Biochem. J. (1967) **103**, 262

The 'Compartmentation' of Choline Acetyltransferase within the Synaptosome

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(*Received* 12 *August* 1966)

1. Choline acetyltransferase may be isolated in either a bound or soluble form after hypo-osmotic treatment of a crude synaptosome fraction, depending on the conditions. 2. In the bound form, the enzyme appears to be associated with the larger membrane fragments rather than with synaptic vesicles. 3. The bound form is predominant at slightly acid pH values and low ionic strength, the soluble form under more physiological conditions of pH and ionic strength. 4. Sodium chloride, potassium chloride, magnesium chloride and calcium chloride at similar ionic strengths solubilize the enzyme. 5. Choline acetyltransferase was found to be soluble under these conditions after release from synaptosomes from rat and pigeon cerebra, guinea-pig cortex and rabbit cortex, caudate nuclei, diencephalon and midbrain. 6. Certain isoenzymes of lactate dehydrogenase behaved similarly.

ChAc† (acetyl-CoA–choline *O*-acetyltransferase, EC 2.3.1.6) is important in nervous tissue as the enzyme synthesizing the cholinergic transmitter acetylcholine from acetyl-CoA and choline. The enzyme is present in relatively high concentration together with acetylcholine within the presynaptic nerve terminals of cholinergic neurones and remains sequestered there when the nerve terminals are detached and isolated by homogenization and centrifugation (Hebb & Whittaker, 1958; Gray & Whittaker, 1962; De Robertis, de Iraldi, Arnaiz & Salganicoff, 1962). Suspension of preparations of detached nerve terminals (synaptosomes) in hypo-osmotic media causes them to burst (Johnson & Whittaker, 1962, 1963), and the soluble cytoplasmic constituents, synaptic vesicles, external membranes and intraterminal mitochondria may then be separated by differential (De Robertis, Arnaiz, Salganicoff, de Iraldi & Zieher, 1963) or density-gradient (Whittaker, Michaelson & Kirkland, 1963, 1964) centrifuging. The latter method gives considerably less cross-contamination of fractions (Whittaker *et al.* 1964; Germain & Proulx, 1965), and the synaptic-vesicle fraction so obtained is remarkably homogeneous (Whittaker & Sheridan, 1965). Both groups of workers agree that bound acetylcholine is present in the synaptic-vesicle fraction; however, there is disagreement about the subcellular localization of the enzyme ChAc within the synaptosome. De Robertis *et al.* (1963) claimed that the enzyme is bound to the synaptic vesicles, and Whittaker *et al.* (1964) claimed that the enzyme is localized in the soluble cytoplasm of the synaptosome. They suggested that the presence of ChAc in the vesicle (M_2) fraction obtained by De Robertis *et al.* (1963) is due to contamination of this fraction by undisrupted synaptosomes. Bull, Feinstein & Morris (1964) found that the rabbit-brain enzyme is a soluble protein of relatively low molecular weight (67000).

Since many low-molecular-weight proteins (e.g. globulins) are relatively insoluble in media of low ionic strength, it was decided to investigate the solubility of ChAc under the conditions likely to prevail within the intact synaptosome and during the hypo-osmotic rupture of synaptosomes. While this study was in progress, several additional reports on the subcellular localization of ChAc appeared. McCaman, Arnaiz & De Robertis (1965) suggested that species differences could account for the disagreements between De Robertis *et al.* (1963) and Whittaker *et al.* (1964). They concluded that ChAc was synaptic-vesicle-bound in rat and rabbit cortex, partly vesicle-bound and partly soluble in the guinea-pig and soluble in the pigeon. The ChAc values reported by these workers were, however, appreciably lower than those found by others where comparison can be made (Fonnum, 1966*a*).

Tuček (1966) found that, in preparations from whole rabbit brain and sheep caudate nucleus that were osmotically disrupted, the enzyme behaved as

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† Abbreviations: ChAc, choline acetyltransferase; LDH, lactate dehydrogenase.

though particle-bound. Density-gradient separation by the method of Whittaker *et al.* (1964) showed, however, that the enzyme was bound, not to the synaptic vesicles, but to larger-particulate material (V. P. Whittaker, personal communication). By contrast, Saelens & Potter (1966), using a different density-gradient procedure, reported that ChAc in rat cerebra was bound to synaptic vesicles.

It is demonstrated below that the fall in ionic strength and pH attendant on hypo-osmotic rupture of a crude synaptosome preparation may lead to a binding of ChAc to membranes that is believed to be non-specific in character. This effect shows species and regional variations and is particularly marked with preparations from rat and rabbit brain. Under conditions of pH and ionic strength approximating those believed to exist within the intact synaptosome, the enzyme becomes soluble irrespective of species or regions. The effect is independent of the nature of the ions added and was observed with Na^+, K^+, Mg^{2+} and Ca^{2+}. Similar effects are also seen with one of the LDH isoenzymes. Most of the experiments were done with rat brain since it is particularly in the rat that ChAc is said to be vesicle-bound. The implications of the results for studies of the 'compartmentation' of enzymes within osmotically sensitive subcellular particles are discussed.

