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ADENOSINE 5′-TRIPHOSPHATE-CITRATE LYASE ACTIVITY IN MAMMALIAN SPERMATOZOA

A NEW RADIOMETRIC METHOD AND CHARACTERIZATION OF THE ENZYME IN SPERMATOZOA

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Abstract—A simple radiochemical method was developed for determining the ATP-citrate lyase activity in mammalian spermatozoa. The determination of enzyme activity was followed by the measurement of the incorporation of the [1-^{14}C]acetyl group from [1,5-^{14}C]citrate into [1-^{14}C]acetylcoenzyme A (ACoA). Separation of ^{14}C-labeled ACoA from the reactants and their products was achieved by rapid anion exchange chromatography. The optimum pH was 6.4 for rat spermatozoal ATP-citrate lyase. The activity was not altered by dithiothreitol. $MgCl_2$ (10 mM) caused a 50 per cent inhibition in the enzyme activity. ATP-citrate lyase activities in rat and human spermatozoa were 154 ± 14 and 90 ± 12 nmoles of ACoA formed/mg of protein/5 min. Citrate may serve as an acetyl source for acetylcholine formation by spermatozoa.

ATP-citrate lyase (EC 4.1.3.8) is a cytoplasmic enzyme that occurs in pigeon liver [1], ox brain, pig heart [2] and certain bacteria [3]. It catalyzes the formation of acetylcoenzyme A (ACoA) in the presence of citrate, coenzyme A, ATP and Mg^{2+} according to the following equation:

$$\text{Citrate} + \text{CoA} + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} \text{ACoA} + \text{oxaloacetate} + \text{ADP} + P_i$$

In general, moderately high specific activities of this enzyme are present in those tissues that have a high capacity for acetyl group utilization. Examples are extracts of rat liver, which has a high capacity for fatty acid synthesis, and rat brain, which uses citrate for acetylcholine (ACh) synthesis [4].

Mammalian spermatozoa contain choline acetyltransferase (ChA), ACh and acetylcholinesterase [5–8]. Sperm ChA catalyzes the synthesis of ACh from its precursors, choline and ACoA. There are probably four sources that supply choline for ACh synthesis in spermatozoa: (a) free choline in seminal plasma (only in some species) [9, 10]; (b) phosphorylcholine in seminal plasma [11]; (c) glycerylphosphorylcholine in seminal plasma [11]; and (d) phospholipids in spermatoza [12]. Nothing is known, however, about ACoA, the second precursor for ACh synthesis, in spermatozoa. Mammalian semen contains citric acid [11], which may serve as a source for the synthesis of ACoA. No studies have been published on ATP-citrate lyase in spermatozoa. Therefore, a radiometric method has been developed for the assay and characterization of ATP-citrate lyase in spermatozoa.

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METHODS

Materials. [1,5-^{14}C]Citric acid (sp. act. 90.5 mCi/mmole) and [acetyl-1-^{14}C]coenzyme A (sp. act. 52.4 mCi/mmole) were purchased from the New England Nuclear Corp., Boston, MA. Coenzyme A was obtained from the Nutritional Biochemical Corp., Cleveland, OH, and ATP and bovine serum albumin were obtained from the Sigma Chemical Co., St. Louis, MO. Anion exchange resin (AG 1-X4, 200–400 mesh, chloride form) was purchased from Bio-Rad Laboratories, Richmond, CA. Dithiothreitol was purchased from CalBiochem, San Diego, CA. All other chemicals used were of analytical grade and were obtained from commercial sources. All solutions were prepared using distilled, deionized water. Aqueous counting scintillant was purchased from the Amersham–Searle Co., Arlington Heights, IL.

Rat and human spermatozoa homogenates. Male Sprague–Dawley rats (250–300 g) were decapitated. The cauda portion of the epididymis was used as the source of mature spermatozoa. Caudae were washed with ice-cold, deionized, distilled water, and 1 ml of ice-cold sucrose solution (0.32 M) was injected directly into each cauda. After 10 min, the cauda was cut into two pieces and pressed with forceps to obtain a suspension of sperm cells in sucrose solution. This suspension was centrifuged for 10 min at 1000 *g* (3000 rpm) in a Sorvall SS34 rotor, and the supernatant fraction was discarded. The pellet was resuspended in ice-cold sucrose solution (1 ml/cauda), and a portion of it was removed for counting cells. In some experiments more sucrose solution was added to dilute the enzyme protein concentration. The

sperm suspensions thus obtained were homogenized with a Polytron PT 10 homogenizer at maximum speed for 20 sec.

