Journal of Pharmacy and Pharmacology

Published by
The Pharmaceutical Society
of Great Britain

Volume 19 No. 9
September 1967

Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN

17 Bloomsbury Square, London, W.C.1. Telephone: 01-405 8967

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J. Pharm. Pharmac., 1967, **19**, 561–589

Review Article

The possible role of conformational isomerism in the biological actions of acetylcholine

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SITES OF ACTION OF ACETYLCHOLINE

SINCE acetylcholine is involved as chemical mediator at synapses between neuron and neuron, between neuron and muscle cell, and between neuron and secretory cell, apparently generating essentially similar bioelectric potentials in all three types of post-synaptic cell (Nachmansohn, 1959), it plays a fundamental role in a number of distinct physiological situations. Thus acetylcholine is involved in the transmission of nerve impulses at both the sympathetic and parasympathetic ganglionic synapses (Kibjakow, 1933; Feldberg & Gaddum, 1934; Feldberg & Vartianen, 1935; Grundfest, 1957), at the synapse between motor nerve and voluntary muscle (Dale, Feldberg & Vogt, 1936; Brown, Dale & Feldberg, 1936; Brown, 1937), at the synapses between autonomic nerves and certain exocrine glands (Dale & Feldberg, 1934; Hurley, Shelley & Koelle, 1953; Goodman & Gilman, 1955a) and at the synapse between smooth muscle and those autonomic nerves which, in consequence, have been termed cholinergic (Loewi, 1921; Chang & Gaddum, 1933; Dale & Feldberg, 1934; Gaddum, 1936) and which correspond to the parasympathetic nervous system. Acetylcholine has long been known to be involved in the release of adrenaline and noradrenaline from the modified ganglion cells constituting the adrenal medulla (Feldberg & Minz, 1933; Feldberg, Minz & Tsudzimura, 1934) and more recently it has been shown to be implicated also in autonomic adrenergic transmission (Burn, 1961; Burn & Froede, 1963; Burn, Rand & Wien, 1963). It may also play a role at the termination of sensory nerves (Brown & Gray, 1948; Douglas & Gray, 1953; Davis, 1961; Koelle, 1961, 1962), while new emphasis has recently been given (Nachmansohn, 1959, 1961, 1962) to older views (Nachmansohn, 1946; Lorente de Nó, 1949; Hodgkin, 1951; Toman, 1952; Eccles, 1953; Tasaki, 1953) that it may participate in the conduction of nerve impulses along axons, although the conclusions concerning its involvement in this situation have been contested (Ritchie & Armett, 1963; Triggle, 1965). The occurrence of acetylcholine within the central nervous system suggests even further physiological significance for this compound but its exact functions in this location are still inconclusively established (e.g. Crossland, 1960), although it is known to be concerned in the release of antidiuretic hormone from the neurohypophysis (Pickford, 1945, 1947; Verney, 1947; Duke, Pickford & Watt, 1950; Harris, 1951; Jewell, 1953) and to have a transmitter role at the Renshaw cells in the spinal cord (Eccles, Eccles & Fatt, 1956). Indeed cortical synapses have been postulated to possess an acetylcholine receptor similar to that of the neuromuscular junction (Feher, Klitina & Molnar,

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1965). Further speculations about the biological significance of acetylcholine are to be found in the various comprehensive reviews concerned with drugs which exert their effects by mimicking or antagonizing the neurohormone (*inter alia* Goodman & Gilman, 1955b; Del Castillo & Katz, 1956; Bovet, Bovet-Nitti & Marini-Bettolo, 1959; Nachmansohn, 1959; Cavallito & Gray, 1960; Crossland, 1960; Waser, 1960, 1961a; Bowman, 1962; Koelle, 1962; Stenlake, 1963; Barlow, 1964; Triggle, 1965).

SELECTIVITY OF ACTION OF MIMETICS AND ANTAGONISTS OF ACETYLCHOLINE

Despite the diversity of sites of action of acetylcholine, with some exceptions, at normal dose levels, drugs which mimic or antagonize the actions of acetylcholine, do so at a limited number of sites only. This has given rise to the established pharmacological classification into nicotinic or muscarinic agents for mimetics and neuromuscular blocking agents, ganglion blocking agents or antispasmodic drugs for antagonists. In part, this relative selectivity to site of action may have its origin in transport and permeability factors, the absence of lipid barriers at the neuromuscular synapse (Couteaux, 1947) adequately explaining the ready access to this site of polyonium salts which are normally unable to penetrate such barriers (Brodie & Hogben, 1957; Walsh & Deal, 1959; Rosenberg & Ehrenpreis, 1961a,b; Rosenberg & Podleski, 1962). Certainly the central actions of neuromuscular blocking agents on intrathecal, intracisternal or intraventricular injection (Wislicki, 1958) help to demonstrate the role of the blood brain barrier where these compounds are administered by other routes, while studies with nerve fibre preparations lacking protective lipids show these agents to be capable of exhibiting activity at yet other sites not affected in the intact animal (Rosenberg & Ehrenpreis, 1961a,b; Dettbarn, 1960).

Consideration of permeability factors alone, however, fails to provide a full explanation as to why purely muscarine-like and atropine-like drugs show little or no action at nicotinic sites, since absence of a permeability barrier at the neuromuscular junction can hardly be invoked to explain absence of activity on grounds of a denial of access to these drugs. Similarly, it is difficult to see, from permeability considerations, why local anaesthetics which appear to occupy certain acetylcholine receptors (Goodman & Gilman, 1955c) exhibit no activity at normal muscarinic or nicotinic receptors. Moreover, the recent demonstrations (Takeshige & Volle, 1963; Jones, 1963) that ganglia appear to contain both nicotinic and muscarinic receptors would also seem to strengthen the view that factors other than permeability effects are involved.

POSSIBLE ROLE OF CONFORMATIONAL ISOMERISM IN THE PHYSIOLOGICAL ACTIONS OF ACETYLCHOLINE

That the conformational flexibility of the acetylcholine molecule could make possible the existence of several distinct types of acetylcholine receptor, which would provide a fundamental basis for the observed selectivities of different groups of acetylcholine mimetics and acetylcholine

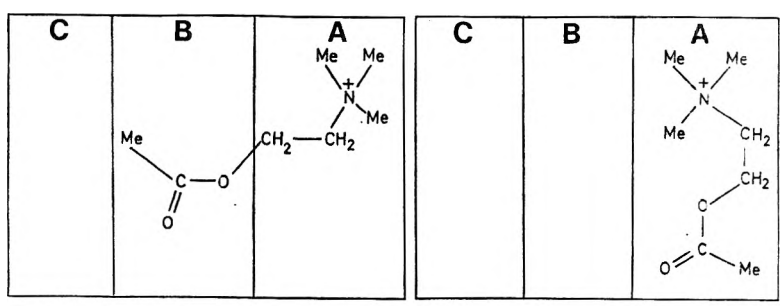
antagonists, has been implied by several workers (Schueler, 1956; Kennard, 1960; Archer, Lands & Lewis, 1962). Indeed, such a situation would not be inconsistent with the somewhat different pictures of the muscarinic and nicotinic receptors emerging from other considerations (*inter alia* Waser, 1961a, 1962; Cavallito, 1962; Triggle & Belleau, 1962; Beckett, Harper & Clitherow, 1963; Belleau & Puranen, 1963; Bebbington & Brimblecombe, 1965), especially since nicotinic activity, in marked contrast to muscarinic activity (Hardeggar & Lohse, 1957; Gyermek & Unna, 1958, 1960; Beckett, Harper & others, 1961; Belleau & Puranen, 1963), does not appear to be greatly influenced by changes in stereochemistry. This is illustrated by the high nicotinic potency present in both enantiomorphs of compounds such as muscarone (Waser, 1961a), lactoylcholine (Rama-Sastry & Auditore, 1963) and nicotine or nornicotine (Hicks, Brücke & Heuber, 1935; Hicks, MacKay & Sinclair, 1947), and by the retention of nicotinic activity in acylcholines where the acid radical has considerable steric bulk (Bergel, 1951; Akcasu, Sinha & West, 1952). Again the conformational flexibility of the acetylcholine molecule could rationalize any differences between the muscarinic and nicotinic receptors on the one hand and the acetylcholinesterase surface (Friess & Baldridge, 1956; Nachmansohn & Wilson, 1959; Krupka & Laidler, 1961; Thomas, 1961; Belleau & Lacasse, 1963; Turpajev, Nistratova & Putintseva, 1963; Wilson, 1963) on the other, although the direct nicotinic action of such typical anticholinesterase drugs as neostigmine and edrophonium (Lewis, 1962) must be taken into account. The hypothesis advanced by Roepke (1937) of the identity of acetylcholinesterase and the cholinergic receptor has since met with both support and opposition and the pertinent evidence has recently been assessed (Holmstedt, 1963; Werner & Kuperman, 1963; Belleau, 1964).

Unfortunately detailed consideration of the molecular features of the many drugs which act as mimetics or antagonists of acetylcholine sheds little light on the possible biological importance of conformational isomerism in the neurohormone for three main reasons.

(i) *Lack of suitable rigid molecules.* Most drug molecules known to mimic or antagonize the biological actions of acetylcholine are themselves conformationally flexible and there is no way of establishing for a given case which one of several possibilities is in fact operative. In some instances a drug may be capable of precise mimicry of only a single biologically-significant conformer of acetylcholine on account of unfavourable bonded or non-bonded interactions within the drug molecule. In other instances, although itself capable of mimicking more than one biologically-significant conformer of acetylcholine, a drug molecule may not be able to attach itself to certain acetylcholine receptors due to interaction between these receptors and molecular units present in the drug molecule but absent in acetylcholine. Again a wide spectrum of ease of conformational interconversion can be expected for different drugs which could underlie observed differences in relative selectivity with respect to site of action.

(ii) *Difficulty in assessing the relative significance of conformational*

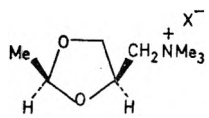
phenomena in mimetics and antagonists of acetylcholine. Even if certain conformational deductions were to prove feasible for drugs interfering with the normal course of events at acetylcholine receptors, the absence of any *a priori* distinction between the molecular features leading to drug receptor interaction (affinity) (Ariëns & Simonis, 1964) and those determining elicitation of a positive biological response (intrinsic activity) makes it difficult to assess the relative significance of conformational phenomena in mimetics and antagonists of acetylcholine. However, in the case of antagonists, it would appear that shielding of the receptor, rather than an exact fit with it, could be the significant factor. Thus many antispasmodic drugs possess somewhat bulky molecules, while increase in the size of the cationic substituents of depolarizing neuromuscular blocking agents (which would be expected to sterically hinder approach to the receptor) is accompanied by a change to non-depolarizing activity. Recently, the hypothesis has been advanced (Belleau & Lacasse, 1964; Belleau, 1964) that affinity may be ascribed to hydrophobic forces serving to transfer a drug from the aqueous phase to non-polar sites on the receptor protein whilst intrinsic activity can be related to the particular conformational perturbation in the tertiary structure of the receptor protein induced through interaction with the drug molecule. Agonistic activity is then correlated with the induction of a unique specific conformational perturbation and antagonistic activity with the induction of any one of a number of non-specific conformational perturbations incapable of inducing biological response. The phenomenon of partial agonism is rationalized by assuming that equilibrium mixtures of receptor protein transformed by the specific and non-specific conformational perturbations are produced by the drug. An extension of this hypothesis has led to the view that the muscarinic receptor is constituted by three non-polar



Nicotinic Receptor		Muscarinic Receptor	
Compartment	*Nature*	*Compartment*	*Nature*
A	Non-polar	A	Non-polar
B	Polar	B	Non-polar
C	Non-polar	C	Non-polar

FIG. 1. Schematic representation of compartmentalization of receptor surfaces (adapted from Belleau, 1965).

compartments whereas the nicotinic receptor is envisaged to be composed of a polar or semi-polar compartment adjacent to two non-polar compartments (Fig. 1), and further that the cholinergic receptor may be an enzyme whose role is to catalyse phosphoryl group transfer (Belleau, 1965). It is to be noted, however, that in the 1,3-dioxolane analogues (e.g. *1*) of acetylcholine, upon which certain of these deductions (Belleau & Lacasse, 1964) concerning the conformation adopted by acetylcholine at the muscarinic receptor and during complex formation with acetylcholinesterase are based, only the equivalent of the $MeCOOCH_2$-fragment of the acetylcholine molecule is locked in a rigid conformation. Free rotation about the remaining bonds in the 1,3-dioxolanes and in acetylcholine itself still permits conformational heterogeneity (*vide infra*).



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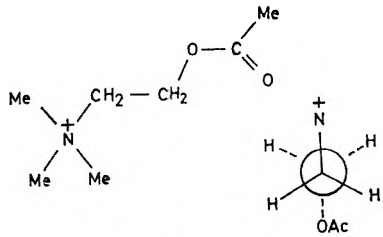
(iii) *Difficulties in determining the focal point of conformational isomerism.* The activity displayed by a compound such as the tetramethylammonium cation, which formally represents a portion of the acetylcholine molecule divorced from conformational significance with respect to the acetoxyethyl moiety, indicates that conformational isomerism within this latter radical cannot exert a crucial influence at all acetylcholine receptors. This raises serious doubts about its exact significance at other centres although the stereochemical requirements of the anionic site in the receptor appear to be reasonably specific (Kellet & Hite, 1965).

In view of these factors, therefore, it would seem necessary to look elsewhere for evidence bearing on the possible role of conformational isomerism in the biological actions of acetylcholine.

THE CONFORMATION OF ACETYLCHOLINE

X-ray studies on crystalline acetylcholine bromide (Sörum, 1959) were originally interpreted to indicate that in the solid state the molecules of acetylcholine coexisted in two separate favoured conformations—the fully staggered conformation (*2*) and the quasi-ring conformation (*3*). The validity of this interpretation was later criticized (Dunitz, 1963) and a reinvestigation of the crystal structure of acetylcholine bromide (Canepa, Pauling & Sörum, 1966) has shown the sole presence of a quasi-ring form (*4*) in which the interatomic angles are such that the methyl group (C–1) seems to form a bent hydrogen bond through one of its hydrogen atoms to the acyloxy oxygen atom (O–1). The structure of acetylcholine in the crystal lattice is thus very similar to those of choline chloride (*5*) (Senko & Templeton, 1960) and L(+)-muscarine iodide (*6*) (Jellinek, 1957). In the latter the stability of the quasi-ring conformation has been attributed to C–H - - - - O bonding (Sutor, 1963).



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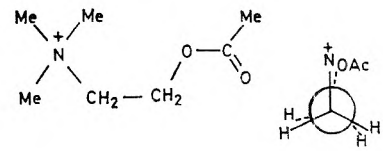

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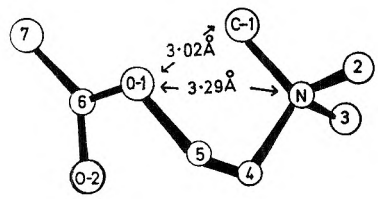

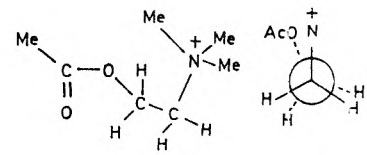

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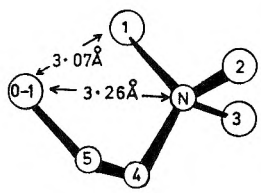

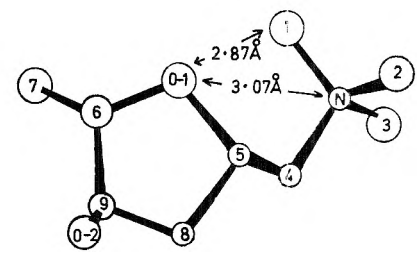

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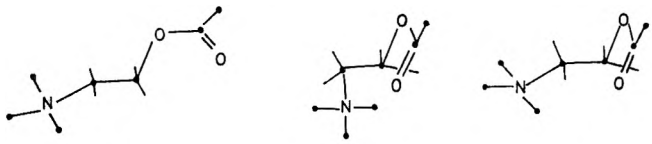
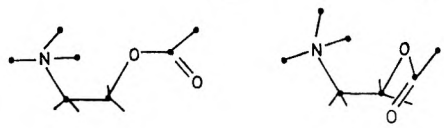
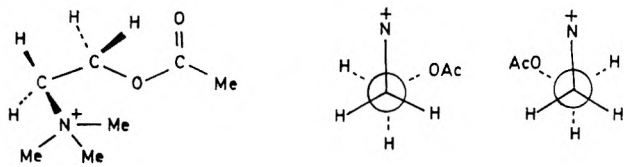
Although the existence of the extended conformation (*2*) of acetylcholine bromide in the solid state has been disproved, this by no means excludes the existence of a fully staggered conformation in other situations. Thus, in the extended conformation (*2*), non-bonded interactions are minimal and at ordinary temperatures it might be expected to be the favoured conformation of the molecule. Conformational isomerism in acetylcholine is dependent upon free rotations about the C–C and C–O bonds of the choline fragment and of the infinite number of conformers possible, nine separate well-defined conformations for acetylcholine must be considered. These are the fully staggered conformation (*2*) and four pairs of identical skewed forms (*2a, b, c, d*). However, the cyclic conformation *2a* may be regarded as being extremely unfavoured from a consideration of steric requirements and interaction energies (Gill, 1965). On the basis of intrachain stereochemical interactions, duly corrected for the dipole interaction between the positive charge of the quaternary nitrogen atom and the ester function, the relative probabilities of the



2 2a 2b



2c (≡ 4) 2d



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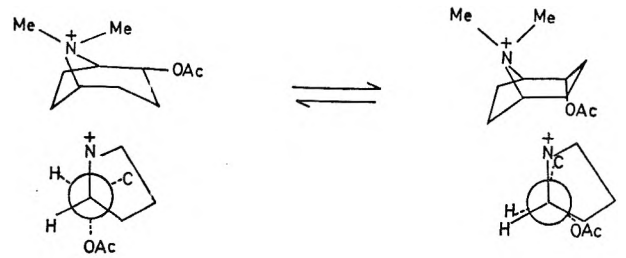
four forms *2*, *2b*, *2c* and *2d* of acetylcholine in solution have been calculated as 1 : 0·55 : 0·08 : 0·02 (Gill, 1965). Despite these considerations however, acetylcholine has been concluded to exist in aqueous solution as a quasi-ring form akin to *3* or *4* (Canepa, 1965). Indeed a nuclear magnetic resonance study of acetylcholine in deuterium oxide has been interpreted as providing evidence that the mean conformation of acetylcholine in aqueous solution is best represented as in *7* (Culvenor & Ham, 1966). This conformation is similar to that pertaining in the crystal lattice (Canepa & others, 1966) in that the $\overset{+}{N}$–C–C–O system is in a *gauche* arrangement but differs (compare *4*) in that the CH_2–O–CO–Me grouping has the normal conformer populations of a primary ester. This does not preclude the possibility that both the extended form and the quasi-ring form of acetylcholine may separately be of biological significance, as has been conjectured by other workers (Schueler, 1956; Kennard, 1960; Archer & others, 1962; Smissman, Nelson & others, 1966), since the receptor may not necessarily complex with the energetically most favoured form of a given molecule (Gill, 1965). Furthermore, if the receptor surfaces are in compartments as envisaged by Belleau (1965) and if the muscarinic and nicotinic receptors differ solely in the polarity of the central compartment (B in Fig. 1) then the proposed polar nature of this compartment in the nicotinic receptor may well accommodate the polar acyloxy function of the extended conformation (*2*) of acetylcholine. It may be significant that effective nicotinic drugs frequently include a polar group at three to four interatomic distances away from the quaternary

nitrogen atom (Rossum, 1962). On the other hand, the central compartment of the muscarinic receptor is considered to be non-polar and would thus be unable to accommodate a polar function. Therefore, the quasi-ring form of acetylcholine (e.g. *4*) could well be involved at the muscarinic receptor.

THE POSSIBLE PHYSIOLOGICAL INVOLVEMENT OF ACETYLCHOLINE CONFORMERS

Archer & others (1962) have advanced the hypothesis that nicotinic activity depends on conformation *4* of acetylcholine and muscarinic activity on conformation *2* since the L- and (±)-2α-acetoxytropane methiodides (*8*) possessed comparable weak muscarinic activity (absent in the L-2β-acetoxytropane methodide) while L-2β-acetoxytropane



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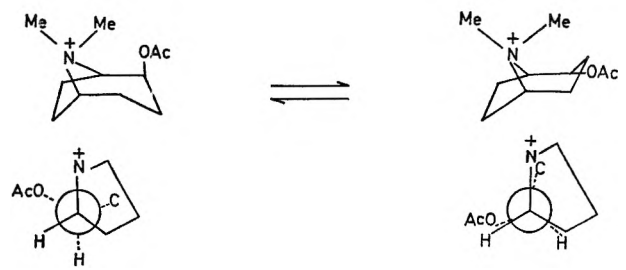

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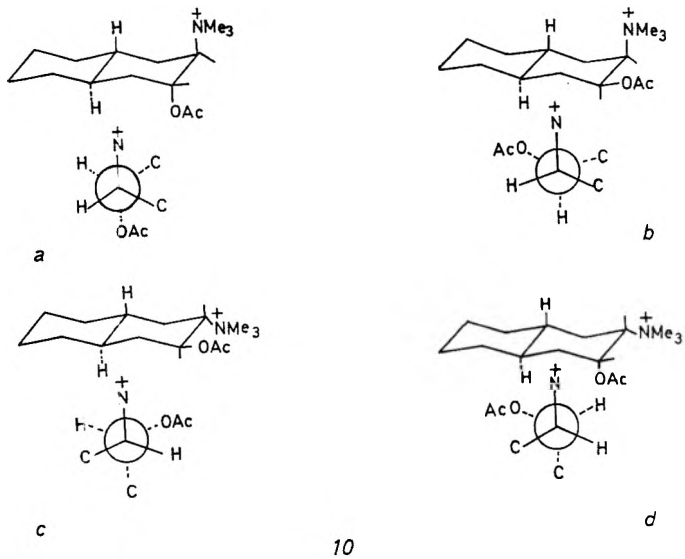
L-Forms depicted

methiodide (*9*) possessed stronger nicotinic activity than the L- and (±)-2α-compounds. Smissman & others (1966) also consider from a study of the isomeric 3-trimethylammonium-2-acetoxy-*trans*-decalin halides (*10a-d*) and the isomeric α,β-dimethylacetylcholine halides (*11a* and *b*) that muscarinic activity depends on conformation *2* of the acetylcholine molecule, but the activities of the compounds were very weak. Unfortunately only the racemic forms of the various isomers were tested and nicotinic activities were not recorded. These hypotheses would also



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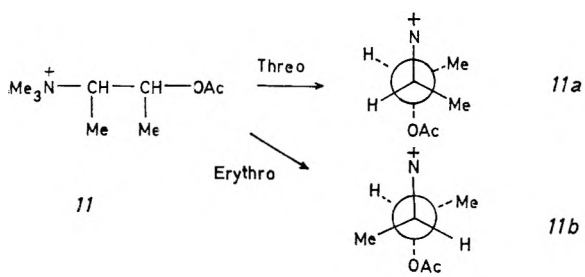
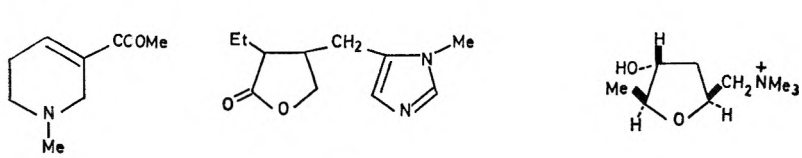
(One enantiomorph only of each compound is shown)

accommodate the pronounced muscarinic properties of arecoline (*12*) which can assume a conformation in which the acyloxy oxygen atom and the tertiary nitrogen atom are spatially disposed in a manner somewhat similar to the corresponding functional groups in conformation *2* of acetylcholine. Arecoline, however, also exhibits significant nicotine-like actions (Goodman & Gilman, 1955). It is to be noted that the muscarinic properties of (+)-pilocarpine (*13*) are difficult to accommodate on the



11 11a 11b



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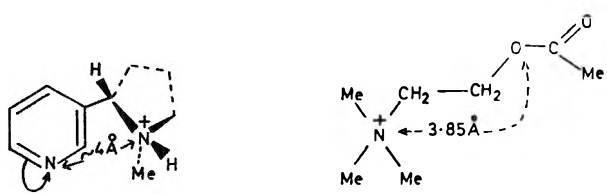
basis of the above ideas. It is of course possible that some, if not all, of these discrepancies arise from the compound concerned inducing acetylcholine release and not exerting a direct action in its own right.

