the fraction of the dose which would eventually be excreted unchanged in the urine, assuming that the biological half-life remained constant throughout the elimination of the drug. From urinary excretion data (see Fig. 2), it appears that absorption of the drug is complete within the first 4 hr after administration; the amount of amphetamine excreted between 4 hr and infinity $\left(\overset{A_e}{4 \rightarrow \infty}\right)$ was therefore calculated using equation (1)

$$\overset{A_e}{4 \rightarrow \infty} = \frac{\overset{A_e}{4 \rightarrow t}}{1 - \exp\text{-}[K_d(t-4)]} \quad .. \quad .. \quad (1)$$

where $\overset{A_e}{4 \rightarrow t}$ is the amount of amphetamine excreted between 4 and t hr after administration of the drug ($t > 4$), and K_d is the first order elimination constant ($0{\cdot}693/t_{\frac{1}{2}}$). Generally t was taken as 16 hr and the amount of amphetamine excreted between 4 and 16 hr determined from the graph of the cumulative excretion of amphetamine. The biological half-life ($t_{\frac{1}{2}}$) was calculated from the slope of the log-urinary excretion rate graph by applying the method of the least sum of squares. The total drug excreted unchanged A_{e_∞} was then given by

$$A_{e_\infty} = \overset{A_e}{0 \rightarrow 4} + \overset{A_e}{4 \rightarrow \infty} \quad .. \quad .. \quad .. \quad (2)$$

where $\left(\overset{A_e}{0 \rightarrow 4}\right)$ is the amount of amphetamine excreted in the first 4 hr following the dose.

CALCULATION OF ABSORPTION RATES

The percentage of the dose absorbed at various times was calculated using equation (3) (Wagner & Nelson, 1964).

$$\% \text{ absorbed} = \frac{A_t}{A_\infty} \times 100 = \frac{\left[\frac{1}{K_d}\left(\frac{dA_e}{dt}\right) + A_e\right]}{A_{e_\infty}} \times 100 \quad .. \quad (3)$$

where A_t is cumulative amount of drug absorbed at time t, $A\infty$ is the total amount of drug finally absorbed, and A_e is the cumulative amount of drug excreted in the urine at time t. The corresponding values of the excretion rate (dAe/dt) and Ae were determined from cumulative urinary excretion plots as described by Wagner & Nelson (1964).

Results

Amphetamine was stable in both acid and alkaline urine for at least 4 days when stored at 4° and was recovered quantitatively from gastric juice, plasma and bile; the contents of these fluids did not interfere with the determination of the drug.

URINARY EXCRETION TRIALS

pH not controlled. Table 1 shows the amount of unchanged amphetamine excreted in 48 hr after the oral administration of 5–15 mg (+)- and 10–15 mg (−)-amphetamine sulphate. The excretion rate showed fluctuations throughout the day (e.g. see Fig. 1); these occurred in all subjects and appeared to parallel changes in urinary pH. An acid urine effected

TABLE 1. URINARY EXCRETION OF AMPHETAMINE 48 HR AFTER ORAL ADMINISTRATION OF (+)- AND (−)-AMPHETAMINE SULPHATE

Subject	Dose (mg sulphate)	Unchanged amphetamine excreted (as % of dose administered)	
		(+)-Amphetamine	(−)-Amphetamine
G.W.	5	18·0	—
A.S.	5	28·0	—
A.S.	10	40·9	57·3
A.T.	10	38·2	66·0
M.B.	10	18·6	51·6
J.W.	10	27·9	29·2
J.W.	10	12·0	—
G.W.	10	31·4	39·4
G.W.	10	35·1	—
G.W.	10	32·7	—
P.T.	10	20·1	—
P.W.	10	36·3	—
C.M.L.	10	32·7	—
M.R.	10	—	41·3
M.B.	15	36·0	—
A.T.	15	34·2	—
M.R.	15	16·0	48·7
E.J.T.	15	44·0	70·7
N.B.	15	27·0	38·3
Mean.. ..		32·9	49·2

a high excretion rate of amphetamine whereas a low excretion rate was obtained when the urine was alkaline. A urine volume effect was sometimes noticeable at urinary pH values of about 7, a high urine flow rate resulting in an increased excretion of amphetamine. Also, during both amphetamine excretion trials, and when no drug was taken, fluctuations in urinary pH occurred (range pH 4·9–8·3). A pH rhythm was generally apparent with high pH values between 8·0 p.m. and midnight and a fall overnight. The excretion rate of amphetamine decreased between 8.0 p.m. and midnight and rose again during the night. Several subjects exhibited an alkaline urine (pH 6·8–7·6) throughout most of the morning and afternoon.

Alkaline urine trials. The mean 16 hr excretion of amphetamine in alkaline urine was 2·6% and 2·2% of the dose after ingesting (+)- and (−)- amphetamine sulphate respectively (Table 2). Significant quantities of amphetamine (2·7–3·6%) were still excreted 40–48 hr after the administration of drug, when the urine had become more acidic (pH 6·3–6·7).

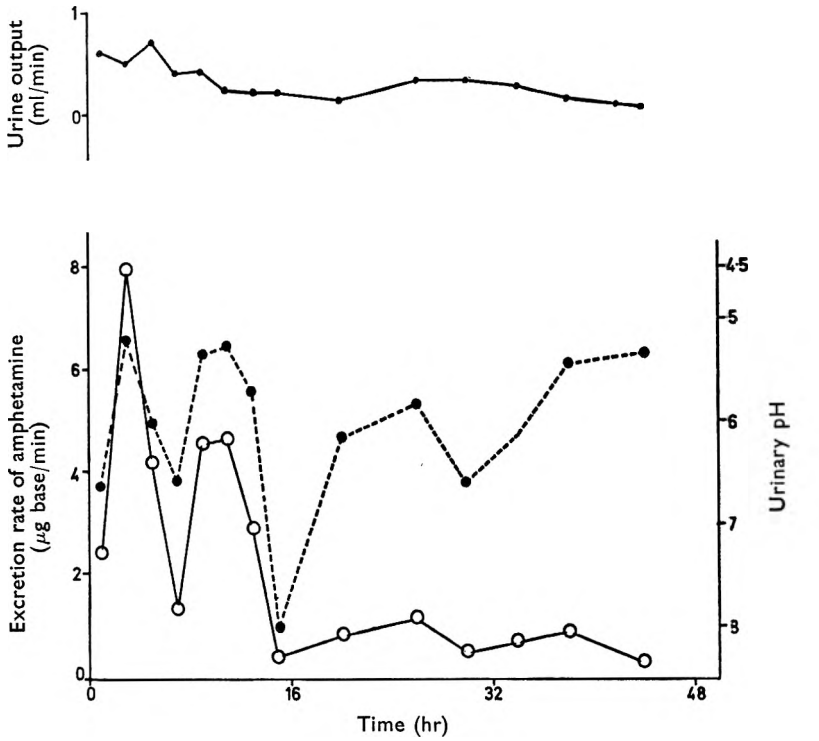

FIG. 1. Influence of urinary pH and urine output on the urinary excretion of amphetamine in man, after oral administration of 15 mg (+)-amphetamine sulphate. Subject E. J. T. (A similar pattern was observed with other subjects) —○— Amphetamine excretion rate. – – –●– – – Urinary pH.

Acidic urine trials. Table 3 gives the 16 hr urinary excretion (mean values 57 and 66%), of unchanged amphetamine after oral administration of (+)- and (−)-amphetamine sulphate to subjects whose urine was

TABLE 2. URINARY EXCRETION OF AMPHETAMINE 16 HR AFTER ORAL ADMINISTRATION OF (+)- AND (−)-AMPHETAMINE SULPHATE AND ALKALINE URINE CONTROL

Subject	Dose (mg sulphate)	Isomer	Amphetamine excreted (% of dose)	Urinary pH
P.T.	10	(+)	2·4	7·8 ± 0·2
P.W.	10	(+)	2·5	7·85 ± 0·2
G.R.W. ..	10	(+)	2·6	8·0 ± 0·2
C.M.L.	10	(+)	3·3	8·1 ± 0·2
M.R.	15	(+)	3·0	7·95 ± 0·25
	15	(−)	1·2	7·80 ± 0·20
E.J.T.	15	(+)	2·2	7·95 ± 0·25
	15	(−)	0·9	8·1 ± 0·2
N.B.	15	(+)	4·2	7·8 ± 0·25
	15	(−)	4·6	7·90 ± 0·25

TABLE 3. TABLE SHOWING AMPHETAMINE EXCRETION AND BIOLOGICAL HALF-LIFE VALUES AFTER ORAL ADMINISTRATION OF (+)- AND (−)-AMPHETAMINE SULPHATE AND INTRAVENOUS INJECTION OF (+)-AMPHETAMINE SULPHATE, URINE BEING MAINTAINED ACID

Subject	Dose (mg sulphate)	Route	Isomer	$t_{\frac{1}{2}}$ (hr)	Amphetamine excreted (as % dose administered)		Urinary pH
					16 hr	Total	
N.B. ..	15	oral	(+)	4·75	55·4	61·5	4·95 ± 0·2
	—	i.v.	(+)	4·50	—	—	5·15 ± 0·2
	15	oral	(−)	5·94	62·7	76·2	5·10 ± 0·2
M.R. ..	15	oral	(+)	5·02	56·5	63·6	4·80 ± 0·2
	—	i.v.	(+)	4·52	—	—	4·90 ± 0·2
	15	oral	(−)	5·94	62·5	74·8	4·95 ± 0·25
E.T. ..	15	oral	(+)	4·93	73·5	83·5	4·90 ± 0·25
	—	i.v.	(+)	4·60	—	—	5·05 ± 0·2
	15	oral	(−)	4·82	71·8*	82·3	4·80 ± 0·2
G.W. ..	10	oral	(+)	6·80	48·0	61·2	4·90 ± 0·2
C.M.L. ..	10	oral	(+)	4·21	54·4	59·0	5·10 ± 0·2

* 15 hr only

maintained at a relatively constant acid pH. The fluctuations in the excretion of amphetamine, observed with no pH control, were abolished. Amphetamine excretion rate reached a maximum about 2 hr after

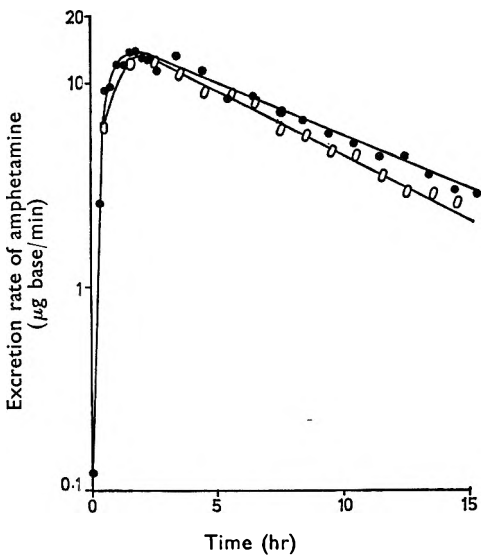

FIG. 2. Urinary excretion of amphetamine after oral administration of 15 mg (+)- and (−)-amphetamine sulphate under acidic urine conditions. Subject M.R. —●— (−)-Amphetamine. —○—(+)-Amphetamine.

administration and, thereafter, fell exponentially (e.g., Fig. 2). Urine flow rate appeared to have little influence on the excretion rate of amphetamine.

INTRAVENOUS INJECTION OF AMPHETAMINE

Acid urine. Fluctuations in the excretion rate of amphetamine occurred during the first 3 hr (e.g. Fig. 3). The amount of amphetamine excreted in the first 15 min urine sample was lower than that in the subsequent

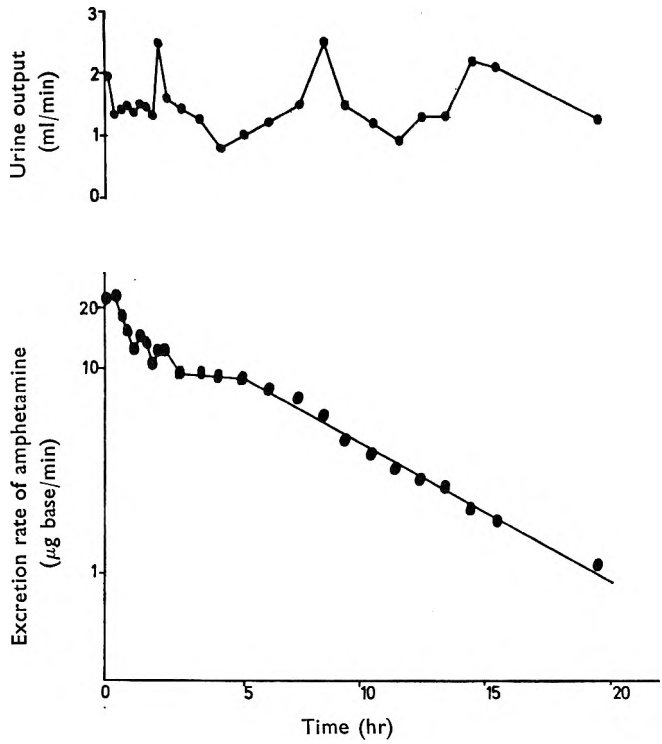

FIG. 3. Urinary excretion of amphetamine and urine output after intravenous administration of 15 mg (+)-amphetamine sulphate under acid urine conditions. Subject N.B.

sample. Also, one individual (see Fig. 3) exhibited a constant excretion rate before a logarithmic fall was observed.

AMPHETAMINE IN BIOLOGICAL FLUIDS

Bile. No amphetamine was found in the bile, before or after digestion of the bile with 2N hydrochloric acid for 1 hr.

Gastric contents. 0·5–1% of the dose administered was found in the stomach contents after 40 min, the contents being acid throughout the trial (pH 1·7–4·5).

Plasma. No amphetamine could be demonstrated in the plasma of the subject taking 30 mg (+)-amphetamine sulphate daily. Amphetamine plasma levels of 0·17 and 0·12 μg base/ml were found ½ and 2½ min after an intravenous injection into the narcoleptic patient.

Clinical effects. Central nervous stimulation and dryness of the mouth were the most common effects experienced when taking (+)-amphetamine

sulphate. These effects were more pronounced under alkaline than normal urine conditions and sometimes resulted in insomnia that night, even though the drug was taken at 8.30 a.m. No effects were observed when the (−)-isomer was administered.

Discussion

The 48 hr urinary excretion of amphetamine after oral administration of (+)- and (−)-amphetamine sulphate (Table 1) is within the range reported by previous workers. The present results with amphetamine (pK_a 9·77, Leffler, Spenser & Burger, 1951; 9·93, Lewis, 1954) may be explained by the passive reabsorption of unionised drug from the kidney, the process being pH and volume dependent (Milne, Scribner & Crawford, 1958; Weiner & Mudge, 1964). The more alkaline the urine the higher the percentage of unionised drug and hence a greater reabsorption of amphetamine with a subsequent decrease in the excretion rate. The increase in duration of pharmacological effects, observed when the urine is maintained alkaline, is probably due to retention of amphetamine in the body.

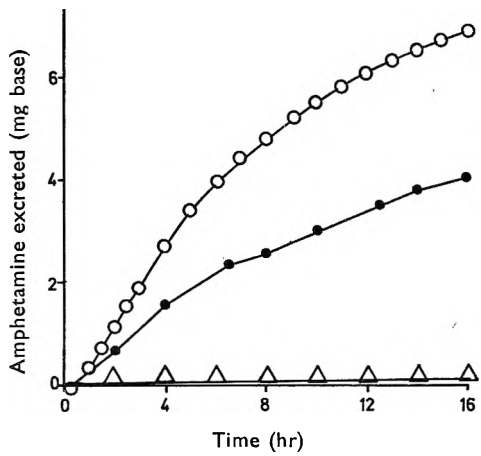

FIG. 4. Cumulative urinary excretion of amphetamine under normal —●—, alkaline —△— and acidic —○— urine conditions after oral administration of 15 mg (−)-amphetamine. Subject M.R.

The difference in excretion of amphetamine in alkaline (Table 2) and acid urine (Table 3) cannot be attributed to changes in urine output since the mean 16 hr urine output was 1,250 and 1,640 ml respectively. These results contrast with those for the excretion of amphetamine under normal urinary pH conditions (e.g. see Fig. 4). Similar results in man and rat have been reported by Asatoor, Galman, Johnson & Milne (1965).

The influence of changes in urine output on the excretion rate of amphetamine at pH 7·0 is probably due to dilution effects of amphetamine in the kidney tubules which alters the rate of reabsorption of the drug.

The observed lack of influence of urine output on the excretion of amphetamine, when urine is maintained acid or alkaline, suggests that reabsorption is respectively negligible and almost maximal under these conditions.

