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A SIMPLE RADIOISOTOPIC ASSAY FOR CHOLINE ACETYLTRANSFERASE AND ITS APPLICATION IN THE *LACTOBACILLUS PLANTARUM* SYSTEM*

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Abstract—A new assay for choline acetyltransferase (acetyl CoA: choline-*o* acetyl transferase) (2.3.1.6) activity has been developed. The Reineckate salt of acetylcholine formed from radioactive acetate or acetyl coenzyme A is assayed for radioactivity.

This assay was used in studies of an acetylcholine-producing strain of *Lactobacillus plantarum*. It was shown that when pantothenate is limiting, appreciable growth can occur with no manifestation of choline acetyltransferase activity. The enzyme was partially purified from extracts of this organism.

CHOLINE acetyltransferase (choline acetylase) is a widely distributed enzyme concerned with the biosynthesis of the neurohormone acetylcholine (ACh).[1] Besides nervous tissue, the enzyme has been found in human placenta, red blood cells, protozoa and bacteria.[2–4]

While studying ACh production in *Lactobacillus plantarum* we developed a rapid, convenient, sensitive radioisotopic assay for choline acetyltransferase, which is described herein. Application of this method revealed that *L. plantarum* is capable of growth under conditions in which choline acetyltransferase is not detectable, suggesting that ACh does not play an essential role in its metabolism.

MATERIALS AND METHODS

Organisms and culture conditions

Several strains of *L. plantarum* (ATCC nos. 8014, 8292, 4008, 10012, 10241) were found to produce ACh in washed suspensions supplemented with glucose and choline. *L. plantarum* no. 8014 (synonym, *L. arabinosus* 17–5) was chosen for these studies since it is widely used for microbiological assays.

Stab cultures of the organism were maintained on a medium containing 2% tryptone, 1% yeast extract, 1% glucose, and 1·5% agar. The same medium without agar was used for liquid cultures.

The synthetic medium consisted of 10 g vitamin-free casamino acids (Difco), 9·8 g sodium acetate, 20 g glucose, 1 g KH_2PO_4, 1 g K_2HPO_4, 100 mg L-cysteine,

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100 mg DL-tryptophan, 5 mg adenine, 5 mg guanine, 5 mg uracil, 5 mg xanthine, 1 mg thiamine HCl, 1 mg riboflavin, 1 mg nicotinic acid, 2 mg pyridoxine HCl, 0·1 mg *p*-aminobenzoic acid, 1 mg folic acid, 0·2 mg Ca pantothenate, 2·5 μg biotin, 400 mg $MgSO_4 \cdot 7\ H_2O$, 20 mg NaCl, 20 mg $FeSO_4 \cdot 7\ H_2O$, 20 mg $MnSO_4 \cdot H_2O$. The pH was adjusted to 6·8 with KOH and the final volume was 1 liter. The cells were grown at 37°.

Chemicals

Acetate-1-^{14}C and acetyl-1-^{14}C-CoA, having specific activities of 2 and 32 μc/μmole respectively, were obtained from the New England Nuclear Corp., Boston, Mass. Acetyl-1-^{14}C-choline (1 μc/μmole) was obtained from Nuclear Research Chemicals, Orlando, Fla. and from the New England Nuclear Corp.

Preparation and incubation of resting cells. The cells were washed three times with water by centrifugation and incubated at 37° in a suspension having the following composition: cells equivalent to 0·5 mg dry weight/ml, sodium acetate-1-^{14}C 0·005 M (0·5 μc/ml final mixture), choline chloride 0·02 M, glucose 0·05 M, Na_2HPO_4 0·003 M. The pH of the mixture was 7·0. After incubation the cells were removed by centrifugation and the ACh determined in the supernatant by one of the methods described below. Total ACh was determined after acidification (2 drops 0·1 N HCl/1 ml supernatant) and heating to 100° for 2 min prior to centrifugation.

