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MECHANISMS OF ACETYLCHOLINE SYNTHESIS

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The demonstration by Feldberg & Mann (1944) that the enzyme system which acetylates choline is present in aqueous extracts of acetone-dried mammalian brain tissue has been the basis of several important discoveries about the properties of the system. The dependence of the enzyme concerned on a co-factor was first clearly demonstrated by the same authors (Feldberg & Mann, 1945, 1946), who showed that the loss of activity which occurs when extracts are dialysed is associated with the disappearance of a heat-stable, dialysable substance which they called the activator but which, following Lipmann & Kaplan's (1946) experiments, can now be identified as coenzyme A. (For discussion of evidence see Feldberg, 1950.)

Investigations of the effects of organic acids which might act as acetyl-donors for the system led to the discovery that citrate accelerates synthesis very strongly, while acetate itself has little or no effect (Feldberg & Mann, 1945, 1946; Nachmansohn & John, 1945; Lipton & Barron, 1946). It is the more remarkable that acetate should be ineffective when incubated with extracts of brain powder since, when the enzyme is purified by fractional precipitation of the extracts, it can synthesize large amounts of acetylcholine in the presence of acetate (Nachmansohn, Hestrin & Voripaieff, 1949). It should be noted, however, that even in this system the substrate role of acetate has not been clearly shown. The mode of action of citrate with the cruder extracts is even less certain, since the rate of synthesis without addition of citrate or other organic acid indicates that the extracts themselves are the source of acetyl groups.

In the experiments now to be described we have found conditions under which the utilization of acetate by simple extracts of acetone-dried mammalian brain tissue occurs, and we have been able to demonstrate that both citrate and acetate act as acetyl-donors. Analysis of the conditions necessary to bring these substances into reaction has led to the conclusion that the enzymes concerned with each are not identical with one another. Further, it has

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enabled us to define more precisely than was possible earlier the part played by coenzyme A in the acetylation of choline. The success of these experiments, some of which have already been published in two preliminary reports (Balfour & Hebb, 1950*a*, *b*), has depended largely on the use of a preparation of coenzyme A which has a high standard of purity. It has been prepared according to a method originally devised by Comline (1948).

METHODS

Preparation of enzymes

Acetone powders, prepared as described by Feldberg & Mann (1945) from the brains of rabbits or, less frequently, guinea-pigs, and stored at low temperature (0–5° C) in a desiccator under vacuum, were extracted and tested under three different conditions of experiment.

Dialysed extracts of acetone powders. The powders were extracted with ice-cold 0·9 % (w/v) NaCl which in some tests contained neutralized cysteine, 0·05–0·2 mM/ml. The weight of powder per ml. of saline was usually 50 mg; but in some experiments it was 125 or 200 mg. The supernatant fluid obtained by centrifuging was dialysed at 0–2° C against 50 to 300 times its volume of saline to which was added in the case of cysteine-containing extracts 1 mM neutralized cysteine per 100 ml. The dialysing chamber was twice recharged with fresh solution as dialysis proceeded. For some tests dialysis was continued for 12–18 hr, but a shorter period of 3½–4 hr gave similar results. The extracts were usually incubated immediately after preparation, but by freezing they could be stored (temperature = – 25° C) without significant loss of activity for several weeks.

Resin-treated extracts. A saline or cysteine-saline extract (50 mg powder/ml. saline) was prepared and mixed with approximately half its volume of an anion-exchange resin. If necessary, a few mg of $NaHCO_3$ were added to the mixture to maintain a neutral reaction. After 30 min at 0° C the mixture was filtered through glass wool. It was then ready for testing.

The anion-exchange resin used in these experiments was De Acidite E (Permutit Water Co.). Before use, it was washed, charged with N-HCl, then re-washed with glass-distilled water until free from acid.

Dialysed fractionated enzyme extracts. Fractionated extracts were prepared by the method described by Nachmansohn *et al.* (1949). The method was modified slightly in that to the extraction fluid containing KCl, $MgCl_2$, NaCl and Na_2HPO_4 we added 4mM cysteine/100 ml. The precipitates formed at 16, 25, 30 or 36 % $(NH_4)_2SO_4$ were recovered either by centrifuging or by filtering through no. 15 Whatman paper.

Each precipitate was taken up in fluid of the same composition as that used for extraction, with the addition of 2 mM of neutralized cysteine, and dialysed as described by Nachmansohn *et al.* (1949) for 3 hr. It was then ready to be tested.

