Biosynthesis of N-Acetyl-L-aspartic Acid*

FRED B. GOLDSTEIN

From the Research Department, Letchworth Village, New York State Department of Mental Hygiene, Thiells, New York

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The occurrence of N-acetyl-L-aspartic acid in mammalian and avian brain at concentrations from 80 to 120 mg per 100 g fresh weight of tissue was demonstrated recently by Tallan et al. (1). The pattern of distribution of acetylaspartic acid in bovine brain was found by Tallan (2) to parallel that of many enzymes and to correlate equally well with both the distribution of respiratory activity and the degree of vascularity of the central nervous system. The metabolic importance and functional role of this compound which appears to occur specifically in nerve cells of higher animals (2) is unknown.

A very small conversion of C¹⁴-labeled glucose or aspartate into the acetylaspartic acid of brain preparations has been reported (3). The present communication describes a study of the enzymatic mechanism involved in the synthesis of acetylaspartic acid by rat brain. The results indicate that acetyl coenzyme A is the activated intermediate in the conjugation of L-aspartate by a specific acylating enzyme present in the supernatant fraction of rat brain homogenates. The participation of acetyl adenosine 5'-phosphate and some properties of the enzyme, aspartic acetylase, have been investigated.

EXPERIMENTAL

Acetyl-CoA, butyryl-CoA, benzoyl-CoA, and phenylacetyl-CoA were prepared by treatment of reduced CoA with the corresponding acid anhydride (4, 5). Fluoroacetyl-CoA and propionyl-CoA were made similarly from the corresponding mixed anhydride of ethyl carbonate (6). Acetyl-AMP derivatives were prepared by the general method of Avison (7), acetyl-AMP and butynyl-AMP according to the procedure of Dorg (8), and benzoyl-AMP and phenylacetyl-AMP from the directions of Moldave and Meister (9). Disodium ATP and AMP were purchased from Schwarz Laboratories, Inc. Reduced CoA, L-aspartic acid, and D-aspartic acid were obtained from Nutritional Biochemicals Corporation. Sodium acetate-1-C¹⁴ and D,L-aspartic-4-C¹⁴ were products of Tracerlab, Inc. Fluoroacetic acid was purchased from K & K Laboratories and sodium fluoroacetate was kindly furnished by Dr. Roscoe O. Brady.

Analytical Procedures—Acetylaspartic acid was isolated by ion exchange chromatography according to the procedure of Tallan et al. (1) with some minor modification. Percollaric acid was used as the protein precipitant. The KOH-neutralized, perchlorate-freed samples were transferred to 0.9 x 15-cm columns of Dowex 1 X8 acetate (-400 mesh). The fore-run was omitted. Ammonium acetate buffers to which one-tenth volume of 95% ethanol was added just before use, were employed throughout. Effluent fractions of approximately 2 ml were collected directly on stainless steel planchets, dried by infrared radiation, and subsequently assayed for radioactivity. For the chemical determination of acetylaspartic acid after measurement of radioactivity, three successive 1 ml portions of distilled water were used to dissolve and transfer the material from the plate to a small (1.0 x 2.5-cm) column of Dowex 50 X8(Na⁺) which retained ammonium ions. To obtain the acetylaspartic acid in the minimum effluent volume, air pressure was applied to the top of the column between additions. When necessary, elution with water was continued until the effluent was free from appreciable radioactivity. Aliquots of the effluents were reacted with ninhydrin directly and after mild acid hydrolysis (1). In most experiments with the partially purified enzyme, the amount of acetylaspartic acid formed was calculated from its radioactivity and a conversion factor relating counts per minute to μmoles of acetylaspartic acid.

Enzyme Preparation—Routinely, the cerebral hemispheres from 15 to 18 young rats (80 to 100 g each) were homogenized with 30 ml of cold sucrose-potassium phosphate-MgSO₄-nicotinamide medium (10) in a Teflon pestle homogenizer. The homogenate was centrifuged at 30,000 x g for 45 minutes at 0-5°C. The supernatant was mixed with saturated ammonium sulfate solution (stirred at room temperature) to produce 20% saturation. After standing 30 minutes in the cold, the precipitate was sedimented and dissolved in 10 ml of cold distilled water. Since either dialysis or repeated freezing and thawing resulted in considerable losses in activity, the solution was used without further treatment and stored at 0-5°C. Maintained under these conditions, the preparations, which contained from 8 to 10 mg of protein per ml, remained stable and demonstrated consistently reproducible acylating activity for weeks.