Preparation of cell-free extracts. After growth for 21 hr the cells were harvested in a Sharples centrifuge, and an acetone-dried powder prepared. Thirty liters of tryptone yeast extract, and glucose medium yielded 5 g of powder. The extraction and fractionation procedures described below were carried out at 0–5°. Five grams of acetone-dried powder was stirred with 50 ml of water. The paste was treated with a Branson Sonifier sonic oscillator for 25 min at setting (7). The sonicate was centrifuged at 23,500 *g* for 40 min and the supernatant decanted. Solid ammonium sulfate was added to the supernatant to bring the solution to 50, 70, and 90 per cent of saturation. After each addition a 40-min interval elapsed before centrifugation. Each fraction was dissolved in water and dialyzed against 4 l. of water for 20 hr with one change of water after 6 hr. Three ml of a 5% solution of streptomycin sulfate was added to 30 ml of the 0–50% ammonium sulfate fraction. After 2 hr the precipitate was removed by centrifugation. The supernatant solution was dialyzed as described above. This fraction has been stored at −20°, with only slight loss of activity, for three months. Further purification was obtained by DEAE chromatography. A DEAE-cellulose column 2·3 × 24 cm was equilibrated with 0·01 M sodium phosphate buffer, pH 7·5. Fifteen ml of the streptomycin-treated enzyme preparation (27·8 mg protein) was adjusted to pH 7·5 with 5% sodium bicarbonate, applied to the column, and washed with 150 ml of buffer. The column was eluted with a NaCl gradient (250 ml buffer in mixing chamber and 250 ml 0·35 M NaCl in buffer in reservoir); 9-ml fractions were collected. Fractions 36 through 51 were combined, lyophilized, and taken up in 1·5ml water. Although individual tubes had specific activities as high as 35, after lyophilization the specific activity was approximately the same as the streptomycin-treated preparation added to the column. Protein was determined by the method of Lowry *et al.*[5]

Determination of acetylcholine

Biological determination. The frog rectus abdominis muscle assay was carried out as described.[6]

Radiochemical determination. (a) Plating method: 0·2 ml of a 1% solution of ACh chloride and 3 ml of a saturated solution of Reinecke salt in 0·5 N HCl, prepared fresh daily, were added to 1 ml of the reaction mixture. After 30 min the precipitate, consisting of the Reineckates of ACh and choline, was collected on a Millipore filter, pore size 0·65 μ. The precipitate was washed four times with 2-ml portions of *n*-propanol. Suction was maintained until excess *n*-propanol had evaporated. The filter was glued to an aluminum planchet with rubber cement, dried under an infrared lamp for 30 min, and radioactivity was then measured with a Nuclear-Chicago thin-window gas-flow Geiger counter. The blank consisting of the incubation mixture without enzyme had negligible radioactivity. A factor which combines a self-absorption correction and a correction for extent of precipitation (80 per cent) was obtained by precipitation of authentic ACh-^{14}C from the same volume of reaction mixture. After correcting for counting efficiency, the amount of ACh could be calculated from the specific activity of the added acetate-^{14}C. When acetate with a specific activity of 0·1 μc/μmole was used, this method detected 2×10^{-10} mole of ACh, a level that is comparable to the threshold dose in the frog rectus abdominis muscle assay.

When no unlabeled ACh was added, the Reineckate precipitated as shiny pink flakes which did not adhere well to the filter. Addition of the prescribed amount of ACh produced a fine precipitate which did adhere well to the filter. Six determinations on a sample of ^{14}C-ACh synthesized by resting cells yielded an average value of 37·3 with a standard deviation of $\pm$ 1·1 (3 per cent) mμmoles/mg/2 hr. MacIntosh and Perry[6] report a standard deviation of $\pm$ 5 per cent for the frog rectus assay.

(b) Scintillation counting method. This procedure is based on that described by McCaman.[7] Twenty μliters of a 1% solution of ACh chloride and 0·2 ml of a saturated solution of Reinecke salt in 0·5 N HCl were added to 0·2 ml of the enzyme incubation mixture in a 0·5-ml centrifuge tube (Misco). After 30 min the precipitate was centrifuged and washed three times with 0·4 ml *n*-propanol. The precipitate was dissolved in 0·2 ml of acetone, and 0·2 ml of silver nitrate solution (5 mg/ml) was added. The resulting precipitate of silver Reineckate was centrifuged, and the supernatant solution containing the radioactive ACh was decanted into a vial for counting. The precipitate was washed once with 0·4 ml 50% acetone in water, and the wash was added to the vial. Fifteen ml of DAM-611 counting fluid[8] was added to the vial, and the radioactivity was measured in the Packard Tri-Carb scintillation counter. The recovery of added ACh-^{14}C was the same as for the plating method (80 per cent).

