REVIEW ARTICLE

A NEW GENERAL CONCEPT OF THE NEUROHUMORAL FUNCTIONS OF ACETYLCHOLINE AND ACETYLCHOLINESTERASE*

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THERE is surely no other compound for which a neurohumoral function has been so well established as it has for acetylcholine (ACh). The early work of Loewi, Dale, Feldberg, and their collaborators which first indicated the occurrence of cholinergic transmission at various sites has been extensively confirmed and amplified by subsequent investigators. The sequence of events which is now generally accepted as taking place during the passage of an impulse across a cholinergic synapse, such as those of the autonomic ganglia, is as follows (Grundfest, 1957): With the arrival of the nerve action potential (NAP) at the terminals of the preganglionic axon, the transmitter, ACh, is liberated from an intra-axonal storage site; it diffuses across the narrow (at most, a few hundred Å) synaptic cleft, and combines with receptor groups on the ganglion cell membrane, causing the development of a localised non-propagated depolarisation, known as the postsynaptic potential (PSP); the latter initiates electrogenically a NAP, which is propagated along the postganglionic fibre; the polarised state of the postsynaptic membrane is restored with the rapid destruction of the synaptic transmitter by the enzyme, acetylcholinesterase (AChE).

The work in our laboratory over the past several years has been concerned chiefly with the correlation of histochemical studies of the cytological localization of neuronal AChE with pharmacological investigations of the effects of anticholinesterase (anti-ChE) agents, with the general aim of elucidating the physiological functions of AChE and ACh. Our findings and those of several other investigators have not been fully consistent with the description of the steps involved in cholinergic transmission given in the foregoing brief account. In order to explain these discrepancies, a working hypothesis was proposed (Koelle, 1961), according to which the ACh liberated by the NAP acts initially at the same presynaptic terminals to bring about the liberation of additional quanta of ACh, and it is the secondarily released, increased amount of ACh which acts at the postsynaptic site to effect transmission. In many types of non-cholinergic neurons, it is equally likely that a similar mechanism is involved, in which the initial liberation of ACh promotes the release of another neurohumoral transmitter from the same nerve endings. Finally, it was suggested that at peripheral sensory receptors the specific stimulus may activate the release of ACh from either the accesssory cells or the

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axonal terminal itself, and that it then acts on the latter to initiate indirectly the nerve impulse.

Here an explanation, and perhaps a word of apology, is in order for the title of this review. The hypothesis outlined above, which will be discussed in the sections which follow, is not new in many of its individual facets. It has been used or implied in part by several authors in order to explain various observations. However, the evidence from many different areas of investigation now seems sufficient to justify its presentation as a general concept or working hypothesis. The findings which have led to this proposal, and the evidence in support of it will now be considered.

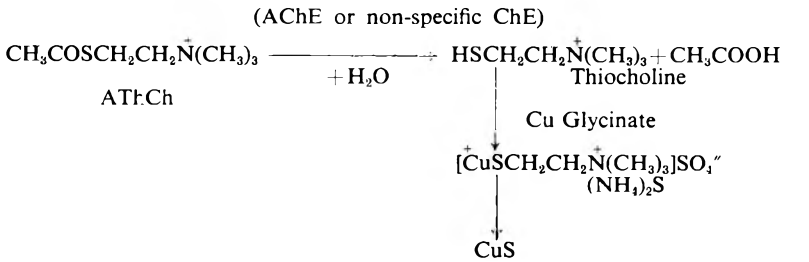
I. Components of Cholinergic Neurons: Choline Acetylase (ChAc), Acetylcholine (ACh), and Acetylcholinesterase (AChE)

All cholinergic neurons contain significant concentrations of three components: choline acetylase (ChAc), ACh, and AChE. Choline acetylase is the enzyme which effects the final step in the synthesis of ACh, namely, the transfer of an acetyl group from acetylcoenzyme A to choline (Nachmansohn, 1962). The enzyme itself is probably synthesized within the neuronal perikaryon, then transferred along the axon to its terminals where the formation of ACh is believed to occur (Hebb and Waites, 1956). At all presynaptic terminals, cholinergic and otherwise, electron microscopic studies have revealed the presence of large numbers of vesicles, approximately 400 Å in diameter (De Robertis and Bennett, 1955; Palade, 1954; Palay, 1954; Sjostrand, 1953). It is likely that these "synaptic vesicles" represent the storage form of ACh (Whittaker, 1959) and other neurohumoral transmitters. In the absence of conducted nerve impulses, ACh is probably liberated continually in small quantities, as indicated by miniature endplate potentials recorded at the motor endplates of skeletal muscle (Fatt and Katz, 1952); following a NAP, a much greater amount of ACh is released. The third component, AChE, is present throughout the entire length of cholinergic neurons (Koelle, 1951; Koelle, 1955). However, as will be shown, there are great differences between its relative distributions at the pre- and postsynaptic membranes at various sites of cholinergic transmission. Although it has been suggested that AChE, like ChAc, is synthesised within the perikaryon and transported along the axon (Dale, 1955; Koelle and Steiner, 1956; Fukuda and Koelle, 1959), a recent study by my associate, E. Koenig (Koenig and Koelle, 1961), failed to confirm this. The major function of AChE is generally considered to be the hydrolysis of ACh following its production of the postsynaptic potential in order to insure the rapid termination of the localised depolarisation. Very little is known about the nature of an additional component of cholinergic systems, the ACh-receptor (Chagas, 1959; Ehrenpreis, 1960) or cholinoceptive site (Dale, 1954), as was pointed out by Waser (1960) in a previous lecture in this series.

II. Cytological Localisation of AChE

Of the foregoing components of cholinergic neurons, the only one which at present can be localised at the cytological level with reasonable

assurance is AChE. There are three general types of methods by which this can be done: ultramicroanalysis, centrifugal fractionation, and microscopic histochemistry. Of the several histochemical procedures which are now available for AChE (Koelle, 1962a), most of the work in our laboratory has been done using modifications of the thiocholine method, which was developed originally by Dr. Jonas Friedenwald and myself (1949). This consists of incubating fresh-frozen sections in a medium containing acetylthiocholine (AThCh) as substrate, magnesium ion as an activator, and copper glycinate; as the AThCh is hydrolysed by either AChE or non-specific (pseudo-, butyro-) cholinesterase (ChE), a white mercaptide salt (Malmgren and Sylvén, 1955) precipitates at the sites of enzymatic activity, and this is converted subsequently to copper sulphide:



The addition of a high concentration of sodium sulphate minimises diffusion of the enzymes and the reaction product (Koelle, 1951), and by the use of selective substrates and inhibitors, sites of AChE or non-specific ChE activity can be visualized individually (Koelle, 1950; 1955).

Results typical of those found in tissues of the cat are illustrated in Fig. 1 (see p. 78*a*) which represents autonomic ganglia stained selectively for AChE (Koelle and Koelle, 1959). In the ciliary ganglion (Fig. 1C), which gives rise to cholinergic postganglionic fibres, all ganglion cells are stained heavily at the cell membrane, throughout the cytoplasm, and along the lengths of the axons and dendrites as far as these can be traced. However, only a few cells are stained similarly in the stellate ganglion (Fig. 1A); these probably represent neurons which give rise to sympathetic cholinergic fibres to sweat glands and the vasodilator fibres (Koelle, 1951; Holmstedt and Sjöquist, 1959; Sjöquist and Fredricsson, 1961). The great majority, which are stained very faintly, probably represent the cells of origin of the adrenergic fibres. However, a small number show intermediate or light staining, the possible significance of which will be considered below. Most of the heavily stained fibres in the stellate ganglion are the preganglionic axons and their terminations, as shown by their disappearance after chronic sectioning of the preganglionic trunk (Fig. 1B). The non-specific ChE of the same ganglia is confined to the glial cells which surround the neurons, and the Schwann sheath cells of the pre- and postganglionic trunks. Cholinergic neurons at other sites (for example, the anterior and lateral horn cells, and cells of the nuclei of motor cranial nerves) show the same intensive staining as the ciliary ganglion cells.

On the other hand, most primary afferent neurons, such as those of the dorsal root ganglia and vagal nodose ganglion, are stained with light or moderate intensity like the small number noted in the stellate ganglion.

The pattern of staining is somewhat different in the sympathetic ganglia of the rabbit and rhesus monkey (Koelle, 1955), the rat (Koelle, 1954), and several other species, including man (Cauna, Naik, Leaming and Alberti, 1961). In all these species, practically all the neurons (including, therefore, those from which most of the adrenergic fibres arise) are lightly or moderately stained.

The limitations of both resolution by light microscopy and the accuracy of staining by the method itself have prevented drawing conclusions directly regarding the orientation of AChE with respect to the neuronal membranes. However, this question has been approached indirectly by administering to anaesthetised cats, lipid-soluble and -insoluble anti-ChE agents, alone and in combination, then removing the autonomic ganglia (normal and chronically denervated), subjecting them to special treatment, and staining for AChE as described above (Koelle, 1957; Koelle and Koelle, 1959). From these and related studies (Koelle and Steiner, 1956) it has been found, in confirmation of earlier proposals (Schweitzer, Stedman and Wright, 1939; Nachmansohn, 1950; Burgen and Chipman, 1952), that the neuronal AChE is separable into internal and external fractions with respect to the relationship of its active sites to the cell membrane. Characteristic effects of anti-ChE agents on ganglionic transmission were obtained by inhibition of only the external fraction, hence this was termed "functional AChE"; the internal fraction, which is probably associated with the endoplasmic reticulum (Fukuda and Koelle, 1959; Toschi, 1959), could be inactivated selectively without immediately apparent effects, and was called "reserve AChE" (McIsaac and Koelle, 1959). The distributions of these two fractions in the stellate and ciliary ganglia are shown in diagram in Fig. 2.

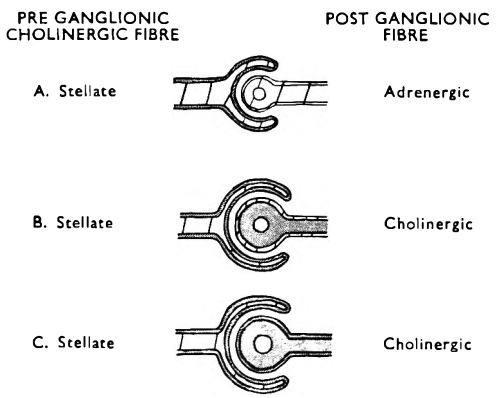


FIG. 2. Diagrammatic representation of distributions of functional (external) and reserve (internal) AChE at synapses of autonomic ganglia. Density of cross-hatching indicates relative concentration of enzymatic activity.

(From Koelle and Koelle, 1959)

A striking difference is apparent between the localisation of the functional AChE of the stellate ganglion and that of the total AChE of the neuromuscular junction, as demonstrated by Couteaux and Taxi (1952) with another modification of the same technique (Fig. 3, see p. 78*a*). At the former site, the enzyme is confined almost entirely to the presynaptic membrane (Fig. 2A, B), whereas at the latter it is mostly postsynaptic (Fig. 3C—G). The ciliary ganglion occupies an intermediate position in this regard (Fig. 2C). On the basis of the assumed primary function of AChE, that is, the rapid destruction of ACh after its activation of the postjunctional membrane, its location at the neuromuscular junctions would seem much more favourable than that in the stellate ganglion.

The foregoing findings presented, then, two apparent inconsistencies with regard to the function of neuronal AChE, in terms of the usual concept of cholinergic transmission: first, the presence of varying concentrations of AChE in presumably non-cholinergic neurons, and secondly, the predominant or exclusive localisation of the enzyme at the presynaptic, rather than the postsynaptic, site in certain ganglia.

III. Differences in Anatomical, Physiological, and Pharmacological Properties at Various Sites of Cholinergic Transmission

The contrasting pre- and postjunctional localisation of AChE is by no means the only striking difference between the sympathetic ganglionic synapse and the neuromuscular junction. The superior cervical ganglion of the cat is composed of approximately 100,000 neurons, closely packed within a volume of a few cubic millimetres and interspersed with innumerable preganglionic terminal arborisations and boutons which synapse with the dendritic ramifications and somata of the ganglion cells. The latter are surrounded individually by capsular glial cells which contain non-specific ChE. In skeletal muscle, the motor endplates are arranged in fairly orderly fashion, and occupy only a minute fraction of the total surface of the individual muscle fibres, with virtually no spatial overlap between neuromuscular junctions. Under physiological conditions, the neuromuscular junctions (Hoff and Grant, 1944) sustain a much higher frequency of transmission of impulses than do the ganglia (Bishop and Heinbecker, 1932). Furthermore, the effects of anti-ChE agents in producing prolongation of the postjunctional or endplate potential (EPP) (Eccles, Katz and Kuffler, 1942; Eçcles and McFarlane, 1949) and repetitive firing (Brown, 1937) are much more pronounced at the former site than at sympathetic ganglia, where the equivalent actions are demonstrated much less readily (Eccles, 1944; Holaday, Kamijo and Koelle, 1954). In fact, it has been suggested that diffusion alone, without the participation of AChE, can account for the termination of the transmitter action of ACh in the superior cervical ganglion (Ogston, 1955; Emmelin and MacIntosh, 1956).

Although one central cholinergic synapse (between the Golgi II collaterals of the anterior horn cells and the Renshaw cells of the spinal cord) has been studied intensively by Eccles and his associates (Eccles, Fatt and Koketsu, 1954; Eccles, Eccles and Fatt, 1956), in general little is known

with respect to the foregoing factors for cholinergic pathways of the central nervous stystem. The same applies to the remaining site of cholinergic transmission, the terminations of postganglionic cholinergic autonomic fibres at smooth muscle and gland cells.

IV. Physiological Function of Sympathetic Ganglionic AChE

In view of the marked differences mentioned above, it is not unlikely that AChE serves different primary functions at various synaptic and neuro-effector sites. Recently, R. L. Volle and I (1961) undertook an investigation of the primary role of the AChE of the cat superior cervical ganglion. Four possibilities were suggested from the earlier literature: (1) temporal and spatial limitation of the transmitter action of ACh at the postsynaptic site (Feldberg and Vartiainan, 1934; Eccles, 1944; Holaday and others, 1954), (2) provision of an immediate source of choline, by the hydrolysis of liberated ACh, for uptake and synthesis of ACh by the preganglionic terminals (Perry, 1953; 1957), (3) prevention of the accumulation of sufficient ACh liberated during the resting stage to activate the ganglion cells (Feldberg, 1945a), and (4) protection of the presynaptic terminals against the effects of ACh released during the resting or active stages (Koelle and Koelle, 1959). Previous reports indicating the first possibility were confirmed by measuring the effects of intra-arterially injected diisopropyl phosphorofluoridate (DFP) on the post-

TABLE I

THRESHOLD DOSES FOR ACTIVATION OF SUPERIOR CERVICAL GANGLION BY INTRA-ARTERIAL INJECTION OF ACH AND CARBACHOL
(from Volle and Koelle, 1961)

	Mean Threshold Dose (mμ mol ± S.D.)	
	ACh	Car
Normal control	27 ± 13 (21)*	2·8 ± 1·1 (8)
Normal post-DFP†	0·73 ± 0·50 (13)	2·2 ± 1·1 (7)
Control: post-DFP	38 : 1	1·3 : 1
Denervated control	36 ± 15 (19)	71 ± 45 (10)
Denervated post-DFP†	3·4 ± 2·8 (14)	6·6 ± 7·2 (8)
Control: post-DFP	11 : 1	11 : 1
Control *denervated: normal*	1·3 : 1	26 : 1
Post-DFP *denervated: normal*	4·6 : 1	3·0 : 1

* Number of experiments in parentheses.
† 2 μ mol, i.a.

ganglionic response to supramaximal stimulation of the partially resected preganglionic trunk. No evidence could be obtained to support the second proposal. The third suggestion was substantiated most dramatically by the appearance of spontaneous postganglionic firing and its persistance for several hours after the intra-arterial injection of high doses of DFP. The likelihood of the major importance of the fourth suggested function, protection of the presynaptic terminals against the action of ACh, was indicated by results obtained from determinations of the threshold intra-arterial doses of ACh and of its much more stable analogue, carbachol, for the production of detectable firing of the post-ganglionic trunk. Such determinations were conducted in normal and

chronically preganglionically denervated ganglia, both before and after inactivation of ganglionic AChE and non-specific ChE by the intra-arterial injection of DFP. The results, which in certain respects were unexpected, are summarised in Table I. First, it will be seen that in normal ganglia DFP caused a 38-fold reduction in the mean threshold dose of ACh, but no significant change in that of carbachol; this is fully in keeping with the susceptibility of only the former to rapid hydrolysis by AChE. On the other hand, in the denervated ganglia the mean threshold doses of both ACh and carbachol were reduced after DFP by 11-fold. Now, if the mean threshold doses of the two compounds are compared in normal and denervated ganglia, it is apparent that that of ACh was not changed significantly, whereas that of carbachol was increased 26-fold; the latter figure is strikingly the reverse of what would have been predicted from the "law of denervation supersensitivity" (Cannon, 1939). Likewise, the threshold doses of both ACh and carbachol were higher (approximately 5-fold and 3-fold, respectively) in DFP-treated denervated than in DFP-treated normal ganglia. The proposed interpretation of these findings is depicted in Fig. 4. If we consider first the denervated ganglia without

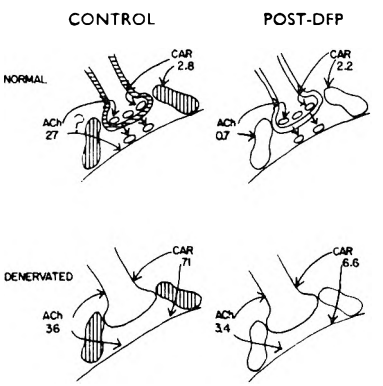


FIG. 4. Diagram depicting threshold intra-arterial doses (in mμ mol) of ACh and carbachol in four situations studied.

Horizontal cross-hatching represents AChE of presynaptic terminals; vertical cross-hatching non-specific ChE of capsular glial cells; vesicles the transmitter.
(From Volle and Koelle, 1961.)

DFP-treatment (lower left), here the two compounds must have acted directly at the only available excitable site, the ganglion cell membranes. The much lower threshold dose of carbachol in the normal ganglia (upper left) suggests that in this situation it acted on the presynaptic terminals, causing them in turn to liberate sufficient ACh to activate the ganglion cells. However, the effectiveness of injected ACh was limited here by the protective presynaptic sheath of functional AChE; hence, its threshold dose (not significantly different from that in the denervated ganglia) might have acted presynaptically, postsynaptically, or at both sites. On the other hand, in the DFP-treated normal (upper right), as compared with the DFP-treated denervated (lower right) ganglia, the threshold doses of both

drugs were significantly lower; hence, it may be assumed that both acted primarily at the presynaptic site in the former group. There are two possible explanations for the 11-fold reduction in the threshold doses of both ACh and carbachol in the denervated ganglia following treatment with DFP: (1) sensitization of the postsynaptic site by the combination of DFP with non-specific "B" groups in the area of the specific receptors, as suggested by Cohen and Posthumus (1955), and (2) the alkylphosphorylation of the non-specific ChE of the glial cells, which might otherwise act as the barrier to both compounds by combining with them without necessarily promoting their hydrolysis significantly (Koelle, 1946; Goldstein, 1951). The foregoing interpretation is not the only possible one for the results obtained, and several alternatives were considered (Volle and Koelle, 1961); nevertheless, at present it seems to be the one which is most consistent with all the observations noted.

