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Choline Acetyltransferase Binding to and Release from Membranes

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1. The binding of non-occluded choline acetyltransferase to synaptosome membranes is a reversible process that is primarily dependent on the pH and ionic strength of the suspending medium. 2. The distribution of soluble enzyme bound to synaptosome membranes was studied by density-gradient centrifuging. 3. Choline acetyltransferase shows enzyme activity both in the free and in the membrane-bound form. 4. Varying the temperature or prolonged hypo-osmotic treatment does not release the membrane-bound enzyme. 5. The release of choline acetyltransferase from membranes by different anions, thiols, adenosine nucleotides and enzyme substrates was studied.

ChAc* synthesizes the cholinergic transmitter acetylcholine from acetyl-CoA and choline. Subcellular fractionation of brain tissue has shown that the enzyme is present together with acetylcholine in the detached nerve terminal (Hebb & Whittaker, 1958; Gray & Whittaker, 1962; De Robertis, de Iraldi, Arnaiz & Salganicoff, 1962). The 'compartmentation' of these two components within the detached nerve terminal (synaptosome) has been studied by disrupting the latter in water (Johnson & Whittaker, 1963) and separating fractions containing soluble cytoplasmic constituents, synaptic vesicles, external membranes and intraterminal mitochondria by means of differential (De Robertis, Arnaiz, Salganicoff, de Iraldi & Zieher, 1963) and density-gradient (Whittaker, Michaelson & Kirkland, 1964) centrifuging.

Hypo-osmotic rupture of synaptosomes prepared from guinea-pig brain and separation of the resulting components by density-gradient centrifuging showed that ChAc was localized in the soluble cytoplasm and acetylcholine in the synaptic vesicles (Whittaker *et al.* 1964). In contrast, differential centrifuging of ruptured synaptosomes from rat brain led to the isolation of both ChAc and acetylcholine in the high-speed pellet, which was believed to be rich in synaptic vesicles (De Robertis *et al.* 1963). When ruptured synaptosomes from rat brain were subjected to density-gradient centrifuging, however, acetylcholine was localized with synaptic vesicles and ChAc with heavier membranes (Fonnum, 1966*a*, 1967*a*; Tuček, 1966*b*). It was further demonstrated that the particulate ChAc could be solubilized by increasing the ionic strength and pH to values closer to those likely to prevail in nerve terminals *in vivo* (Fonnum, 1966*a*, 1967*a*).

The species difference in localization of ChAc between rat, rabbit, guinea pig and pigeon claimed by McCaman, Arnaiz & De Robertis (1965) could be explained by the different affinities of membranes at low ionic strength for ChAc from these species (Fonnum, 1967*a*). In view of the many additional reports of the occurrence of particulate ChAc in preparations of low ionic strength, such as ChAc in disrupted synaptosomes from sheep, cat and dog brain (Tuček, 1966*a*) and in sucrose homogenates from goat ventral root and sciatic nerve (Hebb & Silver, 1963), it was decided to investigate the various factors that may favour the presence of soluble or particulate forms of the enzyme. Such information would be of importance whenever a particulate localization of ChAc is suspected.

Particular attention was paid to the binding of soluble ChAc to synaptosome membranes and to the distribution of the bound enzyme on density-gradient centrifuging. The activity of the enzyme in the free and membrane-bound form is compared. The release of particulate ChAc by time, temperature, ionic environments and different compounds was studied. The results are compared with the behaviour of acetylcholine whenever known. A preliminary report has been given (Fonnum, 1967*b*).

METHODS

Preparation of fractions

Primary fractions. The experiments were carried out with rat cerebra obtained from the whole brain by discarding the parts caudal to the superior colliculi. The subcellular fractions were prepared essentially as described by

* Abbreviation: ChAc, choline acetyltransferase (acetyl-CoA–choline *O*-acetyltransferase, EC 2.3.1.6).

Whittaker *et al.* (1964). The tissue samples (2–4g.) were homogenized in 0·32M-sucrose and the homogenate was centrifuged at 1000*g* for 10min. (The average *g* value is quoted for all experiments.) The pellets were washed by resuspending them in 0·32M-sucrose and recentrifuging under the same conditions. The combined supernatants were centrifuged at 10000*g* for 20min. The pellet was again washed by resuspending it in 0·32M-sucrose and centrifuging at 10000*g* for 30min. The final pellet was called fraction P_2, and has been shown to consist of myelin, membranes, synaptosomes and mitochondria (Whittaker *et al.* 1964; Whittaker, 1968).

Hypo-osmotic treatment. The fraction P_2 pellet was resuspended in cold water (5ml./g. of original tissue), and the pH was adjusted to 7·2 with a few drops of dil. NaOH. The temperature of the suspension was never allowed to rise above 10° during this procedure, which took about 15min.