Preliminary accounts of this work have been given by Michaelson (1967) and by Fonnum (1966*b*).

METHODS

Preparation of primary fractions

Dissection of tissues. With brains of rat, guinea pig and rabbit, the cerebellum and the brain stem caudal to the superior colliculi were discarded, the hemispheres separated from the diencephalon and most of the white matter was removed with a scalpel. With the rat the tissue so obtained was pooled and is referred to as 'cerebra'. With rabbit the neo-cortical grey matter, caudate nuclei, hippocampus, diencephalon and midbrain were separated: in some experiments the caudate nuclei, diencephalon and midbrain were pooled; the tissue so obtained is referred to as 'midbrain'. With pigeon the discarded tissue comprised the cerebellum, optic lobes and subjacent region of the brain stem; the tissue utilized is again referred to as 'cerebra'. With rats, guinea pigs and pigeons tissue from two to four animals was pooled for each preparation.

Preparation of fractions. Primary subcellular fractions were prepared essentially as described by Whittaker *et al.* (1964). The tissue samples (1–4g.) were homogenized in 0·32M-sucrose and the homogenate was centrifuged at 1000*g* for 11min. The pellets were washed by resuspension in 0·32M-sucrose and recentrifuging under the same conditions. The combined supernatants were centrifuged at 12000*g* for 20min. The pellet obtained was washed by resuspension in 0·32M-sucrose and recentrifuging at 12000*g* for 30min. This pellet containing synaptosomes, mitochondria, myelin and some microsomes was called the washed P_2 fraction and contained 20–40% of the total ChAc activity of the original homogenate.

Hypo-osmotic treatment

Procedure A. The washed P_2 pellet was suspended in water (2–4ml./g. of original tissue) and 5ml. of suspension was layered on to a discontinuous sucrose density gradient consisting of five layers of 5ml. each of 0·4M-, 0·6M-, 0·8M-, 1·0M- and 1·2M-sucrose. Separation was achieved after centrifuging for 150min. at 53000*g* in the SW 25 head of the Spinco model L preparative ultracentrifuge. The tube was then sliced with a tube cutter to give seven fractions corresponding to fractions *O*, *D*, *E*, *F*, *G*, *H* and *I* described by Whittaker *et al.* (1964).

Procedure B. The washed P_2 fractions were suspended in various amounts of water to give 5ml. of suspension/g. of original tissue. The suspension was homogenized by sucking it up and down ten times in a pipette. Samples of the suspension (2·0ml.) were transferred to stainless-steel tubes containing 2·0ml. of water or salt solutions (NaCl, KCl, $MgCl_2$ or $CaCl_2$) of various molarities, and the contents well mixed. The salt solutions were adjusted to pH6·5–7·0 before use and because of their low buffering capacity had little or no effect on the final pH (about 6·8) of the suspension. The suspensions were immediately centrifuged at 105000*g* for 30min. The supernatants were separated as completely as possible from the precipitates; the latter were suspended in 4ml. of water and samples of each fraction taken for ChAc, LDH and protein analysis. The supernatant was also analysed for sodium and potassium.

This procedure ensured that all samples in any one experiment were submitted to the same degree of hypo-osmolarity during the initial procedure and thus that the proportion of ruptured synaptosomes in all tubes was the same.

Procedure C. In other experiments the salt solution was replaced by sodium phosphate buffer of constant ionic strength (0·01) and various pH values (the effect of pH on the ionization of the buffer was taken into account when calculating the ionic strength). The final pH of the suspension was recorded immediately before centrifuging. The pH of the supernatants was adjusted to 7·4 before enzyme assay.

Other treatments

Ether. Synaptosome preparations (2·5ml.) were disrupted by the addition of 1ml. of peroxide-free ether at 0°. The tubes were intermittently shaken and after 10min. the ether was removed in a stream of nitrogen. The ionic strength of the suspension was then adjusted if necessary and the suspension separated into a pellet and high-speed supernatant by centrifuging at 105000*g* for 30min.

Incubation. Preparations were incubated at 37° for 30min. and again separated into pellet and high-speed supernatant fractions by centrifuging.

