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Aspartate Carbamyl Transferase from *Escherichia coli*

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Carbamyl phosphate was found to be an intermediate in the biosynthesis of carbamyl aspartic acid (= ureidosuccinic acid). Strong evidence was obtained that enzymically prepared and purified compound X was identical with synthetic carbamyl phosphate.

An enzyme was purified *ca.* 100 fold from extracts of lyophilized *E. coli* which catalyzed the following stoichiometric reaction:
Carbamyl phosphate + L-aspartate → L-carbamyl aspartate + P_i

The name aspartate carbamyl transferase is proposed for this enzyme. The equilibrium of the reaction was shifted very far towards synthesis of CA-aspartate.

Out of 31 tested amino acids only L-aspartic acid served as acceptor for the carbamyl group. The enzyme is distinct from the citrulline forming enzyme and the enzyme forming β-ureidopropionic acid.

The enzyme was inhibited by *p*-chloromercuribenzoate and the inhibition could be completely reversed by glutathione. Aspartate + carbamyl phosphate protected the enzyme from the inhibition, while each substrate alone had no effect.

Inorganic ^{32}P showed no enzymic exchange with the phosphate moiety of carbamyl phosphate in the absence of aspartate, and the enzyme catalyzed no exchange of isotope between L-aspartate-^{14}C and carbamyl aspartate.

When the reaction was carried out in $H_2{}^{18}O$ no isotope was found in the carbamyl group of carbamyl aspartate or carbamyl phosphate.

A probable mechanism for the reaction is discussed.

Extracts from various organs of the rat and from lyophilized *E. coli* contain an enzyme which has the ability to catalyze the formation of carbamyl asparacid (= ureido succinic acid *) from aspartic acid and an activated carbamyl compound, called compound X[1,2]. This reaction shows a close analogy to the previously demonstrated formation [3] of citrulline from ornithine + compound X. With a partially purified enzyme from *E. coli* we could demonstrate the stoichiometric formation of one molecule of CA-aspartate ** per one molecule

*. In earlier communications the name ureidosuccinic acid was used instead of the synonym carbamyl aspartic acid. The enzymic mechanism involved in the formation of this substance makes us now prefer the latter name.

**. The following abbrevations are used in this paper: ATP, adenosine triphosphate; CA, carbamyl; CAP, carbamyl phosphate; CMBA, *p*-chloromercuribenzoate; P_i, inorganic phosphate; tris, trishydroxymethylaminomethane; PCA, perchloric acid.

of aspartate and one molecule of compound X. Since it was believed [3] that compound X contained acetylglutamate and phosphate as well as the carbamyl group, the reaction was tentatively formulated as follows:

$$\text{Compound X} + \text{L-aspartate} \rightleftharpoons \text{CA-aspartate} + \text{acetyl glutamate} + P_i \quad (1)$$

Jones *et al.*[4] have recently synthesized carbamyl phosphate and have presented very strong evidence that this compound is the carbamyl donor in enzymic citrulline synthesis and therefore identical with compound X. Their work prompted us to reinvestigate the formation of CA-aspartate with the bacterial enzyme. It was found that the earlier enzymically prepared and purified compound X was identical with synthetic CAP. The easy availability of the latter compound made feasible a further purification and a closer study of the CA-aspartate forming enzyme and the mechanism of its action. The enzyme was found to catalyze the transfer of a carbamyl group from phosphate to L-aspartate in a stoichiometric manner:

$$\text{Carbamyl phosphate} + \text{L-aspartate} \rightarrow \text{L-carbamyl aspartate} + P_i \quad (2)$$

The name aspartate carbamyl transferase is proposed for this enzyme.

EXPERIMENTAL

L-Acetyl glutamic acid was synthesized according to Karrer *et al.*[5], the labeled *L-aspartic acids* according to Wu and Rittenberg [6], and *carbamyl phosphate* according to Jones *et al.*[4] *DL-CA-aspartate* was synthesized according to Nyc and Mitchell [7]. Since the L-isomer does not crystallize readily it was purified by chromatography as described below, lyophilized and utilized directly for the demonstration of the back reaction. *Compound X* was prepared enzymically as described earlier [8,1].

Enzyme assay. Method 1: L-Aspartate-^{15}N was used as substrate and the CA-aspartate formed was determined after addition of carrier DL-CA-aspartate as described earlier [9]. The reaction was terminated by acidification with PCA instead of heat denaturation used in previous studies. In this way it was possible to avoid significant non-enzymic formation of CA-aspartate at substrate concentrations below 0.1 M. When conditions were the same as described for method 2, identical results were obtained with both methods. In most cases, however, only 0.03 M aspartate-^{15}N was used in order to preserve isotope. Under those conditions the obtained value for CA-aspartate formation could be multiplied by a factor of 1.5 in order to obtain a comparison with standard conditions.

Method 2: This method depended on the direct determination of the inorganic phosphate formed during the enzyme reaction. It was the method of choice with the purified enzyme whenever, in a set of experiments, it was possible to work with a relatively low and constant concentration of CAP, and no substances were present which interfered with phosphate analysis.

The enzyme was added to a solution containing 100 μmoles of L-aspartate, 20—30 μmoles of CAP and 150—500 μmoles of tris buffer, pH 7.5, the final volume being 1.0 ml *. Incubation was carried out for 15 minutes at 37°. The reaction was stopped by immersion of the sample in an ice bath and P_i determined directly on aliquots by the Lowry-Lopez [10] method. A blank containing the same constituents with the exception of aspartate (or enzyme) was carried through the same procedure in order to make possible a correction for the non-enzymic decomposition of CAP. The P_i value of the blank was subtracted from that of the complete sample. Under those conditions proportionality

*. The lithium salt of CAP and the potassium salt of L-aspartic acid were used in all experiments. Li-CAP was dissolved immediately before use in each experiment. No difference was found in the reaction rate when sodium aspartate was used instead of the potassium salt.

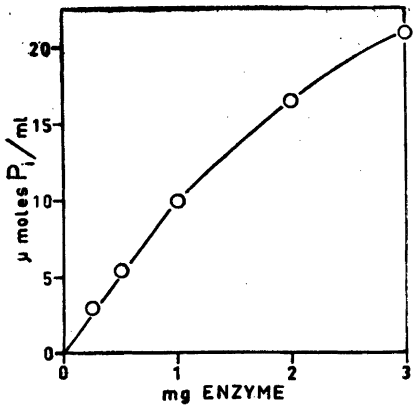


Fig. 1. Formation of P_i from CAP with different amounts of enzyme (ammonium sulfate fraction).

