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THE SPECIFICITY OF BRAIN CHOLINE ACETYLASE

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The relationship between structure and pharmacological action in parasympathomimetic drugs has been extensively studied, notably by Hunt (1915), Hunt and Renshaw (1925, 1929, 1933), Bovet and Bovet-Nitti (1948), and Holton and Ing (1949). These and other studies have shown that few modifications in the acetylcholine molecule are permissible if the same order of pharmacological activity is to be retained. For instance, replacement of one of the methyl groups by ethyl caused little change in activity, but the substitution of *n*-propyl or longer aliphatic groups, or the substitution of more than one methyl by ethyl, led to a pronounced decrease in activity (Holton and Ing, 1949). Substitution of a β-methyl group had little effect on muscarinic activity but almost abolished nicotinic activity (Simonart, 1932). On the other hand α-methyl substitution reduced muscarinic activity without appreciable effect on nicotinic activity. Increase in the separation between the quaternary nitrogen and the ester group to 3 carbons in acetyl-γ-homocholine had little effect on the activity (Hunt, 1915); and finally tertiary, secondary, and primary amino ethyl acetates were much less active than acetylcholine (Stehle, Melville, and Oldham, 1936).

Similar studies on the specificity of the cholinesterases have shown that, although these enzymes are sensitive to the nature of the acyl group, they are extraordinarily unspecific for the alcoholic part of the ester (Adams, 1949), so that even non-basic esters are vigorously split and may sometimes be hydrolysed faster than acetylcholine (e.g., *iso*propenyl acetate—Burgen, unpublished).

Until recently, in default of other evidence, it had been assumed that choline acetylase was specific. Doubt was thrown on this when Banister, Whittaker, and Wijesundera (1953) demonstrated the presence of propionylcholine in ox spleen. Gardiner and Whittaker (1954) and Berman, Wilson, and Nachmansohn (1953) were able to show that acetone powder extracts of pigeon brain and squid ganglia could synthesize propionylcholine or butyrylcholine if propionate or butyrate were added to the incubate in place of acetate. The only suggestion that choline acetylase might also be non-specific for choline, too, was the finding of Korey, Braganza, and Nachmansohn (1951) that choline acetylase from the squid head ganglion was able to acetylate dimethyl ethanolamine as fast as choline, but that ethanolamine, monomethylethanolamine, and diethylethanolamine were not acetylated at all.

The present work was undertaken to study systematically the effects of modification of the choline molecule on the rate of acetylation by choline acetylase and also to attempt to find specific inhibitors of the acetylase.

Since the acetyl esters of many of the bases studied have negligible biological activity it was not possible to use the usual bioassay methods for estimating the rate of acetylation. The most satisfactory solution to this problem was the use of a highly purified preparation of choline acetylase and the measurement of the rate at which the enzyme could transfer acetyl radicles from acetyl-Coenzyme A to the base. This was attained by measuring the rate of deacetylation of acetyl-CoA. The method was applicable to any acetyl acceptor that did not generate a " high energy " acetyl group (it was not applicable for this reason to thiocholine) and did not require modification for the various compounds employed. A further advantage of the method is that it employs the simplest possible acetylation system in which only one enzyme is involved. This eliminates the possibility of indirect inhibition of acetylation which may occur with the usual more complex system in which acetate or citrate must be converted to acetyl-CoA by a phosphokinase system.

For the successful use of this method it is imperative that the enzyme be largely freed of thioesterases. In the presence of these enzymes acetyl-CoA is rapidly split to yield free acetate. When the enzyme is satisfactorily purified there is good agreement between the breakdown of acetyl-CoA and the amount of acetylcholine formed.

