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Radiochemical Micro Assays for the Determination of Choline Acetyltransferase and Acetylcholinesterase Activities

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1. The methods for the assay of choline acetyltransferase were based on the reaction between labelled acetyl-CoA and unlabelled choline to give labelled acetylcholine. 2. Both synthetic acetyl-CoA and acetyl-CoA formed from sodium [1-^{14}C]acetate or sodium [^{3}H]acetate by incubation with CoA, ATP, Mg^{2+} and extract from acetone-dried pigeon liver were used. 3. [1-^{14}C]Acetylcholine was isolated by extraction with ketonic sodium tetraphenylboron. 4. [^{3}H]Acetylcholine was precipitated with sodium tetraphenylboron to remove a ketone-soluble contaminant in sodium [^{3}H]acetate and then extracted with ketonic sodium tetraphenylboron. 5. The values of choline acetyltransferase activity obtained in the presence of sodium cyanide or EDTA and synthetic acetyl-CoA were similar to those obtained with acetyl-CoA synthesized *in situ*. 6. The assay of acetylcholinesterase was based on the formation of labelled acetate from labelled acetylcholine. The labelled acetylcholine could be quantitatively removed from the acetate by extraction with ketonic sodium tetraphenylboron. 7. The methods were tested with samples from central and peripheral nervous tissues and purified enzymes. 8. The blank values for choline acetyltransferase and acetylcholinesterase corresponded to the activities in 20ng. and 5ng. of brain tissue respectively.

ChAc* and AChE are important enzymes in nervous tissue because of their role in the synthesis and hydrolysis of the chemical transmitter substance acetylcholine. ChAc catalyses the formation of acetylcholine from acetyl-CoA and choline; AChE catalyses the hydrolysis of acetylcholine to acetate and choline. The introduction of radioactively labelled substrates has provided sensitive and accurate assay methods for these enzymes.

Radiochemical methods for the assay of ChAc depend on the separation of the synthesized labelled acetylcholine from radioactive substrates such as acetyl-CoA and acetate and from by-products. This has been achieved by precipitation of acetylcholine with reineckate (McCaman & Hunt, 1965), sodium tetraphenylboron (Fonnum, 1966) or periodide (Goldberg, Kaita & McCaman, 1969), by isolation of acetylcholine by electrophoresis (Potter, Glover & Saelens, 1968; Giller & Schwartz, 1968), or by removing labelled anions from acetylcholine with an anion-exchange column (Schrier & Schuster, 1967). The only method that has been applied to microgram quantities of tissue is the method of McCaman & Hunt (1965), and a modification of this (Buckley, Consolo, Giacobini & McCaman, 1967).

Labelled acetylcholine was first used as substrate for AChE by Winteringham & Disney (1964). Sensitive micro methods depending on the separation of labelled acetate from labelled acetylcholine have been devised involving either extraction of acetic acid with pentanol in toluene (Potter, 1967) or removal of the radioactive acetylcholine by precipitation with reineckate (McCaman, Tomey & McCaman, 1968).

To determine the activities of ChAc and AChE in micro-dissected samples from thin freeze-dried sections of brain, it was necessary to develop more sensitive and less cumbrous assay methods for these enzymes. The methods were based on the observation that acetylcholine could be isolated from aqueous solutions by liquid cation exchange by using sodium tetraphenylboron in an organic solvent (Fonnum, 1968*a*, 1969). Three reaction mixtures for ChAc, based on different radioactive substrates, namely [1-^{14}C]acetyl-CoA, sodium [1-^{14}C]-acetate and sodium [^{3}H]acetate, are described. The last two substrates are converted into labelled acetyl-CoA by the inclusion in the incubation

* Abbreviations: ChAc, choline acetyltransferase (acetyl-CoA-choline *O*-acetyltransferase, EC 2.3.1.6); AChE, acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7).