The solutions contained per ml: 100 μmoles of L-aspartate, 25 μmoles of CAP and 150 μmoles of tris buffer, pH 7.5. Incubation for 15 minutes at 37°.

between amount of enzyme added and P_i formed was observed up to *ca.* 10 μmoles (Fig. 1).

One unit of enzyme activity was defined as the amount of enzyme which formed one μmole of P_i or CA-aspartate under the above conditions. The specific activity of the enzyme was defined as units per mg protein. Up to and including the ammonium sulfate fractionation step protein concentration was determined by nitrogen analysis of the PCA precipitate, assuming a nitrogen content of 16 %. Subsequently protein was determined by light absorption measurements at 280 and 310 mμ. It was found that 1 mg of protein of the lyophilized ammonium sulfate fraction dissolved in 1 ml of water showed a value for $E_{280}-E_{310}$ of 0.60. This value was used for calculation of protein concentrations during and after the chromatographic purification step.

The activity of the *citrulline forming* enzyme was measured under identical conditions as described above for CA-aspartate, except that ornithine was substituted for aspartate. The citrulline formed was determined colorimetrically according to Koritz and Cohen [11].

Purification of the enzyme. Lyophilized *E. coli* were handled as forty grams samples. The first steps including extraction, protamine precipitation and ammonium sulfate fractionation were carried out as described earlier [1] with one exception. Incomplete precipitation with protamine was encountered in a few cases. Therefore this precipitation was performed in an ice bath and the solution allowed to stand for 30 minutes in the cold room prior to centrifugation in the Spinco centrifuge (30 000 g for 10 minutes). In this way less than 15 % of the enzyme activity remained in the supernatant in all cases. After the ammonium sulfate step a 7—12 fold purification of the enzyme had been achieved. The enzyme could be lyophilized and stored at —15° without appreciable loss of activity for at least 6 months.

Further purification of the enzyme was carried out by chromatography [13] on DEAE columns *. The column (diameter 2 cm) was prepared from 12 g of DEAE-cellulose (0.9—1.0 % nitrogen, prepared from Whatman's standard grade paper powder). It was washed first with *ca.* 500 ml M KCl, followed by 200 ml of 0.01 M imidazole : HCl buffer, pH 6.5, containing KCl at 0.02 M concentration. The last wash and all the following operations were carried out at 2—4°.

The dialyzed enzyme, after the ammonium sulfate step (from 40 g of bacteria, dissolved in 15—20 ml of 0.01 M phosphate buffer, pH 6.5), was then adsorbed on the column, which was connected with a 1 000 ml mixing flask containing 0.01 M imidazole buffer (pH 6.5) : 0.02 M KCl. This flask received 0.75 M KCl from a reservoir flask. The protein was eluted by gradient chromatography (*cf.* Ref [13]). Half hour fractions with a volume of *ca.* 10 ml were collected.

*. We wish to thank Drs. E. A. Peterson and H. A. Sober who most generously made available to us a manuscript of their work prior to publication.

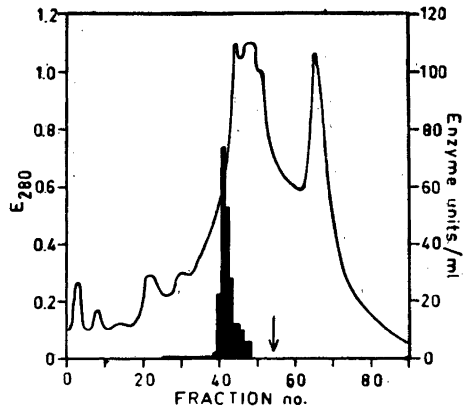


Fig. 2. Chromatography of transferase (560 mg, 5 300 units) on DEAE cellulose.

KCl concentration changed to 2 M at arrow. Stepped curve = enzyme activity, continuous curve = E_{280}.

Protein concentration was determined in each fraction by direct measurement of the light absorption at 280 mμ in a Beckman spectrophotometer, model DU. Furthermore, aliquots from each fraction (usually 0.1 ml) were analyzed for transferase activity according to method 2. A typical diagram obtained in this way is shown in Fig. 2.

The total recovery of enzyme activity after chromatography was *ca.* 75 %. More than 60 % of the recovered activity was located in two or three middle fractions. By chosing only these fractions for further work the chromatographic step resulted in a 8—13 fold purification of the enzyme with a recovery of 45—50 % of the total activity.

To the combined chromatographic fractions were added 1 ml of M K_2HPO_4 and 100 μmoles of neutralized reduced glutathione. The enzyme was then precipitated by addition of solid ammonium sulfate to 65 % saturation. The solution was first attached to a water pump for 15 minutes in order to remove most of the dissolved air and then centrifuged. The resulting precipitate was suspended in 2 ml of 0.1 M tris buffer, pH 7.5, and centrifuged. The supernatant contained 80—100 % of the transferase activity of the combined chromatographic fractions. It was frozen and stored at −15°. Lyophilization could not be carried out at this point without heavy losses. During storage different preparations showed a varying decline of enzyme activity. Addition of glutathione (final concentration 0.05 M) seemed to preserve enzyme activity for a longer time (at least 2—3 weeks).

The results obtained by the chromatographic step were very reproducible. The enzyme could always be localized in an approximate way from the E_{280} curve, since it coincided with a more or less pronounced "shoulder" of this curve before the first large peak.

A summary of a typical purification procedure is given in Table 1.