To extrapolate these essentially pharmacological findings to the question of the physiological function of the ganglionic AChE, the first point to be emphasised is the apparently much greater sensitivity of the presynaptic terminals than of the postsynaptic membranes to carbachol and, after DFP, to ACh. This is consistent with the earlier suggestion that the primary function of the sympathetic ganglionic AChE is to protect the axonal terminals, where the enzyme is almost exclusively localised, against the persistent action of ACh liberated by themselves and adjacent terminals (Koelle and Koelle, 1959). The continuous, spontaneous postganglionic firing which followed inactivation of most of the enzyme by DFP therefore seems most reasonably attributable to continuous re-excitation of the terminals by endogenously liberated ACh, with the consequent accumulation of sufficient ACh to act postsynaptically. Likewise, the temporal and spatial spread of postganglionic activation after preganglionic stimulation which followed moderate doses of DFP can be explained on the basis of extension of the influence of the transmitter at the presynaptic level. One is then faced with the question, what physiological advantage could result from this seemingly reversed order of sensitivity to a presumably junctional transmitter? According to the working hypothesis proposed, this provides a self-amplification mechanism for extension of the depolarising action of ACh across the synaptic cleft. In contrast to the usual concept of the sequence of events in cholinergic transmission, mentioned at the beginning and depicted in Fig. 5A (Grundfest, 1957) let us assume than an additional step is interposed: the ACh first liberated at the terminals by the depolarising action of the NAP (Eccles and Liley, 1959) acts at the same terminals to maintain the depolarised state long enough to release sufficient ACh to produce depolarisation at the postsynaptic site (Fig. 5B) Koelle, 1961). The primarily presynaptic action of the transmitter is then limited or terminated by the AChE located there; its postsynaptic action is terminated immediately by diffusion, although the presynaptically located enzyme probably is responsible for the ultimate hydrolysis of the major part.

Related observations can be found in the literature which are consistent with this concept. Ordinarily, injected ACh or anti-ChE agents do not

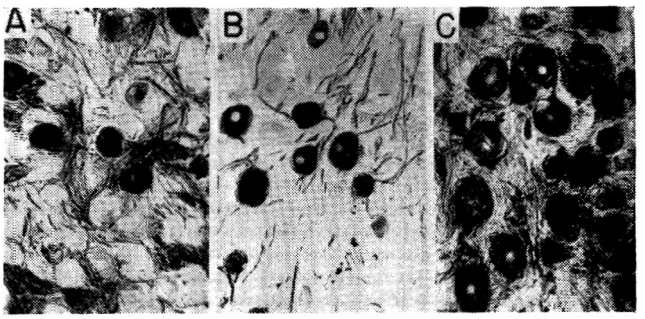


FIG. 1

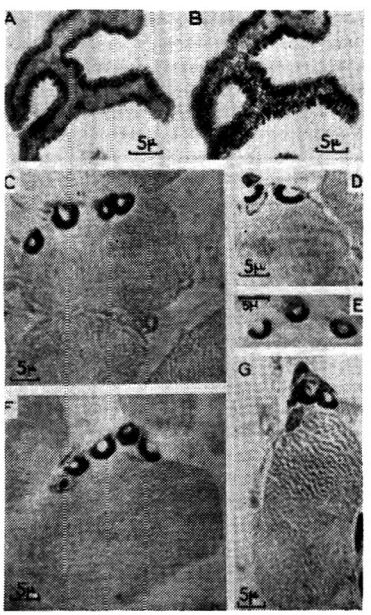


FIG. 3

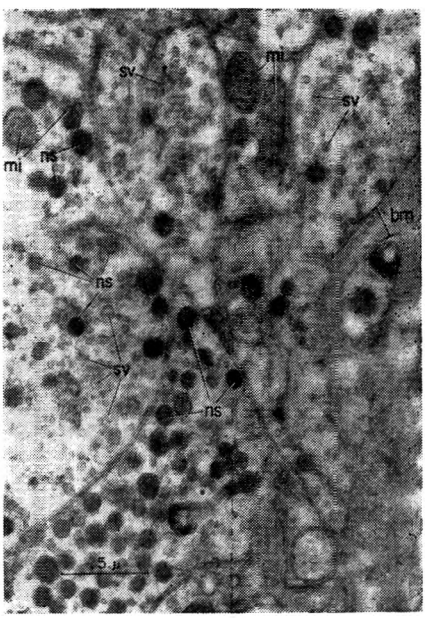


FIG. 6

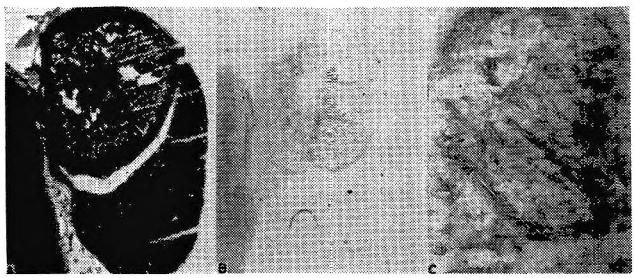


FIG. 7

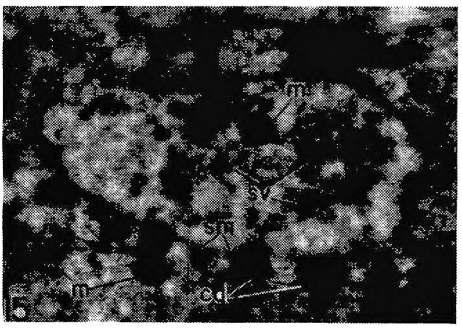


FIG. 8

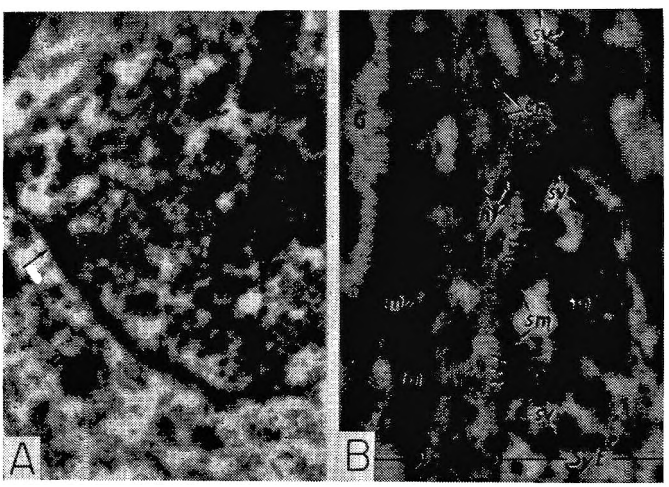


FIG. 12

cause antidromic firing from preganglionic terminals (Volle and Koelle, 1961; Douglas, Lywood and Straub, 1960), as they do from the terminals of motor nerves, to be discussed in the next section. This may be due to the restriction of the chemically excitable region of the preganglionic axons to the fine terminal twigs, where the localised potentials may be of insufficient magnitude to initiate propagated impulses. A comparable situation was encountered at the neuromuscular junction by Riker and his associates in a study, discussed below, of the apparent action of 3-hydroxyphenyltriethylammonium on the motor axonal terminal: in most cases, the drug produced both antidromic firing along the axon and

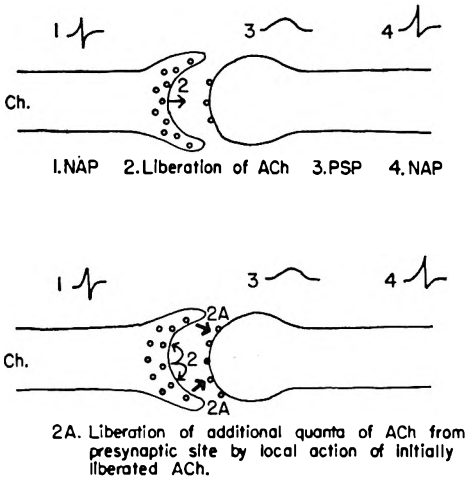


FIG. 5. Functions of ACh in Cholinergic Transmission.

A (upper): Standard concept. (1) Nerve action potential (NAP) causes axonal terminals to liberate (2) ACh, which diffuses across the synaptic cleft and combines with postsynaptic receptors, resulting in (3) localised depolarisation, the postsynaptic potential (PSP), which initiates electrogenically (4) NAP in second axon (Grundfest 1957).

B (lower): Proposed presynaptic function. Acetylcholine acts first at terminal from which liberated to activate release of (2A) additional quanta of ACh, which produce the PSP (Koelle, 1961).

repetitive activation of the muscle fibre; however, in about one-third of the cases only the latter effect was noted (Riker and others, 1959a). Dempsher and his associates have shown that in the pathological condition resulting from infection with pseudorabies virus, spontaneous and synchronous postganglionic and antidromic preganglionic firing arises from the superior cervical ganglion (Dempsher and others, 1955), and that this is probably due to the action of ACh released from the presynaptic terminals (Dempsher and Riker, 1957). Furthermore, they have detected at both poles of such ganglia slow waves of localised depolarisation, on which the propagated spikes are superimposed (Dempsher and Zabara, 1960); the former appear to represent typical generator potentials which have generally been thought to occur only at the postsynaptic side of the

junction. It is possible that the mechanisms underlying these virus-induced phenomena are a combination of destruction of much of the presynaptic AChE, and extension centrally of the area of ACh-excitability of the axonal terminals, comparable to that which occurs distally at the sarcoplasmic membrane of skeletal muscle fibres after denervation (Axelsson and Thesleff, 1959). The complexity of components of the ganglionic potential has recently been analysed by Eccles and Libet (1961), who concluded that the early (N) and late negative (LN) phases are due to the combination of ACh with two distinct types of postsynaptic receptors. However, it seems also possible that the latter phase may reflect a more protracted presynaptic potential, such as that described for the pseudo-rabies-infected ganglia. Its identification by the authors as postsynaptic was based largely on its susceptibility to the action of atropine and other drugs, whereas Laporte and Lorente de Nó (1950) concluded that in the turtle ganglion, (+)-tubocurarine and other drugs act primarily at the presynaptic site.

V. The Roles of AChE and ACh at the Neuromuscular Junction and Other Sites of Cholinergic Transmission

The ability of the neuromuscular junction to sustain a much higher frequency of impulse transmission than the sympathetic ganglia, the more obvious susceptibility of the former to anti-ChE agents, and the predominantly postjunctional localisation of the AChE at the motor endplate all indicate that the primary function of the enzyme here is to terminate the action of the transmitter at the postjunctional membrane, or subneural apparatus. While this is probably true, nevertheless there is convincing evidence that the axonal terminals are also susceptible to the actions of various agents, including ACh, which suggests that here too ACh may have an intermediary presynaptic role in transmission. One of the earliest studies indicating this was the now classic paper of Masland and Wigton (1940). These authors reasoned that the muscular fasciculation which follows the administration of physostigmine or neostigmine must reflect the synchronous firing of entire motor units, and not the unorganised contraction of individual muscle fibres; hence the drug, or the accumulated endogenous ACh, must act at some neural site, directly or indirectly. In support of this, they quoted the much earlier observation of Langley and Kato (1915) that physostigmine does not produce fasciculation in chronically denervated muscle. In a series of well-controlled experiments, Masland and Wigton showed that the bursts of muscle action potentials which followed small intravenous doses of neostigmine were accompanied by discharges, at approximately the same frequency, of antidromic impulses along the motor nerve, as recorded at the anterior root. When the motor nerve was stimulated in the presence of the drug, the resultant tetanic contraction of the muscle was accompanied by repetitive showers of both muscle action potentials and antidromic motor nerve volleys. By sectioning or cocainising the nerve at various levels, the origin of the antidromic volleys was traced to the neighbourhood of the motor axonal terminals. The administration of curare blocked simultaneously both

the muscle fasciculation and the antidromic motor nerve volleys. Similar results were obtained following the intra-arterial injection of ACh, that is, muscle fasciculation, showers of antidromic motor nerve volleys, and the blockade of both by curare. In considering various possible interpretations of their findings, the authors concluded: "It is much more likely that in the same way that acetylcholine stimulates the end plate, it also stimulates the motor nerve ending at the end plate." Likewise, they assigned the blocking action of curare to both sites.

These findings and their interpretation have been quoted in detail both because of their bearing on the present hypothesis, and because they have received considerable amplification and confirmation since then. By means of different techniques, results similar to those obtained with neostigmine have been found with physostigmine (Eccles, Katz and Kuffler, 1942; Feng and Li, 1941) and DFP (Van der Meer and Meeter, 1956). The presynaptic origin of the antidromic firing was questioned on the basis that the muscle action potentials themselves might intiate such activity in the motor nerve terminals (Lloyd, 1942). However, it has been shown recently that retrograde firing of motor fibres can originate from both sources, and that the activity recorded by Masland and Wigton probably arose chiefly, as they had concluded, directly from the terminals (Werner, 1961). Although the competitive antagonism of tubocurarine with ACh at the postjunctional membrane was clearly demonstrated several years ago (Eccles and others, 1942; Kuffler, 1942), several authors have concluded subsequently that tubocurarine acts predominantly at the presynaptic terminal (Abdon and Bjarke, 1945; Storner, 1958a; Lilleheil and Naess, 1961), probably by interfering with the release of ACh (Abdon and Bjarke, 1945; Lilleheil and Naess, 1961). On the other hand, tetraethylammonium may act at the same site to augment ACh-release following the nerve impulse (Koketsu, 1958; Stovner, 1958b).

Of particular note are the recent studies along these lines by Riker and his associates (1957, 1959a, b) with a series of trialkylphenylammonium analogues of neostigmine, in which earlier work had shown that curare-antagonism and cholinesterase-inhibition could be dissociated (Randall and Lehman, 1950; Riker and Wescoe, 1950). With the aim of elucidating the sites and mechanisms of action of these compounds, they compared them for their abilities to (1) potentiate muscle tension following single maximal nerve shocks (through conversion of a single to a repetitive response of the muscle fibre), (2) stimulate denervated muscle fibres (referred to as depolarising potency), (3) antagonise curare, and (4) initiate repetitive retrograde axonal firing of motor nerves following single orthodromic volleys. The results indicated a distinct dissociation between effects on the pre- and postsynaptic membranes. 3-Hydroxyphenyltriethylammonium (3-OH PTEA) was most striking in its potency in three of the four tests, but its action on denervated muscle was negligible. On the other hand, phenyltrimethylammonium (PTMA) was most potent in the latter respect, but weakly active or inactive in the others. It was concluded that both curare-antagonism and twitch-potentiation by these

drugs resulted from actions at the presynaptic terminals, where they were believed to induce repetitive firing in response to single nerve volleys, thus increasing transmitter action. With respect to implications regarding the function of ACh in normal transmission, the authors' final statement in the first paper was: "It may be rather that acetylcholine is released from a bound state in the motor nerve terminal and reacts with presynaptic receptors to initiate the transmitter effect" (Riker and others, 1957). This, as far as it goes, is practically identical with the conclusion reached above for transmission in the sympathetic ganglion; for the present hypothesis, it need be added only that the transmitter effect is the release of further quanta of ACh to activate the postsynaptic membrane. In their subsequent study, in which the actions of 3-OH PTEA were examined more extensively, Riker and associates (1959a) indirectly strengthened this concept by emphasising the probable similarity of the localised events at the pre- and postjunctional membranes during transmission. A similar implication is found in the results obtained by Koketsu (1958) with direct extracellular and intracellular recordings from motor fibres, in the region of their terminals, following the arrival of the nerve impulse. These and related reports have recently been reviewed (Werner and Kuperman, 1962).

There is virtually no directly related information from which any conclusions can be drawn as to whether ACh exerts an intermediary presynaptic effect at the other two sites of cholinergic transmission, that is, at the terminals of certain postganglionic autonomic fibres and of certain fibres of the central nervous system. However, there are several observations which are highly suggestive of an intervening cholinergic mechanism in transmission by analogous non-cholinergic fibres, as will be considered next. On this basis, it follows that the mechanism proposed above for cholinergic preganglionic and neuromuscular transmission may apply also to the remaining types of cholinergic fibres.

VI. Evidence for the Participation of ACh in Transmission by Non-Cholinergic Neurons

Having considered the possible functional significance of the presynaptic location of functional AChE at certain sites of cholinergic transmission, we may now return to the other general histochemical observation for which an explanation was not readily apparent: the presence of moderate concentrations of AChE in various presumably non-cholinergic neurons. These include a small proportion of the sympathetic ganglion cells of the cat (of the remainder, an equally small proportion contain high concentrations, and the great majority only traces), and the afferent neurons of the dorsal root and vagal nodose ganglia of the cat, rabbit, and rhesus monkey. In the latter two species, practically all the sympathetic ganglion cells were shown to contain moderate concentrations of AChE (Koelle, 1955). The same was true also for the superior cervical ganglion of the rat. In the rat's central nervous system, all degrees of neuronal staining were noted, from intense, as in those giving rise to peripheral cholinergic fibres (for example, anterior and lateral horn cells, various

cranial motor and parasympathetic nuclei), to faint, as in certain association centres; however, the overwhelming majority showed some degree of staining for AChE (Koelle, 1954).

It is not possible to draw reasonable conclusions concerning function from isolated histochemical findings such as these. However, they raise questions, the answers to which must be sought from other lines of evidence. The general conclusion, or hypothesis, which seems most satisfactory at present is that in many or all such fibres, the conducted nerve impulse liberates first ACh, and that it in turn acts on the same terminals to release another compound which acts as the junctional transmitter.