media of CoA, ATP, Mg^{2+} and acetyl-CoA synthetase from acetone-dried pigeon liver. The sensitivities and the applications of the three incubation media are discussed. Particular attention is paid to comparison of the values obtained with synthetic acetyl-CoA and acetyl-CoA formed *in situ*. AChE was assayed by measuring the labelled acetate left in the aqueous phase after the quantitative removal of the unhydrolysed [1-^{14}C]acetylcholine by extraction with sodium tetraphenylboron in higher ketones. The assay methods may be used for submicrogram quantities of tissues and advantages over existing methods are discussed.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Acetylcholine chloride (sp. radioactivity 11 mc/m-mole), sodium [1-^{14}C]acetate (sp. radioactivity 52 mc/m-mole) and sodium [^{3}H]acetate (sp. radioactivity 5900 mc/m-mole) were obtained from the Radiochemical Centre, Amersham, Bucks. The radioactive acetates were dissolved in water and stored frozen. Sodium [^{3}H]acetate was in some cases freeze-dried to remove a volatile contaminant. [1-^{14}C]Acetylcholine was dissolved in 2 mM-sodium phosphate buffer, pH 7·4, and chromatographed on a column (1 ml.) of Dowex 1 (X2), equilibrated with the same phosphate buffer, to remove traces of labelled acetate. [1-^{14}C)-Acetyl-CoA (sp. radioactivity 58 mc/m-mole) was supplied by New England Nuclear Corp., Boston, Mass., U.S.A. and was stored at pH 5.

Acetone-dried pigeon liver (Sigma Chemical Co., St Louis, Mo., U.S.A.) was extracted with 20 mM-$KHCO_3$ and the clear supernatant gel-filtered on a column (10 ml.) of Sephadex G-25 (coarse grade) equilibrated with 20 mM-sodium phosphate buffer, pH 7·4. Sodium tetraphenylboron (Kalignost) was obtained from E. Merck A.-G., Darmstadt, Germany.

Enzyme preparations

Homogenates (5%, w/v) of rat cerebrum and cerebellum were prepared in 1 mM-EDTA buffer, pH 7·0, in a Potter–Elvehjem homogenizer. The clearance between pestle and wall was 0·2 mm., and the pestle was rotated at 1440 rev./min. Homogenates (5%, w/v) of rat diaphragm and sciatic nerve were prepared in an Ultra-Turrax homogenizer (Janke und Kunkel K. G., Bremen, Germany). The knife rotated at 20000 rev./min. for three periods of 15 sec. All samples were treated with 0·5% Triton X-100 to release full enzyme activity (Fonnum, 1966). The homogenates were diluted with 1 mM-EDTA to a final concentration of 5 μg. wet wt. of tissue/μl. Below this concentration the homogenate was diluted with heat-inactivated homogenate.

Partially purified ChAc was obtained from guinea pig brain by a modification of the method of Kumagai & Ebashi (1954), or from rat and cat brain by a modification of the method of Potter *et al.* (1968). The enzymes were purified 15–40-fold. Purified AChE (from bovine erythrocytes) was purchased from Sigma Chemical Co.

Standard assay procedure for ChAc

The incubation volume in the macro procedure was 1 ml. and it was 2 μl. in the micro procedures. When the incubation volume was 2 μl., 10 μl. of cyclohexane was added as a cover to prevent evaporation (Buckley *et al.* 1967). The presence of cyclohexane did not affect the enzyme activity nor the extraction procedures. The incubation temperature was 37°.

Macro procedure. The procedure was generally used when reaction volumes were larger than 50 μl. The incubation mixture contained (final concns.): 5 mM-sodium [1-^{14}C]acetate (10^6 c.p.m.), 12·5 mM-choline, 300 mM-NaCl, 50 mM-NaF, 10 mM-sodium phosphate buffer, 0·05 mM-CoA, 10 mM-ATP, 0·1 mM-eserine salicylate, 0·5 mM-KBH_4, 2·5 mM-$MgCl_2$ and extract from 12 mg. of acetone-dried pigeon liver. The pH was adjusted to 7·4. The incubation was carried out in 10 ml. centrifuge tubes with ground-glass stoppers. The mixture was incubated for 10 min. to preform acetyl-CoA and the reaction was started by addition of enzyme. The reaction was stopped by adding 7 ml. of an acetylcholine chloride solution (0·5 mg./7 ml.), followed immediately by 1 ml. of butyl ethyl ketone containing 25 mg. of sodium tetraphenylboron. After being shaken lightly for 4 min., the tubes were centrifuged at 3000*g* for 4 min. in a swing-out head, to separate the aqueous and organic phases. After gentle stirring of the ketone layer, the proteins soon separated out as a flat layer at the interface between the two phases. When more than 10 mg. of brain homogenate was used it was advisable to extract with 2 ml. of ketone. As much as possible of the ketone layer, containing acetylcholine, was transferred by a Pasteur pipette to another tube. The ketone layer was washed once with 4 ml. of 10 mM-sodium phosphate buffer, pH 7·4, containing 2 mg. of sodium tetraphenylboron. After recentrifugation a sample of the ketone layer, usually 0·5 ml., was transferred to a scintillation vial containing 2 ml. of acetonitrile and 10 ml. of toluene scintillation mixture (Fonnum, 1969). The radioactivity was determined in a Packard Tri-Carb model 3003 liquid-scintillation spectrometer. The counting efficiency was 73%.