Table 1. Purification of transferase.

	mg protein	total units	specific activity	recovery %
Extract from lyophilized bacteria (40 g)	12 300	13 900	1.13	100
Extract from protamine precipitate	2 350	12 200	5.2	88
Ammonium sulfate fraction (30—45 % sat.)	675	7 800	11.6	57
After chromatography and ammonium sulfate precip.	28	3 550	127	25

Chromatographic separations. CA-aspartate and aspartate were separated by gradient elution from a Dowex-2 formate column. This separation was carried out both during the demonstration of the back reaction of the enzyme and during the chemical synthesis of L-CA-aspartate. In the latter case the solution (pH 9—10) was applied to a 3 cm^2 × 10 cm column connected with a 500 ml mixing flask containing water. Gradient elution with M formic acid separated aspartate (emerging after *ca.* 6 column volumes) from CA-aspartate (after *ca.* 13 column volumes). A small peak, which appeared at 9 column volumes and coincided with the position of 5-acetic acid hydantoin was completely separated from the other substances. The peaks were localized by their radioactivity and by spot tests with ninhydrin (aspartate) and *p*-dimethylaminobenzaldehyde (CA-aspartate [14]). For the separation of CA-aspartate from aspartate during the demonstration of the back reaction four columns, 0.63 cm^2 × 10 cm, connected with one 500 ml mixer flask were chromatographed simultaneously as described above.

Separation of CAP and P_i by gradient elution was carried out during the isotope exchange experiments. Four columns of Dowex-2 formate, 0.62 cm^2 × 10 cm, were connected with one 1 000 ml mixing flask containing water. During chromatography the mixing flask received M ammonium formate, pH 5.0. P_i appeared after *ca.* 16 column volumes completely separated from CAP, which appeared after *ca.* 24 column volumes. The peaks were localized by determination of radioactivity and by phosphate analysis. The chromatography was carried out at +4°.

Isotope determinations. ^{15}N and ^{13}C were measured as N_2 and CO_2, respectively, in the mass spectrometer. ^{14}C and ^{32}P were determined on infinite thin samples in a Tracerlab SC-18 windowless flow counter.

^{18}O*-analyses* were performed directly on CAP and CA-aspartate. For this purpose *CAP* was isolated from the incubation mixture in the following way. After cooling in an ice bath the solution was acidified with M PCA and evacuated with an oil pump to remove CO_2 as completely as possible. The solution was neutralized with M NaOH (CO_2-free) with phenolphtalein as internal indicator. Then 0.25 ml of 2 M barium acetate were added and the resulting precipitate removed by centrifugation at 0°. Two volumes of alcohol were added to the supernatant and after standing for 5 minutes the resulting barium salt of CAP was collected by centrifugation, washed twice with 2 ml of 50 % alcohol and dried in a desiccator.

The Ba-CAP was finely ground and poured into a glass tube of 3 mm internal diameter which was sealed at one end. It was directly attached to the inlet system of the mass spectrometer by a ground glass joint. After evacuation of the system the bottom of the tube was immersed in a metal bath of 350°. At this temperature Ba-CAP decomposed thermally, while $BaCO_3$ was relatively stable. This was important since CO_2, during incubation, equilibrated with the isotope of $H_2{}^{18}O$ and since Ba-CAP might contain some $BaCO_3$. To ascertain the maximum amount of possible contamination a mixture of 2.5 mg of $Ba^{13}CO_3$ (32 % excess) and 10 mg of non-labeled Ba-CAP were analyzed under the described conditions. The CO_2 contained 0.14 % excess ^{13}C, indicating that less than 0.5 % was derived from the $BaCO_3$.

CA-aspartate for ^{18}O *analyses* was isolated directly from the incubation mixture by chromatography as described earlier. After addition of carrier (DL-form) it was crystallized from water.

CA-aspartate could be thermally decomposed at 200° *in vacuo* in the same way as described for CAP. Since the purpose was to analyze the ^{18}O content of the carbamyl group, it was necessary to determine the contribution of this group to the CO_2 evolved during the thermal decomposition. Thus ^{13}CA-aspartate was synthesized [15,7] from $K^{13}CN$ (atom per cent excess = 5.0) and the gas mixture resulting from the decomposition of ^{13}CA-aspartate was analyzed in a mass spectrometer [16] (Table 2). It can be seen that by far the most intensive peak had a mass number of 44 (CO_2). In a separate experiment the excess ^{13}C was accurately determined from the mass ratio 44/45 and found to be 4.91. This result demonstrated that within experimental error all the carbon of the CO_2 obtained by thermal decomposition of ^{13}CA-aspartate was derived from the carbamyl carbon. Therefore half of the oxygen from this peak corresponded to the carbamyl oxygen.

In the same experiment measurements of the ratio 28/29 (CO and/or N_2) were performed. On the assumption that the ratio was completely due to CO a ^{13}C-value of 4.65 atom per cent excess was obtained (as compared to the theoretical value of 5.0). The presence of either nitrogen or CO arising from another source than the carbamyl group

would have lowered the ^{13}C-value. The observed value therefore demonstrated that 90—95 % of the peaks with a mass number of 28 and 29 consisted of CO derived from the carbamyl group of CA-aspartate. In the case of CO all oxygen arose from the carbamyl oxygen.

Table 2. Thermal decomposition of ^{13}CA-aspartate.

Mass number	Intensity of peak
16	4.1
17	2.2
18	7.7
22	0.9
28	5.6
29	0.4
44	100.0
45	7.0
46	0.47

The analysis of the gas mixture was carried out in a recording mass spectrometer [16]. The carbamyl group of CA-aspartate contained 5.0 per cent excess ^{13}C. Decomposition was carried out at 200° in a high vacuum. The intensity of the peak with a mass of 44 is arbitrarily chosen as 100.

In the actual experiments both the 44/46 and 28/30 ratios were used for the calculations of ^{18}O.

Attempts to characterize the non volatile products of this interesting thermal decomposition have not been successful.

RESULTS

CAP as substrate for CA-aspartate formation

Our earlier experiments [1] had been carried out with enzymically prepared compound X and for further work it was of the greatest interest to establish the possible identity of this substance with synthetic CAP [4], especially in view of differences of opinion in the literature concerning this point [17,18].

For this purpose compound-X-^{14}C was prepared enzymically from labeled $NaHCO_3$ and purified as earlier. It was then co-chromatographed on paper with synthetic nonlabeled CAP in two different solvents and subjected to paper electrophoresis (Table 3). In each experiment one radioactive spot was obtained which coincided with one spot obtained by a phosphate spray [19].

When a small amount of compound-X-^{14}C was co-chromatographed with CAP on Dowex-2 as described in the experimental part for CAP, radioactivity emerged from the column simultaneously with CAP (by phosphate analysis), and a constant ^{14}C/phosphate ratio was obtained in the different fractions (Table 4).

