Biochemical Pharmacology, Vol. 24, pp. 1007–1011. Pergamon Press, 1975. Printed in Great Britain.

MEASUREMENT AND ACTIVATION OF CHOLINE ACETYLTRANSFERASE

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(*Received* 23 *September* 1974; *accepted* 3 *December* 1974)

Abstract—A method for the radiometric assay of choline acetyltransferase, depending for the separation of the product [1-^{14}C]acetylcholine, from the substrate, [1-^{14}C]acetyl-CoA, on elution through an anion exchange resin has been modified so as to increase its sensitivity, reduce the blanks and improve the accuracy. An important step was the purification of the acetyl-CoA to eliminate a radioactive contaminant that was not completely retained by the resin. Increased sensitivity was gained from changes in the incubation medium and also from the methods by which the enzyme was extracted and activated before incubation. During the investigation two artifacts were encountered that could give misleadingly high results. One was due to imidazole, added as buffer, which can itself catalyze the formation of acetylcholine; the other was due to sucrose which appeared to be acetylated itself to some extent, also non-enzymically.

In the original procedure for the radiometric assay of choline acetyltransferase (ChAc*), McCaman and Hunt [1] depended upon reineckate precipitation to separate the radioactive product [^{14}C]acetylcholine, from the radioactive substrate, [^{14}C]acetyl-CoA. With this method blank values were very high; and while other methods of separation subsequently introduced [2–4] have lower blanks, they are still higher than is desirable for an analytical method of this kind. Of the various procedures that had already been tried by others, separation by anion exchange resin [3, 4] seemed to us for a number of reasons to be the method of choice. Accordingly, we set out to modify it so as to increase its sensitivity, reduce the blanks and by this and other means to increase the accuracy of the assay. During our investigation two unexpected sources of error were discovered and will be described.

MATERIALS

Enzyme sources. Rats and rabbits were killed either by an overdose of sodium pentobarbitone or by stunning and decapitation. Each cerebrum was homogenized by hand in either 0·3 M sucrose, 0·155 M NaCl or water. The homogenates were then stored for 16 hr or longer at −18° before use. ChAc in human placental tissue was partially purified from homogenates (100 mg/ml in 0·1 M sodium phosphate buffer pH 6·5) by $(NH_4)_2SO_4$ fractional precipitation at pH 5·5 [5]. This yielded a stable preparation with ChAc activity of 0·8–1·52 units/ml at 39°. This was sometimes followed by gel filtration on Sephadex® G-75.

Chemicals. Coenzyme A (87% pure), *cis*-oxaloacetic acid, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and Tris were products of Sigma Chemical Company, St. Louis, Mo., U.S.A. Sephadex®, G-10, and other sizes, were obtained from Pharmacia, Uppsala, Sweden. Choline chloride, Nonex 501, eserine and imidazole were products of British Drug Houses, Laboratory Chemicals Division, Poole, U.K. All radioactive compounds used were supplied by the Radiochemical Centre, Amersham, Bucks, U.K. Citrate synthase (EC 4.1.3.7) was supplied by Boehringer Corporation, London U.K., bovine plasma albumin by Armour Pharmaceutical Company, Eastbourne, U.K., and the resin DeAcidite, FF-IP SRA63 by Permutit Co. (632/652 London Road, Isleworth, Middx., U.K.).

Chemicals for liquid scintillation counting, diphenyloxazole (PPO), 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP), naphthalene and dioxan and a premixed scintillator (Unisolve) were supplied by Koch Light (Colnbrook, Bucks., U.K.), as were Triton X-100® and DEAE cellulose (DE-52 made by Whatman). The dioxan based scintillator contained 100 g naphthalene, 0·3 g POPOP, 7 g PPO in 1 litre of dioxan. Other chemicals were of the highest purity available.

METHODS AND RESULTS

Choline acetyltransferase assay

(1) *Preparation of* [1-^{14}C]*acetyl-CoA.* Labelled acetyl-CoA was prepared from CoA by treatment (adapted from Simon and Shemin [6]) with [1-^{14}C]acetic anhydride. Residual [1-^{14}C]acetic acid was removed by ether washing at pH 1·0 (recovery, 50–70 per cent) or by fractionation on G-10 Sephadex® (recovery 80–100 per cent). After adjusting its pH to between 4 and 5 the acetyl-CoA was next put on a column (30 × 2 cm) of DEAE–cellulose already equilibrated with 3 mM HCl and eluted with a linear NaCl gradient (0–150 mM) in 3 mM HCl. Two main peaks with an extinction at 254 nm were obtained. In two preparations the first peak (from earlier work [7] thought to be acetyl-dephospho-CoA) represented nearly 30 per cent of the theoretical yield of acetyl-CoA when it was subsequently purified, assayed and