Micro procedure based on [1-^{14}C]*acetate.* This procedure was used for reaction volumes less than 50 μl. The incubation mixture contained (final concns.): 2 mM-sodium [1-^{14}C]-acetate, extract from 2·5 μg. of acetone-dried pigeon liver/μl. of incubation mixture, and otherwise as described under 'Macro procedure'. The incubation mixture (100 μl.) was preincubated for 10 min., and 1 μl. was transferred with a constriction pipette to small conical tubes (2 mm. diam.) containing the enzyme preparation. After incubation the tube was transferred to a 10 ml. centrifuge tube containing 7 ml. of 10 mM-sodium phosphate buffer pH 7·4, and 0·25 mg. of acetylcholine chloride. The phosphate buffer solution was flushed with a Pasteur pipette three times into the conical micro tube, thus washing its contents into the large tube. The acetylcholine was extracted with 1 ml. of butyl ethyl ketone containing 15 mg. of sodium tetraphenylboron. The ketone layer was isolated by centrifugation, transferred to a new tube and washed with 4 ml. of 10 mM-sodium phosphate buffer containing 2 mg. of sodium tetraphenylboron. After recentrifugation the radioactivity of the ketone layer was determined as described above.

Micro procedure based on [1-^{14}C]*acetyl-CoA.* The procedure was used for purified enzymes and samples with low activities. The incubation mixture consisted of (final concns.): 0·1 mM-[1-^{14}C]acetyl-CoA, 10 mM-EDTA (sodium salt) or -NaCN, 10 mM-choline, 300 mM-NaCl, 50 mM-sodium phosphate buffer, 0·1 mM-eserine salicylate and albumin (0·5 mg./ml.). The final pH was 7·4. After incubation the conical tube was transferred to a 10 ml. centrifuge tube

containing 7ml. of 10mM-sodium phosphate buffer, pH7·4, and 0·25mg. of acetylcholine, and the contents were washed into the large tube. The acetylcholine was extracted with 1ml. of butyl ethyl ketone containing 15mg. of sodium tetraphenylboron. The radioactivity in the ketone layer was tested without further washing.

Micro assay based on sodium [^{3}H]*acetate.* The procedure was only used for samples with low activities. The incubation mixture contained (final concns.): 1·3mM-sodium [^{3}H]-acetate, extract from 2·5μg. of acetone-dried pigeon liver/μl. of incubation mixture, and otherwise as described under 'Macro procedure'. The incubation mixture without choline was preincubated for 15min. to preform acetyl-CoA. The reaction was started by adding the incubation mixture containing choline to small conical tubes containing the enzyme preparation. At the end of the incubation the conical tube was transferred to a large centrifuge tube containing 7ml. of acetylcholine chloride solution (0·5mg./7ml.), and the contents were washed into this tube. Then 1ml. of sodium tetraphenylboron in water (15mg./ml). was added and the solution left for 15min. to ensure that all the acetylcholine was precipitated. The precipitate was centrifuged down in a swing-out head at 3000g for 6min. and the clear supernatant discarded. To the tube was added 1ml. of butyl ethyl ketone containing 15mg. of sodium tetraphenylboron and 7ml. of 10mM-sodium phosphate buffer, pH7·4. After shaking for 3min. the precipitate was dissolved and the acetylcholine was extracted into the ketone layer. After centrifugation the radioactivity in the ketone layer was determined as described above. The counting efficiency was about 8%.

Standard assay procedure for AChE

Microassay procedure. The incubation mixture contained (final concns.): 0·5mM-[1-^{14}C]acetylcholine, 20mM-sodium phosphate buffer, pH7·2, and bovine serum albumin (0·8mg./ml.). The final incubation volume was 2·5μl. and the incubation was carried out at 30° for 1hr. After incubation, the contents of the incubation tubes were transferred into small centrifuge tubes containing 0·5ml. of 10mM-sodium phosphate buffer, pH7·4. The acetylcholine was removed by shaking with 0·5ml. of butyl ethyl ketone or di-isobutyl ketone containing 15mg. of sodium tetraphenylboron. After centrifugation the ketone layer was sucked off and the aqueous phase washed once more with ketonic sodium tetraphenylboron. A sample of the final aqueous layer was counted with 4ml. of ethanol and 10ml. of toluene scintillation mixture. The counting efficiency was 78%.