Analysis of fractions

Estimation of choline-acetyltransferase activity. Fractions were treated with Nonex detergent (British Drug Houses Ltd., Poole, Dorset) in a final concentration of 0·5% (w/v) to release any occluded enzyme. Sucrose was largely removed from fractions before assay by gel filtration through Sephadex G-25 (AB Pharmacia, Uppsala, Sweden) or by diluting

the fractions into an equal volume of water and centrifuging at 105000*g* for 1 hr. The enzyme assay was carried out radiochemically as described by Fonnum (1966*a*). Incubation mixture contained (final concentrations): 8 mM-sodium [1-^{14}C]acetate (350000 counts/min.), 8 mM-choline, 8 mM-$MgCl_2$, 200 mM-NaCl, 4 mM-KCl, 40 mM-NaF, 0·5 mM-KBH_4, 0·05 mM-CoA, 16 mM-ATP, 0·1 mM-eserine sulphate and 8 mg. of acetone-dried pigeon liver/ml. In some experiments, thioglycollic acid (1 mM) was present in the assay mixture (cf. Morris, Hebb & Bull, 1966). This resulted in a small increase in total activity but similar subcellular distributions were obtained. Assays were normally carried out immediately the fractions had been prepared.

Estimation of lactate-dehydrogenase activity. The enzyme was activated with Triton X-100 (Lennig Chemicals Ltd., London, W.C. 1) in a final concentration of 1% (w/v) (Fonnum, 1965) and assayed spectrophotometrically as described by Johnson (1960).

Acetylcholine. The acetylcholine content of fractions was assayed on a small strip of the dorsal muscle of the leech (Szerb, 1962) as described by Whittaker *et al.* (1964). The acetylcholine was released by heating sucrose fractions at pH 4·0 in a boiling-water bath for 10 min. Sucrose solutions treated in the same way acted as blanks.

Protein nitrogen. Protein N was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Determination of ionic strength. Sodium and potassium were determined in the 105000*g* supernatant (procedure B) by flame photometry with an EEL flame photometer (Evans Electroselenium Co., Harlow, Essex). Their contribution to the ionic strength of the supernatant was calculated on the assumption that they were present in ionic form. The constituents of electrolytes in brain and cerebrospinal fluids have been summarized by McIlwain (1966), and K^+ and Na^+ are responsible for 98% of all cations. The effect of other cations may therefore be neglected.

Electrophoretic separation of lactate-dehydrogenase isoenzymes on agar gel. This was performed as described by Boyd (1964). Runs lasted 30 min. at a constant potential difference of 150 v between the two sides of a 6 cm. agar plate.

Expression of results. The degree of solubilization of ChAc, LDH or protein in procedure B was expressed by the percentage of the total recovered material appearing in the 105000*g* supernatant. Usually 80–100% of the original material was recovered in the two fractions. The activity of fractions was also expressed in terms of the relative specific activity, i.e. the percentage of the total recovered activity appearing in the fraction divided by the percentage of total recovered protein appearing in the fraction. The absolute activities are given in Table 1.

RESULTS

Density-gradient separation. When hypo-osmotically ruptured washed P_2 fractions from rat or rabbit brain were submitted to density-gradient separation (Methods section: procedure A) the appearance of the gradient after centrifuging was similar to that described for guinea-pig preparations by Whittaker *et al.* (1964). However, the distribution (Fig. 1) of ChAc, LDH, acetylcholine and protein in the gradient differed somewhat. LDH, generally accepted as a cytoplasmic marker, was found mainly in fraction *O* containing soluble cytoplasm; the next richest fraction was that consisting mainly of partially disrupted synaptosomes (*H*). This fraction is at the level in the gradient to which synaptosomes that escaped disruption would mainly migrate, as shown by control experiments. Slightly more LDH was released from rabbit cortex than from rabbit midbrain or rat cerebra. When the results are expressed as relative specific activity no definite secondary peak emerges, fractions *G*, *H* and *I* having approximately equal activity. It seems reasonable to attribute the LDH activity of these fractions to occluded cytoplasm within undisrupted synaptosomes. The proportion of the total LDH recovered in these fractions indicates that most (80%) of the synaptosomes were ruptured and that the cytoplasm was released by the hypo-osmotic treatment.

Bound acetylcholine was measured only in the rat preparation; it was present in highest concentration in fractions *D* and *E* (synaptic-vesicle and

Table 1. *Protein nitrogen content and choline-acetyltransferase and lactate-dehydrogenase activities of washed* P_2 *fractions from rabbit, rat, guinea-pig and pigeon brain*

Fractions were prepared and assayed as described in the text. The amounts of protein and of enzyme activity relate to a volume of the fraction equivalent to 1 g. of tissue.