A. Hypothalamico-Neurohypophysial System

Neurosecretory systems, which play a prominent role in development and metabolic regulation in arthropods, have a mammalian counterpart in the hypothalamico-neurohypophysial system. The neurohypophysis itself probably represents only a collection of terminals of neurosecretory fibres which arise from neurons in the supraoptic and paraventricular nuclei of the hypothalamus and course through the infundibular stalk to the infundibular process of the neurohypophysis. The secretory products, oxytocin and vasopressin, are synthesised at least in part within the hypothalamic cell bodies, then transported to the terminals, from which they are released in response to centrally mediated reflexes (Bargmann and Scharrer, 1951; Scharrer and Scharrer, 1954). From the studies of Pickford (1947) and her associates (Duke, Pickford and Watt, 1950; Abrahams and Pickford, 1956) of the effects of anti-ChE agents, it is likely that the secretion of both oxytocin and vasopressin is mediated by cholinergic fibres. While visiting our laboratory a few years ago, V. C. Abrahams attempted to identify the cholinergic pathways involved by staining serial sections of the dog's hypothalamus for AChE. While the enzyme was found in moderate concentrations in the neurons of the supraoptic and paraventricular nuclei, none of the fibres synapsing with them appeared to contain significant amounts. Along with several alternative possible explanations of these findings, we suggested that the neurosecretory fibres themselves might be cholinergic; that impulses conducted along the hypothalamico-neurohypophysial fibres might liberate ACh at their terminals, and it in turn provide the stimulus for the release of oxytocin or vasopressin (Abrahams, Koelle and Smart, 1957). The same proposal was subsequently made independently by De Robertis and his associates (Gerschenfeld and others, 1960) on the basis of their finding that electron micrographs of the neurohypophysial terminals in the toad, just as in the rat (Palay, 1957), revealed two distinct populations of vesicles. Those of one group (which could be traced back to the hypothalamic nuclei, were relatively electron-opaque, and averaged 1,500 Å in diameter at the terminals) presumably represented the endocrine secretions; the other group (which were confined to the terminals, were less opaque, and had an average diameter of 400 Å) resembled the synaptic vesicles seen at the terminals of cholinergic and other types of fibres throughout the nervous system (Fig. 6, see p. 78*a*). Following this report, the neurohypophysis of

the cat was examined histochemically for AChE activity. As seen in Fig. 7 (see p. 78*b*), the fibres were found to contain moderate concentrations of the enzyme, which was not present in the adjacent adenohypophysis (Koelle and Geesey, 1961).

Here, then, is indirect evidence of cholinergic mediation in the secretion from the same terminals of hormonal agents which act at distant sites. It should be noted that both the electron microscopic and histochemical findings in the neurohypophysis are in distinct contrast to those noted in the adrenal medulla. At the latter site, both AChE activity (Koelle, 1951) and the characteristic synaptic vesicles (De Robertis, 1959) are confined to the presynaptic terminals of the splanchnic fibres, whereas the catecholamine-containing granules are localised postsynaptically in the chromaffin cells (Fig. 8, see p. 78*b*). The latter pattern is consistent with the concept that the nerve fibres represent the preganglionic cholinergic innervation of the chromaffin cells, which embryologically and functionally are the analogues of adrenergic ganglion cells.

B. Adrenergic Fibres

Burn and Rand (1958a, b; 1960) have proposed that a cholinergic mechanism intervenes in the release of noradrenaline by postganglionic adrenergic fibres; they have accumulated considerable evidence to support such a claim. Several years ago, Burn (1932) found that stimulation of the sympathetic fibres to the hind leg of the dog under different conditions could cause either vasoconstriction or vasodilatation; the observations were explained only partially by the assumption that both adrenergic and cholinergic fibres are present in the lumbar sympathetic chain (Bülbring and Burn, 1935). More recently, Burn and Rand (1958a, b) have shown that after the administration of sufficient atropine to block the muscarinic effects of ACh, the intra-arterial injection of ACh

FIG. 1. Autonomic ganglia, cat, stained for acetylcholinesterase activity. Sections (10μ) incubated 80 min. in AThCh medium following selective inhibition of non-specific ChE by DFP. Magnification X 100.

A. Stellate ganglion, normal.
B. Stellate ganglion, preganglionically denervated.
C. Ciliary ganglion.

(From Koelle and Koelle, 1959)

FIG. 3. Localisation of ChE activity at the level of the motor endplate of mouse intercostal muscle. (Formalin fixation: 45 min. incubation with AThCh at pH 4·7.)

A and B. Front view of motor endplate. A, focused at the border of the synaptic gutters and showing the levelling of the subneural apparatus at the surface; B, focused at the base. Magnification × 875.

C to G. Cross sections of muscle fibres of different types, showing the endplates at the level of the synaptic junction. In C, the condenser has been adjusted to render the muscle fibres colourless; in the remainder, secondary staining due to carmine is detectable. Magnification × 750.

(From Couteaux and Taxi, 1952)

FIG. 6. Electron micrograph of a normal toad neurohypophysis. Within the enlarged endings there are neurosecretion granules (ns) surrounded by a membrane, synaptic vesicles (sv), and mitochondria (mi). A thick fibrillar basement membrane (bm) is seen near the capillary. 28,500 ×.

(From Gerschenfeld, Tramezzani and De Robertis, 1960)

or of nicotine produced vasoconstriction and other sympathomimetic effects in various organs. On the other hand, with prior administration of reserpine, which depletes most organs of their noradrenaline content, stimulation of sympathetic nerves caused cholinomimetic responses at several sites; the latter effects were blocked by atropine (Burn and Rand, 1960). From these and related findings, the authors concluded that adrenergic nerve impulses liberate first ACh at the terminals, and that it in turn releases noradrenaline. Confirmatory evidence was published by Chang and Rand (1960), who found that hemicholinium (HC3,α,α′-dimethyl-ethanolamino-4,4-biacetophenone) blocked at several sites the effects of stimulation of sympathetic nerves; this compound can prevent the liberation of ACh by certain cholinergic fibres, presumably by interfering with its synthesis through blockade of choline-uptake (Birks and MacIntosh, 1957; Gardiner, 1957). On the other hand, other investigators have reported that HC 3 failed to prevent contraction of the cat nictitating membrane in response to stimulation of the postganglionic sympathetic fibres, *in situ* (Wilson and Long, 1959) and *in vitro* (Gardiner and Thompson, 1961).

Two possible sites were suggested for the peripheral stores of noradrenaline: the terminals of the adrenergic fibres themselves, and adjacent chromaffin cells (Burn and Rand, 1960). Thus, in relation to the proposed ACh-mediated release mechanism, these would be analogous to the contrasting situations in the neurohypophysis (Fig. 6) and the adrenal medulla (Fig 8), respectively. Inasmuch as sympathetic denervation depletes various organs of most of their noradrenaline content (Euler and Purkhold,

FIG. 7. Sections (15 μ) of cat neurohypophysis (above), adenohypophysis (below), and adjacent hypothalamus (left) stained for AChE activity by 120 min. incubation in AThCh medium preceded by 30 min. incubation with 10^{-3} M DFP for selective inhibition of non-specific ChE.

A. Magnification × 7½, counterstained with H and E. B. Magnification × 7½, no counterstain. C. Portion of neurohypophysis, magnification × 40, no counterstain. All staining in B and C represents AChE activity.

(From Koelle and Geesey, 1961)

FIG. 8. Electron micrograph of a nerve ending of the adrenal medulla of the normal rabbit interposed between chromaffin cells. The ending contains mitochondria (m) and numerous synaptic vesicles (sv); sm, synaptic membrane. In the chromaffin cell large catechol-containing droplets (cd) and mitochondria are seen. (× 28,500.)

(From De Robertis, 1959)

FIG. 12. Specialised features of presynaptic and postsynaptic membranes at presumed sites of transmission.

A. Electron micrograph of a single bouton on the surface of a neuron in the abducens nucleus. The ending is filled by eight mitochondrial profiles and a host of synaptic vesicles. The arrow indicates a synaptic complex. The synaptic cleft is well shown except in its lower portion where the pre- and postsynaptic membranes overlap in the plane of the section. The cytoplasm of the neuron beneath the postsynaptic membrane displays no characteristic differentiation. × 33,450.

(From Palay, 1958)

B. Electromicrograph of a thin section of a normal ventral acoustic ganglion of the guinea-pig. Two synaptic endings (SyE) containing mitochondria (m) and numerous synaptic vesicles (sv) are found in contact with a dendrite (D). The synaptic membrane (sm) shows regions of higher electron density (marked with arrows). Also indicated are a glial cell (G), neuroprotofibrils (nf), and the endoplasmic reticulum (er). × 29,500. (From De Robertis, 1958)

1951; Cooper and others, 1961), the former site probably accounts for the major portion.

Burn and Rand's proposal of the intermediary role of ACh in the release of noradrenaline by adrenergic fibres provides a tentative explanation of the function of the intermediate and low concentrations of AChE observed in various sympathetic ganglion cells. Insofar as they can be traced, the AChE-contents of the postganglionic fibres are proportionate to those of their neurons of origin (Koelle, 1955; Giacobini, 1957). Accordingly, it might be postulated that the AChE concentrations of the neurons reflect the extent of participation of ACh in the release of noradrenaline at their terminals. If this is true, it would follow that the postulated mechanism is a much more important and generalised one in the rabbit and monkey than in the cat. In certain fibres, such as those to the cat nictitating membrane where transmission was reported to be unaffected by HC 3 (*v.s.*), the mediation of ACh in noradrenaline release may be of little or no significance.

C. Primary Afferent Fibres

Several years ago De Castro (1942, 1951) reported some remarkable experiments in which he produced, by means of ingenious nerve transplantations, "synthetic" autonomic reflex arcs entirely apart from the central nervous system. An example is illustrated in Fig. 9, in which the

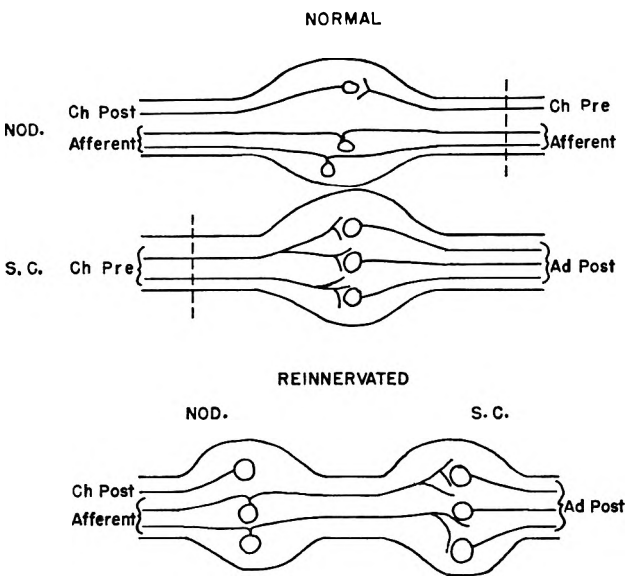


FIG. 9. Diagrammatic representation of synaptic relationships in the normal vagal nodose ganglion (NOD) and superior cervical ganglion (s.c.), and in the cross-anastomosed preparations.

Transverse dotted lines indicate sites of sectioning. Cranial ends to the right, caudal to the left. Fibre types include cholinergic preganglionic and postganglionic (Ch Pre and Ch Post), adrenergic postganglionic (Ad Post), and afferent. (Modified from De Castro, 1951 and Matsumura and Koelle, 1961.)

centrally directed afferent fibres from the vagal nodose ganglion were caused to reinnervate the preganglionically denervated superior cervical ganglion of the cat. On the basis of the miosis and contraction of the nictitating membrane which followed physiological stimulation of the afferent distribution of the vagus or electrical stimulation of the trunk, it appeared that new functional synapses had been established. Application of solutions containing physostigmine to the reinnervated ganglia failed to modify the responses; therefore, De Castro concluded that the newly established preganglionic fibres were non-cholinergic.

Recently, Matsumura (Matsumara and Koelle, 1961) was able to produce similar preparations in our laboratory, and to confirm by the same criteria the establishment of functional reinnervation. He then undertook a comparison of the effects of physostigmine and several selective blocking agents, injected via the arterial supply of the ganglion, on the response of the nictitating membrane to the injection of various ganglionic stimulants by the same route and to stimulation of the artificial preganglionic trunk. To summarise the most significant results, fixed doses of physostigmine caused potentiation, and of tetraethylammonium selective blockade of the responses to both stimulation of the nerve trunk and injection of ACh, propionylcholine, or butyrylcholine. The other blocking agents (for example, lysergic acid diethylamide and carbinoxamide) blocked selectively their stimulant pharmacological counterparts (5-hydroxytryptamine and histamine, respectively) but required much higher doses to block the response to preganglionic stimulation or to ACh. Over a wide range of doses, physostigmine caused only a reduction in the response to the other ganglionic stimulants. Histochemical examination of the reinnervated ganglia revealed that the presynaptic fibres contained relatively low (in comparison with the normal preganglionic fibres) concentrations of AChE, just as the majority of those of the normal vagal trunk. From these findings we concluded that the transmitter involved in the reinnervated ganglia was probably ACh or a closely related compound.

The difference between Matsumura's results and those of De Castro with physostigmine was ascribed to the different methods of administration employed. However, there is another, more serious objection to the foregoing interpretation. On the basis of the relatively low concentration of AChE (Burgen and Chipman, 1951), and the very low amounts of ACh (Loewi and Hellauer, 1938; Lissack and Pasztor, 1940; MacIntosh, 1941) and ChAc (Hebb and Silver, 1956; Cohen, 1956) present in the vagal trunk and the dorsal spinal roots, it has been assumed generally that primary afferent fibres are non-cholinergic. Accordingly, we proposed, along with some other possibilities, that ACh might under normal circumstances function at the central vagal afferent terminals to release another neurohumoral transmitter to which their normal postsynaptic connections, but not the sympathetic ganglion cells, are sensitive. For example, substance P is present in relatively high concentrations in dorsal root fibres and has been proposed as their transmitting agent (Andrews and Holton, 1958); however, it apparently does not stimulate ganglion cells

(Beleslin, Radmanović and Varagić, 1960). The proposed action of ACh at the presynaptic terminals in the spinal cord might be the basis of the relatively prolonged dorsal root potentials described by Barron and Matthews (1938) and Lloyd (1952).

In Fig. 10 is depicted the proposed function of ACh in synaptic transmission by the afferent vagal and other non-cholinergic fibres. With minor modifications, it could represent Burn and Rand's hypothesis of cholinergic mediation in adrenergic transmission. If the distance between the sites of release and action of the transmitter is then increased from a few hundred Å to several dozen centimetres, the further modified diagram would illustrate also the role of ACh in neurohypophysial secretion proposed earlier.

Let us turn from consideration of the proposed function of ACh at the central terminals of primary afferent neurons to its possible role at the peripheral terminations of the same or equivalent neurons. This is by no means a new question, but one which has long been disputed. It is well known that injected ACh and related compounds can stimulate most sensory endings and end-organs, and that this action is blocked by such drugs as hexamethonium (C-6), TEA, and nicotine (Gray, 1959). From such observations it has been argued that ACh is involved in the normal mediation of sensory reception (Liljestrand, 1954), and contrariwise, that these are essentially pharmacological phenomena which do not justify extrapolation to inferences regarding physiological function (Douglas, 1954). An apparently strong piece of evidence for the latter viewpoint is that, whereas relatively low doses of hexamethonium blocked the effect of ACh on the carotid sinus pressoreceptors, doses several

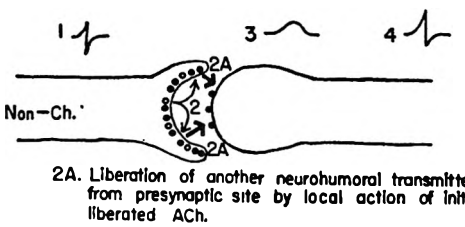


FIG. 10. Proposed presynaptic function of ACh in transmission by non-cholinergic fibres. Compare with Fig. 5.

(1) NAP liberates (2) ACh from presynaptic terminals, which acts at same terminals to effect release of (2A) synaptic transmitter; latter produces (3) PSP, which initiates (4) NAP (Koelle, 1961).

magnitudes higher failed to prevent their response to increased intracarotid pressure (Diamond, 1955; Gray and Diamond, 1957). On the other hand, it is conceivable that both the administered drugs acted at chemosensitive portions of the fibres which were adjacent centrally to sites of physiological release and action of ACh, and that at the latter sites membranous barriers prevented access to compounds coming from the circulation. Suggestive of this is the report that the tertiary base, nicotine, prevented the response of touch receptors of frog skin to both mechanical

stimulation and applied ACh, whereas the quaternary compound, tubocurarine, blocked only activation by the latter (Jarrett, 1956).

The strongest indication of the participation of a chemical-receptor combination in the peripheral initiation of afferent impulses lies in the parallelism between the electrical events recorded at sensory terminals and at various postsynaptic membranes. The initial response to pressure recorded at a Pacinian corpuscle (Gray and Sato, 1953; Loewenstein, 1959), or to tension at the crayfish stretch receptor (Eyzaguirre and Kuffler, 1955), is a localised, relatively slowly developing, graded transducer or generator potential, which in all these characteristics resembles the EPP or PSP produced at the postjunctional site of the motor endplate (del Castillo and Katz, 1955) or anterior horn cell (Coombs, Curtis and Eccles, 1957) in response to a transmitted nerve impulse or the application of minute amounts of ACh. The sensory generator potential, like the EPP or PSP, then initiates a propagated nerve action potential. On the basis of an extensive analysis of this sequence of events in a wide variety of sensory receptors, Davis (1961) has concluded recently that a chemically mediated step is interposed between the physiological stimulus and the initiation of the generator potential.

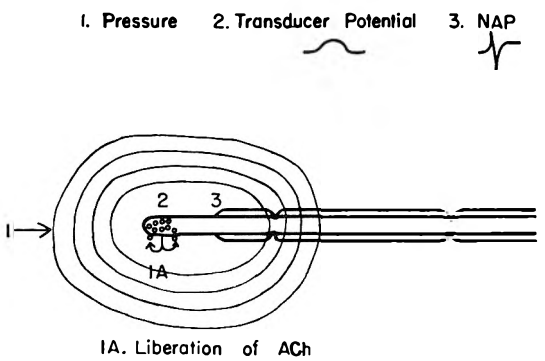


FIG. 11. Proposed function of ACh in sensory reception.
(1) Specific stimulus (e.g., pressure) causes release of (1A) ACh from axonal terminal (as shown) or accessory cells, which causes (2) depolarisation of terminal recorded as transducer potential; latter initiates electrogenically (3) NAP. (Koelle, 1961.)

Electron micrographs have revealed the presence of synaptic vesicle-like inclusions in the axonal terminals within Pacinian corpuscles (Pease and Quilliam, 1957), and in the laminar cells adjacent to the terminals in Meissner's corpuscles (Cauna, 1960). At many sensory receptors, low concentrations of AChE have been noted within the axonal terminals, and in some cases the surrounding accessory cells contain non-specific ChE (Cauna, 1961; Koelle, 1962b). From the foregoing considerations, it seems reasonable to propose that in response to the specific stimulus, such as stretch, ACh or a similar compound is released, either from the axonal terminal itself or from the accessory cells, which combines with receptors in the axonal membrane to initiate the generator potential (Fig. 11). Of possible bearing on this proposal is the conclusion of

Hutter and Trautwein (1956) that the application of stretch to the terminals of motor fibres caused recruitment of ACh release.

D. Central Neurons

It is now generally believed, with considerable justification, that the central nervous system contains both cholinergic and non-cholinergic neurons (Feldberg, 1945b, 1956). Although several other central neurohumoral transmitters have been proposed, none has yet been shown convincingly to function in this capacity (Perry, 1956; Paton, 1958). It must be admitted that at present there is virtually no evidence for a presynaptic role of ACh in either type. However, the assumption of such would provide an explanation of the results obtained in a histochemical survey of the central nervous system of the rat, mentioned previously (Koelle, 1954). Certain known cholinergic central neurons, for example, the anterior horn cells, and others, for example, those of the caudate nucleus, were found to contain high concentrations of AChE, but extremely few appeared to be devoid of the enzyme. In most instances, neuronal staining was noted as "intermediate" or "light." We might then suggest that here, as proposed for adrenergic ganglion cells, the concentration of AChE in a given neuron reflects the extent of the participation of ACh in synaptic transmission, both pre- and postsynaptically in heavily stained, cholinergic neurons, and presynaptically in less heavily stained, non-cholinergic fibres. There is nothing that can be added, excepting perhaps a classic plea: "Data! data! data! . . . I can't make bricks without clay" (Doyle, 1938).