Release of ChAc. Samples (2ml.) of hypo-osmotically treated fraction P_2 were transferred to stainless-steel tubes, the solutions (2ml.) to be investigated were added and the contents were well mixed. The pH was again controlled and adjusted if necessary. The tubes were centrifuged at 105000*g* for 45min. The supernatant was poured off, and the pellet was resuspended in 6ml. of 2mM-EDTA, pH7·4, unless otherwise stated.

Readsorption of ChAc. Fraction P_2 was resuspended in water (5ml./g. of original tissue) as described above; 60mM-NaCl (2·5ml./g. of original tissue) was added and the pH adjusted to 7·2. Under these conditions most of the ChAc (70%) was found to be present in the soluble form. The enzyme was then readsorbed on to membranous material by lowering the pH and ionic strength by gel-filtering samples (3ml.) through a 12ml. column of Sephadex G-25 (coarse grade). The Sephadex column had been equilibrated with either 1mM-sodium phosphate buffers of various pH values or 1mM-sodium phosphate buffer containing 3mM-, 8mM- or 13mM-NaCl, pH7·2. Eluate (4ml.) was collected, and this contained 70–90% of the amount of protein and ChAc applied. The pH and the ionic strength of the eluate were measured, and the sample was centrifuged at 105000*g* for 45min.

Density-gradient centrifugation. A more homogeneous preparation of synaptosomes was obtained by layering 10ml. of fraction P_2 suspended in 0·32M-sucrose on a discontinuous sucrose gradient consisting of 10ml. each of 1·0M- and 1·2M-sucrose. Separation was achieved by centrifugation for 120min. at 53000*g* in the SW 25 head of the Beckman Spinco model L ultracentrifuge. The fraction on top of 1·2M-sucrose was collected, diluted 1:1 with water and centrifuged at 105000*g* for 30min.

After hypo-osmotic treatment of purified synaptosomes or readsorption of ChAc on synaptosome membranes, samples (5ml.) were layered on a discontinuous sucrose 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 centrifugation for 150min. at 53000*g* in the SW 25 head. The tube was sliced by a tube cutter into seven fractions corresponding to subfractions *O*, *D*, *E*, *F*, *G*, *H* and *I* as described by Whittaker *et al.* (1964).

Analysis of fractions

ChAc. This was assayed essentially as described by Fonnum (1966*b*). The incubation mixture contained (final concentrations): 5mM-sodium [1-^{14}C]acetate (600000 counts/min.), 8mM-choline, 5mM-$MgCl_2$, 0·2M-NaCl, 40mM-NaF, 10mM-ATP, 0·05mM-CoA, 0·5mM-KBH_4, 0·1mM-eserine salicylate and extract from 15mg. of acetone-dried pigeon liver (Sigma Chemical Co., St Louis, Mo., U.S.A.). The samples were treated with hydroxylamine and deproteinized with trichloroacetic acid as described by Fonnum (1966*b*). The radioactive acetylcholine was precipitated by adding sodium tetraphenylboron (Kalignost; E. Merck A.-G., Darmstadt, Germany) in 6ml. of 0·1M-sodium acetate. This alters the pH of precipitation to about 5 and decreases the blank value. The precipitate was washed twice with ether containing a drop of acetic acid.

Non-occluded enzyme activity was assayed by adding the resuspended pellet directly to the incubation mixture. Occluded enzyme activity was taken as the increase in activity obtained after adding 0·05vol. of a neutralized solution of 10% (v/v) Nonex (British Drug Houses Ltd., Poole, Dorset) to the resuspension.

The effect of ions on the enzyme activity (Table 5) was tested with an assay mixture based on synthetic acetyl-CoA: 0·15mM-[1-^{14}C]acetyl-CoA (160000 counts/min.), 2mM-choline, 0·1mM-eserine salicylate and various concentrations of NaCl and sodium phosphate buffer. The assay mixture (45μl.) was diluted to 5ml. and the radioactive acetylcholine was extracted into 1ml. of butyl ethyl ketone containing 15mg. of Kalignost (Fonnum, 1968*b*).

Protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Human serum albumin was used as a standard. When interfering substances were present, e.g. thiols, the proteins were first precipitated in 10% (w/v) trichloroacetic acid and redissolved in 0·1N-NaOH. Separate experiments showed that the results were not significantly altered by this procedure.

Ionic strength. The ionic strength was estimated after the determining of the sodium and potassium concentrations by a Perkin–Elmer flame photometer.

RESULTS

Release of ChAc

Effect of time. In the standard procedure adopted in the present paper the hypo-osmotic treatment lasted for 15min. before centrifuging. Acetylcholine leaks out of the synaptic vesicles during their preparation (Whittaker & Sheridan, 1965). The release of ChAc may similarily be expected to increase during prolonged hypo-osmotic treatment. Fraction P_2 was hypo-osmotically treated and diluted 1:1 with 2mM-sodium phosphate buffer at pH7·2, and samples were centrifuged after 0·25, 1·25 and 24hr. (Table 1). Only minor differences were observed. There was a slight increase in bound ChAc after 1·25hr. and a slight decrease after 24hr.