However, the fact that L(+)-muscarine (*14*) in the solid state exists in a quasi-ring conformation (Jellinek, 1957) which corresponds closely with the quasi-ring conformer (*4*) of acetylcholine (Canepa & others, 1966) might suggest that this conformation (*4*) rather than the fully-staggered conformation (*2*) of acetylcholine is more likely to be involved at the muscarinic sites. In addition, should the molecules of both acetylcholine and nicotine have a two point attachment at the nicotinic receptor,



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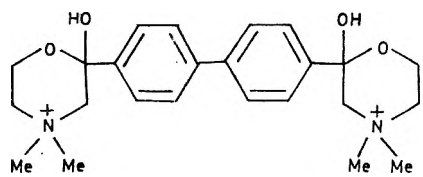

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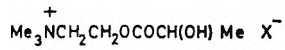
evidence is available that conformation *2* of the acetylcholine molecule may be the nicotinic conformation since examination of molecular models of nicotine shows that in one of the two conformations in which the pyridine and pyrrolidine rings are almost co-planar (*15*) the internitrogen distance of *ca* 4Å closely corresponds with the distance in *2* between the nitrogen atom and the acyloxy oxygen atom. Since the pyrrolidine nitrogen atom of nicotine is generally assumed to be in cationic form at physiological pH (Barlow & Dobson, 1955; Gillis & Lewis, 1956) and the pyridine nitrogen atom can be regarded as being somewhat analogous electronically to the acetoxy oxygen atom in acetylcholine, the purely nicotinic actions of nicotine are fully rationalized in terms of a receptor accepting conformation *2* of acetylcholine, provided both molecules share a common two point attachment involving the atoms just indicated. It is perhaps significant that the hemicholiniums (e.g. *16*) (Schueler, 1955), which possess morpholine rings in which the nitrogen and oxygen atoms are constrained in a steric relationship akin to that pertaining in conformation (*4*) of acetylcholine, do not act at the myoneural synaptic receptors in the same manner as other muscle relaxants, but exert their effect by

preventing the synthesis of acetylcholine (Reitzel & Long, 1959). This could perhaps be taken as evidence that conformation (*4*) is not accepted by the nicotinic receptors. Similarly a number of morpholine and isoxazolidine compounds (Eugster, Haffliger & others, 1958) exhibit high muscarinic activity (Waser, 1961a) although in this case the situation is complicated by the simultaneous presence of a degree of nicotinic activity. Again a series of morpholinium compounds were two to three times less potent than the corresponding piperidinium compounds as neuromuscular blocking agents (Donahoe, Seiwald & others, 1957, 1961).

That a three point or multipoint attachment is not involved in the production of an acetylcholine-like response at the nicotinic receptors would seem to be strongly indicated by the high nicotinic activity of both enantiomorphs of lactoylcholine (*17*), muscarone (*18*), nicotine (*19*, R = Me), and nornicotine (*19*, R = H). This clearly demonstrates that the three point projected asymmetry associated with optical isomerism (compare Beckett, 1959) is of little significance at nicotinic sites. Moreover it is impossible for the muscarones to achieve a three point correspondence with any conformation of acetylcholine as is apparent from inspection of molecular models.



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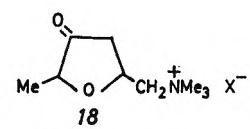

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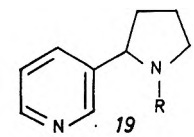

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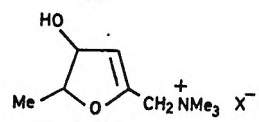

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Conformational isomerism in the acetylcholine molecule may indeed be an important factor in determining its ease of access to a sterically protected anionic receptor where only a one point attachment is involved, but the operation of such a one point attachment does not provide an interpretation of nicotinic activity in other molecules, through the absence of a second point of reference. However the muscarinic receptor, in contrast to the nicotinic receptor, is obviously highly stereoselective as evidenced by the pronounced differences in activity of L(+)- and D(−)-muscarine (Hardeggar & Lohse, 1957; Gyermek & Unna, 1958), of the enantiomorphs of acetyl-β-methylcholine (Beckett & others, 1961, 1963), of 4,5-dehydromuscarine (*20*) (Gyermek & Unna, 1960), and *cis*-2-methyl-4-trimethylammonium-methyl-1,3-dioxolane iodide (*1*) (Belleau & Puranen, 1963; Belleau & Lacasse, 1964), and by the marked effect of the size of the acyl group of the acylcholines upon affinity for the muscarinic receptor (Chang & Gaddum, 1933; Rossum, 1963). The stereoselectivity of the muscarinic site has been further emphasized in various attempts to give a pictorial representation of the receptor (Beckett & others, 1961, 1963; Waser, 1961a; Belleau & Puranen, 1963) which in no way invalidate conformational considerations.

CONFORMATIONALLY RIGID ANALOGUES OF ACETYLCHOLINE

It would appear, in the light of the preceding discussion, that a comprehensive study of conformationally rigid acetylcholine-like molecules in which the quaternary nitrogen atom and the acetoxy group are spatially held in mimicry of *2* and *4* with respect to muscarinic activity, is more likely to yield definite information about the role of conformational isomerism in determining the biological actions of acetylcholine than is any study of nicotinic activity, although the situation is complicated by the possibility that two acetylcholine molecules might react with one receptor (Turpajev & others, 1963). The appropriate absence of nicotinic activity might give supporting evidence provided that unfavourable solubility, permeability, transport or biotransformation phenomena do not prevent such drugs reaching the receptors, and provided that Gill's (1959) contention that conformationally rigid molecules will prove inactive is unfounded.

Few such rigid molecules have so far been examined. The 2-acetoxytropane methiodides (*8* and *9*) examined by Archer & others (1962) are conformationally non-rigid and the four racemic isomeric 2-acetoxy-3-trimethylammonium-*trans*-decalin halides (*10a-d*), although conformationally restricted, can exist in a variety of double boat and boat-chair conformations as well as in the double chair conformations shown. Baldridge, McCarville & Friess (1955) have prepared and tested the racemic forms of the stereoisomeric *cis* and *trans* 2-acetoxycyclohexyltrimethylammonium iodides (*21* and *22* respectively). Fig. 2 shows the theoretically possible extreme conformations of the *cis*-cyclohexane derivative (*21*) and Fig. 3 those of the *trans* isomer (*22*). It is to be noted that in both the *cis* and *trans* isomers of 1-acetoxy-2-trimethylammonium cyclohexane there is a form in which there is a $+60°$ projection angle between the quaternary nitrogen and the acetoxy oxygen atom with the $\overset{+}{N}$–O intergroup distance being 2·94 Å. Unfortunately compounds *21* and *22*, although tested for their ability to function as substrates for acetylcholinesterase and for their activity on the kitten phrenic nerve diaphragm preparation (Standaert & Friess, 1960), have not been investigated for muscarinic actions (S. L. Friess, personal communication). Schueler (1956) prepared and tested the two cyclic analogues *23* (which is optically inactive) and *24* (as the racemate). Compounds *23* and *24* appear to exhibit comparable muscarinic activity but are less potent than acetylcholine. The theoretically possible extreme conformations of the *NN*-dimethyl-3-oxomorpholinium ion (*23*) and of the *NN*-dimethyl-3-acetoxypiperidinium ion (*24*) are shown in Figs 4 and 5 respectively. Since none of the compounds *21–24* approximate to true rigidity they are therefore of little value in delineating the possible role of conformational isomerism at the different sites of action of acetylcholine. The racemic *cis* and *trans* 2-acetoxycyclopentyltrimethylammonium iodides (*25* and *26* respectively) (Friess & Baldridge, 1956) are, however, more nearly rigid, as the cyclopentane ring permits of only limited conformational isomerism (Pitzer & Donath, 1959; Brutcher, Roberts & others, 1959; McCullough, Pennington & others, 1959). Fig. 6 shows an approximate Newman

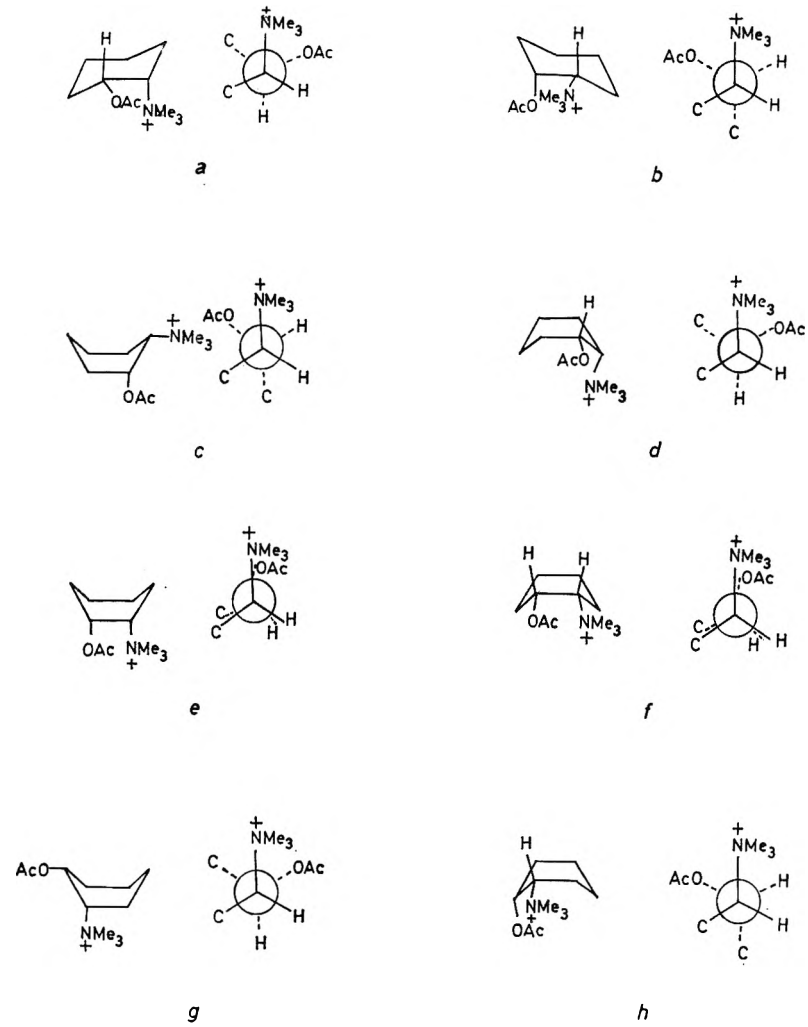

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FIG. 2. Theoretically possible extreme conformations of *cis*-1-acetoxy-2-trimethylammonium cyclohexane. (One optical enantiomorph only is shown.)

(1956) projection formula for one of the optical enantiomorphs of each of the *cis* (*25*) and *trans* (*26*) cyclopentane compounds. In these compounds none of the possible conformers with their partial eclipsing can be expected to correspond exactly to *2* although *25* is very close to *4*. Unfortunately compound *25* has not given as much information as could have been desired since it was prepared solely as the racemate and, moreover, was tested only for its ability to suffer hydrolysis by acetylcholinesterase when it was found to be more effectively hydrolysed than the corresponding *trans* racemate (*26*) (Friess & Baldridge, 1956). To secure

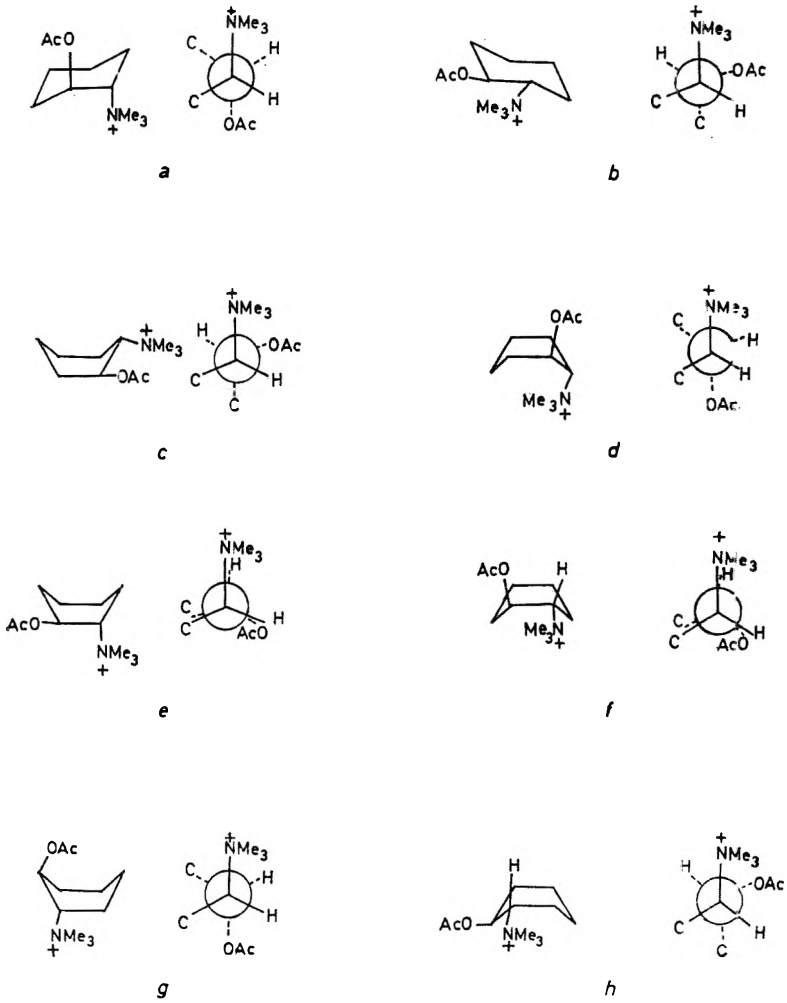

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FIG. 3. Theoretically possible extreme conformations of *trans*-1-acetoxy-2-trimethylammonium cyclohexane. (One optical enantiomorph only is shown.)

meaningful information compounds *25* and *26* should be subjected to pharmacological screening at both nicotinic and muscarinic sites, in the form of each separate pure enantiomorph.

THE POSSIBLE INVOLVEMENT OF INTRAMOLECULAR NC–H - - - - O HYDROGEN BONDING IN DETERMINING THE MUSCARINIC ACTIVITY OF ACETYLCHOLINE AND OTHER COMPOUNDS

Further support that *2* may be the nicotinic conformation and *4* the muscarinic conformation of acetylcholine could be advanced if it could be established conclusively that the stability of the quasi-ring conformations

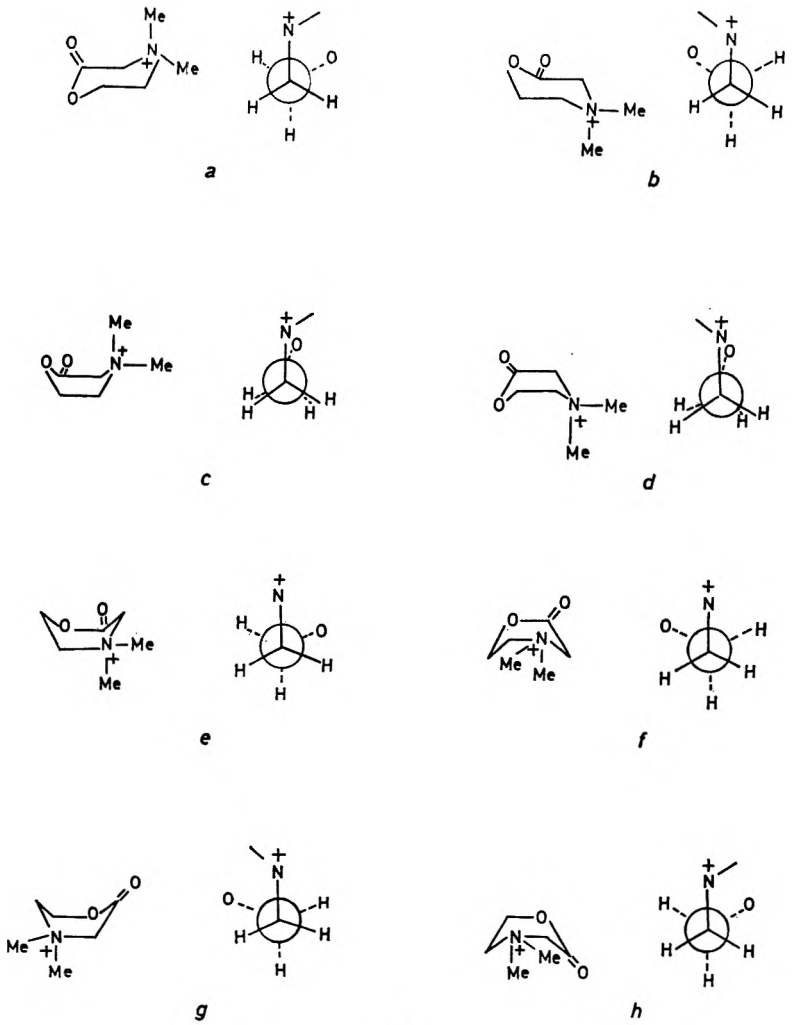


FIG. 4. Theoretically possible extreme conformations of the *NN*-dimethyl-3-oxomorpholium ion.

of acetylcholine (*4*) and muscarine (*6*) has its origin either in intramolecular coulombic attraction between the quaternary nitrogen and ether oxygen atoms or in intramolecular NC–H - - - - O bonding as suggested by Sutor (1962, 1963) since factors tending to reduce the electron density on the ether oxygen atom (or its equivalent)—and hence also to inhibit hydrogen bonding—should then tend to favour nicotinic properties, whilst factors tending to increase electron density at this point, and so favour quasi-ring formation, should tend to favour muscarinic activity.

Comparative infrared studies (Fellman & Fujita, 1962, 1963, 1965, 1966)

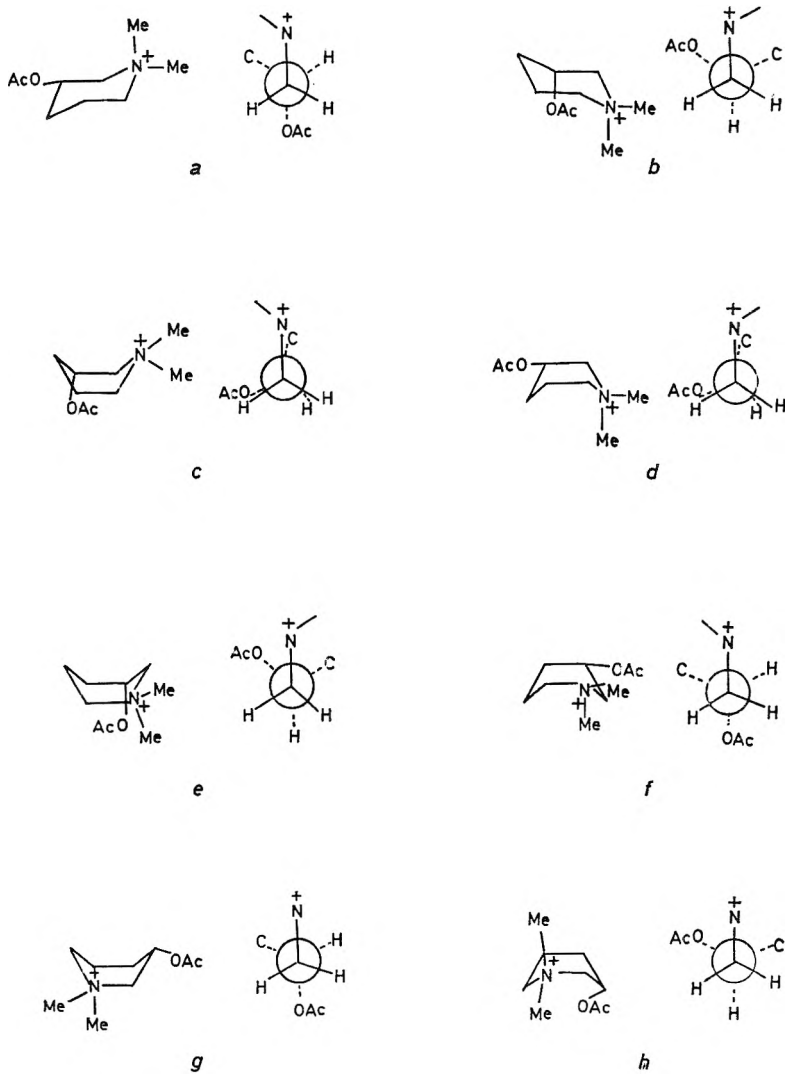


FIG. 5. Theoretically possible extreme conformations of the *NN*-dimethyl-3-acetoxypiperidinium ion. (One optical enantiomorph only is shown.)

and kinetic investigations showing a high electrophilicity for the ester carbonyl carbon atom in acetylcholine (Butterworth, Eley & Stone, 1953; Fellman & Fujita, 1962) have been interpreted in terms of the influence of the inductive effect from the quaternary ammonium group (*27*) although this interpretation has been challenged (Canepa & Mooney, 1965). At the same time the later results of Fellman & Fujita (1963, 1965, 1966), while disproving the existence of the previously postulated conformation

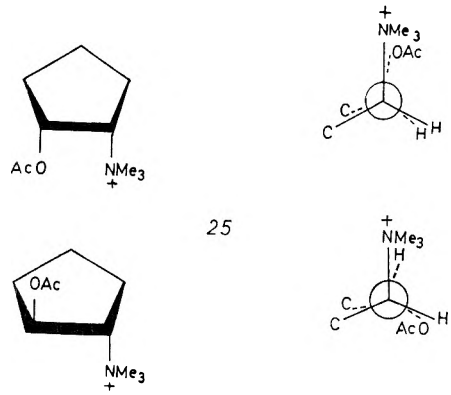
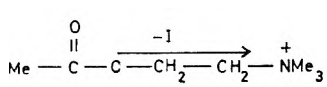


FIG. 6. Newman projection formulae of the *cis*- and *trans*-1-acetoxy-2-trimethylammonium cyclopentones. (One optical enantiomorph only is shown.)

(*28*) (Fellman & Fujita, 1962) which would result in a decreased carbonyl double bond character with consequent lowering of the stretching frequency (Jones & Sandorfy, 1956), would not be incompatible with the existence in solution of conformation *29* of acetylcholine, provided the stability of this conformation does result from intramolecular NC–H - - - - O bonding (Fellman & Fujita, 1966; Martin-Smith, Smail & Stenlake, 1967). In *29* electron withdrawal from the carbonyl group occurs *via* the acyloxy oxygen atom which would serve to depress the permanent polarization of the carbonyl group with consequent rise in its absorption frequency (Henbest & Lovell, 1957; West, Korst & Johnson, 1960; Bruice & Fife, 1962; Biggins, Cairns & others, 1963).

The proposed NC–H - - - - O bonding (e.g. *29* and *30*) could perhaps best be rationalized in terms of the inductive effects shown in *29*. The



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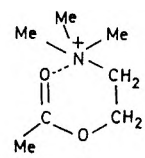

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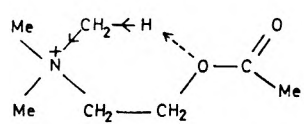

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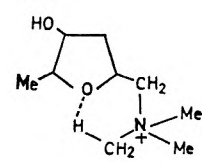

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primary C–N inductive effect is unexceptional and is even paralleled in amines, other than quaternary salts, where electronegativity differences

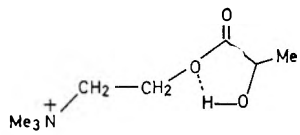
alone are operating, e.g. the increase in nucleophilicity of the nitrogen atom on replacement of the hydrogen atoms in the ammonia molecule by one and then two methyl groups (Sykes, 1961). The weakening of the NC–H bond responsible for the hydrogen bonding would also seem to be a normal phenomenon in *N*-methyl compounds, as is perhaps indicated by the low *N*-methyl C–H stretching frequency in the infrared spectrum of these compounds (Cross, 1960) and by analogy with ylide formation (Cope, LeBel & others, 1961). Relevant to the proposed N–C–H - - - - O hydrogen bonding are the results of an nmr study of *o*-fluoro-*NN*-dimethylbenzamide and *o*-fluoro-*N*-cyclohexyl-*N*-methylbenzamide (Lewin 1964) which would not be incompatible with N–C–H - - - - F hydrogen bonding.

Since sulphur and selenium are less electronegative than oxygen (Pauling, 1944), and do not as readily enter hydrogen bonding or possess the same high point electron charge density as does oxygen, it is instructive to compare the acetylcholine-like properties of the sulphur and selenium isosteres of such compounds as acetylcholine, acetyl-β-methylcholine and muscarine, in which quasi-ring conformations analogous to *29* and *30* are less likely to be favoured, with those of their prototypes. Indeed acetylthiocholine and acetylselenocholine are reported to be weaker muscarinic agents and stronger nicotinic agents than acetylcholine (Renshaw, Dreisbach & others, 1938; Günther & Mautner, 1963) whilst thiomuscarine gives rise to a decrease in muscarinic activity without the production of any marked nicotinic properties (Waser, 1961a). In the latter case steric hindrance similar to that which must pertain for acetyl-β-methylcholine would readily explain the absence of nicotinic potency, although anomalies exist in acetyl-β-methylthiocholine (Renshaw & others, 1938) and in thiomuscarone (Waser, 1961a,b) which show nicotinic activity.