Human ejaculates were collected from a homogeneous group of normal healthy volunteers. Ejaculates were allowed to liquify at room temperature (25°). Samples were pooled and suspended in 10 ml of cold (4°) Norman–Johnson's solution without glucose [13]. The suspension was centrifuged (1000 *g*, 10 min) in a Sorval SS34 rotor. This process was repeated twice more, with the final washing being 10 ml of ice-cold sucrose (0.32 M). After washing, the sperm pellets were resuspended in an appropriate amount of 0.32 M ice-cold sucrose (1–5 ml). The sperm suspensions were then homogenized using a Polytron PT 10 homogenizer at maximum speed for 20 sec.

Radiometric assay for ATP-citrate lyase. The reaction mixture contained KH_2PO_4–K_2HPO_4 buffer (50 mM, pH 6.4), coenzyme A (0.6 mM), ATP (3 mM), $MgCl_2$ (5 mM), bovine serum albumin (1 mg/ml), citrate (3.3 mM, 2.2 × 10^5 dpm [1,5-^{14}C]citric acid), dithiothreitol (5 mM), and enzyme preparation (0.1 ml) in a total volume of 0.9 ml. The enzyme preparation was preincubated with 0.5 per cent (v/v) Triton X-100 in an ice–water bath for 30 min. The reaction mixture was preincubated for 5 min at 37°. The enzyme preparation was added to the reaction mixture, and it was incubated at 37° for specific time periods. The reaction was arrested by immersion of the reaction tubes in boiling water. Heating of the reaction mixture at 100° was continued for 5 min. Blanks contained boiled enzyme preparations. After cooling, the reaction mixture in each tube was poured onto a column (7 × 26 mm) of Bio-Rad AG 1-X4 resin (200–400 mesh, chloride form). Excess citric acid and fatty acids were eluted with 20 ml of formic acid (2 M). Formic acid did not elute ACoA adsorbed on the column. Acetylcoenzyme A was eluted with 4 × 2 ml of HCl (2 M). A 2-ml aliquot of the combined HCl fractions was pipetted into a scintillation vial containing 15 ml of Aqueous Counting Scintillant (ACS), and the radioactivity was measured in a liquid scintillation counter (Beckman CMP-100).

Protein measurements. Protein concentration was measured by the method of Sutherland *et al.* [14]. Bovine serum albumin was used as a standard.

Statistical methods. Wherever possible, values are presented as the mean ± S.E.M. of *N* determinations. The statistical significance of the differences between means was determined by Student's *t*-test. Each mean was obtained from a minimum of six values. The difference between two means was considered significant when $P < 0.05$.

RESULTS

Separation of citrate from acetylcoenzyme A on anion exchange columns and product identification. Citrate and acetylcoenzyme A were separated on columns (7 × 26 mm) of Bio-Rad AG 1-X4 (200–400 mesh, chloride form) by a modification of the method of Reijnierse *et al.* [15]. Ion exchange chromatograms of [^{14}C]citrate and [^{14}C]acetylcoenzyme A standards and of enzymatic products are shown in Fig. 1. In the case of the [^{14}C]citrate standard, 99.5 per cent of the total radioactivity was found in the formic acid fraction, while no significant radioactivity was observed in the HCl fraction. About 92 per cent of the total radioactivity was found in the HCl fraction with the [^{14}C]acetylcoenzyme A standard, but about 8 per cent of the radioactivity was also present in the formic acid fraction (Fig 1A). This may have been due to hydrolysis of the ester bond of acetylcoenzyme A with the [^{14}C]acetic acid that resulted having been eluted by formic acid. With enzymatic products, 3 per cent radioactivity was observed in the HCl fraction, and about 97 per cent of the activity was present in the formic acid fraction.