Effect of temperature. Acetylcholine is easily released from synaptosomes at higher temperatures (Whittaker, 1959) whereas lactate dehydrogenase and ChAc are not (Fonnum, 1967*a*). Also, synaptic vesicles readily release their acetylcholine content on warming (Whittaker *et al.* 1964; Barker, Amaro &

Table 1. *Effect of time on the release of ChAc and protein after hypo-osmotic treatment of fraction P_2*

The fraction was incubated in 1mM-sodium phosphate buffer, pH7·2, at 0°. At the time-intervals indicated the suspensions were centrifuged at +2° and assayed as described in the text. The results are expressed as the percentages of total activity recovered found in the high-speed supernatant.

	Activity released (%)	
Time (hr.)	ChAc	Protein
0·25	38, 45	23, 24
1·25	29, 34	25, 26
24	44, 44	24, 26

Table 2. *Effect of temperature on the release of ChAc and protein after hypo-osmotic treatment of fraction P_2*

The fraction was incubated in 0·5mM-sodium phosphate buffer, pH7·2, at various temperatures for 30min. The suspensions were then centrifuged at +2° and assayed as described in the text. The results from two separate experiments are given, and they are expressed as the percentages of the total activity recovered found in the high-speed supernatant.

	Activity released (%)	
Temperature	ChAc	Protein
0°	29, 27	29, 23
15	35, 21	27, 21
25	36, 25	28, 23

Guth, 1967; Marchbank, 1968). It was therefore decided to study the effect of temperature on the release of the particulate ChAc after hypo-osmotic treatment. Fraction P_2 was treated with water and diluted 1:1 with 1mM-sodium phosphate buffer, and the suspension was incubated at 0°, 15° and 25° for 30min. The samples were then cooled and centrifuged as before. There was negligible effect of higher temperatures on the solubilization of ChAc (Table 2). This finding again points to the large difference between ChAc and acetylcholine in the nature of the binding to components of the disrupted synaptosome. It also shows that temperature differences during and after the hypo-osmotic rupture and the time of resuspension in hypo-osmotic media cannot explain the differences in the proportion of soluble and particulate ChAc observed in different Laboratories.

Effects of sodium salts. The release of ChAc as a function of sodium chloride concentration at pH7·2 is shown in Fig. 1. This curve is used as a reference for the study of the effect of other compounds on the solubilization of ChAc. Hypo-osmotic suspensions of fraction P_2 had pH about 6·8 and the addition of salts frequently lowered this value. Adjustment of the pH was therefore carried out before and after adding the compounds under investigation. ChAc was readily released from rat cerebral synaptosomes at this pH and most of the enzyme was obtained in the soluble form at 15mM-sodium chloride. Further increase of the ionic strength up to 1·0 increased the proportion of soluble ChAc to 80–90%. There was no tendency to rebind at the high ionic strength; rebinding may be found when pH is not adjusted, owing to the decrease in pH on addition of salts.

The proportion of the soluble protein varied very little with the sodium chloride concentration. This indicated that the fraction of synaptosomes destroyed and the fraction of cytoplasmic protein released were easily reproduced from experiment to experiment. The effect of resuspending the fraction P_2 in different media, and the subsequent distribution of soluble, non-occluded and occluded ChAc, are given in Table 3. When fraction P_2 was resuspended in iso-osmotic sucrose, most of the enzyme appeared in an occluded particulate form, which is the intact synaptosome. The low non-occluded soluble and particulate enzyme activities show that very little disruption of synaptosomes occurred on resuspension. A low uptake of substrates into the synaptosome during incubation might account for some of the non-occluded particulate enzyme activity.

When fraction P_2 was resuspended in water and the pH adjusted to 7·2, the enzyme was mainly non-occluded, but it was mostly present in the particulate form. Addition of sodium chloride to this suspension transferred nearly all the enzyme activity into the high-speed supernatant. The hypo-osmotic conditions therefore released the enzyme by disrupting the synaptosome, but owing to the low ionic strength it was still adsorbed to particles. An increase in ionic strength broke the loose bond between the enzyme and the particles.

The sodium salts of several different anions, such as fluoride, bromide, nitrate and nitrite, were tested in 20mM concentrations and released 60–70% of the ChAc. The multivalent anions phosphate, citrate, oxalate and sulphate released 70–80% of the enzyme when tested at the same molar concentrations as the univalent anions. This is expected from the higher ionic strength of their solutions.

Effect of thiols. ChAc is activated and protected by the presence of thiols in the assay mixture (Morris, 1967). It was therefore decided to test whether these compounds had any effect on the solubilization of the enzyme. Table 4 shows that the thiols investigated were less effective than sodium chloride (Fig. 1) at similar concentrations.

