3. Sodium fluoroacetate (1 mM) increases the amount of free ammonia in the brain-cortex slices after incubation in the presence of glucose for 1 hr. at 37°.

4. The yields of ^{14}C -labelled glutamine and ^{14}C -labelled aspartate derived from [$^{14}\text{C}_5$]glutamate by rat-brain-cortex slices, incubated in Krebs-Ringer phosphate medium containing glucose, are diminished in the presence of sodium fluoroacetate (1 mM).

5. The addition of Amytal (0.5 mM) diminishes the enhanced rate of formation of ^{14}C -labelled glutamate from [$^{14}\text{C}_5$]glucose due to 1 mM-sodium fluoroacetate.

6. The results are consistent with the conclusion that sodium fluoroacetate, at concentrations of 1 mM or less, inhibits the utilization of NH_4^+ ions by rat-brain-cortex slices respiring in Krebs-Ringer phosphate medium containing glucose.

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The Chromatographic Behaviour of the Acetylcholine Activity of Brain Extracts

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There is abundant evidence, accumulated over the years, to indicate that the chemical entity acetylcholine acts as a transmitter of peripheral synaptic action and probably serves a similar function within the central nervous system. In this light we have thought it important to re-examine some recent reports which imply that, of the

material which can be extracted from the brain and which reacts as acetylcholine in biological assays, only a small proportion can be accounted for in terms of acetylcholine itself.

Hosein, Proulx & Ara (1962) have claimed that the largest part of the activity of brain extracts is due to the presence of certain betaine esters of

coenzyme A, and possibly also to acetylcholine-CoA (Hosein & Proulx, 1962). These claims are based upon the findings that paper-chromatographic analysis of trichloroacetic acid extracts of brain resulted in the separation of the biological activity into two distinct fractions, of which one apparently contained acetylcholine and smaller quantities of other acylcholine esters and was quantitatively less important. From the second fraction the tetrachloroaurates of certain betaines could be isolated, and there was evidence also for the presence there of CoA. Hosein & McLennan (1959) and Hosein, Ottolenghi & Dorfman (1961) had shown that esters of γ -butyrobetaine possess certain acetylcholine-like properties.

The results obtained in the present experiments do not support these claims. Instead it is suggested that the second chromatographic band of faster mobility owes its activity to the presence of acetylcholine trichloroacetate, the behaviour of which is analogous to that of the histamine complex described by West & Riley (1954).

METHODS

Whole cerebral hemispheres from adult albino rats were used. The animals were killed by decapitation and the brains rapidly removed and weighed. Hosein *et al.* (1962) described an elaborate extraction procedure in which the initial disruption of the tissue was in cold water containing eserine. Thereafter chloroform and trichloroacetic acid were added, and after precipitation with Reinecke salt the acetylcholine activity was brought to a methanol solution. In one experiment we have followed this procedure, which in our hands resulted in a considerable loss of the original acetylcholine activity of the brain. Qualitatively the chromatographic behaviour of the residual material was similar to that of simpler trichloroacetic acid extracts.

The brains were dropped, within 1 min. of their excision, either into sufficient cold 5% (w/v) trichloroacetic acid or into cold water containing eserine sulphate (10 mg./100 ml.) to form a 10% (w/v) tissue suspension, and homogenized. Trichloroacetic acid extracts were centrifuged after standing for 20–30 min.; water extracts were brought to pH 4 with N-HCl and placed for 3 min. in a water bath at 100° before centrifuging. Supernatant fluids containing trichloroacetic acid were exhaustively extracted with ether until the pH had risen above 6. The solutions were evaporated to dryness *in vacuo* at 40° and the residues taken up in small quantities of water.

Descending chromatograms were run on Whatman no. 4 filter paper with water-saturated butan-1-ol as solvent system. When dry the papers were cut into strips and eluted with water if the material was to be further treated, or with the appropriate saline medium if the samples were to be tested for acetylcholine. In all cases authentic samples of acetylcholine chloride and often of choline chloride were run at the same time.

Some concentrated extracts in volumes of 1.5–2 ml. were passed through columns (7 cm. $\times$ 1 cm.) of Dowex 1 (X8; Cl⁻ form). The columns were washed through with water until 30 ml. had passed. These solutions were evaporated and prepared either for further chromatography or for assay as above.

