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.

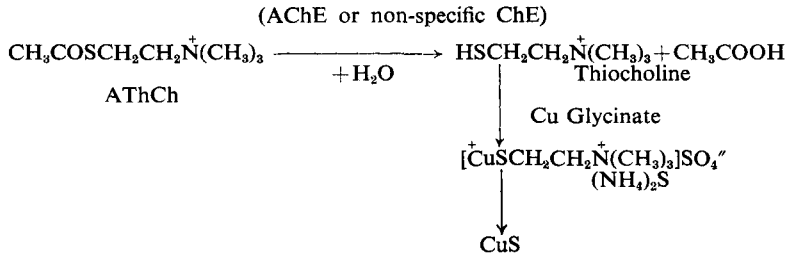
I. Components of Cholinergic Neurons: Choline Acetylase (ChAc), Acetylcholine (ACh), and Acetylcholinesterase (AChE)

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.

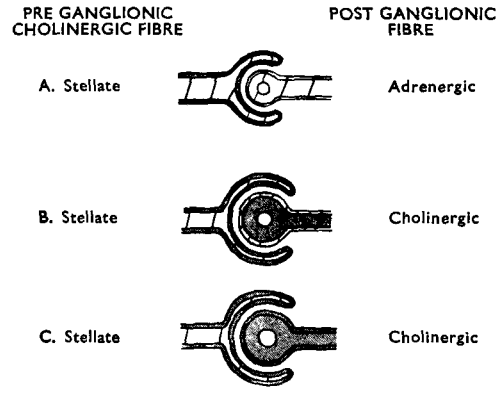


FIG. 2. Diagrammatic representation of distributions of functional (external) and reserve (internal) AChE at synapses of autonomic ganglia. Density of cross-hatching indicates relative concentration of enzymatic activity.

(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

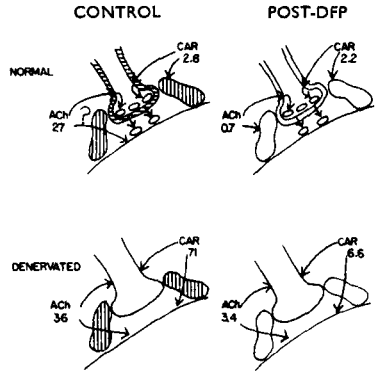


FIG. 4. Diagram depicting threshold intra-arterial doses (in mμ mol) of ACh and carbachol in four situations studied.

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

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

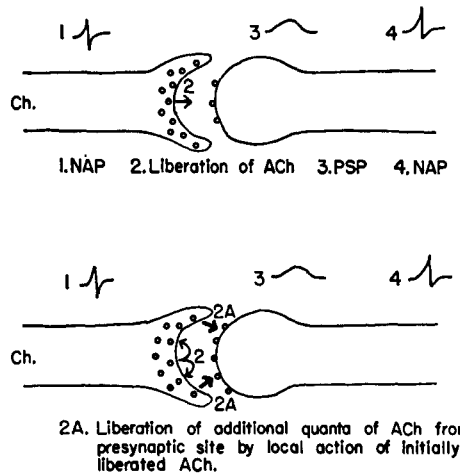


FIG. 5. Functions of ACh in Cholinergic Transmission.

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)