Species	Tissue	Protein N content (mg.)	ChAc activity (μmoles of acetylcholine formed/hr.)	LDH activity (μmoles of $NADH_2$ oxidized/min.)
Rabbit	Cortex	3·7	1·8	9·5
	Caudate nucleus	3·4	5·4	7·6
	Hippocampus	3·4	0·9	6·3
	Medulla + pons	2·5	0·7	6·0
Rat	Cerebra	4·0	2·2	17·0
Guinea pig	Cortex	4·5	1·3	9·9
Pigeon	Cerebra	2·	0·9	2·0

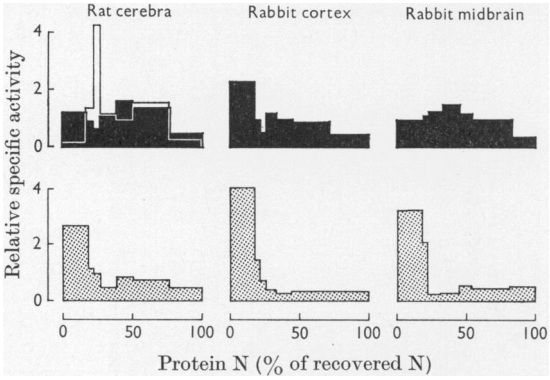

Fig. 1. Distribution of ChAc (■) and LDH (▩) in fractions of hypo-osmotically disrupted synaptosomes separated on a discontinuous density gradient (Methods section: procedure A). In the experiment with rat cerebra, acetylcholine was also determined; this distribution is shown by the black and the white lines superimposed on the corresponding ChAc distribution. The blocks correspond to fractions *O–I* of Whittaker *et al.* (1964), reading from left to right. The width of each block is proportional to the percentage of total recovered protein N found in the fractions; the area of the block is proportional to the percentage of total recovered activity found in the fraction.

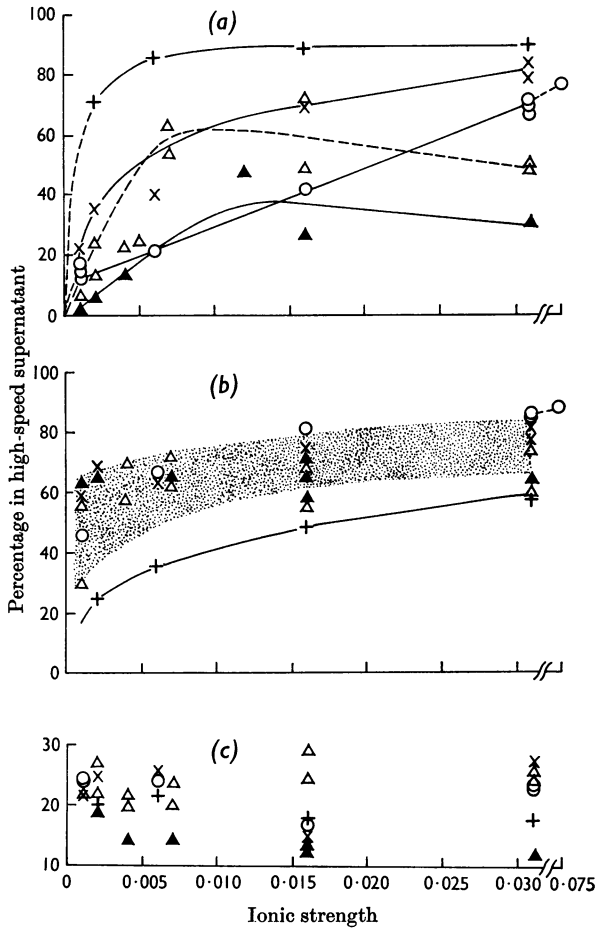

Fig. 2. Release into high-speed supernatant of (*a*) ChAc, (*b*) LDH and (*c*) protein N from crude synaptosome preparations of diverse origin as a result of suspension in water followed by adjustment of ionic strength with NaCl (Methods section: procedure B). The symbols represent the results of two experiments with pigeon cerebra (+), rat cerebra (○), guinea-pig cortex (×) rabbit cortex (△) and rabbit 'midbrain' (▲).

membrane-rich fractions). The acetylcholine-containing vesicles appeared to be slightly denser than those from guinea-pig brain but the bimodal distribution previously noted (Whittaker *et al.* 1964) was again obtained, with a secondary peak in *H*.

ChAc differed from both acetylcholine and LDH in its distribution: from acetylcholine in having its lowest activities in the acetylcholine-rich *D* and *E* fractions, and from LDH in being less completely released into the soluble cytoplasmic fraction and in being associated to a considerable extent with fractions *F* and *G* (large membranes) as well as fraction *H*. The difference between the distribution of LDH and ChAc is particularly striking with rabbit midbrain.