These considerations might thus be taken as suggesting that conformation *2* could be the nicotinic conformer of acetylcholine, a view reinforced by the virtual absence of muscarinic activity in the (+) and (−)-lactoylcholines (*17*) (Rama-Sastry & Auditore, 1963) where hydrogen bonding from the hydroxyl group to the acyloxy oxygen atom (*31*) would stabilize the extended conformer. Further instances of expected destabilization of quasi-ring conformations akin to *4* may be sought in compounds where the thermodynamically favoured six-membered ring present in *29* is not possible or where the electron density on the acyloxy oxygen atom is decreased. The former situation is encountered in higher homologues of acetylcholine possessing more than two methylene groups between the trimethylammonium group and the acetoxy function, and indeed such compounds show markedly reduced muscarinic properties (Barlow, 1964). With respect to the second situation it is interesting to note that stress has been laid by other workers (Beckett & others, 1961, 1963) on the importance of the ether oxygen of the muscarine molecule in terms of a primary binding site in the receptor, whereas the function of this atom with its high electron density could in fact be more concerned in conformational stabilization. Decreased electron density on this atom with

consequent increase in favourability of an open chain conformer would be expected in benzoylcholine, where the benzene ring can act as an electron sink, and in methanesulphonylcholine (*32*) and in fact these compounds show little or no muscarinic activity (Akcasu & others, 1952; Eckhardt & Schueler, 1963). However, the concurrent operation of steric factors cannot be overlooked, as is clear from the pronounced nicotinic properties of trimethylacetylcholine (*33*) (Bergel, 1951) where the inductive effect is in the opposite direction.

The appearance of nicotinic as well as muscarinic activity in the muscarones (e.g. *34*) can perhaps also be attributed to an increased favourability of the extended conformation as compared to the situation in muscarine and it will be interesting to learn whether an X-ray study of crystalline



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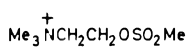

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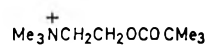

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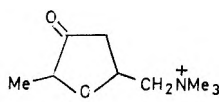

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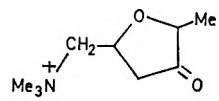

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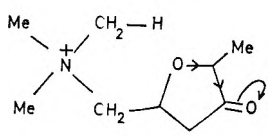

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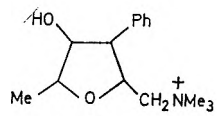

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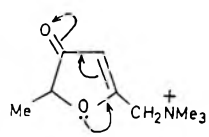

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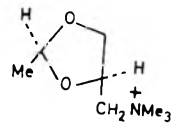
muscarone iodide will demonstrate the existence of such a conformation (*34a*) rather than a quasi-ring conformation (*6*) as is characteristic of muscarine iodide (Jellinek, 1957). If indeed stabilization of the quasi-ring conformation of muscarine is due to intramolecular NC–H - - - - O hydrogen bonding (Sutor, 1962, 1963) then the polarization of the oxo group in muscarone could perhaps destabilize the quasi-ring form through

relayed inductive effects (*35*) but complexities are introduced by the possibility that muscarone could interact with the receptor in its enol form (Waser, 1961a). Similar relayed inductive effects might explain the dual muscarinic and nicotinic properties of 3-phenylmuscarine (*36*) in which a delicate balance between the extended and quasi-ring conformers can readily be envisaged, but once again such electronic considerations cannot be divorced from steric arguments (Waser, 1961a; Beckett & others, 1963; Belleau & Puranen, 1963). With 4,5-dehydromuscarine (*20*) resonance between the π-electrons of the double bond and the p-electrons of the furan oxygen would serve to lower electron density on the latter, again explaining the dual nicotinic and muscarinic properties in terms of virtually equal favourability of an extended conformer and a quasi-ring conformer. The extended conjugation present in 4,5-dehydromuscarone (*37*) would presumably have a similar effect.

On the other hand, factors tending to increase the electron density of the acetoxy oxygen atom of acetylcholine should favour the quasi-ring conformation (*4*) if it is due to coulombic attractions or hydrogen bonding of the types discussed. Such a situation would be expected to pertain in choline ethyl ether (Simonart, 1932) and β-methylcholine ethyl ether (Holton & Ing, 1949) which are potent muscarinic agents. This is in contrast to the aryl ethers of choline in which the electron density on the ether oxygen will be decreased and which are well established as nicotinic agents (Hey, 1949, 1952; Fukui, Chikayoshi & Akira, 1960). The pronounced muscarinic activity of certain ketals (Fourneau, Bovet & others, 1944; Triggle & Belleau, 1962) could also result from the possibility of forming six-membered quasi-rings similar to *4*. Thus, in the 1,3-dioxolane series (Belleau & Lacasse, 1964) where the most active compound was L(+)-*cis*-2-methyl-5-trimethylammonium-methyl-1,3-dioxolane iodide (*38*) which contains only the equivalent of the $MeCOOCH_2$-fragment of acetylcholine locked in rigid conformation, the stereochemical and configurational specificity of this compound is still in accord with the possible existence of the quasi-ring conformation (38*a*).

Sekul & Holland (1961a,b; 1963a,b) have suggested that the activity of muscarinic compounds depends upon a fractional positive charge in the position corresponding approximately to the acetoxy oxygen atom of acetylcholine whereas nicotinic activity requires a fractional negative charge on the carbonyl oxygen atom. This hypothesis with regard to muscarinic activity is certainly in agreement with the concept of NC–H - - - - O hydrogen bonding since, although hydrogen bonding is favoured by increased electron density on the ether oxygen atom, a fractional positive charge could well arise on this atom after the hydrogen bond has formed. In support of their contention Sekul & Holland (1961a,b; 1963a,b) advance the facts that methoxymethylcholine ether (*39*) and propargylcholine ether (*40*) are twice as active as muscarinic agents as the propenyl (*41*) and propyl (*42*) analogues. However, interpretation of these results suffers from the disadvantage that there is no proof that the methoxyl group of *39* and the acetylenic group of *40* are in fact acting as electron withdrawing substituents. A consideration of the primary and

secondary inductive effects in the four choline ethers allows an alternative interpretation of these results. The $-CH_2CH_2\overset{+}{N}Me_3$ moiety is common to all four ethers and therefore its influence on the comparative electron density on the oxygen atom of the ethers may reasonably be ignored. The other primary inductive effects in methoxymethylcholine ether are shown in *39a* and the secondary inductive effects in *39b*. Although no quantitative assessment of electronic effects is possible, the electron density



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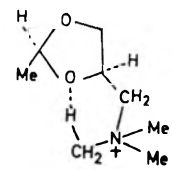

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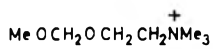

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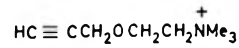

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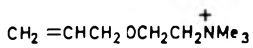

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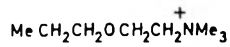

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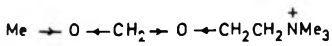
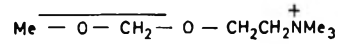


39a



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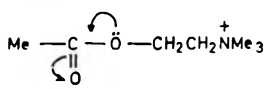

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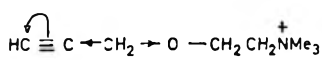

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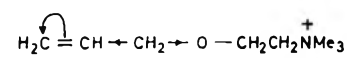

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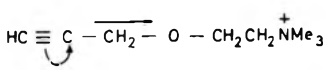

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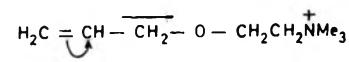

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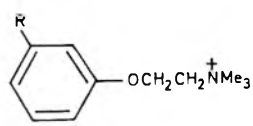


43

on the ether oxygen of methoxymethylcholine ether would *a priori* be expected to be greater than that on the acetoxy oxygen atom of acetylcholine for two main reasons.

(i) Electron withdrawal from the "ether" oxygen is greater in acetylcholine than in methoxymethylcholine ether due to the stronger polarization of the carbonyl group (*39c*) as compared to the weak inductive effect of the methoxymethylene function in *39a*.

(ii) The secondary inductive effect (*39b*) partially restores electron density on the ether oxygen atom of the choline ether.

The lower muscarinic activity of methoxymethylcholine ether as compared to acetylcholine may be rationalized on the basis of the differing stereochemical requirements of the methoxymethylene and acetyl groups respectively.

The primary inductive effects in the propargyl- and propenyl-choline ethers are shown in *40a* and *41a* respectively and the respective secondary inductive effects in *40b* and *41b*. In both of these compounds the secondary inductive effects probably outweigh the primary inductive effects as is perhaps evidenced by the anti-Markownikoff addition of hydrogen halides to allylic alcohols (e.g. Finar, 1959). Once again the secondary inductive effect aids in the restoration of electron density on the ether oxygen atom. The greater muscarinic activity of the propargyl ether (*40*) when compared to the propenyl analogue (*41*) may be on account of the greater mobility of electrons in the acetylenic system.

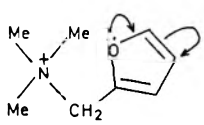
In the propyl ether (*42*) the primary and secondary inductive effects operate in the same direction. This compound would thus be expected to exhibit muscarinic properties (on the basis of NC–H - - - - O hydrogen bond formation) but perhaps weaker than those of *39*, *40* and *41* due to the relatively small inductive effect associated with alkyl groups as shown, for example, by certain infrared studies (Flett, 1957; Stone & Thompson, 1957; Brown, Eglinton & Martin-Smith, 1962).

Again the results obtained from a series of substituted choline phenyl ethers (*43*, R = I,Br,Cl,F,NH_2,H,NO_2), in which the order of nicotine-like stimulant action (*43*, R = I>NH_2>Br>Cl>F>H>NO_2) was the reverse of that expected by Hey's (1949) prediction (Coleman, Hume & Holland, 1965), are not above unambiguous interpretation. The complete delocalization present in the aromatic system makes it difficult to separate the various polarization and polarizability effects (Ingold, 1953) especially when the substituent is in the 3-position. Furthermore, even if electromeric effects could be disregarded, the influence of purely inductive effects on the ether oxygen atom instigated by substituent R (in *43*) must be practically non-existent being three carbon atoms from the oxygen atom. Further objections to the hypothesis of Sekul & Holland (1961a,b, 1963a,b) have been raised by Barlow (1964).

If the stability of quasi-ring conformations akin to *4* is crucially influenced by quite minor changes in the electron density of the ether oxygen atom it becomes possible to reinterpret the Five-Atom Rule (Ing, 1949; Alles & Knoefel, 1939; Ing, Kordik & Tudor-Williams, 1952). For

instance, as Beckett & others (1961) have proposed, the pronounced differences in muscarinic potency between furfuryltrimethylammonium (*44*) and 5-methylfurfuryltrimethylammonium (*45*) could be explained on the grounds that the inductive effect of the 5-methyl substituent in the latter may induce a crucial restoration of electron density on the furan oxygen (Acheson, 1960) which in the former compound will be depleted through resonance interaction with the furan ring. The net result is restoration of the stability of the quasi-ring conformation.

The effect of the proposed NC–H - - - - O hydrogen bonding is to constrain the acetylcholine molecule in the quasi-ring conformation (*4*)



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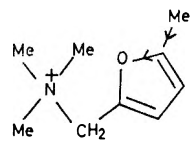

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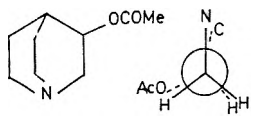

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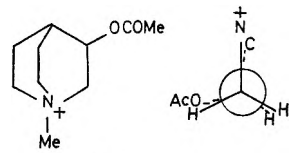

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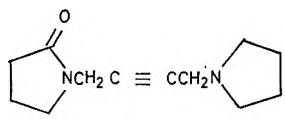

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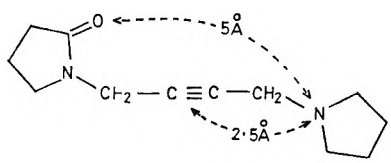

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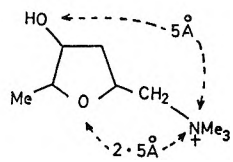

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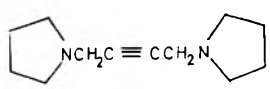
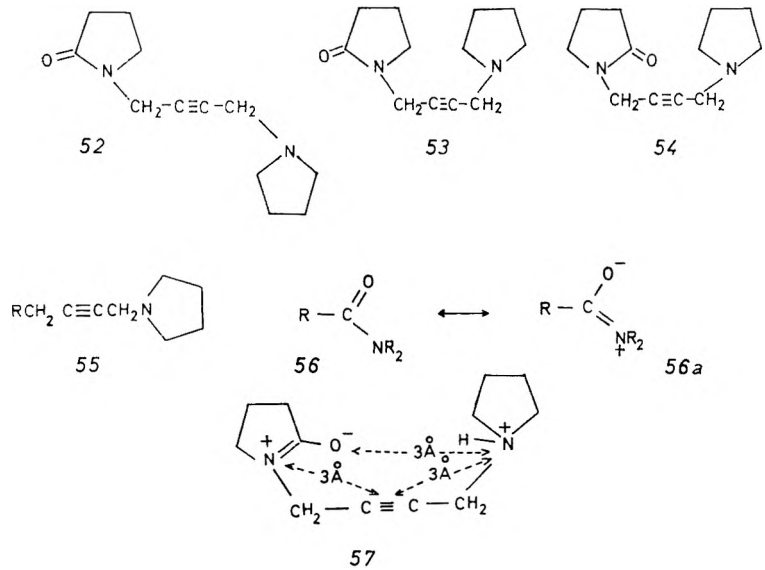


51

in which the N–O acyloxy distance approaches 3·2 Å. This consideration is important with respect to certain quinuclidine derivatives. Thus, 3-acetoxyquinuclidine (*46*) shows high muscarinic activity and almost complete absence of nicotinic properties (Mashkovsky, 1963) and 3-acetoxyquinuclidine methiodide (*47*) has been used to show that the enzyme acetylcholinesterase must favour a transoid conformation of acetylcholine with a similar disposition of the quaternary nitrogen atom and acetoxy group to that which pertains in the rigid quinuclidine salt (Solter, 1965). However, despite this transoid arrangement the N–O acetoxy distance in both *46* and *47* is *ca* 3·4 Å which is in close agreement with the N–O distance in the *gauche* conformer (*4* and *7*) of acetylcholine. In the former the rigid quinuclidine ring system constrains the functional groups in the requisite position whereas in the latter NC–H - - - - O hydrogen bonding might reasonably be supposed to do the same.

The tertiary acetylenic amine, oxotremorine (*48*), has been shown (Cho, Haslett & Jenden, 1962) to be a potent muscarinic agent devoid of nicotinic activity and on the assumption that this compound was acting directly and not via acetylcholine release it was proposed that the high activity could best be explained on the basis of the Koshland (1959) "induced fit" theory. Bebbington & Brimblecombe (1965, 1966) consider from an examination of molecular models that in the planar transoid form (*49*) the distance between possible active centres coincides with that of L(+)-muscarine (*50*). Thus, if the pyrrolidine nitrogen atom is protonated at physiological pH (Cho & others, 1962) then the acetylenic linkage of *49* coincides with the furan oxygen of *50* as a centre of high electron density and the carbonyl oxygen is considered to interact with site 3 (Beckett & others, 1961) of the muscarinic receptor. That the carbonyl group of oxotremorine plays some vital role in its muscarinic activiy would seem to be strongly indicated by the complete absence of any comparable activity in tremorine (*51*) itself (Cho & others, 1962). Although the planar transoid form (*49*) of oxotremorine was chosen by Bebbington & Brimblecombe (1965, 1966) there seems little reason to exclude the other planar transoid form (*52*) or the two planar cisoid forms (*53* and *54*), but in either *52* or *53* the carbonyl oxygen of the pyrrolidone moiety, being some 8 Å from the pyrrolidine nitrogen atom, can no longer be involved in receptor interaction of the type envisaged by these authors. It has further been shown (Bebbington & Brimblecome, 1965, 1966) that within a series of oxotremorine analogues (*55*, R a group containing amide, ester or ketone functions) muscarinic activity was inversely proportional to the infrared frequency of the carbonyl absorption and thus amides, which absorb at lower frequencies, had much greater activity than related esters or ketones. It is well known that the lower frequency of amide carbonyl absorption as compared to esters or ketones is attributable to the greater single bond character of the carbonyl group (Jones & Sandorfy, 1956) and therefore a substantial contribution from structures analogous to *56a* is implied. Application of these arguments to oxotremorine makes it not inconceivable that the molecule exists substantially in the form *57* assuming protonation of the pyrrolidine nitrogen atom at



physiological pH. In this structure there are now the equivalent of two positively charged nitrogen atoms at *ca* 3 Å from two centres of high electron density which again compares favourably with the N–O acetoxy distance in the quasi-ring form of acetylcholine (*4*). Hydrogen bonding between the protonated pyrrolidine nitrogen atom and the negatively charged oxo function of the pyrrolinium group could reasonably be considered as the force serving to constrain the molecule in what, *a priori*, would be considered an unfavoured conformation but what in fact may be a receptor favoured conformation. There is no conformation of oxotremorine complementary to the extended conformation (*2*) of acetylcholine thereby rationalizing the absence of nicotinic activity in the former and, at the same time, lending credence to the view that conformation *2* of acetylcholine is involved at the nicotinic sites.

These arguments seem to indicate that there are at least theoretical grounds for the involvement of conformational isomerism in the biological actions of acetylcholine. The supposition, however, that the fully staggered conformation and the quasi-ring conformation are actually involved at the receptors would be considerably strengthened if it were demonstrated that both are simultaneously present in solution. Studies toward this end have already been instigated (Martin-Smith & others, 1967; Fellman & Fujita, 1966) and future work may provide definitive evidence on the role of conformational isomerism at the cholinergic receptor. Valuable information should also be forthcoming from comparative pharmacological studies with further fully conformationally rigid molecules designed as analogues of various extreme conformations of acetylcholine. Such compounds should be prepared in both enantiomorphic forms, where molecular asymmetry exists, in view of the marked

stereoselectivity of the muscarinic site (Beckett & others, 1961, 1963; Waser, 1961a; Belleau & Puranen, 1963).

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J. Pharm. Pharmac., 1967, **19**, 590–595 Received May 15, 1967

Evaluation of potential antirheumatic drugs *in vitro* using lymphocytes and epithelial cells. The selective action of indoxole, methyl glyoxal and chloroquine

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Anti-inflammatory steroids and non-steroid drugs rapidly inhibit the incorporation of radioactivity from tritiated uridine and thymidine into the nucleic acid fraction of cultured epithelial cells and lymphocytes obtained from sheep, rats, rabbits and chickens. Acidic (uncoupling) drugs were also found to inhibit protein synthesis in these particular cells, and protein and nucleic acid synthesis in other cells which were steroid-insensitive. DNA synthesis in epithelial cells and lymphocytes was inhibited by methyl glyoxal (pyruvaldehyde). Chloroquine selectively inhibited thymidine incorporation by the epithelial cells. Indoxole was a potent inhibitor of the metabolism of circulating lymphocytes incubated in protein-free media. These findings may indicate why some of these drugs suppress adjuvant arthritis in rats.

SYNTHETIC and natural corticosteroids related to cortisol (hydrocortisone) have proved particularly valuable for the topical treatment of inflammatory conditions in epithelial tissue. It is well known that these anti-inflammatory–antirheumatic steroids, when given systemically, cause involution of the thymus gland in small animals and depress the number of circulating eosinophils in the bloodstream. This suggests that these steroid drugs may suppress inflammatory disease states by virtue of their action on lymphoid cells and certain leucocytes. This supposition is reinforced by recent observations that a form of experimental arthritis in rats, induced by injection of dead mycobacteria in liquid paraffin (so-called "adjuvant arthritis") and which is powerfully suppressed by anti-inflammatory steroids, is also inhibited by extirpation of the lymph nodes (Newbould, 1964) or by administration of an anti-lymphocyte serum (Currey & Ziff, 1966). If lymphoid cells are essential for the development of this type of experimental arthritis, it seems logical to enquire if current anti-inflammatory drugs are able to influence the metabolic activity of these cells, when they are present either in the blood-lymph circulation or contained within lymph nodes and other lymphoid tissues. The purpose of this communication is to report that the incorporation of radioactivity from labelled nucleosides into the nucleic acid fractions of various lymphoid cells and certain epithelial cells, maintained *in vitro*, is rapidly inhibited not only by corticosteroids but also by other anti-inflammatory and anti-arthritic drugs including indoxole (2,3-bis *p*-methoxyphenylindole) and ICI-43,823 (2-butoxycarbonylmethylene-4-oxothiazolidine).

The two latter drugs inhibit the chronic (lymphoid mediated?) stage of adjuvant arthritis in rats (Glenn, Bowman & others, 1967; Newbould, 1965) but ICI-43,823 has no effect upon the initial, acute, local inflammatory response to injected adjuvant. Both the acute and chronic

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stages of this experimental arthritis are inhibited by anti-inflammatory corticosteroids and by several non-steroid anti-inflammatory acids such as phenylbutazone, indomethacin, the fenamic acids and high doses of salicylates (Newbould, 1963; Winter & Nuss, 1966; Graeme, Fabry & Sigg, 1966; Ward & Cloud, 1966).

Experimental

A population of sheep lymphocytes, containing less than 2% of other cells, was obtained by centrifuging the lymph collected from a cannula inserted into the efferent duct draining the popliteal lymph node of conscious Merino or Corriedale ewes. The lymph was collected at room temperature over 24 hr periods in sterile plastic bottles containing approximately 1 mg of heparin (150 I.U.) and 2 mg of penicillin G. Circulating lymphocytes were also obtained from the thoracic duct lymph of conscious albino rats and anaesthetized New Zealand/California white rabbits. Tissue lymphocytes admixed with other cells, were obtained by dispersing finely chopped lymphoid tissues from young rats, rabbits and chickens (thymus, spleen, bursa of Fabricius) in chilled 0·3M sucrose or Krebs-Ringer media with the aid of an all-steel homogenizer: tissue debris was removed by very low speed centrifugation and the cells were collected as the fraction sedimenting above erythrocytes (if present) at 700 $\times$ g for 10 min. Three lines of polyploid epithelial cells, H(uman) EP(ithelial)-2, human amnion ("U" cell) and pig kidney (PK), were obtained from continuous cultures maintained by the Microbiology Department, John Curtin School of Medical Research. These different types of lymphocytes and epithelial cells were washed once in sterile Hank medium and resuspended in a Krebs-Ringer phosphate medium (pH 7·4) containing glucose (7mM) to which heparin (2 IU/ml) was added, to facilitate cell dispersion. Calcium was omitted from the medium in experiments with epithelial cells to minimize cell clumping. The phosphate component was replaced by isotonic Trishydrochloride pH 7·4, for experiments with inorganic [^{32}P]phosphate. The drugs were added as solutions in dimethylformamide or dimethylsulphoxide (final concentration of these solvents was 1% v/v) to aliquots of the cell suspension in round-bottomed centrifuge tubes containing 10–15 $\times$ 10^6 cells/ml, 2 min before addition of radioactive substrates. These mixtures of cells, drugs and substrate were incubated at 37° for 30 min, with slow shaking in air. Incubations were terminated by rapid freezing or by addition of strong acid.

Drug action on these cells was detected by measuring the rates of incorporation of [^{14}C]-labelled amino-acids (an algal protein hydrolysate), [5-^{3}H]uridine and [6-^{3}H]thymidine into material insoluble in 10% (w/v) trichloroacetic acid. All the radioactivity incorporated from added [^{3}H]uridine into the trichloroacetic acid-insoluble fraction was soluble in 10% trichloroacetic acid on heating for 10 min at 95° and high resolution autoradiographs (kindly made by Dr. B. Morris) of washed glutaraldehyde-fixed uridine-labelled sheep lymphocytes indicated that the radioactivity was principally located in the nucleus. The radioactivity incorporated from [^{3}H]thymidine was almost exclusively located in the cell

nucleus and the bulk of this radioactivity was soluble in hot 10% trichloroacetic acid (=nucleic acid). With all the lymphoid cells examined, a persistent thymidine-derived radioactive component (approximately 10–15% total incorporated radioactivity) resisted solution in hot acid and radioactivity was also consistently found in chloroform–methanol (2 : 1 v/v) extracts of well-washed cold trichloroacetic acid-insoluble material. Epithelial cells and micro-organisms incorporated very much less radioactivity (<5% total incorporated) from thymidine into this ("protein") fraction, insoluble in hot acid (cf. Tsien, Duncan & Sheppard, 1967).

Results

Table 1 shows that all the anti-inflammatory anti-arthritic drugs investigated rapidly inhibited incorporation of radioactive uridine into nuclear RNA by "pure" sheep lymphocytes. RNA labelling in other "lymphocytes" (from rat and rabbit thoracic lymph and thymus tissue, or chick spleen, thymus and bursa of Fabricius) was also inhibited by the same concentrations of these drugs, except indoxole (see below). Previous *in vivo* stimulation of sheep popliteal lymph lymphocytes (by subcutaneous injection of swine influenza virus below the popliteal node) greatly enhanced DNA synthesis and, after several days, stimulated protein synthesis

TABLE 1. EFFECT OF ANTI-ARTHRITIC DRUGS, CHLOROQUINE AND METHYL GLYOXAL UPON THE *in vitro* LABELLING OF NUCLEIC ACIDS AND PROTEIN IN LYMPHOCYTES (LC) FROM SHEEP POPLITEAL LYMPH AND IN CULTURED HUMAN CELLS OF EPITHELIAL ORIGIN

Figures are percentage incorporation of (i) [5-^{3}H]uridine into RNA (ii) [6-^{3}H]thymidine into DNA and (iii) mixed [^{14}C]amino-acids into protein, by cells co-incubated with drugs for 30 min at 37°, compared with that in drug-free controls (containing 1% v/v dimethylformamide) and are taken from duplicate experiments.

