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INTRACELLULAR DISTRIBUTION OF CHOLINE ACETYLASE

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The experiments to be described deal with the intracellular distribution of choline acetylase in rabbit brain studied by the method of differential centrifugation. This enzyme catalyses the transfer of acetyl groups from acetyl CoA to choline and it is only recently that it has become possible to measure its activity independently of the enzymic synthesis of acetyl CoA (Korkes, Campillo, Korey, Stern, Nachmansohn & Ochoa, 1952; Hebb, 1955; Smallman, 1956). Knowledge of the intracellular distribution of choline acetylase is important for the understanding of the chemical transmission of nerve effects.

METHODS

Rabbits were killed by a blow on the neck; the brains rostral to the superior colliculi were removed, weighed and made into a homogenate. The homogenates were prepared in 0·25 M sucrose solution, 1 ml. of homogenate corresponding to 100 mg of fresh tissue. A Potter Elvehjem all-glass homogenizer cooled in ice was used. The time taken to homogenize the tissue was 1–2 min.

Various fractions of the homogenate were prepared by centrifuging in a Servall (Model SS–1) angle centrifuge at a temperature of +2° C. After centrifugation at a given speed the supernatant was decanted, the residue resuspended in a given volume of sucrose solution and recentrifuged at the same speed. The supernatants were then pooled for the next centrifugation at a higher speed. The time of each centrifugation and the calculated centrifugal force applied are given with the results.

For comparison, acetone powders of rabbit brain were prepared by grinding the tissue in a mortar in a large volume (100 × tissue vol.) of cold (−10° C) dry acetone which was renewed several times. The acetone was filtered through a Buchner filter fitted with a No. 30 Whatman paper. The powder was dried *in vacuo* (300 mm Hg below atmospheric) over P_2O_5 for 3–4 hr. It was extracted in cysteine saline (3 mg cysteine/ml. 0·9% NaCl) solution, in a concentration of 50 mg acetone powder per ml. solution; the suspension was cooled to −15° C and kept frozen until required. Before incubating the extracts were centrifuged at 2800 rev/min for 3 min. In each case where the activity of a homogenate was compared with an extract of acetone-dried brain the two preparations were either from the brains of litter-mates, or from the separated halves of the same brain.

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Choline acetylase determination. The brain extracts were incubated with the following additions: 70 units crude yeast coenzyme A (Hebb, 1955), 0·8 ml. of a reaction mixture (containing in 32 ml. 23·8 ml. of K-ATP solution made from 540 mg BaATP, 2·2 ml. 24% KCl, 4 ml. 4% choline chloride, 2 ml. 0·5% eserine sulphate) 0·1 ml. 2·4% $MgCl_2.6\,H_2O$, 0·2 ml. 6·7% Na citrate, 0·1 ml. 0·5% Na_2HPO_4, 0·1 ml. 2·7% Na acetate. $3H_2O$, 0·1 ml. 6% l-cysteine solution, 0·11–0·12 ml. aged pigeon liver enzyme (Kaplan & Lipmann, 1948); water to give final volume of 2·5 ml.

Incubation was carried out in two stages. In the first stage the above constituents were incubated at 37° C without the brain extract for 10–15 min in order to allow acetyl CoA to form. Then the second stage of incubation was begun with the addition of brain extract; this was continued for 60 min, and the acetylcholine formed was expressed as μg ACh/hr and provided the estimate of the activity of choline acetylase in the brain extract. At the end of incubation, six drops of B.D.H. Universal Indicator and 1 ml. of N/3 HCl were added to each flask. The acidified ncubates were boiled and their acetylcholine content assayed on the frog rectus abdominis following the procedure described by Feldberg & Hebb (1947).

RESULTS

Comparison of homogenates and extracts of acetone-dried brain

Homogenates of brain were much less active than extracts of acetone-dried brain. When, however, the homogenates were treated before incubation with ether or an ether-chloroform mixture, procedures previously shown to increase acetylcholine synthesis in fresh tissue suspensions (Stedman & Stedman, 1937, 1939; Feldberg, 1945), their activity became equivalent to the activity of extracts of acetone powder. This is seen by comparison of columns 1, 2 and 3 in Table 1.