Extraction efficiency

The percentage of acetylcholine extracted may be calculated from the following equation (Fonnum, 1969):

$$\frac{(\%\text{ of acetylcholine})_{\text{organic phase}}}{(\%\text{ of acetylcholine})_{\text{aq. phase}}} = 70 \times \frac{(\mu\text{moles of sodium tetraphenylboron})^{1\cdot18}}{(\mu\text{moles of Na}^+)^{0\cdot94}}$$

RESULTS

ChAc

Extraction procedures. Earlier work (Fonnum, 1969) demonstrated that acetylcholine was extracted by sodium tetraphenylboron in benzyl alcohol, nitriles or higher ketones. Several extraction solvents were therefore compared to see in which the highest sample/blank (heat-inactivated brain homogenate) activity ratio was obtained. Unlabelled acetylcholine was added to avoid adsorption of radioactive acetylcholine on the homogenate. The results showed that butyl ethyl ketone was a slightly better solvent than butyronitrile, benzyl alcohol and di-isobutyl ketone (Table 1). The differences in activities in the samples are due to differences in counting efficiencies. In addition both butyronitrile and benzyl alcohol were much more water-soluble. Since the acetylcholine–tetraphenylboron complex was only slightly soluble in di-isobutyl ketone, this solvent was only considered for micro assays. The extraction procedures were less tedious and gave considerably lower blank values than those obtained by precipitation of acetylcholine with sodium tetraphenylboron and washing the precipitate with ether (Fonnum, 1968*b*). The extracted radioactivity was identified as acetylcholine by electrophoresis (Fonnum, 1969). Also, samples containing purified acetylcholinesterase instead of choline and eserine (physostigmine) gave values corresponding to the blank.

Nearly all the acetylcholine was recovered by the three isolating procedures (Table 2), and the percentages of acetylcholine found (94 and 95%) agreed well with the values of 94 and 96% determined from the above equation. The recoveries of acetylcholine in the macro method were found to be independent of the amount of homogenate. Since the butyl ethyl ketone is slightly soluble in water, and therefore some ketone (about 5%) is lost in the aqueous phase, the c.p.m./ml. of ketone was practically unchanged. The values obtained were therefore always used without compensating for any loss on recovery.

Less than 0·1% of the radioactivities of sodium [1-^{14}C]acetate and of [1-^{14}C]acetyl-CoA was left in the ketone layer after the first extraction (Table 2). Further washing had little effect on the remaining traces of [1-^{14}C]acetyl-CoA. Tritiated compounds are generally less stable than those labelled with ^{14}C (Oldham, 1968). When therefore the same extraction and washing procedure was used for [^{3}H]-acetate, unexpectedly high blanks were obtained. This was due to a contaminant formed in the radioactive acetate on storage and it could in part be removed by freeze-drying the solution before use. The contaminant was partly soluble in ketone and best avoided by precipitating the acetylcholine with sodium tetraphenylboron and then extracting with ketonic sodium tetraphenylboron.

The blank value with labelled acetate (Table 1) was higher than expected from Table 2 and it increased with increasing amounts of acetone-dried pigeon liver, probably owing to traces of ChAc activity in the latter. Experiments with material from two different batches of acetone-dried pigeon liver showed that 41 and 33 pmoles of acetylcholine were synthesized/hr./mg. of powder.

Assay conditions. Lower values for ChAc activity were obtained when the enzyme was assayed with incubation media based on synthetic acetyl-CoA than on acetyl-CoA formed *in situ* (for comparison see Fonnum, 1966). In the attempt to elucidate this discrepancy the effects of different compounds on ChAc activity and also the results obtained with different acetyl-CoA preparations were examined.

Addition of sodium cyanide and EDTA, but not magnesium chloride to the incubation medium (Table 3) increased the ChAc activity in rat homogenate and in purified enzyme preparations from cat, rat and guinea pig brain, to a value similar to that obtained with acetyl-CoA formed *in situ* (see also Table 4). Generally slightly higher values were obtained with sodium cyanide than with EDTA, but their effects were not additive. Higher concentrations of these two compounds did not improve the results further. The effect was most pronounced with unstable enzyme preparations such as dilute solutions of crude homogenate or partially purified enzymes. Sodium cyanide did not have any effect when acetyl-CoA formed *in situ* was used for the assay of ChAc.

When labelled acetyl-CoA (93% pure) was diluted with less pure unlabelled acetyl-CoA (70% pure), the time-course of the reaction was no longer linear (Fig. 1). The synthesis of acetylcholine when assayed with the methods based on labelled sodium acetate was linear with time for 90 min. (Fig. 1).

