A Radiochemical Method for the Estimation of Choline Acetyltransferase

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1. A radiochemical method for the estimation of choline acetyltransferase (choline acetylase) has been devised which involves the formation of labelled acetylcholine from labelled acetate. 2. [1-¹⁴C]Acetate and coenzyme A are pre-incubated in the presence of non-rate-limiting concentrations of acetyl-coenzyme A synthetase to give [1-¹⁴C]acetyl-coenzyme A, which then reacts with choline in the presence of the acetyltransferase to give [¹⁴C]acetylcholine. 3. Any [¹⁴C]-acetyl-coenzyme A remaining at the end of the reaction is destroyed by the addition of excess of hydroxylamine, and [¹⁴C]acetylcholine is freed from other labelled compounds by precipitation with sodium tetraphenylborate (Kalignost). 4. The washed precipitate is dissolved in acetonitrile-benzyl alcohol and estimated by scintillation counting. 5. Advantages over other methods are discussed.

Choline acetyltransferase (choline acetylase, acetyl-CoA-choline *O*-acetyltransferase, EC 2.3.1.6) catalyses the formation of acetylcholine from acetyl-CoA and choline. Synthetic acetyl-CoA may be used as substrate, but higher activities are obtained when acetyl-CoA is formed *in situ* from acetate and acetyl-CoA synthetase or acetyl phosphate and phosphate acetyltransferase (Smallman, 1958; Morris & Tuček, 1966). Choline acetyltransferase is generally assayed by estimating the acetylcholine produced pharmacologically; this is a sensitive but tedious procedure. The pharmacological method is also sensitive to any changes in its medium; this limits the study of the effects of drugs and ions on choline acetyltransferase.

Recently two radiochemical methods have been introduced (Schuberth, 1963; McCaman & Hunt, 1965). Both methods have several disadvantages. Schuberth (1963) states that his method is only suitable for acetone-dried powders or purified enzymes. The method does not remove radioactive by-products like acetyl-CoA, and therefore gives rather high blank values. It makes use of gas-flow counting, which gives lower counting efficiency than scintillation counting. McCaman & Hunt (1965) separated the radioactive acetylcholine from radioactive by-products by reineckate precipitation. The strong colour and low solubility in acetone of the reineckates limit the method to assay volumes of about 10–20 μ l. The method has the

disadvantage of using synthetic acetyl-CoA and therefore gives considerably lower values than the present method. The method described below may be used both on a micro- and macro-scale. It utilizes [1-¹⁴C]acetate and CoA in the presence of acetyl-CoA synthetase to give [1-¹⁴C]acetyl-CoA. The acetyl-CoA is converted by choline acetyltransferase into [¹⁴C]acetylcholine. The acetylcholine is isolated as its tetraphenylborate salt, washed with ethanolic ether, redissolved and estimated by scintillation counting.

MATERIALS AND METHODS

Materials. The following special chemicals were used: CoA (90% pure) and ATP (Sigma Chemical Co., St Louis, Mo., U.S.A.); Triton X-100 (Lennig Chemicals, London, W.C. 1); deoxycholate, saponin and methoxy-polyethylene glycol laurate (Nonex 501) (British Drug Houses Ltd., Poole, Dorset); 2,5-diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene, scintillation grade (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.); [1-¹⁴C]-acetylcholine, acetyl[¹⁴C]choline and sodium [1-¹⁴C]-acetate (The Radiochemical Centre, Amersham, Bucks.); sodium tetraphenylborate (Kalignost) (Koch-Light Laboratories Ltd., Colnbrook, Bucks.).

Enzyme preparations. Acetone-dried pigeon liver was prepared essentially as described by Kaplan & Lipmann (1948) and acetone-dried guinea-pig brain by the method of Berry & Whittaker (1959). Crude brain mitochondrial fractions were prepared in 0.32 M-sucrose as described by Gray & Whittaker (1962). Choline acetyltransferase is present in brain in an occluded form from which it must be released. Brain homogenates were therefore prepared in

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water and full activity was obtained by treatment with ether (Hebb & Smallman, 1956).