The identity of the two substances was also established in enzyme experiments. Using aspartate-^{14}C and ^{13}CAP as substrates the amount of CA-aspartate formed from each precursor could be calculated by the isotope dilution method. Identical amounts were formed from each precursor. In different experiments increasing amounts of compound-X-carbamyl-^{15}N were added simultaneously with constant amounts of ^{13}CAP and aspartate-^{14}C. When the amount of CA-aspartate formed in the different experiments was calculated independently from the three different isotopes the results demonstrated in Fig. 3 were obtained. The total amount of CA-aspartate formed (calc. from

Table 3. Paper chromatography and paper electrophoresis of CAP and compound-X-carbamyl-^{14}C.

Solvent system	cm from start		Front cm
	radioactive spot	phosphate spot	
n-Propanol:water:conc.NH_3:versene [20] 6 : 10 : 3 : 0.01	3.5 – 8.5	3.7 – 8.3	28
Methanol: 2 N NH_3 : versene [22] 7 : 3 : 0.01	22.5 – 27.5	23 – 27	30
Paper electrophoresis (saturated tetraborate in 0.01 M versene) [21]	13.0 – 16.0	13.4 – 15.6	

In each experiment 0.5 – 2 μmoles of CAP were chromatographed together with 0.05 μmoles of labeled compound X (60 000 ct/μmole). At the end of the experiment the position of the radioactive spots was first determined, and then the paper sprayed with a phosphate reagent [19].

Electrophoresis (12 volts/cm) was carried out for 4 hours. All operations were performed at temperatures between 0 and +4°.

^{14}C) was constant in all experiments and equalled the sum of the amounts formed from CAP (calc. from ^{13}C) and from compound X (calc. from ^{15}N). Furthermore, with increasing amounts of compound X, increasing amounts of CA-aspartate were formed from ^{15}N and decreasing amounts from ^{13}C. The two curves were the mirror images of each other. These results are explained, if compound X and CAP are freely interchangeable with each other in the enzyme reaction.

The same type of behaviour was observed when the concentrations of compound X and aspartate were kept constant, while increasing amounts of CAP were added (Fig. 4).

The intermediate position of CAP in the synthesis of CA-aspartate from NH_3, CO_2, aspartate and ATP could be demonstrated in extracts from rat liver mitochondria. In different experiments CA-aspartate was synthesized from constant amounts of $^{15}NH_4Cl$, $NaHCO_3$, aspartate-^{14}C, ATP and acetyl-

Table 4. Ion exchange chromatography of CAP (25 μmoles) + compound-X-carbamyl-^{14}C (1 μmole, 60 000 ct/μmole).

Fraction No.	ct/ml	μmoles of phosphate/ml	ct/μmole
30	1 200	0.52	2 300
31	6 200	2.78	2 230
32	4 100	1.71	2 400
33	1 050	0.47	2 230
34	100	—	—

Phosphate analysis [10] was carried out after heating for 5 minutes in a boiling water bath. For the determination of radioactivity samples of 0.05 ml + 0.05 ml of 0.1 M NaOH were dried and counted.

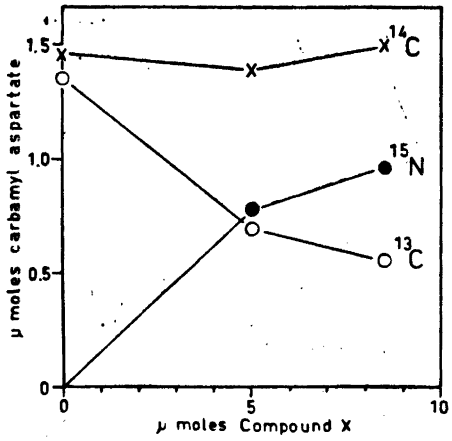


Fig. 3. Influence of compound-X-^{15}N on the synthesis of CA-aspartate from ^{13}CAP.

Substrate (per ml): 30 μmoles of L-aspartate-^{14}C (12.000 ct/μmole), 5 μmoles of CAP (31 atom per cent excess ^{13}C), 150 μmoles of tris buffer, pH 7.5, and 3 units of transferase (spec. activity 10). Compound-X-^{15}N (atom per cent excess $^{15}N = 32$) as indicated on abscissa. Incubation for 15 minutes at 37°.

In each experiment 160 μmoles of carrier DL-CA-aspartate were added, and the content of the different isotopes in the reisolated CA-aspartate was determined.

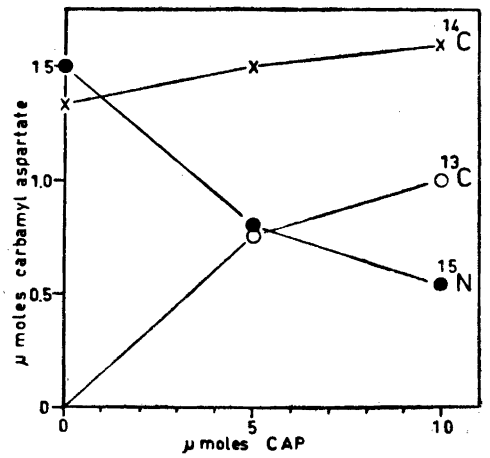


Fig. 4. Influence of ^{13}CAP on CA-aspartate synthesis from compound-X-^{15}N..

Substrate (per ml): 30 μmoles of L-aspartate-^{14}C, 5 μmoles of Compound-X-^{15}N, 150 μmoles of tris buffer, pH 7.5, and 3 units of transferase. ^{13}CAP as indicated on abscissa.. The experiments were conducted as in Fig. 3.

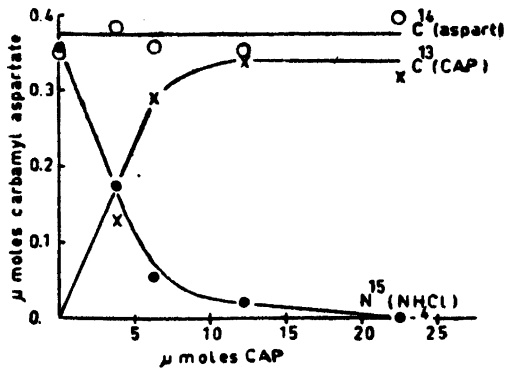


Fig. 5. Influence of ^{13}CAP on CA-aspartate synthesis from $^{15}NH_4Cl$, $NaHCO_3$, ATP and L-aspartate-^{14}C in an extract from rat liver mitochondria.