METHODS

Preparation of the Brain Enzyme

Acetone Powders.—The brains from 40–80 rats were placed in ice-cold 0.9% saline immediately after removal. The brains were then dried on blotting paper, and, after being freed from the blood vessels and membranes, were homogenized in 200–400 ml. of pure dry acetone at 0° C. in a Waring blender. The acetone was purified by adding solid $KMnO_4$, and after 7–10 days' storage was distilled, dried over anhydrous $CaSO_4$, and then redistilled. The brain suspension was filtered immediately on a coarse sintered glass Buchner funnel and the cake washed three times with ice-cold dry acetone. The powder was spread on filter paper for a few minutes to allow most of the acetone to evaporate and then transferred to a vacuum desiccator over P_2O_5 and evacuated with a water pump for about twenty minutes. The desiccator was stored in the refrigerator. One brain yielded about 0.2 g. of powder.

Extraction and Purification of Choline Acetylase.—The acetone powder was extracted by being ground in a chilled mortar with 13.5 ml./g. of ice-cold extraction medium (0.015 M-K_2HPO_4: KH_2PO_4 *p*H 7.0; 0.058 M-NaCl; 0.001 M-disodium ethylenediamine tetracetate (EDTA); 0.004 M-cysteine). The mixture was allowed to stand at 4° C. for 10 min. and then centrifuged. The supernatant fluid was retained and purified by a variation of the procedure described by Kumagai and Ebashi (1954).

The extract was acidified to *p*H 4.5 with acetic acid and, after overnight storage at 4° C., was centrifuged and the precipitate discarded. The supernatant fluid was adjusted to *p*H 6.8 with $NaHCO_3$ and solid ammonium sulphate added to give a concentration of 16%. After 20 min. the mixture was centrifuged and the residue again discarded. More ammonium sulphate was added to the supernatant fluid to bring the concentration to 36%. After 20 min. storage the mixture was centrifuged and the supernatant fluid discarded. The residue was taken up in 6 ml. of extraction medium and acidified to *p*H 5.0 with acetic acid. After 30 min. at 4° C. the insoluble material was removed by centrifugation. The supernatant fluid was adjusted to *p*H 7.0 with $NaHCO_3$ and dialysed for 1 hr. against a medium containing 0.116 M-NaCl, 0.015 M-K_2HPO_4: KH_2PO_4, 0.001 M-EDTA, and 0.004 M-cysteine. This medium was adjusted to *p*H 7. The enzyme preparation was diluted with the dialysis medium to give a volume of 8 ml./g. of acetone powder. If the preparation was not used immediately it was stored at −10° C.

Acetyl-Coenzyme A

Preparation.—The acetyl-CoA was prepared by the thiolacetate method described by Wilson (1952). Batches of 30 mg. of Coenzyme A (Pabst 85% pure) were acetylated. The acetylation was almost theoretical as shown by the change in extinction at 233 mμ. on alkaline hydrolysis (Lynen, 1953) and by hydroxamic assay.

Determination.—The hydroxamic method of Lipmann and Tuttle (1951) was simplified by using only three solutions: (a), Buffered hydroxylamine, prepared by mixing 25 ml. 4 M-hydroxylamine hydrochloride with 25 ml. 35 M-NaOH and 50 ml. 0.1 M-acetate buffer *p*H 5.4; (b), 10% trichloroacetic acid; (c), 10% $FeCl_3$ in 1.5 N-HCl. Ten min. was allowed for hydroxamate formation after 0.3 ml. of the incubate had been added to 0.1 ml. of reagent (a). We then added 0.1 ml. of (b) and finally 0.1 ml. of (c). The tubes were centrifuged at 3,000 rev./min. for 5 min. and the density read immediately in the Beckman DU spectrophotometer at 540 mμ. Pure acetylthiocholine was used as a standard.

Assay of Acetylcholine and Measurement of Rate of Hydrolysis by Cholinesterase

Acetylcholine was assayed on the guinea-pig ileum. The susceptibility of the acetate esters to hydrolysis by cholinesterase was measured in a Warburg apparatus. The details of the methods used are described by Burgen and Chipman (1951).

Acetylation System

The incubation mixture contained: acetyl-CoA 0.75 μM./ml.; choline or test substrate 11.1 μM./ml.; tetraethylpyrophosphate 0.072 μM./ml.; EDTA 2.2 μM./ml.; K_2HPO_4: KH_2PO_4 *p*H 7; 56 μM./ml.; enzyme equivalent to 30–40 mg. acetone powder/ml. In the inhibition experiments, the test substance was added in a concentration of 11.1 μM./ml.