Acetylcholine assays were always performed upon the eserine-treated dorsal muscle of the leech, and in this paper the results are expressed in terms of acetylcholine chloride. In one experiment (Table 2, no. 2), duplicate samples were assayed upon a cat's blood pressure and excellent agreement was obtained in this instance between the two methods.

RESULTS

The location of the acetylcholine activity of three different brain extracts separated on paper chromatograms is given in Table 1. The results are completely in accord with those reported by Hosein (1960) and by Hosein *et al.* (1962), in that the bulk

Table 1. *Distribution of acetylcholine activity of trichloroacetic acid extracts of brain on paper chromatograms developed with butanol-water*

R_F values given were those of the centres of the eluted strips, which extended 0.05 R_F unit on each side of the centre. The R_F of pure acetylcholine in this system is approx. 0.12. Activity is expressed as μ mg. of acetylcholine chloride.

Extract A		Extract B		Extract C	
Total acetylcholine activity applied (μ mg.)					
950		7200		11 600	
R_F	Activity	R_F	Activity	R_F	Activity
0.05	0	0.05	0	0.10	3300
0.10	250	0.10	1000	0.25	0
0.15	0	0.15	0	0.40	0
0.20	0	0.20	0	0.60	8600
0.25	0	0.35	0	0.75	0
0.30	0	0.45	0	0.95	0
0.35	0	0.60	6850	—	—
0.50	700	0.75	0	—	—
0.65	0	0.90	0	—	—
0.90	0	—	—	—	—

Table 2. *Effect of re-chromatography and of mild acid treatment on the breakdown of acetylcholine activity (R_F 0.6)*Quantities are expressed as $\mu\text{mg.}$ of acetylcholine chloride.

Original extract	Expt. 1		Expt. 2	
	16 000		18 200	
Chromatography { $R_F \sim 0.1$ (A) $R_F \sim 0.6$ (B)	2 050		1 700	
	13 800		11 150	
	$R_F \sim 0.1$	$R_F \sim 0.6$	$R_F \sim 0.1$	$R_F \sim 0.6$
Re-chromatography of half-portions of B immediately	—	—	1 100	4 500
After 1 hr. at 22°, pH 7	4 300	2 500	—	—
After 3 hr. at 22°, pH 7	—	—	400	4 600
After 1 hr. at 22°, pH 3	2 400	2 050	—	—

Table 3. *Chromatography of acetylcholine activity (R_F 0.6) after treatment with anion-exchange resin*Quantities are expressed as $\mu\text{mg.}$ of acetylcholine chloride.

Original extract	Expt. 1		Expt. 2	
	6650		8450	
Chromatography { $R_F \sim 0.1$ (A) $R_F \sim 0.6$ (B)	1350		550	
	5100		7150	
	$R_F \sim 0.1$	$R_F \sim 0.6$	$R_F \sim 0.1$	$R_F \sim 0.6$
Re-chromatography after passage through Dowex 1				
A	1100	~ 100	200	~ 100
B	4250	400	3300	1200

Table 4. *Chromatography of water extracts of brain*Quantities are expressed as $\mu\text{mg.}$ of acetylcholine chloride.

	Expt. 1	Expt. 2	Expt. 3
Original extract	10 150	10 600	10 550
Chromatographed			
$R_F \sim 0.1$	6 150	9 350	9 500
$R_F \sim 0.6$	~ 0	~ 50	~ 100

of the material acting as acetylcholine migrated to a position on the chromatogram far removed from that occupied by acetylcholine itself. In the latter position a much smaller amount of activity was located. The R_F which we have found for the faster-running material is slightly higher than that reported by Hosein *et al.* (1962) (R_F 0.6 against R_F 0.45–0.56).

To examine further the nature of the faster-running material, a portion of the R_F 0.6 fraction from chromatogram of extract C (Table 1) was treated at pH 3 for 1 hr. at room temperature. The resulting material, from which some activity had been lost, after neutralization was applied to a new paper and run in the same solvent system. After elution it was found that, of the activity applied, 29 % was recoverable from a band at R_F approx. 0.1 and the remainder as before from R_F 0.5–0.65. It appeared therefore that this mild treatment might have converted some of the material from a 'complex' into a 'free' form of acetylcholine. Stronger acid treatment (5 min. at 100° and at

pH 2) destroyed almost all of the acetylcholine activity.