Since diurnal variations in urinary pH under normal conditions are well known (Brunton, 1933; Kenyon, Wilson & Macy, 1934; Bridges & Mattice, 1940; Elliott, Sharp & Lewis, 1959), it is probable that the rhythmic pattern of amphetamine excretion is caused by this urinary pH

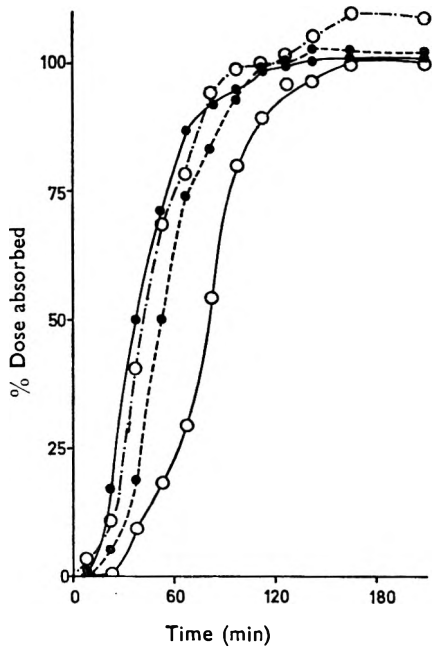

FIG. 5. Amphetamine absorbed (%) as a function of time after an oral dose of amphetamine to 4 subjects. N.B. (−)-isomer — - —○— - —. E.J.T. (−)-isomer - - -●- - -. M.R. (−)-isomer ——●——. G.R.W. (+)-isomer ——○——.

rhythm. These fluctuations in the excretion rate cannot be explained by transfer of drug from plasma to stomach contents with subsequent reabsorption in the gut, as only small amounts appeared in the stomach contents after an intravenous dose of amphetamine, a similar result to that of Jacobsen & Gad (1940). Enterohepatic recycling can also be excluded as no amphetamine was found in the bile either free or conjugated.

KINETIC STUDIES IN ACID URINE

When the urine is maintained acid, urinary excretion is the major route of elimination of amphetamine from the body (Table 3). The maximal subjective effects, which were noted to be $1\frac{1}{2}$–3 hr after ingestion of (+)-amphetamine sulphate, are in accord with the maximum excretion rate of amphetamine occurring at 2 hr (e.g. Fig. 2). Since the excretion rate falls exponentially during the period of observation, K_d is constant

during this time and therefore k_e, k_m ($K_d = k_e + k_m$), the rate constants for excretion and metabolism, are constant. In addition, assuming that K_d remains constant beyond the period of observation, the anticipated total excretion of amphetamine (Table 3) shows that excretion is largely complete within the first 16 hr.

Transposition of urine excretion data to absorption rate data (Fig. 5) shows that absorption of amphetamine was complete within 2½ hr of an oral dose of drug, so that the determination of $t_{\frac{1}{2}}$ values from data obtained after 4 hr was valid; furthermore the absorption was not described by a single first order process. The S-shaped absorption curves (Fig. 5) might be due to the effect of stomach emptying, so that the amount of amphetamine in the gut, where absorption takes place, is not maximal at zero time but at some later time dependent on the rate constants for stomach emptying and absorption of the drug. However, the slow initial rise in the absorption of amphetamine is exaggerated owing to the time necessary for drug to pass through the kidney into the urine, and also to incomplete emptying of the bladder so that, in the first 15 min sample, the measured excretion rate is lower than the true value. The low initial excretion rate observed after an intravenous injection of (+)-amphetamine sulphate may be explained similarly.

The observed fluctuations in the excretion rate during the first 3 hr after intravenous injection of (+)-amphetamine sulphate (e.g., Fig. 3) are at present inexplicable. The fluctuations at constant urinary pH cannot be generally correlated with changes in urine output or explained by the passage of drug from the plasma to stomach or by enterohepatic recycling of the drug. When the excretion rates of the drug had become exponential, $t_{\frac{1}{2}}$ values were then the same as those of the (+)-isomer given orally (Table 3) suggesting that, after equilibrium had been established, the route of administration did not influence the kinetics of elimination of amphetamine from the body. Since variations occurred in the volume injected when 1 ml containing 15 mg (+)-amphetamine sulphate was used, quantitative treatment of the intravenous results has not been attempted.

FINDINGS WITH OPTICAL ISOMERS

In acid urine trials the biological half-life for (+)-amphetamine was slightly lower than that of the (−)-isomer for two subjects (Table 3) whilst in another subject (E.J.T.), who excreted larger amounts of unchanged drug, no difference in the biological life of the two isomers could be seen. Assuming excretion is the same for both isomers, the results suggest that the metabolism of (−)-amphetamine was less extensive than the (+)-isomer, as was also found by Alles & Wisegarver (1961). Under acid conditions, i.e., when excretion of amphetamine is high and metabolism low, any differences in the metabolism of the isomers tends to be obscured. (Further work is in progress to investigate the influence of stereochemistry on the metabolism and excretion of amphetamine and other amines).

Urinary excretion kinetic studies are only valid if excretion rates are indicative of blood concentrations of the drug. This relationship does not appear to be the case with either amphetamine or methylamphetamine (Beckett & Rowland, 1965b) under normal conditions. Cavallito & others (1963) found no relationship between blood levels and excretion rates of tritiated phenylephrine, a result which might in part be due to excretion being pH dependent, as found with the related amine, adrenaline

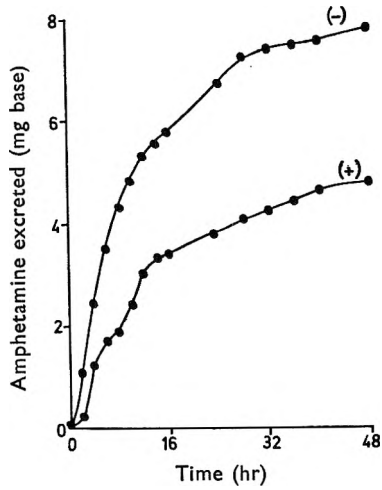

FIG. 6. Cumulative urinary amphetamine excretion after oral administration of 15 mg amphetamine sulphate. Subject E.J.T.

(Braun, 1964). Thus, since the urinary excretion of many drugs is pH dependent and in some cases urine-volume dependent (Milne & others, 1958; Peters, 1960; Weiner & Mudge, 1964; Braun, 1963), and since pH and urine output fluctuate throughout the day, urinary excretion data should be interpreted with caution. Important information may also be obscured by the incorrect times of sampling of urine and by the pooling of data. Cumulative excretion graphs tend to obscure any fluctuations in the excretion rate (e.g., cf. Fig. 6 and Fig. 1).

With drugs like amphetamine, in which there is marked extravascular concentration, as shown by the extremely low plasma levels even when large doses of amphetamine were ingested, urinary excretion studies may be the only practical method of examining the *in vivo* release of drugs from their preparations. If, as with amphetamine, urinary excretion is pH dependent, then a practical solution would be to render the urine acidic with ammonium chloride and thus give high but constant K_d values. Under these circumstances, urinary excretion rates reflect drug levels in the plasma and so would enable the absorption profiles of drugs from conventional and prolonged-release dosage forms to be examined, provided that ammonium chloride does not interfere directly with drug release.

Acknowledgements. The authors thank all those participating in the trials, E. J. Triggs for the blood and bile analyses, Drs. P. Turner and J. Royle for assistance in the intravenous and gastric trials, and Smith, Kline and French Laboratories Limited for the samples of (+)- and (−)-amphetamine sulphate. One of us (M.R.) thanks the Medical Research Council for a grant.

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Impairment of sympathetic nerve responses by dopa, dopamine and their α-methyl analogues

J. B. FARMER

Dopa and dopamine reduced the response of the nictitating membrane to post-ganglionic sympathetic nerve stimulation in cats pretreated with the monoamine oxidase inhibitor pargyline. A similar reduction could be obtained with α-methyldopa and α-methyldopamine in untreated cats. The sensitivity of the membrane to injected noradrenaline, α-methylnoradrenaline or adrenaline was not reduced. The possible mechanism of the anti-hypertensive action of monoamine oxidase inhibitors and α-methyldopa is discussed in the light of these findings.

THE impairment of the response to sympathetic nerve stimulation by indirectly-acting sympathomimetic amines has been described by several investigators (Aström, 1949; Day, 1962; Day & Rand, 1963a,b). Those amines which are substrates for monoamine oxidase are only effective in the presence of a monoamine oxidase inhibitor (Day & Rand, 1963a,b), but amines possessing an α-methyl substituent, which confers immunity to attack by monoamine oxidase (Blaschko, Richter & Schlossmann, 1937) impair sympathetic responses in untreated animals.

Dopamine, the immediate precursor of noradrenaline, has direct sympathomimetic actions, although in some tissues its action is partly direct and partly indirect (Bejrablaya, Burn & Walker, 1958; Strömblad, 1960). Similarly, α-methyldopamine acts both directly and indirectly. Evidence for its indirect action is the marked depletion of tissue stores of noradrenaline it effects (Porter, Totaro & Leiby, 1961). Dopamine is a good substrate for monoamine oxidase (Blaschko & others, 1937) whilst α-methyldopamine is not metabolised by this enzyme (Carlsson & Lundqvist, 1962).

The purpose of this investigation was to determine whether dopamine and α-methyldopamine behaved like other indirectly-acting sympathomimetic amines in impairing responses to sympathetic nerve stimulation. The amino-acid precursors of these amines, dopa and α-methyldopa were also tested.

Experimental

METHODS

Cats, weighing 2–3·5 kg, were used. Anaesthesia was induced with halothane in nitrous oxide and oxygen (3 : 1 v/v) and maintained by intravenous injection of 80 mg/kg chloralose. In some experiments additional injections of pentobarbitone were necessary since infusions of dopamine, and more particularly dopa, lightened anaesthesia in animals pretreated with the monoamine oxidase inhibitor. Injections and infusions were made into the right femoral vein. The infusions of dopa, dopamine, α-methyldopa and α-methyldopamine were made at a constant rate for a period of 50 min. The amounts of these substances referred to

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are the total amounts in mg/kg given within this period. The contractions of the right nictitating membrane were recorded on smoked kymograph paper with an isotonic frontal writing lever of 8 g tension and 15 times magnification. The postganglionic cervical sympathetic nerves were exposed on the right side by partial removal of the trachea and oesophagus; the trachea was cannulated low in the neck. The postganglionic sympathetic nerves were laid over bipolar platinum electrodes and the superior cervical ganglion was crushed. The edges of the wound were stitched to a metal frame and the dissected tissue flooded with liquid paraffin. The nerve was stimulated with rectangular wave pulses from an electronic stimulator. The pulses were of 1 msec duration, 10 V strength, and were given at 1, 5 or 10 pulses/sec. They were applied for 20 sec, which allowed the membrane to reach the full response at each of the frequencies applied. In each experiment the following procedure was observed: injections of noradrenaline, adrenaline, or α-methylnoradrenaline were given intravenously in amounts which produced a well defined contraction of the membrane followed by three periods of nerve stimulation. This sequence was performed in repeated cycles before and after the infusions. The monamine oxidase inhibitor employed in this study was pargyline (Taylor, Wykes, Gladish & Martin, 1960). Cats were pretreated with pargyline, which was administered subcutaneously as a single dose (25–50 mg/kg), or as four divided doses (total 100 mg/kg) during the two days preceding the experiment. Experiments were started 16 hr after the last injection. The decarboxylase inhibitor NSD 1055 (Hansson & Clark, 1962) was given intravenously.

DRUGS

The following drugs were used: (−)-adrenaline acid tartrate, (−)-noradrenaline acid tartrate, L-dopa, dopamine hydrochloride, 4-bromo-3-hydroxybenzyloxyamine dihydrogen phosphate (NSD 1055), L-α-methyldopa, (−)-α-methylnoradrenaline, (±)-α-methyldopamine, pargyline hydrochloride (*N*-benzyl-*N*-methyl-2-propynylamine). Doses were given in terms of these compounds.

Results

There was no difference between control cats and cats pretreated with pargyline in the sensitivity of the membrane to injected noradrenaline, adrenaline and nerve stimulation. The response of the membrane to nerve stimulation and injected noradrenaline and adrenaline was determined for up to 4 hr after the end of the infusions. Within this period, deterioration of the preparation occurred in some experiments, the response to the highest frequency of nerve stimulation being most affected. This effect could be clearly differentiated from the impairment produced by the infusions.

The infusion of dopa at 10 mg/kg and above, produced a sustained contraction of the membrane; this effect was more prolonged and was produced by smaller amounts of dopa in cats pretreated with pargyline. α-Methyldopa, 100 and 200 mg/kg, did not cause the membrane to

contract. Dopamine, 5 and 10 mg/kg, and α-methyldopamine, 10 and 20 mg/kg, caused a prolonged contraction of the membrane; the effect of dopamine, as for dopa, was more prominent in cats pretreated with pargyline. The contraction persisted for up to 1½ hr after termination of the infusions; the responsiveness of the membrane to nerve stimulation or to intravenous injections was not determined until the contraction produced by the infusion had subsided. The administration of the decarboxylase inhibitor NSD 1055, 20 mg/kg, before the infusion of dopa, 10 mg/kg, prevented the appearance of the prolonged contraction. However, a subsequent infusion of dopamine produced a prolonged contraction. It is perhaps of interest that infusions of dopa and dopamine resulted in copious salivation in all cats, particularly those pretreated with pargyline. NSD 1055 prevented this response to dopa but not to dopamine.

EFFECT OF INFUSIONS ON THE RESPONSE OF THE MEMBRANE TO POSTGANGLIONIC NERVE STIMULATION

In three experiments the infusion of dopa, 5, 10 and 20 mg/kg, did not cause impairment of the response to nerve stimulation. In a further two experiments in cats pretreated with pargyline, dopa, 5 and 10 mg/kg, reduced the response to nerve stimulation (Fig. 1A). However, when an injection of NSD 1055 was given before the infusion there was only a slight reduction in the responses (Fig. 1B).

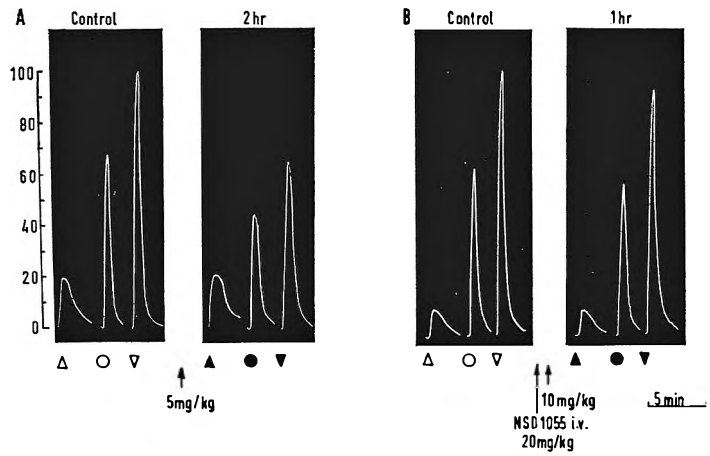

FIG. 1. Infusion of dopa, 5 and 10 mg/kg in 50 min, in two cats pretreated with pargyline. Effect on the response of the cat nictitating membrane to injected noradrenaline (△) and postganglionic nerve stimulation (1 pulse/sec ○; 5 pulses/sec ▽). Infusion made at ↑. In B infusion preceded by injection of 20 mg/kg NSD 1055. Open symbols before, closed symbols at stated time after termination of infusion.

The infusion of dopamine, 5 mg/kg in one experiment and 10 mg/kg in two experiments, did not result in any marked change in the responses to nerve stimulation. In one experiment there was a gradual decline in the responses while in two experiments they were unchanged or increased. In three experiments in cats treated with pargyline, dopamine, 5 mg/kg in

one experiment and 10 mg/kg in two experiments, caused impairment of responses to nerve stimulation (Fig. 2A). In the experiment with 5 mg/kg of dopamine, there was some recovery of the responses within 3 hr. The

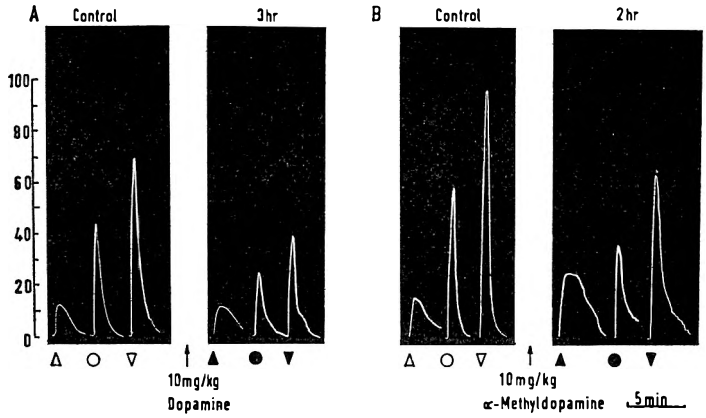

FIG. 2. Infusion of dopamine 10 mg/kg in 50 min in cat pretreated with pargyline (A) and α-methyldopamine 10 mg/kg in 50 min in untreated cat (B). Effect on the response of the cat nictitating membrane to, in A, injected noradrenaline (△) or, in B, α-methylnoradrenaline (△) and postganglionic nerve stimulation (1 pulse/sec ○; 5 pulses/sec ▽). Open symbols before, closed symbols at stated time after termination of infusion.

responses were also impaired in the cat given NSD 1055 before the infusion of dopamine.

In two experiments, infusions of α-methyldopa, 100 and 200 mg/kg, were given. No permanent effect on nerve stimulation was observed with the lower dose; there was some depression immediately after termination of the infusion but the responses then recovered fully. The infusion of 200 mg/kg of α-methyldopa produced a gradually developing impairment of the responses.

In two experiments, α-methyldopamine 10 and 20 mg/kg, caused impairment of the responses to nerve stimulation (Fig. 2B).

EFFECT OF INFUSIONS ON RESPONSE OF THE MEMBRANE TO INJECTED CATECHOLAMINES

After dopa, the responses to noradrenaline and adrenaline were enhanced in normal cats but not in those pretreated with pargyline. After dopamine the responses to noradrenaline and adrenaline were unchanged, either in the presence or absence of pargyline. α-Methyldopa and α-methyldopamine enhanced the response of the membrane to noradrenaline and α-methylnoradrenaline. α-Methylnoradrenaline was included in the experiments in which α-methyldopa and α-methyldopamine were investigated since an approximate estimate of the potency relative to noradrenaline was required. Neither noradrenaline nor α-methylnoradrenaline were examined for chemical purity, but the activity of α-methylnoradrenaline on the membrane was approximately half that of noradrenaline, which is in agreement with Ahlquist (1948).