These results are in contrast with those of Whittaker *et al.* (1964) with guinea-pig brain, who found much closer agreement between the distributions of LDH and ChAc, suggesting that the latter was a soluble cytoplasmic enzyme also. In these and other experiments the amount of soluble ChAc varied much more from experiment to experiment than did that of LDH or glutamate decarboxylase (F. Fonnum, unpublished work). Hypo-osmotic treatment is accompanied by a decrease in ionic strength and a small fall in pH to 6·5–7·0; the extent of these changes depends, among other things, on the amount of dilution. The next step was therefore to examine the percentage of ChAc and LDH released by hypo-osmotic disruption as a function of these two variables.

Effect of varying ionic strength. The results of varying the ionic strength (Methods section: procedure B) are shown in Figs. 2(*a*) and 2(*b*). The soluble protein released into the supernatant was also measured (Fig. 2*c*). The points in the graph of the lowest ionic strength (*I* 0·001) correspond to suspension of an amount of washed P_2 pellet derived from 1 g. of tissue in 10 ml. of water without further addition of electrolyte; these are approximately the conditions used by McCaman *et al.*

(1965). Increases in ionic strength were obtained by the addition of various amounts of sodium chloride. There is some variability in the results, this being most apparent in the rabbit experiments, but certain trends can nevertheless be distinguished. This variability may have been due to: (1) variations between experiments in the number of synaptosomes ruptured; (2) the low buffering capacity of the preparation and consequent small variations in pH between one experiment and another. As shown below (Fig. 4) the effect of pH is far from negligible.

Taking the results from ChAc first (Fig. 2*a*), there was, in all species examined, an increase in the proportion of soluble enzyme as the ionic strength of the suspension was increased. The effect is most pronounced for rat cerebra where the solubility was followed up to 76mM-sodium chloride. The enzyme from guinea-pig cortex gave similar results, but was slightly more soluble at lower ionic strengths. The rabbit-cortex enzyme showed a similar pattern at low ionic strengths, but there were indications of a maximum in the curve; that from rabbit midbrain was generally less soluble than those of the other two species. The pigeon enzyme differed from the rest in being very soluble even at low ionic strengths, though it showed a small further increase at higher ionic strength. The lower dilution of the suspensions used by Whittaker *et al.* (1964) (2ml./pellet equivalent to 1g. of tissue) will thus favour greater solubilization than the conditions of McCaman *et al.* (1965).

LDH also showed an increase in solubility with increasing ionic strength, as demonstrated in Fig. 2(*b*). The effect is, however, not so dramatic as for ChAc. There were no marked species differences between rat, rabbit and guinea pig (shaded area, Fig. 2*b*) but the LDH of pigeon preparations was noticeably less solubilized by increasing ionic strength than the mammalian enzymes

When the LDH-isoenzyme distribution was studied by means of agar-gel electrophoresis, differences were found in the behaviour of the various isoenzymes. An electrophoretogram of fractions from an experiment with rat cerebra illustrates this (Fig. 3). Five bands are present in the supernatants from suspensions adjusted to high ionic strength (*I* 0·031), but the most basic of the isoenzymes is missing from the supernatant of low ionic strength (*I* 0·001); there is also slightly less of isoenzymes 2, 3 and 4 and more of isoenzyme 1. The fact that only the most basic isoenzyme is markedly affected accounts for the much less striking effect of the ionic strength on LDH as compared with ChAc; it may also account for the more marked effect on pigeon preparations compared with mammalian; basic LDH isoenzymes predominate in the pigeon (J. W. Boyd, personal communication).

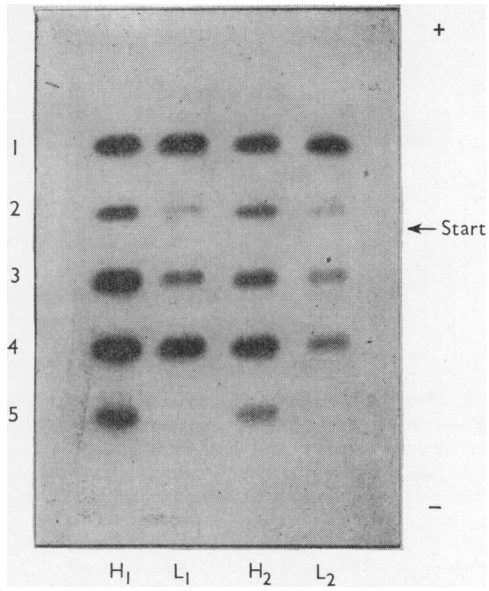

Fig. 3. Electrophoretogram of LDH isoenzymes released from rat cerebral synaptosomes after hypo-osmotic disruption and adjustment to high (0·03) (H_1, H_2) and low (0·001) (L_1, L_2) ionic strength. The isoenzyme with the highest positive charge density is absent from the supernatant with low ionic strength. The separation of isoenzymes was achieved by agar-gel electrophoresis.