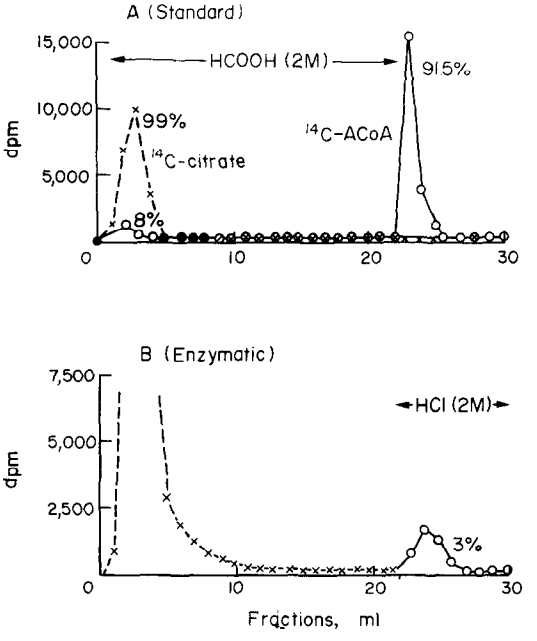

Fig. 1. Ion exchange chromatograms of [^{14}C]citrate standard, [^{14}C]acetylcoenzyme A standard, and enzymatic products. Bio-Rad AG 1-X4 (200–400 mesh, chloride form), 7 × 26 mm columns were loaded with 0.9 ml reaction mixture containing phosphate buffer (50 mM, pH 6.4), coenzyme A (0.6 mM), ATP (3 mM), $MgCl_2$ (5 mM), bovine serum albumin (1 mg/ml), dithiothreitol (5 mM), and 0.1 ml of enzyme preparation. Before loading, the reaction mixture was incubated for 1 hr at 37° and boiled for 5 min. To the standards, 0.1 ml of boiled enzyme preparation was added. Panel A: (×——×) chromatogram of citrate standard (3.3 mM, 1.02 × 10^4 dpm of [1,5-^{14}C]citric acid); and (○—○) chromatogram of acetylcoenzyme A standard (3.3 mM, 1.56 × 10^4 dpm of [^{14}C]acetylcoenzyme A). Citrate was not added to this reaction mixture. Panel B: Chromatogram of enzymatic products. Enzyme preparation (0.1 ml) was present in the reaction mixture. Citrate (3.3 mM, 2.2 × 10^5 dpm of [1,5-^{14}C]citric acid) was present in the reaction mixture. Key: (×———×) unreacted citrate; and (○—○) enzymatically formed acetylcoenzyme A. Elution was first done with 22 × 1 ml of formic acid (2 M) and then with 8 × 1 ml of HCl (2 m). Fractions were collected in a scintillation vial containing 10 ml of aqueous counting scintillant and were monitored by measurement of radioactivity.

Descending paper chromatography of the radioactive product present in the 2 M HCl fractions from

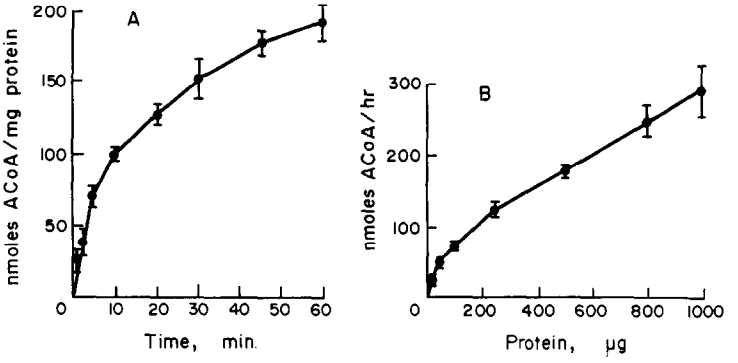

Fig. 2. Formation of acetylcoenzyme A (ACoA) as a function of time (A) and as a function of enzyme concentration (B).

the Bio-Rad AG 1-X4 (200–400 mesh, chloride form) was carried out at 5° for 36 hr on Whatman 3MM (2 × 50 cm) paper using the solvent system: isobutyric acid–sodium hydroxide (1 N)–water (57:35:8). For comparison, the same chromatography was performed using [1,5-^{14}C]citric acid (R_f, 0.46) and [acetyl-1-^{14}C]coenzyme A (R_f, 0.68) standards. On the radiochromatogram of the HCl fractions, the peak at R_f value 0.68 was identified as acetylcoenzyme A. The peak at R_f 0.68 was absent in the 2 HCl fractions when boiled enzyme was used in the reaction.

Linearity of the formation of ACoA with incubation time and enzyme concentration. Panels A and B of Fig. 2 depict the formation of ACoA as a function of time and enzyme concentration, respectively. The rate of formation of ACoA was linear with time for the first 5 min, after which a decrease in the rate was observed. It was linear with enzyme–protein concentrations up to 50 µg per incubation mixture.

