ACTA CHEMICA SCANDINAVICA 11 (1957) 523—536

Ornithine Carbamyl Transferase from Rat Liver

PETER REICHARD

Biochemical Department, Karolinska Institutet, Stockholm, Sweden

An enzyme (ornithine carbamyl transferase) has been purified from rat liver which catalyzes the formation of citrulline according to: L-ornithine + carbamyl phosphate ⇌ L-citrulline + phosphate. The enzyme moved as a single compound during paper electrophoresis between pH 5.0 and 8.5 with an isoelectric point around 8.1. A minor impurity amounting to *ca.* 5—8 % was found during ultracentrifugation.

The reaction greatly favoured citrulline synthesis, the equilibrium constant being *ca.* 10^5. Out of 26 amino acids tested only L-ornithine was able to accept the carbamyl group of carbamyl phosphate. Inhibition by SH-reagents was observed and partial protection from inhibition could be obtained with each of the substrates, tentatively indicating binding of both substrates to SH groups of the enzyme. Isotope exchange experiments indicated that the transfer reaction was of the single displacement type.

The citrulline phosphorylase of Krebs *et al.*[8] has been found to be identical with ornithine carbamyl transferase.

By coupling ornithine carbamyl transferase with aspartate carbamyl transferase [5] synthesis of carbamyl aspartate was obtained from citrulline + aspartate in the presence of phosphate.

The work of Grisolia and Cohen [1] has demonstrated that citrulline is formed enzymically in mammalian liver through a transfer of the carbamyl group of an active carbamyl compound to ornithine. Jones *et al.*[2] recently synthesized carbamyl phosphate and demonstrated its identity with the active carbamyl compound.

Our interest in carbamyl transfer reactions originated from investigations of the enzymic formation of carbamyl aspartate, an intermediate in pyrimidine biosynthesis. We found that this substance was formed from aspartate, CO_2 and NH_3 in the presence of ATP and acetylglutamate and that carbamyl phosphate (CAP*) was an intermediate in the reaction [3-5]. An enzyme (aspartate carbamyl transferase) which catalyzed the transfer of the carbamyl

* The following abbreviations are used in this paper: CA = carbamyl, CAP = carbamyl phosphate, P_i = inorganic phosphate, ATP = adenosine triphosphate, PCA = perchloric acid, tris = tris(hydroxymethyl)amino methane, CMBA = *p*-chloromercuribenzoic acid, EDTA = ethylenediamine tetraacetic acid.

group was purified a hundredfold from *E. coli* [5] and a study of the mechanism of its action was made. The limited supply of the starting material and the relative instability of the purified enzyme made further purification difficult.

The present investigation was started with a twofold purpose. First it was intended to study a carbamyl transfer reaction with an essentially pure enzyme. Second it was of interest to investigate whether the citrulline forming enzyme by a reversal of its action could form carbamyl phosphate which then together with aspartate would give rise to carbamyl aspartate. Such a reaction would represent a second mechanism for the utilization of the carbamyl group of citrulline for pyrimidine synthesis [6], the first being the transfer of the carbamyl group of citrulline to a phosphate group of ATP with the formation of CAP [7]. In this connection it was also of interest to determine whether citrulline phosphorylase as described by Krebs *et al.*[8] was identical with the citrulline forming enzyme.

After purification of the enzyme from rat liver the following stoichiometric reaction could be demonstrated:

$$\text{Carbamyl phosphate} + \text{L-ornithine} \rightleftharpoons \text{L-citrulline} + \text{phosphate} \quad (1)$$

The name ornithine carbamyl transferase is proposed for this enzyme.

MATERIALS AND METHODS

L-Citrulline-carbamyl-^{14}C was synthesized according to Smith [9]. In spite of repeated crystallizations the compound contained a small amount (*ca.* 1 %) of ornithine. For the determination of the equilibrium constant this amino acid was removed by paper electrophoresis (1 h, 12.5 V/cm) in 0.1 M acetic acid. Using acid washed Whatman filter paper (No. 3) 300 μmoles of citrulline could be run on one sheet. A small strip was sprayed with ninhydrin to locate the amino acids. The main amount of citrulline was then eluted with water from the paper. After evaporation to dryness it was redissolved in a small amount of water and used directly. On paper chromatography (phenol:water, 9:1) no ornithine could be detected.

^{14}CA-aspartate was synthesized according to Nyc and Mitchell [10], *CAP* according to Jones *et al.*[2] DL-*Ornithine*, L-*ornithine* and DL-*ornithine-2-^{14}C* were commercial samples (Schwarz laboratories, Inc. and California Foundation for Biochemical Research).

