

Radiochemical Determination of Choline Acetyltransferase

JAN SCHUBERTH

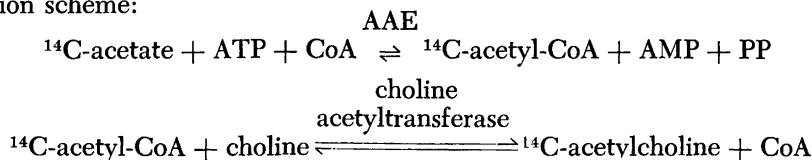
Research Institute of National Defence, Department 1, Sundbyberg, Sweden

A simple and sensitive radiochemical method for analysis of choline acetyltransferase in small samples of tissue extracts is described. In the system used for enzymatic synthesis of acetylcholine, ^{14}C -acetate, in the presence of an acetyl-coenzyme A generating system, is activated to ^{14}C -acetyl-coenzyme A. Acetyl-coenzyme A then reacts with choline, through choline acetyltransferase, to form ^{14}C -acetylcholine. ^{14}C -acetate is completely volatile as acetic acid when the acid sample is evaporated to dryness, whereas the ^{14}C -acetylcholine formed is stable under the same conditions and thus obtainable for radiochemical determination. By this method it is possible to analyze choline acetyltransferase, producing as little as 10 μmoles of acetylcholine with good accuracy and without time-consuming manipulations.

Choline acetyltransferase catalyzes the formation of acetylcholine from choline¹ and acetyl-coenzyme A² (acetyl-CoA). Previously described methods for the assay of choline acetyltransferase activity are based on the determination of acetylcholine formed after incubation of the enzyme with both substrates. The amount of acetylcholine thus formed is determined either by a rapid but insensitive photometric method³ or by a sensitive but rather time-consuming and laborious biological method⁴.

The use of an acetyl-CoA donor system is preferred to the use of stoichiometric amounts of preformed acetyl-CoA because, according to Berman *et al.*⁵ and Smallman⁶, there is good evidence that the latter method does not give as high a rate of acetylcholine synthesis as is obtainable with the donor system. Acetate activating enzyme (AAE) has been used by Hebb⁷ and by Berry and Whittaker⁸, as an acetyl-CoA generating enzyme during acetylcholine synthesis.

The aim of the present paper is to describe a new method for the analysis of choline acetyltransferase in small amounts of crude tissue extracts with good accuracy and without time-consuming manipulations. The complete system used by us for enzymatic synthesis of acetylcholine is described by the following reaction scheme:



The assay method is based on the fact that free acetate is completely volatile when the acid sample is evaporated to dryness, while acetylcholine is stable⁹ and non-volatile and therefore obtainable for radiochemical determination.

EXPERIMENTAL

Material

AAE was prepared from pressed baker's yeast. Disruption of the yeast cells was performed by the quick-freeze method as described by Jones *et al.*¹⁰ and the AAE was fractionated from the crude extract with ammonium sulfate at +4°C and at pH 7.0. The precipitate formed in 35% ammonium sulfate was discarded and the active material was obtained as a precipitate at 45% saturation. The AAE was stable for several months when stored either in saturated ammonium sulfate or in 0.02 M phosphate buffer pH 7.0 at -25°C.

Choline acetyltransferase. Full term human placenta was a convenient source of choline acetyltransferase¹¹. The placenta, trimmed from membranes, large vessels and necrotic areas, was cut in small pieces and homogenized in dry acetone at -15°C (100 × tissue volume). The acetone was filtered, the filter cake washed with ether at -15°C and then left to dry in vacuum for 3–4 h. The enzyme was extracted from the dry powder in 0.02 M phosphate buffer at pH 7.0 (50 mg powder per 1 ml buffer) and fractionated from the clear extract with ammonium sulfate at +4°C. The precipitate obtained at 33% saturation was discarded and choline acetyltransferase was precipitated at 40%. The active material was stable for several months when stored in saturated ammonium sulfate. When the enzyme activity was to be assayed the precipitate in ammonium sulfate was sedimented by centrifugation and dissolved in 0.02 M phosphate buffer at pH 7.0.

Reagents. CoA was obtained from Sigma, USA, and sodium acetate-1-¹⁴C from the Radiochemical Centre, England. Only chemicals of Analytical grade were used.