Stability of sperm ATP-citrate lyase. To study stability, rat sperm homogenates were stored at three different temperatures—0°, 4°, and 25°—and enzyme activities were determined at various time intervals (Fig. 3). After 24 hr at 25°, a 35 per cent decrease in activity had occurred. No significant change in the activity was noticed during the first 24 hr at temperatures 0° and 4°. At 37°, no depression in the enzyme activity was observed during 1 hr of incubation time.

Formation of ACoA as a function of citrate concentration. The formation of ACoA increased up to a citrate concentration of about 5 mM (Fig. 4). Concentrations higher than 5 mM did not inhibit the enzyme. The apparent K_m for citrate was about 2 mM.

pH Optimum of rat sperm ATP-citrate lyase. Rat sperm homogenate was used to determine the optimal conditions for the assay. Phthalate–NaOH (pH 4.1–5.7), K_2HPO_4–KH_2PO_4 (pH 6–8), Tris–HCl (pH 8.4–8.8), and borax–NaOH (pH 9.3–10.1) buffers (50 mM each) were used for determining the optimal pH. The highest activity for ATP-citrate lyase was observed at pH 6.4 (Fig. 5). There is a second broad peak for rat sperm ATP-citrate lyase activity at pH 9.2–9.6, the significance of which is not known. Further studies are necessary to indicate whether a second enzyme form of ATP-citrate lyase is present in rat spermatozoa.

Miscellaneous properties of rat sperm ATP-citrate lyase. Several conditions that influence the assay of ATP-citrate lyase were studied. Addition of bovine serum albumin (BSA), up to 1.5 mg/ml, to the incubation medium significantly increased the enzyme activity (Fig. 6). An increase in the concentration of BSA from 1.5 to 2 mg/ml in the reaction mixture decreased the enzyme activity.

When the enzyme preparations were not pre-

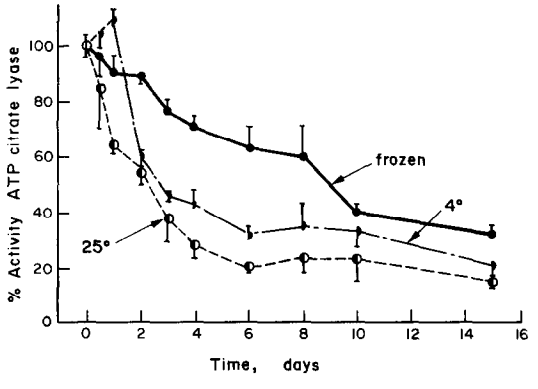

Fig. 3. Effects of storage at different temperatures on spermatozoal ATP-citrate lyase activity. ATP-citrate lyase activity was measured at pH 6.4 as described in Methods.

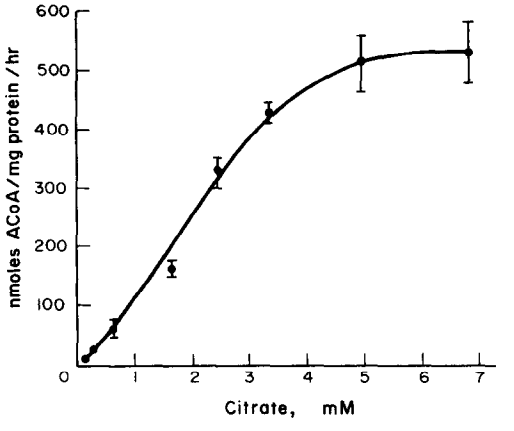

Fig. 4. Formation of acetylcoenzyme A by rat spermatozoal ATP-citrate lyase as a function of citrate concentration.