VII. Other Functions of ACh and AChE

So far, we have considered only the functions of ACh and AChE in neurohumoral transmission, and have proposed that they act in this capacity at both the pre- and postsynaptic sites. Yet the widespread occurrence of ACh and its associated enzymes in both neural and non-neural tissues suggests that its functions are not confined to this role. It has been proposed that ACh modifies ciliary movement in protozoans (Seaman and Houlihan, 1951) and in ciliated membranes of invertebrates (Bülbring, Burn and Shelley, 1953) and vertebrates (Kordik, Bülbring and Burn, 1952), acts as a local hormone in the control of excitability and contraction of smooth (Feldberg and Lin, 1950) and cardiac muscle (Burn and Kottegoda, 1953), is responsible for the initiation of the nerve action potential in axonal conduction (Nachmansohn, 1959), and regulates active or passive transfer of ions in the membranes of erythrocytes (Holland and Greig, 1950), frog skin (Kirschner, 1953), skeletal muscle (Van der Kloot, 1958), and avian salt glands (Hokin and Hokin, 1960). The evidence for and against these proposals has recently been summarised (Koelle, 1962b). It should be noted that in all these cases, just as at the synapse (Eccles, 1959), the primary action of ACh is considered to be the modification of membrane permeability. From an evolutionary standpoint, it is likely that this function of ACh was introduced originally in non-neural tissues, and developed eventually to its highest degree of

specialization and efficiency at the synapse. It is at cholinergic synapses that the direct influence of ACh has been extended from a local to an intercellular sphere.

A synaptic element which has been mentioned only in passing, but which may participate more actively in regulating transmission than is generally considered, is the glial cell (Galambos, 1961). In all autonomic ganglia, and at many other sites, the glia contain high concentrations of non-specific ChE, the function of which is unknown.

VIII. Pharmacological Considerations

The receptor theory, which was developed at the turn of the century by Paul Ehrlich to explain antigen-antibody reactions, was elaborated subsequently by several pharmacologists, in particular A. J. Clark (1933), to account for the actions of various classes of drugs on cells. With the advent and general acceptance of the neurohumoral theory, the receptor theory of drug action could be expressed in much more concrete terms, although the nature of the receptors themselves for the most part has remained unknown (Waser, 1960). Thus, various classes of drugs have been considered to combine with the postjunctional cholinergic receptors (or cholinoceptive sites), and there to mimic or block the actions of the endogenous transmitter, ACh. The actions of anti-ChE agents have been attributed to the production of the former effect indirectly by causing the accumulation of excessive endogenous ACh. The theory can account for drug specificity by the assumption that while all cholinoceptive sites have characteristic groups in common which allow them to combine with ACh, secondary groups or specific features which influence orientation differ at various sites; thus, atropine can combine more readily with the cholinoceptive groups of autonomic effectors, and hexamethonium with those of autonomic ganglion cells. However, specificity is not absolute for either the mimicking or the blocking drugs; with sufficiently high doses, actions become diffuse at virtually all cholinoceptive sites to which the drugs have access, and other actions become manifest as well. Furthermore, a given drug, such as ACh itself, may produce either cholinomimetic or cholinergic blocking actions at certain sites, depending upon the dose, rate of combination with the receptors (Paton, 1961), and other factors. The theory has never been claimed to account for the actions of all types of neurotropic drugs; among the numerous exceptions are the general anaesthetics and anticonvulsant agents. Likewise, there are probably exceptional synapses where transmission is electrogenic rather than neurohumoral (Furshpan and Potter, 1959).

The new hypothesis which has been presented in no way detracts from the current theory, but rather introduces an additional element: the cholinoceptive site at the presynaptic terminals of cholinergic and non-cholinergic fibres. Several examples have been given above of drug actions at autonomic ganglia and the neuromuscular junction which can be explained most reasonably on the basis of a cholinomimetic or cholinergic blocking action at presynaptic receptors of the former type. As a likely example of a drug's acting at the presynaptic cholinergic receptors

of non-cholinergic fibres we might cite, as has Burn (1961), bretylium (*N-o*-bromobenzyl-*N*-ethyl-*NN*-dimethylammonium *p*-toluene sulphonate). This compound, which has a certain structural resemblance to ACh, has as its major action the prevention of the release of noradrenaline by adrenergic fibres (Boura and others 1960). There is a considerable amount of data which suggest that it does so by interfering with the hypothetical cholinergic mechanism involved in adrenergic transmission.

Another possible mechanism of pharmacological action which is inherent in the hypothesis is blockade of transmission by interference with the release of ACh at both cholinergic and non-cholinergic axonal terminals. Paton (1957) and Schaumann (1957) have demonstrated this action of morphine for certain cholinergic fibres. The possibility that morphine might have a similar effect on the central terminals of non-cholinergic vagal afferent fibres was suggested by Matsumura and Koelle (1961).

As mentioned previously, several investigators have demonstrated beyond question the cholinomimetic and cholinergic blocking actions of drugs at post-junctional sites. At the same time, in most cases the demonstrations did not exclude the possibility of a secondary or primary action at the presynaptic site. It is a corollary of the neurohumoral theory that the chemically excitable membrane must differ functionally, and probably structurally, from the electrically excitable conducting membrane of the remainder of the neuron or the muscle fibre (Grundfest, 1957). No direct demonstration of this has been published to date. However, it is worth noting that Palay (1958) and De Robertis (1958) have shown by electron microscopy that at various junctions there are "synaptic complexes" or "active points," characterised by higher electron density of both the presynaptic and postsynaptic membranes and a widening of the synaptic cleft; the synaptic vesicles are concentrated on the presynaptic side of such points, which the authors believe represent the actual sites of transmission (Fig. 12). It is possible that the increased electron density on both sides of the cleft in some way reflects the presence of receptor sites.

To carry the pharmacological implications of the present proposal one step further, the hypothetical presynaptic cholinergic receptors would be expected to vary in their specific characteristics, and hence in their potentialities for selective activation or blockade by drugs, just as do the post-junctional receptors. Such differences might apply to different types of ACh-reactive terminals, and to the presynaptic as compared with the postsynaptic receptors of a given cholinergic synapse. This suggests a possible rational approach to the development of more selectively acting drugs than most of those which are available at present. As just pointed out, bretylium, which has provided a new basis for the treatment of hypertension, is possibly a cardinal example of this type of selectivity.

In an excellent detective story which appeared several years ago, the murderer, a brilliant antiquarian, planted a number of clues which at first sight pointed directly to himself as the culprit. The subtlety behind this was that on more careful analysis, the false clues pointed obliquely to

a third party whom the perpetrator wished to implicate as the offender while giving the impression that the latter had attempted to implicate him. At the denouement, the police sergeant accused the antiquarian on the basis of the superficial evidence, the district attorney accused the third party, while the amateur detective saw through the plot and, like the sergeant, identified the true culprit, but on the basis of an entirely different line of reasoning. I mention this because it parallels in part the stages in the history of our conceptions about the sites of action of many of the drugs that have been considered here. Up to the 1930's, most textbooks of pharmacology stated that drugs such as atropine and curare produce their effects by paralysing the appropriate nerve terminals. With the development of the concept of neurohumoral transmission, it became generally accepted that most synaptotropic agents act by combining with postsynaptic receptors, and there mimicking or blocking the actions of the endogenous transmitter. Here the parallelism with the fictional situation must be modified. It would not be accurate to say that the presynaptic terminals can once again be implicated as the major site of action of most drugs. However, it seems undeniable that in many instances the axonal terminal is at least an accessory before the fact, with the relative importance of drug effects here and at the postsynaptic membrane varying with the drug, its dose, the synapse or junction, and the particular effect which is being studied.

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RESEARCH PAPERS

THE PRESSOR ACTION OF GUANETHIDINE IN THE SPINAL CAT

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Guanethidine has a marked pressor action in the spinal cat. Doses of 2–4 mg./kg. produce a greater increase in blood pressure than doses of 8–16 mg./kg. Large doses of guanethidine (16 mg./kg.) inhibit the pressor action of small doses (2 mg./kg.). Guanethidine potentiated the pressor action of noradrenaline but inhibited that of tyramine. The degree of inhibition of tyramine increased with the dose of guanethidine, but was inversely related to the pressor action of guanethidine.

In the anaesthetised dog or cat guanethidine produced sympathomimetic actions of approximately 45 min. duration (Maxwell, Plummer, Schneider, Povalski and Daniel, 1960; McCubbin, Kaneko and Page, 1961). When the sympathomimetic actions of the drug subsided a prolonged hypotension was observed. This paper describes the pressor action of guanethidine in the spinal cat.

Experimental

Cats were anaesthetised with ether and made spinal as described by Burn (1952). Blood pressure was recorded from the right carotid artery and drugs were injected into a femoral vein.

Drugs: Tyramine hydrochloride, (−)-noradrenaline bitartrate and 2-(octahydro-1-azocinyl)ethyl guanidine sulphate (guanethidine) were dissolved in 0·9 per cent w/v aqueous NaCl before each experiment. Doses have been expressed as weights of base.

Results

Although there was no significant regression of pressor action on dose for guanethidine, a small dose of the drug (2–4 mg./kg.) produced a greater increase in blood pressure than a large dose (8–16 mg./kg.), $P<0{\cdot}001$ (Table I*a* and Fig. 1). In most of these experiments a single injection of guanethidine was made, but when the drug was tested more than once in a cat, the amount injected did not exceed 4 mg./kg. except for the final injection, and injections were separated by intervals of at least 45 min. In one cat a first injection of 4 mg./kg. guanethidine produced a rise in blood pressure of 98 mm. Hg and a second injection of the same dose 45 min. later produced a rise in blood pressure of 95 mm. Hg. In two other experiments, however, 16 mg./kg. guanethidine inhibited the pressor action of a subsequent injection of 2 mg./kg. of the drug, $P<0{\cdot}01$ (Table I*b*).

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In most experiments the pressor actions of tyramine and noradrenaline were recorded before and after the injection of guanethidine. Submaximal doses of tyramine and noradrenaline were injected alternately, the dose of tyramine being selected to produce a rise in blood pressure of between 40 and 80 mm. Hg. When reproducible responses were obtained

TABLE I

EFFECT OF INTRAVENOUS INJECTIONS OF GUANETHIDINE ON SENSITIVITY TO NORADRENALINE AND ARTERIAL BLOOD PRESSURE IN SPINAL PREPARATIONS OF CATS

Numerical values are means ± s.e. of the mean. Number of observations in brackets

Dose (mg./kg.)	Sensitisation to noradrenaline $\left(\frac{\text{Initial dose noradrenaline}}{\text{equipressor dose after guanethidine}}\right)$	Rise in blood pressure (mm. Hg)
a. 2–4	4·8 ± 0·9 (5)	119·0 ± 6·9 (9)
8–16	4·6 ± 1·4 (7)	66·2 ± 7·3 (9)
b. 2	—	123·8 ± 9·5 (5)
2 after prior injection of 16 mg./kg.	—	34·0 ± 2·0 (2)

to these amines guanethidine was injected and the preparation rested until the blood pressure reached a steady level. Half an hour after guanethidine the same dose of tyramine and a reduced dose of noradrenaline were injected and their actions on blood pressure compared with the effects produced by these amines initially. Guanethidine

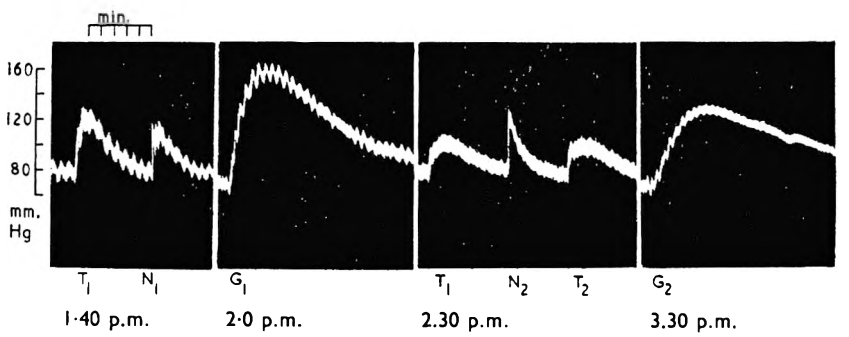

FIG. 1. Arterial blood pressure of spinal cat. The subscripts mark intravenous injections of 250 μg. tyramine (T_1), 2 μg. noradrenaline (N_1), 4 mg./kg. guanethidine (G_1), 0·25 μg. noradrenaline (N_2), 750 μg. tyramine (T_2), and 8 mg./kg. guanethidine (G_2).

potentiated the action of noradrenaline but inhibited that of tyramine, confirming the results of Maxwell, Plummer, Povalski and Schneider (1960). After guanethidine the response to tyramine changed very little with increasing dosage (Fig. 1). The per cent inhibition of the pressor action of tyramine increased with the dose of guanethidine (Fig. 2).

DISCUSSION

McCubbin and others (1961) suggested that the sympathomimetic actions of guanethidine resulted from a release of endogenous catecholamines since the rise in blood pressure and the vasoconstriction in a

denervated perfused hind limb of the dog after guanethidine were abolished with phentolamine or by pre-treatment with reserpine. It has been shown, moreover, in the rabbit and cat that guanethidine selectively depleted heart and spleen noradrenaline (Cass, Kuntzman and Brodie, 1960). However, the sympathomimetic actions of guanethidine were of shorter duration than the depletion of noradrenaline.

The prolonged release of a substance in the body may be mimicked by its infusion. When adrenaline or noradrenaline was infused into an animal for several hours the sympathomimetic actions declined during

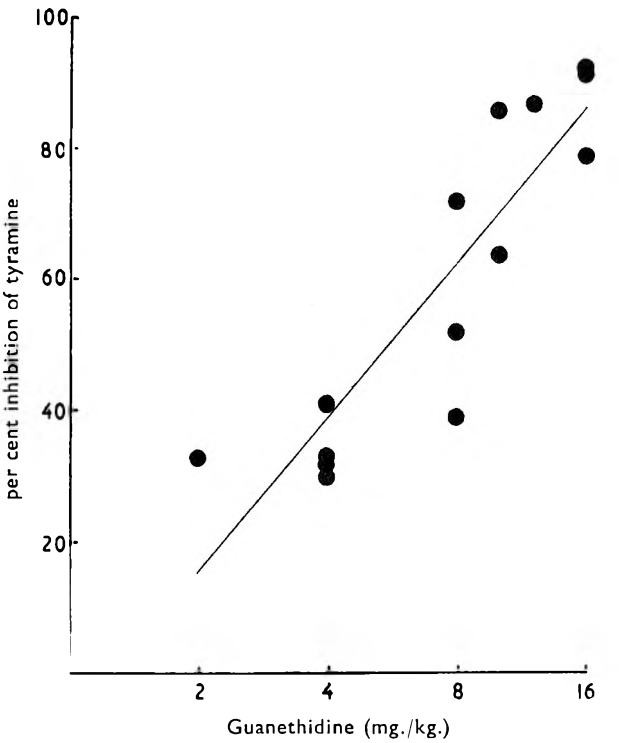

FIG. 2. Inhibition by guanethidine of the pressor action of tyramine in spinal preparations of cats. The calculated regression is significant at the 1 per cent level.

the infusion (Draškoci, Feldberg and Haranath, 1960; Lever, Mowbray and Peart, 1959). The discrepancy between the duration of sympathomimetic actions and the depletion of noradrenaline from heart and spleen did not therefore conflict with the hypothesis of McCubbin and others (1961) that the sympathomimetic actions of guanethidine were the outcome of the release of endogenous catecholamines.

In the present experiments a small dose of guanethidine (2–4 mg./kg.) produced a greater rise in blood pressure than a large dose (8–16 mg./kg.). It seemed unlikely that this was due to an action on catecholamine receptors since large and small doses of guanethidine potentiated the

pressor action of noradrenaline to the same extent (Table Ia). But, it could be explained if the small dose released more catecholamines than the large dose. If this was so, a large dose of guanethidine must inhibit its own action as a catecholamine releaser. The inhibition by a large dose of guanethidine of the pressor action of a small dose of guanethidine lends support to this hypothesis.

The pressor action of tyramine may be produced indirectly by the release of endogenous catecholamines (Lockett and Eakins, 1960a and b). This action would not be seen after a prior release of the amines or in

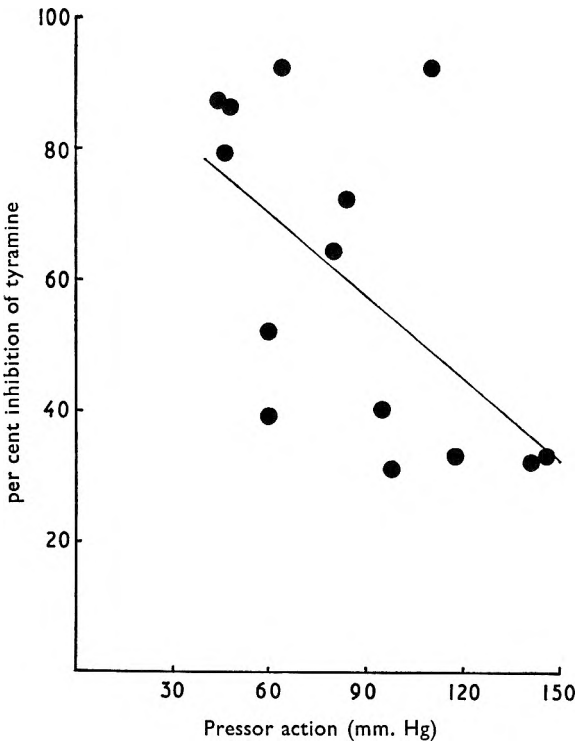

FIG. 3. Relation of pressor action of guanethidine to the ensuing inhibition of tyramine in spinal preparations of cats. The calculated regression is significant at the 5 per cent level.

the presence of an inhibition of catecholamine release. When guanethidine produced a large rise in blood pressure, indicative of a release of catecholamines, the corresponding inhibition of tyramine was small; tyramine was more effectively inhibited by doses of guanethidine which had a smaller pressor effect (Fig. 3). This suggests that inhibition by guanethidine of the pressor action of tyramine was not the outcome of a massive release of catecholamines from the tissues, but was the result of a change in the state of the tissue catecholamines so that their release by tyramine was inhibited.

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ASSAY OF DIGITALIS IN PIGEONS BY INTRAPERITONEAL ADMINISTRATION

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A new biological method of assay for digitalis is proposed. Preparations are administered intraperitoneally and the LD50 estimated by means of the "up-and-down" procedure. The LD50 of the preparation examined is compared with the LD50 of the standard preparation, and the result expressed in international units. The proposed method gives more evidence on the loss in potency of the drug than does the intravenous method in pigeons (U.S.P. XVI).