The details of two further similar experiments are given in Table 2. It is apparent that not only acid treatment but simple re-chromatography of the faster component resulted in the conversion of some into a material which took up the same position as acetylcholine itself on the developed chromatogram. The possibility exists therefore that the active material with R_F 0.6 is a complex form of acetylcholine, probably combined with trichloroacetic acid. Two further sets of experiments suggest that this is the case.

In two experiments (Table 3) trichloroacetic acid extracts of brain were chromatographed as before, and the slower- and faster-moving fractions passed through an anion-exchange resin in the Cl^- form to replace all anions in the effluent by Cl^- ions (Whittaker, 1956). The effluents were then re-chromatographed. It is clear that the slower fraction, presumably acetylcholine itself, was unaffected by this procedure, whereas a considerable proportion of the faster material now migrated only to the low R_F position taken up by pure acetylcholine.

If the postulated acetylcholine complex is one with the trichloroacetate ion, it follows that extract prepared without trichloroacetic acid should not show the same separation of activity into two chromatographically distinct bands. In Table 4 are the results of three experiments in which extracts prepared with aqueous eserine were

analysed. In none was more than a trace of faster-moving material found, and most of the original activity could be recovered from the acetylcholine band.

DISCUSSION

It seems reasonable to interpret the above results in very different terms from those adopted by Hosein *et al.* (1962). The facts that (a) acetylcholine activity moving to a position of R_F 0.6 on paper chromatograms developed with butanol-water can be detected in trichloroacetic acid but not in aqueous-eserine extracts of brain, and (b) most of the faster-moving material can be changed apparently to free acetylcholine by re-chromatography or by removal of all anions but Cl^- ions from the solution, argue in favour of the concept that most of this material is a combined form of acetylcholine and trichloroacetate. Experiments carried out by other workers (Banister, Whittaker & Wijesundera, 1953; Augustinsson & Grahn, 1954; Beznak, 1956; Whittaker, 1956; Henschler, 1957; Levy & Pierron, 1959) in which a faster-moving acetylcholine component has been detected have also all been made upon trichloroacetic acid extracts of various tissues. West & Riley (1954) have described what may be a similar complex of histamine with trichloroacetic acid, which also moves to a more advanced chromatographic position than does histamine itself and which could be broken down by re-chromatography in the same solvent system. Levy & Pierron (1959), using pure solutions of acetylcholine and trichloroacetic acid, have directly demonstrated the formation of a complex which has a greater chromatographic mobility than acetylcholine itself. It must also be said that acetylcholine appears able to form fast-moving complexes with materials other than trichloroacetic acid (Whittaker, 1956).

The possibility that the faster-moving active material is made up of betaine esters of CoA, as Hosein *et al.* (1962) suggest, seems to be supported by their finding that betaines can be isolated from a hydrolysate of the faster-moving band. This would, however, require that, in the re-chromatography experiments of Table 2, where active material appears at R_F 0.1, the unesterified betaines be demonstrated to possess an acetylcholine-like action. Free γ -butyrobetaine and carnitine do show R_F approx. 0.1 in butanol-water; they are, however, devoid of acetylcholine effects (Hosein *et al.* 1961). Further, the absence of the higher R_F band of activity in aqueous-eserine extracts cannot be readily explained if the betaine esters contribute significantly to the total acetylcholine activity of the brain. The suggestion by Hosein & Proulx (1962) that acetylcarnitine may be of importance cannot be decided as yet. E. A. Hosein (personal

communication) has prepared this material, and has informed us that in the free state its position on chromatograms developed with several solvent systems including butanol-water is indistinguishable from that of acetylcholine. However, sufficient material has not yet become available to investigate its pharmacological properties.

SUMMARY

1. The acetylcholine activity of a trichloroacetic acid extract of rat brain can be fractionated on a paper chromatogram developed with butanol-water into two bands. One, R_F approx. 0.1, corresponds to acetylcholine itself, and the second, R_F approx. 0.6, has been claimed by Hosein *et al.* (1962) to owe its activity to the presence of betaine esters of CoA.

2. Re-chromatography of the R_F 0.6 band in the same solvent system results in the appearance of an active material behaving chromatographically as acetylcholine.

3. Passage of the material from the R_F 0.6 band through an ion-exchange resin, such that all anions are replaced by Cl^- ions, causes the appearance of activity behaving chromatographically as acetylcholine.

4. Water extracts of brain show no evidence for the presence of a faster-moving fraction of the acetylcholine activity.

5. It is concluded that most of the faster-moving material is a complex form of acetylcholine itself, presumably with the trichloroacetate ion.

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