Radiochemical assay. The incubation mixture contained (final concentrations): 8mm-sodium [^{14}C]acetate (350000 counts/min.), 8mm-choline, 200mm-NaCl, 4mm-KCl, 40mm-NaF, 0.5mm-KBH₄, 0.05mm-CoA, 16mm-ATP, 0.1mm-eserine sulphate and 8mg. of acetone-dried pigeon liver/ml. The mixture was incubated at 37° and pH 7.2 for 10 min. to preform acetyl-CoA (Smallman, 1958); under these conditions the rate of acetyl-CoA formation is about 1 $\mu\text{mole ml.}^{-1}\text{hr.}^{-1}$. The transferase reaction was then started by adding the brain preparation to give a final volume of 2.5 or 0.25 ml. depending on the amount of material available. The following procedure is given for a volume of 2.5 ml. After incubation for 1 hr., the reaction was stopped by adding 1 ml. of *m*-hydroxylamine hydrochloride adjusted to pH 6.5 with *N*-NaOH. The hydroxylamine reacts spontaneously with the acetyl-CoA present and decreases the blank considerably. The tubes were placed in ice after 5 min. and the protein was precipitated by adding 300 $\mu\text{l.}$ of 100% (w/v) trichloroacetic acid containing 2 mg. of acetylcholine perchlorate. The acetylcholine decreases any adsorption of labelled acetylcholine on to protein and acts as a carrier.

The protein was removed by centrifugation and 3.0 ml. of the clear supernatant was added to 1 ml. of aq. 0.1M-Kalgnost. The precipitate that formed after 1 hr. was collected by centrifugation at 8000g for 10 min. The pellet was washed twice by resuspending in 0.3 ml. of ethanol and then adding 10 ml. of sodium-dried ether. One washing was usually sufficient, but as a routine the precipitate was washed twice.

Finally, the pellet was transferred to the scintillation vial by dissolving it in 3.0 ml. of acetonitrile and 3.0 ml. of benzyl alcohol; 10 ml. of toluene containing 0.5% of (w/v) 2,5-diphenyloxazole and 0.02% (w/v) of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene was added and radioactivity was counted at 1040v with window-setting 10–100 in a Packard scintillation counter. Under these conditions the counting efficiency was 65%. Control incubations containing enzyme preparations previously heated at 100° for 10 min. gave blank values of 200–300 counts/min.

Bioassay. When comparisons were to be made with bioassay, the reaction mixture was divided after incubation and the portion for bioassay acidified to pH 4 with 0.33N-HCl, heated at 100° for 10 min. and assayed by using the eserine-treated frog rectus-abdominis muscle preparation (Chang & Gaddum, 1933).

RESULTS

Experiments with labelled acetylcholine. Marquardt & Vogt (1952) introduced Kalgnost as a precipitating agent for choline derivatives. It was later used by several workers to isolate choline derivatives from different tissues (Fiedler, Hildebrand & Neu, 1953; Augustinsson & Grahn, 1954; Kewitz, 1959).

The precipitate is formed under acid conditions and the solubilities of the tetraphenylborates are comparable with those of the reineckates.

The preliminary experiments (Table 1) were designed to determine the optimum conditions for precipitating acetylcholine and washing the precipi-

Table 1. *Recovery of acetylcholine from choline-acetyltransferase reaction mixtures after precipitation with Kalgnost and washing*

The values are percentages of the added acetyl[^{14}C]choline recovered in the precipitate after washing various numbers of times with ethanol and ether as described in the text.

