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THE SPECIFICITY OF THE TRIMETHYLAMMONIUM GROUP IN ACETYLCHOLINE

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It has long been known that replacement of the three N-methyl groups of acetylcholine, or of other choline esters and ethers, by ethyl or larger alkyl radicals leads to virtual disappearance of stimulant actions on structures with parasympathetic innervation and at ganglion cells; such triethylammonium compounds retain only a paralysing action on ganglion cells (Hunt and Renshaw, 1929, 1933). The similar contrast between tetramethylammonium and tetraethylammonium salts is equally striking and well known (Burn and Dale, 1914), and studies of a more extensive range of " onium salts " suggest that only those containing at least three methyl groups attached to the central atom (which may be N, P, As, or S) retain muscarine-like and stimulating nicotine-like actions (Hunt and Renshaw, 1925; Ing and Wright, 1933). More recently Fourneau, Bovet, Bovet, and Montezin (1944) found that the intense muscarine-like properties of the acetal of 2 : 3-dihydroxypropyltrimethylammonium iodide were entirely abolished when the trimethylammonium group was replaced by triethylammonium. At the same time observations have been recorded which suggest that the integrity of the trimethylammonium group may not be indispensable to the possession of some stimulant actions on parasympathetic endings and on ganglion cells. Thus, Hunt and Taveau (1911) observed that β-acetoxyethyl*iso*amyldimethylammonium chloride (I; R = *iso*amyl) caused a fall (but sometimes a rise) of blood pressure in the cat, which was annulled by atropine; Hunt and Renshaw (1929) made similar observations with β-acetoxyethylbenzyldimethylammonium chloride (I; R = benzyl); both compounds were only feebly active. Hunt and Taveau also described a muscarine-like effect on the circulation of the compound (II), in which one methyl group of acetylcholine is replaced by acetoxyethyl, and some years later Hunt and Renshaw (1929) described a similar effect produced by tetra-β-hydroxyethylammonium chloride (III).

$$CH_3CO_2CH_2CH_2NMe_2R\}Cl$$
(I)
$$(CH_3CO_2CH_2CH_2)_2NMe_2\}Cl$$
(II)
$$(HO.CH_2CH_2)_4N\}Cl$$
(III)

In order to throw more light upon the specificity of the trimethylammonium group in choline derivatives we have studied the effect of successive replacement of the N-methyl groups of acetylcholine by (i) ethyl groups and (ii) acetoxyethyl groups. The names and formulae of the compounds investigated, and the symbols which will be used in referring to them, are given in Table I.

The effect of replacing the methyl groups of acetylcholine successively by hydrogen was studied

TABLE I

Name	$CH_3CO_2CH_2CH_2-N^+$ R	R′	R″	Symbol
Acetylcholine bromide ..	CH_3	CH_3	CH_3	Ach
Acetoxyethyldimethylethylammonium iodide	CH_3	CH_3	C_2H_5	MonoE
Acetoxyethylmethyldiethylammonium iodide	CH_3	C_2H_5	C_2H_5	DiE
Acetoxyethyltriethylammonium bromide ..	C_2H_5	C_2H_5	C_2H_5	TriE
Bis-acetoxyethyldimethylammonium iodide	CH_3	CH_3	$CH_3CO_2C_2H_4$	BisA
Tri-(acetoxyethyl)methylammonium iodide	CH_3	$CH_3CO_2C_2H_4$	$CH_3CO_2C_2H_4$	TrisA

TABLE II

Preparation and effect	Approx. equipotent molar ratios					
	Ach	MonoE	DiE	TriE	BisA	TrisA
Cat; fall of B.P.	1	3	400	>2,000	150	>20,000
Rise of B.P. after atropine	1	>5	No rise	No rise	No rise	No rise
Guinea-pig ileum; contraction	1	2.5	700	1,700	100	>20,000
Frog's heart; slowing and reduction of beat	1	2	1,500	Reduces Ach effect	75	12,500
Rabbit auricles; slowing and reduction of beat	1	1.6	600	Reduces Ach effect	50	25,000
Frog's rectus abdominis; contracture	1	5	300	5,000	164	No contracture; reduces Ach effect

by Stehle, Melville, and Oldham (1936), and their results will be considered later.