One of the main disadvantages of the biological methods for the assay of digitalis is that in cats, guinea-pigs and pigeons the preparations are injected intravenously, whereas clinically they are usually administered by mouth. The activity of glycosides and aglycones introduced directly into the blood is known to differ from that obtained by oral administration. This is because the pathways of absorption by the oral route are by-passed when the drug is given intravenously. Further, some interfering substances from the crude drug, introduced directly into the blood, can influence the activity of glycosides (Hildebrand, 1954) and thus give results different from those obtained by oral administration, where these substances, passing through the gastrointestinal tract do not enter the blood in the same form or in the same quantity.

In the biological assay of digitalis oral administration does not yield constant results as it is greatly influenced by the functional state and content of the digestive tract. Because of the emetic reflex of some species of laboratory animals the oral route is unsatisfactory. Although rats, mice and guinea-pigs do not exhibit this reflex they are unsuitable for this purpose on account of their very high resistance to the digitalis glycosides when given orally. Certain glycosides administered in this way cannot even kill some rodents (for example, strophanthin in rats, Hatcher, 1908). Because of these disadvantages oral administration seems to be generally abandoned in the assay of digitalis (Weese, 1936). Subcutaneous administration of digitalis to warm-blooded laboratory animals appears to be unsuitable since the results obtained are often inconsistent (Weese, 1936).

The overnight frog method, however, partly overcomes the disadvantages of intravenous administration, since the preparations are injected into the lymph sac where conditions for absorption resemble more closely those for oral administration. For this reason the frog method offers more information, with glycoside preparations which have undergone decomposition (Petričić, 1955). However, the main disadvantage of the frog method is that it requires a large number of animals to obtain satisfactory limits of error on the assay (Miles and Perry, 1950).

In order to overcome all these difficulties we chose pigeons as experimental animals because in the intravenous assay of digitalis (Braun and Lusky, 1948), they give results of greater precision than those given by other species of laboratory animals.

In our experiments preparations have been administered intraperitoneally. The LD50 has been determined for both the standard and the test preparations using the procedure of Dixon and Mood (1948). This concentrates testing around the LD50, thereby increasing the accuracy of the estimation. Under these experimental conditions the results obtained have satisfactory limits of error.

Experimental

Adult pigeons of either sex, showing no evident signs of illness, obesity or emaciation and weighing from 250 to 450 g. are used. The animals are divided into two similar groups, according to their weight. One group is used for testing the standard preparation, and the other the sample under examination. The preparations are administered intraperitoneally. Food, but not water, is withheld for about 12 hr. before the assay.

The approximate LD50 and its standard deviation is previously determined. The tincture or glycoside solution, is diluted with physiological saline, in such a way that the animals get from 1 to 6 ml./kg. weight. The doses selected for the assay have an equal logarithmic interval between them (see Table I). If the animal survives the dose given,

TABLE I

Example of the "up-and-down" procedure for the assay of digitalis in pigeons by the intraperitoneal method

Volume of diluted tincture injected (ml./kg. of body wt.)	Calculated amount of undiluted tincture administered (ml./kg. of body wt.)	Log. ml./kg. of undiluted tincture injected	Results												Frequency	
															x's	o's
3·59	0·287	$\bar{1}$·458	x									x			2	0
2·97	0·239	$\bar{1}$·378		x		x		x			o		x		4	1
2·48	0·199	$\bar{1}$·298			o		o		x					o	1	4
2·06	0·165	$\bar{1}$·218								o					0	1

x = animal died. o = animal survived. Log. dose ratio = 0·08.

the subsequent higher dose is injected into the next pigeon, whereas if the animal dies the subsequent smaller dose is given, according to the procedure described in detail by Kimball, Burnett and Doherty (1957). The result is observed after 24 hr. The LD50 obtained for the test preparation is compared with that of the standard preparation and the potency expressed in international units. For digitalis leaf and its tinctures the International Standard of Digitalis is used, whereas for the assay of digitoxin the Digitoxin Authentic Chemical Substance issued by Apotekens Kontrollaboratorium, Stockholm, is used as the reference standard.

A detailed description of the toxicity test as well as the table of the "G" values is given by Kimball, Burnett and Doherty (1957) and a discussion on the statistical analysis of the method is given by Dixon and Massey (1957).

A suitable dilution for the tincture is between 1 : 8 to 1 : 12 with physiological saline. Digitoxin is dissolved in 6 to 8 ml. of 96 per cent ethanol

and then diluted with saline to give a solution containing about 6 mg./100 ml. The convenient logarithmic interval of the doses was found to be from 0·05 to 0·15. Under these conditions this interval is generally lower than two standard deviations, and $NB-A^2/N^2$ is generally higher than 0·3. This satisfies the criteria for the simplified statistical analysis and gives satisfactory limits of error (Dixon and Massey, 1957).

Generally one preliminary test for each kind of preparation is sufficient to obtain information on the logarithmic dose interval, *d*.

Results and Discussion

The reproducibility of the procedure has been previously proved by repeating the test on some smaller groups of animals of approximately equal weight. Thereafter, the same concentration of digitoxin (3 mg. per cent) has been administered to eight groups of pigeons each of various

TABLE II

Estimations of the LD50 of one preparation (tincture)

LD50 ml./kg.	Limits of error per cent (P = 0·95)	Total number of animals used
0·24	92·1–108·6	21
0·23	81·1–123·5	10
0·26	85·0–117·4	13
0·23	95·9–104·2	28
0·23	95·6–104·5	24

weight to establish whether there was a correlation of dose and body weight. The results obtained showed a statistically significant correlation ($r = 0{\cdot}907$, $P < 0{\cdot}01$). Because of the correlation of LD50 and body weight doses were given in proportion to body weight in all subsequent assays.

The estimation of the LD50 of the same preparation was repeated under the same experimental conditions in five groups of pigeons. The results (Table II) show that good reproducibility and satisfactory limits of error were obtained.

TABLE III

Comparison of the loss in potency of a sample of digitalis as shown by the intravenous and intraperitoneal methods

	Potency (Intravenous method)	Limits of error per cent (P = 0·95)	No. of animals per group	Potency (Intraperitoneal method)	Limits of error per cent (P = 0·95)	No. of animals per group
Sample of powdered digitalis leaf	1	95·5–104·7	12	1	86·1–116·0	21
Above sample mixed with water and dried at room temperature	0·48	92·7–107·9	6	0·26	81·7–122·3	19

(The toxicity of the sample of digitalis before treatment is calculated as one in both methods used).

Petričić (1955) proved that the frog method as well as the intravenous method in pigeons are satisfactory procedures for determining the loss

in potency of the drug. To examine the proposed intraperitoneal method for this purpose, a sample of powdered digitalis leaf was mixed with two volumes of water and the liquid allowed to evaporate at room temperature (about 2 weeks). From the dry residue obtained a tincture was prepared and assayed by the intravenous and the proposed intraperitoneal methods. The results compared with those obtained for the untreated sample, are shown in Table III.

The ageing of the tincture was studied by both methods and the results obtained are shown in Table IV.

TABLE IV

COMPARISON OF THE LOSS IN POTENCY OF A DIGITALIS TINCTURE AFTER 9 MONTHS STORAGE AS SHOWN BY THE INTRAVENOUS AND INTRAPERITONEAL METHODS

	Potency (Intravenous method)	Limits of error per cent (P = 0·95)	No. of animals per group	Potency (Intraperitoneal method)	Limits of error per cent (P = 0·95)	No. of animals per group
Fresh tincture	1	95·5–104·7	12	1	86·1–116·0	21
Same tincture after 9 months storage	0·79	93·6–106·8	12	0·36	87·5–114·4	13

(The toxicity of the fresh tincture is calculated as one in both methods used.)

As shown in Tables III and IV the proposed method illustrates better the extent of the loss in potency of digitalis and its tincture than does the intravenous method. (In all intravenous experiments results were statistically treated according to B.P. 1953.)

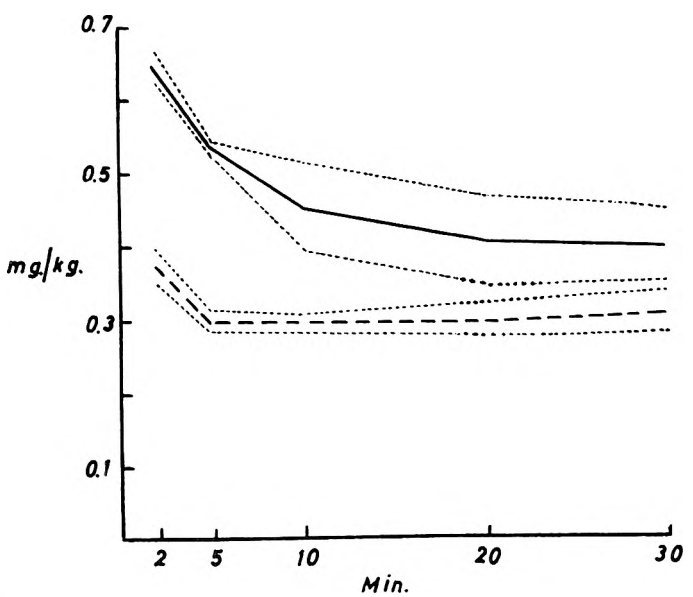

FIG. 1. Relationship of time interval between the intravenous dose of glycoside and the lethal dose in pigeons. Solid line, digitoxin, broken line, lanatoside C. Limits of error are indicated by dotted lines.

The activity of pure glycosides is generally more predictable than that of digitalis which contains a mixture of glycosides and interfering substances. For this reason the activity of two pure glycosides, digitoxin and lanatoside C has been determined by both methods. Digitoxin has marked cumulative properties and acts slowly whereas lanatoside C is only slightly cumulative and acts rapidly.

As can be seen from Table V the same relationship of potency for both glycosides has not been obtained by means of both methods, since the ratio of the lethal dose of digitoxin to that of lanatoside C obtained by the intravenous method is greater than that obtained by the intraperitoneal method. The results shown in Fig. 1 offer an explanation for this effect.

TABLE V

COMPARISON OF RESULTS OBTAINED BY THE INTRAVENOUS AND INTRAPERITONEAL METHODS FOR TWO PURE GLYCOSIDES

	Intravenous lethal dose mg./kg.	Limits of error per cent (P = 0·95)	Intraperitoneal LD50 mg./kg.	Limits of error per cent (P = 0·95)
Digitoxin	0·54	99·1–101·0	0·32	90·1–111·0
Lanatoside C	0·30	94·7–105·6	0·22	91·5–109·4

The lethal dose of digitoxin and lanatoside C has been determined using five groups of pigeons for each preparation, each group containing 12 to 24 animals. The intravenous method was used, but the time interval between single doses varied for each group thus: 2, 5, 10, 20 and 30 min.

From Fig. 1 it is evident that the 5-min. interval (U.S.P. XVI) is sufficient for each dose of lanatoside C to exert its full effect, since after increasing the interval between doses up to 30 min. no further change in lethal dose was observed. With digitoxin, increasing the interval between doses produced a gradual decrease of the lethal dose such that at the 30-min. interval the lethal dose was reduced to 0·40 mg./kg.

The ratio of the intravenous lethal dose (5-min. dose-interval) to the intraperitoneal LD50 is 1·7 for digitoxin and 1·4 for lanatoside C (Table V). However in the case of digitoxin this ratio is reduced to 1·3 if the intravenous lethal dose (0·4 mg./kg.) obtained after injecting doses every 30 min. (Fig. 1) is used.

This suggests that with the intravenous method as recommended in the U.S.P. (XVI) the lowest mean lethal dose for some glycosides is not always obtained. The values for preparations exhibiting a slow onset of action, like digitoxin, are too high.

The choice of the method would be of no special importance if the sample under examination were compared with a standard preparation of the same composition, and the relative potency expressed in terms of this standard. However, this is not usually the case. Various crude preparations often show considerable differences in their chemical composition and in the quantities of glycoside present, and consequently great differences in potency and rate of action are found. The greatest differences are observed with *Digitalis purpurea*, the drug most frequently

assayed. If the standard and the test preparations show considerable differences in the composition of glycosides, and particularly if one of them contains a high percentage of digitoxin, and the other, more glycosides having a rapid action (for example, gitaloxin), the results obtained might be unreliable as the potency of the tested sample might be over or underestimated. This is confirmed in Table VI where results of testing six various samples of digitalis leaf (tincture) are given.

TABLE VI

COMPARISON OF RESULTS OBTAINED BY THE INTRAVENOUS AND INTRAPERITONEAL METHODS FOR SIX DIFFERENT SAMPLES OF DIGITALIS LEAF (FRESH TINCTURE)

Preparation	Intravenous (I.V.) lethal dose ml. tinct./kg.	Limits of error per cent (P = 0·95)	Intraperitoneal (I.P.) LD50 ml. tinct./kg.	Limits of error per cent (P = 0·95)	Ratio LD (I.V.) : LD50 (I.P.)	Relative potency in relation to International Standard (Int. Std. = 1)	
						I.V.	I.P.
1	2	3	4	5	6	7	8
Bilj.236	0·69	96·5–103·5	0·33	88·1–113·5	2·10	1·07	0·94
A	1·37	97·4–105·3	0·52	82·5–121·3	2·63	0·54	0·60
C	1·07	97·6–102·4	0·61	91·4–109·3	1·75	0·69	0·51
-	0·95	97·8–102·2	0·88	74·9–133·5	1·08	0·78	0·35
L	0·73	95·7–104·3	0·31	84·0–119·1	2·26	1·06	1·00
JS	0·67	97·4–102·6	0·22	83·3–120·1	3·05	1·11	1·41

$$\text{Column 7} = \frac{\text{I.V. potency of the Digitalis International Standard}}{\text{I.V. potency of the tested sample}}$$

$$\text{Column 8} = \frac{\text{I.P. potency of the Digitalis International Standard}}{\text{I.P. potency of the tested sample}}$$

The results from assaying several samples of digitalis leaf and tinctures (Table VI) show that there is no constant ratio between the potencies obtained by the intravenous and intraperitoneal methods since the ratio varies between 1·08 and 3·05.

Further, the ratio of the highest and lowest result obtained by the intravenous method is approximately 2, while by the intraperitoneal method it is 4.

Taking into account the limits of error obtained the intravenous method shows significantly smaller differences between the potencies of tinctures than does the proposed intraperitoneal method.

On the basis of the results from the present study it may be concluded that the proposed intraperitoneal method is more suitable than the intravenous method for the assay of most of the preparations used clinically.

The proposed intraperitoneal method, however, requires more animals for each assay, and is more time consuming, because of the sequential design of the assay, than the intravenous method.

As the potency of some tinctures decreases rapidly on storage (Kuševič and Porges, 1956) prolonged assays should be avoided by carrying out several sequences of the test simultaneously and combining their results.

The experimental design described by Brownlee, Hodges and Rosenblatt (1953) can be applied when rapid results are required.

The activity of some digitalis preparations should be determined simultaneously in man and by the proposed intraperitoneal method to asses the suitability of the latter for the routine assay of digitalis.

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THE FUNGISTATIC ACTIVITY OF METHYL AND PROPYL HYDROXYBENZOATES AND A MIXTURE OF THESE AGAINST *PENICILLIUM SPINULOSUM*

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The fungistatic activity of the methyl and propyl esters of *p*-hydroxybenzoic acid and of a mixture of these esters has been investigated. A mucilaginous substance is formed by fungal spores in contact with low concentrations of hydroxybenzoates in liquid medium. A difficulty in reading fungistatic end points from this cause was resolved by using a microscope.

THE use of the esters of *p*-hydroxybenzoic acid as preservatives has long been established in the food and cosmetic industries (Sabalitschka, 1924, 1929). In recent years these esters marketed commercially as "Nipagin M" (methyl ester) and "Nipasol M" (propyl ester), have found increasing use as preservatives for pharmaceutical and related preparations (Gershenfeld and Perlstein, 1939; Lawrence, 1955; Schimmel and Husa, 1956; Boehm and Jones, 1957).

The British Pharmaceutical Codex of 1934 included references to the methyl and propyl esters of *p*-hydroxybenzoic acid, but it was not until the edition of 1949, that these compounds were formulated in a solution for eyedrops. The Codex of 1959 recommends the use of the propyl ester at a concentration of 0·05 per cent w/v for aqueous preparations and 0·1 per cent w/v as a preservative in creams and emulsions and the methyl ester at a concentration of 0·1 to 0·2 per cent w/v for aqueous preparations. The Codex solution for eyedrops contains 0·023 per cent w/v of methyl ester and 0·011 per cent w/v of propyl ester, a mixture thought to show potentiation (Schimmel and Husa, 1956).

The fungistatic action of methyl and propyl hydroxybenzoates, alone, and in admixture has been evaluated in both liquid maltose medium and in solid agar medium using roll tube counts. *Penicillium spinulosum* was chosen as a test organism since its suitability for such purposes has been established (Gerrard, Harkiss and Bullock, 1960).

EXPERIMENTAL

The test organism. Penicillium spinulosum. Strain 42237 of the Commonwealth Mycological Institute.

Preparation of spore suspensions. Suspensions of single spores in sterile water were prepared from 21 day old cultures grown on malt agar slopes by the method previously described by Gerrard, Harkiss and Bullock (1960).

Double strength liquid maltose medium. A 10 per cent w/v solution of maltose in water, distributed in 5 ml. volumes into tubes capped with metal caps and sterilised by autoclaving.

Solid roll tube medium. Malt extract (6 per cent w/v), rose bengal (0·025 per cent w/v) and agar (3 per cent w/v). The medium was distributed into tubes (4 ml. volumes) and sterilised by autoclaving.

Preparation of media containing the esters. Stock solutions in sterile water were prepared with methyl ester 0·15 per cent w/v, propyl ester 0·04 per cent w/v, ester mixture; methyl ester 0·057 per cent and propyl ester 0·029 per cent w/v (combined ester concentration 0·086 per cent w/v). The esters used were of B.P.C. standard.

Final liquid media for use were prepared by mixing suitable volumes of ester stock solution, double strength maltose medium (5 ml.) and sterile water to give 10 ml. volumes containing the following ester concentration ranges; methyl ester 0·07 to 0·007 per cent w/v, propyl ester 0·02 to 0·002 per cent w/v, ester mixture 0·04 to 0·004 per cent w/v combined concentration. *Final solid media* for use were prepared by incorporating the esters into the solid medium to give the following concentration ranges, methyl ester 0·05 to 0·015 per cent w/v, propyl ester 0·042 to 0·015 per cent w/v, ester mixture 0·045 to 0·015 per cent w/v combined concentration.

Fungistatic Activity

Liquid medium. To each tube of medium containing one of the various concentrations of ester was added 0·1 ml. of spore suspension (containing about 7,000 spores per ml.). Each ester concentration was replicated five times and the tubes incubated at 25° and examined for growth every day for 21 days. A viability control on the spore suspension was made simultaneously.