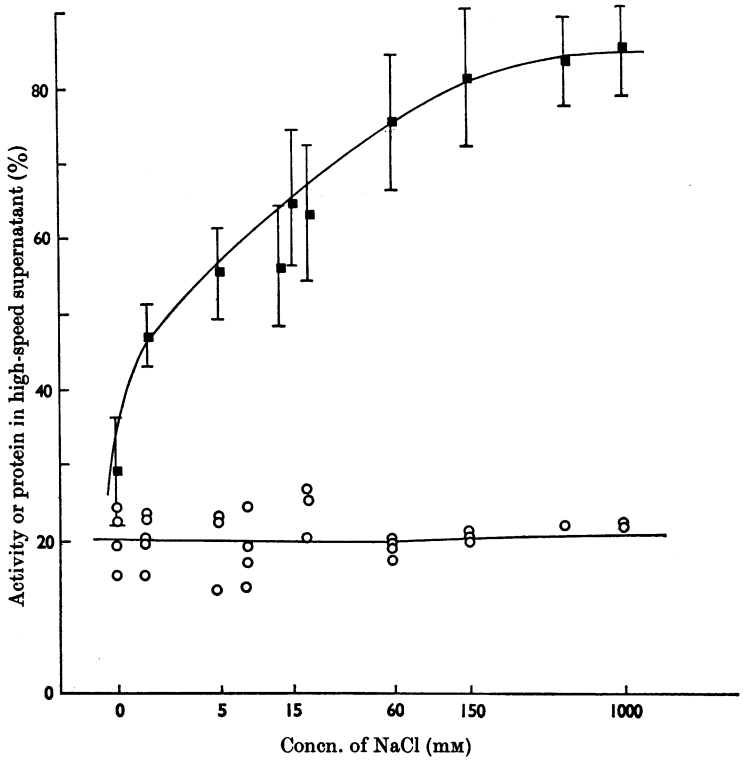

Fig. 1. Release of ChAc (■) and protein (○) from fraction P_2 at pH 7·2 and various NaCl concentrations. The symbols represent the percentages of the total activity recovered found in the high-speed supernatant. The symbols for ChAc represent mean values ± S.D. of three to eight separate experiments; for protein they represent single measurements.

Table 3. *Distribution of soluble and particulate ChAc activity in fraction P_2*

The fraction was prepared as described in the text. It was then resuspended in 0·32 M-sucrose, in water at pH 7·2, or in water followed by adjustment of I to 0·150 with NaCl. After centrifugation, the ChAc activity was assayed in the supernatants, and in the pellets resuspended in 0·32 M-sucrose (non-occluded) and after activation with 0·5% Nonex (non-occluded + occluded). Values are expressed as percentages of total recovered activities, and are means ± S.D. of three observations.

	Activity %								
Suspending medium ...	0·32 M-Sucrose			Water, pH 7·2			Water + 150 mM-NaCl, pH 7·2		
Fraction ...	Soluble	Particulate		Soluble	Particulate		Soluble	Particulate	
		Non-occluded	Occluded		Non-occluded	Occluded		Non-occluded	Occluded
	5 ± 2	16 ± 2	79 ± 2	34 ± 10	59 ± 9	7 ± 3	80 ± 14	7 ± 6	13 ± 6

Dithiothreitol, which is one of the most active thiols known, was the least effective in solubilizing ChAc. A mixture of cysteine and sodium chloride did not enhance the release of the enzyme above that expected.

Effect of adenosine nucleotides. It has been claimed that glutamine synthetase may be specifically released from brain microsomes by adenosine nucleotides (Sellinger, Verster, Sullivan & Lamar, 1966). It might be that these energy-rich compounds were particularly effective in releasing enzymes like ChAc from membranes. The con-

Table 4. *Release of ChAc and protein in the presence of thiols, adenosine nucleotides, and substrates from fraction P_2 after hypo-osmotic treatment*

The fractions were prepared as described in the text. The results are the means of the numbers of experiments given in parentheses.

Group	Compound	Concn. (mM)	Percentage released	
			ChAc	Protein
Thiols	Cysteine	5·0	30 (2)	23 (3)
		10·0	36 (2)	26 (3)
	Glutathione	3·5	43 (2)	25 (2)
		7·0	48 (2)	27 (2)
	Dithiothreitol	5·0	27 (2)	24 (2)
		10·0	34 (1)	24 (2)
	Cysteine + NaCl	5·0 + 5·0	53 (2)	24 (2)
Adenosine nucleotides	ATP*	1·00	42 (3)	27 (3)
		0·50	36 (3)	27 (3)
	ATP†	1·00	70 (2)	26 (3)
	ADP	1·67	61 (3)	27 (2)
		0·83	43 (3)	27 (2)
	AMP	3·30	66 (3)	26 (2)
		1·65	57 (3)	26 (2)
Substrates	Acetylcholine	10·0	60 (3)	23 (3)
	Choline	10·0	66 (3)	24 (3)
	Acetyl-CoA	0·1	26 (3)	22 (3)

* pH 6·8.
† pH 7·0 (in 0·5 mM-phosphate buffer, and with ouabain).

centrations selected for this investigation were those that would give an ionic strength similar to 0·005 and 0·01, assuming that all the phosphate groups were fully ionized.