MATERIALS

The following compounds were prepared:

(1) *Acetoxyethyldimethylethylammonium iodide*, by addition of ethyl iodide to dimethylaminoethyl acetate; crystallized from hot acetone by addition of ethyl acetate; m.p. 81–82°. (Found: C, 33.8; H, 6.2. $C_8H_{18}O_2NI$ requires C, 33.5; H, 6.3 per cent.)

(2) *Acetoxyethylmethyldiethylammonium iodide*, by addition of methyl iodide to diethylaminoethyl acetate; crystallized from methylethyl ketone; m.p. 68°. (Found: C, 35.7; H, 6.7. $C_9H_{20}O_2NI$ requires C, 35.9; H, 6.6 per cent.)

(3) *Acetoxyethyltriethylammonium bromide*, by addition of ethyl bromide to diethylaminoethanol and acetylation of the product; crystallized from acetone; m.p. 131–132°. (Found: C, 44.9; H, 8.1. $C_{10}H_{22}O_2NBr$ requires C, 44.8; H, 8.2 per cent.)

(4) *Bis-acetoxyethyldimethylammonium iodide*. Diethanolamine was methylated with formalin and formic acid and the product acetylated; addition of methyl iodide to the crude ester gave a product which crystallized from acetone, m.p. 113–114°. (Found: C, 34.8; H, 5.8. $C_{10}H_{20}O_4NI$ requires C, 34.7; H, 5.7 per cent.)

(5) *Tri-(acetoxyethyl)methylammonium iodide*, by acetylation of triethanolamine and addition of methyl iodide to the crude ester; crystallized from acetone, m.p. 110–111°. (Found: C, 37.8; H, 5.8. $C_{13}H_{24}O_6NI$ requires C, 37.4; H, 5.8 per cent.)

All these compounds are hygroscopic. Hunt and Taveau (1911) prepared the chloroplatinates of acetoxyethyltriethylammonium and bis-acetoxyethyldimethylammonium.

METHODS

Each substance was compared with acetylcholine on the following preparations:

(1) Blood pressure of the cat under chloralose.
(2) Isolated ileum of the guinea-pig.
(3) Perfused heart of the frog.
(4) Isolated auricles of the rabbit.
(5) Rectus abdominis of the frog.

TriE and TrisA were also tested on the perfused superior cervical ganglion of the cat. TriE was tested for mydriatic activity in mice by Pulewka's method (1932; cf. Ing, Dawes, and Wajda, 1945). TrisA was tested on the denervated gastrocnemius of the cat. In addition the hydrolysis of each substance by the cholinesterases of horse serum, dog's caudate nucleus, and cobra venom was studied manometrically.

RESULTS

The results obtained with the first five preparations mentioned above are summarized in Table II; they are expressed as equipotent molar ratios and the figures are therefore inversely proportional to molar activities. The effects of the substances were qualitatively similar to those of acetylcholine unless otherwise stated.

The equipotent molar ratios were obtained either by matching doses or by plotting the magnitude of an effect against the logarithm of the dose; they are only approximate because some of the preparations were not sensitive enough to distinguish between doses differing by less than 50 per cent. Moreover, the responses to increasing doses of acetylcholine were usually more steeply graded than those to increasing doses of the other compounds, and consequently an arbitrary response had to be chosen for the estimation of equipotent doses; thus with the rectus abdominis a contracture 50 per cent of the maximal was chosen. With the rabbit's auricles linear and parallel relations between percentage inhibition and log. dose were obtained for Ach, MonoE, and DiE.