Method of incubation

The enzyme preparations were incubated in the presence of ATP, eserine sulphate, KCl, cysteine, choline and phosphate (pH 7) for 1 or 3 hr at 37° C. Other additions (coenzyme A, brain activator, citrate, acetate, $MgCl_2$ and $CaCl_2$) are indicated in the text.

As a control, choline was occasionally omitted in order to determine whether the biologically active product of synthesis estimated as acetylcholine by assay on the frog rectus abdominis was dependent upon the presence of choline. This precaution was taken in view of the earlier report by Nachmansohn *et al.* (1949) that in the absence of choline, fractionated extracts could synthesize large quantities of a substance biologically but not chemically similar to acetylcholine. As stated earlier we were not able to confirm this (Balfour & Hebb, 1951*b*), although it is true that if choline is not added crude extracts of acetone powder can synthesize between 2 and 10 % of the amount of acetylcholine (or similar substance) normally formed in the presence of added choline. Such extracts, however, may themselves be a source of choline.

Estimation of acetylcholine

The amount of acetylcholine produced during incubation was estimated by bio-assay on the frog rectus abdominis following the precautions described by Feldberg & Hebb (1947) to control the sensitizing actions of other substances which may be present in the extracts. The results were expressed in relation to the amount of acetone powder from which the extracts were originally made as μg acetylcholine/g brain powder.

Preparation of special reagents

Coenzyme A was extracted from yeast according to a method devised by Balfour & Comline (to be published) and its activity in units/mg determined by the method of Kaplan & Lipmann (1948). Boiled brain extract (brain activator) was prepared as described by Feldberg & Mann (1945, 1946).

ATP was prepared from fresh rabbit muscle as described by LePage (1949). Barium nitrate was substituted for barium acetate in order to avoid contamination of the barium ATP with acetate.

RESULTS

Experiments on dialysed extracts of acetone-dried brain

Acceleration of synthesis by acetate. The action of acetate on acetylcholine synthesis was compared with that of citrate first under conditions of experiment similar to those described by Feldberg & Mann (1945, 1946) and then after modifying the conditions in two different ways. In the unmodified system the usual saline extract provided the enzyme (12·5 mg acetone powder/ml. incubate), reactivation was effected by addition of brain activator,

TABLE 1. Formation of acetylcholine by extracts of rabbit brain

Preparation of enzyme extract	Source of coenzyme	ACh, μg/g brain powder/hr: Control +Ca and Mg	+Acetate +Ca and Mg	+Citrate +Mg
Saline	Brain activator	104	168	600
Cysteine	Brain activator	120	288	560
Saline	Yeast coenzyme A	120	352	704
Cysteine	Yeast coenzyme A	80	640	560

Saline extracts, 12 mg/ml. incubate. Cysteine-saline extracts, 6 mg/ml. incubate. Boiled brain activator, 20 mg/ml. incubate. Purified yeast coenzyme A, 10 units/ml. incubate.

and the ingredients of the incubation mixture included $MgCl_2$ (0·004 M) in addition to those already listed. The two modifications were (1) extraction of enzyme with cysteine-saline solution, and (2) replacement of activator with purified extract of yeast coenzyme A.

The effect of these substituted procedures is illustrated by the experiment shown in Table 1. Without them, as shown by the top row of values, acetate had little, possibly no, significant effect; while citrate exerted the relatively strong accelerating action usually observed. Acetate, too, was able to accelerate acetylcholine production either when the enzyme was cysteine-extracted (2nd row of Table 1) or when purified coenzyme A was the reactivating agent (3rd row); and when both conditions were fulfilled (4th row) its effect was nearly equal to the largest effect of citrate.

In this experiment the yield of acetylcholine per g acetone powder was not so large as in most others in which values of the order of 1000 μg/g/hr were obtained. Differences were due partly to differences in the activity of the extracts and partly to the amount of coenzyme A added. As shown in Fig. 1, increasing the concentration of coenzyme increased the output of acetylcholine. In this as in a later experiment (Fig. 2), the output might have reached still higher values than those shown if higher concentrations of coenzyme had been tested; but the amount available for all purposes was limited and more extensive tests of this kind were impracticable.