The results (Table 4) were obscured by the hydrolysis of the compounds by phosphatases. AMP was the most efficient agent and solubilized ChAc slightly better than did sodium chloride at the corresponding ionic strength. ADP gave slightly lower results, and ATP the lowest values. When ATP was tested in the presence of ouabain, an adenosine triphosphatase inhibitor, and weak phosphate buffer, higher results were obtained.

Effect of substrates. ChAc *in vivo* will be surrounded by acetyl-CoA, choline and acetylcholine. The release of ChAc from a hypo-osmotically treated suspension by these compounds was examined after the acetylcholinesterase activity had first been inhibited by di-isopropyl phosphorofluoridate (10μM for 10 min.). Table 4 shows that the low concentrations of choline and acetylcholine tested were sufficient to solubilize most of the enzyme.

Binding of ChAc

If the particulate ChAc is present only because of the low ionic strength and fall in pH accompanying hypo-osmotic treatment, it should be possible to convert the enzyme from a soluble into a particulate form by changing the pH and ionic strength.

Effect of pH. ChAc was solubilized after hypo-osmotic treatment by bringing the ionic strength to 0·02 with sodium chloride and the pH to 7·2. Under these conditions about 70% of the enzyme was soluble (Fig. 1). The pH and ionic strength of the suspensions were then changed by gel filtration on Sephadex columns that had been equilibrated with 1 mM-sodium phosphate buffer at pH values in the range 6·2–7·4. The results showed (Fig. 2) that after the decrease of the pH and ionic strength there was a substantial increase in particulate ChAc. As expected, a larger increase in bound ChAc occurred at the lower pH values, and the final curve was in agreement with that obtained by studying the release of ChAc as a function of pH (Fonnum, 1967*a*). Separate experiments with gel filtration of soluble enzyme in the absence of membranes, at the lowest pH and ionic strength, showed no conversion of the enzyme into an insoluble form. This showed that there was a binding of enzyme to particulate matter and not a precipitation of the enzyme near its isoelectric point. Gel filtration of enzyme, in the presence of membranes, on Sephadex equilibrated with medium of ionic strength 0·015 and pH 7·2 (Fig. 2*b*) yielded a proportion of soluble ChAc that was in good agreement with that present in the

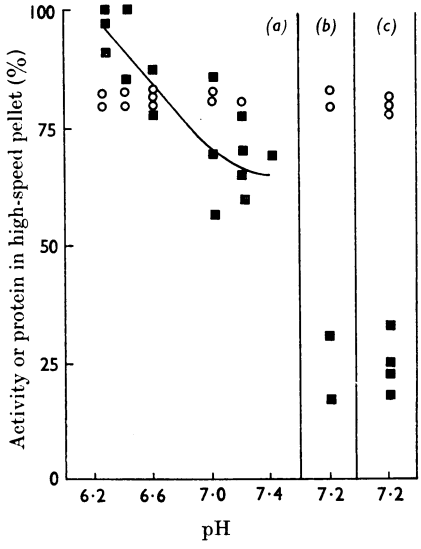

Fig. 2. Binding of ChAc (■) and protein (○) to membranes from fraction P_2 at various pH values. The results are expressed as percentages of the total recovered ChAc activity and protein found in the high-speed pellet. (*a*) Gel filtration on Sephadex columns equilibrated with 1mM-sodium phosphate buffer at various pH values; (*b*) gel filtration on Sephadex columns equilibrated with a solution of ionic strength 0·015 and pH 7·2; (*c*) dilution of sample with water to the elution volume of the Sephadex columns to give final ionic strength 0·015 and pH 7·2 (see the Methods section under 'Readsorption of ChAc'). The recovery of protein and ChAc was 75–95%.