The mixture was incubated at 36° C. for 30 min. ; the tubes were then placed in an ice bath while aliquots for acetyl-CoA determination were removed. The residue was acidified to *p*H 4–5 for acetylcholine assay.

Substrates

The substrates were either purified from commercially available materials or prepared by standard methods. With the exception of the liquid tertiary compounds which were redistilled all the substances were obtained as pure crystalline salts.

RESULTS

With the purified enzyme the agreement between the formation of acetylcholine and the deacetylation of acetyl-CoA was fairly good, but the purified enzyme still contained some enzymes that could deacetylate acetyl-CoA in the absence of choline or other acetyl acceptors (probably " thioesterases "). To obtain accurate results it was therefore necessary to run a tube containing all the system except choline and take the difference between the amount of acetyl-CoA left after 30 min. incubation of this tube and of the complete system. Table I illustrates a typical experiment. Here the presence of choline resulted in the disappearance of an extra 0.135 μM. acetyl-CoA/ml. At the same time 0.127 μM./ml. acetylcholine was formed. When these precautions were taken the agreement between the two values was within ±20%. The high activity of the enzyme will be noted.

TABLE I

DEACETYLATION OF ACETYL-COENZYME A WITH AND WITHOUT CHOLINE

For composition of acetylation system, see Methods. Incubated for 30 min.

	Acetyl-CoA (μM./ml.)	Acetyl-CoA Utilized (μM./ml.)	Acetyl-choline (μM./ml.)
Complete system, not incubated	0·690	—	0
Complete system ..	0·510	0·135	0·127
Complete system minus choline	0·645	—	0

Acetylcholine formed 3·8 μM./g. powder/30 min.

Test substrates were tested in duplicate in groups of three or four. In each run tubes containing choline and with no substrate were included so that the rate of acetylation could be compared with that of choline. The rate of choline acetylation was 2.5–4.7 μM./g./30 min. in different batches of enzyme.

Table II shows the results obtained on choline and 24 other alkanolamines. Successive replacement of methyl groups by ethyl had little effect and indeed the monoethyl derivative (2) was consistently acetylated at a greater rate than choline. Replacement of the methyl group by longer chains had little effect until *n*-hexyl (7) which was not acetylated. This substance is quite surface active, but the lack of acetylation was not due to enzyme denaturation, as the presence of (7) did not affect choline acetylation. It is interesting that even benzyl (8) or *iso*propyl (9) substitution did not greatly reduce acetylation. Substitution by hydroxyethyl groups was somewhat more inhibitory than ethyl groups, but this decreased activity was only marked in tetraethanolamine (12).

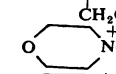
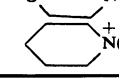
The tertiary, secondary, and primary bases corresponding to choline (13, 14, 15) were all acetylated about one-eighth as rapidly as choline. This change is probably due to the decrease in base strength from the quaternary to the hydrogen bonded lower bases. The differences in base strength between the primary, secondary, and tertiary bases are trivial by comparison. The lack of acetylation of compound 23 may be interpreted in a similar way. This compound, with one methyl group carboxylated giving an electrically neutral betaine, was also devoid of parasympathomimetic activity. The results with the non-quaternary compounds differ from those of Korey *et al.* with the squid enzyme, in which dimethylethanolamine was acetylated as rapidly as choline.

An examination of compounds in which the hydroxyethyl group was modified showed that this was the part of the molecule in which most of the specificity resided. Increase in the hydroxyl-nitrogen separation by one carbon in γ-homocholine (16) abolished acetylation and side chain substitution as in α-methylcholine (18) and β-methylcholine (19) had the same effect. Curiously, increase in the hydroxyl-nitrogen separation to 4 carbons in δ-hydroxybutyltrimethylammonium (17) restored some acetylation.