Discussion

In the presence of a monoamine oxidase inhibitor the response of the nictitating membrane to postganglionic nerve stimulation was impaired after infusion of dopa and dopamine, although the response to injected noradrenaline and adrenaline remained unchanged or enhanced. Such impairment did not occur in normal cats. α-Methyldopa and α-methyldopamine impaired the response to nerve stimulation in normal animals and enhanced the response to injected noradrenaline and α-methylnoradrenaline. A gradually developing impairment observed after α-methyldopa has been reported previously by Day & Rand (1964). This may be explained by the very slow rate of decarboxylation of this substance in tissues (Lovenburg, Weissbach & Udenfriend, 1962). In contrast, dopa in the presence of pargyline produced an immediate impairment in much smaller amounts than those required for α-methyldopa. However dopa did not impair the responses after injection of a decarboxylase inhibitor (NSD 1055) although NSD 1055 did not affect the impairment produced by dopamine. The dose of NSD 1055 employed here prevented the vasopressor action of dopa (Horlington, M., personal communication); this is indicative of the inhibition of decarboxylase activity (Clark, 1959). Similarly, the administration of a decarboxylase inhibitor prevented the hypotensive and catecholamine-depleting action of α-methyldopa (Davis, Drain, Horlington, Lazare & Urbanska, 1963).

The results suggest that α-methyldopa or dopa in the presence of pargyline exert their effects after decarboxylation, and that the metabolites are the active substances. Now α-methyldopa undergoes decarboxylation and subsequent hydroxylation *in vivo* (Carlsson & Lundqvist, 1962) yielding first α-methyldopamine and then α-methylnoradrenaline. α-Methylnoradrenaline has less activity than noradrenaline and could therefore cause impaired responses by acting as a false transmitter (Day & Rand, 1963c, 1964). The impairment of nerve responses produced by α-methyldopamine was approximately 40%, particularly at low stimulation rates, and since α-methylnoradrenaline has one half the potency of noradrenaline on the nictitating membrane of the cat (Ahlquist, 1948), this degree of impairment would require almost total replacement of the noradrenaline store with α-methylnoradrenaline. Observations of the effect of α-methyldopamine on noradrenaline levels in tissues indicate that this amount of depletion does not occur (Porter & others, 1961; Levine & Sjoerdsma, 1964). It must also be taken into consideration that the infusion of α-methyldopamine increased the sensitivity of the membrane to injected α-methylnoradrenaline. It seems unlikely that the formation and utilisation of α-methylnoradrenaline could account completely for the effects of α-methyldopa or α-methyldopamine. Dopa undergoes decarboxylation to form dopamine and is then hydroxylated to form noradrenaline (Blaschko, 1939). The formation of a false transmitter cannot account for the impairment observed in these experiments after infusion of dopa and dopamine. α-Methyldopamine, and dopamine in the presence of pargyline, produced similar degrees of impairment of

the responses and were active in similar doses. Therefore dopamine formed from dopa may accumulate after inhibition of monoamine oxidase, and α-methyldopamine formed from α-methyldopa may be responsible for the effects of the amino-acids. There are grounds for the hypothesis that accumulation of dopamine may account for the anti-hypertensive action of monoamine oxidase inhibitors. In animals treated with monoamine oxidase inhibitors, an increased dopamine content in peripheral tissues and an increased ability to hold injected dopamine has been demonstrated (Harrison, Levitt & Udenfriend, 1963). Extracts of sympathetic nerves incubated with dopa and the monoamine oxidase inhibitor, marsilid, synthesise increased amounts of dopamine (Goodall & Kirshner, 1958). A reduction in the amount of noradrenaline released by nerve stimulation from the isolated perfused spleen of cats pretreated with the monoamine oxidase inhibitor nialamide has been observed (Davey, Farmer & Reinert, 1963); the accumulation of dopamine may account for this finding. Other indirectly acting sympathomimetic amines, derived from naturally occurring amino-acids, which are substrates for amine oxidase, have been shown to produce impairment of sympathetic responses in the presence of a monoamine oxidase inhibitor (Day & Rand, 1963b). However, dopamine formed from dopa in the cytoplasm of adrenergic nerves is taken up by the noradrenaline store and converted to noradrenaline (Schumann, 1960). Excessive amounts of dopamine may be favourably sited to interfere or compete with either uptake or release of noradrenaline. The accumulation of dopamine would be favoured since conversion of the amino-acid to the amine is rapid, whilst hydroxylation of the amines proceeds more slowly (Hess, Connamacher, Ozaki & Udenfriend, 1961).

Similarly there are grounds for the hypothesis that the accumulation of α-methyldopamine may account for the anti-hypertensive action of α-methyldopa. α-Methyldopa is converted to α-methyldopamine and eventually to α-methylnoradrenaline; the presence of α-methyldopamine was detected for up to 24 hr in tissues of animals treated with α-methyldopa, whilst noradrenaline levels were decreased for considerably longer (Carlsson & Lundqvist, 1962). In man, large single doses of α-methyldopa lower blood pressure for approximately 24 hr (Dollery & Harrington, 1962). Therefore a similar mechanism may operate for α-methyldopamine as for dopamine, this substance accumulating because of the failure to be metabolised by monoamine oxidase. In this context, α-methyl-*m*-tyrosine is less efficacious than α-methyldopa in lowering blood pressure in man and animals (Stone, Porter, Watson & Ross, 1961; Horwitz & Sjoerdsma, 1963), whilst both substances produce similar degrees of depletion of tissue stores of noradrenaline in animals (Hess & others, 1961). α-Methyl-*m*-tyrosine is metabolised *in vivo* to α-methyl-*m*-tyramine and metaraminol (Carlsson & Lundqvist, 1962). Metaraminol, but not α-methyl-*m*-tyramine, is easily bound in tissues when injected intravenously (Alpers, Busfield & Shore, 1964) but both substances will deplete tissue stores of noradrenaline (Udenfriend & Zaltzman-Nirenberg, 1962). Now injected tyramine, unlike dopamine, is not easily bound by tissues, but is

rapidly converted to octopamine (Musacchio, Kopin & Snyder, 1964). When α-methyl-*m*-tyramine is generated *in vivo* from α-methyl-*m*-tyrosine, part will be lost from the tissue and excreted and part will be rapidly hydroxylated; thus very little of this substance may accumulate. It may be that α-methyl-*m*-tyrosine does not have marked anti-hypertensive actions since there is failure of the metabolite α-methyl-*m*-tyramine to accumulate in the vicinity of the noradrenaline store.

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The anticoagulant activity of carrageenan

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(With the technical assistance of Miss J. E. Harthill)

λ-Carrageenans and κ-carrageenans from samples of *Chondrus crispus*, carrageenan from *Polyides rotundus* and degraded carrageenan from *Eucheuma spinosum* have anticoagulant activity on intravenous injection in the rabbit. Anticoagulant activity appears to be caused by a general reaction with plasma protein. The undegraded carrageenans are acutely toxic on intravenous injection and form insoluble complexes with fibrinogen in neutral solution. Degraded carrageenan is very much less toxic and, like heparin, forms soluble complexes with fibrinogen. The λ-carrageenans from *C. crispus* have higher sulphate content, consistently prolong the clotting time more, and are more toxic than the corresponding κ-carrageenans. The differences in sulphate content between the various λ-carrageenans, and between the carrageenans from the other seaweeds tested, do not correspond directly with differences in anticoagulant action and toxicity.

THE carrageenans, which occur naturally in the red seaweeds, constitute a closely related group of sulphated galactans. While displaying the biological properties of sulphated polysaccharides in general, quantitative differences exist between the members of the group and between the readily separated κ- and λ-carrageenans which are present in certain of the members. Houck, Morris & Lazaro (1957) examined unfractionated whole extracts of a number of seaweeds for anticoagulant activity which they found only in *Gigartina acicularis*. Hawkins & Leonard (1962, 1963) fractionated one *Chondrus crispus* carrageenan into its κ- and λ-components and found greater anticoagulant activity in the λ-carrageenan which contained more ester sulphate than the κ-carrageenan. Rees (1963) has suggested that λ-carrageenan may be the biological precursor of κ-carrageenan. Whole extracts of seaweeds might therefore be expected to vary in the relative content of κ- and λ-carrageenans and hence in anticoagulant activity. This may explain largely negative findings such as those of Houck & others (1957).

We report a study of the anticoagulant activity of a group of carrageenans differing in ester sulphate content, molecular weight and source.

Materials and methods

Animals. Male New Zealand white rabbits (2–4 kg) were used. Food, but not water, was withheld for 18 hr before testing. Each animal acted as its own control and none was used more than once.

Collection of blood. Blood was allowed to drip freely into Pyrex glass tubes from a small incision made on the marginal vein of the shaved, warmed and solvent-cleaned ear. The first ml of blood was discarded.

All clotting tests were made at 37°. Control results were obtained from blood withdrawn immediately before injection of carrageenan; test bloods were withdrawn 2 hr after the injection of carrageenan, or 0·5 hr in the heparin experiments. Preliminary experiments showed that the greatest anticoagulant effect of the carrageenans occurred 2 hr after injection while that for heparin occurred at 0·5 hr.

Saline. 0·85% w/v sodium chloride in water for injection, B.P.

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Carrageenans. Degraded carrageenan (Ebimar, Evans Medical Ltd.). The other carrageenans, the κ- and λ-fractions (Black, Blakemore Colquhoun & Dewar, 1965), and the data in Table 1 were provided by the Arthur D. Little Research Institute through the courtesy of Dr. E. T. Dewar. The carrageenans were injected intravenously in 8 ml saline solution.

Heparin. Sodium heparin, 150·1 units/mg (Evans), kindly supplied by Dr. C. H. Smith.

Thrombin solution. Thrombin Topical (Maw) 20 u/ml in saline.

Citrated plasma. Blood (9 ml) was allowed to drip into trisodium citrate solution (1 ml; 3·13% w/v), mixed, and centrifuged at 1400 rpm for 10 min.

Fibrinogen. Prepared according to Biggs & Macfarlane (1962). A saline solution containing 1 mg/ml was used in the clotting tests.

Whole blood clotting time. The test of Lee & White (1913) in Pyrex glass tubes (10 × 75 mm) was used.

Thrombin time. Citrated plasma, (0·2 ml) and saline, (0·1 ml) were mixed and thrombin solution, (0·1 ml) was added. The time between addition of thrombin and solid clot formation was noted. Triplicate determinations were done on each plasma and results calculated as follows:

$$\frac{\text{average clotting time of control plasma}}{\text{average clotting time of test plasma}} \times 100$$

In the *in vitro* experiments (Table 3) saline (0·1 ml) in the control was replaced by carrageenan solution (0·1 ml) in the test and normal citrated rabbit plasma used.

One-stage and two-stage prothrombin tests; prothrombin consumption test. The methods of Biggs & Macfarlane (1962) were used and results were expressed as follows:

one-stage: $\dfrac{\text{average clotting time of control plasma}}{\text{average clotting time of test plasma}} \times 100$

(triplicate determinations on each plasma)

two-stage: $\dfrac{\text{area under test curve}}{\text{area under control curve}} \times 100$

prothrombin consumption index:

$$\frac{\text{minimum plasma clotting time}}{\text{minimum serum clotting time}} \times 100$$

In vitro prothrombin times (Table 3) were obtained using a one-stage test modified to include saline (0·1 ml) in the control and carrageenan solution (0·1 ml) in the test. 50% increase in time was taken as standard because certain carrageenans caused precipitation rather than clotting when used in excess of the amount required to prolong the clotting time by 50%.

Fibrinogen-carrageenan precipitation. Aqueous fibrinogen (1 ml; 0·4%) was mixed at room temperature with aqueous carrageenan (1 ml) of various concentrations. pH of the mixture was 6·8. Mixtures were allowed to stand 24 hr before reading against a fibrinogen solution as control.

Carrageenan was determined by the method of MacIntosh (1941).

Platelet counts. The method of Brecher & Cronkite (1950) was used.

Results and discussion

The results are in Tables 2–7. All results from *in vivo* experiments are means from four rabbits.

Anticoagulant action of the carrageenans. In the whole blood clotting test all the λ-carrageenans were, in varying degree, active at 3 mg/kg (Table 2) but toxicity began to appear at 5 mg/kg. Table 2 reveals a trend to greater activity with higher sulphate content (Table 1) amongst the four λ-carrageenans, but the carrageenan with the highest sulphate content did not have the greatest activity, *Polyides* carrageenan having greatest effect. It is nevertheless clear that, for the *Chondrus* carrageenans,

TABLE 1. SOURCES AND PROPERTIES OF THE CARRAGEENANS USED

Carrageenan	Code	Source	SO_3Na(%)		3,6-Anhydro galactose, (%)		Inherent viscosity (dl/g)	
			κ	λ	κ	λ	κ	λ
Chondrus crispus	–CY	Yarmouth, Nova Scotia	28·2	37·3	29·2	3·5	13·7	16·2
,, ,,	–CNS	Northumberland Strait, Nova Scotia	28·4	34·9	25·3	4·1	20·8	21·7
,, ,,	–CSE	Sebasco Estates, Nova Scotia	29·8	32·3	25·2	9·1	14·3	13·8
,, ,,	–CMI	Mud Island, Nova Scotia	27·0	32·2	24·8	9·8	8·6	9·4
Polyides rotundus		Moose Head, Nova Scotia	35·0		2·3		5·1	
Degraded-λ from *C. crispus*-CNS			30·7		—		1·3	
Degraded carrageenan (*Eucheuma spinosum*)		S.E. Asia (Evans Medical Ltd.)	29·0		21·0		0·3	

Inherent viscosity, $\eta_{inh} = c^{-1}\ln(\eta_{soln.}/\eta_{solv.})$ dl/g where c = g solute in 100 ml solution, was measured at 25° in an Ostwald viscometer (M2 BS U/M) using 0·1 M sodium chloride as solvent. For undegraded carrageenans c = 0·02; for degraded carrageenans c = 0·2. Viscosity is taken as a comparative indication of molecular weights amongst these substances which all have similar structure.

TABLE 2. RABBIT WHOLE BLOOD CLOTTING TIMES 2 HR AFTER INTRAVENOUS CARRAGEENAN; LOWEST INTRAVENOUS DOSES OF CARRAGEENANS KILLING WITHIN 24 HR

Carrageenan	Clotting time, min. mg/kg i.v.				Lowest dose killing within 24 hr mg/kg i.v.	
	5		3			
	κ	λ	κ	λ	κ	λ
C. crispus –CY	11	14	6	10	3	1
,, –CNS	8	toxic	9	13	5	1
,, –CSE	7	8	6·5	7·5	10	5
,, –CMI	6	10	5	9	15	5
Degraded λ–CNS		8		9	10	
P. rotundus		—		20	5	
Degraded carrageenan	200 mg/kg 17		100 mg/kg 10		>1000	
Heparin	(200 u/kg) 20		(75 u/kg) 8		—	

Results are averages from four rabbits.

TABLE 3. WEIGHTS OF CARRAGEENANS REQUIRED TO PROLONG THE PROTHROMBIN AND THROMBIN TIMES *in vitro* BY STATED AMOUNTS

Carrageenan	Weight required to prolong the prothrombin time by 50% µg		Weight required to prolong the thrombin time by 100% µg	
	κ	λ	κ	λ
C. crispus –CY	35	5	12	<1
,, –CNS	35	3	7	1
,, –CSE	35	12	8	2
,, –CMI	45	15	30	2
P. rotundus	5		1	
Degraded carrageenan	200		50	

Results are averages from plasmas of four rabbits.

each λ-carrageenan has higher sulphate content and greater effect than its κ-counterpart. Although this confirms the belief that the λ-carrageenans are generally more active anticoagulants than the corresponding κ-carrageenans, the results for all the carrageenans, and also the high dose required for degraded carrageenan, suggest that anticoagulant activity does not depend only on ester sulphate content. This conclusion is supported by the results (Table 3) of *in vitro* experiments in which different stages in the clotting mechanism are examined.

Degraded carrageenan is a degraded κ-carrageenan; it has a high 3,6-anhydrogalactose content and the parent carrageenan extracted from *Eucheuma spinosum* can be degraded by mild mineral acid treatment without serious sulphate hydrolysis. This degradation results in a much smaller molecule, permits a higher dose unaccompanied by the toxicity of the undegraded κ- and λ-carrageenans, and anticoagulant activity can be clearly demonstrated. But with undegraded κ-carrageenan, even at the highest safe dose, anticoagulant activity was either low or absent. Amongst the undegraded κ-carrageenans, the most active (κ-CY) was the most toxic and, conversely, the least toxic (κ-CMI) showed the least activity; it also had the lowest viscosity. It is unlikely that the difference in sulphate content (1%) between these two, accounts for the difference in anticoagulant activity, a conclusion which is supported by the data (Tables 1, 2 and 3) for the other two κ-carrageenans. Discussing a related group of polysaccharide sulphates, the laminarin sulphates, Adams, Heathcote & Walker (1962) stated that the laminarin "with the highest molecular weight and greatest degree of sulphation has high antilipaemic and anticoagulant activity, and toxicity".

λ-Carrageenans have a low, and κ-carrageenans a high, 3,6-anhydrogalactose content, and this is one of the distinguishing features between the κ- and λ-carrageenans (Table 1). That the presence of 3,6-anhydrogalactose is not responsible for lower activity of the κ-carrageenans is shown by the anticoagulant activity of degraded carrageenan at adequate dosage.