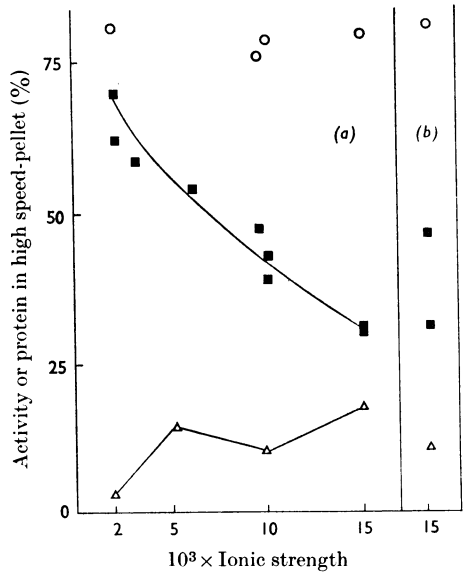

Fig. 3. Binding ChAc and proteins to membranes from fraction P_2 at various ionic strengths. The results show total particulate ChAc (■), occluded particulate ChAc (△) and protein (○), and are expressed as percentages of the total recovered found in the high-speed pellet. The occluded enzyme represents the increase in activity after activation of the resuspended high-speed pellet with Nonex. (*a*) Gel filtration on Sephadex columns equilibrated with 1mM-sodium phosphate buffers plus NaCl to the final ionic strength and pH 7·2; (*b*) dilution of sample with water to the elution volume of Sephadex columns giving final ionic strength 0·015 and pH 7·2 (see the Methods section under 'Readsorption of ChAc'). The recovery of protein and ChAc was 70–90%.

original suspension (Fig. 2*c*). Gel filtration as such did not therefore promote any binding of the enzyme. The percentage of soluble proteins was about 20% in all the fractions, and again this agreed with the results shown in Fig. 1.

Effect of ionic strength. The binding of soluble ChAc to membranous material at various ionic strengths but constant pH is shown in Fig. 3. As expected, more ChAc became attached to the particulate matter when the ionic strength was decreased. The results are in good agreement with those showing the release of ChAc (Fig. 1). The particulate enzyme activity was mainly present in the non-occluded form, and the occluded enzyme activity was similar at all ionic strengths tested except the very lowest (Fig. 3). The particulate enzyme was therefore caused by adsorption of the enzyme on membranes rather than reocclusion of the enzyme into 'ghost' particles formed by membranes.

Binding capacity of fraction P_2. ChAc was released from fraction P_2 as usual, and centrifugation of this fraction gave a pellet containing synaptosome membranes and a high-speed supernatant containing ChAc and other soluble proteins. To membranes derived from 250mg. of original wet tissue was added the high-speed supernatant derived from 125, 250, 375 and 500mg. of original wet tissue. The samples were made up to 3ml. and gel-filtered on Sephadex columns equilibrated with 1mM-sodium phosphate buffer, pH 7·0. The gel-filtered suspensions were centrifuged and ChAc was assayed in the new pellets and supernatants. The results (Fig. 4) show that the membranes had capacity to bind at least twice as much ChAc as that present in the corresponding supernatant. The soluble part of the total ChAc activity was similar in all the fractions: 18, 24, 32 and 28% of the total. This indicated that there might be a fixed proportion of total ChAc that is soluble at a given pH and ionic strength; provided that the membranes are not saturated with the enzyme, this proportion might be independent of the amount of enzyme or of membranes in the suspension.

Active forms of ChAc. Throughout this paper ChAc was assayed in a medium of high ionic strength corresponding to about 300mM-sodium chloride. In this medium the activity was not influenced by the presence of membranes. The recovery of enzyme activity was the same if the enzyme was originally associated with membranes or if it was present in the high-speed supernatant. Under these assay conditions the non-occluded ChAc is completely solubilized (Fig. 1). The enzyme is, however, not only released by increasing ionic strength, but also activated by the presence of a high concentration of sodium chloride (McCaman & Hunt, 1965; Morris & Tuček, 1966; Schuberth, 1966). To determine whether ChAc is more active in the soluble than in the membrane-bound state, the enzyme was assayed in the presence or absence of synaptosome membranes. It was possible to study the enzyme activity with a series of different ratios between bound and soluble enzyme, by assaying the enzyme under different conditions of pH and ionic strength. The enzyme activity found in the presence of 300mM-sodium chloride was taken as 100%. A purified synaptosome fraction was used as enzyme source. Enzyme in the presence of membranes was obtained by hypo-osmotic treatment followed by gel filtration on a Sephadex column equilibrated with 1mM-sodium phosphate buffer. Enzyme in the absence of membranes was obtained by treating the above fraction with 10mM-sodium phosphate buffer, pH 7·2, and centrifuging. The high-speed supernatant was then gel-filtered as above. The maximal enzyme activities of both preparations were in good agreement. The proportion of ChAc bound (Table 5, column 5) was determined separately after incubation of the enzyme preparation in the assay medium devoid of acetyl-CoA and centrifuging. The results (Table 5) showed that the stimulation of enzyme activity by ions was similar for both preparations. There was no suggestion that ChAc became more active when solubilized. Under the present assay conditions (2mM-choline and 0·15mM-acetyl-CoA) the soluble and membrane-bound forms of ChAc were equally active. The degree of activation agreed well with earlier published work on purified enzyme preparations (McCaman & Hunt, 1965; Schuberth, 1966; Morris & Tuček, 1966).