Methods

Assay¹² of AAE. The rate of activation was measured with neutral hydroxylamine as a trapping agent for formed acetyl-CoA; the acetylhydroxamic acid formed was assayed photometrically as a complex with ferric ion.

Photometric assay of choline acetyltransferase. The acetylcholine formed after incubation at 37.0°C was determined by the method of Hestrin³ in 1.0 ml samples of the reaction mixture which in addition to the choline acetyltransferase extract contained in 1.0 ml: potassium phosphate buffer pH 7.0 (50 μmoles), potassium acetate (10 μmoles), prostigmine bromide (0.1 μmole), KBH₄ (1 μmole), CoA (70% pure, 0.25 μmoles), ATP (10 μmoles), KF (30 μmoles), MgCl₂ (3 μmoles), AAE (1.5 units¹²), choline (16 μmoles). Under these conditions, we found that the rate of acetyl-CoA formation and the choline concentration did not limit the rate of acetylcholine synthesis. In the blank choline was replaced with water.

Radiochemical assay of choline acetyltransferase. Ten or twenty μl of enzyme extract were incubated in the same incubation mixture as previously described for photometric analysis of choline acetyltransferase, with the addition of 0.3 μC sodium ¹⁴C-acetate. The final incubation volume amounted to 100 μl. After incubation for one h at 37.0°C the reaction was stopped by the addition of 100 μl of 1% trichloroacetic acid (TCA) in 1 M HCl. To minimize the adsorption of ¹⁴C-acetylcholine on the precipitate formed, the TCA-HCl mixture contained 20 mM unlabelled acetylcholine. After 5–10 min 2.0 ml 0.2 M HCl was added and the precipitate was sedimented by centrifugation. 0.1 ml of the clear supernatant was uniformly spread out on a plate of stainless steel over an area of 2.25 cm² and evaporated at room temperature in a vacuum desiccator containing solid sodium hydroxide. When the sample had been evaporated to dryness, which usually occurred after 15 min, the radioactivity was measured with a flow counter (Frieseke-Hoepfner). Selfabsorption of the sample was less than 0.01% of the total radioactivity. The time for 10⁴ counts was measured. The determination of the standard sample, *i. e.* the radioactivity corresponding to 100% conversion of acetate to acetylcholine, was performed by adding to the incubation mixture 2.1 ml 0.01 M NaOH instead of 2.1 ml of the above mentioned acid solutions. The radioactivity was then determined in the same manner as previously described. Selfabsorption of the standard sample was also less

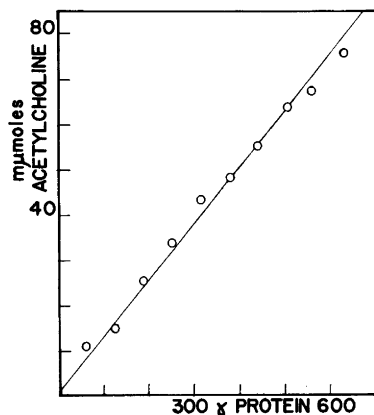


Fig. 1. Relation between the acetylcholine formation and the relative concentration of choline acetyltransferase.

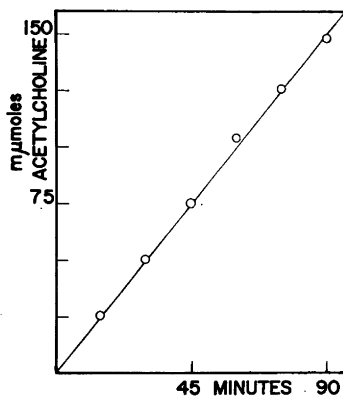


Fig. 2. Relation between acetylcholine formation and the time of incubation.

than 0.01% of the total radioactivity. The amount of formed acetylcholine is obtained by calculating the radioactivity of the specimen corrected for the blank value divided by the standard radioactivity and by multiplying this quotient by the total amount of acetate in the reaction mixture.

RESULTS

Relation between activity and concentration. Different dilutions from a stock solution of choline acetyltransferase were prepared and the protein concentration of each sample was determined according to Warburg and Christian¹⁴. Fig. 1 illustrates the relation between the choline acetyltransferase activity as determined by the radiochemical method described in "methods" and the relative concentration of choline acetyltransferase expressed by the total amount of added protein.