* *Abbreviations used.* ACh—acetylcholine; Acetyl-CoA—acetyl-coenzyme A; ChAc—choline acetyltransferase; C.V.—coefficient of variation; DMG—3,3-dimethyl glutaric acid-NaOH; DTNB—5,5-dithiobis-(2-nitrobenzoic acid); DTT—dithiothreitol; S.D.—standard deviation; S.E.M.—standard error of the mean; TX—Triton X-100®.

its radioactivity measured. Although in both cases it assayed as acetyl-CoA by Chase's [7] method and supported ACh synthesis, it was not well retained by the anion exchange resin; from one nearly 5 per cent of radioactivity and from the other nearly 9 per cent was recovered in the fraction of eluate where ACh would normally be found. From the second much larger peak which yielded the bulk of the acetyl-CoA, the leak was between 0·02 and 0·03 per cent, equivalent to less than 20 pmoles of ACh (see Section 4). Only the second peak was used in all subsequent preparations; by this means alone the blanks were reduced from 1–10 per cent to less than 0·1 of added acetyl-CoA.

The fractions belonging to the second peak were combined and rotary-evaporated (at 50°) to about 3 ml, desalted by gel filtration on G-10 Sephadex® and again evaporated to 3 ml. By flame photometry it was found that the concentration of NaCl in this final preparation was about 40 mM.

Stored at pH 4–5 and at −18°, the acetyl-CoA prepared in this way was very stable over periods of 6 months or longer.

The concentration of acetyl-CoA as nmoles per unit volume was measured by the citrate synthase assay [7]; from this figure and measurement of the radioactivity on a scintillation spectrometer, the specific activity, counts/min per nmole, was calculated. The specific activities of the preparations used varied from 400 to nearly 13,000 counts/min per nmole (i.e. from 0·25 to 8 mCi/m-mole).

All ^{14}C-labelled compounds were counted at an efficiency of *ca.* 73 per cent on a refrigerated Packard Scintillation Spectrometer, Model 3320.

(2) *Incubation system.* The composition of the standard incubation medium is shown in Table 1. EDTA was included in the medium because of evidence that a toxic impurity was present in the incubate in amounts sufficient to cause a significant reduction in the rate of synthesis over an incubation period of 30 min or longer. The evidence was that when the yield of ACh synthesized in 30 min was plotted against varying concentrations of ChAc the shape of the curve was seen to be very like that shown in Dixon and Webb's [8] Fig. IV.4 which illustrates the effect on an enzyme of a toxic impurity present in the incubating medium. On the assumption that this was a heavy metal, EDTA was added as an extra component of the incubation solution. In concentrations of 0·1–0·3 mM, the shape of the curve was improved proportionately and the yield of ACh increased as well, but it required 0·4 mM to achieve the expected straight line coupled with a further increase in the production of ACh. In the light of these tests a concentration of 0·5 mM was chosen to add to the buffer–substrate solution normally used for incubation. This is 5 times the concentration employed by Glover and Potter [9] but only one-twentieth of that used by Fonnum [10]. When bovine plasma albumin was also added, a general increase in the reaction rate was observed; this amounted to an average of 14 per cent in three 30-min incubations.

Table 1. Incubation media for choline acetyltransferase

Substance added	Final concentration (mM) in incubation mixture A	B
NaCl	300	240
Na phosphate buffer (pH 7·5)	33	20
Choline chloride	6–12·5	6·0
Bovine plasma albumin	(0·5 mg/ml)	(0·5 mg/ml)
EDTA	0·5	—
Eserine sulphate	0·13	0·1
Acetyl-CoA	0·5	0·5–0·7
Final volume	(120 μl)	(200 μl)

Incubation medium: A—the standard mixture used in the present research (final pH: 7·3); B—mixture used by Bull and Nowak [4].

Each incubation was started by the addition of acetyl-CoA, to tubes containing the rest of the buffer–substrate mixture and prewarmed to 39°. The tubes were made of polystyrene with internal dimensions 5 × 38 mm (supplied by Luckham Ltd., Burgess Hill, Sussex, U.K.). The incubation was terminated by addition of 80 μl 0·15 M $HClO_4$, rapid mixing of the acidified mixture with a vortex mixer followed by immediate cooling in an ice-bath [11].