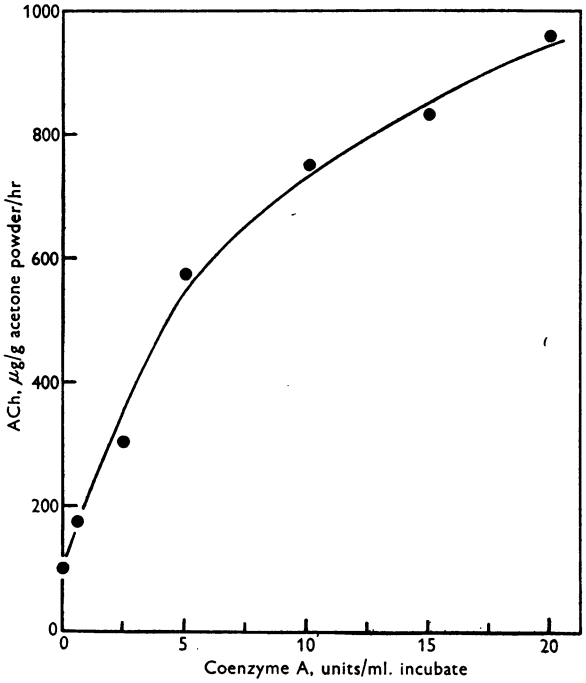

Fig. 1. Cysteine-saline extract of acetone-dried rabbit brain dialysed for 18 hr. Curve to show reactivation by yeast coenzyme A in presence of acetate.

Inorganic requirements of the system. In further experiments it was found that the production of acetylcholine, in the presence of acetate with the modifications of method described, was only slightly affected by Ca or Mg salts (chlorides). Added separately or together, both increased synthesis to a slight extent. The inorganic requirements of the system therefore differed from those necessary for citrate utilization. With both substances, K ions (here added as KCl) were essential; but with citrate, as shown earlier by Feldberg & Hebb (1945, 1947), the acceleration of acetylcholine production was dependent upon Mg ions (for which Mn could be substituted) while Ca ions had a depressant action.

Synthesis of acetylcholine with higher concentrations of enzyme. In a limited number of experiments concentrated dialysed extracts of enzyme (125 mg acetone powder/ml. saline or cysteine-saline solution) were incubated for 3 hr with additions of either citrate with $MgCl_2$, or acetate with $MgCl_2$ and $CaCl_2$. The amount of extract added was equivalent to 37·5 mg acetone powder/ml. incubate. The results are shown in Table 2. From this it will be seen that citrate no longer accelerated synthesis but inhibited it significantly. With acetate, the hourly output of acetylcholine per g acetone powder was of the same order as was found in the experiments with more dilute extracts. Cysteine-extraction again was of importance in this result. It will be noted, however, that the control values (without addition of acetate or citrate) were considerably higher than earlier even when the longer period of incubation was taken into consideration. This result could be explained on the assumption that the extracts themselves contained a source of acetyl groups, and that with higher concentrations of enzyme more of these became available.

TABLE 2. Formation of acetylcholine using high concentrations of dialysed enzyme extracts

	ACh, μg/g brain powder/3 hr		
Enzyme* preparation	Control	+Acetate	+Citrate
Cysteine extracts	850	1600	530
Cysteine extracts	1180	2260	1000
Saline extracts	940	1180	420

* Concentration equivalent to 37·5 mg acetone powder of rabbit brain/ml. incubate. Yeast coenzyme A (12 units/ml.) used in all tests.

Rate of synthesis with different concentrations of acetate. On incubating dialysed extracts made from acetone powder which had been washed with ether it was found that the control rate of synthesis was considerably lower than in other experiments in which an equivalent concentration of enzyme (12·5 mg original acetone powder/ml. incubate) had been used. In one of the experiments in which the rate of acetylcholine production by a cysteine-saline extract of guinea-pig brain powder was 12·5 μg/g the effects of adding increasing concentrations of acetate were determined. The results given in Table 3 showed that increasing the acetate concentration from 0 to 0·5 and 12·5 μM/ml. was accompanied by a steady although not a proportional increase in acetylcholine production. The results given in Table 3 showed that at a concentration of 0·05 μM/ml. one-third of the acetate was utilized; but at 0·5 μM/ml. only about one-tenth was used; while at 12·5 μM/ml. the fraction utilized was less than 1/200.