Solid medium. For each ester concentration six roll tubes were prepared each inoculated with 1 ml. of a spore suspension containing approximately 40 spores per ml. The roll tubes were incubated at 25°, examined daily for 21 days and the highest colony count recorded. Early experiments had shown that no formation of new colonies was apparent after 21 days incubation. Replicate experiments, three in all, were made.

Results

Fungistatic Action in Liquid Maltose Medium

The fungistatic action of the esters against the test organism was assessed by their inhibition of macroscopic, visible growth attributable to mycelium formation. It was found difficult to determine the precise ester concentration range at which no growth occurred. At the lower concentrations full and typical mould growth, comparable with that in the viability control, occurred. As the concentration increased, visible growth decreased but a uniform turbidity developed. At higher concentrations no visible growth occurred. The contents of the media tubes were deposited by centrifuging (3,150 R.C.F. for 15 min.) and examined microscopically. Three conditions were seen. (i) In those tubes which showed no macroscopic visible growth, denoted in Table I by —, the deposit contained mould spores only. (ii) In those tubes which showed a uniform turbidity rather than a typical mould growth, the deposit contained mould spores, irregular in shape and associated with a mucilaginous

material. These tubes are denoted in Table I by ±. (iii) In those tubes which showed macroscopic visible growth, denoted in Table I by +, the deposit consisted of typical mycelium. The source of mucilaginous material would seem to be the mould spores themselves. This material evidently appears as a result of the action of low concentrations of the esters upon the germinating spores.

TABLE I

THE RECOVERY IN MALTOSE BROTH CONTAINING DIFFERENT CONCENTRATIONS OF ESTERS

			Concentration (per cent w/v)	Result
Methyl hydroxybenzoate			0·052	−
			0·045	±
			0·030	±
			0·022	+
			0·015	+
Propyl hydroxybenzoate			0·016	−
			0·014	−
			0·012	−
			0·010	±
			0·008	±
			0·006	+
Ester mixture ..	Me 0·0114	Pr 0·0058	Combined	
			0·0172	−
			0·0129	±
			0·0086	+

+ Presence of mycelium.
± Uniform turbidity.
− No growth, undeveloped spores only present.

The results show that the methyl ester inhibits mycelial growth at a concentration of 0·03 per cent although turbidity is present. A concentration of 0·052 per cent is required to give a clear solution with no turbidity. The propyl ester inhibits mycelial growth at a concentration of 0·008 per cent, and turbidity is absent at a concentration of 0·012 per cent. The mixed esters inhibit mycelial growth and turbidity at a combined concentration of 0·0172 per cent.

Fungistatic Action in Solid Medium

In the control tubes an average of 40 colonies per roll tube developed and maximum colony formation was attained within 21 days incubation. The methyl ester prevented colony formation at a concentration of 0·05 per cent, and allowed 50 per cent colony formation at a concentration of 0·0425 per cent and was ineffective at a concentration of 0·035 per cent. The propyl ester prevented colony formation at a concentration of 0·024 per cent, allowed 50 per cent colony formation at 0·021 per cent, and was ineffective at a concentration of 0·018 per cent. The ester mixture prevented colony formation at a concentration of 0·036 per cent, combined esters, and was ineffective at a concentration of 0·031 per cent.

DISCUSSION

The methyl and propyl esters of *p*-hydroxybenzoic acid and mixtures of these effectively inhibit the growth of *P. spinulosum* in both liquid and solid media.

A mutual potentiating effect is evident in mixtures of the esters investigated (Tables I and II) as has been reported by others (Schimmel and Husa, 1956; Boehm and Jones, 1957). In general the esters are more active in liquid medium than in solid medium and the Codex recommendations allow for this factor.

TABLE II

EFFECT OF ESTER CONCENTRATION IN THE RECOVERY MEDIUM UPON THE COLONY COUNT

	Concentration (per cent)			Viability (per cent)
Methyl hydroxybenzoate ..	0·05			Nil
	0·045			3·0
	0·0425			49·2
	0·040			73·9
	0·0375			90·0
	0·035			100·0
Propyl hydroxybenzoate ..	0·024			Nil
	0·021			50·0
	0·018			100·0
Mixture of esters	Me	Pr	Combined	
	0·024	0·0120	0·036	Nil
	0·021	0·0105	0·0315	100·0

The fungistatic end points recorded in liquid maltose medium (Table I) may be compared with those of Neidig and Burrell (1944), who reported the methyl ester to be fungistatic against *Penicillium glaucum* in senna syrup at a concentration of 0·07 per cent and the propyl esters at a concentration of 0·03 per cent. Boehm and Jones (1957), reported that neither of the esters, when incorporated singly in glucose broth, to a concentration of 0·02 per cent, was active against *Penicillium brevicaule*, whereas a combined ester concentration of 0·02 per cent was fungistatic.

The type of procedure described, in which the fungistat is placed in contact with mould spores in nutrient liquid medium has proved difficult to interpret. The usual method in which visual growth is looked for after adequate periods of incubation is complicated by a turbidity developing in those tubes containing concentrations of ester approaching that which totally inhibits growth. This turbidity is not comparable with the full mould growth seen in the viability control and in those tubes containing totally ineffective concentrations of esters, but is such that it would not normally be recorded as no growth. A turbidity effect has not been reported by other workers referred to here and in comparing their values with those in the present work this factor must be considered. Thus, the actual criterion taken in reporting a fungistatic concentration in this work, has been the presence or absence of mycelium as checked microscopically.

The measurement of fungistatic concentration of the esters incorporated in solid medium gave results which concur with those of Schimmel and Husa (1954). They incorporated the esters in agar medium which was inoculated with *P. glaucum* and plated out. Colony formation was prevented by the methyl ester at a concentration of 0·05 per cent and by the propyl ester at a concentration of 0·025 per cent.

An observation of special interest has been the fall in the percentage of spores developing with small increase in concentration of ester. This was particularly evident in the case of the propyl ester and the ester mixture, where difference in concentration of 0·006 and 0·0045 per cent, respectively represented the difference required to bring sub-fungistatic concentration up to a fully fungistatic concentration. With the methyl ester an increase in concentration of 0·015 per cent was required to give the full fungistatic effect to a sub-fungistatic concentration. This phenomenon is related to the relative efficacy of the esters as fungistatic agents and may represent the presence of different mechanisms of action.

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A COLOUR TEST TO DISTINGUISH BETWEEN THE FRUITS OF *ILLICIUM VERUM* AND *ILLICIUM ANISATUM L.*

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A simple colorimetric method to differentiate the non-poisonous Chinese Star Anise from the poisonous Japanese Star Anise is described.

The fruit of the plant *Illicium verum* (Chinese Star Anise) is widely used as a flavour agent in curries, medicaments and meat dishes in South East Asian countries. There also exists a different specie of the plant, *Illicium anisatum* L. producing the fruit known as skikimmi or skimmi fruits (Japanese Star Anise). Its toxic symptoms have been reported by Guerero (1916). Occasionally, these fruits are substituted for those of non-poisonous Chinese Star Anise, and their ingestion has led to acute poisoning often resulting in death. Greenish (1909) described a means of distinguishing the poisonous and non-poisonous species by their physical appearance, odour and taste. Wagenaar (1936) described the microscopical differences between the two species. Unfortunately, star anise is usually sold in powder form, mixed with other condiments such as clove, cinnamon bark, ginger and pepper, so that such a physical identification is difficult and sometimes impossible. In such instances the need for a chemical method of identification to differentiate between these two species of Star Anise is essential.

We have modified the method of Wagenaar (1936) mainly by using a preliminary steam distillation procedure and by substituting orthophosphoric acid for sulphuric acid during colour development to prevent charring.

Isolation and Purification

Steam distil about 5 g. of the powdered sample mixture and collect about 100 ml. of the distillate. Acidify the distillate with 1–2 drops of dilute hydrochloric acid and extract with an equal volume of ether. Shake the ether extract with 3 × 10 ml. of 0·1N sodium hydroxide, rejecting the alkaline aqueous extract each time. Evaporate the ether extract on a water bath to 2–3 ml. and then at 30° until the odour of ether has disappeared. Dissolve the residue in 1–2 ml. of ethanol (95 per cent) for the colour test.

Colour Development

Introduce 3 ml. of orthophosphoric acid into a test tube and add 1–2 mg. of phloroglucinol. Shake the mixture for 30 sec. and add the ethanolic solution of the extract dropwise with shaking until a slight yellow colour appears. If the yellow colour changes to pink within 5–10 min. Chinese Star Anise is present. The pink colour intensifies on standing. Japanese Star Anise gives only a yellow colour which remains

unchanged. With mixtures of powdered condiments containing star anise powder, Chinese Star Anise gives an orange red colour instead of pink, while Japanese variety still remains yellow. The test cannot detect with certainty a mixture of the two varieties because the orange red coloration of the Chinese Star Anise masks the yellow colour developed by the Japanese variety.

Acknowledgements. We wish to thank the City Analyst Laboratory, Singapore, and The Food and Drugs Inspectorate, Ministry of Health, Singapore, for samples of Japanese Star Anise and Chinese Star Anise. The authors thank the Chief Chemist, Singapore, for his interest in this work.

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AN ANATOMICAL STUDY OF ETHIOPIAN KHAT (LEAF OF *CATHA EDULIS* FORSK)

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Received October 31, 1961

The morphology and anatomy of the leaves of *Catha edulis* Forsk. obtained from Ethiopia is described and illustrated. A list of the characters of powdered Khat is also given.

Khat is the arabic name for the shrub, *Catha edulis* Forsk. (Family Celastraceae), the leaves of which are used as a traditional medicine in various regions of East Africa and Arabia. The leaves are, however, more commonly used for other reasons and the habitual consumption of khat has created deep social problems in many of these countries.

The United Nations Bulletin on Narcotics (1956) includes a comprehensive review on khat which indicates that among the poorer sections of the people of East Africa and Arabian countries, where the leaf is chewed to alleviate the sensations of hunger and fatigue,both its occasional and habitual use may lead to serious illness and even madness or death. In 1957 the Bulletin contained an article which deals specifically with the social and medical problems created by its use in Djibouti. However, the 7th Report of the Expert Committee on Addiction Producing Drugs (1957) did not consider that khat could be classified within the accepted definitions of drug addiction or drug habituation. Nevertheless, the French Government decided to classify Khat as a narcotic and for this purpose defined khat as the leaves only of this plant.

According to Paris (1958) there are several varieties of the plant obtained from different geographical sources but the most esteemed, and, according to some authorities, the only acceptable material is that obtained from Ethiopia where it is specially cultivated in the Harar district. There is certainly a regular air transport of freshly collected khat between Addis Ababa and cities and towns in neighbouring African and Arab countries where it is in great demand in the bazaars.

The identification of the leaves of *Catha edulis* Forsk in the broken or powdered condition as well as when whole, may frequently be necessary for forensic purposes and as previous descriptions appear to be inadequate, poorly illustrated and sometimes inaccurate, it was decided to make a detailed examination of the leaf.

Previous Investigations

The first anatomical description of the leaf was given by Collin (1893) and apart from the inadequate description of the fibres it is substantially

In recording the measurements, the system adopted by Moll and Janssonius (i.e. the dimensions in the radial (R), tangential (T) and longitudinal (L) directions of growth) is used in most instances for the sizes of cells. Where, however, this system might be confusing or ambiguous, the ordinary method of recording the longest and shortest axes irrespective of direction of growth, is given.

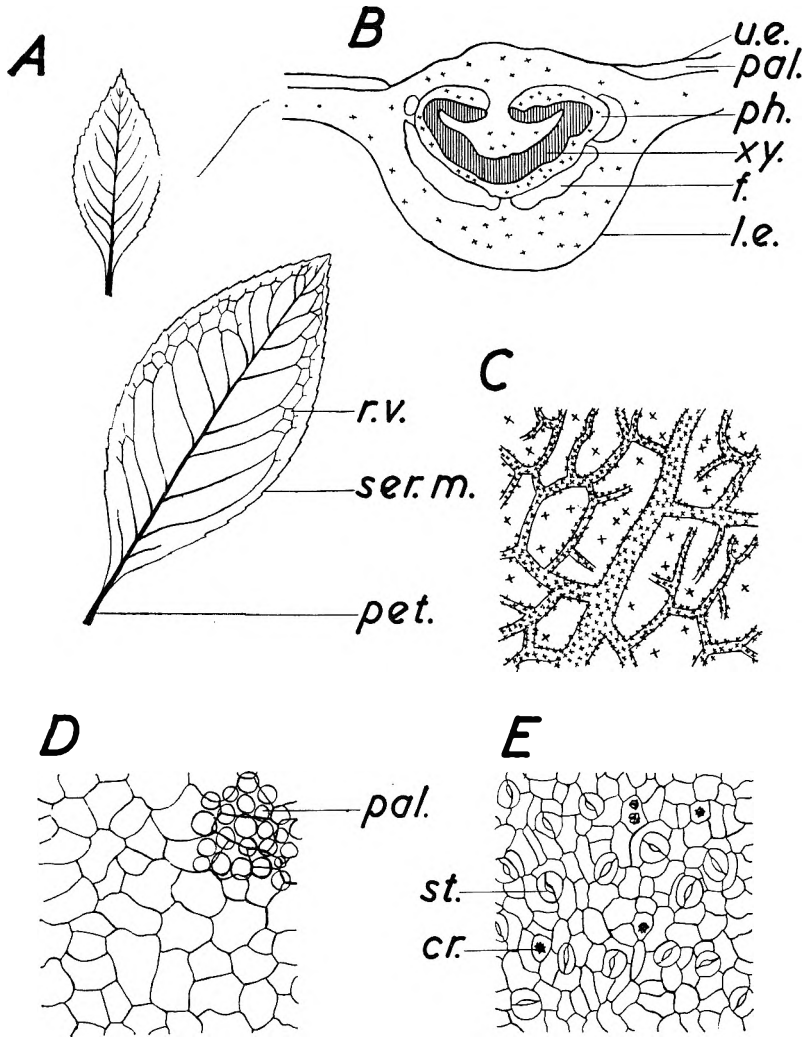

FIG. 1. A, Leaf, natural size. B, Transverse section through midrib × 15. C, Lamina showing venation and distribution of calcium oxalate × 240. The x represents cluster crystals. D, Upper epidermis (surface view) × 240. E, Lower epidermis (surface view) × 240. cr., cluster crystals of calcium oxalate; f., fibres; l.e., lower epidermis; pal., palisade; pet., petiole; ph., phloem; r.v., reticulate venation; ser.m., serrate margin; st., stomata; u.e., upper epidermis; xy., xylem.

correct. The drawings, however, are not good in comparison with present day standards. Perrot (1943–44) gives only a brief and incomplete description in which he refers to the crystals of calcium oxalate as twin crystals. The British Pharmaceutical Codex (1949) includes a short account of the leaf (as an adulterant or substitute for Tea). The crystals of calcium oxalate are correctly described though no sizes are given and reference is also made to tanniferous cells. Paris (1958) gives a very

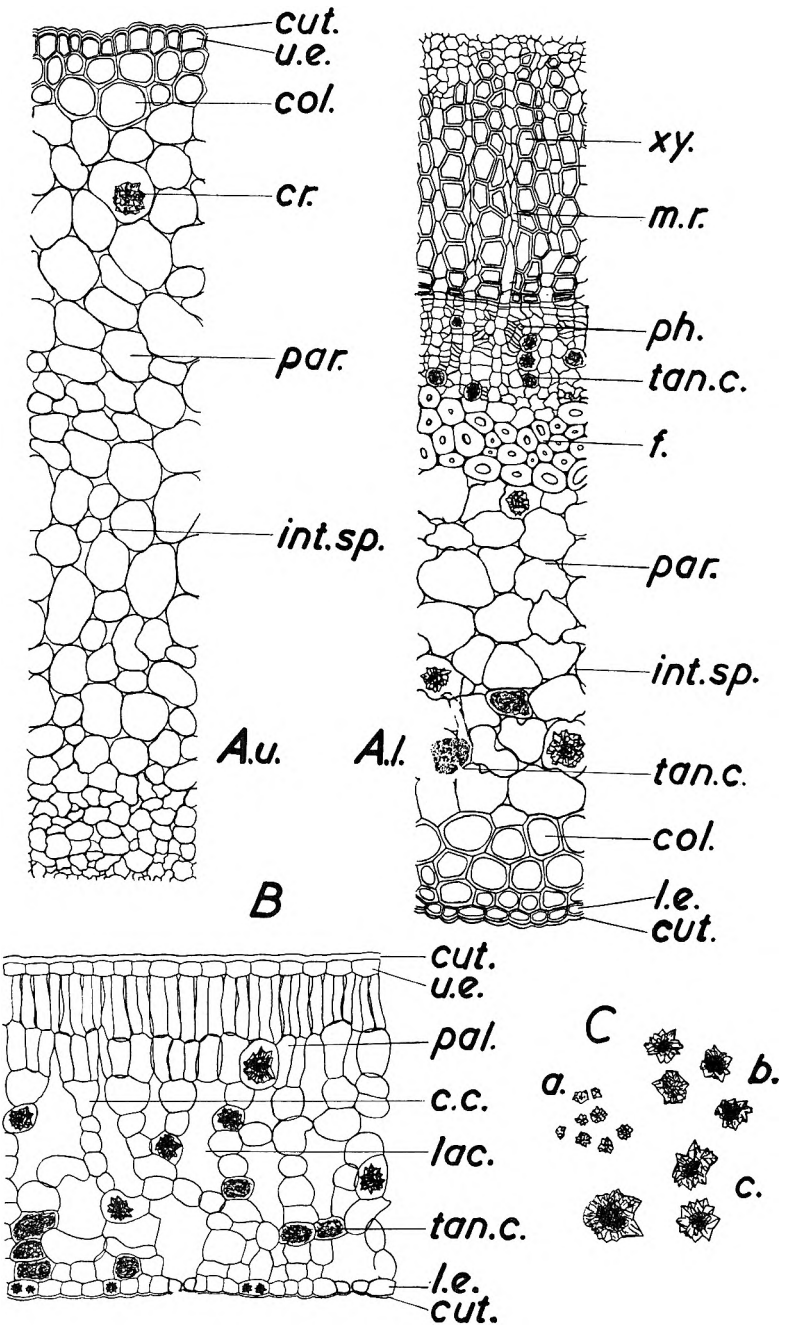

FIG. 2. A, Transverse section through midrib × 180. A u., upper portion, A l., lower portion. B, Transverse section through lamina × 180. C, Cluster crystals of calcium oxalate × 275: a, from the lower epidermis; b, from the phloem; c, from the parenchyma and mesophyll. c.c., collecting cell; cr., cluster crystals of calcium oxalate; col., collenchyma; cut., cuticle; f., fibres; int.sp., intercellular space; lac., lacunae; l.e., lower epidermis; m.r., medullary ray; par., parenchyma; ph., phloem; tan.c., tanniferous cell; u.e., upper epidermis; xy., xylem.

brief description of the features of the powdered leaf with some drawings but totally inadequate to be of great help in identifying the powdered leaf with any certainty.

Materials

The samples of khat used in this investigation were obtained as follows:

(*a*) a sample purchased in a bazaar in Aden in October 1959 by Mrs. V. Stewart and flown to England the same day.