(3) *Separation of [1-^{14}C]acetylcholine from [1-^{14}C]acetyl-CoA.* In the method pioneered by Schrier and Shuster [3], ACh formed by incubation is separated from the residual acetyl-CoA by means of an anion exchange resin. In the present research the resin used was DeAcidite FF-IP SRA 63, chloride form in columns of 0·65 × 10 cm.

Either the total acidified incubate (0·2 ml) or one-half of it was put through the resin column and eluted with further additions of water. By determining the elution patterns of [1-^{14}C]acetylcholine and of [1-^{14}C]acetyl-CoA, it was found that maximum recovery of ACh (98–100 per cent) was ensured, if after discarding the first 1·0 ml eluted, the next 1·6 ml was collected for counting.

The recovery of ACh was checked by determining the elution patterns of both labelled ACh and unlabelled ACh. In the second case the concentration of reagent in successive fractions was measured by bioassay (eserinized frog *rectus abdominis* [12]). Both methods gave a recovery of 98–100 per cent.

The elution of [1-^{14}C]acetyl-CoA by itself led to the appearance of a small amount of radioactivity in those fractions which would normally contain ACh. This was monitored in each incubation by running column controls. Two tubes which were identical in all respects to the test incubation tubes except that they contained inactivated or no enzyme, were carried through the normal procedure and the mean counts obtained from them (uncorrected for background) subtracted from the count recorded for each test incubation. The difference was taken to be the true reading for the ACh synthesized.

Checks with sodium [1-^{14}C]acetate showed that acetate was wholly retained by the resin columns.

(4) *Accuracy of the assay.* When the ACh formed in duplicate samples of incubated enzyme was measured by bioassay [12] as well as radiometrically, the results agreed well within the error of the bioassay, and so confirmed the original measurements by which the specific activity of the [1-^{14}C]acetyl-CoA had been determined.

An improvement over earlier radiometric procedures [3, 4] was that the blank values expressed in terms of the amount of labelled reagent put on

Table 2. Reproducibility of the assay

Batch no.	Acetyl-CoA Sp. act. (counts/min/nmole)	Active enzyme added	Average of counts accumulated	Mean value as pmoles ACh ± S.E.M.	S.D.	C.V. (%)
(All of incubate counted)						
B64	1367	—	5286	17·0 ± 0·26 (6)	0·63	3·7
B62	5182	—	10,000	16·8 ± 0·18 (6)	0·41	2·5
B61	11,400	—	2165	16·1 ± 0·42 (6)	0·94	5·8
B62	5182	+	10,000	316·5 ± 5·0 (6)	11·50	3·6
B64	1367	+	483,000	20,310·6 ± 106·0 (9)	300·00	1·48
(One half of incubate counted)						
B62	5182	—	7418	17·8 ± 1·53 (6)	3·76	21·1
B64	1367	+	10,000	88·8 ± 3·65 (6)	8·93	10·1
B62	5182	+	10,000	142·3 ± 3·23 (6)	7·91	5·6

Mean, S.D. and S.E.M. are all expressed in terms of pmoles of ACh; figures in brackets signify the number of trials. C.V. is the coefficient of variation, that is S.D. expressed as a percentage of the mean. The six trials of B61 (acetyl-CoA) are composed of three sets of duplicates in blanks from three separate incubations. All other figures are based on six simultaneous trials; for the controls water replaced the enzyme. The amount of radioactivity coming through the control columns is the leakage of acetyl-CoA from 60,000 pmoles.

A plus sign indicates that active enzyme was added.

the columns, were considerably lower than those reported earlier, 0·4–0·7 per cent [3] and approaching 1 per cent [4].

In our procedure the amount of acetyl-CoA coming through the control columns and found in the fraction of effluent normally containing ACh was only 16–17 pmoles per 60,000 pmoles put on the column (Table 2) or 0·026–0·028 per cent. The data in the table for tests in which the whole of the acidified incubate (0·2 ml) was processed show the close similarity in molar terms of the blanks for three preparations of [1-^{14}C]acetyl-CoA which had widely different specific activities. The data for preparations B62 and B64 were each obtained from the incubation of six comparable controls; the data for B61, however, were obtained from three pairs of controls belonging to three separate incubations. The coefficient of variation (S.D. as per cent of mean) for this set of blanks is highest possibly in part because of differences in treatment but more certainly because the number of counts accumulated was lowest and the expected counting error would accordingly be highest. On the other hand, when a high concentration of enzyme was incubated and the number of counts accumulated was larger (483,000) the variability then observed was lowest and within the range we were hoping to achieve.