Drug	Conc. (×10^{-4}M)	Radioactivity % in Sheep LC** RNA	Sheep LC** DNA	Sheep LC** Prot.	"HEP-2" RNA	"HEP-2" DNA	"HEP-2" Prot.	"U-cell" RNA	"U-cell" DNA	"U-cell" Prot.
None	—	100	100	100	100	100	100	100	100	100
Indoxole	0·02	65	85	85						
	0·1	30	36	75	90	100	94	90	82	100
	0·5	15	12	40	68	76	83	52	39	80
Bis-2,3-phenylindole	0·1	50	43	35						
	0·5	8	7					75	85	76
ICI-43, 823	2·5	50	61	100	54		80	55		86
	5·0	38	42	92	24		68	27	27	47
2,5-Diphenyloxazole	0·5	38	12	40						
	1·0							38	50	100
Cortisol	2·5	52	70	90	56	55	100	39	35	65
Cortisone	1·0	23	62	96	20	25	95	26	21	80
Cortisol-21-aldehyde	1·0	31	47	65	51	57	63	30	36	67
Cortisol-FPP*	0·25	25	33	80						
	0·5	5		25	19	34	100	7	15	68
Tetrahydrocortisol	2·5	28	42	65	50	44	74	40	32	64
Desoxycorticosterone	1·0	20	48	73	29	24	68	19	12	37
Phenylbutazone	1·0	14	22	62	70	91	81	46	75	74
Indomethacin	1·0	20	38	70	72	93	75	83	47	52
Flufenamic acid	1·0	11	12	17	66	39	34	61	8	13
2,4-Dinitrophenol	1·0	22	61	35	54	65	49	59	16	29
Methyl glyoxal	10	76	10	18	100	29	15	100	53	49
Chloroquine phosphate	10	93	90	84	87	55	92	100	75	94

* FPP = 2'-(*p*-fluorophenyl)-[3,2-C]-pyrazole.

but did not appreciably alter the pattern of drug sensitivity of these circulating cells.

Those drugs that were the most powerful inhibitors of uridine incorporation also significantly inhibited amino-acid incorporation into protein and the incorporation of thymidine into DNA by these lymphocytes, within 30 min. The anti-inflammatory steroids and indoxole (at concentrations indicated in Table 1) had no effect on nucleic acid and protein biosynthesis in spermatozoa, *Tetrahymena pyriformis*, baker's yeast or *Aerobacter aerogenes*, and somewhat variable activity in inhibiting protein and nucleic acid synthesis in three different types of continuously cultured cells of epithelial origin (see Table 1). In PK cells, uridine incorporation, but not protein synthesis, was inhibited more than 50% by 100 μM steroids, >50 μM indoxole and 250 μM ICI-43,823. ICI-43, 823 also inhibited nucleic acid synthesis in spermatazoa, *Tetrahymena* and *Aerobacter*. Anti-inflammatory drugs which uncouple oxidative phosphorylation (Whitehouse, 1965) and 2,4-dinitrophenol, inhibited protein and nucleic acid biosynthesis in all lymphoid and epithelial cells examined and also in other cell types, e.g. spermatazoa, *Tetrahymena*, yeast and *Aerobacter*. Inhibition of protein synthesis in lymphocytes and epithelial cells by these uncoupling drugs (100 μM phenylbutazone, indomethacin and flufenamic acid) was detectable after short incubation periods (1 to 8 min) and was probably not merely a consequence of reduced (messenger) RNA synthesis. Reduction of RNA synthesis by 50% in sheep lymphocytes and human amnion epithelial ("U") cells by actinomycin D (2 μg/ml) had almost no effect on the incorporation of amino-acids into protein within 30 min of drug addition, suggesting that the natural life of the messenger RNA molecules in these particular animal cells was not particularly short (also see Allfrey & Mirsky, 1963).

Relatively high concentrations of cortisol (>100 μM) were required to inhibit RNA synthesis in these short-term experiments with lymphocytes. Less polar steroids such as tetrahydrocortisol, cortisone, corticosterone, desoxycorticosterone, and even 5α-pregnan-3,17,20-triol, were more potent in inhibiting uridine incorporation than cortisol itself. The absolute potency of these individual steroids was diminished, but their relative potency was not altered, when lymph proteins were added to the incubation medium. Prednisolone, cortisol-21-aldehyde (from G. D. Searle and Merck) and cortisol-2′-(*p*-fluorophenyl)-[3,2-C]-pyrazole, FPP (Hirschmann, Steinberg & others, 1963) were notably more potent than cortisol as inhibitors of protein and nucleic acid synthesis in epithelial cells and in lymphocytes obtained from both lymph and the fixed lymphoid tissues.

Indoxole was remarkably potent in suppressing the metabolism of circulating lymphocytes, being effective at 2–5 μM in the absence of protein. At least 10 times these concentrations of indoxole were needed to suppress protein and nucleic acid synthesis in epithelial cells and tissue lymphocytes (from thymus, bursa). Indole and anisole which each represent $\frac{1}{3}$ of the indoxole molecule, singly and together (0·5 mM) had no effect on lymphocyte RNA synthesis. The 2,3-bis-phenylindole analogue showed similar

activity to indoxole but successive replacement of the phenyl groups by methyl groups much diminished the drug acitivity *in vitro*. 2,5-Diphenyloxazole (PPO) was much more potent than ICI-43,823 *in vitro*.

None of the neutral anti-inflammatory drugs listed in Table 1 impaired the uptake or oxidation of [(1,6 or U)-^{14}C] glucose to lactate and CO_2 and of [^{14}C]acetate to CO_2 by sheep lymphocytes at drug concentrations inhibiting uridine incorporation by 60–80%. The "spurious" activity of desoxycorticosterone could however be largely attributed to its effect on energy-yielding reactions in these cells as this particular (non anti-inflammatory) steroid inhibited glucose and acetate oxidation and also depressed the incorporation of inorganic [^{32}P]phosphate into organic phosphates.

Chloroquine phosphate (1 mM), a slow-acting anti-arthritic drug, did not inhibit lymphocyte metabolism in these short-term experiments, but did partially inhibit thymidine incorporation by the epithelial cells and by a protozoon, *Tetrahymena pyriformis*. This parallels its relatively selective effect upon DNA synthesis in chloroquine-sensitive bacteria (O'Brien, Olenick & Hahn, 1966). Chloroquine does not inhibit adjuvant arthritis in rats (Newbould, 1963; Graeme & others, 1966; Ward & Cloud, 1966). This is understandable if this drug is unable to suppress DNA biosynthesis and the consequent proliferation of lymphocytes, following stimulation of lymphoid cells by adjuvant passing into the lymph nodes.

Methyl glyoxal (pyruvaldehyde) which may act as an anti-cancer agent (Szent-Györgyi, Együd & McLaughlin, 1967), selectively inhibited DNA synthesis in these lymphoid and epithelial cells and in *Tetrahymena*. (The apparent suppression of protein synthesis may be the result of combination of this aldehyde with [^{14}C]amino-acids.) This action of methyl glyoxal contrasts with that of cortisol-21-aldehyde, another α-ketoaldehyde, which apparently suppresses both RNA and DNA synthesis in these cells. Neither cortisol-aldehyde (0·5 mM) nor methyl glyoxal (3 mM) uncouples oxidative phosphorylation in rat liver mitochondria (under conditions given by Skidmore & Whitehouse, 1965) which supports the inference that both these ketoaldehydes are relatively selective inhibitors of polymer biosynthesis. Diacetyl (1 mM), the simplest diketo analogue of methyl glyoxal, had no effect on thymidine incorporation by the lymphoid and epithelial cells but actually stimulated uridine incorporation by these cells.

Disussion

Measurements of the incorporation of tritiated uridine and thymidine by lymphoid cells (and by certain lines of epithelial cells) *in vitro* would seem to provide a suitable metabolic system for examining potential antirheumatic drugs, especially since both acidic (uncoupling) and neutral drugs may give a positive response in the one system. In this respect this system has an advantage over other proposed *in vitro* biochemical assays for potential anti-inflammatory activity, which either primarily respond to the acidic drugs (Mizushima & Nakagawa, 1966; Skidmore & Whitehouse, 1966) or respond to steroids only after a long time lag (Whitehouse & Boström, 1962). However, a structure-action relationship

for corticosteroid derivatives obtained from such *in vitro* studies may be misleading as it has been found that all the types of lymphocytes examined, respond both to those steroids which strongly inhibit respiration, e.g. desoxycorticosterone (Jensen & Neuhard, 1961) as well as to those which do not, e.g. cortisol. In this respect these findings are at variance with recent observations upon rat "thymocytes" preincubated with steroids for 3 hr before measuring nucleic acid synthesis (Makman, Dvorkin & White, 1966).

The present findings also indicate that indoxole is probably more cell-specific than the anti-inflammatory steroids and so might possibly be considered a forerunner of a new class of fairly selective drugs, whose action is primarily directed against certain lymphoid cells (in this case, circulating lymphocytes). Such drugs could have important implications for immunobiology, organ transplantation and the treatment of auto-immune diseases. Powerful though they are, the cytotoxic alkylating agents and the currently available anti-inflammatory acidic drugs and steroids do not attain this ideal of selective anti-lymphoid activity.

Acknowledgements. Drugs were generously provided by Merck, Sharp and Dohme, Upjohn, G. D. Searle, J. R. Geigy and I.C.I. Pharmaceutical Division. I am particularly grateful to Professor F. C. Courtice, F.A.A. for providing facilities for this work, to Drs. B. Morris, M. Simpson-Morgan and R. Fraser for cannulating animals to provide lymph, Mr. A. J. Brand for providing epithelial cells, to the Royal Society for a Nuffield Commonwealth Bursary and the Australian National University for a Visiting Fellowship. This work was carried out whilst the author was on study leave from the University of Oxford.

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J. Pharm. Pharmac., 1967, **19**, 596–602 Received February 15, 1967

Mechanism of the protective effect of reserpine on aggregated mice treated with (+)-amphetamine

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Experiments were made to see if the effect of reserpine in protecting aggregated mice from the toxic effect of (+)-amphetamine depended on the hypothermia or on the depletion of brain noradrenaline it induces. In aggregated mice, a 7 mg/kg dose of amphetamine elevated body temperature, lowered the level of brain noradrenaline and caused 100% mortality. In reserpinized aggregated mice, amphetamine did not cause hyperthermia or any further depletion of brain noradrenaline. Prevention of the hypothermic effect of reserpine by keeping amphetamine-treated reserpinized animals at a higher environmental temperature markedly lowered the protective effect of reserpine. When also the depletion of noradrenaline by reserpine was antagonized by dopa, reserpine no longer protected aggregated mice from the toxic effect of amphetamine. The lowering of 5-hydroxytryptamine content brought about by reserpine remained unaltered during these procedures. Complete protection against amphetamine toxicity was also offered by α-methyl-l-tyrosine in doses which lowered brain noradrenaline to almost the same extent as reserpine, but which did not lower temperature or brain 5-hydroxytryptamine. When the body temperature of aggregated mice with brain noradrenaline lowered by α-methyl-l-tyrosine was elevated by subjecting the animals to heat stress, the protective effect was reduced. Hypothermia induced by reserpine could thus be related to its noradrenaline-depleting action. The results show that both properties contribute to reserpine's protective action. However, the abolition by dopa of this protective effect of reserpine and the complete protection offered by α-methyl-l-tyrosine without hypothermia suggest that depletion of brain noradrenaline plays the more important role in the protective effect of reserpine.

SINCE Chance (1947) reported that the toxicity of amphetamine is markedly enhanced in aggregated mice, the increased mortality has been attributed to enhanced motor activity and excitement (Greenblatt & Osterberg, 1961), hyperthermia (Greenblatt & Osterberg, 1961; Askew, 1962; Fink & Larson, 1962), excessive noradrenaline liberation (Maxwell, 1959; Weiss, Laties & Blanton, 1961; Moore, 1963) and enhanced brain excitability (Swinyard, Clarke & others, 1961).

The importance of hyperthermia as a contributing factor has been stressed by Askew (1962) and by Hardinge & Peterson (1963, 1964), although Wolf & George (1964) did not find this to be so. Amphetamine depletes brain noradrenaline (McLean & McCartney, 1961; Sanan & Vogt, 1962) and this is enhanced in aggregated mice (Moore, 1963). Since adrenergic blocking agents protect aggregated mice from the toxic effect of amphetamine (Maxwell, 1959; Weiss & others, 1961), excessive noradrenaline release by amphetamine could be considered a factor in the mechanism of amphetamine toxicity in aggregated mice.

Reserpine protects aggregated mice from the lethal effect of amphetamine (Burn & Hobbs, 1958). We have evaluated the relative importance of the two actions of reserpine, which could be interrelated, namely, hypothermia (Lessin & Parkes, 1957) and brain noradrenaline depletion (Holzbauer & Vogt, 1956) in reducing the toxicity in aggregated mice. The role of hypothermia was assessed in amphetamine-treated reserpinized mice kept at a higher environmental temperature to counteract the

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hypothermic action of reserpine without altering its noradrenaline-depleting activity on the brain. Experiments were also made in the reserpine treated mice in which both the hypothermic and the noradrenaline lowering effect of the tranquillizer were antagonized by DL-3,4-dihydroxyphenylalanine (dopa). Since reserpine is known to liberate 5-hydroxytryptamine (5-HT) as well as noradrenaline and the tranquillizing effect has been correlated with the change in 5-HT (Brodie, Tomich & others, 1957) brain 5-HT levels were measured in the brains of these animals. α-Methyl-l-tyrosine, the tyrosine hydroxylase inhibitor (Nagatsu, Levitt & Udenfriend, 1964) which causes a specific lowering of brain noradrenaline without affecting brain 5-HT (Spector, Sjoerdsma & Udenfriend, 1965) was also used.

Experimental

METHODS

Reserpine (Serpasil, Ciba), α-methyl-l-tyrosine (Merck, Sharp & Dohme) DL-dihydroxyphenylalanine (Nutritional Biochemical Corporation) and (+)-amphetamine sulphate (SKF), doses of which refer to the salt, were used. The solution of α-methyl-l-tyrosine was prepared according to Spector & others (1965). Reserpine was dissolved in a few drops of glacial acetic acid and the volume made up with distilled water. Other drugs were dissolved in distilled water. All the injections were made intraperitoneally and the volume of injected solutions was 0·01 ml/g body weight. Controls in which the animals were treated with the particular solvent were run simultaneously.

Albino mice (CDRI strain) were kept in identical conditions for a week before the experiments. These were made at a room temperature of $29 \pm 2°$. Aggregated mice (groups of 10) were kept in covered metallic cages (25 cm × 13 cm × 12 cm). Animals to be kept at an elevated temperature were put in a well aerated chamber at $38·5 \pm 0·5°$ immediately after amphetamine treatment. The same groups of animals were used to study changes in mortality rate and for determining the rectal temperature. Noradrenaline determinations were made in different groups of aggregated mice.

Influence of drugs and other procedures on the lethal effect of amphetamine in aggregated mice. Immediately after treating groups of animals with doses of amphetamine ranging from 3 to 10 mg/kg, the mice were aggregated and the number of animals dying hourly during the first 4 hr was determined. A dose of 7 mg/kg was lethal to all the mice and 6 mg/kg was lethal only to 50%. The 7 mg dose was used in experiments assessing protective effects and a 4 mg dose in those procedures where marked potentiation of amphetamine toxicity had to be demonstrated. This dose was lethal to 20% of the control animals.

Experimental procedures are described in Table 1. Immediately after amphetamine treatment, the animals were aggregated and were observed hourly for 4 hr. The mice not treated with amphetamine (groups I, III, V, VII, X and XII), were kept together in large cages, one for each

TABLE 1. PROCEDURES USED AND RESULTS OBTAINED IN THE EXAMINATION OF THE MODIFICATION OF AMPHETAMINE TOXICITY IN GROUPS OF 10 AGGREGATED MICE. Rectal temperatures were taken 18 hr after reserpine or its controls or 4 hr after the last dose of α-methyl-l-tyrosine, or its control, and measurements repeated at hourly intervals for 4 hr. Animals were killed for the estimation of amines 20 hr after reserpine, or its controls, or 6 hr after the last dose of α-methyl-l-tyrosine or its control. Heat-stressed groups (H.S.) were subjected to the elevated temperature (38·5°) 18 hr after reserpine, or its controls, or 20 hr after α-methyl-l-tyrosine or its control.

Group No.	Pretreatment	Amphetamine (7 mg/kg)	Animals dead within 4 hr/ Total No. of animals employed	No. animals	Whole brain content of noradrenaline 5-HT (μg/g wet tissue)	
I	Solvent of reserpine	Not given	0/10	6	0·401 ± 0·06	0·418 ± 0·03
II	Solvent of reserpine ..	18 hr later	10/10	9	0·313 ± 0·044 ($P < 0·05$) when compared with group I	0·410 ± 0·03
III	Reserpine[1] ..	Not given		6	0·148 ± 0·016 ($P < 0·001$) when compared with group II	0·108 ± 0·009 ($P < 0·001$) when compared with group II
IV	Reserpine ..	18 hr later	0/10 ($P < 0·001$) when compared with group II	6	0·152 ± 0·011	0·119 ± 0·008
V	Reserpine ..	Not given (H.S.)		6	0·149 ± 0·009	0·113 ± 0·01
VI	Reserpine ..	18 hr later (H.S.)	11/20 ($P < 0·001$) when compared with group IV	6	0·139 ± 0·011	0·113 ± 0·01
VII	Reserpine + dopa[2] ..	Not given		6	0·291 ± 0·028 ($P < 0·01$) when compared with group III	0·099 ± 0·02
VIII	Reserpine + dopa[2] ..	15 min after dopa	20/20 ($P < 0·001$) when compared with group IV	6	0·278 ± 0·019	0·141 ± 0·002
IX	Solvent of α-methyl-l-tyrosine ..	4 hr after the last dose	10/10	3	0·308 ± 0·04 ($P < 0·05$) when compared with group I	0·401 ± 0·01
X	α-Methyl-l-tyrosine[3] ..	Not given		6	0·150 ± 0·01 ($P < 0·001$) when compared with group I	0·389 ± 0·04
XI	α-Methyl-l-tyrosine[3] ..	4 hr after the last dose	0/20 ($P < 0·001$) when compared with those given only amphetamine 7 mg/kg	6	0·159 ± 0·008	0·400 ± 0·03
XII	α-Methyl-l-tyrosine[3] ..	Not given (H.S.)		6	0·149 ± 0·02	0·398 ± 0·01
XIII	α-Methyl-l-tyrosine[3] ..	4 hr after the last dose (H.S.)	7/20 ($P < 0·02$) when compared to group XI	6	0·148 ± 0·017	0·410 ± 0·04

[1] Dose of reserpine was 1 mg/kg in all these experiments.
[2] Dopa was administered in a dose of 200 mg/kg 18 hr after reserpine.
[3] Dose of α-methyl-l-tyrosine, 3 doses of 80 mg/kg each over 24 hr.

treatment, and were aggregated into groups of ten the day after drug or solvent administration and after taking the rectal temperature.

Rectal temperature of mice. Rectal temperature was taken hourly (Table 1) for 4 hr by introducing a probe 1·5 cm into the rectum.

Influence of various procedures on the noradrenaline and 5-HT *contents of whole brain of mice.* The noradrenaline content of whole brain was extracted and estimated at the appropriate time (Table 1) according to Shore & Olin (1958) and 5-HT according to Mead & Finger (1961). Deaths were highest during the second hour after amphetamine treatment,

so amine estimations were made during this hour. Animals already dead were not used for neurohormonal estimations.

Results

Influence on the mortality of aggregated mice treated with amphetamine. Pretreatment with reserpine (1 mg/kg) offered complete protection to grouped mice treated with lethal dose of amphetamine (Table 1). This protective effect of reserpine was reduced when the animals were kept at a higher environmental temperature after amphetamine administration and was completely abolished when pretreatment included both reserpine and dopa and the animals were not sedated. Pretreatment of animals with α-methyl-l-tyrosine did not produce any obvious sedation, but was as effective as reserpine in preventing deaths due to the lethal dose of amphetamine. In these animals also, exposure to elevated environmental temperature reduced the protective effect. Preliminary work also showed that when grouped mice were treated either with reserpine or α-methyl-l-tyrosine (both without amphetamine) and kept at the elevated environmental temperature no mortality occurred over 8 hr.

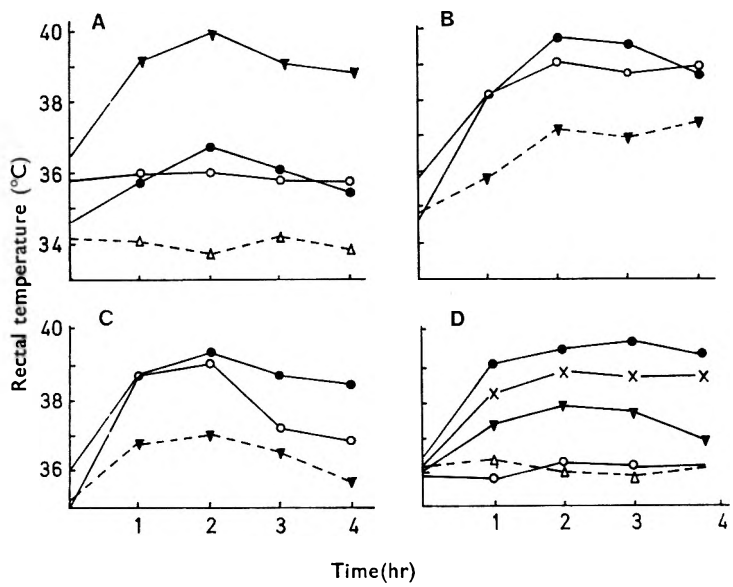

FIG. 1. The influence of reserpine on the effect of amphetamine on the rectal temperature of albino mice kept at an elevated temperature of 38·5°, or pretreated with dopa or α-methyl-l-tyrosine. A. ○—○ Solvent, ▼—▼ solvent + amphetamine, ●—● reserpine + amphetamine, △—△ reserpine. B. ○—○ Solvent + elevated temperature, ▼—▼ reserpine + elevated temperature, ●—● reserpine + amphetamine − elevated temperature. C. ○—○ Solvent + dopa, ▼—▼ reserpine + dopa, ●—● reserpine + dopa + amphetamine. D. ○—○ Solvent of α-methyl-l-tyrosine (AMT), △—△ AMT, ▼—▼ AMT + amphetamine, ×—× AMT + elevated temperature, ●—● AMT + amphetamine + elevated temperature.

Influence on the amphetamine-induced hyperthermia in aggregated mice. (Fig. 1). Amphetamine (7 mg/kg) produced profound hyperthermia in aggregated mice and the animals died when the body temperature exceeded 40°. Reserpine alone produced marked hypothermia in mice and this effect was antagonized by amphetamine, but no hyperthermia was observed in this group of mice. When the reserpinized animals were subjected to elevated environmental temperature, the rectal temperature began rising within the first hour and a rise of 2° in temperature above normal values was observed in 2 hr. No further increase was noted. When the reserpinized animals were treated with amphetamine and then subjected to elevated temperature, the rise in body temperature was comparable to those groups of animals treated with amphetamine only. Administration of dopa caused the body temperature of reserpine-treated mice to return to normal; administration of the 7 mg/kg dose of amphetamine to these grouped mice caused a hyperthermia which was less intense than the control group of animals treated with amphetamine only.

Treatment with α-methyl-l-tyrosine did not produce any significant change in the rectal temperature of aggregated mice. Administration of amphetamine to α-methyl-l-tyrosine-treated animals caused only mild hyperthermia. When these animals were kept at a higher environmental temperature, hyperthermia was observed, the effect being comparable to those obtained in unprotected aggregated mice treated with amphetamine.

Noradrenaline and 5-HT *content of whole brain of aggregated mice* (Table 1). Reserpine (1 mg/kg), 20 hr after administration, caused a 60% reduction of the noradrenaline content of whole brain of aggregated mice (group III). This effect remained unchanged when the reserpinized animals were treated with amphetamine (group IV) or subjected to elevated temperature (group V) or a combination of these two (group VI). Treatment of reserpinized mice with dopa (group VII) partially replenished the noradrenaline content. In these aggregated mice the noradrenaline content of brain was only 25% below control values (group I). Amphetamine (group VIII) did not cause any further change in the above group. α-Methyl-l-tyrosine caused a lowering of brain noradrenaline by 60% (group X) and, as with reserpine, when the α-methyl-l-tyrosine treated animals were given amphetamine (group XI) subjected to elevated temperature (group XII), or were treated with a combination of these two procedures (group XIII), no further change in the noradrenaline level was observed.

Reserpine, but not α-methyl-l-tyrosine, lowered brain 5-HT and other procedures did not alter this amine content further.

Discussion

In aggregated mice, the 7 mg/kg dose of amphetamine killed all the animals. These animals showed marked hyperthermia and the time of death in most instances coincided with the rise of temperature beyond 40°. A significant decrease in brain noradrenaline occurred. Elevation of body temperature has been reported by Greenblatt & Osterberg (1961),

and Askew (1962), and decreased brain noradrenaline content by Maxwell (1959) and Moore (1963) in amphetamine-treated aggregated mice. Although the possibility that these two factors are interrelated cannot be excluded, there is some purpose in seeking which of the two is more directly responsible for the mortality of the animals.

