ENZYMATIC SYNTHESIS OF LABELED CARBAMYL-ASPARTIC ACID*

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Summary. A method for the enzymatic preparation of radioactive carbamyl-aspartic acid (ureidosuccinic acid) from radioactive aspartic acid and carbamyl phosphate is described. It involves the use of extracts from the trypanosomid flagellate, Crithidia fasciculata, which contain active aspartate transcarbamylase (aspartate carbamoyltransferase). Two methods for the isolation of the carbamylaspartate from the incubation mixtures are described, one involving paper chromatography or paper electrophoresis and the other making use of ion-exchange chromatography on Dowex 50W (H^+). The incubation allows the conversion of aspartate to carbamylaspartate of 90-98% within 2 hours and recovery of as much as 73% of the original aspartate carbons.

The lack of a commercial source of isotopically labeled carbamylaspartic acid (ureidosuccinic acid) and the necessity of its use in studies of the enzymes of pyrimidine biosynthesis prompted our working out a simple enzymatic method for its production. The method is based upon the activity of aspartic transcarbamylase (aspartic carbamoyltransferase, 2.1.3.2) catalyzing the nonreversible reaction

Carbamylphosphate (CAP)** + L-Aspartate (Asp) → Carbamyl-aspartate (CAA) + Pi.

This communication describes the use of extracts of the trypanosomid flagellate, Crithidia fasciculata, which contain high aspartic transcarbamylase activity,*** for the preparation of [^{14}C]-labeled CAA from [^{14}C]-labeled

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**Abbreviation: CAA = carbamylaspartate (ureidosuccinate); DHO = dihydroorotate; OA = orotate; CAP = carbamylphosphate; Asp = aspartate.

***Details of the Crithidia transcarbamylase are to be reported elsewhere.

aspartic acid and non-labeled carbamylphosphate (both commercially available). This method was found to be better suited to our needs than the chemical methods previously published (1,2,3) as it could be carried out on a micro scale and the product was always of the natural configuration.

Materials. [U-^{14}C] L-aspartic acid was purchased from Schwarz-Mann. All other chemicals, unless otherwise noted, were purchased from Sigma.

Preparation of cell extracts. Crithidia fasciculata was grown in either defined Medium II of Kidder and Dutta (4) or in a crude medium made up of 2% Proteose-peptone (Difco); 1% Liver Fraction L (Nutritional Biochemicals); 1% glucose (separately sterilized); 250 µg% folic acid and 1 mg% hemin (filter sterilized). The cells, in the exponential growth phase, are obtained as previously described (5,6), are harvested by centrifugation at 15,000 g for 10 min, and, following one wash in 0.05 M phosphate buffer + 0.02% Na azide (pH 7.0), are subjected to lyophilization and stored until ready for use.

Lyophilized cells (50 mg) are suspended in 3 ml of pH 7.0 phosphate buffer and sonicated in an ice bath for 1 min at 20 mA in a Wave Energy Systems Ultra Tip sonicator. Cell debris is removed by centrifugation for 20 min at 48,000 g. The extract contains, as well as the aspartic transcarbamylase, a CAA cyclizing enzyme which forms dihydroorotic acid (DHO) from CAA and the DHO hydroxylase (7) which leads, after dehydration, to orotic acid (OA) production. While the cyclizing enzyme has very weak activity at the high pH (8.6) used for CAA synthesis, even this becomes inacceptable for CAA production when DHO hydroxylase is present to "draw" the reaction to OA. The DHO hydroxylase is inactivated by freezing, however, and while the specific activity of the aspartic transcarbamylase is also diminished the frozen preparation is entirely satisfactory for the purpose of CAA production.

Pilot assay. Before an extract is used for a preparative run it is assayed as follows: into a small (10 x 45 mm) tube is pipetted 0.05 ml of a

0.4 M Tris-Bis (equimolar 2-amino-2-hydroxymethyl-1,3-propanediol and 2-amino-2-methyl-1,3-propanediol) buffer, pH 8.6; 0.01 ml of a freshly prepared solution of carbamylphosphate (1 mg/ml); 0.01 ml of [^{14}C] L-aspartic acid (containing approximately 1 x 10^6 cpm/ml); 0.01 ml cell extract. Incubation is at 37° for 2 hrs. The reaction is stopped with a drop of glacial acetic acid and the mixture is spotted on either Whatman #1 or 3MM filter paper, together with standards, and subjected to descending chromatography with n-butanol : acetic acid : water (20:3:7 v/v) as the solvent. Alternatively the paper is moistened with an electrolyte (formic acid : water, 8:300 v/v) and subjected to electrophoresis at 500 V and 12 mA. Satisfactory separation of Asp, CAA, DHO and OA is accomplished in 18 hrs. by paper chromatography and 2 hrs. by paper electrophoresis. Radioactivities are determined by use of a scanner (Tracerlab, 4π) and the percent of conversion by planimetry.

Preparation of CAA. To minimize manipulations plastic Eppendorf centrifuge tubes (1.5 ml) are used as the incubation vessels. To each tube is added 0.4 ml of the Tris-Bis pH 8.6 buffer containing 25 µCi (0.24 µmoles) of [U-^{14}C] L-aspartic acid. This has been prepared by evaporating the radioactive aspartic acid solution (in 0.1 N HCl) to dryness in vacuo and dissolving it in the buffer. Then 0.2 ml of a freshly prepared solution of carbamylphosphate (2 mg/ml) and 0.4 ml of the frozen cell extract (10-15 mg protein) are added. (The amount is doubled when extract from cells grown in the crude medium is used.) After incubation for 2 hrs. at 37° the reaction is stopped and the protein precipitated by the addition of 0.05 ml of 4 N perchloric acid. The tubes are centrifuged for 2 min in an Eppendorf 3200 Micro Centrifuge and the supernate is transferred with a pasteur pipet to fresh 1.5 ml centrifuge tubes. Saturated KOH is added to precipitate excess perchloric acid and the potassium perchlorate removed by centrifugation. The supernate is transferred to a small (10 x 45 mm) glass tube and, depending upon the method of CAA isolation to be employed, either used without further treatment or adjusted to pH 6.6-6.8.