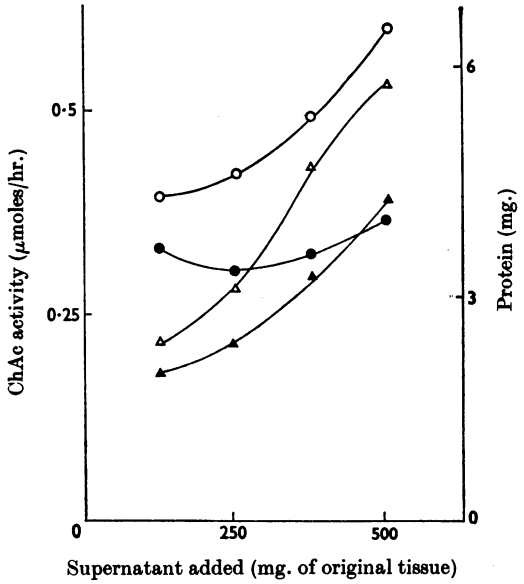

Fig. 4. Capacity of membranes from fraction P_2 (from 250mg. of original tissue) to bind soluble ChAc (from 125–500mg. of original tissue). △, Total ChAc; ▲, membrane-bound ChAc; ○, total protein; ●, membrane-bound proteins. For experimental details see the text.

Subcellular distribution

After it has been established that soluble ChAc could be bound to membranes by decrease in ionic strength and pH of the suspending medium, it was

Table 5. *Enzyme activity of free and membrane-bound ChAc*

ChAc was assayed with synthetic acetyl-CoA as described in the text. All buffers were sodium phosphate.

Assay conditions		Activity (% of maximum)		
pH	Concn. of buffer (mM)	Membrane absent	Membrane present	Non-occluded particulate ChAc (% of total activity)
7·0	1	7	7	78
6·5	5	12	11	70
6·5	10	17	20	60
7·0	5	18	17	42
7·1	10	24	24	30
7·2	10*	100	100	15

* With 300mM-NaCl.

decided to investigate which membranes had affinity for the enzyme. In particular, the distribution of ChAc after the binding of soluble enzyme to membranes was compared with the distribution after hypo-osmotic rupture of synaptosomes, since this would demonstrate the extent to which binding and redistribution of ChAc are of importance.

To decrease the contamination of non-synaptosomal constituents, the experiments were carried out on a pellet of purified synatosomes. The hypo-osmotic treatment was carried out by resuspending the pellet in 1 mM-sodium phosphate buffer, pH 6·8. The binding was carried out, after release of the enzyme (ionic strength 0·02 and pH 7·2), by gel filtration on a Sephadex column equilibrated with 1 mM-sodium phosphate buffer, pH 6·8. The gradient appeared similar to that described by Whittaker *et al.* (1964) and Fonnum (1967*a*).

The distribution of ChAc after hypo-osmotic treatment is shown in Fig. 5(*a*) and after binding of soluble enzyme in Fig. 5(*b*). The most striking feature is the similarity between Figs. 5(*a*) and 5(*b*). In both cases the highest specific activity of ChAc was exhibited at about 0·8 M-sucrose (fraction *F*). The specific activity of this fraction was five to seven times that in the original brain homogenate. Slightly more ChAc was present in fraction *G* than in fraction *E*. The ChAc associated with the membranes was in both cases mainly of the non-occluded form. The results showed that ChAc was mainly localized on membranes larger than the synaptic vesicles. The high capacity of fraction P_2 (Fig. 4) for ChAc does not exclude the possibility that vesicle membranes may have capacity to bind an appreciable part of the ChAc present in the synaptosome.

When fractions *E*, *F* and *G* were pooled and treated with an iso-osmotic solution of sodium chloride at pH 7·2, most of the non-occluded ChAc was solubilized and the relative specific activity of the supernatant was 14 times that of the brain homogenate. This is a significantly higher specific activity for ChAc than that reported in previous publications on subcellular fractionation of this enzyme (Fonnum, 1967*a*; Tuček, 1966*b*; McCaman *et al.* 1965).

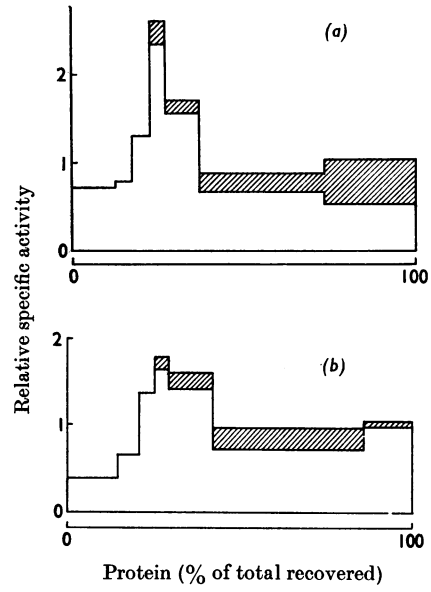

Fig. 5. Distribution of non-occluded (□) and occluded (■) ChAc in fractions separated by discontinuous density-gradient centrifuging. (*a*) Suspension of hypo-osmotically disrupted synaptosomes; (*b*) after the binding of soluble ChAc to synaptosome membranes. The blocks correspond to fractions *O–I* described by Whittaker *et al.* (1964), reading from left to right. The width of each block is proportional to the percentage of total recovered protein found in the fraction; the area of each block is proportional to the percentage of total recovered activity found in the fraction.