Reserpine offered complete protection to aggregated mice against the toxic effect of amphetamine. In reserpine-treated animals amphetamine did not produce hyperthermia. Amphetamine did not affect the brain noradrenaline concentration already lowered by reserpine. It seems that pretreatment with reserpine prevented the amphetamine-induced release of noradrenaline from the storage sites. These findings suggest that the protective effect of reserpine is dependent on one or both of these factors.

After amphetamine treatment and aggregation, when the reserpinized animals were kept at an elevated environmental temperature, the noradrenaline content of brain did not change, but the animals showed hyperthermia, and this change markedly reduced the protective effect of reserpine. Administration of dopa effectively counteracted the hypothermic effect of reserpine and also partly replenished the brain noradrenaline. Although in these animals there was no hyperthermia and the brain noradrenaline was only 75% of normal values, the amphetamine toxicity was markedly increased.

It can therefore be concluded that if the hyperthermic effect of amphetamine is blocked, a return to normal of the noradrenaline content in reserpine-treated mice abolished the protective effect offered by this tranquillizer against amphetamine toxicity to aggregated mice.

Further support for the important role played by the lowered brain noradrenaline in the reserpine effect is provided by the experiments with α-methyl-l-tyrosine. Both reserpine and α-methyl-l-tyrosine lowered the noradrenaline level to the same extent whereas reserpine also caused a lowering of brain 5-HT. The degree of protection offered to mice by both these drugs was the same. These results indicate that in its protective action against amphetamine toxicity in aggregated mice, reserpine's lowering of brain noradrenaline alone is sufficient. These findings, as well as our recent experimental data showing that the pharmacological effects of reserpine are enhanced in α-methyl-l-tyrosine-treated animals (Menon, Dandiya & Bapna, 1967), support the contention of Carlsson & others (1957) and Carlsson (1961) that reserpine sedation may probably be more directly related to the loss of adrenergic transmitter from the hypothalamus reticular formation and other areas in the brain.

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J. Pharm. Pharmac., 1967, 19, 603–607 Received April 25, 1967

The fate of [^{14}C]thalidomide in the pregnant hamster

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The embryotoxicity and fate of [^{14}C]thalidomide in the pregnant European golden hamster have been investigated. Daily administration of thalidomide (1 or 2 g/kg orally) to pregnant hamsters on days 4–12 inclusive of pregnancy was not embryotoxic. [^{14}C]Thalidomide (150 mg/kg) administered on the 204th hr of pregnancy is well absorbed and about 84% of the ^{14}C is excreted in the urine and 9% in the faeces in the 3 days after dosing. The urinary ^{14}C consists of thalidomide (3% of dose), α-(*o*-carboxybenzamido)glutarimide (26%), 2- and 4-phthalimidoglutaramic acids (8%), 2-phthalimidoglutaric acid (0·2%) and 2- and 4-(*o*-carboxybenzamido)-glutaramic acids plus 2-(*o*-carboxybenzamido)glutaric acid (27%). ^{14}C is present in the embryo and the relative concentrations of radioactivity in the embryo and plasma are about the same at 4, 12 and 24 hr after dosing. At 4 hr after dosing the embryo contains mainly thalidomide, but at 12 hr this has largely disappeared and the ^{14}C consists of seven hydrolysis products. The lack of embryotoxicity of thalidomide in the hamster is thus not due to an inability of the teratogen to penetrate to the conceptus.

THALIDOMIDE is teratogenic in man (Lenz, 1961, 1962; McBride, 1961), monkey (Delahunt & Lassen, 1964), rabbit (Felisati, 1962; Giroud, Tuchmann-Duplessis & Mercier-Parot, 1962; Seller, 1962; Somers, 1962; Spencer, 1962), rat (Bignami, Bovet & others, 1962; King & Kendrick, 1962; Bignami, Bovet-Nitti & Rosnati, 1964), mouse (Giroud & others, 1962; Di Paolo, 1963), and chicken (Kemper, 1962; Boylen, Horne & Johnson, 1963; Ehmann, 1963; Yang, Yang & Liang, 1963). However, thalidomide does not appear to be teratogenic in the hamster (Somers, 1963; Fratta, Sigg & Maiorana, 1965) although Homburger, Chaube & others (1965) have reported some embryotoxicity in certain inbred strains of hamsters, but not in randomly bred strains.

Thalidomide is teratogenic in the pregnant New Zealand white rabbit when administered only during the morphogenetic phases of embryonic development (Fabro & Smith, 1966). Furthermore, when thalidomide is administered to the mother it penetrates into the conceptus and the teratogenicity appears to be due to thalidomide itself rather than to one of its metabolites (Fabro, Smith & Williams, 1967). The resistance of the hamster to the teratogenic effects of thalidomide could be due to an inability of the teratogen to penetrate into the embryonic tissues when given to the mother; we have therefore investigated the fate of [^{14}C]-thalidomide when administered orally to pregnant hamsters.

Experimental

MATERIALS AND METHODS

Thalidomide, m.p. 272°, was a gift from the Lilly Research Laboratories Ltd. [*Carbonyl*-$^{14}C_1$]thalidomide (m.p. 270°; specific activity 0·75 μc/mg) was synthesized from [*carbonyl*-$^{14}C_1$]phthalic anhydride according to the method of Beckmann (1962). (±)-α-(*o*-Carboxybenzamido)glutarimide, (±)-2- and 4-phthalimidoglutaramic acids, (±)-2-phthalimidoglutaric

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acid, (±)-2- and 4-(*o*-carboxybenzamido)glutaramic acids and 2-(*o*-carboxybenzamido)glutaric acid were samples previously prepared (Schumacher, Smith & Williams, 1965; Fabro & others, 1967).

ANIMALS

Pregnant European golden hamsters (random breed; 7 years closed colony; 100–150 g) were purchased (A. F. Longmoor, 63, Sherrard Road, Forest Gate, London, E.11). They were mated at the breeding centre at night between 11 p.m.–2 a.m. and were sent to our animal department the following morning. They were kept in individual cages and maintained on Diet No. 41B (E. Dixon & Son, Ware) with water *ad lib.*

For the teratogenic testing, thalidomide was administered orally each day as a suspension (in 0·5% carboxymethylcellulose) at dose levels of 1 or 2 g/kg to groups of pregnant hamsters from the 4th to the 12th day inclusive, of gestation. The controls were given 1 ml 0·5% carboxymethylcellulose orally. On the 15th day the hamsters were killed and the number of implantations, resorptions and viable foetuses, both normal and abnormal, were counted. Viable foetuses were weighed and examined for external malformations and then fixed in 95% ethanol. After one week the foetuses were dissected for internal malformations. The skeletons were stained with alizarin according to the method of Chaube (1965).

[^{14}C]Thalidomide (150 mg/kg; 20 μc/kg) was also administered orally as a single dose to 19 pregnant hamsters on the 204th hr of pregnancy. At this time morphogenesis is occurring and 15–20 pairs of somites are present (Waterman, 1948). One group of three treated animals were housed individually in metabolism cages and their urine and faeces collected. Blood samples (0·4 ml) were withdrawn by heart puncture from a second group (4 animals) at 4, 8, 12 and 24 hr after dosing, and the plasma separated by centrifugation at 2000 rev/min for 10 min. Three groups each of 4 treated animals were killed at 4, 12 and 24 hr respectively after dosing, and blood samples and embryos removed. Embryos free of blood and uterine tissue were isolated by placing the uterus containing the embryos in hexane at −40°. The embryo and trophoblast were then isolated by peeling off the uterine tissue. The embryos were quickly washed in ice-cold saline, dried on blotting paper, weighed and homogenized in groups of three in a mixture of equal parts of methanol and dioxan and the volume adjusted to 10 ml. Portions (2 ml) were transferred to counting vials containing a scintillation fluid consisting of a mixture of dioxan–ethylene glycol–methanol (88:2:10 by vol.) containing naphthalene (6%), 2,5-diphenyloxazole (0·4%), 1,4-bis-(5-phenyloxazolyl)benzene (0·02%) and 5% thixotropic gel powder (Cab-o-sil). ^{14}C in urine, faeces and blood was estimated as previously described (Fabro & others, 1967). ^{14}C was counted in a Packard Tricarb Liquid Scintillation Spectrometer (Model No. 3214) and counting efficiency was measured by the twin-channel ratio method (Bush, 1963) or by internal standards. The nature of the ^{14}C compounds in the urine, plasma and embryo was determined as previously described (Fabro & others, 1967).

Results and discussion

EFFECT OF THALIDOMIDE ON THE HAMSTER PREGNANCY

Table 1 shows that in the European golden hamster the average number of implantation sites, the litter size, the mean body-weight of the

TABLE 1. EFFECT OF THALIDOMIDE IN PREGNANT HAMSTERS. Thalidomide was orally administered on days 4–12 inclusive of pregnancy. Animals were killed on the 15th day of pregnancy. Foetuses were examined for external and internal malformations.

Treatment	No. of animals	Total no. of implantations	Average no. of implantations ± s.d.	Resorptions	Average litter size ± s.d.	Mean foetal body wt (g ± s.d.)	Normal foetuses	Malformed foetuses
Controls* ..	44	370	8·4 ± 3·0	62 (16·7%)	7·0 ± 2·4	1·8 ± 0·4	308	0
Thalidomide (1 g/kg) ..	24	224	9·3 ± 2·7	30 (13%)	8·1 ± 3·2	1·7 ± 0·3	194	0
Thalidomide (2 g/kg) ..	22	188	7·7 ± 3·1	26 (16%)	7·4 ± 2·5	2·0 ± 0·5	162	0

* Controls were given 1 ml 0·5% carboxymethylcellulose.

15-day old foetus, and the incidence of resorptions and malformed foetuses was not affected by oral administration of thalidomide (1 or 2 g/kg) daily on days 4–12 of pregnancy. These findings are similar to those found by Somers (1963) who administered doses of up to 8 g/kg throughout pregnancy without causing a reduction in litter size, malformations of the young or a significant increase in resorptions. Similar results have been found by Fratta & others (1965) and by Homburger & others (1965) in certain random bred strains of hamster.

FATE OF [^{14}C]THALIDOMIDE IN THE PREGNANT HAMSTER

Table 2 shows that the greater part of an oral dose of thalidomide in pregnant hamsters is absorbed and excreted mainly (84%) in the urine

TABLE 2. EXCRETION OF ^{14}C BY PREGNANT HAMSTERS AFTER A SINGLE ORAL DOSE OF [^{14}C]THALIDOMIDE. [^{14}C]Thalidomide (150 mg/kg; 20 μc/kg) was administered orally to pregnant hamsters on the 204th hr of pregnancy.

	% of doses of ^{14}C found in				
	Urine		Faeces		
Hamster no.	0–24 hr	24–72 hr	0–24 hr	24–72 hr	Total
18	78·4	7·4	2·8	5·3	93·9
19	68·5	2·7	2·8	9·0	83·0
20	77·0	10·4	0	7·6	95·0

with only about 9% in the faeces in the 3 days after dosing. The urinary ^{14}C in the 24 hr urine consists of thalidomide (3% of dose), α-(*o*-carboxybenzamido)glutarimide (26%), 2- and 4-phthalimidoglutaramic acids (8%), 2-phthalimidoglutaric acid (0·2%) and 2- and 4-(*o*-carboxybenzamido)glutaramic acids plus 2-(*o*-carboxybenzamido)glutaric acid (27%). These values are the means of three experiments. Fig. 1 shows the ^{14}C plasma levels at various times after an oral dose of [^{14}C]thalidomide.

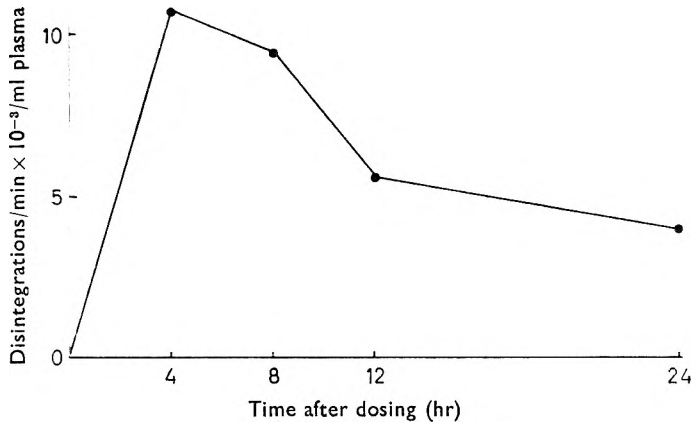

FIG. 1. Plasma ^{14}C levels after a single oral dose of [^{14}C]thalidomide (150 mg/kg) to pregnant hamsters.

It reaches a maximum at about 4 hr after dosing and then steadily declines; at 24 hr the level is about 40% of that at 4 hr. It can be seen from Table 3 that radioactivity passes to the embryo and that the relative concentrations of ^{14}C in the embryo and plasma are about the same at 4, 12 and 24 hr after dosing. At 4 hr after dosing about 50% of the plasma ^{14}C is thalido-

TABLE 3. DISTRIBUTION OF ^{14}C IN THE MATERNAL PLASMA AND EMBRYO AFTER THE ORAL ADMINISTRATION OF [^{14}C]THALIDOMIDE TO PREGNANT HAMSTERS. [^{14}C]Thalidomide (150 mg/kg; 20 μc/kg) was administered orally to groups of pregnant hamsters on the 204th hr of pregnancy and they were killed at the times shown. Values are means; figures in parentheses refer to ranges.

Time after dosing (hr)	No. of animals	Disintegrations/min/g	
		Plasma	Embryo
4	4	10,176 (8,532–14,820)	11,363 (4,992–19,980)
12	4	6,762 (5,956–6,044)	7,741 (6,213–7,992)
24	4	3,190 (2,850–3,624)	2,921 (2,675–3,079)

TABLE 4. CONCENTRATION OF THALIDOMIDE AND ITS METABOLITES IN THE EMBRYO AND MATERNAL PLASMA AFTER THE ADMINISTRATION OF [^{14}C]THALIDOMIDE TO THE PREGNANT HAMSTER. See Table 3 for dose. Values are the means of three experiments.

Compound	Concentration (μg/g) in			
	plasma		embryo	
	4 hr	12 hr	4 hr	12 hr
Thalidomide	17·2	5·7	19·1	4·4
α-(*o*-Carboxybenzamido)glutarimide	14·1	8·3	15·0	7·2
2- and 4-Phthalimidoglutaramic acids	1·3	4·4	0·1	2·5
2-Phthalimidoglutaric acid	<0·1	<0·1	<0·1	<0·1
2- and 4-(*o*-Carboxybenzamido)glutaramic acids + 2-(*o*-carboxybenzamido)glutaric acid	0·8	2·2	0·1	10·8

mide, the rest is largely α-(*o*-carboxybenzamido)glutarimide with small amounts of other hydrolysis products. At 12 hr after dosing the plasma level of thalidomide has declined to about 30% of that at 4 hr, and the rest of the ^{14}C consists of hydrolysis products. Similarly, the embryo contains at 4 hr mainly thalidomide and α-(*o*-carboxybenzamido)-glutarimide but at 12 hr the level of thalidomide has declined to 20% of that at 4 hr and the rest of the ^{14}C consists of seven hydrolysis products (Table 4).

It is thus clear that an oral dose of thalidomide given to a pregnant hamster during the period of morphogenesis is able to penetrate to the conceptus and to persist for more than 12 hr as such. Therefore, the lack of embryotoxicity of thalidomide in the hamster is not due to the inability of the teratogen to reach the embryo.

Acknowledgement. This work was supported by a grant from the Association for the Aid of Crippled Children, New York. The Packard scintillation spectrometer used in this work was purchased by means of a U.S. Public Health Service research grant AM 06772-01 from the National Institutes of Health. The authors thank Professor R. T. Williams for his interest and encouragement.

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J. Pharm. Pharmac., 1967, **19**, 608–611 Received April 7, 1967

The tissue distribution and metabolism of amidopyrine in the rat and pregnant and non-pregnant rabbit

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The tissue distribution of amidopyrine and its metabolites 4-amino-antipyrine and *N*-acetyl-4-amino-antipyrine has been determined at intervals following oral doses of 300 mg/kg of amidopyrine in pregnant, non-pregnant and foetal rabbits, and also in adult non-pregnant rats. Except in lung, their concentration was higher in the normal rabbit than the rat. In both species amidopyrine levels were maximal at 2 hr, but the metabolite levels varied with tissue, species and time interval. The dominant pathways were demethylation in the rabbit and acetylation in the rat. Plasma and tissue levels of amidopyrine were higher in non-pregnant than pregnant rabbits, whereas those of the metabolites varied. Studies indicate that 25-day, but not 14-day foetuses may metabolize amidopyrine.

SPECIES differences in the metabolism of drugs is well known (Burns, Ross & others, 1953; Brodie, Burns & others, 1953; Perel, Chen & others, 1961), so too is the fact that drug metabolism varies with pregnancy and in neo-nates (Brodie & Maickel, 1962; Hartiala, Pulkkinen & Rauramo, 1963; Lessel & Cliffe, 1964).

In a systematic investigation on drug metabolism and tissue distribution (Crema & Berté, 1960; Mascherpa, 1963; Mascherpa & Berté, 1966), the behaviour of amidopyrine in different species, and in pregnancy and pre-natality, was examined because it is still used as an analgesic and antipyretic; its main metabolic pathway is known and involves two serial steps (demethylation to 4-aminoantipyrine and acetylation of this compound to *N*-acetyl-4-aminoantipyrine), other metabolites are also present with or without ring cleavage, and its physiological disposition is known in man and dog. The present paper describes the tissue distribution of amidopyrine and two metabolic products (4-aminoantipyrine and *N*-acetyl-4-aminoantipyrine) in the pregnant rabbit and in the rabbit foetus, and also in the adult non-pregnant rat and rabbit at different times after drug administration. The metabolic transformations in some rabbit adult and foetal tissues have also be examined.

Experimental

Tissue distribution of amidopyrine. Fifty-four adult female Wistar rats (220 ±5 g), 12 adult female Dutch rabbits (2·0 ±0·18 kg) and 12 female Dutch rabbits at the 25th day of pregnancy (2·6 ±0·24 kg) were used; amidopyrine was administered orally by stomach tube to the fasting animals at the dose of 300 mg/kg. The animals were killed by bleeding 1, 2, or 4 hr after drug administration and the lung, liver, kidney, brain, muscle, and placenta were immediately homogenized for 5 min with saline solution (200 mg of tissue/ml of homogenate). Amniotic fluid and heparinized blood were centrifuged for 20 min at 2,000 rev/min. The concentration of amidopyrine, 4-aminoantipyrine and *N*-acetyl-4-aminoantipyrine in the biological materials were estimated by the method of Brodie & Axelrod (1950).

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Amidopyrine metabolism. The organs of 12 pregnant Dutch rabbits (2·8 ±0·4 kg) were used; the animals were killed by bleeding at the 25th day of pregnancy and the kidney, liver, lung and brain were removed immediately from mother and foetus. The organs were homogenized with 3·3 volumes of potassium chloride (1·49% for the brain and 1·15% for the other organs) using a Potter type homogenizer. The homogenates (corresponding to 3·0 g of brain and 1·5 g of the other organs) were centrifuged at 9,000 rev/min for 15 min. The supernatants were mixed with 0·4 μmole NADP, 20 μmole glucose 6-phosphate, 50 μmole nicotinamide, 0·1 ml of M potassium chloride solution, 0·1 ml of 7·14% magnesium chloride solution, 0·5 ml of amidopyrine solution (600 μg/ml) and phosphate buffer (pH 7·4) to a final volume of 6 ml. The mixtures were incubated at 37° in an atmosphere of oxygen and shaken at 50 cycles/min. Every 15 min, for 1 hr, a sample of each organ was removed from the shaking incubator and stored at −10°.

The determination of amidopyrine, 4-aminoantipyrine and *N*-acetyl-4-aminoantipyrine was made (Brodie & Axelrod, 1960). The extinction was measured against a blank containing buffer solution instead of supernatant from the homogenates.

Similar experiments were made using the organs from five foetuses at the 14th day.

Results and discussion

The difference in the species in distribution and metabolism of amidopyrine is seen in Fig. 1. With exception of lung, the concentrations of

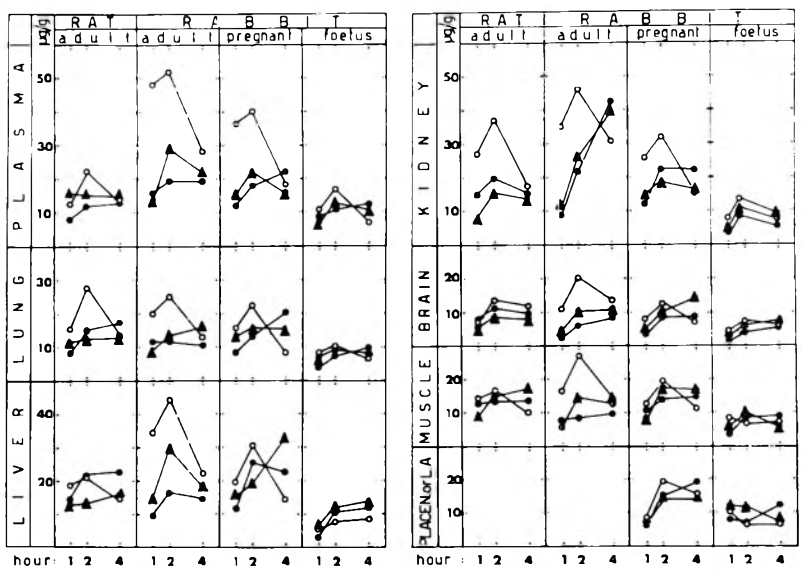

FIG. 1. Distribution (μg/g, on ordinate) of amidopyrine (○—○), 4-aminoantipyrine (●—●) and *N*-acetyl-4-aminoantipyrine (▲—▲), at 1, 2, and 4 hr (on abscissae) after oral administration of 300 mg/kg of amidopyrine to non-pregnant adult rats and rabbits and to rabbits on the 25th day of pregnancy, Placen. = placenta; L.A. = amniotic fluid.

the substance are much higher in organs of the normal rabbit than in the rat. In both animals the amidopyrine concentrations of the organs have a maximum value at the second hour, whereas the 4-aminoantipyrine and the *N*-acetyl-4-aminoantipyrine distribution differs greatly in the various tissues at the different examination times. Also, except in kidney, the acetylated metabolite concentrations are higher in rat than in rabbit, in spite of the lower amidopyrine concentration in plasma. Furthermore, with the exception of plasma and muscle, the 4-aminoantipyrine/*N*-acetyl-4-aminoantipyrine ratio concentration is reversed in rabbit compared to the rat. These data confirm the high activities of demethylation in rabbit and acetylation in rat.

The distribution of amidopyrine and its two metabolites in the tissues including the foetus of the 25-day pregnant rabbit is also shown in Fig. 1. The plasma and tissue levels of the drug are higher in non-pregnant than in the pregnant rabbit, while the concentrations of the two metabolites in the various organs show many differences at the different times of examination. The diffusibility of amidopyrine across the placenta to the foetus is indicated by the presence of the drug in the foetal tissues, although the concentrations are constantly lower than those of the mother. The presence of metabolic products in the organs of the foetus may be due to maternal metabolism, or to placental metabolic activity, or to foetal drug metabolism. To explain this last possibility, we have investigated the amidopyrine metabolism by some organs from mother and 25 day foetus, *in vitro* as indicated in Fig. 2. These foetal tissues can metabolize the

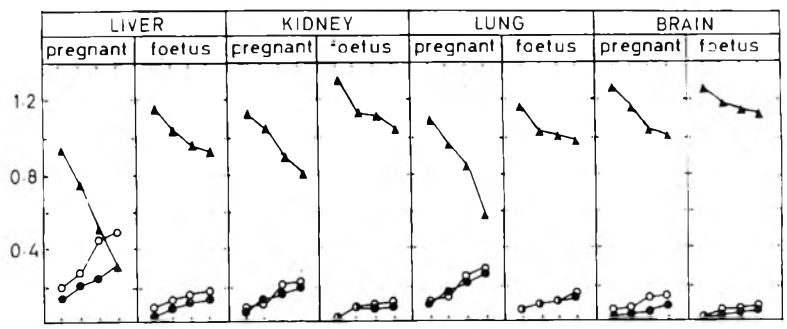

FIG. 2. Metabolism *in vitro* of amidopyrine, for 1 hr at 15 min periods (on abscissae) after substrate activation. The ordinate shows the μmole of amidopyrine (initial value = 1·29 μmole) (▲—▲), 4-aminoantipyrine (●—●) and *N*-acetyl-4-aminoantipyrine (○—○). The data relate to organs from rabbits on 25th day of pregnancy.

amidopyrine although their activity is much lower than that of the mother's organs. On the contrary, no metabolic activity appeared in the organs of 14-day foetuses.