Resin-treated extracts

Reactivation of extracts. The fact that extracts dialysed for as long as 18 hr showed some residual activity (see Fig. 1) without re-addition of coenzyme A indicated that dialysis was not an efficient method for its

removal. Accordingly, some other means of separating the coenzyme from the protein fraction was sought.

Methods used for the extraction and purification of coenzyme A from liver (Lipmann, Kaplan, Novelli, Tuttle & Guirard, 1950) and yeast (Balfour & Comline, unpublished data) had provided evidence of its acidic properties. It seemed possible, therefore, that treatment of the extracts with a suitable anion-exchange resin would remove the coenzyme. By experiment it was found that De Acidite E did accomplish this satisfactorily. All of the coenzyme, as determined by the sulphanilamide assay method of Kaplan & Lipmann (1948), was removed and the treated extracts were unable to synthesize any acetylcholine except when reactivated by addition of coenzyme. We were thus able to demonstrate for the first time the complete dependence of the system on the coenzyme (Balfour & Hebb, 1951*a*).

TABLE 3. Acetylcholine formation at different concentrations of acetate

Amount of acetate added (μM/ml. incubate)	Amount of acetylcholine formed per hr	
	(μM/ml. incubate)	(μg/g acetone powder)
0	<0·001	12·5
0·05	0·0181	261
0·1	0·024	352
0·2	0·0288	414
0·35	0·043	624
0·5	0·047	680
12·5	0·056	800

Enzyme, cysteine-saline extract of ether-washed, acetone-dried guinea-pig brain; coenzyme, 15 units/ml. incubate (yeast extract).

Fig. 2 shows reactivation curves of the enzymes for the acetylation of choline with citrate and acetate. With citrate, reactivation was nearly maximal at relatively low concentrations of coenzyme A; but the requirements of the system utilizing acetate were much higher and reactivation was probably incomplete even at 15 units/ml. From reactivation curves of this kind it has been estimated that dialysed extracts probably retained sufficient coenzyme to provide a concentration of about 0·5 unit/ml. in the incubate. This amount would account for the synthesis of as much as 700 μg acetylcholine/g with citrate, according to the curve shown in Fig. 2. Accordingly, the retention of coenzyme by dialysed extracts provides an explanation of the relatively large values obtained by Feldberg & Mann (1945) on incubating dialysed extracts with citrate (see Table 4).

As shown in Fig. 3, brain activator was considerably less effective than yeast coenzyme in reactivating the acetate-utilizing system. The two curves have been plotted so that at each point along the abscissa the concentration of coenzyme added in the form of purified yeast extracts or in the form of brain activator should be of the same order. The lower rate of synthesis with

boiled brain extract therefore was probably due to some inhibitory component.

Acetate and citrate as acetyl-donors for the system. Another difference between resin-treated and dialysed extracts was that the resin-treated extracts were unable to synthesize any acetylcholine unless acetate or citrate had been added to the incubate. This is illustrated by the data of Table 4 in which

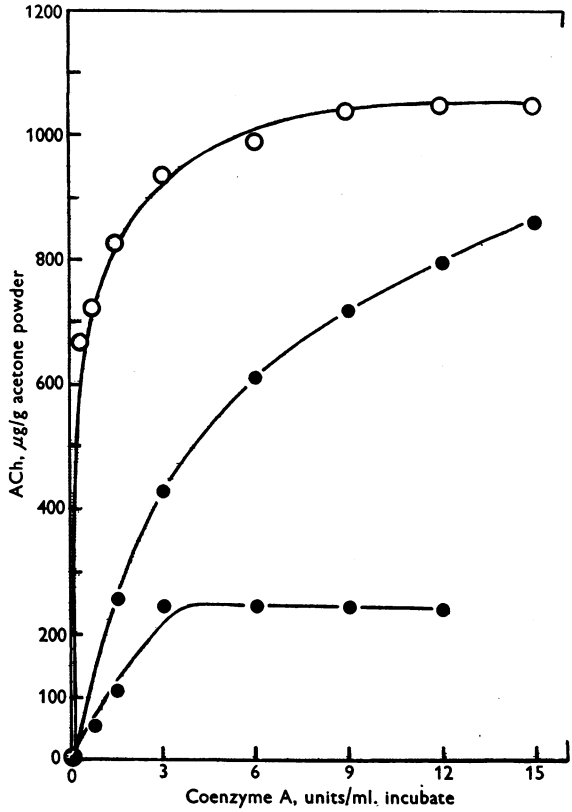

Fig. 2. Reactivation of resin-treated extracts of guinea-pig brain powder in presence of citrate (○——○) and acetate (●——●) by coenzyme A. In the case of citrate and lower acetate curve the extracts were made with saline. In the case of upper acetate curve the extract contained 6 mg. cysteine/ml.