Substrate (per 3 ml): 20 μmoles of L-aspartate-1,4-^{14}C (12 000 ct/μmole), 20 μmoles of $^{15}NH_4Cl$ (32 atom per cent excess), 100 μmoles of $NaHCO_3$, 10 μmoles of ATP, 60 μmoles of 3-phosphoglycerate, 20 μmoles of acetylglutamate, 100 μmoles of $MgSO_4$, 200 μmoles of phosphate buffer, pH 7.5, and 5 mg of a muscle protein fraction (Ratner, S. and Pappas, A. *J. Biol. Chem* **179** (1949) 1183). ^{13}CAP (31 atom per cent excess) as indicated on the abscissa.

The mitochondrial enzyme (1.51 mg nitrogen per experiment) was prepared as described earlier [1].

Incubation for 60 minutes at 37°. Carrier added as in Fig. 3.

glutamate, while increasing amounts of ^{13}CAP were added. When the formation of CA-aspartate from the different isotopic substances was studied (Fig. 5), the results demonstrated that CAP had a large dilution effect on the ^{15}N-incorporation. At the highest CAP concentration studied practically no CA-aspartate was formed from ammonia, while identical amounts were formed from CAP and aspartate.

The experiments are in full agrement with the idea that CAP is the active carbamyl compound in the bacterial enzyme reaction and that it is also an intermediate in CA-aspartate formation in rat liver.

Properties of the transferase reaction

The *stoichiometry* of reaction (2) could be demonstrated with the ammonium sulfate fractionated enzyme (Table 5). The slightly larger formation of P_i than CA-aspartate is fully explained by some non-enzymic decomposition of CAP under the experimental conditions.

Isolation of L-CA-aspartate. Only L-aspartate could act as substrate (see below) and the CA-aspartate formed during the reaction was also the L-isomer. This was demonstrated by the isolation of its barium salt from a large scale experiment. Two mmoles of L-aspartate and 3 mmoles of CAP were incubated with 520 units of enzyme (specific acitivity = 11) at 37° for 60 minutes. After chromatography on Dowex-2 the fractions containing CA-aspartate were combined and the formic acid was removed by evaporation *in vacuo*. The

Table 5. Stoichiometry of transferase reaction.

Expt. No.		CAP	P_i	L-aspartate	CA-aspartate
			μmoles		
1	Initial	31.2	3.0	30.0	0
	Final	11.9	23.1	13.6	16.8
	Δ	19.3	20.1	16.4	16.8
2	Initial	31.2	2.1	30.0	0
	Final	26.9	6.8		
	Δ	4.3	4.7		

Incubation was carried out at 37° for 15 minutes in 1 ml volume.

Experiment 1: 1.5 mg of ammonium sulfate fractionated enzyme, experiment 2: no enzyme.

CAP + P_i was determined by heating an aliquot of the sample in 0.01 N HCl at 100° for 1 minute [4] before phosphate analysis [18].

L-Aspartate (32 atom per cent excess ^{15}N in substrate) was determined by the isotope dilution method. One mmole of carrier L-aspartate was added to an aliquot of the solution. After acidification the aspartic acid was crystallized to a constant isotope content.

CA-aspartate was determined by method 1 and according to Koritz and Cohen [11].

residue was dissolved in a minimum amount of water and the barium salt (160 mg) prepared as described earlier [23]. It showed $[\alpha]_D^{25} = +25.7°$ (as compared to the earlier obtained value of +24.1° for synthetic L-CA-aspartate).

A *time curve* of the reaction is given in Fig. 6.

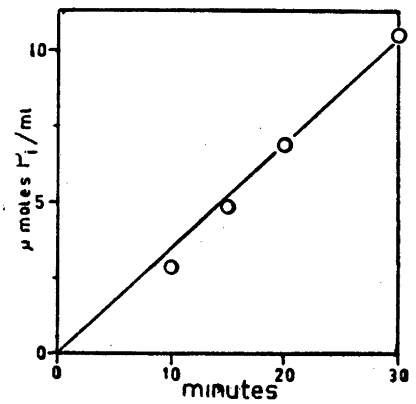


Fig. 6. Time curve of transferase action.

In each experiment volume = 1.0 ml. 5 units of transferase (spec. activity = 11) were incubated with 100 μmoles of L-aspartate, 30 μmoles of CAP and 150 μmoles of tris buffer, pH 7.5. Enzyme assay according to method 2.

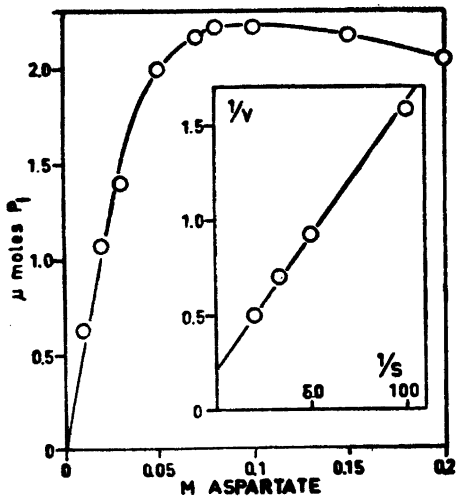


Fig. 7. Influence of aspartate concentration.

Transferase (2 units, spec. activity = 90) was incubated for 15 min. at 37° with 8 μmoles of CAP, 150 μmoles of tris buffer, pH 7.5, and L-aspartate as indicated on the abscissa. Final volume = 1.0 ml. Enzyme assay by method 2.

Dependance on substrate concentration. The influence of *aspartate concentration* on the reaction rate is shown in Fig. 7. As indicated earlier [1] large amounts of substrate were necessary for enzyme saturation. Furthermore, at concentrations above 0.1 M a clear inhibition by aspartate did take place. When a Lineweaver-Burk plot was made from the values obtained below 0.05 M aspartate concentration a value of $K_M = 0.065$ was obtained. In a different experiment at higher CAP concentration (0.1 M) and using method 1 for the determination of reaction rate the value for K_M was 0.060.