The identity of the labeled material excreted by *L. plantarum* with ACh was verified by ascending chromatography of the cell-free medium on Whatman 3 paper, with *n*-butanol : acetic acid : water (4 : 1 : 1), as the solvent. Its R_f value (0·55) coincided with authentic ACh, determined either with radioactivity or by the color produced on spraying the chromatogram with 0·5% iodine in absolute ethanol.[9] Furthermore, the radioactive material synthesized by *L. plantarum* yielded no radioactive material in the plating assay after alkaline hydrolysis. This is the expected result, because ACh is destroyed by alkaline hydrolysis.[10]

RESULTS

Assay of ACh

The recovery of added authentic ACh in the plating method is shown in Fig. 1. The recovery of added radioactivity agrees with the ACh concentration, as determined with the frog rectus assay.

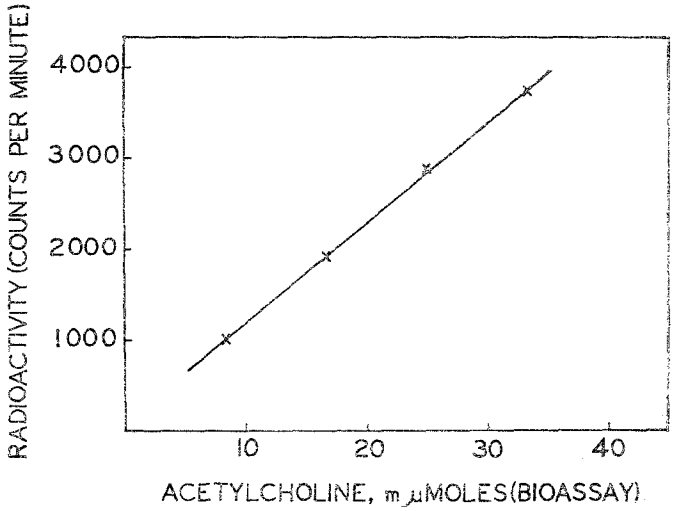

FIG. 1. Recovery of ACh by Reineckate precipitation.

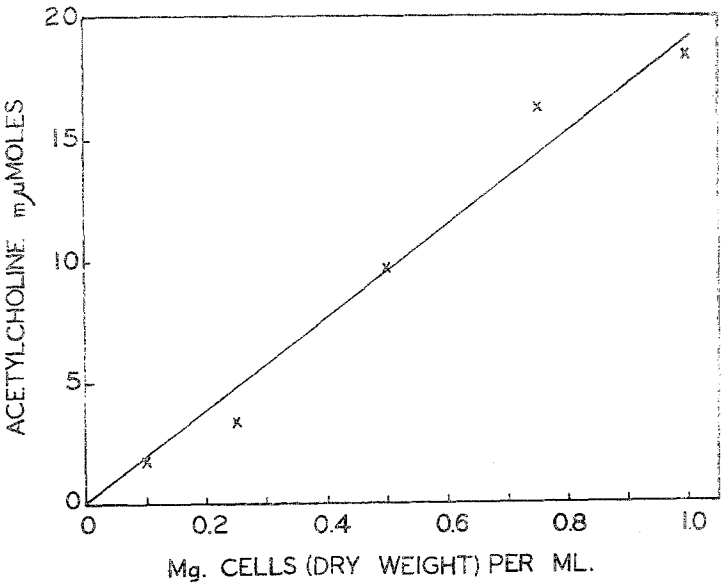

FIG. 2. Formation of extracellular ACh as a function of cell density. Incubation, 2 hr at 30°. ACh determined by scintillation counting method.