results obtained by Feldberg & Mann with dialysed extracts of guinea-pig brain powder are compared with our results on resin-treated extracts prepared from the same species. The fact that citrate (with Mg ions) could be substituted for acetate established with certainty that it too could act as an acetyl-donor for the system. The suggestion that citrate played this part in acetylcholine synthesis was made by Lipton & Barron (1946), but it remained doubtful until other sources of acetyl-precursors had been excluded. One possibility

was that the addition of citrate stabilized a system containing other sources of acetate. This suggestion had also been made by Kaplan & Lipmann (1948)

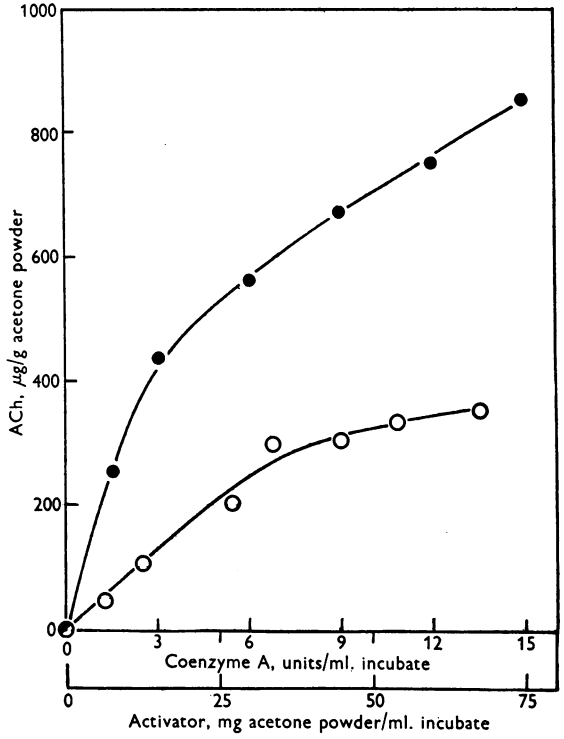

Fig. 3. Reactivation of a resin-treated cysteine-saline extract of guinea-pig brain powder by yeast coenzyme A (●——●) and boiled brain extract (○——○) or 'activator'.

TABLE 4. Comparison of data from Feldberg & Mann (1946) on dialysed enzyme extracts with data now obtained on resin-treated extracts

Preparation of enzyme*		
Dialysed	Resin-treated	Additions†
20	0	ATP
—	0	ATP, acetate
470	0	ATP, CoA
—	250–850	ATP, acetate, CoA
40	0	Citrate
400–900	0	Citrate, ATP
310	0	Citrate, CoA
1100	1040	Citrate, CoA, ATP

* From guinea-pig brain tissue.

† Other additions were those listed in 'Methods', and $MgCl_2$.

ACh formation, μg/g acetone powder/hr.

for the role of citrate in the acetylation of sulphanilamide by liver extracts. Nachmansohn & Weiss (1948), in discussing experiments on acetylcholine synthesis by extracts of squid head ganglia (acetone powders) which acetate

accelerated and citrate inhibited, expressed disagreement with Lipton & Barron and drew attention to Ochoa's suggestion that contaminated ATP might be a source of acetyl groups under some conditions. Although we have found evidence that contaminated ATP could increase acetylcholine synthesis its possible effects were eliminated in our experiments by using acetate-free ATP. The synthesis of some acetylcholine by dialysed extracts without additions of either acetate or citrate must therefore have been due to acetyl-precursors present in the extracts themselves.

Evidence that our ATP was effectively free of acetate will be found in Table 4 (compare figures in 3rd and 4th rows) which also shows that acetylcholine synthesis is dependent not only upon the coenzyme and an acetyl-precursor but upon ATP as well.