Intravenous toxicity of carrageenans. Large doses of sulphated polysaccharides can be given orally to man and animals, little or no absorption occurs, and direct systemic toxicity by this route has never been observed.

TABLE 4. CARRAGEENAN-FIBRINOGEN REACTION IN WATER (pH 6·8)

Carrageenan	μg carrageenan mixed with 4 mg fibrinogen in 2 ml mixture								
	160	80	40	20	10	5	2·5	1·25	0·6
C. crispus –λ–CY	—	—	—	+	+	++	+++	+++	+++
,, λ–CNS ..	—	—	—	0	0	++	+++	+++	+++
,, λ–CSE	—	—	—	—	0	+	++	++	++
,, λ–CMI ..	—	—	—	—	—	0	0	+	+
,, κ–CY	—	—	0	+	+	+	+	+	+
,, κ–CNS	0	0	0	+	+	+	+	+	+
,, κ–CSE	—	—	—	—	—	0	+	+	+
,, κ–CMI	—	—	—	—	—	0	0	0	+
P. rotundus	—	—	—	—	0	+	++	++	
Degraded carrageenan ..	—	—	—	—	—	—	—	—	—
Heparin	—	—	—	—	—	—	—	—	—

— = clear solution after mixing (see text); 0 = mixture has same opalescence as control (i.e. no evidence of complex formation); + = increased opalescence (finely dispersed precipitate); ++ = flocculated precipitate; +++ = copious flocculated precipitate.

Nevertheless, these substances are toxic when administered intravenously. Astrup (1953) and Walton (1954) found toxicity by this route in certain high molecular weight dextran sulphates and concluded that the toxic reactions were initiated by formation, in the circulation, of insoluble fibrinogen complexes which entrapped platelets and blood cells. The carrageenans can be grouped according to the nature of their reaction with fibrinogen at pH 6·8, which is above its isoelectric point. In distinction from the less active, less toxic, κ-carrageenans which formed small particle dispersions with fibrinogen, the λ-carrageenans formed coarser precipitates which were flocculent or coagulated. Both types of precipitate were soluble in excess of the respective carrageenan. Table 4 shows that insoluble complex formation was much more pronounced with the CY-carrageenans, and slightly more pronounced with the CNS-carrageenans, than with the other carrageenans. They were also the most active and toxic of the *Chondrus* group. The λ-carrageenans did not all form similar types of precipitate: thus λ-CY and λ-CNS formed fibrous floating coagula immediately, but λ-CSE and λ-CMI required up to 0·5 hr to form particulate precipitates. Assuming Walton's (1953) conclusions regarding the trapping of platelets by dextran sulphate-fibronogen precipitates to be relevant to the present case, the differences in character of the formed precipitates could be related to the different activities and toxicities of the λ-carrageenans. With the κ-carrageenans, on the other hand, there was little or no evidence to suggest a relationship between sulphate content, precipitation, and anticoagulant activity.

Maximum precipitation occurred for most carrageenans when 2·5 μg reacted with fibrinogen (4 mg; in 2 ml); greater concentrations of carrageenan resulted in less precipitation and eventually in soluble complex formation, indicated by the clearing of the opalescence contributed by the fibrinogen, and by results of estimation of free carrageenan in solution.

Soluble complex formation, seen at all concentrations with degraded carrageenan and heparin is associated in these two substances with relative absence of toxicity. Toxicity is associated with sulphated polysaccharides which can form insoluble complexes with fibrinogen at neutral pH.

The relationship of fibrinogen precipitation *in vitro* to toxicity and

activity *in vivo* is obscure. Concentrations in the precipitation experiments were chosen to simulate *in vivo* concentrations as far as was possible; thus a carrageenan dose of 3 mg/kg, given intravenously to a rabbit having

TABLE 5. PERCENTAGE REDUCTION OF PLATELETS IN THE RABBIT 2 HR AFTER INTRAVENOUS CARRAGEENANS

Carrageenan	Dose mg/kg i.v.	Percentage reduction in platelet count
C. crispus κ–CY	5	24
,, κ–CNS	5	39
,, κ–CSE	5	25
,, κ–CMI	5	0
,, λ–CY	5	62
,, λ–CNS	5	59
,, λ–CSE	5	42
,, λ–CMI	5	31
P. rotundus	3	42
Degraded carrageenan	200	32
Heparin	200 u/kg	0

Results are averages from four rabbits.

a blood volume of 200 ml, would, if completely mixed with the blood, give 90 μg carrageenan in 2 ml blood. It was below this concentration that precipitation *in vitro* began, and maximum precipitation was only seen when the carrageenans were even more dilute (Table 3).

In similar experiments thrombin-carrageenan complexes also precipitated, but with thrombin, as distinct from fibrinogen, heparin and degraded carrageenan formed insoluble precipitates at appropriate concentrations. Carrageenan affected all the tests of clotting examined (Tables 2, 6, 7) and this suggests that their reaction with plasma proteins, especially those concerned with clotting, is a general one and not restricted to fibrinogen; it is possible that insoluble complex formation with other proteins, whether engaged in the clotting or not, could also contribute to toxicity by embolism formation. Complexes formed with clotting proteins need not, on the other hand, be insoluble to affect the clotting reaction.

Platelet counting showed (Table 5) that injection of λ-carrageenans resulted in lower platelet counts than the injection of κ-carrageenans, and amongst the λ-carrageenans the most active anticoagulants caused greatest reduction in count. Although such a trend was obscure amongst the κ-carrageenans, it is noteworthy that κ-CMI caused no reduction in platelet count and was the least active and the least toxic of the κ-carrageenans. Heparin and degraded carrageenan, both relatively non-toxic, caused little or no reduction in platelet count. When the platelet counts were very low, agglutinated platelets were never seen, but with normal or slightly reduced counts (as seen with the κ-carrageenans) agglutination was occasionally seen. It is appropriate to speculate that the structure of the κ-carrageenan-plasma protein complex is unable to trap platelets which are nevertheless caused to agglutinate by the κ-carrageenans. The structure of the λ-carrageenan-plasma protein complex is able to trap the agglutinated platelets, resulting in depleted numbers of unagglutinated platelets in the circulation. Such differences

in structure are more likely to be due to difference in configuration of the sulphated polysaccharides rather than to relatively small differences in sulphate content and molecular weight. This view is supported by the fact that degraded λ-carrageenan is much more toxic than degraded

TABLE 6. EFFECTS OF INTRAVENOUS CARRAGEENANS ON THROMBIN AND PROTHROMBIN TESTS IN THE RABBIT

Carrageenan	Thrombin time				One-stage prothrombin				Two-stage prothrombin			
	mg/kg i.v.				mg/kg i.v.				mg/kg i.v.			
	5		3		5		3		5		3	
	κ	λ	κ	λ	κ	λ	κ	λ	κ	λ	κ	λ
C. crispus –CY	83	68	91	76	78	58	95	69	55	38	61	50
,, –CNS	85	toxic	85	87	84	toxic	81	49	63	toxic	56	36
,, –CSE	87	55	82	74	79	62	78	75	66	57	59	59
,, –CMI	102	67	97	94	92	63	87	70	71	68	82	56
P. rotundus			72				58				43	
Degraded carrageenan 200 mg/kg		60				69				17		
Heparin 200 u/kg		<1				86				9		
75 u/kg		8				100				24		

κ-carrageenan even though both have similar sulphate content and only a small difference in viscosity. The different effect on platelets could also be causally concerned in the different anticoagulant activities of the κ- and λ-carrageenan, although this suggestion is, with present knowledge, not amenable to searching experimental examination.

The general pattern of the fibrinogen reaction of the carrageenans therefore resembles that of certain dextran sulphates, and the picture of respiratory and circulatory collapse with generalised congestion of abdominal organs, in rabbits given a fatal dose, conforms to the description of dextran sulphate overdosage described by Walton (1954) and by others who have observed the toxic effects of parenterally-administered macromolecules.

Localisation of anticoagulant action. The reaction of the carrageenans with plasma protein can explain their toxicity; fibrinogen depletion, caused by its reaction with carrageenan, could affect the final clotting stage. However, the rapid removal of some fibrinogen from the circulation, cannot wholly account for the anticoagulant action which was not fully developed in the rabbit for any of the carrageenans until 2 hr after injection, whereas that of heparin was present at 0·5 hr.

Hawkins & Leonard (1962) deduced that the anticoagulant action of carrageenan was antithrombic in nature on the basis that thrombin time increased with increasing plasma concentration of carrageenan; in agreement with the same authors (1963), Table 3 shows that more carrageenan was required to prolong prothrombin than thrombin time *in vitro* but no quantitative relationship can be obtained from this comparison. Antithrombin action could obviously affect all clotting tests but this does not appear to be the sole mode of action. Thrombin time (Table 6)

was only slightly affected by the carrageenans as compared with heparin which gave a marked effect even at the low dose. This is in contrast to the

TABLE 7. PROTHROMBIN CONSUMPTION INDEX BEFORE AND AFTER INTRAVENOUS INJECTION OF CARRAGEENANS

Carrageenan	mg/kg i.v.							
	5				3			
	κ		λ		κ		λ	
	before	after	before	after	before	after	before	after
C. crispus –CY	6	31	11	42	11	30	5	49
,, –CNS	7	46	toxic		9	72	6	52
,, –CSE	5	42	24	115	13	22	4	29
,, –CMI	3	19	7	66	9	29	14	56
P. rotundus					before 13		after 146	
Degraded carrageenan	200 mg/kg							
	before 11				after 105			
Heparin	200 u/kg				75 u/kg			
	before 5		after 335		before 5		after 5	

similarity in effect in the whole blood clotting time, between *Chondrus* carrageenans and the low dose of heparin, and between degraded carrageenan and the high dose of heparin. This difference between carrageenan and heparin suggests that carrageenan may affect the earlier stages of clotting which precede prothrombin conversion. These earlier stages are associated with prothrombin activator (thromboplastin) formation which can be followed by the prothrombin consumption test. Reduced prothrombin consumption (Table 7) is compatible with the actions of carrageenan already shown, namely platelet count reduction, and effects on fibrinogen and probably on other clotting factors.

The results of the one-stage and two-stage tests (Table 6) support the suggestion that carrageenans interfere with clotting factors in a non-specific manner.

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Estimation of barbiturates by quantitative thin-layer chromatography

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A method for separating and analysing quantitatively, mixtures of barbiturates by thin-layer chromatography is described. The barbiturates are eluted from the silica gel and coupled with mercury which is then estimated by dithizone. The method can be used to assay commercial pharmaceutical preparations.

NUMEROUS chromatographic methods have been used to analyse and identify barbiturate mixtures. Paper chromatography has been widely employed in separations, with analysis times ranging from 4 to 12 hr (Neil & Payton, 1961). Phenobarbitone has been separated from other barbiturates and barbiturates isolated from common analgesics by column partition chromatography (Sabatino, 1954). More recently, gas chromatography has been utilised to separate and identify individual barbiturates and has allowed a quantitative study to be made of their metabolism and excretion in man (Svendsen & Brochmann-Hanssen, 1962). Although rapid, this latter method is still relatively sophisticated and requires expensive apparatus.

Thin-layer chromatography has been used by Cochin & Daly (1963) to identify 16 commercially available barbiturates after their extraction from urine into methylene chloride, and Machata (1960) separated various barbiturates from common pharmaceutical mixtures. A simple method for the identification of barbiturate mixtures when present in blood was devised by Petzold, Camp & Kirch (1963), a procedure which was especially useful in differentiating between amylobarbitone and pentobarbitone. Besides being rapid, the degree of resolution achieved by thin-layer chromatography is usually high and it was thought that a quantitative assay procedure using this technique would be of value. We report the application of such a technique to barbiturate mixtures and to preparations containing barbiturates commercially available in Canada.

Experimental

PREPARATION OF PLATES

Mix 25 g of silica gel with 60 ml of 0·1 N sodium hydroxide and spread the resulting slurry over five 20 × 20 cm glass plates to give a thickness of approximately 0·25 mm. Activate the plates at 110° for 1 hr before use.

REAGENTS

Dithizone solution. Dissolve 150 mg of dithizone in 1000 ml of chloroform. Store the solution in a refrigerator and protect from light.

Mercuric chloride solution. Dissolve 300 mg mercuric chloride in 1 ml of 0·1 N hydrochloric acid and dilute to 100 ml with distilled water.

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Mercuric nitrate solution. Dissolve 1·5 g mercuric nitrate in 5 ml of 2 N nitric acid and dilute to 100 ml.

Buffer solution pH 8·0. Mix 50 ml of 0·2 N potassium dihydrogen phosphate with 46·8 ml of 0·2 N sodium hydroxide and dilute to 200 ml with distilled water.

Barbiturate standards. Prepare a solution containing 20 mg/ml of the relevant barbiturate in 95% ethanol.

Solvent system 1. Mix together isopropanol, chloroform and 25% ammonium hydroxide (60:30:10 ml). Shake well.

Solvent system 2. Water saturated isopropyl ether.

GENERAL PROCEDURE

Samples of the barbiturate standards and of the barbiturate mixtures (in ethanol) were applied in 5 μl and 10 μl quantities to the silica gel plates from self-filling lambda pipettes to give spot sizes of 6–8 mm.

For commercial preparations, three or four tablets were crushed and extracted with 10 ml of 0·1 N sodium hydroxide. The filtrate was acidified with 2 ml N hydrochloric acid and the barbiturates extracted with two 4 ml portions of chloroform which were later diluted to 10 ml exactly. A similar procedure was followed for capsules.

Ten μl of these chloroform extracts was then applied to the thin-layer plates: spot sizes were again 6–8 mm. The plates were placed in tanks which had been equilibrated overnight with the appropriate solvent system and allowed to develop about 15 cm from the starting line. The plates were then removed from the tanks, air-dried, and sprayed with 1·5% aqueous mercuric nitrate solution. The barbiturates appeared as white spots on a pale yellow background. On drying, the relevant areas of silica gel were removed from the plates by an apparatus composed of a small inverted chromatographic tube, the end of which was attached to a vacuum line. The other end was sealed with a rubber stopper through which was placed a right-angled piece of glass tubing (7 mm ext. diam.). The silica gel was sucked through this glass tubing into the column from which the barbiturates could be easily eluted.

The barbiturates were extracted by allowing 30 ml water to filter through the silica gel slowly. To this filtrate, 2 ml of the mercuric chloride solution was added, followed by 2 ml of the phosphate buffer. The resulting opalescent solution was then extracted with one 10 ml portion of chloroform followed by two further 15 ml portions. Two ml of dithizone solution was added, and the solution diluted to 100 ml. The solution was shaken vigorously and allowed to stand for at least 2 hr after which the barbiturates were estimated by measuring the extinction at 475 mμ and comparing with an appropriate standard.

Discussion

According to Truter (1963), one of the main sources of error with elution techniques in thin-layer chromatography is the elution of impurities from the adsorbent. These impurities absorb ultraviolet radiant energy,

particularly from 250–380 mμ, and so interfere with the spectrophotometric assay of many drugs. This was confirmed in the present technique, since attempts to estimate the barbiturates directly in the ultraviolet region following elution from the silica gel gave recoveries ranging as high as 600%. In an attempt to eliminate the need for colorimetric assays

TABLE 1. QUANTITATIVE RESULTS ON BARBITURATE MIXTURES

Drugs	Amount added mg	Number of determinations	Recovered %*	Solvent system	Rf values
Single drugs—					
Amylobarbitone	0·10	4	102·5 ± 1·3	1	0·86
Secobarbitone	0·10	4	102·2 ± 2·1	1	0·83
Barbitone	0·10	8	97·0 ± 2·5	1	0·72
Phenobarbitone	0·10	8	99·8 ± 1·5	1	0·60
Simulated mixtures—					
Secobarbitone	0·20	4	103·0 ± 2·0	1	0·83
Barbitone	0·20	4	100·0 ± 2·5	1	0·72
Phenobarbitone	0·20	4	100·0 ± 2·1	1	0·60
Amylobarbitone	0·20	4	100·0 ± 2·6	1	0·86
Barbitone	0·20	4	101·0 ± 2·2	1	0·72
Phenobarbitone	0·20	4	102·2 ± 2·0	1	0·60
Butabarbital	0·20	12	95·0 ± 4·6	2	0·48
Pentobarbitone	0·20	12	97·0 ± 4·1	2	0·56
Phenobarbitone	0·20	12	100·0 ± 5·2	2	0·37
Secobarbitone	0·20	12	96·0 ± 4·4	2	0·63

* "Recovered %" is expressed as the mean of all determinations ± one standard deviation.

the silica gel was extracted with chloroform, water, decinormal sodium hydroxide and acetone to remove all the absorbing materials before spreading the plates. This proved unsuccessful as recoveries still showed gross overestimation up to 400%. Similarly, using an extract from the silica gel as a blank and treating this under the same conditions as the sample gave results which were very erratic. Interference by impurities still occurred despite these precautions.

The method reported here has been adapted from that of Björling, Berggren & Willman-Johnson (1959), who reported that as little as 0·1 μg/ml of barbiturate could be detected in solution. The present method was found to be quantitative when the amount of the barbiturate applied to the plate was in the range of 50–250 μg. When quantities smaller than 50 μg were applied recoveries were high, and for quantities larger than 250 μg recoveries were low. The orange-coloured solution which results in the mercury-dithizone assay was found to have an absorption peak at 475 mμ. Repeated scanning of the solutions revealed no absorbance maxima in the region reported by Björling & others (1959). By coupling the barbiturates with mercury and subsequently estimating the mercury with dithizone, the wavelength used in the determination of the barbiturates is shifted from 258 to 475 mμ and errors resulting from any absorbing substances in the silica gel are eliminated. Recoveries from simulated mixtures as well as from pharmaceutical preparations are shown in Tables 1 and 2 respectively.