RESULTS

Studies on Acylating Enzyme System—Preliminary studies with sliced rat brains demonstrated that young or adult tissue incubated aerobically in glucose-phosphate medium would incorporate radioactivity from acetate-1-C¹⁴, pyruvate-2-C¹⁴, or uniformly labeled aspartate-Cl⁴ into acetylaspartic acid, although at a feeble rate. The presence of potassium chloride at a final concentration of 0.05 to 0.1 M almost doubled the rate of acetylaspartate synthesis without affecting the incorporation of acetate into the cholesterol of the same tissue samples. The acylating activity of homogenates was confined to the supernatant phase which, however, displayed limited acetate-activating ability. Added acetyl-AMP or acetyl-CoA rapidly disappeared in these extracts without concomitant acetylation. Coupling of the brain supernatant solution with an extract of acetone-dried
pigeon liver in the presence of C¹⁴ acetate, ATP, Mg²⁺, KCl, and aspartate produced an enhanced system (11) and facilitated fractionation of the brain preparation. On the basis of acylating activity per mg of protein, the 0 to 20% ammonium sulfate fraction represented a 21-fold purification with respect to the original homogenate and a 3-fold purification with respect to the supernatant solution. Recovery of the enzyme appeared to be complete.

The formation of acetylaspartate from acetyl-CoA and L-aspartate was directly proportional to enzyme concentration and did not occur in the presence of boiled enzyme. The optimal pH range for enzymatic activity was found to be from pH 6.0 to 6.8 with activity diminishing sharply on either side of the peak (Fig. 1). Acetyl-AMP reacts nonenzymatically with amino acids and certain other nitrogenous substances to form N-acetyl derivatives (12). Compared with other amino acids tested, aspartate was very slowly acylated nonenzymatically, but at a rate sufficient to interfere in studies on the enzymic utilization of acetyl-AMP at pH values above 6.0 (Fig. 1). The time course of the acetylation of aspartate by acetyl-CoA showed a significant lowering of rate after the first hour. This may have been due to the comparatively rapid change in acetyl-CoA concentration in the system employed with the enzyme extract, since results with slices and acetate-1-C¹⁴ indicated a constant rate over a 3-hour interval studied. The effect of L-aspartate and acetyl-CoA concentrations is shown in Fig. 2; maximal activity was observed with 40 pmoles of aspartate. The system appeared to be saturated with 2.5 to 3.0 pmoles of acetyl-CoA.

**Specificity-Studies with the isomers of aspartic acid, L-glutamic acid, and a number of acyl-CoA derivatives indicated an absolute specificity of the acylating enzyme for acetyl-CoA and L-aspartic acid. No evidence for the conjugation of aspartate was obtained after incubation with fluoroacetyl-CoA, propionyl-CoA, butyryl CoA, benzyloxy-CoA, or phenylacetyl-CoA. The rate of acetylation of L-aspartate was not influenced by the presence of α-aspartate or L-glutamate. Table I summarizes the data concerning specificity with respect to amino acid.**

**Studies with Acetyl-AMP** —In the presence of reduced CoA,
acetyl-AMP could substitute for acetyl-CoA with this preparation. However, the rate of synthesis of acetylaspartate from acetyl-AMP and reduced CoA was less than half of that observed with acetyl-CoA under similar conditions. The utilization of acetyl-AMP was completely dependent upon the addition of reduced CoA (Fig. 3). The absence of activity with boiled enzyme and requirement for reduced CoA exclude any contributory nonenzymatic acetylation. The addition of free AMP produced significant inhibitions of acetylaspartate formation from acetyl-AMP (40 and 66% by 10 and 20 μmoles of AMP, respectively) but not when acetyl-CoA was the precursor. These findings clearly indicate that the conversion of acetyl-AMP to acetyl-CoA represented the rate-limiting step in this system.