There was good correlation between the amount

Table 1. *Comparison of the values obtained by different extraction solvents for the determination of ChAc in brain tissue*

A represents the results obtained from 5mg. of brain tissue with the macro procedure and *B* represents the results obtained from 2 μg. of brain tissue with the micro procedure based on [1-^{14}C]acetate. Acetylcholine was isolated either by extraction with sodium tetraphenylboron in organic solvents or by precipitation with sodium tetraphenylboron as described by Fonnum (1968*b*). The results are expressed as c.p.m. found in total volumes of samples and blanks (heat-inactivated tissue).

Isolation method	Radioactivity (c.p.m.) Sample (X)	Blank (Y)	X/Y ratio
A Extraction with butyl ethyl ketone	5922	100	59
Extraction with butyronitrile	5722	98	58
Precipitation	5300	430	12
B Extraction with butyl ethyl ketone	800	36	22
Extraction with di-isobutyl ketone	860	46	19
Extraction with benzyl-alcohol	900	70	13

Table 2. *Recoveries of acetylcholine, sodium acetate and acetyl-CoA isolated from ChAc incubation mixture by the different standard procedures*

The recoveries are expressed as percentages of the intial concentration (allowing for loss in volume) and as c.p.m./0·5ml. of ketone (final vol.). The results are mean values ± S.D. from five samples.

	Macro procedure		Micro procedure based on (1-^{14}C]Acetate				
	[1-^{14}C]Acetylcholine		[1-^{14}C]Acetylcholine		[1-^{14}C]Acetyl-CoA	[1-^{14}C]Acetate	[^{3}H]Acetate
	(c.p.m.)	(%)	(c.p.m.)	(%)	(c.p.m.)	(c.p.m.)	(c.p.m.)
Initial concn.	60800	100	60800	100	12500	350000	400000
1st ketone extraction	59700 ± 500	97	61000 ± 1400	98	14 ± 1	18	550 ± 500
2nd ketone extraction	59700 ± 800	94	62000 ± 1000	95	12 ± 1	5>	300 ± 50

	Micro procedure based on [^{3}H]acetate		
	[1-^{14}C]Acetylcholine		[^{3}H]Acetate
	(c.p.m.)	(%)	(c.p.m.)
Initial concn.	60800	100	400000
Precipitation	60333 ± 1000	98	12000 ± 2000
1st ketone extraction	64200 ± 1500	96	35 ± 5

Table 3. *Effect of substances on the rate of synthesis of acetylcholine assayed with an incubation mixture based on* [1-^{14}C]*acetyl-CoA*

The results are obtained with 5 μg. of rat brain homogenate or 2 μl. of diluted solutions of partially purified enzyme from rat, cat and guinea pig brain. The incubation volume was 7 μl. The results from different brain homogenates are expressed as means ± S.D. Numbers of different brain homogenates tested are given in parentheses.

	Acetylcholine synthesized (pmoles)			
		Partially purified enzymes		
Substance	Rat brain homogenate	Rat	Cat	Guinea-pig
None	24·4 ± 5·5 (5)	128	21·9	41·2
EDTA (10 mM)	28·3 ± 4·8 (5)	144	24·7	67·1
NaCN (10 mM)	32·9 ± 8·1 (5)	181	32·8	61·2
EDTA (10 mM) + NaCN (10 mM)	34·1 (2)	154	—	—
$MgCl_2$ (20 mM)	23·5	131	—	—
[1-^{14}C]Acetate procedure	—	164	28·9	76·3

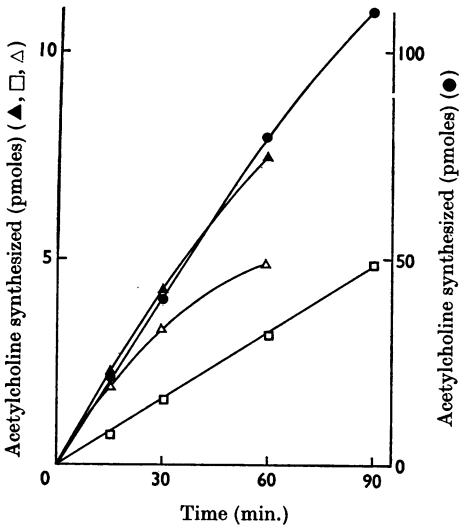

Fig. 1. Synthesis of acetylcholine determined at various times with the three microassay methods. The symbols represent the different micro assay procedures and the corresponding amounts of rat brain homogenate used: ●, [1-^{14}C]acetate, 9·5 μg.; □, [^{3}H]acetate, 0·5 μg.; ▲, [1-^{14}C]acetyl-CoA, 1 μg.; △, 1 part of [1-^{14}C]acetyl-CoA + 3 parts of acetyl-CoA, 1 μg.

