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Isolation, Extraction, and Measurement of Acetylcholine from *Lactobacillus plantarum*

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The isolation, extraction, and spectrophotometric determination of acetylcholine from *Lactobacillus plantarum* ATCC 10241 is described. Acetylcholine was extracted with a mixture of sodium tetraphenylboron-butylethylketone-acetonitrile and was measured enzymatically at 340 nm.

Acetylcholine (ACh) extracted from biological tissues has usually been determined by bioassay methods because of their range of sensitivities, yet bioassay methods usually lack specificity and may be affected by interfering substances (7). For this reason several attempts have been made to devise chemical methods of sufficient sensitivity for the analysis of ACh and related substances in tissues. Gas chromatography and an integrated gas chromatography/mass spectrometer system have been employed by Hammer et al. (3) to measure Ach. Gas chromatography can be as effective as the most sensitive bioassay procedure, but expensive and sophisticated equipment is required. Conversely, inexpensive enzymatic spectrophotometric procedures provide sufficient sensitivity and high specificity.

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Micro Inoculum broth (Difco) and Micro Assay culture agar (Difco) were used to grow the stock culture of *L. plantarum* ATCC 10241. The cells were collected in a Sorvall refrigerated centrifuge, washed, and suspended in 5 to 8 ml of water to give a bacterial concentrate. Turbidity measurements were made on a Coleman Junior spectrophotometer at 650 nm, and dry-weight determinations were done on duplicate portions dried to constant weight at 65°C. One milliliter of concentrated *L. plantarum* was placed in 9 ml of stationary-phase incubation medium, which contained the following: 2.0 ml of McIlvaine buffer (pH 7.5), 1.0 ml of 20% glucose, 1.0 ml of 0.5% choline-hydrochloride, 1.0 ml of concentrated bacterial suspension (10 mg [dry weight]) and 5.0 ml of double-distilled water. McIlvaine buffer, consisting of 0.2 M disodium phosphate and 0.1 M citric acid, was adjusted to pH 7.5. After incubation at 25°C for 20 h, the sample was centrifuged, and the supernatant was acidified with 1 N HCl to approximately pH 4 and lyophilized. A 10-ml portion of lyophilized supernatant was reconstituted with 1.0 ml of water; 0.6 ml of 0.6 M perchloric acid (PCA) was added, and the cells were collected by centrifugation (2,000 rpm, 10 min). The PCA supernatant was neutralized with 0.18 ml of 2 M potassium bicarbonate, the pH was adjusted to approximately 4.0 with 1 N HCl, and the preparation was placed in the freezer overnight.

Tetraphenylboron-ACh complexes are more soluble in organic solvents than in aqueous solutions (1, 2). Hydrochloric acid dissociates the complex and reextracts the ACh into the aqueous layer, thus removing tetraphenylboron, which is inhibitory in the enzyme assay.

The bacterial supernatant was diluted to a working volume of 5.0 ml, and an equal volume of a mixture of sodium tetraphenylboron (Kalignost, 100 mg), butylethylketone (8 parts), and acetonitrile (2 parts) was added to glass-stoppered tubes. Tubes were shaken for 10 min on a wrist-action shaker, and the phases were separated by brief centrifugation (2,000 × *g* for 2 min). After the aqueous phase was extracted two additional times, the three extractions were pooled. In the re-extraction procedure, the ACh was recovered in an aqueous phase by shaking 1.0 ml of the ketone layer with an equal volume of 0.2 N HCl for 10 min. After brief centrifugation, the ketone phase was re-extracted with a second volume of 0.2 N HCl. To remove residual ketone from the aqueous pooled extracts, 1.0 ml of cyclohexane was added, mixed, and removed with a disposable Pasteur pipette. The extract was adjusted to pH 4 to 5 with 3.5 N NaOH and lyophilized.

The neutralized aqueous extract was passed over a Sephadex G-10 column to separate ACh from NaCl, other salts, and low-molecular-

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weight compounds, which interfered with the spectrophotometric assay.

The enzymatic fluorometric method described by O'Neill and Sakamoto (6) was adapted to the spectrophotometer. The reagent mixture contained 200 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0), 0.3 mM oxidized nicotinamide adenine dinucleotide (NAD^+), 4.0 mM potassium L-malate, 0.25 mM coenzyme A (CoA) 4.0 mM cysteine hydrochloride, 37.0 mM KCl, 5.0 mM $MgCl_2$, and 1.0 mM tris(hydroxymethyl)aminomethane-adenosine 5'-triphosphate (ATP). Since CoA was unstable in the complete mixture, it was kept cold and added immediately before the assay. Before use, cysteine-hydrochloride was neutralized with 1 M KOH. The enzyme mixture contained citrate condensing enzyme (0.7 U), malate dehydrogenase (0.7 U), acetyl-CoA synthetase, and acetylcholinesterase (1 to 2 U). To three quartz cuvettes, 500 μl of the reagent mixture was pipetted and the optical density (OD) was read at 340 nm with a spectrophotometer (Zeiss PMQ11). One to fifty microliters of sample or Ach standard was added to each cuvette, and the OD was recorded. Malate dehydrogenase was added to all tubes and mixed, and any change in OD was noted. Citrate condensing enzyme was added at 3 min and mixed, and the absorbance was again read after 2 min. Acetylcholinesterase was added to two cuvettes, with the third serving as a sample blank. After 8 min of incubation, acetyl-CoA synthetase (the rate-limiting enzyme) was added, and the maximum absorbance was recorded. Readings were taken at intervals after the addition of all enzymes, and final readings were taken when the standards and samples showed a maximum change in absorbance.