The influence of *CAP concentration* is demonstrated by Fig. 8. No inhibition was observed up to 0.3 M concentration. $K_M = 0.45 \times 10^{-3}$.

The *pH-curve* of the reaction is shown in Fig. 9. This curve differs significantly from the one given earlier [1], which had been obtained under somewhat different conditions. The reason for the discrepancy is not understood. In the present investigation a pH optimum of 7.5 was obtained in five different experiments with both methods of enzyme assay, using tris or imidazole buffer.

Specificity of the reaction. Ureidopropionic, ureidoglutaric and ureidosuccinic acids, citrulline and urea could not serve as carbamyl donors in place of carbamyl phosphate.

With a 90 fold purified enzyme L-aspartate alone could serve as acceptor for the carbamyl group of CAP. 31 amino acids and related compounds were tested for their ability to bring about the enzymic dephosphorylation of CAP. Furthermore, the appearance of a new carbamyl compound was sought by the method of Koritz and Cohen[11]. Those non reacting compounds were: L-α-alanine, β-alanine, DL-α-aminoadipic acid, α, β, and γ-aminobutyric acids,

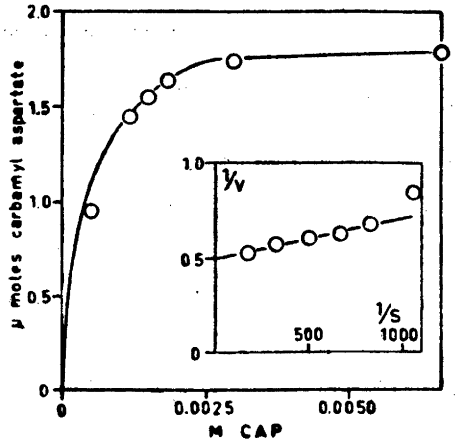


Fig. 8. Influence of CAP concentration.

Incubation (volume = 3.0 ml): 2 units of transferase (spec. activity = 90) were incubated for 15 min. at 37° with 300 µmoles of L-aspartate-^{15}N (32 atom per cent excess ^{15}N), 400 µmoles of tris buffer, pH 7.5, and CAP as indicated on abscissa. Enzyme assay by method 1.

L-arginine, L-asparagine, D-aspartic acid, L-citrulline, L-cysteic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, DL-homoserine, L-hydroxylysine, L-*iso*leucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-serine, taurine, L-threonine, L-tryptophane and L-valine*.

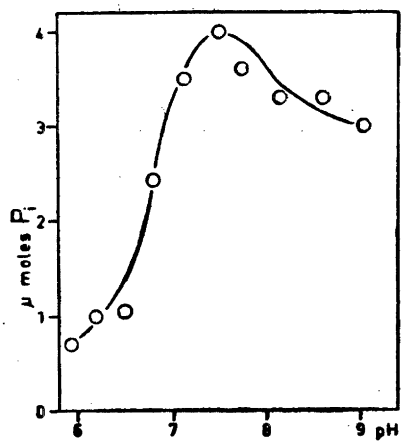


Fig. 9. pH curve.

Transferase (4 units, spec. act. 90) was incubated for 15 min. at 37° with 100 µmoles of L-aspartate, 20 µmoles of CAP and 300 µmoles of tris (pH range 7—9) or imidazole (pH range 6—7.5) buffers. Volume = 1.0 ml. Enzyme assay by method 2.

*. The amino acids were commercial products. We wish to thank Drs. G. Ågren and Z. Bánhidi for gifts of some of these amino acids.

Table 6. Aspartate and ornithine carbamyl transferase activity during enzyme fractionation

	μmoles CA-aspartate formed per ml	μmoles citrulline formed per ml
Bacterial extract	18	14
Supernat. from protamine	3.4	5.6
Extract of protamine precipitate	22	9.4
Ammonium sulfate 0—30 %	9	0
» » 30—45 »	87	10
» » >45 »	0	4.2
After DEAE chromatography	52	0

The CA-aspartate forming enzyme was determined by method 1.
The substrate for the citrulline forming enzyme contained 60 μmoles of DL-ornithine. instead of 30 μmoles of L-aspartate, but apart from this conditions were the same.
Protamine precipitation in this preparation was performed at room temperature.

The ammonium sulfate fractionated enzyme showed activity in catalyzing the reaction between CAP and L-ornithine to form citrulline. It was thought of interest to make a comparison between CA-aspartate formation and citrulline formation at various stages of purification of the enzyme. The result, summarized in Table 6, clearly demonstrated the existence of two different carbamyl transferases, one forming CA-aspartate and the other forming citrulline.

Inhibition of transferase activity. Several amino acids showing structural relationship to L-aspartate were investigated as possible inhibitors of the reaction. In these experiments 30 μmoles of L-aspartate and 20 μmoles of CAP were used as substrate together with 5—10 units of transferase. Usually 150 μmoles of the substance under investigation were added and the formation of P_i measured by method 2. No inhibition was observed with, α-, β- or γ-aminobutyric acids, L-asparagine, D-aspartate, L-cysteic acid, L-glutamate and DL-homoserine.

Table 7. Inhibition of transferase reaction.

Inhibitor	concentration (M)	% inhibition
NaCN	0.01	
Versene	0.05	
Arsenate	0.1	
Phosphate	0.1	
Hydroxylamine	0.1	
Phenylhydrazine	0.01	0
Semicarbazide	0.01	
Bisulfite	0.01	
Arsenite	0.01	
Ethyldichloroarsin	0.0005	
Iodosobenzoate	0.004	
Iodoacetate	0.01	5—10
CMBA	0.003	90—95
CMBA	0.001	45

The inhibitor was added to the buffered enzyme solution (5—10 units, spec. activity 90) at room temperature (volume 0.8 ml). After 10 minutes the solution was cooled in an ice bath for 5 minutes. Then 100 μmoles of L-aspartate + 25 μmoles of CAP were added, final volume 1.0 ml. Incubation for 15 minutes at 37°.

Table 8. Protection from CMBA-inhibition by substrates.