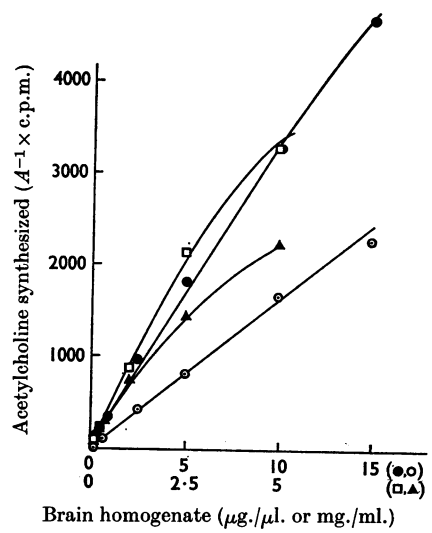

Fig. 2. Synthesis of acetylcholine by various concentrations of brain homogenate determined with the macro and micro assay methods. The enzyme activities are expressed as total c.p.m. of acetylcholine recovered in the ketone layer from 1 μl. or 1 ml. of incubation mixture. The c.p.m. in the sample are obtained by multiplying scale c.p.m. by the factor A. The symbols represent the different assay procedures and the corresponding factor A: ○, macro procedure, $A = 10$; ●, micro procedure with [1-^{14}C]acetate, $A = 1$; □, micro procedure with [^{3}H]acetate, $A = 5$; ▲, micro procedure with [1-^{14}C]acetyl-CoA, $A = 0·5$. On the abscissa, brain homogenate concentrations are given as mg./ml. for the macro procedure and μg./μl. for the micro procedure.

of acetylcholine synthesized and the amount of brain homogenate used (Fig. 2). The enzyme activities were expressed in c.p.m. to give an impression of the radioactivity in samples compared with the blank value. In the macro procedure a linear correlation was obtained for 0·1–15 mg. of brain homogenate. The corresponding ranges were 0·1–10, 0·05–2·5 and 0·025–1 μg. of brain wet wt./μl. of incubation mixture for the methods based on [1-^{14}C]acetate, [^{3}H]acetate and [1-^{14}C]acetyl-CoA respectively.

The macro and the three micro methods were compared by determining the ChAc activity in the

Table 4. *Comparison of values obtained by different ChAc assay procedure*

The activities are expressed as μmoles/hr./g. of tissue. All assay procedures were carried out as described in the Materials and Methods section.

Tissue	Macro procedure	Micro procedure		
	[1-^{14}C]Acetate	[1-^{14}C]Acetate	[^{3}H]Acetate	[1-^{14}C]Acetyl-CoA
Rat cerebrum	7·2	7·0	7·6	6·0
	7·9	8·0	7·8	5·6
Rat cerebellum	0·96	1·02	0·84	1·00
	1·00	0·86	0·80	0·72
Rat sciatic nerve	9·5	7·5	7·6	7·2
	9·2	8·2	7·9	7·6

Table 5. *Activity of AChE as a function of substrate concentration and ionic strength*

The results were obtained with purified AChE. Enzyme activity is expressed as a percentage of maximum activity.

Concn. of acetylcholine (mM)	AChE activity (relative values)	
	100 mM-Phosphate buffer	20 mM-Phosphate buffer
3·40	92	—
2·00	100	—
1·00	89	77
0·50	80	85
0·37	—	83
0·25	—	70

same samples from different tissues (Table 4). There was good agreement between the results obtained by the methods based on labelled acetate. This showed that the difficulties that had arisen from working with small volumes had been overcome. Slightly lower values, 100–75% of those from methods based on [1-^{14}C]acetate, were obtained with the method based on synthetic acetyl-CoA. The values agreed well with those published earlier (Hebb, 1963; Fonnum, 1966).

The blank values obtained on replacing the enzyme with heat-inactivated tissue were similar to those obtained on replacing it with water. The blanks were equivalent to 0·5 nmole of acetylcholine/ml. of incubation mixture for the macro method and about 0·30, 0·25 and 0·15 pmole of acetylcholine/μl. of incubation volume for the micro procedures based on [1-^{14}C]acetate, [^{3}H]-acetate and [1-^{14}C]acetyl-CoA respectively.