The actions of MonoE, DiE, and BisA.—The effects of these three substances resembled those of acetylcholine closely, except that a nicotine-like rise of blood pressure after atropine was not obtained with DiE (4 mg.) or BisA (2 mg.). The replacement of one methyl group of acetylcholine by an ethyl group produces a relatively small decrease in activity, MonoE being between a half and a third as active

as Ach in its muscarine-like effects and about a fifth as active in its nicotine-like effects; but the replacement of two methyl groups by ethyl produces a decline in activity of an entirely different order, DiE having on the average about one five-hundredth of the activity of acetylcholine.

The introduction of a second acetoxyethyl group in place of methyl into acetylcholine is a more radical chemical change than the replacement of one methyl by ethyl and it produces a more pronounced decrease in activity, BisA being about one-hundredth as active as acetylcholine, but still much more active than DiE.

The action of TriE.—The replacement of all three methyl groups of acetylcholine by ethyl reduces the activity on all five preparations by a factor of at least a thousand; moreover it alters the kind of activity on the heart and circulation. In cats under chloralose large doses of TriE (20 mg.) produced a fall of blood pressure which developed more slowly and was more prolonged than that produced by Ach and could be obtained after atropine. Similar doses also reduced the pressor effect of small doses of nicotine. It seems probable that the fall of blood pressure is due to a paralysing action on sympathetic ganglia. Hunt and Renshaw (1929, 1933) also concluded that TriE had a paralysing nicotine-like action; they used cats in which the C.N.S. had been destroyed and, probably for this reason, they observed no fall in blood pressure, but after atropine TriE diminished or annulled the pressor effect of tetramethylammonium. In order to demonstrate this paralysing nicotine-like action the effect of TriE on the perfused superior cervical ganglion of the anaesthetized cat was investigated. As Fig. 1 shows TriE in doses of 1–10 mg. abolished or reduced the contractions of the nictitating membrane in response to preganglionic stimulation, but the effect was short-lived. It is interesting that although TriE had no stimulant effect on sympathetic ganglia it did cause contracture of the frog's rectus abdominis; it did not antagonize Ach in this muscle, nor did it show any curariform action on the rat's phrenic nerve-diaphragm in concentrations so high as 30 mg./100 ml. (one experiment).

Hunt and Renshaw observed no reduction in heart rate in their experiments with TriE; in our experiments also the heart rate in anaesthetized cats was unaltered by TriE. TriE had no effect on the rate of the perfused frog's heart, but it did prevent or reduce the effect of subsequent doses of Ach; when 0.1 ml. $10^{-6}M$ Ach was given at one-minute intervals, 0.1 ml. $10^{-1}M$ TriE prevented the effect of Ach for two minutes and the full effect was only restored five minutes after the administration of TriE. Smaller doses of TriE had a similar effect but o shorter duration (Fig. 2). Somewhat similar effects were also observed with the isolated rabbit auricles, although large doses were required; e.g., 8 mg. TriE nearly but not quite abolished the effect of 11 μg. Ach. It is doubtful whether these blocking

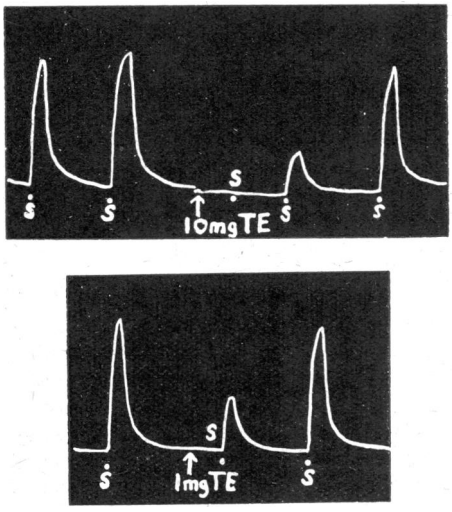

FIG. 1.—Perfused superior cervical ganglion of cat under chloralose. Record of contractions of nictitating membrane in response to stimulation of preganglionic fibres for 4 sec. every 3 min. at S. TriE (given at TE) reduced the response to stimulation.