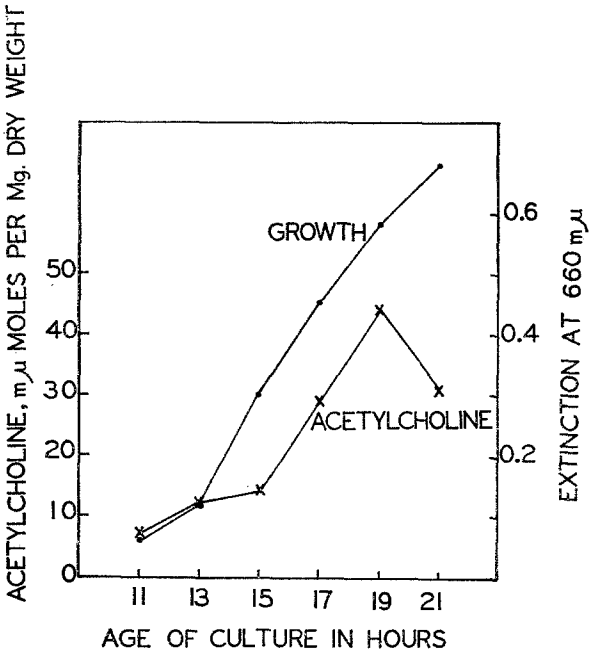

FIG. 3. Formation of ACh as a function of age of culture. Cells harvested from synthetic medium containing 500 μg Ca pantothenate/l. were incubated 1 hr. Growth at time of harvest indicated by turbidity at 660 mμ. ACh determined by scintillation counting method.

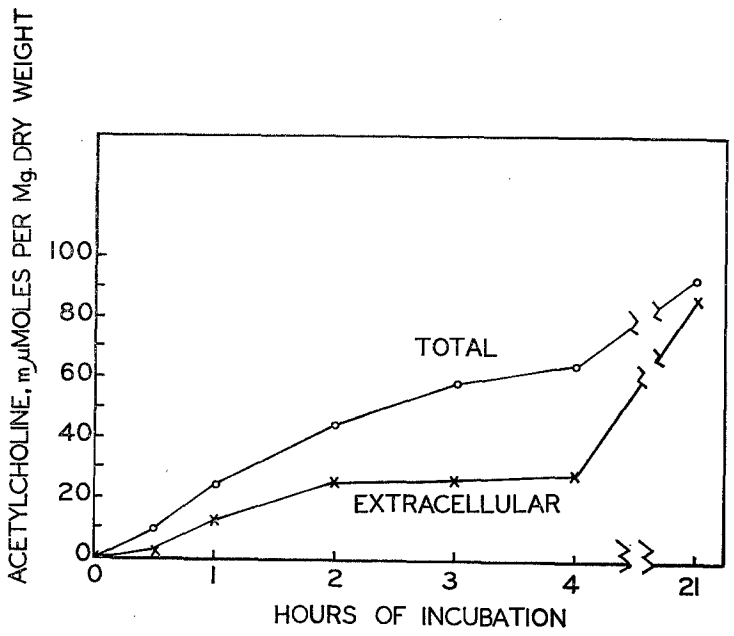

FIG. 4. Rate of ACh production by resting cells. Cells were grown on synthetic medium containing 200 μg calcium pantothenate/liter. Total ACh was determined (scintillation counting method) after adding 0·05 ml 1 N HCl/ml incubation mixture and keeping samples at 100° for 3 min.

ACh production by resting cells under various conditions

As shown in Fig. 2, ACh production is a linear function of cell density. Figure 3 shows that cells harvested in the later stages of growth have a greater ability to synthesize ACh than have those harvested at earlier stages. The rate of ACh synthesis by resting cells is shown in Fig. 4. Up to 4 hr of incubation, about half the total amount synthesized remains inside the cells. By 21 hr, most of the ACh is extracellular.