Attempts to estimate phenacetin and aspirin in some of the mixtures analysed were unsuccessful since no satisfactory colorimetric procedure

was found for these drugs. Consequently it was not possible to shift the absorption peaks from the region of 260 mμ.

It was found necessary to prepare the silica gel with decinormal sodium hydroxide, which gave the slurry an almost neutral pH of 6·7 as opposed to a pH of 5·4 when prepared with water. Barbiturates did not separate with sufficient sharpness to allow their removal from plates prepared with water. Despite the use of several solvent systems, no separation of secobarbitone and amylobarbitone was achieved under any set of conditions.

TABLE 2. QUANTITATIVE RESULTS ON BARBITURATES IN PHARMACEUTICALS

	Labelled strength mg	Number of determinations	Recovered %*	Solvent system	Rf value
Luminal—					
Phenobarbitone	100	14	97·6 ± 2·1	2	0·37
Twin-Barb (Tablets)—					
Sod. Secobarbitone ..	65	13	96·3 ± 3·2	2	0·63
Butabarbital	50	13	92·0 ± 1·7	2	0·48
Twin-Barb (Capsules)—					
Sod. Secobarbitone.. ..	65	8	107·1 ± 1·6	2	0·63
Butabarbital	50	12	98·2 ± 1·3	2	0·48
Tri-Barb—					
Sod. Secobarbitone ..	45	12	95·6 ± 2·4	2	0·63
Butabarbital	30	12	101·3 ± 1·1	2	0·48
Phenobarbitone	45	12	98·1 ± 1·8	2	0·56
Multi-Barb—					
Sod. Secobarbitone ..	150	8	93·6 ± 4·8	2	0·63
Sod. Pentobarbitone ..	150	8	93·1 ± 2·9	2	0·56
Sod. Butabarbital ..	48	8	96·9 ± 5·1	2	0·48
Phenobarbitone	48	8	95·4 ± 3·4	2	0·37
Somnol—					
Hexobarbitone	50	8	94·7 ± 2·7	1	0·85
Phenobarbitone	25	9	99·3 ± 3·3	1	0·60
Sedadrops—					
Phenobarbitone	120	4	97·1 ± 3·4	2	0·37
Sod. Pentobarbitone ..	80	4	94·6 ± 3·8	2	0·56

* "Recovered %" is expressed as the mean of all determinations ± one standard deviation.

Tablet or capsule excipients present in the preliminary extraction do not interfere with the separation of the barbiturates. Similarly, some colouring material present in the Sedadrops elixir did not affect the assay and no preliminary extraction proved necessary.

It would appear that the method could be adapted readily to the quantitative analysis of barbiturates in blood and urine.

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Synthesis of pyridine-2-sulphonhydrazide 1-oxide and α-(2-pyridylthio)acethydrazide and its 1-oxide*

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The title compounds and some of their alkylidene and acyl derivatives have been prepared. A preliminary examination of representative compounds revealed negligible antibacterial activity against selected Gram-positive and Gram-negative organisms.

THE introduction of isoniazid as a tuberculotherapeutic agent (Robitzek & Selikoff, 1952) originated from the observations that nicotinamide (Chorine, 1945), 3-aminoisonicotinic acid (Fox, 1952) and *p*-acetamidobenzaldehyde thiosemicarbazone (Domagk, Behnisch, Mietzsch & Schmidt, 1946) were tuberculostatic. Structural modification of the isoniazid molecule designed to discover new tuberculostatic drugs and to delimit its activity followed, leading subsequently to the discovery of 2-ethyl-isonicotinthioamide (ethionamide) (Rist, Grumbach, Libermann, Moyeux, Cals & Clavels, 1956).

Molecular modifications which have been explored include substitution of acyl, alkyl and alkylidene groups on the hydrazide moiety (Offe, Siefken & Domagk, 1952; Bernstein, Jambor, Lott, Pansy, Steinberg & Yale, 1953; Fox & Gibas, 1953), replacement of the carbonyl group by a sulphonyl group (Talik & Plazek, 1955; Comrie & Stenlake, 1958; Angulo & Municio, 1960), separation of the pyridine ring from the hydrazide group by a methylene or ethylene group (Katritzky, 1954) or by a thiomethylene group (Takahashi, Shibasaki & Uchibayashi, 1954), and modification of the ring nitrogen atom by quaternisation and *N*-oxidation (Bernstein & others, 1953). Examination of the isomeric picolinic acid hydrazide showed that it was active but too toxic for clinical use (Fox & Gibas, 1952) and that 1-oxide formation resulted in concomitant reduction of activity and toxicity (Bernstein & others, 1953). In the present work it was decided to examine the effect of (*a*) replacing the carbonyl group in picolinic acid hydrazide 1-oxide by a sulphonyl group and (*b*) separating the pyridine ring from the hydrazino-group in both picolinic acid hydrazide and its 1-oxide by a thiomethylene group.

2-Mercaptopyridine 1-oxide (Shaw, Bernstein, Losee & Lott, 1950) was converted by low temperature chlorination into pyridine-2-sulphonyl chloride 1-oxide which reacted with hydrazine to give the sulphonhydrazide (I; R = R′ = H) using a previously described method (Comrie & Stenlake, 1958). Arylidene derivatives (I; RR′ = ArCH:) were readily obtained from aromatic aldehydes in methanol.

Condensation of 2-mercaptopyridine 1-oxide and ethyl bromoacetate in ethanol gave the hydrobromide of the ester (II; X = OEt), which reacted

* For previous paper see Comrie & Stenlake (1961).

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with either water, ammonia, hydroxylamine or hydrazine to give respectively the acid (II; X = OH), the amide (II; X = NH_2), the hydroxamic acid (II; X = NH·OH), and α-(2-pyridylthio)acethydrazide 1-oxide (II; X = NH·NH_2). The hydrazide reacted with aldehydes and ketones to give sparingly soluble crystalline alkylidene derivatives (Table 1) (II; X = NH·N:CRR′), with acid anhydrides to give acyl derivatives (II; X = NH·NH·CO·R), and with phenyl isocyanate to give the semicarbazide (II; X = NH·NH·CO·NH·Ph).

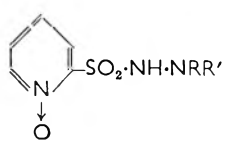
TABLE 1. *N′*-ALKYLIDENE-α-(2-PYRIDYLTHIO)ACETHYDRAZIDES (III; X = NH·N:CRR′)

Derivative (:CRR′)	M.p. °C	Yield %	Formula	Found %			Required %		
				C	H	N	C	H	N
Salicylidene	200–201 (decomp.)	70	$C_{14}H_{13}N_3O_2S$	58·25	4·5	14·6	58·5	4·6	14·6
Piperonylidene	167–170	60	$C_{15}H_{13}N_3O_3S$	57·4	4·3		57·1	4·2	
Veratrylidene	138–141	60	$C_{16}H_{17}N_3O_3S$	57·6	5·4		58·0	5·2	
p-Dimethylaminobenzylidene	186–188	92	$C_{16}H_{18}N_4OS$	60·9	5·7		61·1	5·8	
Cinnamylidene	135–136	54	$C_{16}H_{15}N_3OS$	64·2	4·9	14·7	64·6	5·1	14·1
Vanillylidene	165–167	40	$C_{15}H_{15}N_3O_3S$	57·3	5·2		56·8	4·8	
α-Phenylethylidene	142–144	74	$C_{16}H_{16}N_3OS$	63·1	5·55	15·0	63·15	5·3	14·7
Phenethylidene	152–153	70	$C_{16}H_{15}N_3OS$	62·9	5·4	15·4	63·15	5·3	14·7
Isopropylidene	128–130	86	$C_{10}H_{13}N_3OS$	53·5	5·8	19·0	53·8	5·9	18·8
Cyclohexylidene	112–114	72	$C_{13}H_{17}N_3OS$	59·35	6·6		59·3	6·5	
p-Methoxybenzylidene	136–138	33	$C_{15}H_{15}N_3O_2S$	59·5	5·1		59·8	5·0	
p-Hydroxybenzylidene	194 (decomp.)	52	$C_{14}H_{13}N_3O_2S$	56·7	4·7		56·3	4·7	
Hexahydro-2,4,6-trioxo-5-pyrimidinylidene*	290 (decomp.)	62	$C_{11}H_{11}N_5O_5S$	40·8	3·9	21·9	40·6	3·4	21·5

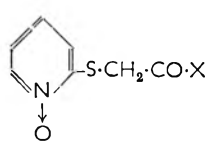
N′-ALKYLIDENE-α-(2-PYRIDYLTHIO)ACETHYDRAZIDE 1-OXIDES (II; X = NH·N:CRR′)

Salicylidene	230–232 (decomp.)	68	$C_{14}H_{13}N_3O_3S$	55·55	4·0	13·9	55·4	4·3	13·9
Piperonylidene	207–208	62	$C_{15}H_{13}N_3O_4S$	54·2	3·9	12·1	54·4	3·9	12·7
Veratrylidene	213–214 (decomp.)	61	$C_{16}H_{17}N_3O_4S$	54·8	5·1		55·3	4·9	
p-Dimethylaminobenzylidene	218–220 (decomp.)	56	$C_{16}H_{18}N_4O_2S$	58·6	5·45	16·2	58·2	5·5	17·0
Cinnamylidene	220–223 (decomp.)	66	$C_{16}H_{15}N_3O_2S$	61·55	4·9	13·3	61·3	4·8	13·4
Vanillylidene	198–200 (decomp.)	46	$C_{15}H_{15}N_3O_4S$	54·2	4·55	12·8	54·1	4·5	12·6
α-Phenylethylidene	201–203 (decomp.)	70	$C_{15}H_{15}N_3O_4S$	60·5	5·0	13·5	59·8	5·0	13·9
Phenethylidene	190–191	52	$C_{15}H_{15}N_3O_2S$	60·0	5·2		59·8	5·0	
Isopropylidene	210–211 decomp.)	83	$C_{10}H_{13}N_3O_2S$	50·3	5·3	17·7	50·2	5·5	17·6
2-Acetyl-1-methylethylidene (acetylisopropylidene)	175 (decomp.)	57	$C_{13}H_{15}N_3O_3S$	51·3	5·7	15·4	51·25	5·3	14·9
Furfurylidene	180–182	72	$C_{12}H_{11}N_3O_3S$	51·9	4·2	15·8	52·0	4·0	15·2
Hexahydro-2,4,6-trioxo-5-pyrimidinylidene*	183 (decomp.)	59	$C_{11}H_{11}N_5O_6S$	37·8	3·8	19·5	37·3	3·2	19·8

* Monohydrate.



(I)



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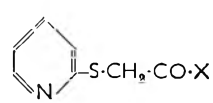

(III)

2-Mercaptopyridine reacted with ethyl bromoacetate giving the ester (III; X = OEt) hydrobromide, which was hygroscopic, and although it failed to give a satisfactory analysis the crude product reacted with hydrazine to give α-(2-pyridylthio)acethydrazide (III; X = $NH{\cdot}NH_2$) in good yield (66%). The acethydrazide formed alkylidene derivatives (Table 1) (III; X = $NH{\cdot}N{:}CRR'$), acyl derivatives (III; X = $NH{\cdot}NH{\cdot}CO{\cdot}R$), and with allyl isothiocyanate gave the thiosemicarbazide (III; X = $NH{\cdot}NH{\cdot}CS{\cdot}NH{\cdot}CH_2{\cdot}CH{:}CH_2$). In an excess of acetic anhydride it gave the diacetyl derivative (III; X = $NH{\cdot}NAc_2$). With toluene-*p*-sulphonyl chloride, α-(2-pyridylthio)acethydrazide and its 1-oxide gave respectively the derivatives (III) and (II) (X = $NH{\cdot}NH{\cdot}SO_2{\cdot}C_6H_4{\cdot}Me$-*p*).

Attempts to prepare alkyl derivatives (III; X = $NH{\cdot}NRR'$) were unsuccessful. Catalytic hydrogenation of alkylidene derivatives led to hydrogenolysis giving 2-mercaptopyridine, while chemical reduction in acid solution regenerated the acethydrazide and carbonyl compound. Condensation of ethyl α-(2-pyridylthio)acetate and *NN*-di-isopropylhydrazine, and the base-catalysed condensation of α-(2-pyridylthio)-acethydrazide and benzyl bromide gave grossly impure products which could not be purified for characterisation.

BACTERIOLOGICAL RESULTS

We thank Mr. Malcolm S. Parker, M.Sc., M.P.S. of this Department for the bacteriological examination of *N'*-benzylidene-*N*-(pyridine-2-sulphon)hydrazide 1-oxide and several representative alkylidene and acyl derivatives, and also the toluene-*p*-sulphonyl derivatives of α-(2-pyridylthio)acethydrazide and its 1-oxide. None of the compounds exhibited activity against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, or *Bacillus subtilis*.

Experimental

Melting points are uncorrected.

Pyridine-2-sulphonhydrazide 1-oxide. Pyridine-2-sulphonyl chloride 1-oxide obtained by chlorination of 2-mercaptopyridine 1-oxide (1·27 g) at −5° and extracted into cold chloroform (120 ml) (Comrie & Stenlake, 1958) was added portionwise to hydrazine hydrate (1·0 g), and the mixture vigorously shaken after each addition and left at *ca.* 0° overnight. The precipitate was filtered off, suspended in ice-cold water (10 ml), filtered and dried *in vacuo*, giving the *sulphonhydrazide* as the monohydrate (0·3 g), m.p. 96–98° (decomp.) (from methanol). Found: C, 28·8; H, 3·9. $C_5H_9N_3O_4S$ requires C, 28·9; H, 4·3%.

*N'-Benzylidene-*N*-(pyridine-2-sulphon)hydrazide 1-oxide.* A solution of pyridine 2-sulphonhydrazide 1-oxide (0·189 g) and benzaldehyde (0·106 g) in methanol (10 ml) was vigorously shaken and the precipitate washed with a small volume of methanol and ether. The *benzylidene derivative* (0·16 g) was obtained as needles, m.p. 145–147° (decomp.) (from methanol). Found: C, 52·6; H, 4·1; N, 15·4. $C_{12}H_{11}N_3O_3S$ requires C, 52·0; H, 4·0; N, 15·2%.

N-(*Pyridine-2-sulphon*)*hydrazide*-N′-*veratrylidene* 1-*oxide*. Pyridine-2-sulphonhydrazide 1-oxide (0·189 g) and veratraldehyde (0·166 g) similarly gave the *veratrylidene derivative* (0·15 g), m.p. 146–148° (decomp.) (from ethanol). Found: C, 49·2; H, 4·1; N, 12·7. $C_{14}H_{15}N_3O_5S$ requires C, 49·85; H, 4·5; N, 12·5%.

Ethyl α-(2-*pyridylthio*)*acetate hydrobromide*. 2-Mercaptopyridine 1-oxide (3·8 g) and ethyl bromoacetate (5·0 g) in ethanol (50 ml) were refluxed for 1½ hr and the solvent removed under reduced pressure to give the *hydrobromide* (3·9 g), m.p. 120° (decomp.) (from ethanol-ether). Found: N, 5·2. $C_9H_{12}BrNO_3S$ requires N, 4·8%.

α-(2-*Pyridylthio*)*acethydrazide* 1-*oxide*. The crude product from the preceding experiment was dissolved in ethanol (40 ml), anhydrous hydrazine (0·9 g) added and the mixture refluxed for 5 hr. The solvent was removed under vacuum and the residue recrystallised from ethanol to give the *acethydrazide* 1-*oxide* (2·5 g), m.p. 200–201° (decomp.). Found: C, 42·2; H, 4·4; N, 20·5. $C_7H_9N_3O_2S$ requires C, 42·2; H, 4·6; N, 21·1%.

N′-*Benzylidene*-α-(2-*pyridylthio*)*acethydrazide* 1-*oxide*. α-(2-Pyridylthio)acethydrazide 1-oxide (0·398 g) and benzaldehyde (0·212 g) were shaken in methanol (10 ml) to effect solution, and then left at *ca.* 0° overnight. The precipitate was filtered off, washed with a small volume of methanol and ether to give the *benzylidene derivative* (0·4 g), m.p. 202–203° (decomp.) (from methanol). Found: C, 58·5; H, 4·55; N, 14·95. $C_{14}H_{13}N_3O_2S$ requires C, 58·5; H, 4·6; N, 14·6%.

Other *N*′-alkylidene derivatives (Table 1) (II; X = NH·N:CRR′) were similarly prepared.

α-(2-*Pyridylthio*)*acetic acid* 1-*oxide*. A solution of 2-mercaptopyridine 1-oxide (1·27 g) and ethyl bromoacetate (1·67 g) in ethanol (10 ml) was refluxed for 1½ hr and the solvent removed under reduced pressure. The residue was refluxed with water (10 ml) for 1½ hr and the solution evaporated to dryness. Recrystallisation from aqueous methanol gave α-(2-*pyridylthio*)*acetic acid* 1-*oxide* (1·0 g), m.p. 288° (decomp.). Found: C, 45·6; H, 4·0; N, 7·7. $C_7H_7NO_3S$ requires C, 45·4; H, 3·8; N, 7·6%.