Protein determinations were made by the method of Bücher [11]. When checked by nitrogen analysis of PCA precipitates at different points during the preparation good relative agreement was obtained. During zone electrophoresis and chromatography protein was measured by light absorption at 280 mμ.

Determination of enzyme activity. Forward reaction. The enzyme was incubated for 15 min at 37° with 30 μmoles of CAP, 100 μmoles of DL-ornithine and 100 μmoles of tris buffer, pH 7.3—7.4, in a final volume of 1.0 ml. The tubes were then immediately immersed in an ice bath and the amount of inorganic phosphate present was determined directly on 0.02 ml aliquots by the Lowry-Lopez [12] method. Readings were made after 5 min. A blank without enzyme was treated in the same manner.

Under those conditions the difference between the amount of inorganic phosphate formed in the sample containing enzyme and the blank was proportional to the amount of enzyme added (Fig. 1) up to a value of *ca.* 6—8 μmoles.

One unit of enzyme activity was defined as a difference of 1 μmole of P_i formed under the above conditions. The specific activity of the enzyme was defined as units/mg protein.

Sometimes the amount of citrulline formed was determined rather than P_i. In this case the enzyme reaction was stopped by the addition of 0.1 ml of 4.4 M PCA and citrulline [13] determined directly on aliquots. This method was more time consuming but was used whenever high accuracy was desired or when substances were present which inter-

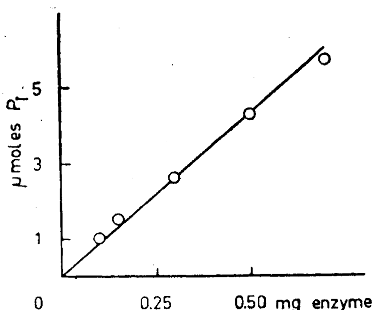

Fig. 1. Enzymic formation of P_i from CAP. Standard conditions as described in text.

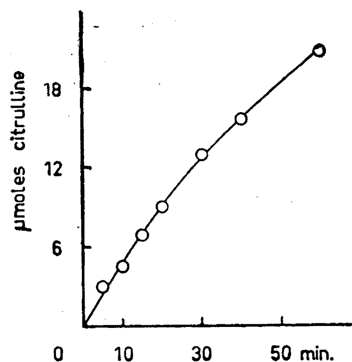

Fig. 2. Time curve of transferase action. Standard conditions with citrulline determination.

fered with phosphate analysis. Identical results were obtained with both methods. A *time curve* of the enzyme reaction is demonstrated in Fig. 2 (citrulline determinations).

Back reaction: It could be demonstrated that the arsenolysis of citrulline [8] is carried out by ornithine carbamyl transferase. The rate of the back reaction was measured by the formation of $^{14}CO_2$ from citrulline-carbamyl-^{14}C in the presence of arsenate. In principle the method was the same as the one used earlier for the determination of CAP formation from citrulline [7]. The enzyme was incubated with 6 μmoles of citrulline-^{14}C (130 000 ct/min/μmole) and 100 μmoles of arsenate buffer, pH 6.7, for 15 min at 37° in a stoppered Warburg vessel, containing 4 N NaOH in the center well and 9 N H_2SO_4 in the side arm. The vessel was cooled on ice, 100 μmoles of $NaHCO_3$ were added and the acid tipped into the main compartment. After shaking for 30 min at 37° the alkali from the center well was precipitated with barium hydroxide, the formed $BaCO_3$ was washed with hot water, filtered on to a paper disk of standard size, and counted as an infinitely thick sample in an end window Geiger-Müller counter. The number of μmoles $^{14}CO_2$ formed could then be calculated from an experimentally determined conversion factor.

One unit of enzyme activity for the back reaction was defined as the amount of enzyme which under the above conditions formed 0.1 μmoles of CO_2.

The same general technique was used during the determination of the equilibrium constant, with the exception that phosphate buffer was used instead of arsenate. In this case the purified (paper electrophoresis) citrulline was used.

Ion exchange chromatographies. The chromatographic separation of CAP from P_i was performed as described earlier [5]. CA-aspartate was separated from citrulline by gradient elution chromatography on Dowex-2-formate (diam. 0.9 cm, length 11 cm) with a 250 ml mixing flask receiving 4 N formic acid. Citrulline was eluted with water, while CA-aspartate appeared after 7—8 column volumes.

RESULTS AND DISCUSSION

Purification of the enzyme

The work of Grisolia and Cohen [1] has demonstrated the localization of the enzyme in the particulate fraction of rat liver. In conformity with these authors the starting material for our enzyme preparation was an acetone powder of "rat liver residue". Essentially no activity was found in the supernatant fraction.