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J. Pharm. Pharmac., 1967, **19**, 612–616 Received March 6, 1967

Influence of the route of administration on the toxicity of some cholinesterase inhibitors

I. L. NATOFF

The acute median lethal doses of a series of cholinesterase inhibitors have been estimated in mice for different routes of administration Differences in the LD50 values obtained for intraperitoneal and oral routes ("hepatic" routes) and subcutaneous and intravenous routes ("peripheral" routes) suggest that the availability of the compound for metabolism by the liver is a major factor in their toxicity.

DIFFERENCES in the route of administration of biologically active compounds may influence their quantitative effects. Thus, tyramine has a more potent pressor effect in cats after intravenous injection than after intraportal injection (Natoff, 1965). Gaines, Hayes & Linder (1966) showed that solutions of some cholinesterase inhibitors produce muscular fasciculation more rapidly when infused into rats by the femoral vein than by the intestinal vein. Ramachandran (1966a,b) showed that di-isopropylphosphorofluoridate (DFP) is more toxic to mice when injected subcutaneously than when injected intraperitoneally.

These observations suggest that the availability of these compounds to the liver may account for the differences in their quantitative effects. To substantiate this, some cholinesterase inhibitors have been administered to mice by different routes and their median lethal doses (LD50) estimated.

Experimental

MATERIALS

Animals. Female albino mice, C.F.E. No. 1 strain, weighing 19 $\pm$5 g, were used at each dose level in groups of 10.

Drugs. The carbamates examined were physostigmine salicylate B.P. (B.D.H.) and neostigmine methylsulphate B.P. (Roche).

The organophosphates examined were Azodrin, Bidrin, chlorfenvinphos, Ciodrin, Phosdrin, parathion (Cheminova) and paraoxon (Baywood Chemicals Ltd.), the structural formulae of which are in Table 1.

TABLE 1. CHEMICAL FORMULAE OF THE ORGANOPHOSPHORUS CHOLINESTERASE INHIBITORS EXAMINED

$(RO)_2P(O)OC(R')=CH(R'')$

Compound	R	R′	R″
Azodrin	Me	Me	–CO·NH·Me
Bidrin	Me	Me	–CO·NMe$_2$
Chlorfenvinphos	Et	2,4-dichlorophenyl	Cl
Ciodrin	Me	Me	–CO·O·CH(Me)Ph
Phosdrin	Me	Me	–CO·OMe
Paraoxon	$(EtO)_2P(O)O(p\text{-}NO_2C_6H_4)$		
Parathion	$(EtO)_2P(S)O(p\text{-}NO_2C_6H_4)$		

From "Shell" Research Ltd., Tunstall Laboratory, Sittingbourne, Kent.

TABLE 2. 24 HOUR MEDIAN LETHAL DOSES OF CHOLINESTERASE INHIBITORS FOLLOWING ADMINISTRATION BY DIFFERENT ROUTES IN FEMALE MICE

Compound	Molecular weight	Active material %	LD50, mg/kg body weight (95% fiducial limits)				LD50, μmole/kg body weight			
			Hepatic routes		Peripheral routes		Hepatic routes		Peripheral routes	
			Intra-peritoneal	Oral	Sub-cutaneous	Intra-venous	Intra-peritoneal	Oral	Sub-cutaneous	Intra-venous
Physostigmine salicylate ..	413·45	100	*ca* 1·0 (—)	5·50 (4·02–7·83)	1·12 (0·85–1·65)	0·46 (0·38–0·56)	2·42	13·30	2·71	1·11
Neostigmine methylsulphate ..	334·39	100	0·62 (—)	>5·0† (—)	0·66 (0·56–0·80)	0·47 (0·28–0·80)	1·85	>15·00†	1·97	1·41
Azodrin	223·17	100	8·91 (4·31–18·4)	14·4 (9·80–21·3)	8·71 (6·06–12·5)	*ca* 9·2 (—)	39·92	64·52	39·03	41·22
Bidrin	237·20	86·8	11·8 (10·5–14·6)	20·0 (15·8–25·2)	11·5 (9·60–13·7)	*ca* 9·9 (—)	43·18	73·19	42·08	36·23
Chlorfenvinphos	359·59	92	87·0* (71·0–112·0)	398* (340–466)	339* (256–448)	87 (64–118)	222·59*	1018·27*	867·32*	222·59
Ciodrin	314·28	86	70·8 (51·1–98·0)	186·2 (171·7–201·9)	15·1 (—)	4·5 (3·9–5·2)	193·73	509·52	41·32	12·31
Phosdrin	224·14	100	2·51 (2·20–2·86)	12·30 (10·85–13·95)	1·18 (0·77–1·80)	0·68 (—)	11·20	54·88	5·26	3·03
Paraoxon	275·21	99	2·29 (—)	12·80 (—)	ca. 0·6 (—)	0·59 (0·53–0·66)	8·24	46·08	2·16	2·12
Parathion	291·27	97	15·1 (12·8–17·6)	25·7 (—)	21·4 (18·0–25·4)	17·4 (11·8–25·7)	50·29	85·59	71·27	57·95

*Vehicle = Dimethylsulphoxide. † Available material did not allow examination of higher concentrations.

These compounds were dissolved or suspended by ultrasonic agitation in physiological saline immediately before administration. Dimethylsulphoxide was used as the vehicle for estimating the LD50 of chlorfenvinphos by the oral, intraperitoneal and subcutaneous routes because of the poor stability of ultrasonic dispersions in saline, but the compound was suspended in physiological saline for intravenous administration and injected immediately.

METHODS

Median lethal doses were estimated in groups of mice by different routes of administration on the same day. The routes examined were classified as "hepatic" (oral and intraperitoneal) and "peripheral" (subcutaneous and intravenous). Preliminary experiments revealed the order of magnitude of the LD50 and the required logarithmic dose interval. The mice were observed for 24 hr after injection, and the LD50 values calculated with subsequent application of fiducial limits.

The volume of material injected was 10 ml/kg body weight for all routes of administration.

Results

Table 2 shows the estimated median lethal doses for each compound by different routes of administration. The values are expressed as mg of original compound per kg body weight, with 95% fiducial limits, and as μmol/kg body weight after correction for the proportion of active material. The term "active material" relates to the proportion of the original sample having biological activity.

In some instances, fiducial limits could not be calculated because of the high gradient of the log dose-mortality curve preventing a sufficient number of observations between 0 and 100% mortality.

Discussion

The toxicity and metabolism of organophosphorus and carbamate cholinesterase inhibitors have been examined by many workers. Although metabolic detoxification products have been identified, the site of their production within the body has not always been specified. Mazur (1946) demonstrated the occurrence of "DFPase" in the liver, and DFP has been shown to be more toxic to mice on intravenous or subcutaneous injection than on intraperitoneal injection. This suggested that DFP is detoxified by the liver (Ramachandran, 1966a).

Compounds administered intravenously or subcutaneously ("peripheral" routes) would enter the peripheral venous circulation directly and only about 27·5% would traverse the liver during the first passage through the body (Gaines & others, 1966). Administration of the compounds orally or intraperitoneally ("hepatic" routes) results in their access to the peripheral venous circulation being predominantly by way of the hepatic portal system. Differences in LD50 values between the "peripheral" and the "hepatic" routes therefore indicate the effect of the liver on the

biological activity of the compound. Moreover, differences between the intravenous and subcutaneous toxicities, or between the intraperitoneal and oral toxicities, indicate variations in the degree or rate of absorption from the appropriate sites. Any reduction in the rate of absorption would allow a greater net efficiency of the detoxifying process. Estimates of the LD50, which are similar between subcutaneous and intravenous routes but different between oral and intraperitoneal routes, suggest poor absorption of the compound from the alimentary tract, or its breakdown within the lumen

Ciodrin, Phosdrin and paraoxon were more toxic by the "peripheral" routes than they were by the "hepatic" routes, suggesting detoxification during passage through the liver. Ciodrin and Phosdrin were also more toxic intravenously and intraperitoneally than they were subcutaneously and orally. This indicates poor absorption both from the intestine and from the subcutaneous site.

Paraoxon was equitoxic subcutaneously and intravenously. However, not only was it less toxic on "hepatic" administration, but the high LD50 value orally suggests poor absorption from the alimentary tract, or breakdown of the compound within the tract.

The carbamates physostigmine and neostigmine were more toxic intravenously than intraperitoneally, suggesting their detoxification by the liver. This agrees with the findings of Roberts, Thomas & Wilson (1965), who showed that within 10 min of the intramuscular injection of [^{14}C]neostigmine into rats, approximately 98% of the radioactivity in the liver was due to a metabolite, the production of which could be reduced by pretreatment with the liver microsome inhibitor SKF 525-A. Although the LD50 values of neostigmine and physostigmine were greatest after oral administration (Table 2), there is evidence of poor absorption of each compound because of differences in the subcutaneous and intravenous LD50 values.

The toxicity of Azodrin, Bidrin, and chlorfenvinphos did not appear to be reduced by "hepatic" administration. Azodrin and Bidrin are chemical congeners and Bidrin is *N*-demethylated *in vivo* to yield Azodrin (Bull & Lindquist, 1964; Menzer & Casida, 1965). The order of toxicity on a molar basis was similar by all four injection routes; oral administration caused the lowest toxicity.

Chlorfenvinphos was the only organophosphate studied which showed no evidence of a lower toxicity after administration by "hepatic" routes in mice. Hutson & Hathway (1967) found in the dog that the concentration of extractable chlorfenvinphos in the portal venous blood after oral dosing exceeded that in the peripheral venous blood, and that the compound had a lower toxicity in the dog than in either the mouse or the rat. Chlorfenvinphos therefore appears to be detoxified by the liver of the dog, but not by that of the mouse. The LD50 values obtained on "peripheral" and "hepatic" administration were similar, but differences for the values obtained for the individual routes within these groupings suggest that chlorfenvinphos is not readily absorbed from the alimentary tract and the subcutaneous site.

Parathion is a potent inhibitor of cholinesterase *in vivo* but not *in vitro*. It is oxidized by the liver to yield the active metabolite, paraoxon (Gage, 1953). Gaines & others (1966) found less parathion was required to produce muscular fasciculation in anaesthetized rats when it was infused into an intestinal vein than when it was infused into the femoral vein, suggesting that the liver is involved in the production of the biologically active product. Although parathion appeared to be more toxic to mice on intraperitoneal injection (Table 2) than on intravenous injection, this difference was not significant. As observations with paraoxon indicate that it is inactivated by the liver, the toxicity of parathion will depend on the resultant of the rate of hepatic conversion to paraoxon and the rate of inactivation of this compound.

Acknowledgements. The author wishes to thank Mr. G. R. Ellender for skilled technical assistance, and the Statistics Unit of Woodstock Agricultural Research Centre for the computer assessment of the median lethal doses, and of the fiducial limits.

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J. Pharm. Pharmac., 1967, **19**, 617–620 Received February 27, 1967

Antagonism of anti-inflammatory drugs on bradykinin-induced increase of capillary permeability

E. ARRIGONI MARTELLI

Phenylbutazone, sodium salicylate, calcium acetylsalicylate and sodium mefenamate each antagonize the bradykinin-induced increase of capillary permeability in the rat paw. The method described was not affected to any extent by urethane anaesthesia nor by mecamylamine, papaverine, reserpine or chlorothiazide. Hexobarbitone partially inhibited the bradykinin effect, as did bretylium and acetic acid.

A possible method for evaluating the anti-inflammatory action of drugs is to test them as specific inhibitors of the humoral mediators of inflammation such as bradykinin. This plasma kinin provokes vasodilatation, increased capillary permeability, pain, and accumulation and migration of leucocytes (Lewis, 1963, 1964; Rocha e Silva, 1964). Bradykinin, when administered intravenously to guinea-pigs, also increases resistance of the lungs to inflation (Collier, 1963). The bronchospasm is inhibited by drugs like salicylate and phenylbutazone (Collier & Shorley, 1960, 1963). Increase of capillary permeability is an action of primary importance in the development of the inflammatory reaction; for this reason it seemed of interest to examine the effect of some anti-inflammatory drugs on the capillary permeability induced by bradykinin in the rat paw and to assess to what extent non-specific pharmacological effects interfered with this action of bradykinin.

Experimental

METHODS

Female Sprague Dawley rats weighing about 150 g were used.

The drugs (phenylbutazone, sodium salicylate, calcium acetylsalicylate, sodium mefenamate) were administered intraperitoneally dissolved in saline; 1 hr later bradykinin, histamine, or 5-hydroxytryptamine (5-HT) was injected dissolved in saline (pH 6·5–7·5) in a volume of 0·05 ml through a 26-gauge needle into the plantar tissue of the posterior right paw. Immediately after, azovan blue 1% solution (5 ml/kg) was administered intravenously. One hr later the animals were killed by exsanguination, and the paws were amputated at tarso-crural level and weighed. The dye content of the tissues was determined as described by Beach & Steinetz (1961).

Results

Phenylbutazone, sodium salicylate, calcium acetylsalicylate and sodium mefenamate antagonized the bradykinin-induced increase of capillary permeability. The effect of these drugs could be overcome by higher doses of bradykinin, the amount needed to produce an effect similar to that observed in control animals (i.e. the same content of dye in the tissues of the rat paw) increasing with the dose of antagonist administered.

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Phenylbutazone, sodium salicylate, and calcium acetylsalicylate also antagonized the effects of histamine and 5-HT, but to a lesser extent, whereas sodium mefenamate at higher dose enhanced these effects. A similar result was described by Collier & Shorley (1963) in experiments on guinea-pig lungs *in vivo*. Table 1 shows the results obtained.

TABLE 1. ANTAGONISM OF PHENYLBUTAZONE, SODIUM SALICYLATE, CALCIUM ACETYLSALICYLATE AND SODIUM MEFENAMATE TO THE BRADYKININ, HISTAMINE, OR 5-HT INDUCED INCREASE OF THE CAPILLARY PERMEABILITY IN RAT PAW

Antagonist	Dose mg/kg i.p.	Bradykinin (μg) needed to restore response (95% fiducial limits)*	Histamine	5-HT
Phenylbutazone	50	0·11 (0·07–0·15)	—	—
"	100	0·24 (0·19–0·27)	1·23 (0·96–1·57)	0·13 (0·09–0·17)
"	200	0·29 (0·24–0·32)	2·56 (1·97–3·12)	0·20 (0·15–0·24)
Sodium salicylate	100	0·07 (0·05–0·12)	—	—
"	200	0·28 (0·20–0·36)	1·47 (1·05–2·12)	0·07 (0·04–0·90)
"	400	0·41 (0·36–0·48)	4·56 (3·72–5·24)	0·23 (0·19–0·30)
Calcium acetylsalicylate	100	0·07 (0·04–0·10)	—	—
"	200	0·24 (0·18–0·30)	1·36 (1·02–1·85)	0·09 (0·10–0·13)
"	400	0·41 (0·35–0·48)	3·95 (3·15–4·71)	0·29 (0·22–0·37)
Sodium mefenamate	50	0·10 (0·07–0·16)	1·49 (1·18–1·97)	0·08 (0·04–0·10)
"	100	0·20 (0·13–0·24)	—	—
"	200	0·21 (0·16–0·26)	0·46 (0·32–0·68)	0·02 (0·01–0·05)

* After obtaining in untreated rats a standard response (a threefold increase of azovan blue dye content relative to controls) to 0·05 μg of bradykinin or to 1·0 μg of histamine or to 0·05 μg of 5-HT, a dose of antagonist was administered and the dose of bradykinin or histamine, or 5-HT giving a response comparable to the standard was then determined. The results reported are the means of 20 determinations.

Section of the spinal cord at various levels and general anaesthesia are known to reduce the development of local oedemas (Domenjoz, 1954). Hexobarbitone (70 mg/kg i.p.) produced a threefold increase in the dose of bradykinin needed to produce an extravasation of azovan blue dye comparable to that observed in non-anaesthetized rats, but the barbiturate did not affect the antagonistic potency of the anti-inflammatory drugs examined. In fact the ratios between the threshold dose of bradykinin before and after administration of test compounds in normal and anaesthetized rats were similar Urethane did not interfere with the action of bradykinin. The results are in Table 2.

TABLE 2. INFLUENCE OF URETHANE OR HEXOBARBITONE ANAESTHESIA ON THE "BLUEING" OF THE RAT PAW CAUSED BY BRADYKININ AND ON THE ANTAGONISTIC EFFECT OF ANTI-INFLAMMATORY DRUGS

Treatment	Threshold dose of bradykinin μg*		
	Normal	Urethane 700 mg/kg i.p.	Hexobarbitone 70 mg/kg i.p.
Saline 3 ml/kg i.p.	0·05 (0·02–0·09)	0·06 (0·02–0·09)	0·13 (0·07–0·02)
Phenylbutazone 200 mg/kg i.p.	0·28 (0·21–0·37)	0·31 (0·02–0·42)	0·59 (0·32–0·78)
Sodium salicylate 200 mg/kg i.p.	0·28 (0·19–0·37)	0·30 (0·21–0·43)	0·73 (0·06–0·95)
Calcium acetylsalicylate 200 mg/kg i.p.	0·23 (0·16–0·32)	0·25 (0·18–0·35)	0·69 (0·49–0·85)
Sodium mefenamate 200 mg/kg i.p.	0·20 (0·13–0·36)	0·23 (0·14–0·39)	0·59 (0·29–0·74)

* The threshold dose is the dose of bradykinin which provokes a threefold increase in azovan blue dye content of the rat paw relative to control animals. In brackets the range of dose in 10 determinations.

As a number of non-specific pharmacological effects are known to interfere with development of local oedemas (Garattini, Jori & others,

1964) the effects of an antihypertensive drug (bretylium), a ganglion-blocking drug (mecamylamine), a vasodilator (papaverine), a catecholamine-depleting agent (reserpine), a diuretic drug (chlorothiazide), and a general irritant (acetic acid) have been evaluated on the bradykinin-induced blueing of the rat paw. The results in Table 3 show that, of the

TABLE 3. EFFECT OF VARIOUS AGENTS ON THE INCREASED CAPILLARY PERMEABILITY INDUCED BY BRADYKININ

Compounds	% Inhibition of azovan blue dye extravasation in rat paw induced by bradykinin (0·05 μg)	No. of experiments
Bretylium 20 mg/kg i.p.	47 (32–59)*	15
Mecamylamine 5 mg/kg s.c.	7 (2–13)	15
Papaverine 10 mg/kg i.p.	8 (3–16)	15
Reserpine 5 mg/kg i.p. (for 3 consecutive days)	12 (7–19)	20
Chlorothiazide 20 mg/kg s.c.	11 (5–19)	20
Acetic acid 0·6–10 ml/kg i.p.	65 (31–83)	15

* Figures in parentheses give range.

compounds examined, bretylium and acetic acid inhibit significantly the extravasation of dye induced by bradykinin whereas the other drugs were without effect.

Discussion and conclusion

The anti-inflammatory drugs examined inhibited the bradykinin-induced increase of capillary permeability.

Over the dose range used, the order of antibradykinin potency was: phenylbutazone > mefenamate > salicylate > acetyl salicylate. This order agrees with the findings of Collier & Shorley (1963) in the guinea-pig bronchospasm test and is similar to that obtained in the skin erythema test (Winder, Wax & others, 1963).

The results reported here also demonstrate differential effects on different agonists when the dose of antagonist is raised.

The capillary permeability induced by bradykinin appears to be less sensitive to systemic pharmacological effects than do local oedemas induced by various agents; changes in cardiovascular function, depletion of tissue catecholamines and induced diuresis do not affect the test. The inhibitory action of bretylium could be ascribed to its enhancing effect on free or circulating adrenaline and noradrenaline which are likely to be acting as local anti-inflammatory hormones (Spector & Willoughby, 1964). The effect of acetic acid is a particular aspect of a known "inhibitory" effect of the general irritation on the inflammatory processes (Garattini & others, 1964).

Urethane anaesthesia does not influence the capillary permeability induced by bradykinin, whereas hexobarbitone anaesthesia partially inhibits this effect of bradykinin, possibly through depression of the central

nervous system, the role of which in the inflammatory response has been pointed out by Chapman & Goodell (1966). However the barbiturate does not modify the antagonistic action of anti-inflammatory drugs expressed in terms of dose-ratios.

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The binding of salicylate to plasma proteins in different species

SIR,—In man, salicylate is extensively bound to serum proteins, and the extent of binding decreases with increasing salicylate concentration. Davison & Smith (1961), using an equilibrium dialysis technique, found that human albumin showed a greater affinity for salicylate at low concentrations than bovine albumin. Potter & Guy (1964) employed a dextran-gel filtration system and reported that dog plasma bound much less salicylate than human plasma. These results suggested that there may be some difference in the affinities between salicylate and plasma proteins in different species. We have therefore investigated the binding curves of salicylate and plasma from man, baboon, rhesus monkey, horse, dog, rabbit, guinea-pig, rat, mouse, turkey and the toad.

Blood samples were collected in lithium heparin bottles, centrifuged at 2000 *g* for 30 min and the separated plasma either used immediately or stored at −20°. Sodium salicylate was added to 0·5 ml aliquots of the plasma samples to give final concentrations up to 2 mM and the mixtures allowed to equilibrate at room temperature for at least 60 min. The protein-bound and free salicylate fractions (see Potter & Guy, 1964) were separated by passing 0·1 ml of each sample through a Sephadex G25 (fine beaded form) dextran gel and subsequently eluting with 2·8 and 5·0 ml quantities of 0·1M potassium phosphate buffer, pH 7·4. The salicylate in the separated fractions was determined with an Aminco-Bowman spectrophotofluorometer using an activating wavelength of 310 mμ and detecting fluorescence at 420 mμ, both values being the maxima for salicylate with the particular instrument employed.

The results showed that the various species differed in the extent to which salicylate was bound by their plasma proteins. The species could be separated into two main groups. The first, comprising baboon, horse, dog, rat, mouse, turkey and toad exhibited a low protein-binding capacity for the drug (Fig. 1). The second group (rhesus monkey, rabbit and guinea-pig) resembled man in

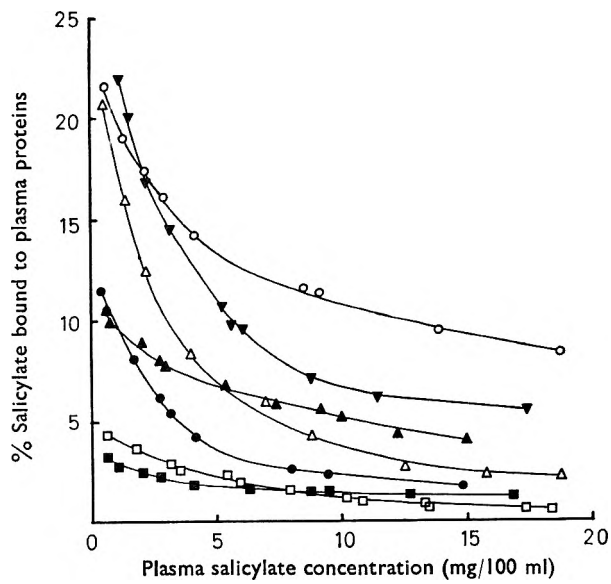

FIG. 1. Binding curves of salicylate to plasma proteins. ●, Baboon; ■, rat; ▲, mouse; ▼, horse; ○, dog; □, toad; △, turkey.

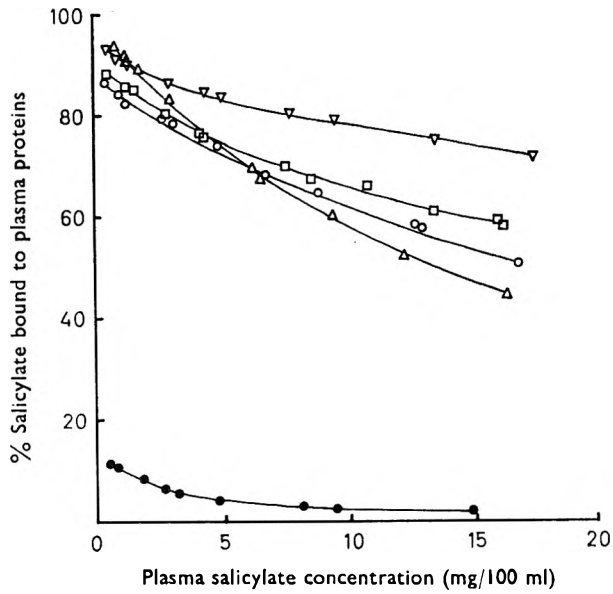

FIG. 2. Binding curves of salicylate to plasma proteins. ●, Baboon; ○, monkey; □, man; △, guinea-pig; ▽, rabbit.

possessing a much greater affinity for binding salicylate to the plasma protein (Fig. 2). The binding curve for the baboon, one of the first group, has been included in Fig. 2 to illustrate this point. No species giving intermediate values for protein binding of salicylate was found in the present work and there was an apparently clear differentiation between the two groups.

In human plasma, salicylate principally binds to the albumin fraction (Reynolds & Cluff, 1960) and there is some evidence that this involves the ϵ-amino- and possibly the guanidino-group of the protein (Davison & Smith, 1961). The present results show that the plasma proteins of some species are less capable of binding salicylate and this may be due to a relative deficiency of ϵ-amino- and guanidino-groups in the molecules of their albumin fractions.