α-(2-*Pyridylthio*)*acetamide* 1-*oxide*. 2-Mercaptopyridine 1-oxide (1·27 g) and ethyl bromoacetate (1·67 g) were refluxed in ethanol (10 ml) as above and the solvent removed. The residue was redissolved in ethanol (10 ml) and shaken with an excess of ammonia solution (d. 0·88) and the solution evaporated to dryness. The *amide* (1·0 g), m.p. 215°, was recrystallised from methanol. Found: C, 45·6; H, 4·3; N, 15·4. $C_7H_8N_2O_2S$ requires C, 45·65; H, 4·3; N, 15·2%.

α-(2-*Pyridylthio*)*acethydroxamic acid* 1-*oxide*. 2-Mercaptopyridine 1-oxide (1·27 g) and ethyl bromoacetate (1·67 g) in ethanol (10 ml) were refluxed as before and after removing the solvent the residue was added to hydroxylamine hydrochloride (1·1 g) in methanol (15 ml) containing sodium methoxide (1·1 g). The precipitate was filtered off and the filtrate concentrated at room temperature under reduced pressure. The *hydroxamic acid* (0·5 g) m.p. 195–197° (decomp.), slowly separated. Found: C, 41·9; H, 4·2. $C_7H_8N_2O_3S$ requires C, 42·0; H, 4·0%.

δ-Phenyl-α-[α-(2-pyridylthio)acetyl]-semicarbazide 1-oxide. α-(2-Pyridylthio)acethydrazide 1-oxide (0·398 g) and phenyl isocyanate (0·2 g) were shaken in acetonitrile (10 ml) for 1 hr and the solvent removed under reduced pressure. The residue was recrystallised from ethanol to give the *semicarbazide* (0·2 g), m.p. 194–195°. Found: C, 52·7; H, 4·2; N, 18·0. $C_{14}H_{14}N_4O_3S$ requires C, 52·8; H, 4·4; N, 17·6%.

N'N'-Diacetyl-N-[α-(2-pyridylthio)acetyl]hydrazine 1-oxide. α-(2-Pyridylthio)acethydrazide 1-oxide (0·398 g) was added in small portions to acetic anhydride (5 ml) and warmed to complete solution. The solid which separated on cooling was washed with ether, dried *in vacuo* and twice crystallised from methanol to give the *diacetyl derivative* (0·4 g), m.p. 135° (decomp.). Found: C, 46·5; H, 4·8; N, 14·9. $C_{11}H_{13}N_3O_4S$ requires C, 46·7; H, 4·6; N, 14·8%.

N'-(β-Carboxypropionyl)-N-[α-(2-pyridylthio)acetylhydrazine 1-oxide. α-(2-Pyridylthio)acethydrazide 1-oxide (0·398 g) was added to succinic anhydride (0·2 g) in methanol (10 ml). The precipitate was recrystallised from methanol to give the *product* (0·4 g), m.p. 200–201° (decomp.). Found: C, 44·4; H, 4·55; N, 14·85. $C_{11}H_{13}N_3O_5S$ requires C, 44·1; H, 4·4; N, 14·05%.

N'-(β-Carboxyacryloyl)-N-[α-(2-pyridylthio)acetyl]hydrazine 1-oxide. α-(2-Pyridylthio)acethydrazide 1-oxide (0·398 g) and maleic anhydride (0·2 g) reacted as described above to give the *product* (0·3 g), m.p. 110–113° (from methanol). Found: C, 44·8; H, 4·2; N, 14·8. $C_{11}H_{11}N_3O_5S$ requires C, 44·4; H, 3·7; N, 14·1%.

N-[α(2-Pyridylthio)acetyl]-N'-(toluene-p-sulphonyl)hydrazine 1-oxide. Toluene-*p*-sulphonyl chloride (0·38 g) was added to α-(2-pyridylthio)-acethydrazide 1-oxide (0·398 g) in dry pyridine (10 ml) and heated on a water-bath for 15 min. The solution was cooled, water added, and set aside for 3 hr. The precipitate was washed with a small volume of water and dried to give the *toluene-p-sulphonyl derivative* (0·29 g), m.p. 242° (decomp.) (from ethanol). Found: C, 47·4; H, 4·5. $C_{14}H_{15}N_3O_4S_2$ requires C, 47·6; H, 4·3%.

α-(2-Pyridylthio)acethydrazide. 2-Mercaptopyridine (1·11 g) was refluxed with ethyl bromoacetate (1·67 g) in dry ethanol (30 ml) for 2 hr and the solvent removed under reduced pressure, leaving a viscous oil which set to a hygroscopic solid (1·3 g). This was dissolved in dry ethanol (30 ml) and refluxed with anhydrous hydrazine (1·0 g) for 5–6 hr and again evaporated to dryness *in vacuo*. The solid residue was suspended in ice-cold water (10 ml), filtered and recrystallised from ethanol to give the *acethydrazide* (1·2 g), m.p. 90–92°. Found: C, 46·0; H, 4·9; N, 22·2. $C_7H_9N_3OS$ requires C, 45·9; H, 4·9; N, 22·9%.

N'-Benzylidene-α-(2-pyridylthio)acethydrazide. α-(2-Pyridylthio)acethydrazide (0·183 g) and benzaldehyde (0·106 g) were dissolved in methanol (10 ml). The solid separating was washed with a little methanol and ether, and recrystallised from methanol to give the *benzylidene derivative* (0·2 g), m.p. 191–192°. Found: C, 62·0; H, 4·7; N, 14·9. $C_{14}H_{13}N_3OS$ requires C, 62·0; H, 4·8; N, 15·5%.

Other *N'*-alkylidene derivatives (Table) (III; X = NH·N:CRR') were similarly prepared.

N'N'-*Diacetyl-α-(2-pyridylthio)acethydrazide.* α-(2-Pyridylthio)acethydrazide (0·183 g) was added in small amounts to freshly distilled acetic anhydride (5 ml). The mixture, which partially solidified, was dried at the pump, washed with ether and recrystallised from ethyl acetate to give the N'N'-*diacetyl derivative* (0·19 g), m.p. 141–143°. Found: C, 49·7; H, 4·9. $C_{11}H_{13}N_3O_3S$ requires C, 49·4; H, 4·9%.

N'-*Acetyl-α-(2-pyridylthio)acethydrazide.* α-(2-Pyridylthio)acethydrazide (0·183 g) and acetic anhydride (0·18 g) were dissolved in dry pyridine (5 ml) and the precipitate recrystallised from ethyl acetate to give the N'-*acetyl derivative* (0·1 g), m.p. 138–140°. Found: C, 48·0; H, 5·1; N, 18·1. $C_9H_{11}N_3O_2S$ requires C, 48·0; H, 4·9; N, 18·7%.

N'-(β-*Carboxypropionyl*)-N-[α-(2-*pyridylthio)acetyl*]*hydrazine.* α-(2-Pyridylthio)acethydrazide (0·183 g) and succinic anhydride (0·116 g) in methanol (10 ml) gave the *product* (0·2 g) m.p. 150–151° (from isopropanol). Found: C, 46·2; H, 4·6. $C_{11}H_{13}N_3O_4S$ requires C, 46·7; H, 4·7%.

N-[α-(2-*Pyridylthio)acetyl*]-N'-(*toluene*-p-*sulphonyl*)-*hydrazine.* Toluene-*p*-sulphonyl chloride (0·38 g) and α-(2-pyridylthio)acethydrazide (0·366 g) in dry pyridine (10 ml) were heated on a water-bath for 10 min and then cooled. The *toluene*-p-*sulphonyl derivative* (0·1 g) was isolated by adding water (50 ml) and on recrystallisation from ethanol had m.p. 165–167°. Found: C, 49·3; H, 4·6. $C_{14}H_{15}N_3O_3S_2$ requires C, 49·85; H, 4·5%.

δ-*Allyl-α-*[α-(2-*pyridylthio)acetyl*]-*thiosemicarbazide.* α-(2-Pyridylthio)-acethydrazide (0·366 g) and allyl isothiocyanate (0·2 g) in acetonitrile (5 ml) were heated on a water-bath for 10 min and then cooled to room temperature to give the *thiosemicarbazide* (0·32 g), m.p. 116–117° (from ethanol). Found: C, 46·3; H, 5·0. $C_{11}H_{14}N_4OS_2$ requires C, 46·8; H, 5·0%.

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Letters to the Editor

Intraperitoneal ether for anaesthetizing small laboratory animals

SIR,—Ether has been used for many years to anaesthetize small laboratory animals despite the inconvenience of its administration by inhalation. In the present experiments, ether was given intraperitoneally in rats as a 40% solution in peanut (arachis) oil. Table 1 summarises the results of experiments made with groups of 10 rats (170–180 g) at each ether dose. Arachis oil controls are designated as ether 0·0 ml/kg. For assessment purposes animals were considered to be anaesthetized if they met the following criteria: (1) loss of righting reflex, (2) lack of response to pinching of the tail, and (3) lack of spontaneous voluntary movements.

As expected, all deaths were attributable to respiratory failure and could have been prevented by respiring the rats artificially, by intubation with a blunted 18 gauge hypodermic needle. Artificial ventilation regularly permitted complete recovery after the administration of otherwise lethal doses (5·00 ml/kg) of ether. Similar results were obtained in 8 cats using 2·5 and 5·0 ml/kg of ether.

TABLE 1. ETHER ANAESTHESIA IN RATS

Dose of ether ml/kg	Number anaesthetized	Duration of anaesthesia min ± s.e.	Number of deaths
0·0	0/10	—	0/10
0·62	0/10	—	0/10
1·25	4/10	2·85 ± 1·7	0/10
2·50	9/10	14·07 ± 2·0	0/10
3·00	9/10	25·12 ± 3·6	4/10
3·75	10/10	50	8/10
5·00	10/10	—	10/10

Approximately 1–2 min after injection of an anaesthetizing dose of ether, the rats became ataxic, then lost the righting reflex with attendant analgesia. Considerable muscle tone persisted as indicated by marked resistance to manual extension or flexion of the limbs. This stage was followed by loss of all voluntary movement and usually by muscle flaccidity. During recovery the animals moved their heads and attempted to right themselves. Righting efforts were generally unsuccessful for as long as 15 min after the initial head movements were noted. During this period muscle tone returned but analgesia was still marked.

Noteworthy in these experiments was the lack of "second stage excitement" which is commonly observed with inhaled ether. Moreover excessive salivation was not observed after intraperitoneally administered ether, whereas salivation usually was profuse after inhaled ether. In contrast, cats after either inhalational or intraperitoneal ether, salivated markedly.

Anaesthesia produced by the intraperitoneal administration of ether in arachis oil has been found to be entirely satisfactory for a variety of surgical procedures. Since the duration of anaesthesia is dose-related, the convenience afforded by this method of administration is apparent.

The author thanks Miss Joan Bayliss for technical assistance.

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Concentrations of amphetamine in the brain in normal or aggressive mice

SIR,—It was recently reported that amphetamine is consistently more toxic in aggressive than in normal mice whether the animals are kept either grouped or isolated (Consolo, Garattini & Valzelli, 1965). To find a possible reason for this increased toxicity, experiments were made to measure the concentration of amphetamine in the brain in this experimental situation.

As previously described, male Swiss albino mice weighing about 20 g were usually kept 6 per Makrolon cage with a floor surface of about 40 cm^2 at a room temperature 22° and a relative humidity of 60%. Mice were made aggressive (Yen, Stanger & Millman, 1959) by isolation for 4 weeks in individual cages of the same size, but with opaque walls.

Dexamphetamine was administered intraperitoneally at 10 mg/kg to all the animals. Brain dexamphetamine was estimated as described by Axelrod (1954), 15, 30, 45 or 60 min after administration, and the results are summarized in Table 1.

TABLE 1. BRAIN AMPHETAMINE IN NORMAL AND AGGRESSIVE MICE ISOLATED (I) OR GROUPED (G) AT DIFFERENT TIMES AFTER ADMINISTRATION OF DEXAMPHETAMINE, 10 MG/KG/I.P.
(Each figure represents the average of 8 determinations)

Time after administration (min)	Amphetamine in brain (μg/g ± s.e.)			
	Normal		Aggressive	
	I	G	I	G
15	10·7 ±0·6	17·4(°) ±1·1	10·8 ±1·4	12·6 ±0·7
30	8·5 ±0·6	15·0(°) ±1·7	6·4 ±0·7	12·4(°) ±1·0
45	7·8 ±1·0	10·4(°) ±0·8	7·3 ±0·4	12·8(°) ±1·0
60	3·5 ±1·0	9·4(°) ±1·4	3·4 ±1·0	11·0(°) ±1·1

(°) $P < 0.01$ (G versus I).

It is evident that grouped animals, either normal or aggressive, have a higher and longer lasting level of amphetamine in the brain, but there are no significant differences between normal and aggressive animals.

It is concluded that the different metabolism of amphetamine between grouped or isolated animals may be a contributing factor in explaining the different toxicities of amphetamine under these two experimental conditions.

On the other hand a difference in amphetamine metabolism does not seem to play the major role which would justify the more pronounced sensitivity to amphetamine of aggressive mice compared with normal animals.

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A simplified method for preparing a pithed cat

SIR,—Most investigators who use the spinal cat follow Dale's method as described by Burn (1952), which involves section of the highly vascularised dorsal spinal muscles, which cling tenaciously to the vertebrae, and resection of the vertebral bone to expose the spinal cord. Recently, Zarro & DiPalma (1964) described another method for preparation of the spinal cat for use in studies on the superior cervical ganglion or the nictitating membrane. The spinal cord was transected through the anterior atlanto-occipital aperture which is readily accessible after the surgical procedures in the neck necessary to expose the superior cervical ganglion. However, since the surgical procedures are extensive, the method is not convenient when a study is to be made involving organs or tissues not located in the neck.

In addition to the disadvantages listed above, it was discovered that when spinal cats in which the spinal cord had not been pithed were used for prolonged experiments (up to 8 hr), large cyclic changes in blood pressure sometimes occurred (4 out of 13 cats). These changes appeared and disappeared at random intervals, the mean blood pressure varying as much as 100 mm Hg. When they occurred, they interfered with the orderly course of the experiment. The cyclic blood pressure changes could be eliminated by an injection of procaine into the spinal cord, which suggests that they were due to activity of spinal vasomotor centres. The following procedure eliminates some of these disadvantages.

The cat is anaesthetized with diethyl ether. After tracheotomy the cat is maintained under light ether anaesthesia by appropriate adjustment of a valve on an etherising bottle which is connected to the tracheal cannula. The head is flexed forward maximally and a midline incision of the skin of the nape of the neck is made. The superficial platysma muscles directly caudad to the lambdoidal ridge of the cranium are doubly ligated and severed and the underlying occipital muscles are scraped from the occipital bone with the blunt end of a scalpel holder, exposing the posterior atlanto-occipital membrane. The membrane is slit and pushed aside to expose the spinal cord. Ether administration is discontinued, and artificial respiration is started. A metal hook is passed around the spinal cord and quickly pulled upward to sever the cord. The brain is destroyed by passing a blunt probe through the foramen magnum. The spinal cord is destroyed by passing a flexible probe into the spinal column. A suitable probe can be cut from a spiral wire spring 4·0 mm in diameter, of the kind commonly sold to clean obstructed sink drains. A cotton wad saturated with petroleum jelly is packed into the spinal column, and the atlanto-occipital opening is corked. After placing a pledget over the wound, the cut edges of the skin are stitched together.

The method described here for pithing the spinal cord proved to be notably satisfactory with 116 cats. Cyclic changes were observed in only 3 of these cats, possibly because there was incomplete destruction of the spinal cord. The mean blood pressure of this group was 69 $\pm$ 14 (s.d.) mm Hg. This method has several advantages over those commonly used. It requires a minimum of surgery, and hence the cat is subjected to less trauma and a shorter period of hazardous ether anaesthesia. In experienced hands, the entire procedure can be completed within 15 min. The method has a further advantage over the anterior approach, even when the preparation is to be used for studies on the superior cervical ganglion, since the spinal cord can be pithed more readily through the posterior atlanto-occipital opening than the anterior.

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Concerning the regulation of some diverse biochemical reactions, underlying the inflammatory response, by salicylic acid, phenylbutazone and other acidic antirheumatic drugs

SIR,—Recent *in vitro* studies of the properties of non-steroid anti-inflammatory: antirheumatic drugs have indicated that, in addition to their analgesic properties, they might inhibit several chemical reactions *in vivo* which probably participate in the overall development of *signa inflammationis*, the subsequent formation of granulation (scar) tissue and wound repair (Spector, 1964; Garattini & Dukes, 1965; Whitehouse, 1965). The biochemical reactions include (i) the mitochondrial biosynthesis of adenosine-5′-triphosphate(ATP) ("oxidative phosphorylation"); (ii) the formation of histamine by substrate-specific histidine decarboxylase(s); (iii) the hydrolysis of proteins or amino-acid esters, or both, by enzymes resembling trypsin (EC no. 3.4.4.4) in their substrate-specificity, for example, the Hageman factor (Schoenmakers, Matze, Haaner & Zilliken, 1964), the kinin-forming enzymes (Webster & Pierce, 1961), and thrombin and plasmin: fibrinolysin (Scheraga, Ehrenpreis & Sullivan, 1958).

These three particular processes, though superficially unrelated in their chemistry, appear to have at least two features in common: firstly their sensitivity to mM concentrations of salicylic acid and certain other acidic anti-inflammatory drugs such as phenylbutazone, cinchophen, indomethacin and flufenamic acid; and secondly, the involvement of an ϵ-amino-group (belonging to a lysine residue in the enzyme protein or protein substrate) in the enzyme-substrate interaction. Where the relation between chemical structure and the ability to inhibit these enzyme reactions has been investigated (see Whitehouse, 1965), it is notable that non-acidic derivatives of these drugs, such as salicylamide, *N*-arylanthranilamides and the amide of indomethacin, are unable to substitute for the parent acid as effective anti-inflammatory drugs or inhibitors of these enzyme systems (although they may still carry analgesic activity). We therefore believe that some of the apparently diverse effects of salts of salicylic acid and other anti-inflammatory acids upon enzyme systems, especially those implicated in the inflammatory response of animal tissues, are due to "neutralisation" of essential lysyl ϵ-amino-groups by the anionic form of these drugs. The evidence for this hypothesis is summarised, as follows.