No. of washings	Recovery of acetylcholine (%)	
	Expt. 1	Expt. 2
—	85.5	93.1
1	89.6	90.1
2	85.0	89.1
5	83.6	91.3
10	76.8	87.5

Table 2. *Comparison of the radiochemical and bioassay methods for determining choline acetyltransferase under conditions of various enzyme concentrations*

Experimental details are given in the text.

Acetone-dried guinea-pig brain (mg.)	Acetylcholine formed ($\mu\text{moles/g. of acetone-dried tissue/hr.}$)	
	Bioassay method	Radiochemical method
7.5	17.9	18.5
15.0	20.8	15.9
30.0	17.3	16.2
50.0	20.7	16.8
Average	19.2	16.8

tate. Acetyl[^{14}C]choline was used, since acetylcholine and choline gave mixed precipitates. The results were later confirmed with [^{14}C]acetylcholine. The radioactive acetylcholine was added to the choline-acetyltransferase assay mixture and the proteins were then removed by trichloroacetic acid. The supernatant was then treated with Kalgnost, the acetylcholine precipitated and the precipitate washed as described above. After the precipitate had been washed twice, 85–90% of the acetylcholine was recovered. This value corresponded well with the difference between the values obtained with the radiochemical and bioassay methods as shown in Tables 2 and 3.

The precipitate could be washed up to ten times with ethanolic ether with only minor losses of radioactivity. This is very important for studies where a large amount of radioactive acetate is being used to increase the sensitivity.

The same precipitation and washing procedure could be applied equally well if heating at pH 4 and 100° or if perchloric acid treatment had been used to remove proteins.

Table 3. *Comparison of values obtained by different methods for the determination of choline acetyltransferase in brain tissues*

Experimental details for the bioassay and radiochemical methods are given in the text. Figures in parentheses are values expressed as percentages of those obtained by bioassay.

Species	Tissue	Choline-acetyltransferase activity (μ moles of acetylcholine/g. of fresh tissue/hr.)			
		Bioassay method	Radiochemical method	McCaman & Hunt (1965) method	Other methods
Rat	Forebrain	7.05	6.62 (94)	5.4*	6.8†
	Cortex	6.05	5.05 (84)	3.48‡	
		7.60	6.35 (84)		
	Cerebellum	0.76	0.84 (111)		
Guinea pig	Forebrain	3.95	4.80 (122)		4.5-5.45
		4.00	3.50 (88)		
	Cortex	4.54	3.80 (84)	2.67‡	6.0
		4.00	3.22 (80)		
Pigeon	Cerebellum	0.70	0.64 (91)		0.5§, 0.65
	Optic lobe	11.2	10.6 (95)	8.1*	7-11.3¶, 8.5**
		16.3	13.0 (80)		
	Forebrain	6.3	4.2 (67)	4.4*	
Rabbit		3.6	3.96 (110)	2.16‡	4.5**
	Cortex	5.10	4.10 (81)	3.26‡	4.5¶
	Caudate nucleus	13.9	10.8 (78)	12.5*	13.2¶
	Thalamus + hypothalamus	2.4	2.1 (88)		2.2¶
	Corpora quadrigemina	3.5	2.8 (80)		

* McCaman & Hunt (1965).

† Dauterman & Mehrotra (1963).

‡ McCaman, Arnaiz & De Robertis (1965) (hemispheres).

§ Hebb (1955).

|| Beani, Bianchi & Megazzini (1964).

¶ Hebb & Silver (1956).

** Aprison, Takahashi & Folkerth (1964).

Variation in the final volume from 2.0 to 7.5 ml. did not lead to any significant variation in recovery.

Acetone-dried brain preparations. The radiochemical method was first tested with acetone-dried guinea-pig brain. Table 2 shows a linear correlation between enzyme concentration and the acetylcholine formed. The pharmacological and radiochemical methods agreed well, and the latter gave values of 88% compared with the other method. In Fig. 1 the time-course of the reaction was studied simultaneously by both methods. There was a linear correlation between the counts/min. of the isolated tetraphenylborate and the $m\mu$ moles of acetylcholine formed. In neither case could the formation of acetyl-CoA have been rate-limiting and in the subsequent experiments with brain tissue activities measured were within the maximum activity shown.