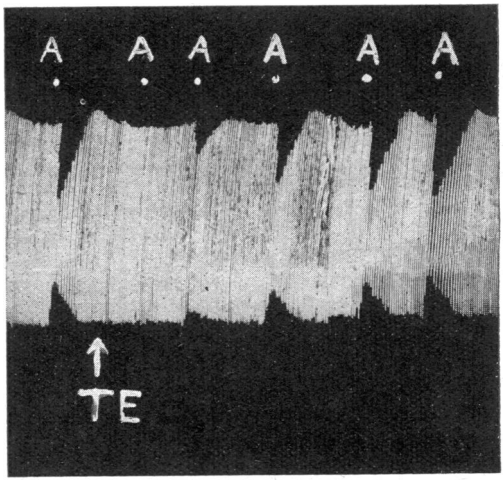

FIG. 2.—Perfused frog's heart; 0.1 ml. $10^{-6}M$ Ach given at intervals of 1 min. (at A); 0.05 ml. $10^{-2}M$ TriE (given at TE) reduced response to subsequent doses of Ach.

effects are truly atropine-like, because TriE did not reduce the effects of Ach on the cat's blood pressure or the guinea-pig's ileum; on the latter TriE had a weak stimulant action. An attempt was made to discover whether TriE had any mydriatic activity, but the results were inconclusive. Groups of five mice were injected intraperitoneally with varying doses of TriE and the pupil diameters measured at intervals. Some dilatation was observed but only after toxic doses; thus a fivefold mean dilatation was obtained with 2 mg. TriE per mouse, but three out of five mice died; 1 mg. TriE produced a threefold dilatation but killed one out of five mice.

The action of TrisA.—The introduction of two acetoxyethyl groups in place of methyl into acetylcholine reduces activity on the five preparations by a factor of at least ten thousand. Nevertheless the effects of TrisA on the cat's blood pressure, the guinea-pig's ileum, and the frog's heart are truly

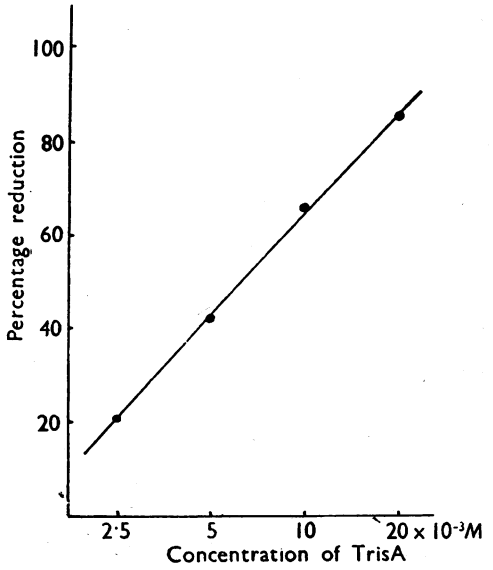

FIG. 3.—Antagonism between TrisA and Ach on rectus abdominis (frog). Ordinates: percentage reduction in contractures produced by $0.75 \times 10^{-6} M$ Ach. Abscissae: molar concentration of TrisA: logarithmic scale.

muscarine-like since they are reduced or abolished by atropine. TrisA did not appear to have any stimulant nicotine-like actions, either on the blood pressure or on striated muscle; it produced no contracture of the frog's rectus abdominis, but it antagonized the effect of Ach on this muscle, and this antagonism was linearly proportional to the logarithm of the concentration of TrisA (Fig. 3).

Similarly TrisA reduced the effect of Ach on the cat's denervated gastrocnemius. Fig. 4 is a record of the contractions of a cat's gastrocnemius, which had been denervated eight days previously; A_1, A_2, and A_3 were contractions in response to the injection of 10 μg. Ach into the iliac artery; at TS 25 mg.