Table 1 shows that the radiochemical plating method accounts for 76% of the ACh as measured by bioassay. The lower values obtained with the plating method are probably due to dilution of added acetate-^{14}C with nonradioactive acetate produced

TABLE 1. A COMPARISON OF THE PLATING METHOD AND BIOASSAY (FROG RECTUS)[6] FOR THE DETERMINATION OF ACH PRODUCED BY RESTING CELLS

Incubation time (hr)	Total ACh		Plating method / Bioassay
	Bioassay (μmoles/mg dry weight)	Plating method (μmoles/mg dry weight)	
0·5	8·5	6·5	0·76
1·0	18·1	15·0	0·83
2·0	39·9	30·2	0·76
3·0	70·0	47·4	0·68
4·0	83·3	58·6	0·70
21·0	160·6	137·2	0·86

from glucose during the incubation. When acetate was omitted, bioassay revealed that 11·1 mμmoles of ACh were synthesized per mg in 2 hr. This is 28 per cent of the amount synthesized when acetate is included (39·9 mμmole/mg/2 hr; Table 1) which would account for the lower values found in the plating method.

The marked influence of pantothenate on the choline acetylase activity of resting cells is shown in Fig. 5. In the critical range of pantothenate concentration (0·03–0·1 μg/ml), an increase in growth of approximately 25 per cent is accompanied by a 30-fold increase in choline acetyltransferase activity. It is noteworthy that 40 to 60 per cent of the maximal growth could be attained with very little manifestation of choline acetyltransferase activity.

Synthesis of ACh in cell-free extracts

The striking dependency of ACh synthesis on the amount of pantothenate added to the culture reflects the availability of CoA. This was shown to be the case by adding CoA to an enzyme preparation from cells grown on a low pantothenate medium (Table 2). In the absence of added CoA the choline acetyltransferase activity was very low. Addition of CoA increased acetyltransferase activity to a level similar to that found in extracts from cells grown with yeast extract.

Partial purification of choline acetyltransferase

After ammonium sulfate fractionation and removal of nucleic acids by treatment with streptomycin, a preparation was obtained which synthesized 3·5 μmoles ACh/mg protein/hr. This represents a sevenfold increase in specific activity over the starting extract (Table 3). Preliminary work with DEAE-cellulose chromatography has

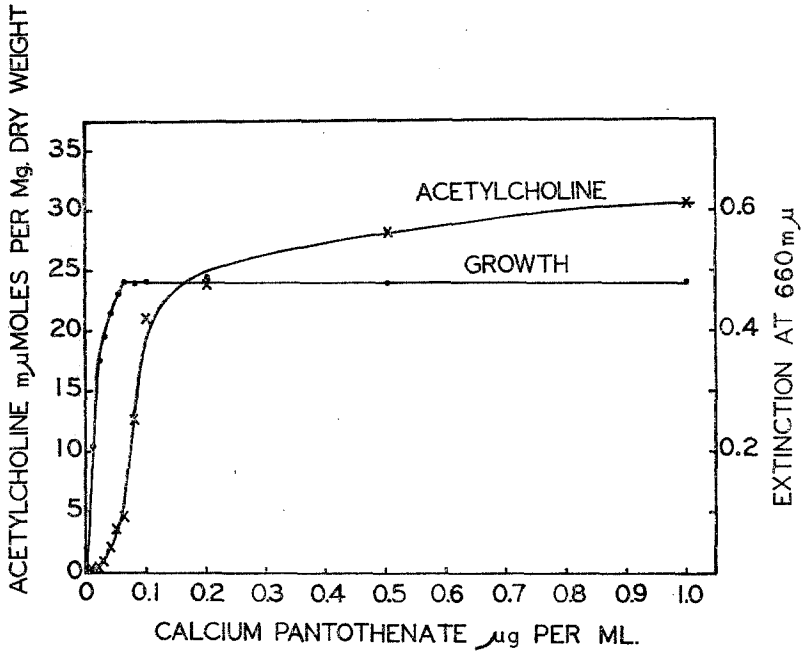

FIG. 5. Influence of pantothenate concentration during cell growth on ACh production by resting cells. The cells used as an inoculum in this experiment were depleted of pantothenate by two transfers through synthetic medium from which pantothenate had been omitted. ACh was determined by scintillation counting method.