The proportion of protein released (Fig. 2*c*), though variable, did not show any obvious correlation with species, region or ionic strength. It is therefore unlikely that the results with ChAc or LDH can be due to systematic variations in the number of synaptosomes remaining intact at the centrifugation stage.

Effect of pH. To obtain an idea of the solubilities of the enzymes at physiological pH values and to exclude the possibility that the desired effects of varying ionic strength were due simply to changes in pH, the release of ChAc, LDH and protein from rat-cerebra synaptosomes was studied as a function of pH (Methods section: procedure C). The results are summarized in Fig. 4, where the method of presentation (use of relative specific activities) takes into account variations in the percentage release of soluble protein. They show that ChAc was more soluble at physiological pH values than at the pH values obtained after hypo-osmotic treatment in an unbuffered medium. LDH again showed much less variation than ChAc.

In one experiment (shown in Fig. 4) two samples of high and one with low ionic strength were included. These confirmed that the solubilities of the enzymes are functions of both pH and ionic strength.

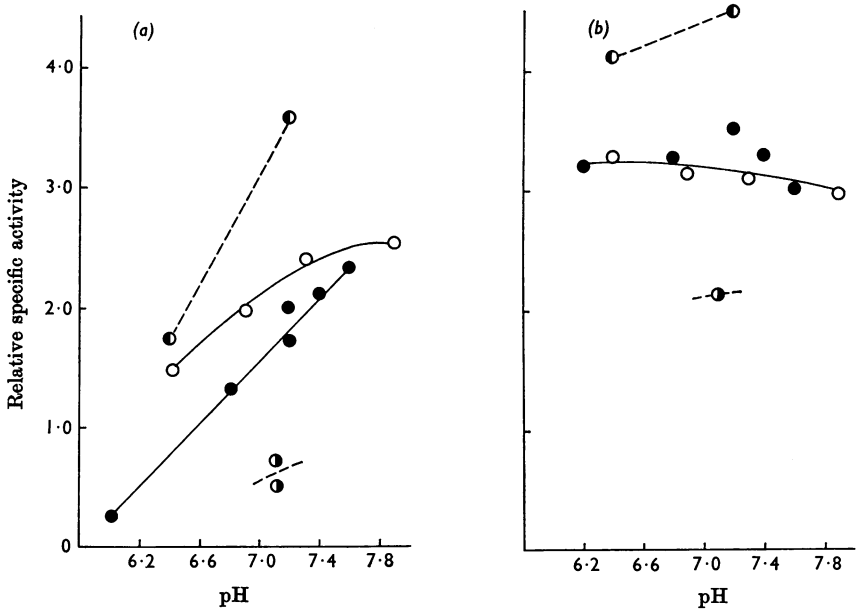

Fig. 4. Relative specific activity of (*a*) ChAc and (*b*) LDH in supernatants from a crude preparation of rat cerebral synaptosomes disrupted by hypo-osmotic treatment at various pH values. The variation of pH was obtained by addition of phosphate buffers of various pH values but constant final ionic strength (*I* 0·006) to the water suspension (Methods section: procedure C). The results were obtained in two separate experiments (○ and ●). For comparison, points showing the effect of high (*I* 0·036) (◐) and low (*I* 0·001) (◑) ionic strength were determined in one of the experiments (●).

Table 2. *Effect of various cations on the solubility of choline acetyltransferase and lactate dehydrogenase and protein nitrogen after hypo-osmotic treatment of a crude synaptosome preparation from rat cerebra*

Fractions were prepared and tested as described in the text. The ions were all added as chlorides. Recoveries were all 90–100%.

Ion added			Proportion solubilized (% of total recovered)					
			Expt. 1			Expt. 2		
Ion	Concn. (mM)	Approx. ionic strength of suspension	ChAc	LDH	Protein N	ChAc	LDH	Protein N
—	—	0·001	17	63	24	14	65	22
Ca^{2+}	5	0·015	60	77	19	—	—	—
Ca^{2+}	10	0·031	70	68	19	64	67	14
Mg^{2+}	10	0·031	—	—	—	67	—	20
K^+	30	0·031	—	—	—	54	75	17
Na^+	30	0·031	70	85	23	—	—	—
Na^+	75	0·076	78	88	22	—	—	—

Effect of various ions. It was next necessary to find out whether the effect of electrolyte was simply a function of ionic strength or involved specific ion effects.

The results in Table 2 showed that K^+, Mg^{2+} and Ca^{2+} at similar ionic strengths gave results comparable with those obtained with Na^+. There was thus no evidence for a specific effect of Mg^{2+} or Ca^{2+} on the binding of the enzymes to membranes. Slight differences of final pH may explain any small differences between the various ions.