DISCUSSION

The present work has demonstrated that the particulate ChAc obtained after hypo-osmotic treatment could have been formed by binding of soluble enzyme to synaptosome membranes. The non-occluded character of the enzyme activity also favours such a binding. Separate experiments showed that the particulate form was not caused by precipitation of the soluble enzyme due to the low ionic strength and pH, neither was it caused by reocclusion of enzyme within 'ghost' particles formed from membranes. The binding of ChAc was found to be reversible, and the proportion bound was determined primarily by the pH and ionic strength of the suspension rather than by the amount of enzyme or the amount of membranes present.

The distribution of ChAc between the soluble and particulate forms did not depend on the previous form of the enzyme. Soluble and particulate enzyme gave remarkably similar distributions when either released or bound under the same conditions of ionic strength and pH. The ratio of soluble to bound enzyme found at various ionic strengths at pH 7·2 was remarkably similar after release of ChAc from membranes (Fig. 1) and binding of ChAc to membranes (Fig. 3). The curve representing the binding of ChAc (Fig. 2) at various pH values at constant ionic strength was also similar to that obtained by releasing ChAc from membranes under similar conditions (Fonnum, 1967*a*).

The enzyme was active in both the soluble and membrane-bound forms. Hence the active site of

the enzyme was not involved in the binding, neither was the configuration of the molecule changed.

The release of non-occluded particulate ChAc was independent of the incubation temperature between 0° and 25° and nearly independent of the incubation time between 15min. and 24hr. None of the many compounds examined was distinctly more efficient than sodium chloride in releasing ChAc. The effects of the different anions tested correlated well with their ionic strengths. Thiols had very little effect on the release of ChAc. Adenosine nucleotides behaved similarly to sodium chloride and their effects did not correlate with their energy levels. A re-examination of the findings of Sellinger *et al.* (1966) might show that the adenosine nucleotides, which were compared only on a molar basis, were equally effective when compared on an ionic strength basis. A noteworthy finding is that acetylcholine and choline solubilize ChAc. This means that, if the enzyme operates in a closed compartment like the synaptic vesicle in the presence of high choline and acetylcholine concentrations, it must be soluble there. It has been suggested that the acetylcholine concentration inside the synaptic vesicle is about 150mM (Whittaker & Sheridan, 1965).

The subcellular localization of ChAc after binding of the enzyme to isolated synaptosome membranes gave results similar to those obtained on hypo-osmotic rupture of purified synaptosomes. The highest affinity for ChAc was found in both cases in fractions containing large membranes with densities similar to that of 0·8M-sucrose. This finding supports the theory that the particulate localization of ChAc after hypo-osmotic treatment is due to a redistribution of the enzyme and does not necessarily bear any relation to its location *in vivo*.

It is possible that the high affinity for ChAc shown by membranes within the synaptosome might have some physiological meaning. If the synthesis of acetylcholine takes place outside the vesicles, the enzyme might be involved in the transport of acetylcholine into the vesicle. So far, however, the uptake of acetylcholine to a pure vesicle fraction has not been reported.

The results are noteworthy in relation to other findings of particulate ChAc. They indicate that, whenever a non-occluded particulate ChAc is suspected, the sample should be treated with a solution of sodium chloride or phosphate buffer at pH 7·4. The effect of pH is of particular importance for ChAc. The effects of temperature, time, degree of hypo-osmoticity and amounts of enzyme or membranes are of less importance. Other enzymes, at first thought to be particulate, are released by similar treatment. They include lactate dehydrogenase from chicken skeletal muscle (Hultin & Westort, 1966) and from pigeon brain (Fonnum, 1967*a*), hexokinase from rat brain (Biesold & Teichgräber, 1967) and glutamine synthetase (Verster, Sellinger & Harkin, 1965).

The effect of ions on the solubilization of ChAc is very different from their effect on glutamate decarboxylase, another enzyme present within the synaptosome. This enzyme is particulate in the presence of calcium chloride (Salganicoff & De Robertis, 1965; Fonnum, 1968*a*) and sodium chloride and potassium chloride (Fonnum, 1968*a*). ChAc is stimulated by sodium chloride and potassium chloride (McCaman & Hunt, 1965; Schuberth, 1966; Morris & Tuček, 1966) whereas glutamate decarboxylase, synthesizing γ-aminobutyric acid, is inhibited by these compounds (Susz, Haber & Roberts, 1966; F. Fonnum & A. Winsnes, unpublished work). The physiological functions of these two enzymes are also completely different, since acetylcholine is a probable candidate for a central excitatory transmitter and γ-aminobutyric acid for an inhibitory transmitter.

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