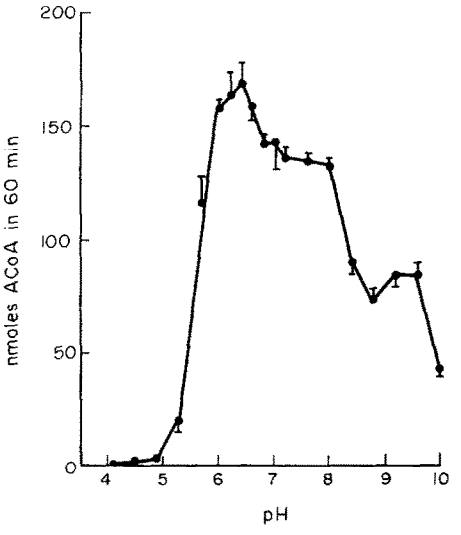

Fig. 5. Rat spermatozoal ATP-citrate lyase activity at various pH values. The medium contained coenzyme A (0.6 mM), ATP (3 mM), $MgCl_2$ (5 mM), bovine serum albumin (1 mg/ml), citrate (3.3 mM, 2.2×10^5 dpm of [1,5-^{14}C]citric acid), dithiothreitol (5 mM), 0.1 ml of spermatozoa homogenate and appropriate buffer (50 mM). Buffers used in this study were: phthalate–NaOH (pH 4.1–5.7); K_2HPO_4–KH_2PO_4 (pH 6.8); Tris–HCl (pH 8.4–8.8); and borax–NaOH (pH 9.3–10.1). The reaction mixture was preincubated for 5 min and then, after the addition of homogenate, for 60 min. An average of 562 ± 28 μg of the enzyme protein was present in the incubation mixture.

treated with 0.5 per cent (v/v) Triton X-100, ATP-citrate lyase activity was 66 per cent of that found with Triton X pretreatment, indicating that at least part of the enzyme activity was present in an aggregated form.

Reducing agents are generally used in enzyme reactions to protect the thiol group of coenzyme A. Dithiothreitol (2–10 mM) and sodium bisulfite (5 mM) did not alter the activity of the enzyme. Sucrose gradients are generally employed for the determination of the subcellular distribution of enzymes. Sucrose concentrations varying between 35 and 70 mM in the reaction medium did not significantly alter the enzyme activity. Variations in the concentrations of the phosphate buffer between 10 and 100 mM did not significantly alter the enzyme activity.

By increasing the ATP concentrations from 1 to 6 mM, the formation of ACoA was increased 2-fold. Further increases in ATP concentration did not increase the ACoA formation. The maximum yield of ACoA was obtained at a Mg^{2+} concentration of 1 mM. Higher concentrations of Mg^{2+} (4–10 mM) caused significant inhibition in the formation of ACoA. A 50 per cent inhibition in the formation of ACoA was observed at a Mg^{2+} concentration of about 9 mM.

Dialysis of enzyme preparations did not influence the ACoA formation by rat ATP-citrate lyase under optimal conditions. The optimal conditions for the radiometric assay of ATP-citrate lyase are summarized in Table 1.

ATP-citrate lyase activity in spermatozoa. ATP-citrate lyase activities were determined under optimal conditions (Table 2) with rat spermatozoa and human spermatozoa. Rat spermatozoa were found to contain activities of ATP-citrate lyase higher than in human spermatozoa. The ATP-citrate lyase activities of spermatozoa and placenta were lower than those in rat brain homogenates (refs. 15 and 17, and Table 2).

DISCUSSION

Several methods to measure ATP citrate lyase activity in tissues have been reported. These include: (a) spectrophotometry, when the amount of acetylcoenzyme A [20] or the amount of unreacted coenzyme A [21–23] is known; (b) hydroxamic acid-trapping of acetylcoenzyme A as the acetylhydroxamate, with measurement of the latter by the color produced with $FeCl_3$ [1]; (c) reaction of the enzyme product, oxaloacetate, with NADH in the presence of malic dehydrogenase, with measurement of the change in optical density [24]; and (d) coupling with carnitine acetyltransferase [25] or with choline acetyltransferase [18, 19]. These methods are not satisfactory for the measurement of the low activities of ATP-citrate lyase in subcellular fractions of tis-