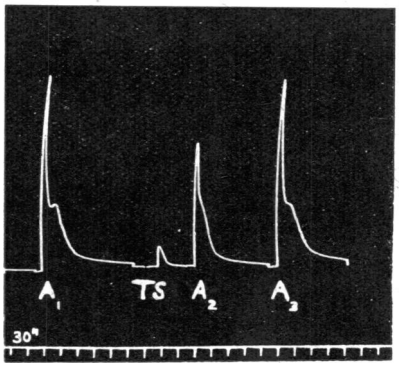

FIG. 4.—Cat's denervated gastrocnemius. Record of contractions in response to 10 μg. Ach injected into iliac artery at A_1, A_2, and A_3; 25 mg. TrisA, injected at TS, reduced response to next injection of Ach.

TrisA were injected; one minute elapsed between TS and A_2, eight minutes between TS and A_3. It will be seen that TrisA produced a small contraction itself but reduced the response to the next injection of Ach by about 40 per cent.

A similar kind of effect was observed in the cat's superior cervical ganglion, perfused with Locke's solution (Fig. 5): injection of 8 mg. TrisA produced no contraction of the nictitating membrane, but diminished its response to stimulation of the

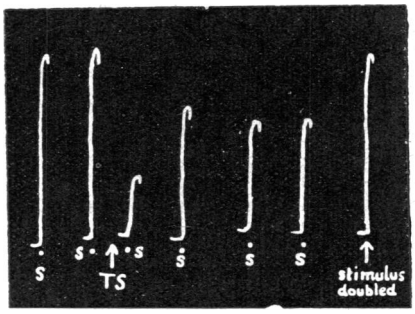

FIG. 5.—Perfused superior cervical ganglion of cat under chloralose. Record of contractions of nictitating membrane in response to preganglionic stimulation at S. At TS 20 mg. TrisA injected 20 sec. before next stimulus.

preganglionic fibres 20 seconds later; the intensity of the stimulus had to be doubled in order to obtain a contraction as large as before the injection of TrisA.

Enzymic hydrolysis

All the substances were hydrolysed by horse serum and a preparation of dog's caudate nucleus; the rates of hydrolysis are given in Table III. It will be noticed that the mono-, di-, and tri-ethyl compounds were hydrolysed by dog's caudate nucleus at about the same rate as acetylcholine, but with horse serum the rate declined with successive substitution of methyl by ethyl groups. With BisA the volume of CO_2 evolved indicated that both ester groups were hydrolysed, but the hydrolysis of TrisA was too slow to be followed to completion.

Since a specimen of cobra venom was available the rates of hydrolysis of the compounds by this preparation were also measured, and the results are included in Table III.

TABLE III

COMPARISON OF RATES OF ENZYMIC HYDROLYSIS OF ESTERS

Substrate concentrations were $10^{-2}M$ for horse serum and $6 \times 10^{-2}M$ for dog's caudate nucleus and cobra venom

Substrate	μl. CO_2 evolved in 15 min. (no correction for retention)		
	0.2 ml. horse serum	4 mg. caudate nucleus	0.2 mg. cobra venom
Ach ..	61	49	87
MonoE	44	53	77
DiE ..	30	47	65
TriE ..	16	46	66
BisA ..	51*	52*	104*
TrisA ..	5	11	12

* Both ester groups hydrolysed

In order to make sure that the observed hydrolysis was due to the cholinesterases of horse serum and caudate nucleus the effect of specific inhibitors of these preparations on the hydrolysis was studied. The hydrolysis of each compound by horse serum was inhibited by the dimethylcarbamic ester of 2 - hydroxy - 5 - phenylbenzyltrimethylammonium bromide (Nu 683), which is a fairly specific inhibitor of the enzyme known as pseudo-cholinesterase (Hawkins and Gunter, 1946). The percentage inhibitions with $10^{-8}M$ inhibitor are given in Table IV; they suggest that the cholinesterase of horse serum is responsible for the hydrolysis of these esters. This conclusion was supported by observations with a purified preparation of horse serum cholinesterase, which hydrolysed all five esters, as well as acetylcholine, at approximately the same relative rates as the unpurified horse serum. We are indebted to Dr. J. W. Legge for this enzyme preparation.