Effect of Anions—The stimulation of acetylaspartate synthesis by potassium chloride first observed with brain slices early in these studies persisted in every system investigated. The results shown in Table II demonstrate that the effect is mediated by the anion and, is also observed upon the addition of bromide, iodide, chloride, nitrite, nitrate, or thiocyanate. Acetate, phenylacetate, phosphate, citrate, tartrate, oxalate, arsenite, and arsenate were ineffective under similar conditions. Although concentrations of chloride or bromide in the range of 0.01 to 0.025 M almost doubled the rate of acetylaspartate synthesis, maximal effects were observed with concentrations approaching 0.08 M.

Competitive Inhibition by Acyl-CoA Derivatives—All of the fatty acid-CoA derivatives examined competed with acetyl-CoA for the acylating enzyme (Figs. 4 and 5). From the data obtained from a number of experiments, the $K_m$ for acetyl-CoA was ascertained to be $5.4 \times 10^{-4}$. Fluorocetyl-CoA, propionyl-CoA, and benzoyl-CoA were found to be very weak inhibitors with considerably lower affinities for the enzyme than that of the substrate (Table III). However, in a coupled system composed of the acylating preparation and a dialyzed extract of acetone-
dried rabbit kidney which activated both acetate and fluoroacetate (13), a 46% inhibition of acetylaspartate synthesis was observed with acetate and fluoroacetate present at equimolar concentration. With acetyl-AMP as substrate and other acyl-AMP derivatives tested at a concentration of $2.0 \times 10^{-3}$ M, inhibitions of 24 and 51% were observed with butyryl-AMP and phenylacetyl-AMP, respectively. Benzoyl-AMP was not inhibitory under the same conditions.

**DISCUSSION**

The results obtained in this study suggest that the synthesis of acetylaspartic acid in brain may be represented by the following reactions:

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\text{Acetate} + \text{ATP} \rightarrow \text{acetyl-AMP} + \text{PP}
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\text{Acetyl-AMP} + \text{CoA-SH} \rightarrow \text{acetyl-CoA} + \text{AMP}
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\[
\text{Acetyl-CoA} + \text{L-aspartate} \rightarrow \text{acetylaspartate} + \text{CoA-SH}
\]

Reactions 1 and 2 represent the activation of acetate as originally proposed by Berg (8). The requirement for ATP in the utilization of acetate (11) and the reduced CoA-dependent participation of acetyl-AMP in the conjugation of aspartate support the involvement of the two step mechanism. Reaction 3 is analogous to that previously designated for acylations catalyzed by animal tissues (5, 9, 14-17).

The experimental findings indicating an absolute specificity of the enzyme for acetyl-CoA and L-aspartate are in agreement with the results of Tallan et al. (1) who found N-acetyl-L-aspartic acid as the only conjugated amino acid present in significant amounts in protein-free brain extracts. Moreover, the average rate of synthesis of acetylaspartate from acetyl-AMP, reduced CoA, and L-aspartate (Fig. 3) corresponds closely to the average rate of deposition of acetylaspartic acid in the brains of young rats from the 10th to the 20th day of life (2), the approximate values being 4.8 and 6.5 mg per 100 g of fresh weight of tissue per 24 hours, respectively.

In discussing the possible role of acetylaspartic acid, Tallan (2) considered its contribution in making up a part of the anion deficit known to exist in nervous tissue and the striking correlation of acetylaspartic acid distribution with the sites of high respiratory activity and increased blood supply in the brain. The possible involvement of acetylaspartic acid in the transmission of the nerve impulse was discounted largely on the basis of its absence in detectable amounts in the brains of lower species and in the electric organ of the electric eel. The present study, unfortunately, offers little further indication of the functional role of acetylaspartic acid in mammalian brain. That a mechanism exists in rat brain for the hydrolysis of acetylaspartic acid became apparent during the course of these studies. The almost complete disappearance of the acetylaspartate contained in rat brain supernatants was found to occur during 2 to 3 hour incubations at 37°C. Further work on the hydrolysis of acetylaspartic acid is in progress.

**SUMMARY**

An enzyme, aspartic acetylase, which catalyzes the acetylation of L-aspartic acid by acetyl coenzyme A to form N-acetyl-L-aspartic acid, has been obtained in a partially purified state from rat brain. With this preparation, acetyl adenosine 5'-phosphate in the presence of coenzyme A could be substituted for acetyl

**REFERENCES**

Biosynthesis of N-Acetyl-l-aspartic Acid
Fred B. Goldstein


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