(*b*) a sample collected in January, 1960, from cultivated plants of *Catha edulis* Forsk. in Ethiopia by J. Donner, Esq.

Both samples were presented to the Pharmacognosy Museum of the Chelsea School of Pharmacy.

Macroscopical Features

The slender twigs have from 10 to 16 leaves arranged in opposite pairs with occasional displacement at the base of the stem so that the lower leaves may appear to be arranged alternately. The leaves are simple and measure 4 to 11 cm. long and 1·8 to 5 cm. wide at the widest part. They have a short, round petiole, 3 to 7 mm. long.

In the fresh condition the leaves are bright green and the surfaces are glossy. When dry the leaves are paler, the colour of both surfaces being similar. The lamina is glabrous and slightly coriaceous. It is oval-lanceolate in shape with an acute or sometimes slightly acuminate apex and an acute, symmetrical base. With the exception of a small portion near the base which is entire the margin is serrate.

The upper surface shows distinct pinnate venation but this is more marked on the lower surface, the midrib being prominent, round and yellow to reddish brown in colour. A few of the lateral veins leave the midrib at a fairly wide angle (45°) but the majority leave at a narrow angle (25 to 30°) and immediately curve upwards. Near the margin the lateral veins anastomose to form a reticulate venation (Fig. 1,A).

The leaves are odourless and have a slightly astringent taste.

Microscopical Features

Lamina. The upper epidermis consists of a single layer of polygonal cells covered with a fairly thick, smooth cuticle. The outer walls of the cells are thickened with cellulose but the anticlinal walls which are somewhat sinuous are not thickened to any great extent. No trichomes or stomata are present (Figs. 1,D and 2,B).

The cells measure about 12 to 55 μ long, 10 to 36 μ wide and 10 to 16 μ high.

The appearance of the epidermal cells over the veins is similar to the remainder of the upper epidermal cells.

The mesophyll is clearly differentiated into palisade and spongy mesophyll. The palisade consists of two rows of thin walled cylindrical cells, those of the inner row being usually shorter and wider than those of the upper row.

The Palisade Ratio is 2·75–**3·18**–4·0.

Apart from the chloroplasts there are no distinctive cell inclusions in the upper row but occasionally the cells of the lower row are enlarged and contain a cluster crystal of calcium oxalate. Frequently the cells of the lower row are in groups of 2 or 3 with spaces between them. Sometimes a cell of the inner row is "Y" or funnel shaped and probably functions as a collecting cell from the adjacent cells of the upper row.

The spongy mesophyll which consists chiefly of thin walled rounded to elongated cells show three interesting features: (i) the cells adjacent to the palisade cells are often larger and "Y" shaped, each being in contact with 2 or 3 palisade cells and probably function as collecting cells ("sammezellen" Haberlandt, 1909). (ii) the cells are arranged to show a pattern of regularly spaced large air spaces (lacunae). (iii) the cells adjacent to the lower epidermis are more closely arranged in 2 or 3 rows and except where there are stomata in the epidermis show no large air spaces.

Some of the cells of the mesophyll contain cluster crystals of calcium oxalate measuring 12 to 32 μ in diameter. Other cells, particularly those near to the lower epidermis contain slightly granular contents which stain brown with dilute solution of iodine, greenish black with solution of ferric chloride and orange red with Sudan III. Sometimes the contents are coloured pale brown and in such cases the colour reaction with Sudan III is not obvious. These are the cells referred to in the British Pharmaceutical Codex as "tanniferous cells" but it is probable that the contents are resinous since they are not readily soluble in water but are soluble in ethanol (90 per cent).

The lower epidermis consists of a single layer of polygonal cells, the walls of which are thickened in a similar manner to those of the upper epidermis. The anticlinal walls are only slightly sinuous. The epidermis is covered with a fairly thick, smooth cuticle. Some of the cells contain one or two small crystals of calcium oxalate which are either clusters or conglomerates of few components; these crystals are smaller than those in the mesophyll and measure 4 to 8 μ in diameter. No trichomes are present but there are a large number of anomocytic stomata surrounded by 3 or 4 (occasionally 5) cells. The epidermal cells measure about 10 to 40 μ long, 8 to 20 μ wide and 8 to 12 μ high. (Figs. 1,E and 2,B.)

The Stomatal Index is 10·4–**14·2**–18·8.

The appearance of the lower epidermal cells over the veins differs from the remainder of the epidermis. There are no stomata, no cells containing crystals of calcium oxalate and seen in surface view the cells are irregularly rectangular in shape.

When a cleared piece of lamina is examined microscopically cluster crystals of calcium oxalate are seen to accompany all the veins (Fig. 1,C). The crystals measure 7 to 25 μ in diameter. Examination of the transverse sections of the lamina through the lateral veins show them to have similar features to the midrib, the crystals of calcium oxalate being in the phloem parenchyma.

The Vein Islet Number is 4·0–**5·1**–7·0.

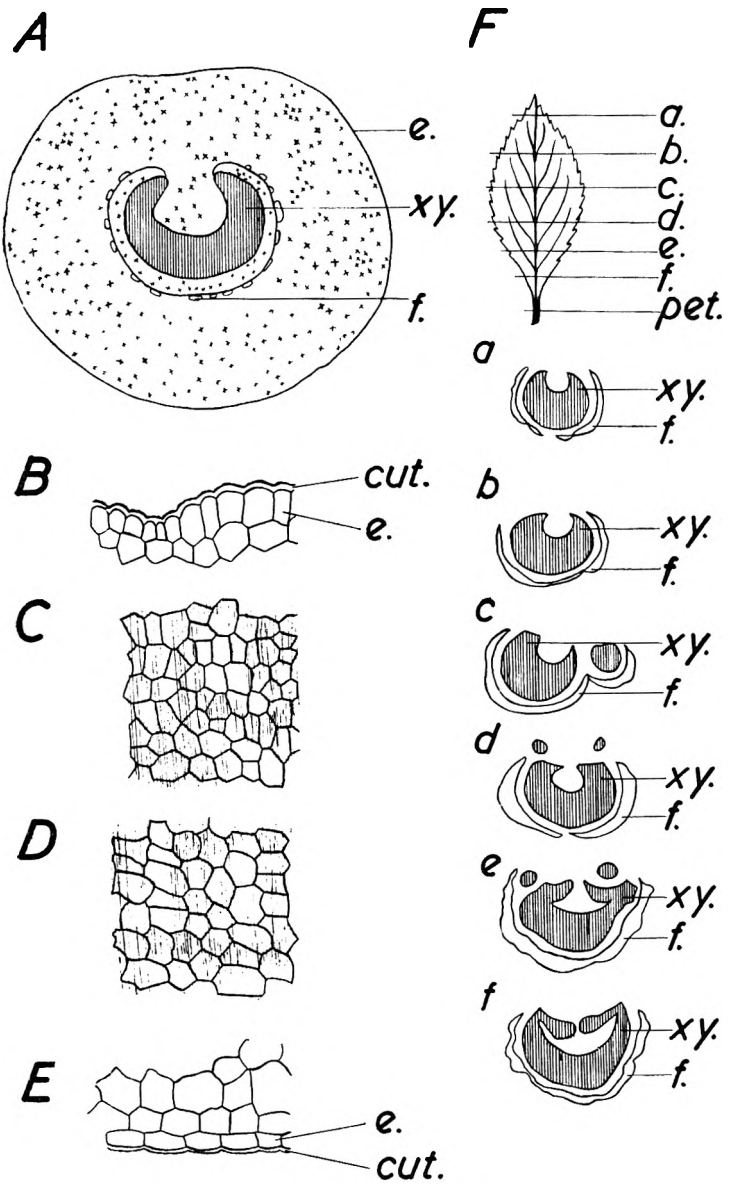

FIG. 3. A, Transverse section through petiole × 25. B, Transverse section through upper epidermis of petiole × 180. C, Surface view of upper epidermis of petiole × 180. D, Surface view of lower epidermis of petiole × 180. E, Transverse section through lower epidermis of petiole × 180. F, Transverse sections through midrib of leaf in position as shown (a to f) × 10. cut., cuticle; e., epidermis; f., fibres; xy., xylem. The x represents cluster crystals of calcium oxalate.

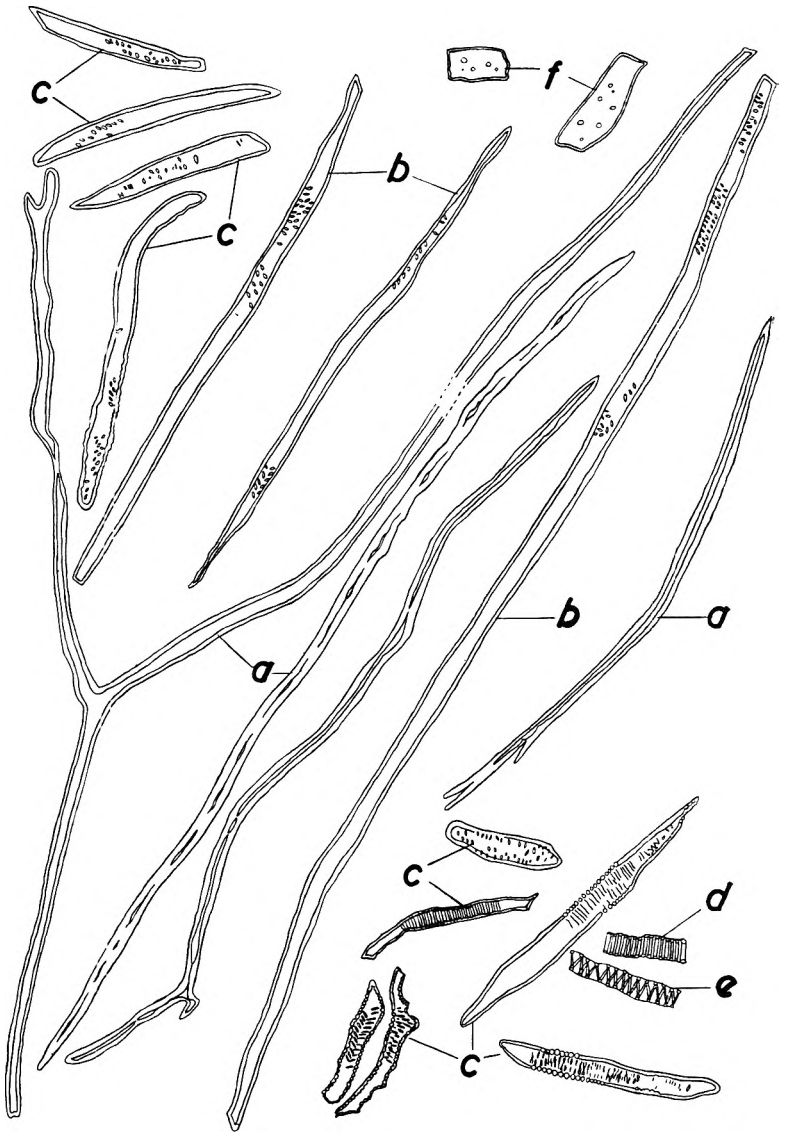

FIG. 4. Isolated elements from the veins × 180: a, fibres from pericyle; b, fibre tracheids; c, tracheids; d, annular vessels; e, spiral vessels; f, parenchyma from medullary rays.

Midrib. Both upper and lower epidermises are similar to those already described as covering the veins of the lamina except that the cell walls are thicker and that over the lower portion of the midrib they gradually change in appearance to resemble the epidermises of the petiole.

The palisade of the lamina is discontinuous over the midrib; collenchyma occurs beneath the epidermis and in older leaves may be quite extensive though in younger leaves may only constitute a few rows of cells. The remainder of the midrib cortex consists of large, rounded parenchymatous cells with small intercellular spaces. In the younger leaves the cell walls are thin but in the older leaves they may be thickened with cellulose. Tanniferous cells are present, particularly near to the lower epicermis.

No starch sheath (endodermis) appears to be present.

The meristele is arcuate in shape but the actual shape of the xylem and the presence or absence of additional meristeles depends upon the position along the midrib at which the section is cut (Fig. 3,F). The phloem consists of sieve tubes, companion cells, phloem parenchyma and some small tanniferous cells. No fibres are present in the phloem which is subtended by a crescent of pericyclic fibres. The extent of the development of this fibrous tissue depends upon the age of the leaf—the older leaves containing many fibres and the younger leaves having fewer fibres occurring in groups of 4 to 10 fibres only. The individual fibres have thick, highly refractive walls up to 5 μ thick. The walls give intense colours with cellulose stains but they are also slightly lignified. The lumen varies in diameter and in places may be entirely obliterated. Many of the fibres are branched tripartly and the ends of the fibres are frequently bifurcated (Fig. 4,a). The fibres have the following dimensions: R and T = 8 to 18 μ, L = frequently greater than 1,000 μ.

The xylem consists of radial rows of vessels, tracheids and fibre tracheids with well defined medullary rays (Fig. 2,A). The cell walls of all the xylem elements are lignified. No crystals of calcium oxalate occur in the cells of the medullary rays. The vessels show spiral and annular thickening and may be up to 20 μ in diameter (Fig. 4,d and e). Most tracheids have elongated oval bordered pits though some tracheids show reticulate and scalariform thickening. These are more common in the xylem of the capillary veins. The tracheids measure R and T = 15 to 20 μ, L = 100 to 200 μ (Fig. 4,c). The fibre tracheids also have elongated bordered pits and measure R and T = 10 to 18 μ, L = 270 to 850 μ (Fig. 4,b).

Petiole. The upper epidermis consists of a single layer of cells covered with a fairly thick, rough cuticle which gives to the epidermis in surface view, a striated appearance. The outer walls are convex and the anti-clinal walls are straight. No trichomes or stomata are present. The cells measure about 8 to 24 μ long, 6 to 18 μ wide and 8 to 24 μ high. The lower epidermis is similar in appearance to the upper epidermis except that the outer walls are not so convex and, seen in surface view the cells are larger. The cuticle is striated; and trichomes, stomata and crystals of calcium oxalate are absent. The cells measure about 12 to 48 μ long, 8 to 24 μ wide, and 4 to 8 μ high (Figs. 3,D and E).

The cortical region consists of parenchymatous cells, some of which are tanniferous cells. A large number contain cluster crystals of calcium oxalate the maximum size of which is 40 μ in diameter.

The stelar region is similar to that in the midrib, the only difference being in the smaller number of pericyclic fibres which occur in groups of 2 to 8 fibres arranged as a crescent shaped tissue. All the cells of the meristele are similar to those already described for the midrib (Fig. 3,A).

Characters of Powdered Khat

1. Fragments of lamina showing veins accompanied by cluster crystals of calcium oxalate measuring 7 to 25 μ in diameter.
2. Fragments of lamina showing the upper epidermal cells with sinuous anticlinal walls. Stomata are absent and cells of the palisade may be present.
3. Fragments of lamina showing the lower epidermal cells with slightly sinuous anticlinal walls. Numerous anomocytic stomata surrounded by 3 or 4 (occasionally 5) cells; some cells may contain one or two cluster crystals of calcium oxalate 4 to 8 μ in diameter.
4. Parenchymatous cells containing cluster crystals of calcium oxalate measuring chiefly 12 to 32 μ in diameter with some up to 40 μ.
5. Cluster crystals of calcium oxalate 4 to 40 μ in diameter.
6. Fragments of thick walled, slightly lignified pericyclic fibres with lumen of variable width, often obliterated. The cell walls also show intense colouration with cellulose stains. The fibres may show tripartite branching and the ends may be bifurcated.
7. Tanniferous cells, the contents of which stain brown with dilute solution of iodine, greenish black with solution of ferric chloride and orange red with Sudan III.
8. Fragments of striated, straight walled epidermal cells from the petiole.
9. Fibre tracheids with lignified walls and having elongated oval bordered pits.
10. Tracheids, some of which show scalariform and reticulate thickening and others with elongated bordered pits.

Acknowledgements. Our thanks are due to Mrs. V. Stewart and to J. Donner Esq. for the gifts of khat. One of us (P.S.) would like to thank the British Council for a scholarship which enabled her to undertake this investigation at the Chelsea School of Pharmacy.

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THE LOCAL ANAESTHETIC PROPERTIES OF ESTERS OF *N*-SUBSTITUTED α-AMINOPHENYLACETIC ACIDS

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The local anaesthetic properties of a series of esters of *N*-substituted α-aminophenylacetic acids are described. Compounds with an ethyl ester group showed little activity, but replacing this group by an isopentyl group produced local anaesthetic action. The cyclohexyl and 3,5,5-trimethylcyclohexyl esters induced a prolonged surface and infiltration anaesthesia; this was more pronounced in those compounds in which the central amino-group was tertiary. The relationship between structure and activity is discussed.

Edwards, Goldberg and Wragg (1960) have described the preparation and spasmolytic properties of esters of *N*-substituted α-aminophenylacetic acids; the local anaesthetic properties are now reported.

Experimental

Surface Anaesthesia in Guinea-pigs

0·1 ml. of a 2 per cent solution of each compound was instilled into the conjunctival sac and the eye-lid held closed for 30 sec. The duration of anaesthesia was evaluated by applying pressure with a fine camel hair brush to the cornea at regular intervals until the return of the corneal reflex.

Infiltration Anaesthesia in Mice

This was determined in 18 to 20 g. male albino mice by the method of Bianchi (1956). A bull-dog artery clip with its blades covered with thin rubber tubing was applied to the base of the tail; if the animal made repeated attempts to remove the clip within 30 sec. they were then used for the test.

Each compound was dissolved in saline and 0·1 ml. of a 2 per cent solution was injected subcutaneously about 1 cm. from the root of the tail; 15 min. later the clip was applied to the tail of each mouse in turn. If the animal made no attempt to remove the clip anaesthesia was present and the test was then repeated at 15 min. intervals until the return of the reflex.

Results

Only results for those compounds that produced local anaesthesia in more than 50 per cent of the animals are given in Table I; activities are expressed as the mean duration and the range.

The compounds have been classified according to the nature of the ester group which is either ethyl, isopentyl, cyclohexyl or 3,5,5-trimethylcyclohexyl.

Ethyl series. Only two of the four compounds examined had local anaesthetic activity. Compound 3012 had no surface anaesthetic

properties, but subcutaneous injection into the tails of mice produced anaesthesia lasting for 30 min. in 2 out of 5 of the animals. Compound 3015 produced brief surface anaesthesia in 3 out of 5 mice and infiltration anaesthesia, similar to that found with compound 3012, in 2 out of 5 mice.

Isopentyl series. The three compounds examined induced topical anaesthesia when tested in guinea-pigs. Maximum activity within this group was exhibited by compound 3000, the surface anaesthetic effect of which had a mean duration of 40 min. This was approximately 3 to 4 times the duration for compounds 3001 and 3002.

Less than 50 per cent of the mice injected with compounds 3001 and 3002 developed local anaesthesia. The injection of compound 3000 was followed by prolonged local anaesthesia in 9 out of 10 mice.

Cyclohexyl series. The members of this series produced surface anaesthesia of about the same potency as the isopentyl series. The longest duration of anaesthesia was given by compound 3006 and that with the minimum activity was compound 3005. Compounds 3003 and 3004 showed intermediate activity.