A. Drugs known to selectively inhibit mitochondrial ATP biosynthesis without affecting mitochondrial respiration (so-called "uncoupling agents") are either (i) acids able to partition from an aqueous phase into the mitochondrial lipid phase, for example, 2,4-dinitrophenol and the acidic anti-inflammatory drugs considered here, or (ii) compounds able to interact with an amino-group adjacent to a thiol group, for example, certain trivalent arsenicals, and carbonyl-cyanide-phenylhydrazones (Heytler, 1963; Whitehouse, 1965). We discovered that several other compounds able to react with free amino-groups under mild conditions (quasi-physiological pH, room temperature) in an aqueous medium,

selectively uncouple oxidative phosphorylation at concentrations which have little or no effect upon either electron transport (succinate oxidation) by rat liver mitochondria or on the activity of added yeast hexokinase (used to trap newly-synthesised ATP) under the experimental conditions described elsewhere (Skidmore & Whitehouse, 1965a). These uncoupling "amine reagents" include 2,4,6-trinitrobenzene sulphonic acid (4 mM), 1-fluoro-2,4-dinitrobenzene (FDNB, 0·8 mM), 2-nitrobenzaldehyde and cinnamaldehyde (5 mM), 2,4,6-trinitrobenzaldehyde (0·5 mM), and also ninhydrin (0·2 mM). Furthermore, the uncoupling effect of some of these reagents (e.g. 5 mM 2-nitrobenzaldehyde or FDNB, 2·5 mM trinitrobenzaldehyde) was not wholly reversed by tenfold dilution after preincubation with buffered mitochondria alone (7 min at 30°); by contrast, the uncoupling action of acidic drugs (80 μM 2,4-dinitrophenol, 3 mM salicylic acid, 0·5 mM indomethacin) and carbonylcyanide *m*-chlorophenylhydrazone (4 μM) was completely reversed under these conditions. This suggests that these "amine reagents" were not inhibiting phosphorylation merely as a consequence of oxidation or hydrolysis to carboxylic or phenolic acids within mitochondria. Preliminary chemical analyses support this conclusion. Other experiments indicated that the 6-amino-group of the adenine moiety (of ATP-precursors) was not blocked by these particular amine reagents under conditions that brought about uncoupling of oxidative phosphorylation.

It seems reasonable to infer that a lysyl ϵ-amino group might be implicated in mitochondrial ATP biosynthesis. This process probably provides the bulk of the ATP utilised in various endergonic reactions associated with the inflammatory response, for example, in histamine release (Uvnäs, 1964), or in connective-tissue biosynthesis.

B. We have observed that salicylic acid, indomethacin, phenylbutazone and flufenamic acid (at $\leq$ 1 mM) all inhibit histamine formation *in vitro* by animal enzymes (from rat pyloric stomach, or foetal liver) which specifically decarboxylate L-histidine and also have a relatively low affinity for the coenzyme, pyridoxal phosphate ($K_m \geq 0{\cdot}1$ μM). At concentrations below 10 mM these drugs do not inhibit the non-specific animal L-aromatic amino-acid decarboxylase (for example, from guinea-pig kidney) or a bacterial histidine decarboxylase (from *Cl. welchii*) which have a higher affinity for pyridoxal phosphate. This drug inhibition of the animal substrate-specific enzymes is non-competitive with respect to the substrate and these drugs appear to act by releasing bound pyridoxal phosphate from the decarboxylase enzyme. Chemical analogues of these drugs which are devoid of anti-inflammatory activity (such as benzoic acid, phenazone, 5-methoxyindole-3-acetic acid) have little or no effect upon histamine formation *in vitro*.

Transaminases, which contain pyridoxal phosphate linked to a lysyl ϵ-amino-group by an azomethine linkage (Braunstein, 1964), are also inhibited by salicylic acid, cinchophen and phenylbutazone (Pulver, Exer & Herrmann, 1956; Huggins, Smith & Moses, 1961; Hänninen & Hartiala, 1965). Pyridoxal phosphate binds to plasma albumen through a similar linkage (Dempsey & Christensen, 1962) and may be displaced from the albumen amino-groups by these acidic anti-inflammatory drugs (Skidmore & Whitehouse, 1965b).

C. Trypsin and non-enteric enzymes with a similar substrate specificity (for example, cathepsin B, Hageman factor, plasmin) may provoke inflammation (Domenjoz & Mörsdorf, 1965; Graham, Ebert, Ratnoff & Moses, 1965). They all hydrolyse peptide or ester linkages in which an L-arginine or L-lysine residue contributes the carbonyl group (Neurath & Schwert, 1950; Scheraga & others, 1958; Schoenmakers & others, 1964). Tryptic digestion *in vitro* of certain proteins, (like diazotised collagen or rat paw homogenates, is partially

inhibited by salicylic acid, phenylbutazone, indomethacin and flufenamic acid and also by amidopyrine (Bertelli, Donati & Rossano, 1965; Mörsdorf, 1965). Domenjoz and Mörsdorf (1965) reported that these acidic drugs also inhibit trypsin-induced oedema in rats.

We found that anti-inflammatory acids lower the initial rate of tryptic digestion of ethanol-denatured horse heart cytochrome *c* (Sigma Chemical Co., London) and urea-denatured globin (Edmundson & Hirs, 1962) prepared from horse heart myoglobin (British Drug Houses Ltd., Poole = B.D.H.). The ratio of lysine residues to arginine residues is 8·5 in purified myoglobin (Holleman and Biserte, 1959) and 9·5 in the cytochrome *c* (Margoliash, Kimmel, Hill and Schmidt, 1962). Tryptic digestion of a commercial protamine preparation (salmine sulphate, B.D.H.), containing 53% arginine and only 0·2% lysine, was far less sensitive to these drugs. With 0·14 mM globin (2·5 mg/ml), equivalent to 2·4 mM lysyl ϵ-amino-groups, and 10 μg/ml crystalline trypsin (B.D.H.) in 60 mM Tris hydrochloride, pH 7·4 or 7·9, the initial rate of proteolysis (up to 4 min) was ⩽ 50% of that in drug-free controls in the presence of 15 mM sodium salicylate or 3 mM phenylbutazone or 2 mM indomethacin or 4 mM 2,4,6-trinitrobenzaldehyde (all added in pre-neutralised solution at pH 7·4 or 7·9). With 1·3 mg/ml salmine sulphate and 5 μg/ml trypsin, the same concentrations of these drugs lowered the initial rate of tryptic protamine digestion by not more than 20% at pH 7·9 and even less at pH 7·4. Three basic anti-inflammatory drugs, chloroquine (20 mM), amidopyrine (20 mM) and hexadimethrine hydrobromide (Polybrene, 2 mg/ml), had no effect on tryptic digestion of protamine and globin.

Presumably concentrations of the acidic drugs lower than those given above would appreciably inhibit non-enteric tryptic enzymes which hydrolysed lysyl (rather than arginyl) linkages in proteins, if either their protein substrates were available only at concentrations giving an effective concentration of lysyl ϵ-amino-groups less than this figure (2·4 mM) or these non-enteric enzymes had lower affinities for their substrates than trypsin has for (myo)globin, or both. Rather high K_m values (70, 17 mM) have been reported for the hydrolysis of lysine ethyl or methyl esters by thrombin and plasmin respectively (Scheraga & others, 1958). We determined the K_m for tryptic hydrolysis of lysine methyl ester to be 0·2 mM at pH 7·4 or 7·9 and 20°, which suggests that trypsin may have a higher affinity for lysyl protein substrates than do non-enteric enzymes such as thrombin or plasmin. If so, these latter enzymes would compete less successfully with an acidic drug for the lysyl (amino-group) binding sites than does trypsin; the drugs should then be more effective in controlling hydrolysis of peptide linkages adjacent to lysine residues by non-enteric (pseudo) tryptic enzymes than they are in inhibiting protein digestion by trypsin itself.

To summarise our hypothesis: the lysyl ϵ-amino-groups of certain proteins appear to be important binding sites for acidic anti-inflammatory drugs, where they may then interfere with enzymic reactions dependent upon the availability of these ϵ-amino-groups, either for pyridoxal phosphate binding (for example, histamine formation), or directing the enzyme reaction (for example, tryptic-like proteolysis, or mitochondrial phosphorylation).

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Effect of non-steroid anti-inflammatory drugs on aldehyde binding to plasma albumen: a novel *in vitro* assay for potential anti-inflammatory activity

SIR,—Dempsey & Christensen (1962) showed that pyridoxal phosphate binds to the lysyl ϵ-amino-groups of plasma albumen and that certain aromatic acids, including salicylic acid, partly reversed this association. We have confirmed and extended their observations as follows. When bovine plasma albumen (Armour Pharmaceuticals Ltd., Eastbourne) was treated with 2,4,6-trinitrobenzene sulphonic (picryl sulphonic) acid (TNBS) in aqueous solution at pH 7·5, at least 50 amino-groups per albumen molecule (number average) were substituted by the trinitrophenyl (TNP) group. The product, "TNP-albumen", no longer bound salicylate anions or pyridoxal phosphate. A similar loss of binding capacity occurred when albumen was treated with 1-fluoro-2,4-dinitrobenzene (FDNB, Sanger's reagent) in dilute sodium bicarbonate solution at pH 8·5. Albumen which had been dialysed against a solution of sodium salicylate at pH 7·5 and had bound *several* molecules of salicylate per albumen molecule, lost all the bound salicylate on treatment with TNBS or FDNB. These findings establish the lysyl ϵ-amino-group as a common binding site for both the salicylate ion and pyridoxal phosphate. The single *N*-terminal aspartyl α-amino-group which also reacts with TNBS and FDNB, could only bind one molecule of drug or coenzyme. [TNBS and FDNB do not react with the guanidino-group of arginine, the only other basic residue in the albumen molecule].

Many acidic anti-inflammatory drugs inhibit mammalian substrate-specific histidine decarboxylases *in vitro* (Whitehouse & Skidmore, 1965); this is believed

to occur by displacing the pyridoxal phosphate coenzyme from the apoenzyme, to which it is probably bound through a lysyl ϵ-amino-group. Furthermore, chemical analogues of these drugs with little or no anti-inflammatory activity do not inhibit these enzymes. We have now found that these anti-inflammatory acids (histidine decarboxylase inhibitors) will displace pyridoxal phosphate from some of its binding sites on bovine plasma albumen (Table 1). The effectiveness of these drugs and their chemical analogues in displacing albumen-bound pyridoxal phosphate largely parallels their relative activity in inhibiting histamine formation *in vitro*.

TABLE 1. EFFECT OF ANTI-INFLAMMATORY ACIDS ON BINDING OF PYRIDOXAL PHOSPHATE AND 2,4,6-TRINITROBENZALDEHYDE TO BOVINE PLASMA ALBUMEN

Drug added	% inhibition with pyridoxal phosphate		% inhibition with trinitrobenzaldehyde	
	at 332 mμ	% shift (410 − 387 mμ	at 425 mμ	at 526 mμ
Sodium salicylate	80	72	31	31
Sodium *p*-hydroxybenzoate	40	55	22	20
Sodium gentisate	50	61	31	27
γ-Resorcylic acid	100	83	41	42
Sodium benzoate	60	55	0	3
Acetylsalicylic acid	80	61	9	9
Phenylbutazone	62	72	45	37
Oxyphenbutazone	100	50	50	44
Sulphinpyrazone	50	61	0	0
Phenazone	0	0	5	5
Amidopyrine	5	0	0	0
Flufenamic acid	100	70	74	73
Mefenamic acid	100	50	57	55
N-Methylanthranilic acid	100	50	35	30
Anthranilic acid	70	—	9	6
(5-Methyoxy)indomethacin (IM)	100	100	75	81
5-Fluoro analogue of IM	100	90	70	76
Indomethacinamide*	0	0	0	0
5-Methoxyindole-3-acetic acid	0	10	23	23
Cinchophen	100	61	54	55
Hydrocortisone*	0	0	8	12
Chloroquine phosphate	100	0	18	22

*Sat. soln., insoluble at 1 mM.

0·67% Albumen (0·1 mM), 0·1 mM aldehyde, 1·0 mM drugs all in 0·1 M sodium phosphate pH 7·5. Inhibition of pyridoxal phosphate (PXALP) binding given as percentage decrease in light absorption at 332 mμ (drug free controls as 0% and unbound PXALP as 100%) and also by the percentage shift of the absorption maximum of bound PXALP (at approximately 410 mμ) towards the absorption maximum of free PXALP (at 387 mμ). Inhibition of trinitrobenzaldehyde (TNBAL) binding given as the percentage decrease in light absorption at 425 mμ and 526 mμ due to bound TNBAL. All values were corrected for light absorption by plasma albumen. Where necessary, drugs were added in solution in *NN*-dimethylformamide (DMF) giving a final DMF concentration of 3% v/v. (also in controls).

These findings indicate the possible value of an *in vitro* assay for potential anti-inflammatory activity, based on spectrophotometric measurements of the extent of pyridoxal phosphate binding to plasma albumen in the presence of the drug. However, the combination of pyridoxal phosphate with plasma albumen involves the formation of several complexes with different absorption characteristics, some of which overlap the absorption bands of aromatic anti-inflammatory drugs. We therefore devised a simpler assay for determining the ability of a drug to bind to protein lysyl ϵ-amino-groups using 2,4,6-trinitrobenzaldehyde (Aldrich Chemical Co., Milwaukee), which rapidly forms a reddish complex with plasma albumen (but not with TNP-albumen or *N*-acetyl-albumen) exhibiting two absorption maxima in the regions 425-435 mμ and 525-535 mμ at pH 7·5. The formation of this complex is prevented by many drugs (see Table 1) and this drug effect is readily seen by eye.

These experiments indicate that there is a remarkable variation in the capacity of individual aromatic acids to displace these two aldehydes when they are bound to albumen, and, also, indomethacin is particularly effective and must have a high affinity for protein lysyl ε-amino-groups. Neutral and basic anti-inflammatory drugs such as amidopyrine, hydrocortisone and chloroquine, did not appreciably affect the binding of these aldehydes to albumen. We have the impression that drug antagonism of trinitrobenzaldehyde binding corresponds more closely with clinical antirheumatic activity (*vide* inactivity of benzoic and anthranilic acids) than does the effect of these acids upon pyridoxal phosphate binding to plasma albumen.

Mizushima (1964; 1965) reported that 1 mM antirheumatic (acidic) drugs and sodium dodecyl (lauryl) sulphate, stabilised a bovine plasma albumen fraction against heat coagulation. Dodecyl sulphate binds to at least 14 lysyl ε-amino groups per molecule of bovine plasma albumen (Markus, Love & Wissler, 1964). We found that both TNP- albumen and *N*-acetyl-albumen could not be protected from heat denaturation in this way and, furthermore, neither of these modified proteins would react with trinitrobenzaldehyde. We therefore believe that Mizushima's method of screening for potential anti-inflammatory drugs *in vitro* affords a measure of the protein-binding, or more specifically the lysine-complexing, ability of the compounds being tested. Measuring aldehyde binding in the presence of potential anti-inflammatory drugs affords another quantifiable index of potency in associating with protein (lysyl) ε-amino-groups, the importance of which is discussed in another communication (Whitehouse & Skidmore, 1965).

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8β-Carbobenzyloxyaminomethyl-1,6-dimethyl-10α-ergoline

SIR,—We wish to draw attention to an error in the chemical name of the compound we examined in our paper entitled: Antagonism of 5-hydroxytryptamine-induced bronchospasm in guinea-pigs by 8β-carbobenzyloxyaminomethyl-l-methyl-10α-ergoline (*J. Pharm. Pharmacol.*, 1965, **17**, 423–428).

We are informed that the stated compound should be 8β-carbobenzyloxyaminomethyl-1,6-dimethyl-10α-ergoline.

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The regulatory role of vitamin C on the adrenal function and resistance to histamine aerosol in the scorbutic guinea-pig

SIR,—Numerous controversial reports concerning the effect of vitamin C on the functional state of the adrenal cortex in the scorbutic guinea-pig have appeared. Bacchus & Heiffer (1953) reported a decrease in the urinary corticoid excretion; Done, Ely, Heiselt & Kelley (1953) found increased adrenocortical activity, while Nadel & Schneider (1952) reported a decrease in the early stage and an increase in the late stage of scurvy. The adrenal function and its possible effect on the resistance to histamine aerosol in the scorbutic guinea-pig is here reported.