TABLE IV

THE EFFECT OF SPECIFIC INHIBITORS OF CHOLINESTERASES

Substrate	Percentage inhibition of	
	horse serum with $10^{-8}M$ Nu 683	caudate nucleus with $3 \times 10^{-6}M$ eserine
Ach ..	71	90
MonoE ..	74	93
DiE ..	68	87
TriE ..	58	95
BisA ..	69	82
TrisA ..	57	80

Table IV also includes data on the percentage inhibition of the hydrolysis of these esters by dog's caudate nucleus in the presence of 3×10^{-6} eserine. These observations indicate that the esters were also substrates of the enzyme known as true cholinesterase.

In accordance with these manometric experiments it was observed that the effects of the five esters on the cat's blood pressure were all increased and prolonged by the previous administration of a large dose (0.5 mg.) of eserine.

Discussion

The main conclusions to be drawn from the results described above are: that the presence of three N-methyl groups is not indispensable to the typical pharmacological properties of acetylcholine; that replacement of one methyl group by ethyl produces a relatively small decrease in activity; but that replacement of two methyl groups by ethyl leads to a profound decline in activity. Similarly, although the replacement of one methyl group by acetoxyethyl reduces activity more than replacement by ethyl, the substitution of a second methyl group by acetoxyethyl has proportionately a very much greater effect. These conclusions suggest that two N-methyl groups play an important part in the structural specificity of molecules of this type.

The specificity of the acetoxy group is well known, and if it be assumed that at the site of its action acetylcholine is attached (e.g., by van der Waals forces) to some component molecule of the excitable structure not only by this group but also by two methyl groups, and the tetrahedral arrangement of the 4-covalent nitrogen atom would allow this, then replacement of one methyl group by ethyl will

reduce the probability of the correct orientation for attachment but will not make it impossible, as the replacement of two methyl groups by ethyl would do. The probability of attachment of an NMe_2R-group will not depend solely upon the geometry of the structure—if it did, it would be one-third that of the NMe_3- group—but it is impossible to predict to what extent the radical R, which may well have its own affinity for the excitable structure, will influence the ease of attachment. We have already noted that replacement of one methyl in acetylcholine by acetoxyethyl diminishes activity much more than replacement by ethyl; replacement by benzyl (Hunt and Renshaw, 1929) appears to reduce the activity of the molecule (on the cat's blood pressure) to about one-thousandth that of acetylcholine, and Hunt and Taveau (1911) described acetoxyethyldimethyl*iso*amylammonium chloride as comparable in activity with choline. The effect of replacement by hydrogen is interesting; Stehle, Melville, and Oldham (1936) studied the effect of successive replacement of the methyl groups of acetylcholine by hydrogen, and their results have been converted in Table V into the form adopted by us for recording our own results in Table II. The progressive decline in activity as methyl groups are successively replaced by hydrogen is not unexpected, but it will be noticed that the effect of replacing one methyl by hydrogen is at least ten times greater than that of replacing one methyl by

TABLE V

Data from Stehle, Melville, and Oldham (1936)

Substance	Approx. equipotent molar ratios		
	Cat's blood pressure	Rabbit intestine	Frog's heart
$AcO.C_2H_4NMe_3\}Cl$ ..	1	1	1
$AcO.C_2H_4NMe_2H\}Cl$ ..	50	>40	>50
$AcO.C_2H_4NMeH_2\}Cl$ ..	>500	>1,000	>500
$AcO.C_2H_4NH_3\}Cl$ ..	>2,000	20,000	40,000

ethyl; the former change, however, alters the character of the cation, which ceases to be a stable structure.