The present work also suggests that greater proportions of free salicylate would occur in the circulation of the low-binding species after the administration of the drug. In these species there would be an increased entry of free salicylate into the cells from the blood and an increased rate of elimination of the drug from the circulation. Thus the species which show a low capacity to bind salicylate to their plasma proteins may be at higher risk, i.e. the LD50 values for salicylate would be lower, and may show lower values for the circulating half-life of salicylate than the species which possess a much greater affinity for binding salicylate to the plasma proteins.

Acknowledgements. We are grateful to Professor P. M. Daniel and Miss M. Sandiford for some of the samples of plasma and to the King's College Hospital and Medical School Research Committee for financial assistance.

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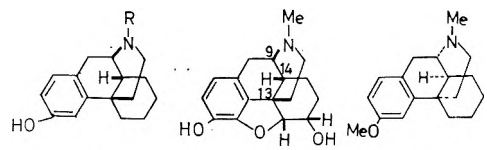
Steric correlation between (−)-3-hydroxy-*N*-methylmorphinan and (−)-morphine and related compounds

SIR,—The recently published investigation (Casy & Hassan, 1967) of the optical rotatory dispersion characteristics of (−)-3-hydroxy-*N*-methylmorphinan (levorphanol, I; R = Me) and (−)-morphine (II) gave strong evidence that the configuration of the C-9, 13 and 14 asymmetric centres of I are the same as those of the corresponding centres of II. These results are in agreement with previous conclusions (Beckett & Anderson, 1960) based on work involving stereoselective adsorbents.



I II III

May we draw your attention to the fact that the stereochemical problem discussed above has already been unambiguously solved by chemical degradation studies (Corrodi, Hellerbach & others, 1959). These findings proved further that the morphine antagonist (−)-3-hydroxy-*N*-allylmorphinan (levallorphan, I; R = $-CH_2-CH=CH_2$) has the same configuration as levorphanol (I; R = Me), while the cough-relieving compound (+)-3-methoxy-*N*-methylmorphinan (dextromethorphan) corresponds to the enantiomeric structure III.

In addition, the degradation experiments showed that the structural formulae I, II, III represent the absolute configurations, thus providing a more fundamental basis for understanding of the biological actions of these substances and the responses of their biological receptors.

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An acetylcholine-like effect of 2′(3-dimethylaminopropylthio)cinnamanilide

SIR,—The anti-5-hydroxytryptamine substance 2′(3-dimethylaminopropylthio)cinnamanilide (or Squibb compound 10,643), has been shown to be highly specific on the isolated rat uterus preparation (Rubin, Piala & others, 1964). It also has an anti-5-HT action on the bioluminescence of *Meganyctiphanes norvegica* (Doyle, 1966), but it has an acetylcholine-like effect on the isolated heart of the mollusc, *Mya arenaria*.

Work at the Squibb laboratories showed that this antagonist had about the same anti-5-HT activity as lysergic acid diethylamide on the isolated rat uterus and did not inhibit acetylcholine contractions in doses which were much larger than those sufficient to block the effect of 5-HT (Krapcho, Rubin & others, 1963; Rubin & others, 1964).

Recent work in this laboratory has shown that compound 10,643 has an acetylcholine-like effect on the isolated heart preparation of *Mya arenaria*. This mollusc heart responds to 5-HT by an increase in frequency and an increase in amplitude of heart beat, with sometimes an increase in tone. Acetylcholine causes a depression of the heart beat and a relaxation of the heart. Similarly Squibb 10,643 causes a depression of the heart beat and a relaxation of the heart. All these effects are easily removed by flushing the isolated organ bath with fresh isotonic solution or with filtered sea water. Doses of 5-HT and of Squibb 10,643 can be found which separately cause stimulation and depression, but which, given together, have no resultant effect on the recorded heart beat.

Concentrations of atropine which decrease the great sensitivity of the oestrous rat uterus preparation to acetylcholine fail to modify the sensitivity of the uterus to 5-HT (Amin, Crawford & Gaddum, 1954). Atropine has no anti-acetylcholine effect on the mollusc heart. Benzoquinonium chloride (Luduena & Brown, 1952) is an antagonist of the effect of added acetylcholine on several mollusc heart preparations, including that of *Mya arenaria*. Stimulation of the cardiac nerve of the clam, *Venus mercenaria*, during perfusion of the heart with benzoquinonium chloride caused acceleration of the heart beat, showing a blocking of the depressive action of acetylcholine released at the nerve endings (Welsh, 1953).

It has been reported that compound 10,643 inhibits the effects of 5-HT endogenously produced after the administration of its precursor, 5-hydroxytryptophan, in rats and mice, because it antagonizes the development of gastric erosions in rats and the cerebral excitation found in mice after the dosing of these animals with 5-hydroxytryptophan (5-HTP) (Rubin & others, 1964). Heart muscle of the mollusc, *Venus mercenaria*, has been shown to be capable of decarboxylating 5-HTP to 5-HT (Welsh & Moorhead, 1959). It is therefore possible that, in the heart preparation of *Mya arenaria*, the acetylcholine-like effect of this anti-5-HT compound is an unmasking of the action of an intrinsic acetylcholine-like substance by inhibition of the conversion of endogenous 5-HTP to 5-HT. But it must not be forgotten that the compound 10,643 can also counteract added 5-HT as in the rat isolated uterus preparation, so it may not, on the *Mya* heart be interfering with 5-HT metabolism, but simply with the action of already formed endogenous 5-HT.

Acknowledgements. Compound 10,643, was a gift from the Squibb Institute for Medical Research, New Brunswick, U.S.A. I thank the boat crews of the Marine Station, Millport for collecting the animals used.

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Release of [^{3}H]noradrenaline from vasoconstrictor nerves

SIR,—The release of noradrenaline by nerve impulses from vasoconstrictor nerves is of great cardiovascular significance. This has been examined in the perfused hind limb of the dog (Rosell, Kopin & Axelrod, 1963) and more extensively in the cat spleen (see reviews by Brown, 1965; Gillespie, 1966). However, these experiments are complicated by the presence of non-vascular smooth muscle and concurrent fluctuations of perfusion parameters during nerve stimulation.

We have investigated the noradrenaline release in the structurally simple rabbit pulmonary artery which is adrenergically innervated (Bevan & Su, 1964; Verity & Bevan, 1966). This artery was cut spirally into a strip as small as 3 × 40 mm, weighing 40 mg. It was connected under a 2 g tension to an isometric strain gauge transducer, and mounted in a 2 ml tissue bath containing Krebs solution at 37° which was constantly stirred by bubbles of oxygen 95% and carbon dioxide 5%. The intramural adrenergic nerves were stimulated by a 2 min train of square wave impulses (0·3–1 msec duration, 10 cycles/sec, near maximal voltage), using platinum wire electrodes placed on either side of the strip. The contraction following stimulation was registered on a pen recorder.

The artery strip was initially incubated in Krebs solution containing 5 μc/ml (0·486 μM) of [(±)-7-^{3}H-noradrenaline]* hydrochloride (specific activity 10·28 c/mmole) for 30 min. This medium was then flushed and plain Krebs solution introduced into the tissue bath at a constant rate (1–2 ml/min). The overflow was collected in 1 ml aliquots for assay of tritium activity by scintillation spectrometry.

Thirty min after commencement of washing out, the first period of nerve stimulation was applied, and this elicited a sharp rise in the tritium outflow and muscle contraction (Fig. 1). Both responses returned to the baseline levels within 20 min. At this interval, stimulations were repeated up to 14 times with consistent contractile responses and a constantly diminishing but significant rise in tritium outflow. The first period of stimulation brought about a disproportionately great tritium output as compared to subsequent stimulations. Thus, in Fig. 1, the first peak represented a total of 93·0 nc tritium output, whereas the second, third and fourth peaks amounted only to 19·4, 11·8 and 9·9 nc, respectively. It is possible that some [^{3}H]noradrenaline was initially present either in the extracellular space or in an easily releasable form and was expelled by the first period of stimulation. The [^{3}H]noradrenaline

* 2-Amino-1-(1,3-dihydroxyphenyl)-[1-^{3}H]ethanol.

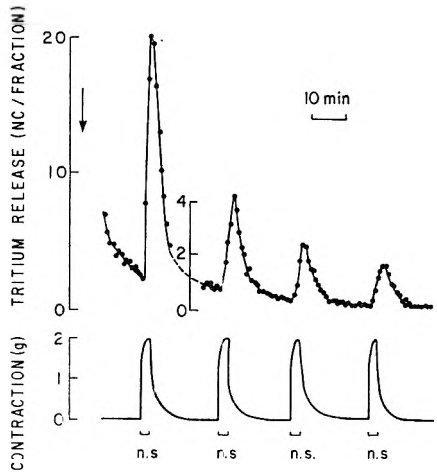

FIG. 1. Isolated rabbit pulmonary artery. After pretreatment of the artery with 5 μc/ml of [^{3}H]noradrenaline for 30 min, continuous flow (1·3 ml/min) of Krebs solution was introduced at arrow. The outflow was collected in 1 ml fractions for assay of tritium activity. Muscle tension was simultaneously recorded, and nerve stimulation applied at n.s.

subsequently released may have mixed more thoroughly with the endogenous noradrenaline and thus better represented the latter.

In several experiments, the pulmonary artery strip was incubated with [^{3}H]noradrenaline at 37° and then irrigated at 25°. When nerve stimulation was applied, delayed and diminished contraction resulted compared to that observed at 37°. The tritium outflow was also delayed and prolonged, and the total outflow per stimulation period reduced as the result of cooling.

Although the [^{3}H]noradrenaline released from the adrenergic nerves is probably in part metabolized and rebound (Axelrod, 1965), the tritium outflow under the above conditions may serve as an indicator of the neural release of endogenous noradrenaline. In view of the simplicity of the preparation and the high sensitivity of the technique, this appears to be a useful method for investigating transmitter release by vasoconstrictor nerves in particular, and the adrenergic neuroeffector transmission in general.

This investigation was supported by Grant H-8359 from the U.S. Public Health Service.

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Evidence for dopamine receptor stimulation by apomorphine

SIR,—Recently, Ernst (1967) has reported that the apomorphine-induced compulsive gnawing in rats is not mediated via the release of catecholamines, since it is not reduced by the catecholamine synthesis inhibitors α-methyl-3,4-dihydroxyphenylalanine and α-methyltyrosine. On the other hand, the gnawing seen after treatment with (+)-amphetamine is blocked by these synthesis inhibitors. Since the apomorphine-induced gnawing requires an intact corpus striatum and gnawing can also be produced by the catecholamine precursor dihydroxyphenylalanine, Ernst (1967) suggested that apomorphine acts on the dopamine receptors whereas amphetamine acts by releasing dopamine. In the present paper supporting evidence for this view is given by further functional, biochemical and histochemical studies.

The functional influence of apomorphine on dopamine neurotransmission in the corpus striatum was examined after unilateral removal of the corpus striatum of adult hooded rats weighing about 200 g (Andén, Dahlström & others, 1966a). A possible action of apomorphine on the noradrenaline receptors of the spinal cord was tested in acutely spinalized adult hooded rats by evaluating the changes in the flexor reflex evoked by pinching the hind limbs.

The effect of apomorphine on the dopamine and noradrenaline levels of the brain and spinal cord was determined biochemically (Bertler, Carlsson & Rosengren, 1958; Carlsson & Waldeck, 1958) and histochemically (Falck, Hillarp & others, 1962; Dahlström & Fuxe, 1964; Hamberger, Malmfors & Sachs, 1965).

Function. These studies were made mainly on rats which had been pretreated with reserpine (10 mg/kg i.p., 3 hr) plus α-methyltyrosine methylester (H 44/68, 500 mg/kg, i.p., 2 hr) after removal of the left corpus striatum by suction. After this treatment all the operated animals turned towards the unoperated side (cf. Andén & others, 1966a). After injection of apomorphine (1–25 mg/kg s.c.) these rats changed their position and turned or rotated towards the operated side. This effect began about 5 min after the injection and was evident for about 45–60 min. If apomorphine was given to operated rats not pretreated with reserpine–H 44/68 combination, this action of apomorphine, like the gnawing, seemed to be less pronounced. If haloperidol (5 mg/kg i.p.) was given 15–20 min after apomorphine all the rats turned from the operated towards the unoperated side in about 15 min and the gnawing ceased.

(+)-Amphetamine (0·5–25 mg/kg s.c.), like apomorphine, made the rats turn or rotate towards the operated side. In contrast to apomorphine, however, this action of amphetamine was not seen after pretreatment with reserpine plus H 44/68 (cf. Weissman, Koe & Tenen, 1966; Hanson, 1967; Ernst, 1967).

Apomorphine (25 mg/kg s.c.), in contrast to (+)-amphetamine (0·5–25 mg/kg s.c.) and L-3,4-dihydroxyphenylalanine (50–75 mg/kg i.v. 2 hr after nialamide 50 mg/kg i.p.), did not cause a definite increase of the flexor reflex in spinalized rats.

Chemistry. The biochemical results obtained in unoperated adult hooded rats are presented in Table 1. Apomorphine caused a retardation of the depletion in brain dopamine produced by H 44/68. The difference between the dopamine levels in the apomorphine–H 44/68 group and in the H 44/68 group is statistically significant ($P < 0.001$, Student's t-test). This action of apomorphine on the brain dopamine was blocked by haloperidol. The disappearance of noradrenaline from the brain and the spinal cord after H 44/68 did not seem to be influenced by apomorphine. (+)-Amphetamine did not cause any significant retardation of the dopamine and noradrenaline loss after H 44/68.

TABLE 1. LEVELS (μG/G; MEAN ± S.E.M.) OF BRAIN DOPAMINE, BRAIN NORADRENALINE AND SPINAL CORD NORADRENALINE IN THE RAT AFTER TREATMENT WITH APOMORPHINE 25 MG/KG S.C., 45 MIN; H 44/68 250 MG/KG I.P., 1 HR; HALOPERIDOL 5 MG/KG I.P., 2 HR; (+)-AMPHETAMINE 25 MG/KG S.C., 45 MIN).

	Dopamine in brain	Noradrenaline in brain	Noradrenaline in spinal cord
No drug treatment	0·80 ± 0·029 (4)*	0·40 ± 0·033 (4)	0·28 ± 0·021 (4)
Apomorphine	0·76 ± 0·034 (4)	0·38 ± 0·022 (4)	0·30 ± 0·019 (4)
H 44/68	0·40 ± 0·015 (11)	0·30 ± 0·012 (11)	0·23 ± 0·012 (11)
H 44/68 + Apomorphine ..	0·55 ± 0·019 (11)	0·28 ± 0·008 (11)	0·21 ± 0·010 (11)
Haloperidol + H 44/68 ..	0·35 ± 0·016 (8)	0·23 ± 0·014 (8)	0·19 ± 0·010 (8)
Haloperidol + H 44/68 + Apomorphine	0·36 ± 0·014 (8)	0·19 ± 0·006 (8)	0·16 ± 0·010 (8)
H 44/68	0·42 ± 0·006 (4)	0·27 ± 0·019 (4)	0·24 ± 0·012 (4)
H 44/68 + Amphetamine ..	0·45 ± 0·015 (4)	0·23 ± 0·018 (4)	0·24 ± 0·017 (4)

* No. of experiments.

In the histochemical studies male Sprague-Dawley rats were used. The rats treated with apomorphine (25 mg/kg s.c. 4½ hr plus 10 mg/kg, s.c. 2 hr before death) plus H 44/68 (250 mg/kg, i.p. 4 hr before death) showed a higher fluorescence intensity in the dopamine terminals of the nucleus caudatus and putamen, nucleus accumbens and the tuberculum olfactorium than did the rats treated with H 44/68 alone. On the other hand, the dopamine terminals of the median eminence, like all the noradrenaline terminals, appeared unaffected by apomorphine. After haloperidol pretreatment (5 mg/kg i.p. 2 hr before death) the dopamine terminals of the rats treated with apomorphine plus H 44/68 seemed to be as depleted as those of the rats treated with H 44/68. As in the biochemical experiments, there was a tendency towards an acceleration of the noradrenaline and dopamine loss after H 44/68 in the haloperidol-treated rats. The reason for using different time-intervals was that in the biochemical experiments it is easier to detect a difference when the amine levels are high, whereas in the histochemical experiments the same is true for low levels.

The apomorphine-induced retardation of the dopamine depletion after H 44/68 is in all likelihood due to reduced activity in the dopamine neurons since a similar retardation after H 44/68 is observed in the noradrenaline nerve terminals lacking an impulse flow such as those in the spinal cord caudal to a transection (Andén, Corrodi & others, 1966b). Such reduced activity in the dopamine neurons can be explained by a negative feed-back mechanism due to a dopamine receptor activation. Such a finding was indicated in the functional studies. The absence of functional and chemical changes by apomorphine after haloperidol treatment may be due to a blockade of the central catecholamine receptors by the latter drug.

Acknowledgements. This work was supported by the Swedish Medical Research Council (K67-14x-502-03, B67-12x-715-03), "Svenska Livförsäkringsbolags nämnd för medicinsk forskning" and "Therese och Johan Anderssons minne". For generous gifts of drugs we thank the Swedish Ciba Ltd., Stockholm (reserpine), Dr. H. Corrodi, AB Hässle, Göteborg (H 44/68) and AB Leo, Hälsingborg (haloperidol). For technical assistance we thank Miss Birgitta Nilsdotter-Högberg, Miss Inger Karlsson, Miss Barbro Lindberg, Miss Ulla Lidgren and Mrs Mirta Baidins.

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α-Adrenergic blocking action of propranolol

SIR,—The inhibitory action of catecholamines on the rabbit aorta may be observed *in vitro* in strips pretreated with phenoxybenzamine and contracted by adding carbachol to the bathing fluid. When propranolol was used to block this inhibitory action of the catecholamines, the original excitatory action of these compounds was observed by us. This unexpected effect was of interest because similar concentrations of phenoxybenzamine caused complete blockade of the excitatory action of catecholamines in untreated strips. We are investigating the mechanism of this anti-α-adrenergic blocking action of propranolol and have observed that the drug has an α-adrenergic blocking action.

All experiments were done on spirally cut rabbit aortic strips suspended in Krebs-Henseleit solution maintained at 38° bubbled with 5% carbon dioxide in oxygen. Isotonic contractions against 2 g tension and magnified tenfold were recorded on a kymograph.

In these experiments increasing concentrations of propranolol caused an increasing degree of blockade of the excitatory action of noradrenaline. Propranolol at 10^{-6} g/ml produced 0–45%, and at 10^{-5} g/ml produced 30–72% inhibition of noradrenaline (10^{-8} g/ml). Inhibition of 10^{-7} g/ml noradrenaline was also studied; at 3×10^{-5} g/ml, propranolol caused 20% inhibition, while at 10^{-4} g/ml it caused 67–89% inhibition. Complete recovery from the blocking action of a single dose of propranolol occurred in 60–75 min at all levels of testing. The effect of multiple concentrations of noradrenaline in the presence of propranolol was compared with the effect of a unit concentration of noradrenaline without propranolol. At 3×10^{-5} g/ml of propranolol the dose-ratio was between 3 and 5, at 5×10^{-5} g/ml it was between 5 and 10, and at 10^{-4} g/ml of propranolol the dose-ratio was between 30 and 100. The pA_{10} of propranolol (exposure time 5 min) against noradrenaline, derived from seven experiments, was $3{\cdot}7 \pm 0{\cdot}03$ compared with $6{\cdot}2 \pm 0{\cdot}04$ for phentolamine (exposure time 3 min).

These observations suggested that propranolol had an α-adrenergic blocking action. The nature of this action of propranolol was examined further by

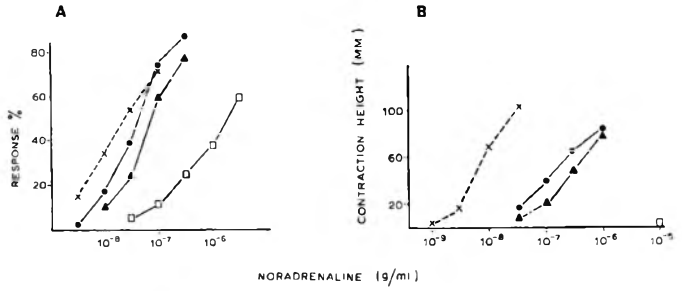

FIG. 1. A. Dose-response curves for noradrenaline with and without propranolol. Concentration of propranolol in g/ml: × - - - ×, 0 (control); ●—●, 10^{-5}; ▲—▲, 3×10^{-5}; □—□, 10^{-4}. B. Protection of noradrenaline responses against blockade by phenoxybenzamine 10^{-7} g/ml (exposure time 5 min). × - - - ×, Control; ●—●, noradrenaline protected; ▲—▲, propranolol protected; □, unprotected. Strips were protected by exposure to the protecting agents for 5 min before and during exposure to phenyoxybenzamine.

making dose-response curves and, also by assessing the ability of propranolol to protect noradrenaline responses against blockade by phenoxybenzamine.

Dose-response curves: control responses of each strip to noradrenaline were obtained at 3–4 dose levels. Each strip was then exposed to a given concentration of propranolol for 5 min and the response to noradrenaline was tested in the presence of the antagonist. Every 25–30 min the same concentration of propranolol was repeated to obtain the dose-response curve of noradrenaline in the presence of propranolol. Propranolol was then discontinued and 60–75 min after washing out the last dose of propranolol, the response of each strip to noradrenaline 3×10^{-5} g/ml was recorded. In all experiments, each response was expressed as % of this near-maximal response. The results of 3–5 experiments at each level of propranolol have been averaged and plotted in Fig. 1A. A progressive shift to the right of the dose-response curve was observed with increasing concentrations of propranolol. Moreover the slope obtained in the presence of 10^{-4} g/ml propranolol was less steep than that of the control.

The ability of propranolol to protect noradrenaline responses against blockade produced by phenoxybenzamine was tested in six experiments. Each experiment was made on three strips taken from the same aorta. A control dose-response curve was obtained for each strip. Two of the three strips were then exposed for 5 min to the protecting agents: one strip was exposed to noradrenaline (3×10^{-5} g/ml) and the other to propranolol (10^{-4} g/ml). All strips were then exposed (without washing) to phenoxybenzamine (10^{-7} g/ml) for the next 5 min. The protecting drugs and the antagonist were then washed out; washings were repeated every 15 min for 75–90 min, at the end of which responses of each strip to increasing doses of noradrenaline were tested. Fig. 1B illustrates the results of a typical experiment. In all six experiments the responses to noradrenaline (10^{-5} g/ml) were fully blocked by phenoxybenzamine in the unprotected strips, while responses to noradrenaline were retained to a variable degree in the strips protected with either noradrenaline or propranolol during exposure to phenoxybenzamine.

These observations indicate that propranolol, considered to be a prototype β-adrenergic blocking drug (Moran, 1967), may also interact reversibly with the α-adrenoceptive receptors and, may cause a reversible and surmountable type of α-adrenergic blockade when used in concentrations higher than those required to produce blockade of β-adrenoceptive receptors.

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Comparative investigation of the effect of cocaine and desipramine on bronchospasm in guinea-pigs

SIR,—Pharmacological examinations of cocaine and desipramine have shown similarities in some of their properties. The essential characteristic common to both drugs is that both inhibit the uptake of noradrenaline and simultaneously potentiate its effect. Yet there are differences in the activity of the two drugs, for while cocaine inhibits the uptake of noradrenaline to a lesser degree than desipramine (Iversen, 1965) it increases more powerfully the activity of exogenously applied noradrenaline. Taking into consideration these common properties we have examined whether any parallelism exists in the effect of two drugs upon bronchospasm in guinea-pigs. We consider this technique appropriate since adrenergic substances block more or less intensively the bronchial spasm provoked by some spasmogens. The investigation of the influence on the bronchospasm was by plethysmography combined with artificial respiration while the thoracic muscles were relaxed by intravenous injection of suxamethonium in doses of 0·1–0·2 mg/kg (Gjuriš, 1965). The animals were anaesthetized with urethane 1·5 g/kg s.c.

The effect of cocaine and desipramine was tested in bronchial spasm provoked by 5-hydroxytryptamine (5-HT), beginning the experiments at the time when the registered reaction to the spasmogen was 50 % of the initial values (5-HT was given in doses 10–20 g/kg, i.v.).

Cocaine, which resembles desipramine in some ways, differs completely in its influence on bronchospasm under these conditions. Thus cocaine in doses of 0·09, 0·2 and 5 mg/kg, i.v. increased bronchospasm by 31, 73 and 93% respectively. On the other hand desipramine inhibited bronchospasm in doses of 1, 5 and 10 mg/kg, i.v. by 11, 48 and 98% respectively. In contrast to our experiments, Foster (1964) found that cocaine potentiated the relaxant effect provoked by transmural stimulation of the trachea at doses less than 25 μg/ml. The two experiments differ in essential ways; we induced bronchospasm with 5-HT in the intact animal whereas Foster stimulated the isolated trachea electrically. The nervous pathways involved may differ.