AChE

Assay conditions. The assay of AChE was based on the quantitative separation of labelled acetylcholine from the enzymically formed acetate, by extraction with sodium tetraphenylboron and di-isobutyl ketone or butyl ethyl ketone. A single extraction removed 99·7–99·8% of the acetylcholine. This result agrees with the theoretical value. For most purposes a single extraction was therefore sufficient. A second extraction of the aqueous phase increased this value to more than 99·9%. More than 99·5% of the labelled acetate was left in the aqueous layer, and the activities of acetate in the aqueous phase per unit volume were 99–103% of the initial values.

The only factor limiting the sensitivity of the AChE assay was the contamination of acetylcholine by radioactive acetate as a result of the spontaneous hydrolysis of acetylcholine during incubation and of slow hydrolysis during storage. The latter source of acetate could be excluded by chromatography of the acetylcholine solution before assay. The non-enzymically formed acetate is proportional to the concentration of radioactive substrate and could be decreased by selecting an assay mixture of low ionic strength. The optimum substrate concentration with 100 mM-sodium phosphate buffer was 2·5 mM-acetylcholine, whereas with 20 mM-phosphate buffer it was lowered to 0·5 mM (Table 5). The enzyme activity under the latter conditions was 85% of that with the 100 mM-phosphate buffer, and the corresponding blank was decreased to 20%. Under the latter assay conditions the enzymic hydrolysis was linear for 3 hr. (Fig. 3*a*) and linear for 0·005–0·1 μg. wet wt. of brain tissue. About 50% of the total substrate may be hydrolysed without any significant effect on enzyme activity (Fig. 3*b*).

Similar blank values were obtained when samples from rat cerebra, diaphragm and sciatic nerve were replaced with heat-inactivated tissue or water or inhibited with 0·1 mM-eserine. The AChE activities found in rat cerebrum, diaphragm and sciatic nerve by this method were 600, 95 and 120 μmoles/hr./g. of wet tissue respectively.

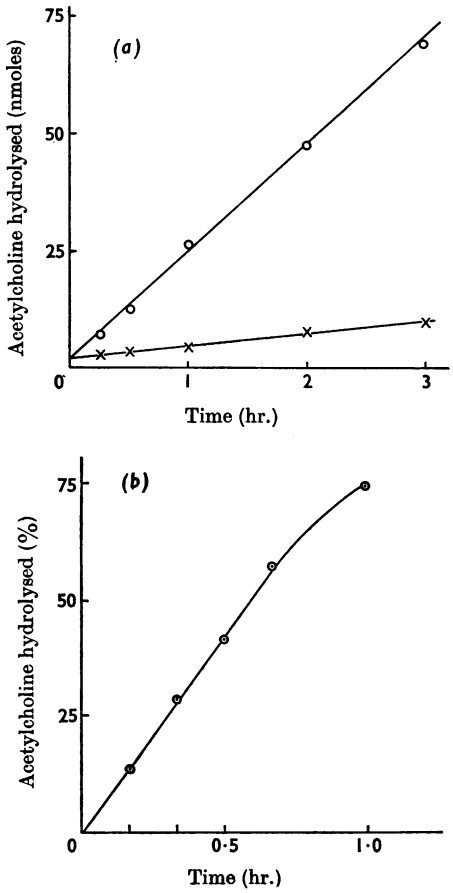

Fig. 3. (*a*) Enzymic hydrolysis (○) of acetylcholine by 40ng. of rat brain homogenate and spontaneous (×) hydrolysis at various times. (*b*) Variation of AChE activity with the amount of ACh hydrolysed. The curve shows the percentage of acetylcholine hydrolysed by 10ng. of purified AChE at various times.

DISCUSSION

Liquid cation exchange, with sodium tetraphenylboron in butyl ethyl ketone, constitutes a reproducible, rapid and selective way of obtaining acetylcholine from a ChAc-assay mixture. The extraction volume in the micro procedures was similar to that of the macro method; thus the handling of small volumes was limited to the incubation stage. Twenty-four samples from the [^{3}H]acetate incubation mixture, the more complicated of the procedures, could be treated within 1hr. after the incubation was finished. The methods gave reproducible results. When 12 portions of a sample containing low ChAc activity were determined with the [^{3}H]acetate procedure, the results were 0·73 ± 0·07pmole of acetylcholine synthesized (mean ± S.D.)