Added at time 0 min.	Added at time 15 min.	μmoles P_i formed	% inhibition
Enz.	CAP + asp.	7.9	
Enz. + CMBA	CAP + asp	4.0	49
Enz. + CAP	asp.	8.1	
Enz. + CAP + CMBA	asp.	4.0	51
Enz. + asp.	CAP	7.6	
Enz. + asp. + CMBA	CAP	4.1	46
Enz. + CAP + asp.	—	8.8	
Enz. + CAP + asp. + CMBA	—	7.3	17

The experimental conditions were as described in Table 7 with additions as indicated. Final CMBA concentration = 0.001 M.

Table 7 demonstrates the results obtained with different types of inhibitors. In all cases, except with versene, phosphate and arsenate, method 2 was used for enzyme assay. Significant inhibition was only observed with CMBA and only at relatively high concentration of the inhibitor.

When, in an experiment at 0.001 M CMBA concentration, 5 μmoles of reduced glutathione were added 5 minutes before the start of the incubation at 37° (*cf.* conditions of Table 7), no inhibition was observed. This experiment demonstrates that inhibition with CMBA could be completely reversed by glutathione.

No protection of the enzyme wat observed by CAP or L-aspartate alone However, a mixture of both substrates partially protected the enzyme from the action of the inhibitor (Table 8).

Reversibility of transferase reaction. The equilibrium of the reaction was shifted very far towards synthesis of CA-aspartate. Unsuccessful attempts

Table 9. Formation of aspartate from CA-aspartate.

Experimental conditions	total counts in aspartate fractions	μmoles aspartate formed
Complete	730	0.76×10^{-2}
No enzyme	515	0.54×10^{-2}
No phosphate	525	0.55×10^{-2}
No enzyme or phosphate	540	0.56×10^{-2}

The complete experiment was carried out with 30 μmoles of CA-aspartate-^{14}C (0.96×10^5 ct/μmole), 50 μmoles of phosphate buffer, pH 7.5, 100 μmoles of tris buffer, pH 7.5, and 80 units of transferase (spec. activity 130). Final volume: 1.0 ml. Incubation for 30 minutes at 37°. The reaction was terminated by addition of 0.4 ml of M PCA. Three ml of water were added together with 30 μmoles of non-labeled aspartate. The pH was adjusted to 9—10 (phenolphtalein). Chromatographic separation of aspartate and CA-aspartate was then carried out as described in the experimental part. Aspartate was localized in the effluent by spot tests with ninhydrin. The total radioactivity in the pooled aspartate fractions was determined, and the amount of aspartate formed calculated from this value.

were made to measure the enzymic formation of aspartate from CA-aspartate and P_i (back reaction) by coupling the reaction with glutamo-oxaloacetic-transaminase [24] and malic dehydrogenase [25] after addition of α-ketoglutarate and DPNH.

The reversibility of the reaction could be demonstrated and measured by the use of carboxyl labeled CA-aspartate-^{14}C as substrate. It could be demonstrated that maximum amounts of aspartate-^{14}C were formed only in the presence of both enzyme and P_i (Table 9). The "background" formation of aspartate resulted from the presence of small amounts of this amino acid in the synthetic CA-aspartate and to a lesser extent from non enzymic breakdown during experiments of long duration. A further complication for any quantitative determinations of the equilibrium position was the chemical lability of CAP, the other product formed during the back reaction. Attempts to determine the equilibrium constant for the transferase action from the back reaction using CA-aspartate-^{14}C did not show conclusive results. It was evident, however, that the equilibrium position was shifted very far towards synthesis of CA-aspartate.

The mechanism of the reaction

Isotope exchange experiments. It was thought possible that the carbamyl group of CAP was first transferred to the enzyme, followed by a carbamyl transfer from the enzyme to aspartate:

$$\text{CAP} + \text{Enzyme} \rightleftharpoons \text{CA-Enzyme} + P_i \quad (3)$$

$$\text{CA-Enzyme} + \text{L-aspartate} \rightleftharpoons \text{L-CA-aspartate} + \text{Enzyme} \quad (4)$$

This mechanism was tested by isotope exchange experiments. According to reaction (3) an enzymic exchange of $^{32}P_i$ with phosphate in CAP should take place in the absence of aspartate. Table 10 demonstrates that such an *enzymic* exchange does not take place. Some ^{32}P was found in CAP in the absence of the enzyme, and addition of enzyme did not increase the specific acticity of the

Table 10. Isotope exchange experiments with $^{32}P_i$.

	Reisolated P_i		Reisolated CAP	
	μmoles	ct/μmole	μmoles	ct/μmole
Complete	59	0.62×10^6	10	2.6×10^3
No aspartate	48	0.77×10^6	18	1.3×10^3
No enzyme	47	0.71×10^6	23	1.5×10^3
No enzyme and asp.	50	0.70×10^6	16	1.7×10^3

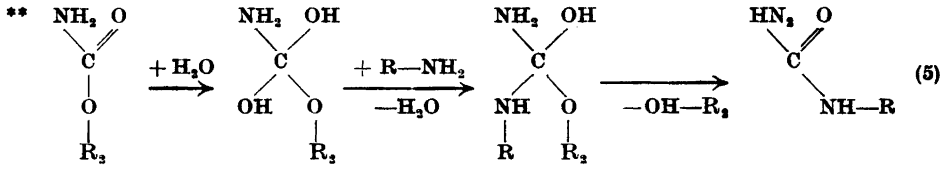
The complete experiment contained 35 μmoles of CAP, 40 μmoles of $^{32}P_i$ (0.83×10^6 ct/μmole), 15 units of transferase (spec. act. 90), and 250 μmoles of tris buffer, pH 7.5. Incubation for 15 min. at 37°. The solution was then cooled in ice, adsorbed directly on a Dowex-2-formate column and chromatographed (see experimental part). Fractions containing P_i and CAP respectively were pooled, and the radioactivity and phosphorus content were determined on aliquots.

reisolated CAP. However, after addition of L-aspartate a small but significant increase was observed. Table 10 demonstrates results typical of 5 different experiments at pH 7.5 and 6.5. The enzymic isotope exchange in the presence of aspartate again demonstrated the reversibility of the reaction while the absence of enzymic isotope exchange without aspartate made reaction (3) seem unlikely.