Three dimeric molecules (20, 21, 22) were tested, and of these the C_{10} member was acetylated at 63% of the rate of choline, but the C_3 and C_5 members were not acetylated at all. Barlow (1955) found that the pharmacological activity of these

TABLE II

ACETYLATION OF ALKANOLAMINES BY RAT BRAIN ENZYME

	Compound R = CH_2CH_2OH	Acetylation
1	$(CH_3)_3N^+R$	100
2	$(CH_3)_2(C_2H_5)N^+R$	143
3	$(CH_3)(C_2H_5)_2N^+R$	102
4	$(C_2H_5)_3N^+R$	95
2	$(C_2H_5)(CH_3)_2N^+R$	143
5	$(n\text{-}C_3H_7)(CH_3)_2N^+R$	74
6	$(n\text{-}C_4H_9)(CH_3)_2N^+R$	67
7	$(n\text{-}C_6H_{13})(CH_3)_2N^+R$	0
8	$(C_6H_5.CH_2)(CH_3)N^+R$	67
9	$(iso\text{-}C_3H_7)(CH_3)_2N^+R$	67
10	$(CH_3)_2N^+(R)_2$	79
11	$CH_3N^+(R)_3$	63
12	$N^+(R)_4$	16
13	$(CH_3)_2N^+(R)_2$	11
14	$(CH_3.NHR$	15
15	NH_2R	15
16	$(CH_3)_3N^+CH_2R$	0
17	$(CH_3)_3N^+CH_2CH_2R$	29
18	$(CH_3)_3N^+CH(CH_3)CH_2OH$	0
19	$(CH_3)_3N^+CH_2CH(CH_3)OH$	0
20	$RN^+(CH_3)_2(CH_2)_3N^+(CH_3)_2R$	0
21	$RN^+(CH_3)_2(CH_2)_5N^+(CH_3)_2R$	0
22	$RN^+(CH_3)_2(CH_2)_{10}N^+(CH_3)_2R$	63
23	$(CH_3)_2NR^+CH_2COO^-$	0
24		0
25		0

compounds showed a sharp peak at a 10 carbon separation with rapid decline in activity on either side.

Finally two compounds in which the quaternary nitrogen was part of a ring structure (24, 25) were not acetylated.

Inhibition of Choline Acetylation

Inhibition of acetylation was tested by adding an equimolar amount of choline and the test substance to the system and comparing the rate of acetylcholine synthesis with that occurring when choline alone was added. The acetylcholine formation was measured by bioassay. In addition to the compounds listed in Table II, the following were tested: tetramethylammonium, glycine betaine hydrazide, and dicholine ether.

Of the substances that were themselves acetylated only ethyldimethylethanolammonium (2) was an effective competitor. This enzyme appears to acetylate this compound preferentially, but differential assay was inadequate for arriving at an accurate quantitative measure of the relative proportions of acetylcholine and acetoxyethylethyldimethylammonium formed. The most satisfactory method would be chromatographic separation, but this was not attempted. Three other compounds produced a significant degree of inhibition. Carboxymethyldimethylethanolammonium (23) inhibited by 40%, betaine hydrazide 28%, and α-methylcholine 25%. No other substances produced appreciable inhibition. There was never a significantly greater total acetyl ester formation in the presence of a pair of substrates than when either choline or a single substrate was present by itself. This makes it unlikely that any of the substrates are acetylated by an enzyme unable to acetylate choline. We feel justified, therefore, in attributing the acetylations described here to the enzyme commonly called choline acetylase, although this may indeed represent a family of enzymes analogous to the situation with cholinesterases.

Discussion

The present work demonstrates that choline acetylase is by no means specific for choline, but indeed can acetylate many analogues of choline. The effect on enzymic acetylation of replacing the methyl groups of choline is surprisingly trivial in comparison with the marked decrease in pharmacological activity that accompanies the same structural change in acetylcholine. Table III summarizes results obtained in this laboratory and those obtained by Holton and Ing. There is no correlation between the pharmacological activities of the acetic esters and either the acetylation of the alkanolamines or the hydrolysis of the acetic esters. The two enzymic reactions run parallel and so do the two pharmacological properties, but there is no concurrence between the pharmacological and enzymic actions in this series of compounds.