The method based on [1-^{14}C]acetate may be used to determine ChAc activity in any tissue and at a relatively wide range of ChAc activities. The compound may be diluted with unlabelled acetate and used for larger incubation volumes. [^{3}H]-Acetate has the advantage over [^{14}C]acetate that a much higher specific radioactivity may be obtained. It is, however, less stable, and therefore a more complicated isolation procedure is recommended. The method is therefore only useful for samples with low ChAc activities. The blank values for the two methods based on labelled acetate were mostly due to traces of ChAc activity in acetone-dried pigeon liver. This is avoided by using synthetic acetyl-CoA, but this modification suffers from the disadvantage of giving slightly lower values; also, the high cost of the reagent limits its use to micro assays only.

The methods based on labelled acetate were successfully applied in the topographical localization of ChAc in the various layers of rat brain hippocampus, for assay of 0·02–0·5μg. dry wt. (F. Fonnum, unpublished work). The macro procedure was used to study the distribution of ChAc in rat muscle and electric fish (*Gnathonemus petersii*) (Israel & Gautron, 1969). The modification based on acetyl-CoA is particularly suitable for studying enzymic properties such as the activation of ChAc by ions in the absence or presence of membranes (Fonnum, 1968*b*).

The blank values were independent of the tissues studied and seem to be lower than for other methods published. They are: for the macro procedure, 0·5nmole of acetylcholine/ml. of incubation mixture, and for the micro methods, in the range of 0·1–0·3pmole of acetylcholine/μl. of incubation mixture, which corresponds to 0·02–0·05μg. wet wt. of brain tissue.

The use of synthetic acetyl-CoA in the assay of ChAc activity has particularly been advocated by McCaman. The values of ChAc activity published by McCaman and co-workers (McCaman & Hunt, 1965; Buckley *et al.* 1967; Goldberg *et al.* 1969) were, however, much lower (2–4μmoles of acetylcholine/hr./g. of rat brain) than those obtained with acetyl-CoA formed *in situ* (5–8μmoles of acetylcholine/hr./g.) as demonstrated in this paper and found in the literature (summarized by Fonnum, 1966). Morris (1967) was successful in obtaining similar values for ChAc activity with incubation media containing thioglycollate or sodium cyanide and synthetic acetyl-CoA as with a medium where acetyl-CoA was formed *in situ* by the phosphate acetyltransferase system. The results were obtained with enzymes purified from human placenta and from the elasmobranch *Torpedo*.

The present work suggests that the low values previously obtained with synthetic acetyl-CoA

might be due to an inhibitory contaminant in some acetyl-CoA preparations (Fig. 1) and to the absence of a protective compound such as sodium cyanide or EDTA in the assay medium (Table 3). The latter two compounds, particularly sodium cyanide, enhanced ChAc activity when assayed with synthetic acetyl-CoA but not when assayed with acetyl-CoA formed *in situ*. The effects of these compounds are most probably due to protection of the enzyme in the presence of synthetic (impure) acetyl-CoA, perhaps by the formation of complexes with metal ions.

The present assay method based on synthetic acetyl-CoA is less laborious than those based on isolation of acetylcholine by precipitation (Buckley *et al.* 1967; Goldberg *et al.* 1969). The two latter methods gave blank values which corresponded to 0·15 and 0·10 μg./μl. respectively, compared with 0·02 μg. of brain tissue/μl. of incubation mixture in the present method. About 0·9% of the radioactivity in the acetyl-CoA preparation contaminated the acetylcholine after reineckate precipitation (Buckley *et al.* 1967) compared to 0·1% in the present method.

The sensitivity of the AChE assay method is comparable to those of other recently published methods (Potter, 1967; McCaman *et al.* 1968). The labelled acetate is contaminated by 0·2–0·4% of the original concentration of labelled acetylcholine after a single extraction with pentanol and toluene (Potter, 1967) or 1% after removal of acetylcholine by a single precipitation with reineckate (McCaman *et al.* 1968), whereas in the present method the contamination is 0·2–0·3%. The method of Potter (1967) suffers the disadvantage of recovering only 80–90% of the acetate each time, whereas the method of McCaman *et al.* (1968), which gives a high recovery of acetate, is more laborious. The present method, which easily removes the acetylcholine and recovers a high percentage of the acetate seems to offer an advantage in methodology. The use of a phosphate buffer of low molarity, which lowers the optimum substrate concentrations, gives a higher degree of sensitivity than the other methods. The effect of ionic strength on the kinetics of AChE has been studied in detail by Myers (1952). The method has been used to study the topographical distribution of AChE in various layers in rat hippocampus with samples of 0·02–0·1 μg. dry wt. (J. Storm-Mathisen, unpublished work).

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