TABLE 1. ACh synthesis, expressed as μg/g fresh brain/hr; activity of extracts of acetone-dried brain compared with that of untreated and treated samples of homogenate

Extracts of acetone-dried brain	Treatment of whole homogenates			
	None	Ether-chloroform	Ether	Acetone
850	120–170	800–920	—	—
1000–1200	320–360	1120	—	—
730–850	220–280	680	720–920	760–880

Acetone treatment also activated the homogenized tissue. Its effect is shown by the following experiment. Samples of homogenate were precipitated in 100 vol. of cold (−10° C) acetone after which they were shaken vigorously and centrifuged at 4800 *g* for 5 min. The supernatant acetone was discarded and the sediments, after being dried for 3–5 hr *in vacuo* (300 mm Hg below atmospheric pressure) over P_2O_5, were taken up quantitatively in known amounts of water and then incubated in the normal way to determine their choline acetylase activity. As is shown by columns 4 and 5 of Table 1, this was found to be the same as the activity of ether-treated samples of homogenate.

A comparison of the activating effect of ether with that of chloroform-ether mixture or chloroform alone showed that ether by itself was a more effective activating agent than chloroform. Since treatment with ether was shorter and more convenient than treatment with acetone, ether was the agent routinely employed to activate preparations used in subsequent experiments.

In detail the procedure was as follows. When the extracts were very active they were first diluted 2–4 times with distilled water. 0·4 ml. ether was added to 1 ml. of extract or diluted extract, the mixture shaken vigorously and stored at 0–3° C for 10–15 min; then after shaking once more, the ether was blown off by bubbling air through it rapidly for 1 min.

TABLE 2. Effect of various treatments on the choline acetylase activity in fractions of two brain homogenates (A and B)

Centrifugal force, $g \times$ time (min)	Fraction	Homogenate	ACh synthesized, μg/g brain tissue: Untreated	Treated with ether	Treated with acetone
600 × 10	Sediment	A	0	0–18·5*	—
800 × 10	Sediment	B	62·5	50	—
1,500 × 10	Sediment	B	—	28–34	—
12,000 × 20	Sediment	B	177–267	400	177
12,000 × 20	Sediment	B	—	409	153
15,000 × 30	Sediment	A	58–116	367*–441	151
25,000 × 60	Sediment	A	5·2	42	—
25,000 × 60	Sediment	B	10	64	—
25,000 × 60	Sediment	B	84	106	—
25,000 × 60	Supernatant	A	145	134	—
25,000 × 60	Supernatant	B	183	214	—

* Treated with ether-chloroform mixture.

Differential centrifugation

Ether-treatment of separate fractions. Homogenates of brain were fractionated by differential centrifugation and the sediments, as well as the final supernatant, were tested for their choline acetylase activity. Control, untreated samples were compared with ether-treated samples of each fraction. The results which are given in Table 2 show that the treatment with ether activated the particulate fractions which sedimented at 12,000 *g* or higher, but had little or no effect on the activity of the final supernatant. This strongly suggests that the state of the enzyme in the final supernatant was different from that in the particulate fractions. It may be in fact that it was in solution but we cannot be certain of this since the maximal speed of centrifugation used was not sufficient to ensure that all particulate matter was eliminated from the final supernatant.

Whereas whole homogenates could be activated by treatment with acetone as well as with ether, the particulate fractions brought down at 12,000 and 15,000 g could not be activated by acetone. These fractions, both of which probably contained only mitochondria, were fully activated by ether treatment but were unaffected by treatment with acetone. This is shown by a comparison of columns 3 and 5, Table 2. Loss of enzyme did not account for this result since the acetone-precipitated fractions could be activated by ether in the same way that the untreated fractions had been. We have no explanation of the difference between the effect of acetone precipitation on the whole homogenate

and its effect on the mitochondrial fraction. As will be shown, the mitochondrial fraction accounted for a great part of the activity of the whole homogenate; and some activation of the enzyme of the isolated fraction was to be expected.

Distribution of enzyme. The histogram of Fig. 1 summarizes the findings of one experiment and shows the partition of enzyme activity between sediment and supernatant at each successive centrifugation. With the first and second centrifugations very little enzyme was present in the particulate fractions; with the third about 50% of all the activity of the homogenate was in the sediment; the final centrifugation brought down only a little more enzyme and left the larger proportion in the supernatant.

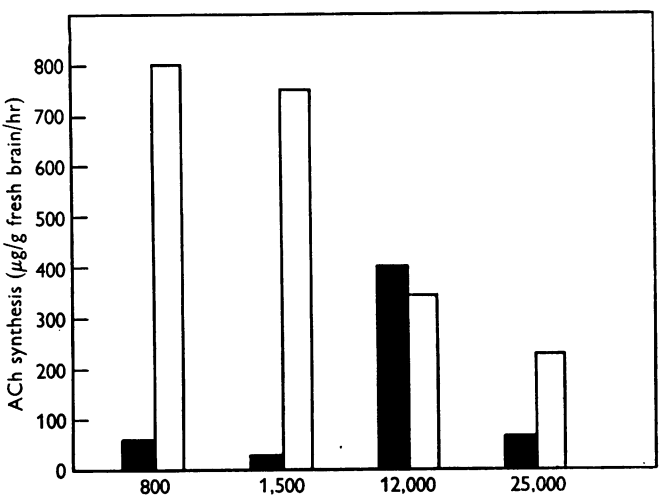

Fig. 1. Histogram showing the partition of choline acetylase activity between centrifugates ■ and supernatants □ at the centrifugal forces indicated along the abscissa. The ordinate represents ACh synthesized per g brain tissue per hr. Times of centrifugation at the successive speeds are the same as those given in Tables 2 and 3.