Isolation of CAA. Paper. The supernate is streaked by multiple applications on a line near one end of a large (46 x 59 cm) sheet of Whatman #1 or 3MM filter paper which is chromatographed, together with standards, with the n-butanol : acetic acid : water (20:3:7 v/v) as the solvent. The paper is air dried and narrow strips are cut from each edge for determination of the location of radioactive products. The position of the CAA is marked on the main body of the paper, and that segment is cut out and extracted twice with 10 ml volumes of hot water. After the macerated paper is removed on a sintered glass filter under reduced pressure, the volume of the extract is reduced in vacuo (concentration over steam results in extensive CAA destruction) until the desired molarity of the CAA is reached, as judged by radioactivity.

Alternatively sheets of 3MM paper, of a size suitable for the frame of a paper electrophoresis apparatus (we use 30 x 17.5 cm) are streaked with the supernate along a line near the cathode, the paper is dipped in the electrolyte (formic acid : water, 8:300 v/v), allowing the electrolyte to approach from each side of the line of application by capillarity, blotted and subjected to a 500 V current of 12 mA. The CAA is located as above and extracted in the same way.

The paper methods of isolation yield extremely clean preparations. The recovery is between 40% and 60% of the CAA of the incubation mixture, depending upon the width of the segment taken and therefore upon the inclusion of tailing and leading edges of the peak.

Ion exchange. When ion exchange chromatography is to be used for the separation of CAA from the residual Asp (paper chromatography must reveal no DHO or OA in the incubation mixture) it is of utmost importance that the incubation mixture be adjusted to just under neutrality (6.6-6.8). This can be achieved conveniently by the addition of 1 N HCl in 0.005 ml aliquots (Eppendorf pipet), testing with indicator paper after each addition. The treated incubation mixture is then added to a small (60 x 5 mm) column of

TABLE 1

Balance Sheet for the Preparation and Isolation of Carbamylaspartate

	Radioactivity[a] (Total cpm)	Remarks
Reaction Mixture[b]	18,500,000	
Deproteinized Mixture (after 2 hr. Incubation)	13,600,000	26% loss due to manipulation during deproteinization. 98% conversion[c] of ASP to CAA (0.173 μmoles, Calc.). No DHO nor OA.
Dowex 50W (H^+) Col. Water eluate (1 ml fractions)		
Fraction 1	80	
2	3,356,000	
3	9,732,600	
4	160,000	
5	14,000	
6	6,000	
7	10	
Total	13,268,690	99.5% of calc. CAA (0.172 μmoles)
0.1 N HCl eluate (10 ml)	280,000	Residual Aspartate
Grand total of radioactivity recovered from deproteinized mixture	13,458,700	99.6% recovery from deproteinized mixture. 73% of original Asp radioactivity.

a. Aliquots plated on planchets and counted in a Tracerlab gas flow counter equipped with an ultra-thin window detector and a Multi-Matic sample changer. Counting efficiency was 22.5%.

b. [U-^{14}C] Asp, 0.24 μmoles in 0.4 ml of 0.4 M Tris-Bis buffer at pH 8.6; 0.2 ml CAP (2 mg/ml); 0.4 ml cell extract frozen (1.12 mg protein/ml, from cells grown in defined medium).

c. Aliquot (0.01 ml) subjected to paper electrophoresis, scanned for radioactivity and percentage of conversion calculated by determining (by planimetry) the areas under the peaks.

Dowex 50W (H^+) previously treated with 0.1 N HCl and washed exhaustively (until the effluent is neutral) with 2X distilled water. Aspartic acid is quantitatively absorbed while the CAA appears in the water effluent. When the pilot assay reveals only CAA as the product of the incubation, the ion exchange isolation is the method of choice. The purity of the CAA is easily demonstrated by co-chromatographing in a number of solvents, with authentic CAA. Recoveries are excellent, as can be seen from Table 1. These results are typical of a number of preparations.

One disadvantage of this method of isolation is that small amounts of other anions, such as perchlorate, phosphate, etc., may be present with the CAA. Passage through a small column of Sephadex G-100, which is then washed with 2X distilled water, overcomes this difficulty. The CAA usually must be concentrated in vacuo to the desired molarity (as judged by radioactivity).

Discussion. Cells grown in defined medium (which contains no pyrimidine) have a higher (about twice) yield of aspartic transcarbamylase per unit of protein than those grown in crude medium. The latter cultures yield more protein per unit volume, and due to the lower expense in time and materials, may be preferred.

Cleaner enzyme preparations, with much higher specific activities for aspartic transcarbamylase, are produced by subjecting fresh sonicate to gel filtration on a column of Sephadex G-200, and eluting with either 0.05 M phosphate or 0.1 M Tris buffer at pH 7.0. The aspartic transcarbamylase peaks well before the other enzymes of the early pyrimidine pathway. Peak fractions can be used without further treatment. The gel filtration method is somewhat more laborious, requires more equipment and the enzyme is somewhat less stable when partially purified.

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