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ACTIVATION OF CHOLINE ACETYLTRANSFERASE IN SUBCELLULAR FRACTIONS OF THE BRAIN BY HYPOSMOTIC TREATMENT AND BY ETHER

S. TUČEK*

Department of Physiology, Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge

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Abstract—Experiments with crude mitochrondrial fractions of dog and rat brain homogenates indicate that treatment with ether brings about more complete activation of choline acetyltransferase contained in the particles than hyposmotic treatment with water. Some features of the activation with ether, including the changes in the appearance of the particles under the electron microscope, support the view that the action of ether is on permeability barriers.

IT HAS been known for a long time that the synthesis of acetylcholine (ACh) in brain homogenates may be increased by treating them with chloroform or ether.[1–3] Even in media with a composition optimal for the activity of the choline acetyltransferase (ChAc, acetyl-CoA:choline O-acetyltransferase, EC 2.3.1.6), the synthesis of ACh in ether-treated homogenates or in extracts from acetone-dried tissue is much greater than in untreated homogenates prepared in isotonic media.[4]

Recently, McCaman *et al.*[5, 6] described the activation of ChAc in brain homogenates and subcellular fractions by treatment with pure water (hyposmotic treatment) and claimed that this treatment gives higher activities of ACh synthesis than the ether treatment described by Hebb and Smallman.[4] They attributed part of the difference between their results and those of other workers[7] to the fact that other workers using ether treatment did not reveal full ChAc activity. This finding if confirmed would mean that in many previous researches, the true maximal ChAc activity in different parts of the brain has not been determined; it would also mean that the distribution of ChAc in different subcellular fractions found in previous investigations such as those of Hebb and Smallman[4] might be misleading or wrong. It seemed desirable, therefore, to make a direct comparison of the two methods of activation of ChAc and this has been done in the experiments described here on crude mitochondrial fractions prepared from homogenates of dog and rat brains.

The treatment of subcellular fractions with ether has been performed in the way which is currently used in the laboratory of Dr. C. O. Hebb.[8] Cold (0°–5°C) peroxide-free ether was used; 0·25 ml of it were added to 1 ml of the subcellular fraction suspended in a solution of sucrose or in water; the mixture was shaken by hand 2 min, and afterwards kept on ice in a refrigerator for another 18 min with occasional shak-

* Wellcome Research Fellow. Present address: Department of Physiology, Charles University Medical Faculty, Plzeň, Czechoslovakia.

ing. Then, the ether was removed by a stream of nitrogen blowing for 90 sec on the surface of the fraction so as to stir without causing frothing.

The crude mitochondrial fraction (P_2), known to consist of mitochondria, pinched-off nerve endings and fragments of myelin[9] was used as the starting material. It was obtained in the way described by Hebb[8] and treated hyposmotically as described by McCaman *et al.*,[5, 6] i.e. by suspending and rehomogenizing the pellet in cold (0°–4°C) water, using 9–10 ml of water per 1 g of original tissue.

In the experiments on dog brain material, the P_2 pellet was treated in the following ways:

(*a*) one part was resuspended in isotonic (0·32 M) sucrose; some of this isotonic suspension was left untreated and the remainder treated with ether.

(*b*) one part was hyposmotically treated as described above; again a part of this was treated with ether and a part left untreated.

The activity of ChAc was then measured in the samples with the incubation system described elsewhere.[10] The amount of ACh synthesized was measured by assay on the rectus abdominis muscle of the frog. The activities found are given in Table 1.

TABLE 1. COMPARISON OF THE EFFECTS OF ETHER AND OF HYPOSMOTIC TREATMENT ON THE ACTIVITY OF CHOLINE ACETYLTRANSFERASE IN THE CRUDE MITOCHONDRIAL FRACTION (P_2) PREPARED FROM DOG BRAIN

Material and treatment	Activity (mμmoles/g original tissue per hr)	
P_2 Suspended in isotonic sucrose	579	573
	568	
P_2 Treated hyposmotically	1112	1112
	1112	
P_2 Treated hyposmotically and then with ether	1390	1397
	1404	
P_2 Suspended in isotonic sucrose and treated with ether	1504	1513
	1522	

When compared with the fraction which was simply suspended in isotonic sucrose, hyposmotic treatment increased the activity of ChAc by 94 per cent; hyposmotic treatment combined with ether treatment by 144 per cent; ether treatment by itself by 164 per cent.

In the next part of the investigation the hyposmotically treated P_2 was separated into several subfractions by centrifuging over a discontinuous density gradient of 0·4 M, 0·6 M and 0·8 M sucrose (see Tuček[10]) and four subfractions analogous to those described by Whittaker *et al.*,[7] O, D, E and F–I were recovered. Of these, O was on the top of the gradient and corresponded in its location to the original position of the P_2 fraction when layered upon the top of the gradient. D corresponded to the upper part of the 0·4 M sucrose layer, E to 0·4–0·6 M sucrose layer, F–I to 0·6–0·8 M sucrose layer and included a pellet. The activity of ChAc was now examined in all subfractions and the effect of ether treatment investigated on all except the subfraction O. Subfractions O prepared in this way are known to contain enzymes which

have been released from the particles into solution by hyposmotic treatment, and to lack particulate material; and ether treatment has no effect on the ChAc present in solution.[4] The activities found are given in Table 2.