TABLE I

LOCAL ANAESTHETIC ACTIVITY OF ESTERS OF *N*-SUBSTITUTED α-AMINOPHENYLACETIC ACIDS (DIHYDROGEN OXALATES)

$C_6H_5CH.(COOR').NR''.CH_2CH_2R.$

Cmpd.	R″	R′	R	Surface anaesthesia in guinea-pigs: Mean anaesthetic time in min. and range			Infiltration anaesthesia in mice: Mean anaesthetic time in min. and range		
3012	H	ethyl	piperidino	(0/5)	0		(2/5)	0	
3013	H	,,	pyrrolidin-l-yl	(0/5)	0		(0/5)	0	
3014	H	,,	diethylamino	(0/5)	0		(1/5)	0	
3015	Me	,,	,,	(3/5)	7	(0–15)	(2/5)	0	
3001	H	isopentyl	piperidino	(7/10)	13	(0–30)	(2/5)	0	
3002	H	,,	pyrrolidin-l-yl	(6/10)	9	(0–20)	(4/10)	0	
3000	Me	,,	diethylamino	(15/15)	40	(30–45)	(9/10)	64	(0–105)
3003	H	cyclohexyl	piperidino	(8/10)	10	(0–30)	(3/5)	45	(0–60)
3004	H	,,	pyrrolidino-l-yl	(6/10)	13	(0–35)	(3/10)	0	
3005	H	,,	diethylamino	(3/10)	0		(8/10)	98	(0–195)
3006	Me	,,	,,	(15/16)	23	(0–45)	(10/10)	300	(120–430)
3007	H	3,5,5-trimethyl-cyclohexyl	piperidino	(20/20)	74	(60–95)	(1/5)	0	
3065	Me	,,	,,	(16/16)	83	(30–100)	(9/10)	82*	
3008	H	,,	pyrrolidino-l-yl	(6/10)	36	(0–40)	(10/15)	74	(0–150)
3009	H	,,	diethylamino	(3/5)	22	(0–40)	(7/10)	66	(0–120)
3064	Me	,,	,,	(16/16)	62	(40–100)	(10/10)	99**	

( ) No. of animals showing anaesthesia/no. tested.
* 6/10 still showing local anaesthetic activity.
** 7/10 still showing local anaesthetic activity.

The outstanding compound after the subcutaneous injection into mice was 3006. Examination of Table I shows that this compound induced a prolonged local anaesthesia. After injection recovery from the effect of this drug was slow and it was not until 430 min. that the response to pain was normal in all the animals. The injection of compound 3005 was followed by a less powerful effect and its duration was one-third of that

found with 3006; however, 195 min. after injection the reaction to pain in all the mice was found to be normal.

3,5,5-*Trimethylcyclohexyl series.* The instillation of the members of this series into the eye of the guinea-pig produced a prolonged anaesthesia. Table I shows that the mean duration of anaesthesia was approximately two to four times longer than for the corresponding cyclohexyl series.

The three outstanding surface anaesthetics in this series were compounds 3007, 3064 and 3065: the mean duration of anaesthesia ranged from 60 to 83 min.

With the exception of compound 3007, all were able to induce anaesthesia after the subcutaneous injection into the tails of mice. The mean duration of anaesthesia was similar for compounds 3008 and 3009. The injection of compounds 3064 and 3065 was followed by prolonged local anaesthesia and at the end of 105 min. it was found that 60 to 70 per cent of the mice still failed to respond to the pain stimulus. If the experiment had continued until all the mice had recovered, the mean duration of anaesthesia for these two compounds would have exceeded the values given in the Table.

Local Tissue Reactions

Local reactions after the instillation into the eye of the guinea-pig and infiltration into the mouse tail was not seen with compounds 3001, 3002, 3003 and 3004, 3012, 3013, 3014 and 3015. All the other compounds at 2 per cent concentration produced a slight discharge and irritation in the eye, and oedema and inflammation at the site of the injection; these were more severe with compounds 3009, 3064 and 3065.

Discussion

Analysis of the experimental data reveals that maximum local anaesthetic activity was related to the nature of the esterifying group and to the conversion of the secondary amino-group to a tertiary group.

The influence of the ester group on local anaesthetic activity is clearly illustrated in Table I. Compounds which were ethyl esters had poor anaesthetic activity compared with those in which isopentanol was the esterifying substance. Replacement of the isopentyl group by either a cyclohexyl or 3,5,5-trimethylcyclohexyl group produces long acting topical and infiltration anaesthesia in guinea-pigs and mice. In general, members of the 3,5,5-trimethylcyclohexyl series were capable of producing anaesthesia of longer duration than that given by a cyclohexyl group in this position.

Throughout the series it was found that conversion of the secondary α-amino-group into a tertiary group was followed by increased activity. This was well illustrated by comparing the behaviour of compound 3005 with 3006 and compound 3007 with 3065. Those compounds in which the α-amino-group was tertiary (3006 and 3065) were found to induce a more prolonged action than those in which this group (3005 and 3007) remained secondary.

From the evidence available it was not possible to establish the part played by the terminal nitrogenous group in influencing local anaesthetic activity.

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A MODIFIED PROCEDURE FOR THE DETERMINATION OF ISONIAZID IN MIXTURES WITH SODIUM *p*-AMINOSALICYLATE

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A modification of a routine method for the determination of isoniazid in mixtures with sodium *p*-aminosalicylate in tablet form is described. After the removal of interfering tablet excipients with hydrochloric acid the isoniazid is determined by reduction of the hydrazine group with a zinc-copper couple in potassium hydroxide to ammonia which is absorbed in standard acid the excess of which is titrated with standard alkali.

Mitchell, Haugas and McRoe (1957) described a method for the determination of isoniazid based on the reduction of the hydrazine group with zinc-copper couple in potassium hydroxide solution to give ammonia which is determined after distillation into standard acid solution. It was claimed that excess *p*-aminosalicylate did not interfere with the determination of isoniazid. This method was studied and it was found that when the conditions described were strictly adhered to, particularly the volume of distillate to be collected, this method gave satisfactory results when applied to mixtures of pure drugs. But when applied to tablets erratic results were obtained. Considerable frothing occurred making it very difficult to control the rate of distillation. The frothing was caused by the presence of starch and magnesium stearate.

The simplicity of the method described by Mitchell, Haugas and McRoe (1957) makes it very attractive as a routine method, provided the substances that cause frothing can be removed.

It was found that treatment with concentrated hydrochloric acid hydrolysed the starch and the magnesium stearate was removed with the *p*-aminosalicylate hydrochloride by filtration. The isoniazid could be determined in the filtrate by a slight modification of the procedure described by Mitchell, Haugas and McRoe (1957). No frothing was encountered during the distillation. The results were independent of the volume of the distillate so long as it is not less than 100 ml.

Experimental

Reagents

Zinc powder, 25 per cent w/v aqueous copper sulphate solution, potassium hydroxide pellets, 0·02N sulphuric acid, 0·02N sodium hydroxide, and 15 per cent sodium chloride solution.

Preparation of Zinc-Copper Couple

About 2 g. of zinc powder and 5 ml. of the copper sulphate solution are shaken in a 50 ml. flask until the supernatant liquid is nearly colourless. This liquid is decanted and the residue washed three times with 10 to 15 ml.

portions of water, decanting each washing. The zinc-copper couple is then ready for use.

Procedure

Twenty tablets are weighed and powdered. About 0·6 g., accurately weighed, of the sample, containing 15–30 mg. of isoniazid, is placed in a 50 ml. beaker together with 10 ml. of water and 1 ml. of concentrated hydrochloric acid. The solution is cooled in ice for 15 min. and filtered through a Whatman No. 1 filter paper. The residue is washed three times with 10 ml. portions of 15 per cent sodium chloride solution. The combined filtrate is placed in a 1 litre flask together with 10 g. of potassium hydroxide, zinc-copper couple and 350 ml. water. This mixture is distilled into 25 ml. of 0·02N sulphuric acid until not less than 100 ml. has been collected. The excess acid is titrated with 0·02N sodium hydroxide solution using methyl red–methylene blue as an indicator.

Each ml. of 0·02N sulphuric acid used is equivalent to 0·00137 mg. of isoniazid.

Recoveries from synthetic mixtures of isoniazid with sodium *p*-aminosalicylate were 100·9 per cent (range +1·2–0·9) and 100·2 per cent (range +1·0–0·7). The figures of analyses of seven commercial samples indicated that the isoniazid contents of some of the commercial samples were lower than the amounts claimed. To ensure that true values had been obtained, recovery experiments were conducted and the results are shown in Table I.

TABLE I

RECOVERY OF ISONIAZID ADDED TO COMMERCIAL SAMPLES CONTAINING ISONIAZID AND SODIUM *p*-AMINOSALICYLATE

Weight of isoniazid per tablet (mg.)		Weight of isoniazid (mg.)		
Stated	Found previously	Added	Total	Amount recovered
15·0	13·8	10	25·0	10·2
15·0	14·5	10	24·4	9·9
15·0	15·1	10	25·0	9·9
25·0*	23·7	10	33·4	9·7
25·0*	25·0	10	34·9	9·9

* The ammonia from these samples was distilled into 30 ml. of Standard 0·02N H_2SO_4.

Calcium phosphate, alginic acid, sugar, starch, magnesium stearate and lactose have been added and do not interfere with the modified procedure of analysis for isoniazid.

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LETTERS TO THE EDITOR

Synthesis and Anticonvulsant Activity of some *N*-phenethylacetamides

SIR,—We would like to report the synthesis of α-chloro-*N*-phenethyl-, α-chloro-*N*-*m*-chlorophenethyl-, α-chloro-*N*-*p*-chlorophenethyl-, α-chloro-*N*-*o*-methoxyphenethyl- and α-chloro-*N*-*p*-methoxyphenethyl-acetamide. These were prepared by us as intermediates for the synthesis of *N*-aminoalkylphenethylamines. However, the unexpected discovery of significant anticonvulsant activity in two of these chloroacetamides has prompted us to make this preliminary communication.

Chloroacetyl chloride (0·11 M) was added slowly to a cooled and well-stirred suspension of the desired phenethylamine (0·10 M) in 10 per cent aqueous sodium hydroxide solution (200 ml.). Stirring was continued for 2 hr. and the solid which separated was triturated with hydrochloric acid (1 :1), washed with water and recrystallised from aqueous ethanol.

(I) α-Chloro-*N*-phenethylacetamide m.p. 70–71° (Child and Pyman, 1931). (II) α-Chloro-*N*-*m*-chlorophenethylacetamide m.p. 66°, found C, 51·62; H, 4·70; N 6·12 per cent. (III) α-Chloro-*N*-*p*-chlorophenethylacetamide m.p. 91°, found C, 51·74; H, 4·71; N, 5·80 per cent; (II) and (III) $C_{10}H_{11}Cl_2NO$ require C, 51·72; H, 4·74; N, 6·03 per cent. (IV) α-Chloro-*N*-*o*-methoxyphenethylacetamide m.p. 84°, found C, 57·87; H, 6·22; N, 6·25 per cent; (V) α-chloro-*N*-*p*-methoxyphenethylacetamide m.p. 102°; found C, 57·94; H, 6·45; N, 6·4 per cent; (IV) and (V) $C_{11}H_{14}Cl\,NO_2$ require C, 58·02; H, 6·14; N, 6·15 per cent.

These compounds, administered orally in suspension in 10 per cent gum acacia, were screened for their anticonvulsant activity in albino rats given maximal electroshock seizures from corneal electrodes dipped in normal saline (Goodman, Brown and Swinyard, 1952). α-Chloro-*N*-phenethylacetamide (I) was the most active and its anticonvulsant activity was, therefore, compared to phenobarbitone, phenytoin sodium and troxidone. The ED 50 of these compounds administered orally was found to be 5 mg./kg. for α-chloro-*N*-phenethylacetamide, phenobarbitone and phenytoin sodium and 100 mg./kg. for troxidone. The percentage of protection against electroshock seizures in rats by oral doses of 5, 10 and 15 mg./kg. of the acetamide was 64, 80 and 96. The corresponding figures for phenobarbitone were 48, 76, 96; for phenytoin sodium 52, 64, 76; for troxidone 52, 64 and 72. The Peak Effect was found to be 4, 3, 4 and 2 hr. respectively.

Thus compound I appears to be as potent as phenobarbitone against electroshock seizures in rats, while α-chloro-*N*-*m*-chlorophenethylacetamide (II) was found to be about two-thirds as potent as (I). The other three compounds reported showed no significant anticonvulsant activity. None of these compounds afforded protection against leptazol induced seizures.

The pharmacological screening described here has been carried out at the Pharmacology Department of the Osmania Medical College, Hyderabad, and we record out thanks to Prof. M. Y. Ansari and Dr. P. Pentiah for this.

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December 18, 1961.

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The effect of Imipramine on the Reserpine Toxicity in Adrenalectomised Rats

SIR,—Reserpine is highly toxic when administered to adrenalectomised rats (Gaunt, Renzi, Antonchak, Miller and Gilman, 1954; also Pennefather and Rand, personal communication). Garattini, Giachetti, Jori, Pieri and Valzelli (1961) have shown that hypothermia, blepharospasm and sedation induced by reserpine are prevented by pretreatment of rats with imipramine, a new antidepressant drug, as also are the occurrence and severity of gastric ulcer in restrained reserpine-treated rats. The present experiments were carried out to study the action of imipramine on the reserpine toxicity in adrenalectomised rats.

Reserpine in graded doses (1, 2·5, 4, 5 mg./kg.) was injected intraperitoneally into groups of rats adrenalectomised 6 days previously. The LD50 calculated by the method of Litchfield and Wilcoxon (1949) was 1·75 (0·49–6·30) mg./kg. Other groups of rats adrenalectomised 6 days previously were given imipramine 25 mg./kg. intraperitoneally 30 min. before similar doses of reserpine. The LD50 was 4·1 (2·2–7·4) mg./kg. Imipramine alone was not toxic to adrenalectomised rats. The confidence limits of these two LD 50 values overlap.

A comparison was next made of the effect of imipramine (25 mg./kg.) on the survival times of adrenalectomised rats receiving reserpine (5 mg./kg.). Male rats of the same weight and adrenalectomised 6 days previously were distributed in matched pairs into two groups each of 12 animals. The test rats were injected with imipramine. After 30 min. both the test group and the control group of the pairs were injected with reserpine. The mean survival-time was determined by inspecting the rats every 30 min. until the last had died. The results are shown in Table I. The significance was tested statistically by the British Pharmaceutical Codex (1959) method.

TABLE I

PROTECTION BY IMIPRAMINE OF RESERPINE TOXICITY IN ADRENALECTOMISED RATS

Drug and dose	No. of rats	Mean survival time in hr.	P
Reserpine 5 mg./kg.	12	7.9	
Imipramine 25 mg./kg. + Reserpine 5 mg./kg.	12	20·4	<0·001

These results show imipramine to delay the reserpine toxicity. Owing to the incompletely established pharmacological activities of imipramine and furthermore to the uncertainty of the causes of reserpine toxicity, it is difficult to define the mode of action of this protection. Nevertheless Garattini, Lamesta, Mortari, Palma and Valzelli (1961) showed that the toxicity of 5-HT is increased in adrenalectomised rats, and Sulser and Brodie (1961) suggested that imipramine or one of its metabolites acts by blocking free 5-HT. It may be that imipramine protects the adrealectomised rat from the toxic effects of reserpine by inactivating the 5-HT which is released.

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6-Methylcortisone Acetate 3-Enol Ethers—A New Group of Anti-inflammatory Agents

SIR,—Following our discovery of a new and highly efficient route to 6-methylated steroids, it has become possible to extend our work on corticoids to the 3-enol ethers of the now readily accessible 6α-methylcortisone acetate (**I**). The ethers listed in Table I were prepared by adaptation of known methods [cf. Ercoli and Gardi (1960)] viz. (*a*) reaction of (I) with the alkyl orthoformate/alkanol in the presence of toluene *p*-sulphonic acid and (*b*) by ether exchange. Anti-inflammatory activity was estimated by the turpentine-agar pellet assay described in an earlier communication [Bianchi, David and others (1961)]. The results in the Table were obtained by oral administration, employing prednisolone acetate as standard.

TABLE I

3-Enol ether	Anti-inflammatory activity Prednisolone acetate = 1
Ethyl	1·2
n-Propyl	2·2
i-Propyl	1·7
n-Butly	2·0
i-Butyl	2·1
n-Pentyl	1·1
Cyclopentyl	1·3
n-Hexyl	0·8
Cyclohexyl	1·7
n-Heptyl	0·8
n-Octyl	1·7
Benzyl	0·9
3'-Phenylpropyl	0·4

Maximal anti-inflammatory activity was shown by 6-methyl cortisone acetate 3-enol n-propyl ether and this compound is being examined further.

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Surface Activity of Lysophosphatidylethanolamine

SIR—Uziel and Hanahan (1957) have reported that the fatty acid present in lysolecithin is capable of migration in the presence of either migratase enzyme or acid. Surface tension studies which we have made with aqueous sols of lysophosphatidylethanolamine have shown that after initial falls the surface tensions remain fairly constant and then commence to rise slowly. This rise in surface tension with time may be due to a migration of the fatty acid from the α to the β position in the absence of any enzyme or acid.

A static method (Wilhelmy Plate) has been used to measure surface tension. Concentrations of 0·01 per cent (w/v) and below were used since lysophosphatidylethanolamine, unlike lysolecithin, is only slightly soluble in water.

The surface tension changed considerably with time, after the plate had been immersed in the surface of the sol. It fell rapidly for about 1 hr., and then more slowly; the rate of change was least at 5–6 hr. For example a 0·005 per cent sol gave an initial reading of 56·23 dynes cm., after 2 hr. it was 39·63 and after 6 hr. it was 37·33. With further increases in the time the surface tension started to rise slowly. On repeating the readings on the same sol on subsequent days a similar pattern was followed but the values obtained 6 hr. after immersing the plate in the surface of the sol were higher on each succeeding day. With the above sol, the surface tension after 6 hr. on the second day was 44·80, and after 6 hr. on the fourth day was 58·93.

On experimenting with a 0·005 per cent (w/v) sol of phosphatidylethanolamine the surface tension fell initially with time and eventually a constant value was reached which remained unchanged for 4 days.

It seems from these results that a possible explanation of the change in surface tension with time with lysophosphatidylethanolamine sols is due to a migration of the fatty acid from the α to the β position. Such a migration could not occur in phosphatidylethanolamine, since both the hydroxyl groups are esterified so that once an equilibrium is set up between the concentration of phosphatidylethanolamine in the surface and the concentration in the bulk of the phase no change in the surface tension occurs. Further investigations are being made and will be reported more fully later.

On plotting the values obtained after 6 hr. with lysophosphatidylethanolamine sols against concentration, a sharp change in the slope of the graph is obtained within the concentration range of 0·001–0·002 per cent (w/v), indicating that the critical micelle concentration for lysophosphatidylethanolamine occurs within this concentration range.

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