Identification of the radioactive compound precipitated by Kalignost. To verify that labelled acetylcholine was the only radioactive compound isolated by Kalignost when crude brain homogenate was assayed, a sample of rat-brain homogenate was incubated for 2 hr. under standard conditions but with radioactive acetate in five times its usual con-

centration. After the addition of hydroxylamine the protein was removed by heating at 100° and pH 4.0 for 10 min. A sample (1 ml.) of the supernatant was chromatographed on a column of Amberlite XE-97 that had been equilibrated with 0.1 M-sodium dihydrogen phosphate at pH 4.3 (Gardiner & Whittaker, 1954). Fig. 2 shows that two radioactive peaks were obtained. The first peak emerged in the void volume of the column and contained most of the radioactivity, but only traces of acetylcholine as measured by bioassay. Less than 1% of this radioactivity could be precipitated with Kalignost. The next radioactive peak corresponded well with the acetylcholine present as estimated by bioassay. The radioactivity could be precipitated by Kalignost. The column was finally eluted with 0.1 N-hydrochloric acid, but only traces of radioactivity were recovered. Any single radioactive peak containing 5-10% of the radioactivity of the acetylcholine peak could have been detected under these conditions. The recovered radioactivity from fractions 10-19 represents 104.5% of the theoretical radioactivity corresponding to the acetylcholine present. It may therefore be concluded that there were no other

Fig. 1. Comparison of the radiochemical (x) and bioassay (o) methods of determining choline-acetyltransferase activity at various times.

enzymes in the crude homogenate that could interfere with the assay.

Comparison with other methods. Table 3 shows the correlation found in single experiments between values from the radiochemical and bioassay methods. There is good agreement between the two methods but, as expected from Tables 1 and 2, the bioassay method gives 10–20% higher values. If radioactive products other than acetylcholine had interfered with the radiochemical method, the values from this method would have been expected to be higher than with the bioassay method. This should especially be apparent in tissues with low choline-acetyltransferase activity like cerebellum. Both rat and guinea-pig cerebellum, however, gave normal activities with the radiochemical method.

The values obtained with the present method are consistently higher than those reported by the method of McCaman & Hunt (1965). The values obtained with the present method from rat, guinea-pig, rabbit and pigeon cortex are 50–100% higher than values reported by McCaman *et al.* (1965). This confirms the general belief that synthetic acetyl-CoA gives lower results than acetyl-CoA prepared *in situ*. Another discrepancy may have been insufficient activation by these authors, although they claim that their activation method gives higher values than any existing method. Most authors have expressed the tissue contents of choline acetyltransferase as activity/mg. of acetone-dried powder. On the assumption that 200mg. of

Fig. 2. Chromatographic identifications of Kalignost-precipitated components of choline-acetyltransferase reaction mixtures. x, Radioactivity in effluent; □, radioactivity precipitated by Kalignost; o, acetylcholine determined by the bioassay method. Experimental details are given in the text.

acetone-dried powder is derived from 1g. of fresh tissue, I have recalculated values from Hebb (1955) and Hebb & Silver (1956). In these papers the acetyl-CoA-donor system utilized acetone-dried pigeon liver and citrate and acetate as substrates. Their values agree well with those observed with the present method. Hebb (1962) has introduced acetyl phosphate and CoA in the presence of phosphotransacetylase to form acetyl-CoA. This system is said to give slightly higher values than extracts from acetone-dried pigeon liver.