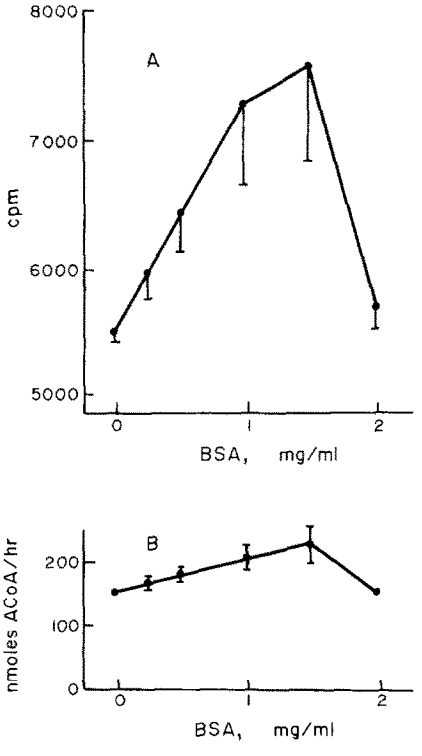

Fig. 6. Effects of bovine serum albumin (BSA) on rat spermatozoal ATP-citrate lyase. Reaction medium: KH_2PO_4–K_2HPO_4 (50 mM, pH 6.4), coenzyme A (0.6 mM), ATP (3 mM), $MgCl_2$ (5 mM), citrate (3.3. mM, 2.2×10^5 dpm [1,5-^{14}C]citric acid), spermatozoa homogenate (580 ± 50 μg protein), dithiothreitol (5 mM) and the appropriate concentration of BSA. Incubation time was 60 min. (A) Counts per minute as a function of BSA concentration. (B) Acetylcoenzyme A (nmoles) formed during 60 min of incubation, as a function of BSA concentration.

Table 1. Conditions for spermatozoal ATP-citrate lyase assay*

Variable	Optimal condition
pH	6.4
K_2HPO_4–KH_2PO_4 (mM)	50
ATP (mM)	4.5
$MgCl_2$ (mM)	0.5
Coenzyme A (mM)	0.6
Citrate (mM)	6.6
Bovine serum albumin (BSA, mg/ml)	1
Reaction time (min)	5†
Enzyme (μg protein)	50‡

* These conditions satisfy the requirements described by Reiner [16] for an enzymatic assay,

† Period for calculating the initial linear velocities that are used in the calculation of kinetic constants. For determining the tissue levels, the yield of ACoA could be increased using the reaction periods of 60 min, when the maximal yield of ACoA was observed.

‡ As tissue homogenate. At 60-min incubation periods, the counting error was less than 1 per cent.

sues. The first and second methods are not sensitive. The third and fourth methods have limitations owing to special reaction conditions of individual enzymes. The method described in the present investigation is rapid, very sensitive, and measures directly the ACoA formed. The method can be adapted to the measurement of subcellular fractions and enzyme preparations separated by sucrose density gradients.

Although many factors contribute to effective sperm motility (cells showing a progressive, straight-line unidirectional motility pattern), the ChA–ACh–AChE system seems to play a significant role. In addition to the occurrence of the above three components [5, 6], the presence of a nicotinic type cholinergic receptor has been demonstrated in sea urchin sperm [26] and in bull sperm [27]. All of these four components of the cholinergic system are localized in the tail fraction of bull spermatozoa [5, 27]. There are no membrane stores of ACh in spermatozoa. Therefore, ChA inhibitors should exhibit dramatic effects on sperm motility if ACh is involved in this motility. Selective inhibitors of ChA, 2-benzoylethyltrimethylammonium and related compounds [28, 29] inhibit human sperm ChA and sperm motility [30]. There is a direct relation between the inhibition of sperm motility and the inhibition of ChA. All of the above observations indicate a specific role for the ChA–ACh–AChE system in sperm motility.

The lack of membrane stores for ACh in spermatozoa indicates that ACh synthesis, the stimulation of the receptor by ACh, and the hydrolysis of ACh by AChE are closely linked and may be localized in the same compartment. With such unique organization, there will be continuous rapid turnover of ACh. To maintain the synthesis of ACh, the precursors of ACh synthesis, choline and ACoA, must be readily available. There are several sources of choline available in spermatozoa. Furthermore, choline formed by the hydrolysis of ACh can be reutilized. ACoA, however must be supplied continuously for the synthesis of ACh. The present demonstration of ATP-citrate lyase in spermatozoa indicates that ACoA can be formed from citrate.

Table 2. ATP-citrate lyase activity in spermatozoa, brain and placenta

Source of enzyme (homogenate)	Acetylcoenzyme A (nmoles/mg protein/5 min)
Rat spermatozoa*	153.5 ± 13.5†
Human spermatozoa*	89.9 ± 11.9
Rat brain‡	517.0 ± 40.0
Human placenta§	58.4 ± 8.0

* Enzyme activity was measured under optimal conditions, described in Table 1, for spermatozoal ATP-citrate lyase. When the samples were analyzed by the coupled ATP-citrate lyase–choline acetyltransferase assay [18, 19] under the same conditions, the values for ACoA were not significantly different from those reported above.

† This was lower by about 64–65 times than that in rat liver cytoplasm (9.8 μmoles acetylcoenzyme A/mg/5 min).

‡ Analyzed in the rat brain homogenates according to the conditions described by Reijnierse *et al.* [15].

§ Quoted from Sastry and Sadavongvivad [17].

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