Regional and species variations. Table 3 shows that ChAc from different parts of the brain and different species is much more soluble at pH 7·4 and

Table 3. *Release of choline acetyltransferase, lactate dehydrogenase and protein from synaptosomes isolated from different parts of the rabbit brain and from different species after hypo-osmotic treatment*

A crude synaptosome pellet was disrupted by suspension in water (10 ml./g. of original tissue); samples were either (*a*) centrifuged immediately or (*b*) adjusted to pH 7·4 and *I* 0·03 with NaCl and then centrifuged. Recoveries were 90–100%.

		Proportion solubilized (% of total recovered)					
		(*a*)			(*b*)		
Species	Tissue	ChAc	LDH	Protein N	ChAc	LDH	Protein N
Rabbit	Cortex	35	55	18	64	80	17
	Caudate nuclei	15	42	22	60	73	22
	Hippocampus	22	48	14	84	78	25
	Medulla + pons	28	82	17	57	72	12
Rat	Cerebra	15	55	24	70	85	23
Guinea pig	Cortex	25	64	24	82	81	22*
Pigeon	Cerebra	72	26	20	90	59	18*

* pH not checked.

Table 4. *Release of choline acetyltransferase, lactate dehydrogenase and protein from synaptosomes after ether activation and after heating*

For experimental procedure see the text. Recoveries were 85–100% unless otherwise stated. Numbers of experiments averaged are given in parentheses.

				Proportion solubilized (% of total recovered)		
Treatment	Solute	Concn. (mM)	Tissue	ChAc	LDH	Protein N
Ether	Sucrose	320	Rat cerebra	20 (3)	77 (3)	36 (1)
			Guinea-pig cortex	73 (1)	82 (1)	32 (1)
Ether	Sucrose + NaCl	300 + 30	Rat cerebra	78 (2)	96 (2)	31 (1)
			Guinea-pig cortex	97 (1)	96 (1)	25 (1)
Heated at 37° for 30 min.	Sucrose	320	Rat cerebra	1* (1)	14 (1)	—

* Recovery 68% (low owing to temperature denaturation).

relatively high ionic strength (*I* 0·03) than under the conditions prevailing in hypo-osmotic treatment. If we regard the LDH released as an indicator of the proportion of synaptosomes disrupted, then more than 80% of the ChAc of disrupted synaptosomes was released in a soluble form. ChAc from caudate nucleus and medulla and pons, earlier regarded as rather insoluble, is, in fact, as soluble as that in other brain regions at pH 7·4 and *I* 0·03.

Effect of other treatments. Table 4 shows that ChAc is 'bound' within the synaptosome in a different manner from acetylcholine. Ether treatment was demonstrated by Whittaker (1959) to release all acetylcholine, but in rat-brain preparations ChAc remained to a considerable extent bound after this treatment. The addition of sodium chloride releases the ChAc. Ether treatment disrupts organized membrane structures and the results with this substance serve to emphasize the unphysiological nature of the binding at low ionic strength. Guinea-pig cortex showed the same effect but to a smaller extent.

Warming at 37° was also shown by Whittaker (1959) to release acetylcholine. LDH and ChAc were not released (Table 4). This observation confirms the general findings that ChAc must be activated before assay (Hebb & Smallman, 1956) and that only part of the enzyme activity is released by heat treatment (Bull & Hemsworth, 1965; Fonnum, 1966*a*).

DISCUSSION

The experiments demonstrate that ChAc can be isolated from synaptosome preparations in both a particle-bound and a soluble form depending on the

final pH and ionic strength of the suspension after hypo-osmotic rupture of crude synaptosome preparations. The bound form is favoured by the fall in ionic strength and pH that accompanies hypo-osmotic treatment; extrapolation of the results to ionic strengths and pH values likely to exist in the intact cell indicates that much if not all of the ChAc must be in a free form within the undisrupted ending.

It was not possible to isolate the particle or particles binding ChAc in a specific fraction, though, as seen in Fig. 1, they could be clearly separated from the acetylcholine-containing synaptic vesicles. The fractions having the highest relative specific activity were those containing relatively large (about 0·5μ diam.) membranes (mainly external presynaptic membranes), myelin fragments and partially disrupted synaptosomes. It is not at present possible to identify the membrane properties that are of importance in the binding, such as concentration of SH groups, surface charge density or simply a large surface area/mg. of protein; however, it can be stated that Ca^{2+} and Mg^{2+} are not involved.

LDH, generally recognized as a soluble enzyme, could also be partially bound under conditions of low ionic strength. Electrophoretic examination of the isoenzyme pattern showed that the charge on the protein was an important factor, the more basic isoenzymes binding the most. This is comprehensible when one considers that lipoprotein membranes are themselves negatively charged. Initially it was thought that the relatively small extent to which LDH was solubilized in the pigeon was due to the presence of osmotically stable particles containing occluded cytoplasm. However, the predominantly basic character of the LDH isoenzymes from pigeon tissues is a sufficient explanation of this species difference.