Cysteine requirements of citrate- and acetate-utilizing systems. Although, with the omission of cysteine, significant quantities of acetylcholine were found on aerobic incubation of the extracts with citrate, the rate of synthesis was much higher with its addition (1·5 mg/ml. incubate). Extraction of the powders with

TABLE 5. Effect of cysteine on acetylcholine formation in presence of acetate by resin-treated extracts of rabbit brain powder

Concn. of cysteine in enzyme-extraction fluid (mg/ml.)	Cysteine added separately on incubation (mg/ml. incubate)	Final concn. of cysteine in incubate (mg/ml.)	ACh formed (μg/g)
None	None	None	9
None	3·0	3·0	640
None	3·75	3·75	540
3	None	0·75	1080
3	1·5	2·25	1190
3	3·0	3·75	1200
9	None	2·25	1200
9	1·5	3·75	1080
9	3·0	5·25	1160

cysteine-saline solution, however, did not make citrate more effective. On the other hand, the amount of choline acetylated by acetate was dependent both on the extraction of the powders with cysteine and on the total amount present in the incubate. When cysteine was omitted both from the extraction fluid and from the incubation mixture practically no acetylcholine was produced. This is illustrated by the experiment of Table 5 which also shows that while cysteine added during incubation could to some extent make up for its omission from the extraction fluid, the greatest activity of the enzyme was obtained only when cysteine had been used for extraction.

Inorganic requirements of the acetate system. The effects of K, Ca and Mg ions on synthesis of acetylcholine by resin-treated extracts in the presence of acetate are shown in Table 6. Of the three ions, K (0·05 M) was the most important in maintaining the activity of the enzymes. Added singly Ca (0·002 M) or Mg (0·005 M) increased synthesis more than when added together.

Experiments on fractionated enzyme preparations

We reported earlier that enzyme preparations obtained from extracts of acetone-dried brain tissue by fractional precipitation according to the method of Nachmansohn *et al.* (1949) could synthesize acetylcholine in the presence of citrate and Mg or acetate, Ca and Mg. Three successive precipitates formed at 16, 25 and 36% (by volume) of $(NH_4)_2SO_4$ were taken up in a solution containing Na_2HPO_4, NaCl, KCl, $MgCl_2$ and cysteine. The precipitate formed at 16 % was, as Nachmansohn *et al.* had shown for acetate, inactive. The 16–25 % fraction, when separated by centrifuging, utilized citrate much more readily than acetate; but the remaining fraction utilized acetate as well as citrate. When the 16–25 % fraction was separated by filtration, however, it produced rather more acetylcholine in the presence of acetate than in the presence of citrate, although the utilization of both substances was relatively large (125 and 90 μg ACh formed per ml. incubate with acetate and citrate respectively). When the precipitate brought down by 25 % $(NH_4)_2SO_4$ was separated in this way it contained almost all of the active material.

TABLE 6. Effects of K, Ca and Mg ions on synthesis of acetylcholine by resin-treated cysteine extracts of acetone-dried brain powder (rabbit brain)

Salts added	μg ACh/g acetone powder/hr
$KCl + CaCl_2 + MgCl_2$	900
$KCl + CaCl_2$	1080
$KCl + MgCl_2$	1080
KCl	1008
$CaCl_2 + MgCl_2$	370
$CaCl_2$	420
$MgCl_2$	544

In two other experiments in which the extraction was carried out at reduced temperatures (0° C) we succeeded in obtaining extracts which were relatively inactive on addition of citrate (17–27 μg ACh/ml. incubate) and very active on addition of acetate (115–165 μg ACh/ml.). In this case the 16–25 % fraction had been separated by centrifuging at low temperatures; and a further precipitate was obtained by increasing the concentration of $(NH_4)_2SO_4$ to 30 % and then filtering. The figures given above were obtained from this second fraction. Contrary to our previous experience, the first fraction was very active with addition of acetate as well as citrate. The reason for this is still uncertain. It may have been that the acetate enzyme appeared in this fraction because it was more easily precipitated at a lower temperature; but further experiments will be required before this can be determined.

The observations made on extracts prepared from fractional precipitates may be briefly summarized. As we reported earlier (Balfour & Hebb, 1951*b*) only negligible quantities of acetylcholine were formed on incubation of the extracts without choline. Similarly, in the absence of acetate or citrate, less

than 10% of the amount of ester normally formed was produced. Contrary to the finding of Nachmansohn *et al.* (1949) Ca was not, in our experience, necessary for acetylation by acetate. Moreover, results of one experiment indicated that Ca did not reduce synthesis in the presence of citrate. The effect of Mg ions was not determined, since $MgCl_2$ was a constituent of the enzyme extracts. More Mg was not required, however, for incubation with acetate.