Comparison of photometric and radiochemical method. The choline acetyltransferase activity of the stock solution described above in "experimental and results" was assayed by the photometric method³ and the protein concentration determined¹⁴. Five independent determinations on each 0.2 ml of the stock solution gave a mean value of 796 mμmoles of synthesized acetylcholine after one hour's incubation. The specific activity of choline acetyltransferase as defined by Berman *et al.*⁵ was calculated to be 0.0125 ± 0.0002 (\pm standard error). The mean specific activity⁵ calculated from the values in Fig. 1 was 0.0126 ± 0.0003 (\pm standard error of estimate) which value is not significantly different from the specific activity assayed spectrophotometrically.

Kinetics of reaction. Choline acetyltransferase was incubated in a total of 1.0 ml of the incubation mixture as described in methods for "radiochemical assay of choline acetyltransferase". 0.1 ml of the mixture was withdrawn at subsequent times and radioassayed for choline acetyltransferase activity according to the description in methods. The result is illustrated in Fig. 2.

Precision of method. The coefficient of variation (standard deviation as percentage of the mean) was obtained as 4.3% when determined on 12 replicate determinations of choline acetyltransferase activity.

Choline acetyltransferase activity in the brain of rat. Three albino rats were killed by decapitation and the whole brains were quickly removed. The brains were separately treated with acetone, ether and extracted as described in "material" for preparation of choline acetyltransferase from placenta. The crude extracts were radioassayed for choline acetyltransferase and the mean value was found to be 22.8 μ moles of synthesized acetylcholine per gram of powder.

Chromatographic identification of acetylcholine: To verify acetylcholine as the radioactive product after the incubation of choline acetyltransferase, AAE system, ^{14}C -acetate and choline, paper chromatographic identification was carried out. Acid specimens, containing enzymatically formed ^{14}C -acetylcholine as described in "radiochemical assay of choline acetyltransferase", were pooled, evaporated to dryness in vacuum and dissolved in 0.5 ml of water. 40 μ l of the clear solution was concentrated in small spots on each three filter papers (Whatman no 1) and chromatographed by descending method in three solvent systems¹³: butanol saturated with water, propanol- H_2O (9:1) and propanol-formic acid- H_2O (8:1:1). The spots containing radioactive material, identified by scanning the paper strips with a flow counter, overlapped perfectly the spots containing unlabelled acetylcholine identified by spraying the paper with hydroxylamine and FeCl_3 ¹³. The following R_F values of the added acetylcholine as well as of the ^{14}C -acetylcholine were obtained: butanol saturated with water 0.11, propanol- H_2O 0.33 and propanol-formic acid- H_2O 0.45.

DISCUSSION

The radiometric method described has been worked out by measuring the enzyme activity of partially purified choline acetyltransferase from human placenta. The author has thus shown that by the radiochemical determination of choline acetyltransferase there is a strict linear relationship between the enzyme concentration and the amount of formed acetylcholine in the range of 10 to 80 $\text{m}\mu$ moles of acetylcholine. The radiochemical method is thus about 50 times more sensitive than the photometric method³ but about 5 times less sensitive than the biological method⁴. By the radiometric method about 15 double determinations per person per day can be performed with an accuracy of 4.3% (coefficient of variation).

It has also been shown that the formation of acetylcholine in the system used by the author follows a zero order kinetics in the range of zero to 90 min of incubation time and zero to 150 $\text{m}\mu$ moles of synthesized acetylcholine.

The metabolism of acetate to nonvolatile substances different from acetylcholine can evidently occur, and this has been corrected for by subtracting the blank value from the specimen value. The validity of this correction, however, requires that the enzyme is free from choline which might not be the case when using crude extracts or homogenates from brain tissue. K_m for choline is 5×10^{-4} ¹⁵ which means that fairly small amounts of choline in the enzyme extract might give too high blank values and thus too low choline acetyltransferase activity. To obtain full choline acetyltransferase activity from brain tissue, the homogenate

must, before extraction, be treated with an organic solvent¹⁶, *e. g.* ether or acetone. The advantage of using acetone to ether is, in accordance with the above discussed demand for choline free enzyme extracts, that choline is fairly soluble in acetone and therefore removed from the tissue powder before the enzyme is extracted. To test the radioassay method on a crude extract of brain tissue the determination of choline acetyltransferase activity in the brain of rat was performed as described in experimental and results. The value thus obtained was in good agreement with that obtained by Berry and Whittaker⁸.

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