Further evidence against reaction (3) was obtained from experiments with arsenate. If reaction (3) took place in the presence of arsenate one might expect that the CA-enzyme formed would give rise to CA-arsenate, which in turn would decompose to carbamate + arsenate. The net effect would be an accelerated breakdown of CAP in the presence of enzyme and arsenate. Such an effect was not observed when 40 μmoles of CAP, 50 units of transferase and 100 μmoles of arsenate were incubated for 15 minutes at 37° *.

Reaction (4) was made improbable by an exchange experiment with L-aspartate-2,3-^{14}C (50 μmoles, 19 000 ct/μmole), CA-aspartate (48 μmoles) and enzyme (50 units). After incubation for 1 hour at 37° no radioactivity was found in the reisolated CA-aspartate.

Experiments with $H_2{}^{18}O$. Another possible mechanism involved the hydration of CAP as a first step, followed by condensation of the hydrate with aspartate by removal of water and subsequent dephosphorylation of the formed intermediate to give rise to CA-aspartate:



(5)

The participation of water in such a mechanism could be investigated by carrying out the transferase reaction in $H_2{}^{18}O$. In such an experiment no ^{18}O was incorporated into the carbamyl group of CA-aspartate, nor was there any significant enzymic exchange of the oxygen of water with that of CAP (Table 11).

DISCUSSION

CA-aspartic acid is known to be an intermediate in the biosynthesis of orotic acid both in microbial[26,27] and mammalian[21,28] systems. The demonstration that L-aspartate and the carbamyl group of CAP are the two groups from which the pyrimidine ring originates links together pyrimidine synthesis with both the tricarboxylic acid cycle and the urea cycle. The occurrence of the enzyme in various mammalian organs and in microbial extracts together with the fact that the equilibrium of the reaction is shifted far towards synthesis of

*. The decomposition of CAP in these experiments was measured by the formation of ammonia. The reaction was stopped by acidification with PCA. After neutralization the mixture was adsorbed on a small Dowex-2-formate column (0.62 cm² × 5 cm). The column was washed with 15 ml of water and aliquots of the eluant were analyzed for ammonia.

**. $R_2 = H_2PO_3$; $R—NH_2$ = L-aspartic acid.

Table 11. Transferase reaction in $H_2{}^{18}O$.

	Atom per cent excess ^{18}O in		
	CAP	Carbamyl group of CA-aspartate	
		from CO	from CO_2
Complete	0.003	0.000	0.001
No aspartate	0.001		
No enzyme	0.005		

Complete experiment: 100 μmoles of CAP, 100 μmoles of L-aspartate-^{14}C (10^3 ct/μmole), 125 μmoles of tris buffer, pH 7.5, 30 units of transferase (spec. act. 130). Incubation for 15 min. at 37° in 1 ml of $H_2{}^{18}O$ (1.4 atom per cent excess).

Two complete experiments were conducted, one for the isolation of CAP, the other for the isolation of CA-aspartate.

Ba-CAP was isolated and analyzed for ^{18}O as described in the experimental part.

CA-aspartate was separated by chromatography and localized by its radioactivity (26 μmoles of L-CA-aspartate). After addition of 80 μmoles of carrier DL-CA-aspartate, the substance was crystallized from water, decomposed and analyzed for ^{18}O.

CA-aspartate makes it very likely that this reaction represents a general first step in pyrimidine biosynthesis.

The purified enzyme showed the very specific capacity to catalyze a transfer of a carbamyl group attached to phosphate in "high energy" linkage to the amino group of L-aspartate. The extreme position of the equilibrium towards CA-aspartate synthesis together with the low affinity of the enzyme for aspartate and the instability of CAP made it impossible to measure the equilibrium constant of the transferase reaction. The existence of a back reaction could be demonstrated with the aid of $^{32}P_i$ (Table 10) and CA-aspartate-carboxyl-^{14}C (Table 9). Although a quantitative determination of the equilibrium position could not be carried out, it is clear from the data of Tables 9 and 10 that the back reaction proceeds at a very slow rate, and that the transferase reaction greatly favours synthesis of CA-aspartate.

Only one enzyme seemed to be involved in the reaction and no evidence could be obtained for the participation of a prosthetic group or an activator. The enzyme was named aspartate carbamyl transferase since this name seems to describe its function properly, although previously described transferases do not generally show such extreme equilibrium positions. The enzyme is distinct from the citrulline forming enzyme[3] and the enzyme forming β-ureidopropionic acid[29]. The latter two enzymes have as yet not been described in detail, but it seems highly probable that they also belong to the class of carbamyl transferases.

The isotope exchange experiments gave no evidence for the formation of a carbamylated enzyme. The experimental conditions allowed the demonstration of the reversibility of the sum of reactions (3) and (4), *i.e.* enzymic exchange of ^{32}P between P_i and CAP in the presence of aspartate. This makes

it improbable that the failure to find evidence from isotope exchange experiments for reaction (3) *or* reaction (4) depended on a limited reversibility of these two reactions, but rather argues against the actual existence of reactions (3) and (4).

The experiments with $H_2{}^{18}O$ speak against the participation of water and the formation of a phosphorylated intermediate as depicted in reaction (5). Such a type of mechanism can, however, not be completely ruled out if the whole reaction sequence were to be considered as taking place with enzyme bound intermediates and if the same molecule of water were first added and then removed.

It would appear, however, that the most probable explanation for all of the experimental results is that the transferase reaction involves a single displacement mechanism of the same type as earlier suggested for phosphate transferases[30]. According to this view both CAP and L-aspartate are first attached to the enzyme. By a nucleophilic attack of the nitrogen atom of aspartic acid on the carbon atom of CAP the carbon-oxygen bond is broken simultaneously with the formation of the new carbon-nitrogen bond.

Inhibition of the enzyme by CMBA could be demonstrated. Glutathione completely reversed the inhibition, which was probably due to the interaction of CMBA with active SH-groups. The absence of inhibition by less powerful SH-reagents does not necessarily speak against the concept that inhibition by CMBA was due to combination with SH groups (*cf.* Refs. [31,32]). Of interest for the understanding of the transferase mechanism was the finding that CAP + aspartate showed the ability to protect the enzyme against inhibition, while CAP or aspartate alone showed little effect. This finding is explained if both substrates compete with CMBA for SH groups of the enzyme. The results therefore tentatively indicate that both aspartate and CAP, during the action of the enzyme, are attached to SH groups.

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