TABLE 2. THE EFFECTS OF A TREATMENT WITH ETHER ON THE ACTIVITIES OF CHOLINE ACETYLTRANFERASE (mμmoles/g original tissue per hr) IN THE HYPOSMOTICALLY TREATED CRUDE MITOCHONDRIAL FRACTION (P_2W) FROM THE DOG BRAIN AND IN THE SUBFRACTIONS OBTAINED BY THE CENTRIFUGATION OF P_2W ON A SUCROSE DENSITY GRADIENT

Fraction	Treatment	Activity		Treatment	Activity		Percentage change due to ether treatment
P_2W	Hyposmotic	1112 1112	1112	Hyposmotic + ether	1390 1404	1397	+25·6
O	Hyposmotic	254 218	236	Hyposmotic	254 218	236	
D	Hyposmotic	116 109	112	Hyposmotic + ether	99 106	103	− 8·7
E	Hyposmotic	115 113	114	Hyposmotic + ether	122 126	124	+ 9·0
F–I	Hyposmotic	666 666	666	Hyposmotic + ether	874 903	889	+33·5
Total recovery			1128			1351	
Percentage recovery			101·4			96·7	

It may be seen from Table 2 that treatment with ether had little additional effect on the activity of ChAc in the particulate material contained in the upper layers of the gradient, but substantially increased the activity of the enzyme in the lowest layer.

The effect of ether was also tested on fractions prepared from rat brains. The starting material was the hyposmotically treated crude mitochondrial fraction. After suspension in water it was separated into three subfractions following the procedure of De Robertis *et al.*[11] and McCaman *et al.*[6]: M_1 (pellet at 11,500 $g \times 20$ min), M_2 (pellet at 100,000 $g \times 30$ min), M_3 (final supernatant). The activities in the starting material and in the subfractions with and without ether treatment are given in Table 3.

As may be seen from Table 3, treatment with ether brought about a further increase in the activity of ChAc in the hyposmotically treated crude mitochondrial fraction and in all its subfractions. The greatest increase was in M_2.

It is interesting to note that the highest degree of activation by ether was not observed in the fractions containing the heaviest and biggest particles. This is apparent from data in Table 3 (particles in M_2 being smaller and lighter than those in M_1), and it was confirmed in several experiments with fractions of rabbit homogenates (Table 4). This observation is in accord with the view that it is not the size of the particles, but the character of their membranes which brings about the need of activation.

No conclusive explanation of the action of ether has been given so far, but the analogy with some occluded enzymes and their activation by means of detergents has suggested to many that the activation of ChAc by ether may be due to its effect on the

membranes, particularly on the external membranes of the nerve endings. It was shown by Hebb and Smallman[4] that the ChAc remains bound to the particles even after the treatment with ether. It is conceivable that the treatment with ether decreases the permeability barriers and renders the enzyme more easily accessible to substrates.[12]

TABLE 3. THE EFFECTS OF A TREATMENT WITH ETHER ON THE ACTIVITIES OF THE CHOLINE ACETYLTRANSFERASE (mμmoles/g original tissue per hr) IN THE HYPOSMOTICALLY TREATED CRUDE MITOCHONDRIAL FRACTION (P_2W) FROM THE RAT BRAIN AND IN THE SUBFRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF P_2W

Fraction	Treatment	Activity	Treatment	Activity	Percentage change due to ether treatment
P_2W	Hyposmotic	2768, 2768 } 2768	Hyposmotic + ether	3280, 3330 } 3305	+19·4
M_1	Hyposmotic	1812, 1691 } 1751	Hyposmotic + ether	1905, 1885 } 1895	+ 8·2
M_2	Hyposmotic	767	Hyposmotic + ether	1066, 992 } 1029	+34·2
M_3	Hyposmotic	177, 183 } 180	Hyposmotic + ether	207, 185 } 196	+ 8·9
Total recovery		2698		3120	
Percentage recovery		97·5		94·4	

TABLE 4. DIFFERENCES IN THE DEGREE OF ACTIVATION BY ETHER OF CHOLINE ACETYLTRANSFERASE IN FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF RABBIT BRAIN HOMOGENATES*

Pellet at $g \times$ time (min)	Activity after the treatment with ether expressed as percentage of activity found in untreated fraction Experiment No.			
	1	2	3	4
10,000 g (5)	143	219	151	169
Between 10,000 g (5) and 29,800 g (30)	451	616	349	585
Between 29,800 g (30) and 112,000 g (60)	320	390	312	254
Whole homogenate	200	249	163	173

* The four experiments included in the table differed in that the homogenizers used varied in the clearance of their pestles between 0·125 mm and 0·375 mm; the homogenates and fractions were kept frozen at −18°C for 1 day between the fractionation and the incubation.

In the present investigation, the effect of ether treatment on the crude mitochondrial fraction was examined under the electron microscope by negative staining with sodium phosphotungstate after fixation with formaldehyde.[7] After ether treatment the number of small fragments of myelin appeared to be diminished, the contours of the particles became less sharp and, most conspicuously, the particles were penetrated and darkly stained by the sodium phosphotungstate. This observation supports the view that the activating effect of ether is due to changes of permeability.

An attempt to use a detergent (Triton) for the activation of ChAc failed because of unfavourable effects of the detergent during the biological assay of ACh. This method of activation of ChAc might prove useful when chemical measurements of the ACh formed can be used instead of bioassay.

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