The greater variation in both blank and test counts when only half of the incubate was analysed will be noted. This is a more convenient procedure but is obviously unsuitable for studies where accuracy is important.

(5) *Column controls and specificity of the assay.* The column controls were useful in providing a check on the specificity of the assay. Artifacts introduced by two substances (a) sucrose and (b) imidazole, differing from each other in their mode of action, were detected in this way.

(*a*) *Sucrose.* The addition of sucrose to the incubation medium led to an apparent increase in the production of ACh of 18–24 pmoles/hr per μmole sucrose when incubated at pH 7·5 and at 39°. The radioactivity produced increased linearly with increments of sucrose (see Fig. 1). Its production occurred in the absence of choline or enzyme and required only acetyl-CoA and buffer. This was needed because the reaction was sharply pH-dependent and fell off rapidly as the pH was reduced to 7 and lower. The effect, assumed to be due to acetylation of some sucrose, was consistently observed with three batches of the sugar. Heat-treated solutions of sucrose (90° for 1 hr) reacted in the same way.

(*b*) *Imidazole.* Because imidazole activates ChAc [13, 14] we used it in a number of incubations as buffer (33·3 mM) but as Burt and Silver [15] discovered earlier in this laboratory, imidazole can itself catalyse the formation of ACh from acetyl-CoA and choline in the absence of enzyme. The rate of synthesis found on incubation, at pH 7·5 and at 39° increases with increments of imidazole and has a dependence on pH similar to that of the sucrose effect. The rate of production of ACh (the identity of which we have confirmed by bioassay) was found to be 150 pmoles/hr per μmole imidazole. Figure 2

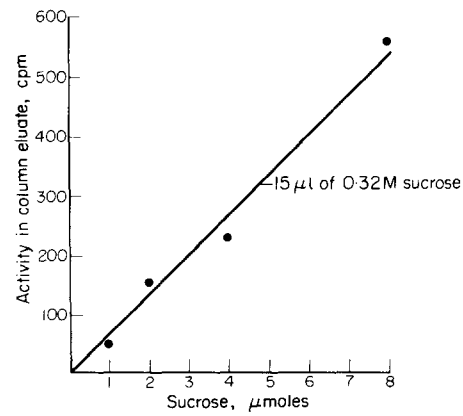

Fig. 1. Increasing sucrose concentrations were incubated for 30 min at 39° and pH 7·5 with an acetyl-CoA that had a sp. act. of 5182 counts/min/nmole. No choline or enzyme was added. The arrow shows how much radioactivity would be due to sucrose if 15 μl of a 0·32 M sucrose homogenate were being incubated.

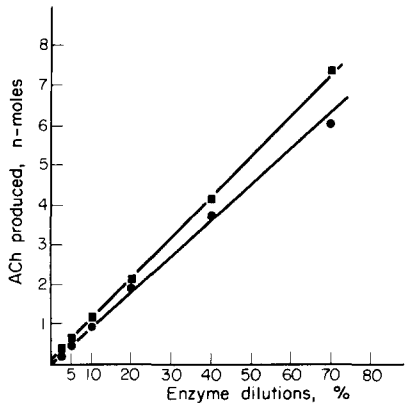

Fig. 2. Incubation of placental ChAc with two different buffers, ■ imidazole and ● phosphate. The imidazole line indicates some ACh synthesis at zero enzyme concentration.

compares two enzyme concentration curves obtained with phosphate and imidazole as buffers. The slightly steeper rise of the imidazole line suggests that some activation of the enzyme may have occurred. In other tests of imidazole its activating effect on the enzyme was regularly observed and sometimes amounted to 80 or 100 per cent; this was in addition to its own production of ACh.