Acetyl-CoA synthetase was prepared from beef heart mitochondria according to the method of Webster (8). It was further purified by column chromatography on Sephadex G-100 a second time after fractionation on triethanolaminoethyl cellulose (7).

In the development of the extraction procedure, a [^{14}C]ACh standard was used to estimate the efficiency of the method. For this purpose, 20 μl of an ACh solution containing 60 nmol and approximately 20 nCi of radioactivity was added to 5.0 ml of a suitable buffer and extracted. The standard contained a mixture of [^{14}C]ACh (70%) and [2-^{14}C]acetate (30%). Based on these considerations, as shown in Table 1, 77 to 89% of the ACh radioactivity was extracted into the organic phase. In Table 2, approximately 73% of the ACh initially present was accounted for in the aqueous extracts after the re-extraction procedure. These calculations were based on the amount of isotope present and on the purity of the [^{14}C]ACh added.

TABLE 1. *Extraction of sodium tetraphenylboron [^{14}C]ACh complex from the aqueous phase with butylethylketone/acetonitrile*[a]

ACh solution	ACh extracted in organic phase (dpm)	ACh remaining in aqueous phase (dpm)	ACh extracted in organic phase (%)	Activity extracted (%)[b]
McIlvaine medium	27,789	8,624	62	89
	25,423	9,750	57	81
	26,909	8,252	60	86
DDW	24,015	9,389	54	77
	25,700	9,447	57	81

[a] Twenty microliters of [^{14}C]ACh standard (44,720 dpm) – [^{14}C]acetate (13,300 dpm) = [^{14}C]ACh (31,420 dpm). Therefore, 70% of dpm = [^{14}C]ACh in [^{14}C]ACh standard, and 30% of dpm = [^{14}C]acetate in [^{14}C]ACh standard.

[b] Calculated according to the formula: (% activity of ACh into organic phase)/(% activity of ACh in [^{14}C]ACh standard in organic phase [70%]) = % of activity extracted.

TABLE 2. *Effect of acidity on recovery of [^{14}C]ACh from butylethylketone/acetonitrile*

ACh extracted in organic phase (dpm)	ACh re-extraction into aqueous HCl phase (dpm)	Re-extraction into aqueous HCl phase (%)	Overall[a] recovery
26,909	20,920	78	91 (0.2 N HCl)
26,909	21,337	79	92 (0.4 N HCl)
26,909	19,340	72	84
26,909	20,234	75	87
26,909	17,701	66	77 (pH 7)
26,909	19,519	73	85
26,909	18,565	69	80

[a] Calculated according to: (% activity re-extracted into aqueous-HCl layer)/(% activity extracted into organic layer [86%]) = overall recovery (percent of activity extracted into organic layer is based on 86% activity extracted, as shown in Table 1).

The spectrophotometric adaptation of the enzymatic fluorometric method permitted the measurement of 20 to 100 nmol of ACh (Fig. 1). Over this concentration range, linear relationships exist between ACh concentration and OD. In our studies, *L. plantarum* 10241 produced 44 μg of ACh per 10 mg (dry weight) of cells and, according to this calculation, there were 24 nmol of ACh per ml of incubation medium per mg (dry weight). Our results are in

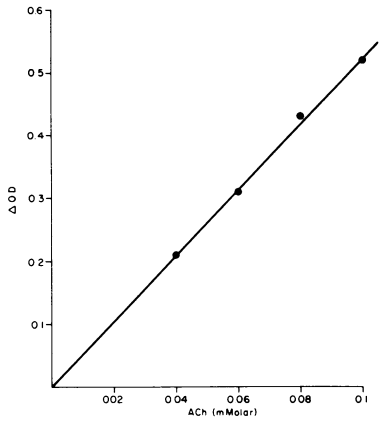

FIG. 1. *Spectrophotometric measurement of standard ACh.*

agreement with Keil and Kritter (5), who reported 40 μg of ACh per ml in sauerkraut juice.

Bacterial supernatants were acidified, freeze-dried, and lyophilized before assay; these steps eliminated free acetate. Adequate control of pH minimized the nonenzymatic formation of acetate from ACh and other acetyl derivatives, e.g., acetyl phosphate, during preparation of the samples for assay. The PCA step was inserted before ACh extraction to precipitate interfering proteins and to remove any potassium in the supernatant. Residual PCA interferes with the enzymatic assay; therefore, neutralization with potassium bicarbonate is necessary. Although potassium has a low solubility in PCA, potassium perchlorate does not precipitate completely from solution at room temperature. The samples were placed in the freezer overnight to facilitate complete precipitation of potassium perchlorate, which interferes in the extraction and measurement of ACh.

The enzymatic method described by O'Neill and Sakamoto (6) reliably measures small amounts of ACh (ca. 10^{-9} mol) from tissue extracts with less than 15% error. The method depends on differences in the properties of $NADH_2$, and NAD^+. ACh is selectively hydrolyzed by acetylcholinesterase to acetate in the presence of other acetyl compounds of biological importance. Specificity is retained in the conversion of acetate to acetyl-CoA by acetyl-CoA synthetase.

The spectrophotometric method of measurement is advantageous in comparison to the fluorometric method in that concentrations can be calculated directly from the extinction coefficient for NADH at 340 nmol, i.e., 6,273 $\times$ 10^{-3} mol/cm^2 (4), thus eliminating the need for running standards. However, ACh standards were carried through the entire procedure to ensure that all enzymes were functioning properly and were not adversely affected by the extraction procedure (7); such standards also served as an estimate of extraction efficiency.

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