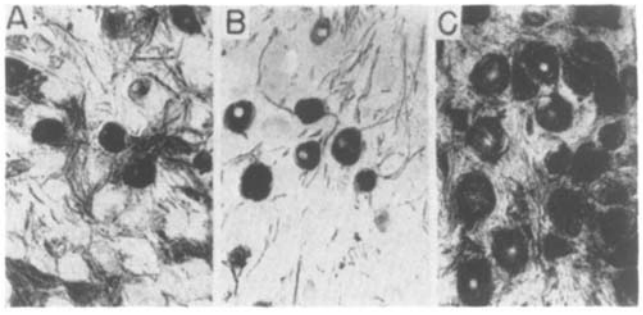


FIG. 1

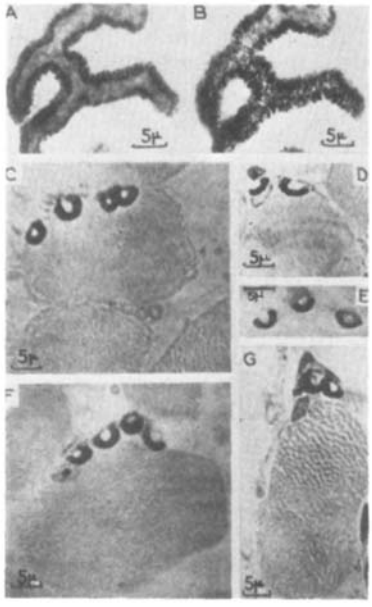


FIG. 3

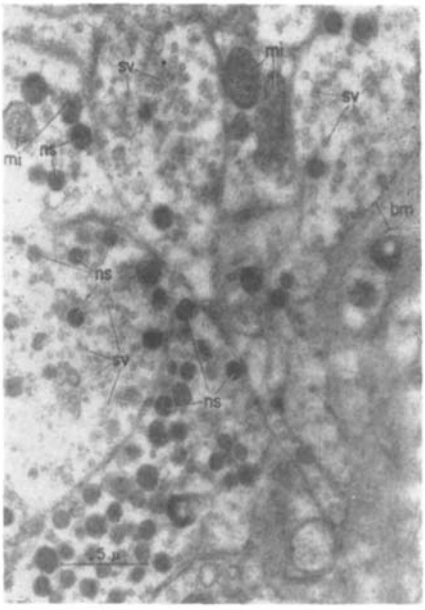


FIG. 6

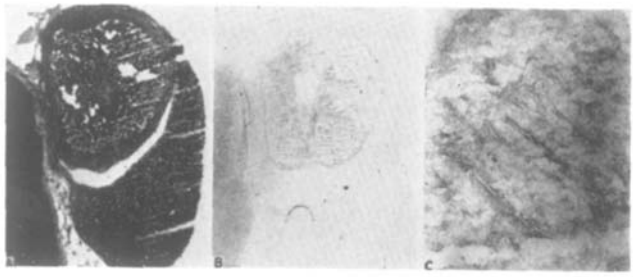


FIG. 7

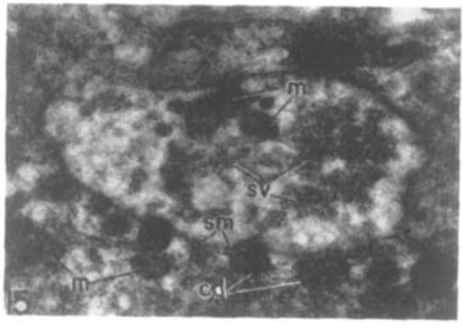


FIG. 8

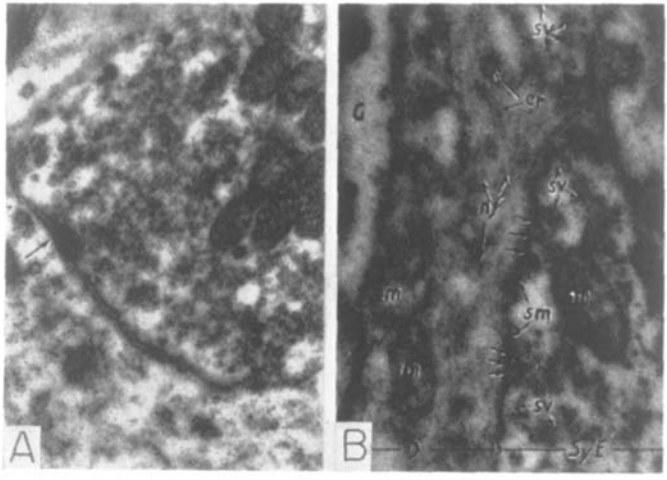


FIG. 12

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′-dimethylethanolamino-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^{-8} 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

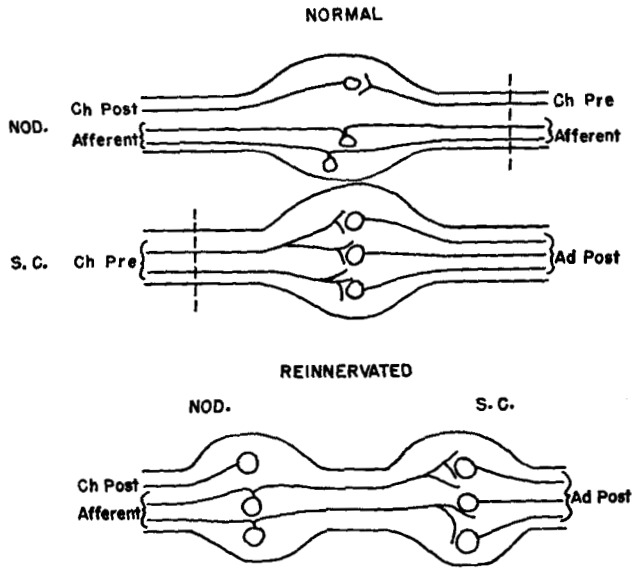


FIG. 9. Diagrammatic representation of synaptic relationships in the normal vagal nodose ganglion (NOD) and superior cervical ganglion (S.C.), and in the cross-anastomosed preparations.

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

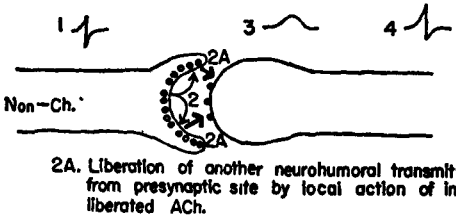


FIG. 10. Proposed presynaptic function of ACh in transmission by non-cholinergic fibres. Compare with Fig. 5.
(1) NAP liberates (2) ACh from presynaptic terminals, which acts at same terminals to effect release of (2A) synaptic transmitter; latter produces (3) PSP, which initiates (4) NAP (Koelle, 1961).

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.

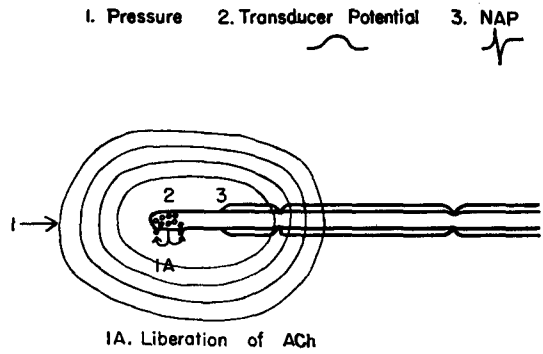


FIG. 11. Proposed function of ACh in sensory reception.
(1) Specific stimulus (e.g., pressure) causes release of (1A) ACh from axonal terminal (as shown) or accessory cells, which causes (2) depolarisation of terminal recorded as transducer potential; latter initiates electrogenically (3) NAP. (Koelle,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