(6) *Extraction and activation of the enzyme.* (*a*) *Effects of ether, Nonex* 501, *Triton X-100® and water.* Occluded ChAc in brain homogenates can be fully activated by treatment with ether, chloroform or ultrasonic vibrations [16]. Fonnum [17] commends Triton X-100® (TX) and Nonex in preference to ether. On the other hand, Bull and Nowak [4] found that TX had no activating effect on homogenates of rabbit brain and Nonex a very slight one. Our own experience is different. As shown in Table 3, TX causes a substantial release of activity (so too does Nonex) from rat and rabbit brain homogenates, although in our hands ether is still better as an activator. The explanation of the earlier negative results is that the NaCl concentration in the incubation medium (B in Tables 1 and 3) was 20 per cent lower than optimal and whereas the deficit was made up in ether-treated samples since they were routinely diluted 1:1 with 2 per cent NaCl before treatment [18], it was not made up in the detergent-treated samples which were diluted with water or more sucrose. These conditions are reproduced in the second incubation on rabbit brain, in Table 3 and give results similar to the earlier ones [4]. The effectiveness of water in activating homogenates can also be judged from Table 3; it is the least effective of the three procedures tested. On the other hand the ether-treated samples incubated under our conditions yield rates of synthesis of up to 25 μmoles/g per hr for rat brain and 17·6 μmoles/g per hr for rabbit brain. This is double the rate of synthesis for rabbit brain observed earlier [4]. That in turn was 2–3 times higher than had been observed previously for mouse brain [3]. The "sucrose artifact" would not have played any part in determining our higher results because the sucrose was diluted to 0·08 M in the most active samples and in any case because relative to the rates of synthesis being measured any error introduced by sucrose would be infinitely small.

In dealing with tissues that are difficult to homogenize and therefore difficult to sample accurately, we have found that adding TX or Nonex (5–10 μl/ml) followed by storage at −18° for some hrs will in many cases solubilize most of the enzyme and hence make it easier to pipette quantitatively.

(*b*) *Activation by sulphydryl reagents.* Both cysteine and dithiothreitol (DTT) activated soluble ChAc. We confirmed earlier observations [4] that the amount of sulphydryl reagent added to the incubation mixture with the enzyme extracts (*ca.* 0·1 or 0·2 μmoles) which increased considerably the rate of synthesis of ACh did not have a significant effect on the rate when added separately to the incubation mixture. This and other control observations eliminated the possibility that the increase in radioactivity recovered was due to acetylation of the sulphydryl reagent [19].

DTT was most effective in concentrations of 3–5 mM. Cysteine was required in concentrations of 19–38 mM. Other advantages of DTT over cysteine are that its activating effect, up to 5 or 10 times increase in the output of ACh, was more consistently observed

Table 3. Rates of synthesis of acetylcholine: the activating effects of ether, Triton X® and water

Incubation medium	Species	Suspending medium	Activating reagent	ACh synthesized (μmoles/15 min per g)	(% of control)
A	Rat	Sucrose	—	2·2	100
			TX	5·6	255
			ether	6·2	282
		Water	—	4·0	186
			TX	5·6	255
A	Rabbit	Sucrose	—	1·8	100
			TX	3·4	189
			ether	4·2	233
		Water	—	3·2	176
			TX	3·4	189
B	Rabbit	Sucrose	—	0·86	100
			TX	0·89	103
			ether	1·89	220

Incubation mixtures A and B correspond to A and B in Table 1.

and lasted over much longer periods of storage. One of its uses, replacing cysteine, was in extracting acetone powders of brain. It was not added to homogenates but was added to partially purified preparations derived from homogenates of placenta or other tissue.

DISCUSSION

In this research our objective has been to improve both the accuracy and the sensitivity of the radiometric assay of ChAc introduced by Schrier and Shuster [3]. To the first end, one step has been to reduce the column blanks to low and predictable levels by purifying the acetyl-CoA, by the use of larger resin columns and by discarding parts of the effluent which are identifiable as containing little or no ACh and may contain other radioactive substances. This has meant that synthesis of quantities of ACh of less than 100 pmoles can be measured with reasonable accuracy although there is still room for improvement if the method is to be used systematically for analysis at this level, in investigation of denervated tissues for example. One of the points to be stressed here is that the acetyl-CoA is at a saturating level in our standard incubation medium and this needs to be taken into account when comparing the results with earlier work [1–3]. It is desirable to use a saturating level in the interests of improving the sensitivity of the assay. This is one of the factors which have enabled us to increase above earlier levels the rate of synthesis of ACh by brain tissue. Other factors were the methods of extraction or activation of the tissues, and some changes in incubation mixture (See Table 1).

Acknowledgements—We wish to thank the nursing staff of the Labour Ward, Mill Road Maternity Hospital, Cambridge, for the placentas used, and our colleagues, Miss H. Banns, Mr. G. Bull, Miss T. Chapman and Mrs. J. Farrimond, for assistance in some of the experiments. Mrs. Janet Hood and Mrs. Sally Rochead gave expert secretarial assistance.

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