The amount of soluble protein released varied little from one sample to another, indicating that the proportion of synaptosomes disrupted and cytoplasm released was approximately the same from one experiment to another. However, Le Baron & Folch (1959) have stated that the amount of protein that can be extracted from whole brain depends on the pH and ionic strength, so that small differences in solubilization could well occur here as with LDH, owing to binding of the more basic proteins to membranes. If so, one would expect to see differences in electrophoretic pattern at low and high ionic strength, but these have not so far been looked for.

The results strongly suggest that the differences between the findings of Whittaker *et al.* (1964) and McCaman *et al.* (1965) with regard to the subcellular state of ChAc may be explained on the basis of differences in the degree of dilution used in hypo-osmotic treatment, in the choice of species and in the degree of homogeneity of the fractions assayed. The choice of the rat and the higher dilution of the synaptosome preparation by McCaman *et al.* (1965) (9ml./wt. of pellet derived from 1g. of tissue compared with 2ml./g. of tissue used by Whittaker *et al.* 1964) both favour binding of the enzyme to membranes. The vesicle (M_2) fraction is stated to be rich in cholinesterase (De Robertis *et al.* 1963) and Na^+-plus-K^+-stimulated adenosine triphosphatase (Albers, Arnaiz & De Robertis, 1965), enzymes that are also present in the microsome (Toschi, 1959; Aldridge & Johnson, 1959; Järnefelt, 1961) and external synaptosome membrane (Whittaker *et al.* 1964; Hosie, 1965) fractions, but not in the purified synaptic-vesicle fraction (Whittaker *et al.* 1964; Hosie, 1965; Germain & Proulx, 1965). Thus those particles in the M_2 fraction binding ChAc may not be synaptic vesicles but contaminating microsomes or fragments of the external synaptosome membrane or both. It is therefore unfortunate that McCaman *et al.* (1965) did not submit their M_2 fraction to density-gradient separation. The hypothesis put forward here, that the species differences in the distribution of the enzyme in the M_1, M_2 and M_3 fractions are due to species differences in its solubility, seems intrinsically more probable than the one advanced by McCaman *et al.* (1965), namely that there are variations in the 'compartmentation' of the transmitter storage mechanism in cholinergic neurones from different species.

The result of Saelens & Potter (1966) may well be explained in a similar way to those of McCaman *et al.* (1965), but insufficient details of their work have been published for an adequate critique. Tuček's (1966) results agree with those presented here, in that he finds that ChAc is bound, not to synaptic vesicles, but to larger particles. He used whole rabbit brain, which would be expected to give results similar to those of my 'midbrain' experiments. I have not studied sheep caudate nucleus, the other tissue used by Tuček (1966), but this may well behave like rabbit caudate nucleus. The tendency of ChAc to bind to membranes may also be relevant in interpreting the results of Hebb & Silver (1963), who found with goat ventral spinal roots and sciatic nerve that ChAc was bound to the microsome fraction in a non-occluded form.

The present study shows some of the difficulties likely to be encountered when attempting to establish the subcellular localization of substances within osmotically sensitive structures, or, indeed, whenever conditions of low ionic strength prevail. Unfortunately, subcellular fractionations cannot be carried out at electrolyte concentrations approaching those of the extracellular fluid or cell sap owing to coacervation of particulate material (Gray & Whittaker, 1962). Attempts were made in the

present study to fractionate osmotically disrupted synaptosome preparations on a density gradient in the presence of 30mM-sodium chloride. Excessive coacervation occurred and most of the particulate material travelled to the bottom of the gradient.

Ionic-strength effects and the presence or absence of electrolytes are known to determine the subcellular distribution of several other enzymes such as glutamine synthetase (Verster, Sellinger & Harkin, 1965) and choline phosphate cytidylyltransferase (Schneider, 1963); a differential binding of LDH isoenzymes to ribosomes has also been noted (Keck & Choules, 1962). The subcellular distribution of low-molecular-weight enzymes will, in future, have to be examined with particular care before definite conclusions can be drawn.

This work was supported in part by U.S. Public Health Service grant no. NB-03928 to Dr V. P. Whittaker. The author expresses his sincere thanks to Dr V. P. Whittaker for continuous encouragement and helpful advice during the investigation, to Mr G. H. C. Dowe for skilful technical assistance, to Miss Lesley Swales for performing the acetylcholine estimations and to Mr J. W. Boyd for help with the electrophoretic separation. The author thanks the Norwegian Council for Scientific and Industrial Research for a fellowship during the investigation.

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