Table 3 summarizes the results of four experiments and gives the rates of acetylcholine synthesis by various fractions expressed as μg/g fresh brain/hr and as percentages of the total activity of all the fractions. The results show first that about 90% of the activity of the unfractionated homogenate is accounted for as the sum of the separate fractions, and secondly that the fractions which contain the mitochondria are the most active.

In the two experiments in which we followed the procedure of Brody & Bain (1952) the mitochondrial fraction sedimented at 12,000 *g*, and had an activity of more than 50% of the total activity recovered. An even higher proportion of activity was found in the mitochondrial fraction when this was isolated by centrifugation at 15,000 *g* applied immediately after centrifugation

at only 600 *g*. The higher activity of the mitochondrial fraction isolated in this way may well be due to a smaller loss of mitochondria to other fractions.

Histological examination of the fractions sedimented at 12,000 and 15,000 *g* revealed no morphological entity other than mitochondria. They were identified by their staining reactions to Janus Green and to aniline acid fuchsin with methyl green as counterstain; and were shown to be strongly sudanophilic (Sudan III). The particulate fractions brought down at 12,000 and at 15,000 *g* were similar to one another. The careful biochemical and histological examinations done by Brody & Bain (1952) had already established that with their procedure the material spun down at 12,000 *g* gives a pure mitochondrial preparation from rabbit brain homogenates.

TABLE 3. Distribution of choline acetylase in fractions of rabbit brain homogenates

	Acetylcholine synthesis by centrifugates and final supernatant							
Centrifugal force, *g* × time (min)	μg/g fresh brain	% of total	μg/g fresh brain	% of total	μg/g fresh brain	% of total	μg/g fresh brain	% of total
600 × 10	18	3	59	7	—	—	—	—
800 × 10	—	—	—	—	62	8	57	7
1,500 × 10	—	—	—	—	28	4	—	—
12,000 × 20	—	—	—	—	400	52	409	54
15,000 × 30	441	69	469	59	—	—	—	—
25,000 × 60	42	7	42	6	64	8	106	14
Final supernatant	134	21	225	28	214	28	189	25
Sum of fractions	635	100	795	100	768	100	761	100
Whole homogenates	705	—	880	—	880	—	880	—

In the mitochondrial fraction most of the enzyme was firmly attached to the particulate matter. Even treatment with ether did not free it, since, although it was then activated, it could still be brought down by centrifugation at the relatively low gravitational force of 1600 *g*. In addition, when a small amount of the mitochondrial fraction was washed twice with half its volume of 0·25 M sucrose, the combined washings showed an activity of only 3–10% of the activity in the unwashed fraction.

DISCUSSION

So far few studies have been concerned with the intracellular distribution of enzymes in nervous tissue, and none of these has dealt with choline acetylase. In general, the distribution pattern of enzymes in nervous tissue is the same as that in liver and kidney; the glycolytic enzymes appear to be soluble, and the enzymes responsible for oxidative phosphorylation are associated with particles having the histological and sedimentation characteristics of mitochondria (Abood, Gerard, Banks & Tschirgi, 1952; Brody & Bain, 1952). The present experiments show that a large part of the choline acetylase, an enzyme associated with the unique function of nervous tissue, that of transmission of

nerve impulses to effector organs, also sediments with the mitochondrial fraction. In fact, 52–69% of the total enzyme of the homogenates could be accounted for by that in the mitochondria.

It remains uncertain whether choline acetylase is associated with the mitochondrial fraction alone. The slight activity present in the fractions sedimented at 600, 800 and 1500 *g* probably resulted from the contamination of them with mitochondria; indeed, we have observed microscopically, as have Brody & Bain (1952), that some mitochondria are present in these fractions. The activity in the fraction sedimented at 25,000 *g* may be similarly explained as the result of contamination with mitochondria which occurred while decanting the supernatant after centrifugation at 12,000 or 15,000 *g*. However, about 25% of the total activity was always found in the final supernatant after centrifugation at 25,000 *g*. The present data do not enable us to decide whether this activity results from release of a portion of the mitochondrial enzyme to the suspension medium during fractionation, or whether the enzyme is in fact present in a soluble form or in cellular components, other than mitochondria, which were ultimately separated in our final supernatant.