Activation of bound choline acetyltransferase. It was established by Hebb & Smallman (1956) that 60–70% of the choline acetyltransferase in brain is present in a bound form in sucrose homogenates. They also showed that the bound activity could be activated by organic solvents. Ether has been regarded as the most satisfactory solvent by most workers. I have previously (Fonnum, 1965) stressed the importance of activating enzymes in the crude mitochondrial fraction by Triton X-100. Ether activation was found in my hands to be time-consuming and to give inconsistent results. Triton activation gave consistent results and similar values to ether activation. This finding prompted me to study the effect of several other detergents on the

Table 4. *Comparison of different methods of activating choline acetyltransferase from crude mitochondrial rat-brain fraction*

Experimental details are given in the text. The numbers of experiments averaged are given in parentheses. The results with ether are taken as 100%.

Activating agent	Concn. (%, w/v)	Activity (%)
Ether	—	100 (4)
Triton X-100	0.5	113 (3)
Triton X-100	1.0	90 (1)
Nonex 501	0.5	118 (4)
Nonex 501	1.0	112 (2)
Deoxycholate	0.5	19 (1)
Deoxycholate	1.0	0 (1)
Saponin	0.5	94 (2)
Not treated	—	24 (2)

activation of choline acetyltransferase. Table 4 shows that Nonex 501 gave the best results. Saponin gave quite promising results, but deoxycholate was found to be unsuitable.

Factors inhibiting acetyl-CoA synthetase. Fluoride ions have to be present in the incubation mixture to ensure a maximum rate of acetyl-CoA formation by the extract of acetone-dried pigeon liver. Presumably the fluoride acts by inhibiting interfering enzymes. This effect was first noted by Nachmansohn & Machado (1943). Recently ouabain has been found to be partly protective (R. J. A. Hosie, personal communication). Under the conditions of my experiments the sodium fluoride present was adequate to suppress completely the effect of these interfering enzymes. However, with very high tissue concentrations, ouabain had a further protective effect that was additional to that of fluoride.

DISCUSSION

A radiochemical method has been described for choline acetyltransferase. The method gives values in agreement with those reported in the literature. One person may perform 50 assays in a day with an accuracy of $\pm 5\%$. The method therefore compares favourably with the existing assays for choline acetyltransferase.

It is much more rapid than the elaborate biological assay. The effective washing procedure allows one to use high initial concentrations of radioactive acetate so that a high sensitivity is obtained. No difficulties were experienced in estimating activities in the range 2–4 μmoles of acetylcholine/hr., which correspond to less than 1 mg. of brain cortex.

It also compares favourably with Schuberth's (1963) method since it removes most of the radio-

active by-products like acetyl-CoA and therefore gives considerably lower blank values. It has also been shown to work with crude tissues, whereas Schuberth (1963) only claims that his method works with purified acetone-dried preparations.

The introduction of reineckate precipitation to isolate the labelled acetylcholine by McCaman & Hunt (1965) represented a considerable improvement in choline-acetyltransferase assays. As already pointed out, the strong colour and use of acetone makes this method of value only for micro-estimations. McCaman & Hunt (1965) used only 10–20 μl . final assay volumes, which are, however, not very convenient for ordinary purposes. Their method gives considerably lower values than the one described in the present paper. I suggest that this is mainly due to the use of synthetic acetyl-CoA. So far the reason for the low values with synthetic acetyl-CoA is unknown. It could be due to an inhibitor in the acetyl-CoA preparation, or to interference by substances in tissues or sub-cellular fractions. If the latter is true, then different tissues or fractions might inhibit the assays to varying degrees. Results with synthetic acetyl-CoA would therefore not be directly comparable for different subcellular fractions or tissues. The increased activity of choline acetyltransferase reported in the presence of high salt concentrations with synthetic acetyl-CoA is also true for the coupled system.

The blank values for the two methods are of the same order. The radiochemical method has enabled better means of releasing bound choline-acetyltransferase activity to be found. The detergent Nonex 501 was found to give the most satisfactory results. The method has been used to compare the subcellular distribution of choline acetyltransferase with glutamate decarboxylase in nervous tissue (F. Fonnum, unpublished work).

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