DISCUSSION

The finding that enzyme preparations recovered by fractional precipitation can utilize both citrate and acetate in the acetylation of choline, and that the enzymes concerned can be partially separated has been the chief reason for our reinvestigation of the properties of unpurified extracts of acetone-dried brain tissue. Direct evidence that citrate accelerates synthesis by providing acetyl groups has now been obtained by the use of resin-treated enzyme preparations. With the finding that added acetate as well as citrate can increase ester production in unpurified extracts the analogy between dialysed, resin-treated and fractionated enzymes is complete.

The system utilizing acetate is distinguished from that using citrate by its high cysteine and coenzyme requirements at least in the crude state, since comparable observations have not been made on fractionally precipitated enzymes. Evidence obtained in other experiments by one of us (W.E.B.) suggests that one function of cysteine is to maintain the coenzyme in an active state but this does not provide an adequate explanation of the necessity of extracting the acetate-utilizing enzyme with cysteine. The part played by cysteine in the system therefore remains obscure.

The term 'choline acetylase', which was first suggested by Nachmansohn & Machado (1943) to describe the enzyme concerned in acetylcholine formation, has been avoided in this paper since the evidence which we have been discussing clearly indicates that more than one enzyme is necessary to account for acetylation by two different organic acids. Even in the simplest scheme for acetylation by one of these substances it seems that at least two successive reactions are involved, the first leading to the acetylation of the coenzyme and the second to the transfer of the acetyl groups to choline. The question of terminology has recently been reopened by Korey, Braganza & Nachmansohn (1951), who have suggested that the term 'choline acetylase' should only be applied to the enzyme concerned in the final step of acetylation. This might then be regarded as a 'final common pathway' for all substances acting as acetyl-donors.

The evidence that acetate, as well as citrate, can take part in the formation of acetylcholine is of importance in determining what parts of the nervous system normally produce the ester. If the citrate method alone were used for this purpose the results might be misleading since a positive result would

depend upon the presence of an enzyme catalysing the initial breakdown of citrate to acetate and oxaloacetate as well as one or more enzymes concerned in the terminal process of acetylation. A negative result, however, need not mean that the tissue could not produce acetylcholine from acetate or possibly some other precursor. An enzyme causing the breakdown of citrate to acetate and oxaloacetate has been found in nervous tissue (Balfour, unpublished experiments) but its distribution has not as yet been studied. Until it is known that it is not a limiting factor for acetylation by any particular extract it would be unwise to deduce that a tissue is incapable of synthesizing acetylcholine because it does not do so *in vitro* when incubated with citrate. On the other hand, if the distribution of the synthesizing systems is studied using the conditions necessary to bring acetate into reaction, the results will not necessarily be more significant. The coenzyme requirements of the system we have described are about ten times higher than those of citrate, and if this is not an artifact it would indicate that citrate rather than acetate is the normal source of acetyl groups. This is evidence of an indecisive kind, however; and it is unlikely that the problem of the acetyl-donor will be solved until more is known about the normal metabolic turnover in nervous tissue of both citrate and acetate.

SUMMARY

1. Conditions have been demonstrated under which acetylcholine synthesis by extracts of acetone-dried mammalian brain can be accelerated by acetate as well as by citrate.

2. Both substances are shown to act as acetyl-donors for the system.

3. Complete dependence of the enzymes concerned in synthesis of acetylcholine on coenzyme A has been demonstrated for the first time.

We wish to express our thanks to Dr Philip Eggleton for advice and assistance received from him in the course of numerous discussions during the progress of this work; and also to Dr R. S. Comline for instruction in preparing coenzyme A from yeast. Miss Margaret Forfar and Mr W. T. Hunt gave us valuable help in a number of the biochemical preparations. Grants to each of us from the Trustees of the Moray Fund of Edinburgh University provided some of the equipment used in this research; while the purchase of materials has been aided by a grant from the Royal Society. Their help is gratefully acknowledged.

Note added in proof. Recently one of us (C.O.H.) has found that it is not necessary to extract the enzyme in the presence of cysteine to obtain maximal synthesis in the presence of acetate; cysteine is equally effective if it is added to the incubating tubes with, or immediately after, the enzyme solution and before any other constituents.

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