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Effect of *p*-chlorophenylalanine on brain noradrenaline in mice

SIR,—*p*-Chlorophenylalanine is an effective inhibitor of the biosynthesis of 5-hydroxytryptamine (5-HT). Many investigators have viewed it as a relatively specific tool for studying the relation of 5-HT to behaviour and to various physiological functions (Koe & Weissman, 1966; Jéquier, Lovenberg & Sjoerdsma, 1967). However, we find that *p*-chlorophenylalanine slightly but consistently lowers brain noradrenaline at various dosages and time periods, in both sexes, and in several different strains of mice.

DL-*p*-Chlorophenylalanine was prepared as described by Koe & Weissman (1966) and administered intraperitoneally in 0·2 ml of a 0·9% saline suspension (facilitated by a drop of Tween 80), at pH 1·8 for Jackson Laboratory C_3H mice and at pH 7·0 for all other strains. Controls were injected with 0·9% saline at the same pH as their experimental congeners, and they were alternated with them in all experimental manipulations. Mice were killed by decapitation and their brains removed, weighed and frozen on dry ice within 2 min of death. The brains were homogenized in 0·01 N hydrochloric acid, extracted by the method of Shore & Olin (1958), and analysed for adrenaline by the method of Crout (1961). *p*-Chlorophenylalanine added to tissue homogenates offered no appreciable interfering fluorescence.

The data in Table 1 illustrate reductions in brain noradrenaline at times varying from 2 hr after a single dose to 24 hr after three consecutive daily doses of *p*-chlorophenylalanine in both males and females, and in five strains of mice.

Brain noradrenaline, in the whole brains of male Jackson BALB/C and C_3H mice, and in the telencephalon, diencephalon + mesencephalon, and pons + medulla of male Cumberland C_3H mice, was measured after the mice had been given 360 mg/kg of a neutral suspension of *p*-chlorophenylalanine either 10 min (Jackson) or 15 min (Cumberland) before being killed. There was a

TABLE 1. EFFECT OF *p*-CHLOROPHENYLALANINE ON BRAIN NORADRENALINE IN MICE

Strain dose, and time of death after dose of *p*-chlorophenylalanin	No of analysis	Noradrenaline† ng/g ± s.e.	P <
Dublin DUB/ICR ♂			
Control	32	501 ± 11	
360 mg/kg, 6 hr.	32	464 ± 9	0·001
Cumberland C_3H ♂			
Control	19	446 ± 18	
350 mg/kg, 4·5 hr.	20	413 ± 14	0·05
Jackson C_3H ♂			
Control	10	549 ± 30	
100 mg/kg, 24 hr. × 1	9	496 ± 20	n.s.
× 2	9	488 ± 8	n.s.
300 mg/kg, 24 hr. × 1	8	497 ± 18	n.s.
× 3	9	497 ± 18	n.s.*
Jackson C_3H ♀			
Control	9	543 ± 32	
300 mg/kg, 2 hr.	10	462 ± 20	n.s.
4 hr.	10	448 ± 12	0·05
18 hr.	10	429 ± 21	0·025
Jackson SWR/J ♀			
Control	12	330 ± 11	
115 mg/kg, 3 hr.	12	305 ± 10	0·05
Charles River HaM/ICR ♂			
Telencephalon			
Control	18	409 ± 9	
360 mg/kg	18	396 ± 8	n.s.
Brainstem			
Control	18	515 ± 16	
360 mg/kg	18	489 ± 18	n.s.

* When all dosages and time periods are pooled, Jackson C_3H mice receiving the drug differ significantly from their controls, $P < 0·025$.

† Whole brains of individual mice were analysed except where brain parts are indicated.

significant fall in noradrenaline in the whole brains of Jackson C_3H mice ($P < 0.05$; 15 mice control, 15 inhibitor) and in the pons + medulla of the Cumberland mice ($P < 0.025$; 30 mice control, 30 inhibitor; brain parts of 2 mice were pooled for each analysis). At this time, there were no significant changes in brain 5-HT or dopamine. This immediate effect of *p*-chlorophenylalanine upon brain noradrenaline was, however, paralleled by an effect upon behaviour. There was invariably a marked reduction of exploratory and motor activity, although the animals remained fully capable of coordinated motor activity if disturbed. The extent of this reduction in activity is illustrated by the fact that, in the 15 min experiment, all 30 saline controls, but not one of the 30 drug-injected mice, climbed out of a small (7 in × 3 in × $2\frac{1}{2}$ in deep) refrigerator tray in which they were individually placed during the 15 min period between injection and death. Beyond this, however, the effect upon individual mice was highly variable. Some individuals evidenced mild hyperpnoea and were only moderately responsive to stimuli; others showed tachycardia, mild piloerection, assumed stereotype postures, and became super-sensitive to external stimuli such as blowing or snapping of the fingers. We were unable to predict in advance the response of particular individuals or of groups of mice. The initial behavioural effect usually passed within 30 min and the behaviour of the mice became grossly normal.

Koe & Weissman noted that *p*-chlorophenylalanine had an *in vitro* inhibitory effect against tyrosine hydroxylase about one-fortieth as great as its effect upon tryptophan hydroxylase, that it markedly inhibited phenylalanine hydroxylase, and that it caused a small reduction in brain catecholamines in mice, rats and dogs; however, they discounted this effect as being functionally unimportant compared with the quantitatively greater effect upon brain 5-HT.

We think that the effect which *p*-chlorophenylalanine has upon brain catecholamine levels may be functionally important, and must be considered in the interpretation of any studies made with this drug.

This contention is supported (1) by the small but consistent reduction of brain noradrenaline that is produced by *p*-chlorophenylalanine at various doses and time periods, in both sexes and in several strains of mice; (2) by the immediate reduction of brain noradrenaline, correlated with a behavioural effect, that occurs within 10 min after drug administration, at which time brain 5-HT is not detectably altered; and (3) by observations made in this laboratory that pretreatment of mice with *p*-chlorophenylalanine can prevent stress-induced increases in both brain dopamine and 5-HT and favour stress-induced reductions of brain noradrenaline and dopamine.

Acknowledgements. Supported by grants from the Air Force Office of Scientific Research, the U.S. Army Medical Research and Development Command, and the National Institute of Mental Health. We thank Dr. V. Cremata of Chas Pfizer & Co. for the *p*-chlorophenylalanine (CP-10, lot 3555–50B).

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Effects of glyceryl trinitrate on the aortic strip of rabbits

SIR,—We have investigated the effect of glyceryl trinitrate in relaxing the vascular spasm produced by a maximal dose of noradrenaline in various ionic media, under different conditions of temperature and hydrogen ion concentration.

The experimental technique for the aortic strip preparation was similar to that previously described (Lorenzetti, Tye & Nelson, 1966). All solutions were in 0·9% sodium chloride solution with the pH adjusted to 7·3 ± 0·1. Bathing media were prepared with triple glass distilled water as in Table 1. Drug solutions were prepared daily and kept in an ice bath during use. All doses were added to the bath in a volume of 0·05 ml. Two to four doses of agonist were used until uniform maximal contraction was observed, with 30-min wash periods between doses. When uniform contractions were assured, the agonist was introduced and the tissue allowed to attain peak contraction, passing through the fast and slow phase of contraction (Brodie, Bohr & Smit, 1959). At the height of contraction glyceryl trinitrate was introduced, starting at 4×10^{-6} μM/ml; subsequent increasing log doses of glyceryl trinitrate were tested after washing the strip for 30 min. A plot of 3 to 5 points on the curve of log concentration against response was made. From this graph the relaxant dose 50 (RD 50), i.e., the dose of glyceryl trinitrate needed to reduce the contraction induced by the agonist by 50%, was determined.

Raising or lowering the pH of the bathing media with the temperature constant at 37° decreased the sensitivity of the preparation (Table 2) to glyceryl trinitrate. Lowering of the temperature below 36° decreased the sensitivity of the preparation to glyceryl trinitrate. But in decreasing the temperature toward 36°, the

TABLE 1. COMPOSITION OF BATHING MEDIA USED FOR AORTIC STRIP EXPERIMENTS (IN MMOLE/LITRE) AND CONCENTRATION OF GLYCERYL TRINITRATE REQUIRED TO REDUCE THE MAXIMUM CONTRACTION TO 0·0059 μMOLE NORADRENALINE BY 50% (RD50) IN THESE SOLUTIONS

Solution	Norm Krebs HCO_3	Ca-Ca-Free	High Ca	K Free	High K	Mg-Free	High Mg	High Barium	Mg-Ca Free
Sodium chloride	118·5	120·0	110·0	123·0	110·0	120·0	115·0	110·0	128·0
Potassium chloride ..	4·8	4·8	4·8	—	13·3	4·8	4·8	4·8	4·8
Calcium chloride.$2H_2O$	1·9	—	6·9	1·9	1·9	1·9	1·9	1·9	—
Potassium dihydrogen phosphate ..	1·2	1·2	1·2	—	—	1·2	1·2	1·2	—
Sodium dihydrogen phosphate ..	—	—	—	1·2	1·2	1·2	—	—	—
Magnesium sulphate·$7H_2O$	1·2	1·2	1·2	1·2	1·2	—	5·2	—	—
Sodium bicarbonate	25·0	25·0	25·0	25·0	25·0	25·0	25·0	25·0	—
Glucose.. ..	10·0	10·0	10·0	10·0	10·0	10·0	10·0	10·0	10·0
Trizma base (Tris) ..	—	—	—	—	—	—	—	—	40·0
Barium chloride	—	—	—	—	—	—	—	2·5	—
Glyceryl trinitrate RD 50 (µmole/ml ± s.e.)	0·0064 ±0·0009 (16)*	0·0037 ±0·0012 (8)	† (9)	0·0035 ±0·0015 (12)	0·00061 ±0·00008 (12)	† (9)	0·004 ±0·001 (9)	† (8)	† (9)

Parameters: ionic strength range, 0·168 to 0·174; osmolarity range, 314·5 to 317·5 milliosmoles; pH range, 7·28 to 7·44, unless indicated. The solutions were gassed for 1 hr with 100% carbon dioxide before adding to the reservoir, then gassed with 95% oxygen-5% carbon dioxide throughout the experiment. All reagents are analytical grade.

* No. of experiments.

† Relaxation not greater than 25%.

TABLE 2. CONCENTRATION OF GLYCERYL TRINITRATE REQUIRED TO REDUCE THE MAXIMUM CONTRACTION[1] BY 50% (RD 50 μMOLE/ML ± S.E.) AT VARIOUS TEMPERATURES AND HYDROGEN ION CONCENTRATION

Test situation[1]	No. of experiments	RD 50 (μmole/ml ± s.e.)
Temp = 37° ± 1° pH = 7·35 ± 0·5	16	0·0064 ± 0·0009
Temp = 36° ± 1° pH = 7·35 ± 0·5	18	0·0012 ± 0·0003
Temp = 33° ± 1° pH = 7·35 ± 0·5	8	0·0181 ± 0·0017
Temp = 27° ± 1° pH = 7·35 ± 0·5	9	0·35 ± 0·0025
Temp = 37° ± 1° pH = 6·9 ± 0·5	9	0·011 ± 0·0008
Temp = 37° ± 1° pH = 7·75 ± 0·5	9	0·013 ± 0·0059

[1] Glyceryl trinitrate administered after peak response to 0·0059 μmole of noradrenaline.

RD 50 for glyceryl trinitrate was significantly lower ($P < 0·001$) than at 37°. This temperature difference gave a consistantly optimal response to the effect of glyceryl trinitrate against the maximum contraction induced by noradrenaline. At 27° the tissue changed in physical appearance from its normal pink colour to white. Temperatures of 40 and 42° made the tissue unresponsive. Below 36° the response to noradrenaline consisted mostly of the slow phase of response and required 7 to 9 min longer than the usual 2 to 4 min to attain its peak.

Removal of all traces of calcium from the Krebs-bicarbonate media decreased the contraction induced by noradrenaline by more than 50% (Table 1). The absence of calcium also increased the sensitivity of the aortic tissue to glyceryl trinitrate (0·0064 versus 0·00037 μM/ml). The only other altered electrolyte medium which required less glyceryl trinitrate to produce a 50% response was the high (13·3 mM) potassium medium. With both the high potassium and high calcium media, the contractile response to noradrenaline was increased by 30 to 40%.

The dual contractile response of the aorta consists of a fast (F) response followed by a slower yet sustained contraction, referred to as the slow (S) response. The F-response occurs within the first 2 min while the S-response may extend another 1½–2 min. The S-response was eliminated by absence of potassium or magnesium or by the high calcium medium while the F-response was little altered by these conditions. The differentiation of the S- and F-response which occurred with no magnesium or high calcium was seen consistently in 18 aortic strip preparations.

Strips which were equilibrating in high K^+, Ba^{2+} or Ca^{2+} Krebs-bicarbonate media consistently displayed a more rapid increase in tone. Absence of these ions as well as high Mg^{2+} Krebs-bicarbonate caused a rapid loss in tone during the equilibration period as well as a 10 to 15% decrease in the noradrenaline response. Absence of calcium gave the greatest sensitivity to glyceryl trinitrate (Table 1), although the contraction induced by noradrenaline was reduced 12 to 14%. High Mg^{2+} (5·2 mmole) and absence of K^+ required more glyceryl trinitrate to produce a 50% relaxant effect. The effect of altering other ions shown in Table 1 was not determinable because of the insensitivity of the tissue. High concentration of Ca^{2+}, K^+, Ba^{2+}, as well as absence of Mg^{2+} potentiated the noradrenaline response 30 to 45%. Much of the potentiation occurred in the S-response with some enchancement appearing in the F-response also. Absence of Ca^{2+} and Mg^{2+} caused a reduced response to noradrenaline of the F-response only, in seven out of nine trials. A reduction in the S-response

was also seen with the calcium free Krebs-bicarbonate. It seems that glyceryl trinitrate relaxation response on vascular muscle may be influenced by the ionic media in isolated tissue studies. Possibly muscular relaxation induced by glyceryl trinitrate is accompanied by ion fluxes.

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Effect of adrenal demedullation and adrenalectomy on amphetamine toxicity in isolated and aggregated mice

SIR,—Amphetamine toxicity is greater in aggregated than in isolated mice (Thiessen, 1964), and heightened adrenal secretory activity (medullary or cortical, or both) in aggregated mice has been implicated as a causative factor.

D'Arcy & Spurling (1961) found that amphetamine toxicity in isolated mice was increased by pretreatment with cortisol or corticotrophin. Moreover, Weiss, Laties & Blanton (1961) reported that adrenalectomy reduced the enhanced toxicity of amphetamine in isolated mice subjected to unavoidable foot-shock. Foot-shock also increases amphetamine toxicity in individual as well as aggregated mice; moreover, the events leading to death in amphetamine-treated, shocked, isolated mice appeared to be the same as those observed in aggregated mice treated with this drug (Weiss & others, 1961; Askew, 1962). Most recently, Richards, Nicol & Young (1966) reported that adrenalectomy reduced the enhanced toxicity of desoxyephedrine in aggregated mice. In contrast, Mennear & Rudzik (1965) observed that amphetamine toxicity in aggregated mice was not altered by adrenalectomy. We have now made some experiments designed to determine the significance of the adrenal in the "amphetamine aggregation effect".

Novice, male, albino mice of a random bred Swiss strain (Maxfield; Cincinnati, Ohio) were injected intraperitoneally with an aqueous solution of (−)-amphetamine sulphate (1 ml/100 g body wt), and were either isolated or aggregated (3 per cage) in metal cages (7 × 7 × 7·5 cm), one side of which was wire mesh to permit observation. Aggregated mice that died during the 3 hr observation period were replaced by untreated mice to maintain aggregation. Ambient temperature was 24 ± 1°.

To evaluate the significance of the adrenal medulla in the amphetamine aggregation effect, (+)-amphetamine toxicity was measured in demedullated, sham-operated and non-operated mice. Enucleation was done under ether anaesthesia via bilateral incisions in the lumbar musculature. Each adrenal capsule was incised and the medulla with most of the attached cortical parenchyma gently squeezed out with small forceps. Histology showed regeneration of the cortex, but not the medulla, to take place within 21 days. The adrenal capsule was not incised in sham-operated mice; non-operated mice remained caged throughout the operative period. Post-operatively, mice were housed in their home cages (45 × 24 × 12 cm) in groups of 15 for not less

than 30 days with Purina laboratory chow and water available *ad libitum*. At the time of drug administration, mice ranged in weight from 25 to 35 g and were 9 to 12 weeks of age. The results obtained from this study are presented in Table 1.

In a second series of experiments, (+)-amphetamine toxicity was measured in mice kept in their home cages in groups of 15 for 16–29 days and then bilaterally adrenalectomized, sham-operated, or non-operated. Post-operatively, these mice were maintained under conditions described above except that the adrenalectomized mice had 1% saline as drinking water. At the time of drug administration (48 hr post-operatively), mice weighed between 20 and 30 g and were 7 to 9 weeks of age. The results from this study are in Table 1.

It is evident from the results that the susceptibility of mice (isolated or aggregated) to (+)-amphetamine- (30 or 100 mg/kg) induced lethality was not significantly altered by prior demedullation. Similarly, the lethal effects of (+)-amphetamine were not antagonized by adrenalectomy (Table 1). In fact, at the 30 mg/kg dose level, adrenalectomy slightly increased the incidence of

TABLE 1. EFFECT OF ADRENAL DEMEDULLATION AND ADRENALECTOMY ON (+)-AMPHETAMINE LETHALITY IN ISOLATED AND AGGREGATED MICE

Treatment	% Dead 3 hr after (+)-amphetamine			
	30 mg/kg		100 mg/kg	
	Isolated	Aggregated	Isolated	Aggregated
Adrenal demedullation				
Non-operated	6·7 (30)*	51·7 (60)	31·4 (118)	66·7 (63)
Sham-operated	3·3 (30)	52·1 (48)	31·4 (102)	61·9 (63)
Demedullated	6·7 (30)	59·5 (42)	25·2 (115)	69·3 (75)
Adrenalectomy				
Non-operated	8·3 (12)	30 (30)	25 (12)	70 (30)
Sham-operated	0 (12)	26·6 (30)	16·6 (12)	70 (30)
Adrenalectomized	8·3 (12)	46·6 (30)	33·3 (12)	63·3 (30)

* Number of mice tested in parentheses

lethality in aggregated mice (sham-operated compared to adrenalectomized, $P = 0{\cdot}10$). The fact that aggregation enhanced (+)-amphetamine toxicity in the absence of the adrenal medulla, and also in the absence of the entire gland, does not prove conclusively that the adrenal does not participate in the amphetamine aggregation effect. It does, however, prove that the adrenal is not essential for this phenomenon. It seems reasonable to conclude that the enhanced toxicity of (+)-amphetamine in aggregated mice is mediated through some mechanism which does not involve adrenal activity.

The ability of adrenalectomy to protect mice against the enhanced toxicity of amphetamine produced by foot-shock (Weiss & others, 1961), but not by aggregation, suggests that the increased toxicity observed in these situations is mediated through different mechanisms. However, this discrepancy in results may also be due to differences of method. As to the protection provided by adrenalectomy against desoxyephedrine toxicity in aggregated mice (Richards & others, 1966), initial consideration must be given to possible pharmacological differences between these two agents. However, the results reported by these authors might very well reflect the fact that LD50 values were employed for quantitation of desoxyephedrine toxicity. Dose-lethality relationships, described by George & Wolf (1966) and Gardocki & others (1966a,b) raise serious questions concerning the validity of employing LD50 values to quantitate amphetamine toxicity in mice. Perhaps similar limitations apply to the use of this expression as a measure of desoxyephedrine toxicity.

In view of recent evidence which indicates that death resulting from amphetamine at high doses follows a different physiological train of events than death from lower doses (George & Wolf, 1966; Gardocki, Schuler & Goldstein, 1966a, b), two dose levels of (+)-amphetamine were used by us, the lower, 30 mg/kg, being intended to reflect the actions of low doses and the higher, 100 mg/kg, to be representative of higher doses. Although aggregation significantly increased the toxicity of (+)-amphetamine at both doses ($P < 0.05$), the effect was more marked at the lower dose. Furthermore, symptoms preceding death and the time of death of the animals depended not on the environmental conditions imposed on the mice or their prior surgical treatment but on the dose of (+)-amphetamine they received. Almost all deaths resulting from the higher dose occurred within 60 min of drug administration and were associated with convulsions. In contrast, deaths from 30 mg/kg dose were preceded by lethargy and coma, and nearly always occurred within 90 to 180 min of injection. These observations again suggest different causes of death at the two dose levels studied.

Acknowledgements. This research was supported by a grant from the National Institute of Mental Health (MH-07397-03) U.S. Public Health Service. The participation of D. J. George was supported in part by a fellowship from the American Foundation for Pharmaceutical Education.

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The Extra Pharmacopoeia - Martindale

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"An authoritative reference work on drugs and medicines in current use throughout the world" is the sub-title on the cover. British reference books on drugs have a deservedly high reputation, not only in the United Kingdom. Regular revisions prevent stagnation, and even with a new B.P. and B.P.C. every five years as is today's established practice, addenda appear periodically to keep these volumes up to date. Such provision involves a vast and continuing labour and those sharing in this toil make a substantial contribution to the promulgation of standards for, and useful knowledge about, medicines. It is nine years since we had a new E.P., and this book is more comprehensive, even encyclopaedic, in its scale and outlook, compared with the B.P. and B.P.C. Yet it is produced by a small devoted team rather than by a series of working-parties, committees and sub-committees, and to this team of ten and especially to the editor, Mr. R. G. Todd, F.P.S., a tribute should be paid—they have done a magnificent job. When Martindale produced his first E.P. in 1883 his was a pocket-book of 313 pages, and he had medical help from Dr. Westcott in the shape of concise abstracts of papers from medical journals. Since the book was taken over by the Pharmaceutical Society of Great Britain it has grown both in size and authority. The previous ten editions appeared in two volumes. The new single-volume E.P. claims to contain 80% more material than the two-volumed edition of 1958. Yet much of the analytical and biochemical information of Volume II has had to be ruthlessly pruned—it is hoped that the lost material will appear in "Companion Volumes" and that amendments and supplements will be issued from time to time. One "Companion Volume" has already appeared.

"When in doubt, see what the E.P. has to say" is good practice. Professor Witts has called Martindale "a great contribution to medical science and scholarship—regarded with affection by all who use it". The B.P and the Codex hardly inspire affection. But the E.P. is basically "Extra" in that it deals with many drugs which no longer qualify for inclusion in the B.P. or B.P.C., and with a few which may be in course of qualifying. Thus Part 2 of the book has in 67 pages short notes on 600 preparations, in alphabetical order, in these categories. One finds here "old, forgotten, far-off things" like rosemary and rue. But here too are notes and recent references to dopa and dopamine, to etorphine (M.99) and the various related analgesics and antagonists, to recent introductions such as "Ponstan" and "Extil". Part 3 (pp. 46) offers the formulae for 1,600 proprietaries advertised to the public and usually supplied over the counter on demand—an increase of 600 over the 24th edition. These are introduced by reminders about the law on advertising proprietaries and the advice given by the Council of the Pharmaceutical Society of Great Britain to its members on what not to supply without a prescription. There follow a "Directory of Manufacturers", an "Index to Clinical Uses" (about which some may have reservations—it covers everything from "Hair—to remove" to "Hepatic Coma"—with references to the appropriate page in the text) and finally a monumental "General Index"—164

* pp. xxviii + 1804 (including index). The Pharmaceutical Press, London, 1967. 150s. (postage: 4s. 6d.)

pages with three columns to a page. This index adds up to some 32,000 entries. So far it has not failed any test applied.

Most of this is very good and very useful, but the cream of the E.P. is found in Part 1. Here in 1498 pages are monographs on 2,600 substances—concentrated information, no frills, little history, arranged in 169 "chapters". Most chapters deal with groups of pharmacologically-related drugs and vary in length—132 pages on "Penicillin and other antibiotics", 28 on "Adrenaline and other sympathomimetics", 28 on "Morphine and other analgesics", 6 on "Alcohol"—including benzyl, isopropyl, methyl, methylated and proof spirits, 5 on "Oxygen and other gases" and so on. Chapters are separated by cross-column headings which may come anywhere on a page, drugs by a line. Pages are about 50% bigger than in the earlier editions—margins are narrow—paper tough but thin—two columns and about 1250 words to a page. There is certainly no wasted space, no "vain repetitions" and even the small type used for abstracts is clear. There are over 10,000 references and abstracts, mostly since 1958. 1,200 new drugs and 2,400 new proprietaries have been added in the present revision. Inevitably, much of the old has gone—it is no good looking for Barger on ergot or Dale on amines and one may sigh over the loss of such classics. But this is a book of first things, first, and the necessary pharmaceutical, pharmacological and therapeutic facts are set out concisely, with these recent abstracts to expand and illustrate and provide for more study in depth.

My copy suffers from some blurring of part of the printing of the monograph on phenelzine and this word is misspelled on p. 1045 but, so far as my perusal of about one-fifth of this text goes, the proof-reading and the production have been meticulous. To consider in detail such chapters as are of special interest to oneself might be unfair to the fields so far unexplored. The price of 150s. may provoke comment—it represents about 12,000 words for each shilling, and this is unusual value!

A. D. MACDONALD

Journal of Pharmacy and Pharmacology

SEPTEMBER 1967 VOL. 19 No. 9

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PRINTED BY W. HEFFER & SONS LTD., CAMBRIDGE, ENGLAND.