TABLE III

SUMMARY OF CHANGES BROUGHT ABOUT BY REPLACING THE METHYL GROUPS IN CHOLINE AND IN ACETYLCHOLINE

	Acetic Esters			Alkanolamines
	Muscarinic Activity Cat: Fall of B.P.	Nicotinic Activity Frog Rectus	Hydrolysis by ChE	Acetylation by Rat Brain Enzyme
R R′ R″				
CH_3 CH_3 CH_3	100	100	100	100
CH_3 CH_3 C_2H_5	33*	20*	108*†	143
CH_3 C_2H_5 C_2H_5	0·2*	0·3*	96*†	102
C_2H_5 C_2H_5 C_2H_5	<0·05*	0·02*	94*†	95
CH_3 CH_3 C_3H_7	0·15	0·45	65	74
CH_3 CH_3 *iso*C_3H_7	0·43	0·50	101	67
CH_3 CH_3 C_4H_9	0·08	—	53	67
CH_3 CH_3 $C_6H_5.CH_2$-	0·06	—	39	67

* Results from Holton and Ing (1949).
† Source of enzyme dog caudate nucleus. The remaining measurements were made with human red blood cell enzyme.

The failure of any base that is itself acetylated, with the exception of the monoethyl substituted one, to compete effectively with choline, shows that with this exception choline appears to have a smaller dissociation constant for the enzyme-choline complex than the others tested. The Michaelis constant for choline acetylase is not known, but it is very difficult to *abolish* acetylcholine synthesis by quite prolonged dialysis aimed at removing the intrinsic choline. This suggests that the K_m may be quite small. It is therefore likely that the concentration of choline employed in our experiments is very much higher than the level required to saturate the enzyme.

It is of considerable interest to know whether any of the substrates that are acetylated *in vitro* are likely to be acetylated *in vivo* in nerve endings and might thus interfere with normal acetylcholine formation.

The level of choline in the blood varies little and is some 1–2.5 mg./l. (10–25 μM./l.) (Bligh, 1952), but it is by no means certain that the choline concentration in the tissues is similar to that in the blood, or that acetylcholine in tissues is formed from free choline. For instance, Brown and Feldberg (1937) found that the choline content of sympathetic ganglia was 243–520 mg./kg.—some 200 times the blood level—but of this only about 10–20% appeared in the perfusion fluid on prolonged stimulation. Nevertheless very small amounts of choline added to the perfusion fluid increased the

output of acetylcholine in half the preparations tested and also increased the ganglion acetylcholine content. Hutter (1952) has also pointed out that quite small amounts of choline can modify neuromuscular transmission in the cat, and suggests that part of this effect may be connected with acetylcholine synthesis.

These findings suggest that it is possible that other alkanolamines may be able to enter the *in vivo* acetylcholine synthetic pathway. The success of such a substance may well depend on its biological half life and its lack of toxicity. Bligh (1953) has shown that choline disappears from the circulation very rapidly as a result of destruction by choline oxidase. It is desirable therefore that the inhibitor should not be attacked by choline oxidase. Wells (1954) examined the action of choline oxidase on a number of the substrates we are interested in and found that it would not attack non-quaternary alkanolamines or triethyl choline, but readily attacked α and β substituted cholines. Triethylcholine and some of the longer chain aliphatic derivatives might therefore be promising. From the point of view of acute toxicity these are also favourable because of their very low parasympathomimetic activity. Triethylcholine is known to have a low toxicity and will in part replace choline in nutrition (see Sebrell and Harris, 1954).

Summary

1. The activity of purified rat brain choline acetylase was examined on 25 substrates related to choline.

2. The rate of acetylation was little affected by change in the alkyl group substitution, but was abolished or greatly reduced by modification of the hydroxyethyl group or decrease in the basicity of the molecule.

3. Four substances have been demonstrated to inhibit acetylcholine synthesis.

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