The form of attachment envisaged in our hypothesis—viz., by means of short-range atomic forces—implies a high degree of fit between the drug molecule and the site of action, and structural changes which, for example, alter the mean distance between the methyl groups might be expected to decrease the probability of attachment and hence the activity of the drug. This effect is illustrated by the results of Welch and Roepke (1935) with the phosphorus and arsenic analogues of acetylcholine. In Table VI we have converted their figures for molar potency into the form adopted in Table II. It will be seen that, for muscarine-like effects, acetylphosphocholine is approximately one-twelfth, and acetylarsenocholine one-eightieth, as active as acetylcholine. The replacement of nitrogen by phosphorus or arsenic

TABLE VI

Data from Welch and Roepke (1935)

Substance	Approx. equipotent molar ratios			
	Cat's blood pressure	Rabbit's intestine	Frog's heart	Frog's rectus
$AcO.C_2H_4NMe_3\}Cl$	1	1	1	1
$AcO.C_2H_4PMe_3\}Cl$	13	12	12	6
$AcO.C_2H_4AsMe_3\}Cl$	66	90	83	37

will not alter the angle between the valencies, but owing to the larger size of the phosphorus and arsenic atoms it will increase the mean distance between the methyl groups. The best available values for the N–C, P–C, and As–C links in methyl compounds are 1.47, 1.87, and 1.98 Å respectively (Pauling, 1945). The tetrahedral angle between the valencies of 4-covalent N, P, or As is 109° 28′ and hence the distance between the carbon centres of two methyl groups will be $2\,y \sin 54° 44'$, where y is the length of the link between carbon and the central atom. The calculated distances are: for nitrogen, 2.40; for phosphorus, 3.05; and for arsenic, 3.23 Å. Thus in acetylphosphocholine the mean carbon-carbon distance between two methyl groups will be 27.1 per cent greater than in acetylcholine; in acetylarsenocholine it will be 34.6 per cent greater. It is interesting that acetylphosphocholine is less active than MonoE in spite of the presence of three methyl groups, and this may well be due to the greater separation of the methyl groups, an effect which is further intensified in acetylarsenocholine.

Another result of increasing the size of the cationic head by replacing the nitrogen atom by phosphorus or arsenic will be to diminish the Coulomb attraction between the cation and an anion in the excitable structure, but the potential function of this attraction is inversely proportional to the distance between the ions, and will consequently decrease much less rapidly with increase in the ionic radius than the van der Waals forces, which are inversely proportional to the sixth power of the distance between atoms.

Summary

1. The effect of successive replacement of the N-methyl groups of acetylcholine by (i) ethyl groups and (ii) acetoxyethyl groups has been studied on the cat's blood pressure, guinea-pig's ileum, the frog's heart, rabbit auricles, and the frog's rectus abdominis.

2. The replacement of one methyl group of acetylcholine by ethyl does not alter the pharmacological activities qualitatively and only reduces them to between a half and a fifth of those of acetylcholine. The replacement of two methyl groups by ethyl produces a drastic reduction in the activities, without altering their nature.

3. The replacement of all three methyl groups by ethyl alters the kind of pharmacological effect produced. Acetoxyethyltriethylammonium has paralysing nicotine-like properties on the cat's blood pressure and superior cervical ganglion; it also antagonizes acetylcholine in the frog's heart and the rabbit's auricles.

4. The replacement of one methyl group by acetoxyethyl does not alter the kinds of activity, but it reduces them more than replacement by ethyl. Replacement of two methyl groups by acetoxyethyl produces a very much greater decline in activities than replacement of one methyl group.

5. The mono-, di-, and tri-N-ethyl analogues of acetylcholine are hydrolysed by the cholinesterases of dog's caudate nucleus and cobra venom at about the same rates as acetylcholine. The rate of hydrolysis by the cholinesterase of horse serum declines with successive substitution of methyl by ethyl groups.

6. Both ester groups of bis-acetoxyethyldimethylammonium iodide are hydrolysed by the cholinesterases of horse serum, dog's caudate nucleus, and cobra venom. Tri-(acetoxyethyl)methylammonium iodide is hydrolysed much more slowly by all three enzyme preparations.

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