The sedimentation characteristics of homogenates of brain clearly differ from those of liver. Liver mitochondria are already sedimented at 8500 *g* for 10 min (Schneider, 1948), whereas brain mitochondria are only sedimented at a gravitational force of 12,000 *g* or higher (Abood *et al.* 1952; Brody & Bain, 1952). In our experiments we have found that these higher gravitational forces are necessary to sediment brain mitochondria. Abood *et al.* (1952) have suggested that the high lipid content of nervous tissue is responsible for the greater difficulty in sedimenting its particulate components.

The finding that choline acetylase is associated with the mitochondria is of interest in connexion with the observation of Jordan (1955) that the mitochondria are concentrated at the synapses of the central nervous system, and raises many questions about the physiological significance of this location of the enzyme. On the assumption that it is active within the mitochondria and synthesizes acetylcholine, it would be expected that these cell particles are also the main repository of the acetylcholine found in the nervous system. On the other hand, the few experiments so far done to determine the location of cholinesterase in nervous tissue indicate that this enzyme is not in the mitochondria but the nuclei (Richter & Hullin, 1951), on the cell membrane (Nachmansohn, 1946) or on the larger cell fragments and microsomes (Smallman & Wolfe, 1956). However, these indications of a spatial separation of choline acetylase and cholinesterase within the nerve cell require confirmation.

In its natural condition in the mitochondria choline acetylase is firmly bound and relatively inactive. In order to reveal its full activity in this situation, it was necessary to treat the mitochondria with ether, a treatment which, however, did not liberate the enzyme from its attachment to the mitochondria.

These findings recall early experiments on the activating effects of ether and of chloroform on the synthesis of acetylcholine by brain suspensions (Stedman & Stedman, 1937; Feldberg, 1945) and suggest that the activation produced in these brain suspensions is also due solely to an action on mitochondria.

Although the enzyme in the mitochondrial fraction required treatment with ether for full activity, the activity of the enzyme contained in the final supernatant was fully realized whether it was treated with ether or not. This observation may afford an explanation of the difference between our results and those of Hagen (1956), who reported that most of the choline acetylase activity found after differential centrifugation of homogenates of human placenta was contained in the high-speed supernatant. From the preliminary account of his work it appears that the separated fractions were used without further treatment. If this is the case, then only the supernatant fraction may have been capable of full activity, while the activity of the mitochondrial fraction would appear to be low. Treatment with ether might reveal the enzyme associated with the mitochondria of human placenta, as it does with the mitochondria of rabbit brain.

It seems that the action of ether on mitochondria, whether separated or contained in homogenates or brain fractions, is to make the enzyme more accessible to its substrates. It may be that ether releases the enzyme from a lipid complex, since the lipid content of mitochondria is high (Abood *et al.* 1952). A similar interpretation seems applicable to the finding of Girvin & Stevenson (1954) that the aqueous extraction of choline acetylase from bacteria depends on the preliminary extraction of lipid material from the cellular debris with *n*-butyl alcohol. Alternatively, the effect of ether may be to disrupt the mitochondrial membrane by a solvent action on its lipids. In this connexion it is interesting to note that Williams (1955), from his observations on the oxidation of choline by liver mitochondria, concluded that they have a permeability barrier for choline. If the effect of ether on the activity of choline acetylase is due to its effect on the permeability of the mitochondrial membrane this would be further support for the view that synthesis of acetylcholine occurs within the mitochondrion.

SUMMARY

1. In homogenates of rabbit brain choline acetylase activity is lower than in extracts of acetone-dried brain made from equivalent amounts of fresh tissue; but the activity of the homogenates can be increased to the level of extracts of acetone-dried tissue by treatment with ether or acetone. Chloroform also has an activating action but is less effective.

2. On differential centrifugation of the homogenates from 52 to 69% of the choline acetylase is found in the mitochondrial fraction brought down at 12,000 or at 15,000 *g*. The only other large single fraction of the enzyme is that

which remains in the final supernatant recovered after centrifugation at 25,000 *g*. The two fractions differ from one another in that the mitochondrial enzyme is largely inactive until treated with ether while the enzyme in the final supernatant is fully active without ether treatment.

3. Most of the mitochondrial fraction of enzyme is firmly bound to the particulate matter and is not detached from it even by ether treatment. It is suggested that the effect of ether is to make the enzyme accessible to its substrates possibly by disruption of lipids forming the mitochondrial membrane.

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