Guinea-pigs, 375–425 g, were put separately in metabolic cages and 24 hr urine specimens collected in bottles containing 50% sulphuric acid (0·5 ml). After collecting 3 or 4 normal samples, the diet was replaced by a scorbutic diet and the daily urine specimens were collected for 21 days. The fluorimetric technique of Silber, Busch & Oslapas (1958) for the estimation of 11-hydroxycorticosteroids was applied using a sulphuric acid : ethanol mixture (75 : 25 v/v) for developing fluorescence. The fluorescence was measured after 15 min in an Aminco–Bowman spectrophotofluorimeter. The peak activation (470 mμ) and fluorescence (530 mμ) wavelengths of all urine samples coincided with those of the hydrocortisone standard. Recovery of hydrocortisone from the urine samples ranged from 94 to 105%. Twenty-four hr after bilateral adrenalectomy the residual non-specific fluorescence amounted to 2·5 to 3·6 μg/day. The amount of 11-hydroxycorticosteroids excreted was dependent on diuresis and therefore the daily water intake was regulated. The average urinary daily corticoid excretion was 98 μg. On the 3rd day of scurvy there was a significant but transient rise in corticoid excretion, possibly due to the rise in urine volume. On the 12th day the average daily excretion was 54 μg. Oral treatment of the scorbutic animals with 50 mg vitamin C daily for 5 days raised the lowered urinary corticoids to normal. If the guinea-pigs were left till the late stage of scurvy, there was a gradual increase in the corticoid excretion till on the 21st day it reached 2·5 times the normal level. Treatment with 50 mg of vitamin C orally for 7 days lowered the elevated corticoid excretion to normal.

The relation between adrenal function and resistance to histamine aerosol was striking. The drop in the resistance of the guinea-pig to histamine aerosol on the 12th day of scurvy and the marked rise on the 21st day (Guirgis, 1965) coincided with the stages of adrenal insufficiency and adrenal hyperfunction respectively. Vitamin C treatment at both stages restored to normal not only the impaired adrenal function but also the resistance to histamine aerosol. The disturbance in histamine metabolism in the scorbutic guinea-pig as reported by Dawson & West (1965) may also have some effect on the resistance of the animal to histamine aerosol.

The mechanism of the anti-anaphylactic effect of vitamin C in the normal guinea-pig is still uncertain. Vitamin C injected in a dose of 100 mg, 20 min before challenge did not protect the guinea-pig from histamine aerosol. Such treatment, however, increased 2·9 times the mean preconvulsion time of sensitized guinea-pigs subjected to antigen aerosol. In this connection Goadby & Smith (1964) reported an increase of 2·1 times with hydrocortisone hemisuccinate.

It is concluded that the functional state of the adrenal cortex in the scorbutic guinea-pig determines the resistance of the animal to histamine aerosol. The

effect of vitamin C on anaphylaxis in the normal guinea-pig is worthy of further investigation.

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The pharmacology of amygdaloid neurones

SIR,—The amygdala contains several potential neurotransmitter substances together with their enzymes of synthesis, for example, 5-hydroxytryptamine, noradrenaline (Vogt, 1954; Kuntzmann, Shore, Bogdanski & Brodie, 1961) and acetylcholine (Hebb & Silver, 1956). However, the direct response of single cells in the amygdala to these substances is unknown. This letter describes the response of amygdaloid neurones to various biogenic substances introduced into their environment by microelectrophoresis.

Four cats anaesthetized with chloralose or diallylbarbituric acid and urethane were used. The skull was opened on one side and the structures overlying the amygdala aspirated. The exposed area of brain was then covered with 3% agar in Ringer's solution to prevent drying. Using the stereotaxic co-ordinates of Jasper & Ajmone-Marsan (1960) the surface of the agar was now marked in several places overlying different areas of the amygdala. Five barrelled glass micropipettes (tip diameter 4 to 8μ) were now inserted through the marks in the agar to the required depths in the amygdala. At the end of the experiments the position of the micropipette tracks was checked histologically in celloidin sections. Eleven out of the fourteen tracks were in the amygdala.

The technique for preparing and using the micropipettes for microelectrophoresis was essentially that described by Krnjević & Phillis (1963). The four outer barrels contained aqueous solutions of the various drugs to be tested, whose pH was adjusted to give maximal ionisation compatible with stability. Drug ions were expelled from the tip of the pipette by appropriate currents. Extracellular spike responses from single cells were recorded simultaneously through the saline-filled central barrel of the pipette. After amplification these spikes were displayed on an oscilloscope and counted on a ratemeter. The ratemeter output was then displayed on a penwriter.

One hundred and thirteen cells were studied; two thirds of these were in the lateral or basomedial complex of the amygdaloid nucleus, the rest in the amygdaloid area. Some cells were firing spontaneously, or could be evoked synaptically through stimulation of the olfactory bulb. Otherwise quiescent cells were

excited by L-glutamate discharged into their vicinity by microelectrophoresis. L-Glutamate excited and γ-aminobutyric acid depressed cell firing in all the cells tested, as shown in Fig. 1. These effects had a rapid time course and were essentially similar to those seen elsewhere in the central nervous system (e.g. Curtis, 1965).

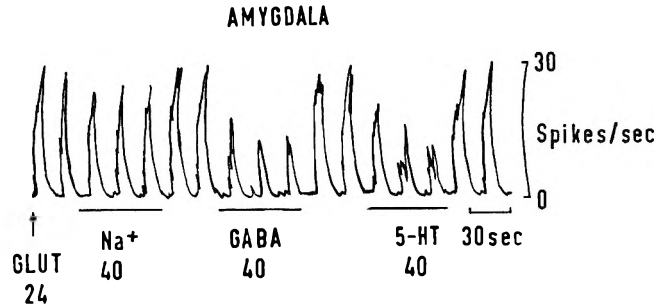

FIG. 1. Ratemeter record of firing of a cell in the lateral amygdaloid nucleus of the cat. This cell was quiescent but could be fired by the regular application of L-glutamate 24nA for 5 sec in every 15 sec (only the first application is indicated at ↑). A background current of 40nA through the saline control barrel (Na^+ 40) indicated by bar below record, had only a slight depressant effect on glutamate-induced excitation. The application of γ-aminobutyric acid with a current of 40nA (GABA 40 and bar below record) had a strong depressant effect which developed and reversed quickly.

The application of 5-hydroxytryptamine with a current of 40nA (5-HT 40 and bar below record) also depressed glutamate-induced excitation, and this depression was progressive.

N.B. When this unit was evoked synaptically by stimulation of the olfactory bulb, the depressant action and time course of γ-aminobutyric acid and 5-hydroxytryptamine was almost identical to that shown in this figure.

The scale to the extreme right of the record shows the number of extracellular spike potentials counted per second by the ratemeter. Time calibration 30 sec.

5-Hydroxytryptamine depressed 32 out of the 42 cells tested. This effect is shown in Fig. 1 and it can be seen that the onset of depression was typically slower than that seen with γ-aminobutyric acid. A similar depression was produced by noradrenaline and dopamine. The depression produced by 5-hydroxytryptamine would appear to be on the post-synaptic membrane since cells were similarly affected whether activated synaptically or with L-glutamate. Excitation by 5-hydroxy-tryptamine was not seen.

The proportion of acetylcholine-sensitive cells was low; thus only 8 cells were excited out of the 48 cells tested. These amygdaloid neurones were also directly excited by acetyl-β-methylcholine and carbachol as shown in Fig. 2. The time course of excitation by these three choline esters was generally slower than shown in this figure, so that recovery might take several minutes after stopping the expelling current.

In general the cells in the amygdala respond to the various potential neurotransmitter substances used, in much the same way as the cells in the pyriform cortex (Legge, Randić & Straughan, 1965). It is of interest that 5-hydroxytryptamine and acetylcholine-sensitive cells appear to be distributed throughout the amygdala and are not concentrated in the basomedial complex. For the concentration of 5-hydroxytryptamine-containing nerve terminals and acetylcholinesterase-containing fibres in the basomedial complex (Carlsson, Falck & Hillarp, 1962; Krnjević & Silver, 1965) would suggest that the 5-hydroxy-

tryptaminergic and cholinergic innervations (if they exist) are confined to that area. Current experiments are concerned with the effect of specific pharmacological antagonists on the synaptic activation of amygdaloid cells. It is hoped that these studies will throw some light on the function of 5-hydroxytryptamine, noradrenaline and acetylcholine in this region.

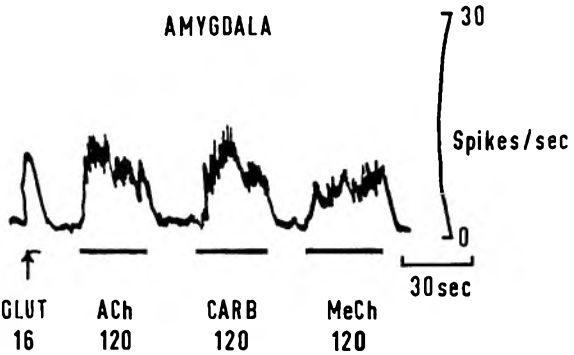

FIG. 2. Ratemeter record from cell in the basomedial complex of the amygdala. On the left of the record L-glutamate applied for 5 sec with a current of 16nA (GLUT 16 and ↑ below the record) rapidly excited the cell. Acetylcholine was then applied with a current of 120nA (ACh and bar below record) and this also excited the cell. Carbachol and acetyl-β-methylcholine caused similar excitation. Note that the onset of excitation with L-glutamate was immediate, while there was a characteristic delay before the choline esters induced firing.

The scale to extreme right of the record shows the number of extra-cellular spike potentials counted per second by the ratemeter. Time calibration 30 sec.

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Pharmacopoeias and Formularies

THE PHARMACOPEIA OF THE UNITED STATES OF AMERICA. SEVENTEENTH REVISION. Pp. lxvi + 1156. Distributed by Mack Publishing Company, Easton, Pa, U.S.A. $12.50, U.S.A.; $13.00 elsewhere.

The Seventeenth Revision of the Pharmacopeia of the United States of America, official from September 1, 1965, reflects in many respects the concern that has arisen during the past five years about the efficacy of drugs and the unavoidable risks of side-effects. This concern has influenced the selection of drugs to be included and the determination of what constitutes an adequate standard for the purposes of a pharmacopoeial monograph. The new revision contains 898 monographs and 85 chapters of general tests. Of the 156 new monographs, 76 are for basic drugs. Monographs omitted total 201.

The selection of drugs and preparations for inclusion and the deciding of acceptable standards for them raises the question—what is the purpose of a modern pharmacopoeia? The objects of the United States Pharmacopeia as determined by the Convention of March, 1960, are stated as "to provide authoritative standards for substances and their preparations that are used in the practice of the healing arts; the establishment of titles, definitions, descriptions, and standards for identity, quality, strength and purity, and also, where practical, methods for their examination and formulas for manufacturing".

Perusal of this revision clearly reveals that these declared objects have been only partially fulfilled. This sad fact inevitably leads one to ask whether such worthy objects can now be fulfilled in a book revised at five yearly intervals, even when supplements are issued between revisions.

The difficulty does not arise merely from the introduction of new drugs and preparations, from the development of new knowledge and new analytical techniques, or from the problems of selection and of drafting standards for many preparations. It arises from the need to provide meaningful standards desirably to be met by manufacturers issuing a particular drug or preparation and standards to which the drug or preparation must conform in order to protect the consumer against its deterioration, or the presence of harmful or potentially harmful impurities in pharmacologically significant amounts. These standards can rarely be precisely the same and it is the purpose of a pharmacopoeia to define the minimum standard that can be allowed for protection of the consumer both as regards potency and freedom from harmful impurities. But the setting of such minimum acceptable standards leaves undefined the standards to which manufacturers must work to afford that protection. There is a "twilight zone" with which a pharmacopoeia cannot cope in its traditional form. This is not of therapeutic significance for most drugs and preparations but it is so for many potent drugs. Among these are the antibiotics, quality control of which can only partially be effected by a written specification. In the United Kingdom this difficulty is overcome, so far as materials for parenteral injection are concerned, by the licensing procedure and regulations made under the Therapeutic Substances Act. But the Act does not overcome the problem of quality control for antibiotics destined for use by any other route. The dilemma has been met in the U.S.A. by the operation of amendments to the Food, Drug and Cosmetic Acts. Under them, all antibiotics intended for use in man are subject to production and testing controls under federal regulation, including batch certification before distribution. The U.S.P. monographs for antibiotics therefore merely refer to this fact and include only those aspects of identity, purity, potency and

packaging and storage that are of special interest to the physician and pharmacist. References to identity, purity and potency of antibiotics have in consequence become simplified and are no longer definitive as standards.

Will the procedure adopted for antibiotics be adopted in future for steroids, for psychotropic drugs, and for other potentially dangerous substances? If so, what remains the purpose of a pharmacopoeia? Surely not merely to provide information on available dosage forms, on 'categories of action' or on general methods of testing? Clearly a pharmacopoeia is still needed to ensure uniformity of content of active ingredient in preparations and to recognise and limit potentially harmful impurities whether these arise from manufacturing procedures or from changes subsequent to release.

An important new inclusion in the U.S.P. is a requirement for the uniformity of content of medicament in each tablet of a representative sample. This requirement is introduced for tablets containing a small amount of medicament, as, for example, digoxin or prednisolone. Each of 10 tablets from a sample of 30 is required to be assayed and the requirement for content uniformity is met if the results for each fall within 85 to 115% of the average of the tolerances specified in the monograph. If one of the 10, but not more than one, falls outside those limits, the remaining 20 tablets must be assayed individually and the requirements are met if not more than one of all the 30 tablets lies outside the limits of 85 to 115% of the declared content.

This edition of the U.S.P., like that of any modern pharmacopoeia, reveals the lack of molecularly specific methods for determining many active ingredients and a limited knowledge of the impurities that arise in the manufacture and storage of many drugs. It reflects the extent to which every modern pharmacopoeia is dependant upon knowledge possessed by the manufacturer of a particular drug for the compilation of specifications that will satisfactorily protect the consumer from the hazards of lack of uniformity and harmful impurities. Thus it is the case for monographs such as that on prednisone, in which the content of required steroid is assessed by comparing the amount of material with a reducing functional group with that of a standard preparation, and the drug's content of related foreign steroids is compared with that of a reference preparation (itself not wholly pure material) on a chromatogram simultaneously with cortisone and hydrocortisone. Infrared and ultraviolet spectra as well as optical rotation are used to confirm the identity of the material but much additional data is required to limit the content of the many other related steroids resulting from the preparation of such drugs. All too little is known of the specific toxic effects that arise clinically from such undetected impurities. But a pharmacopoeia cannot set standards beyond those based on the information made available to it.

It is interesting to note that the steroids selected for inclusion in the U.S.P. XVII do not include dexamethasone, betamethasone or fludrocortisone and that estradiol benzoate, ethisterone, methyltestosterone, progesterone, among other included in the U.S.P. XVI, have now been omitted. The omission of ferrous gluconate, globin zinc insulin injection, nicotinic acid, novobiocin and chlorothiazide may come as a surprise for British readers, but such is the changing choice of medicaments by physicians in different countries.

The task of revision of the U.S.P. as of any pharmacopoeia becomes ever more formidable. It is the dedicated efforts of a vast team of workers and collaborators that have alone made possible even the partial fulfillment of their objectives. There is much to admire and to learn from the outcome.

FRANK HARTLEY.

Book Review

MANUAL FOR THE IDENTIFICATION OF MEDICAL BACTERIA. By S. T. Cowan and K. J. Steel. Pp. x + 217 (including Index). Cambridge University Press, London, 1965. 50s.

This very useful and relatively inexpensive book may be described as a working guide to identification which all microbiologists will welcome. The book is a development of an earlier publication of the authors—*Diagnostic Tables for the Common Medical Bacteria*—originally published in the *Journal of Hygiene.*

There is a common sense approach to the various problems of identification and a refreshing lack of dogmatism in dealing with subtle shades of bacterial variation. The authors set out clearly what they hope to accomplish and give useful references to standard works on those aspects they choose to neglect.

The main substance of the book is contained in the Tables, or rather Table-figures since they combine both functions. The authors have compiled two groups of tables, to be used according to whether the organism is Gram-positive or Gram-negative. If the organism is Gram-negative then a first-stage diagnostic table is used which distinguishes genera on the basis of shape, motility, growth in air, catalase reaction, oxidase reaction, carbohydrate breakdown, and the Oxidation-Fermentation (O–F) test. Gram-positive genera are distinguished by similar tests with the additions of spore production and acid-fastness.

After this preliminary diagnosis, more precise identification is achieved by the use of second stage, and in some cases, third stage tables. The second stage tables contain many more tests which are mainly biochemical. For example the table for *Pseudomonas*, *Chromobacterium*, *Flavobacterium* and *Acinetobacter* species contains 18 tests.

These tables are preceded by a useful, critical section on the tests used and should serve to dispel at least some illusions. It is surprising to find no mention of the usefulness of phase contrast microscopy in establishing the presence of spores. This method is rapid but depends, of course, on the possession of phase contrast facilities. It is interesting to note the means of identification explicitly not used. Early in the book the reader is informed that he will not find diagrams of the different shapes, edges, surfaces, and elevations of colonies, and of the shapes of liquefaction seen in gelatin stab cultures. Such characteristics are indeed referred to as "relics of nineteenth-century bacteriology".

The section on Theory and Practise of Bacterial Identification is crammed with information of particular relevance to the medical bacteriologist and the usefulness of multitest media and microtests is discussed.

The treatment of sterilization of media does not quite match the standard of the rest of the book. The relationship between volume of medium and time to achieve a given temperature in an autoclave could be given quantitatively. It seems inadequate merely to suggest increasing the time of autoclaving when the volume of medium exceeds 1 litre. Reproducibility of heat treatment would seem to be necessary for reproducibility of medium. The usefulness of membrane filters might well have received more attention than the 3 lines allotted in the section on sterilization by filtration. The sections on culture media, staining, biochemical methods and particularly micromethods are most useful.

The style of writing makes for easy reading as does the excellent general layout of text and tables. The index is comprehensive and there are about 700 references to original work. This book is indeed a fitting memorial to Dr. Steel whose death in 1964 was a loss not only to Bacteriology but also to Pharmacy where his career began.

M. R. W. Brown.

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Review Article

Research Papers

Letters to the Editor

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