TABLE 2. THE EFFECT OF CoA ON THE SYNTHESIS OF ACh BY EXTRACTS OF *L. plantarum*

Addition	ACh (mμmoles/mg protein)
None	0·1
CoA, 0·02 μmole	3·3
CoA, 0·06 μmole	7·6

Reaction mixture: Choline chloride 0·02 M, cysteine HCl 0·05 M, sodium acetate-1-^{14}C 0·01 M (0·4 μc), ATP 0·01 M, sonic extract of acetone-dried *L. plantarum* (grown on synthetic medium containing 0·0075 μg Ca pantothenate/ml), 3·2 mg protein. Final volume 2 ml; pH 6·0. After incubation for 2 hr at 30°, ACh was assayed by the plating method.

yielded preparations with a further fivefold increase in specific activity. The results shown in Fig. 6 demonstrate that ACh production is a linear function of protein concentration.

DISCUSSION

The Reineckate plating method is a rapid and simple means of assaying choline acetyltransferase activity. Unlike the frog rectus abdominis assay, it is not affected by the presence of choline and potassium ions.

TABLE 3. PURIFICATION OF CHOLINE ACETYLTRANSFERASE

	Volume (ml)	Protein (mg/ml)	Specific activity*	Units†
Sonicate	40·5	12·5	0·547	270
Ammonium sulfate				
0–50%	31·5	6·7	0·922	190
50–70%	34·0	5·2	0·528	92
70–90%	30·0	1·1	0·018	
Streptomycin supernatant	30·0	1·8	3·52	190

The incubation mixture, containing choline chloride 0·02 M, cysteine HCl 0·05 M, sodium phosphate (pH 6·0) 0·05 M, acetyl-1-^{14}C CoA 0·001 M (0·05 μc), enzyme solution 10μliters, in a total volume of 0·2 ml at pH 6·0, was incubated for 60 min at 37°. ACh was determined by the scintillation counting method.

* Specific activity = μmoles ACh/mg protein/hr.

† Unit = 1 μmole ACh produced/hr.

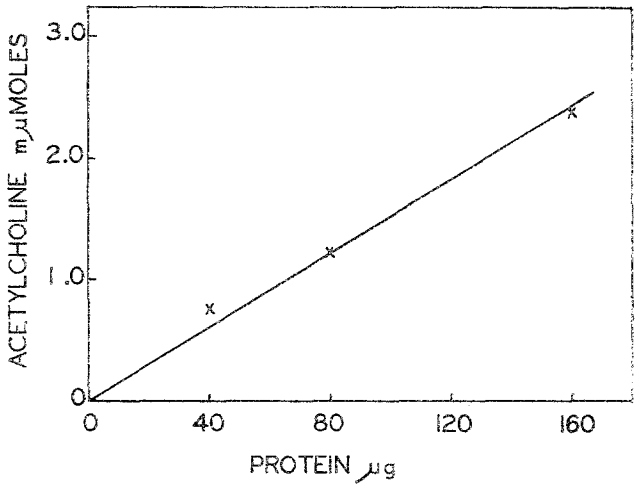

FIG. 6. Synthesis of ACh as a function of enzyme concentration. Assay conditions as in Table 3. The enzyme used was obtained from lyophilized DEAE-cellulose fractions, as described under Materials and Methods.

As a measure of ACh produced by whole cells, the plating method is indicative only of the amount derived from externally supplied acetate. Endogenous sources of acetate would be progressively less significant as the enzyme extract is purified. For whole cells the maximal dilution is 24 per cent of the total amount synthesized.

Wacker *et al.*[11] observed that pantetheine permitted growth and production of ACh in *Streptobacterium plantarum* 10s. Novelli and Lipmann observed the proportionality of pantothenic acid content of the growth medium and CoA content in *L. plantarum*.[12] Since growth proceeds at pantothenate levels capable of supporting only extremely low choline acetyltransferase activity, it appears that this enzyme is not essential for the growth of the organism.

The specific activity of the choline acetyltransferase preparations obtained from *L. plantarum* in the present studies is slightly higher than that reported by others.[3, 4]

The choline acetyltransferase activities of various tissues have been summarized by Hebb.[2] Berman *et al.*[13] purified the choline acetyltransferase from squid head ganglia, obtaining a preparation which synthesized 58 μmoles ACh/mg protein/hr. The most active preparation obtained in the present study was obtained after DEAE chromatography of the partially purified enzyme. It synthesized 35 μmoles ACh/mg/hr. Thus *L. plantarum* provides a convenient source for preparing large amounts of choline acetyltransferase with high specific activity.

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