Preliminary experiments were carried out with rat, rabbit and beef liver. By far the highest activity was observed in crude extracts of rat liver and it was also found that fractionation with ammonium sulfate gave better results in this case. Further work was therefore carried out with rat liver.

Preparation of the acetone powder. All work was performed in a cold room at +2°. The livers (500—600 g) from 80 rats were homogenized in a Waring blendor (low speed) in 3 liters of 1.15 % KCl. The solution was poured through a double layer of cheese cloth and then centrifuged in a Sharples centrifuge (rotor size 200 ml) at 25 000 r.p.m. The flow through the rotor was adjusted to a rate of 50—60 ml/min.

The sediment was removed from the rotor and suspended in a minimum amount of 0.05 M phosphate buffer, pH 7.4. Liver residues from six centrifugations may be conveniently pooled at this point.

The suspension was mixed in a Waring blendor with a five to ten fold excess of acetone (—15°). After filtration the residue was once more treated with cold acetone, filtered again and dried in a desiccator.

The livers from 100 rats corresponded roughly to 40—50 g powder.

Extraction of the acetone powder was carried out with 0.05 M tris buffer, pH 7.4, containing 0.001 M EDTA *. It was found that the enzyme was not appreciably inactivated by heating at 52° for 1 h (*cf.* Ref.[1]). The extraction was therefore directly carried out at this temperature.

The acetone powder (205 g) was ground in a mortar with 2 050 ml of buffer. The suspension was immersed in a water bath of 55° and stirred continuously. When the temperature inside had reached 52° the outside temperature was lowered to the same value and heating was continued for one hour. The solution was then cooled in ice water. From this point all operations were performed at 0—4°.

The suspension was centrifuged in the No. 21 head of a Spinco centrifuge (15 min at 12 000 r.p.m.). The residue was washed with 600 ml of buffer. By addition of more buffer the protein concentration was adjusted to 1 %. Volume 3 150 ml.

Solid *ammonium sulfate* (880 g) was added slowly and under stirring. The solution was allowed to stand overnight and the clear supernatant was decanted. The cloudy bottom layer was centrifuged and the precipitate was discarded. To the combined supernatants (3 500 ml) was added 585 g of solid ammonium sulfate and precipitation allowed to take place overnight. The precipitate was centrifuged in the Spinco centrifuge (12 000 r.p.m. at 15 min), and dissolved in 0.05 M tris buffer, pH 7.4, final volume 210 ml. The clear solution was dialyzed against a total of 9 liters of 0.01 M tris buffer for 16 h.

Alcohol precipitation. The volume of the dialyzed solution was adjusted to 1 120 ml with 0.01 M buffer, giving a final protein concentration of 1 %, and 28 ml of 2 M KCl was added.

The solution was placed in a cooling bath and 50 % ethanol (360 ml) was added slowly under constant stirring. During the operation the temperature of the bath was lowered to —3°. After standing for 1 h the formed precipitate was centrifuged and discarded.

* All solutions throughout the whole preparation contained 0.001 M EDTA.

To the clear supernatant more 50 % ethanol (560 ml) was added slowly while the temperature of the cooling bath was lowered to —5°. After standing for 1 h the precipitate was centrifuged (P_2). A third precipitate (P_3) was obtained after addition of 250 ml of 50 % ethanol and standing for 3 h at —10°. To the supernatant after centrifugation was added 100 ml of 50 % ethanol and the solution was allowed to stand at —10° overnight, yielding a fourth precipitate (P_4). The clear supernatant after centrifugation always contained very little activity. Each precipitate (P_2, P_3 and P_4) was dissolved separately in *ca.* 150 ml of 0.01 M tris buffer, and all solutions were assayed for protein and enzyme activity. The bulk of activity was located in P_2 and P_3*.

Second ammonium sulfate fractionation. P_2 and P_3 were combined (290 ml) and 5 ml of M tris buffer, pH 8.5, was added. A saturated solution of ammonium sulfate (pH 8.0, 320 ml) was added dropwise under constant stirring. After 30 min the solution was centrifuged and the precipitate discarded. Solid ammonium sulfate (54 g) was added to the supernatant and precipitation allowed to take place overnight.

After centrifugation the resulting precipitate was dissolved in 0.01 M tris buffer, pH 7.4, and dialyzed against a total of 6 liters of the same buffer for 24 h.

DEAE adsorption. Attempts to chromatograph the enzyme on DEAE cellulose columns [14] were unsuccessful. It was difficult to obtain complete adsorption of the enzyme, a large amount of activity disappeared during the procedure and the results were not very reproducible. By batchwise treatment with DEAE cellulose in the presence of phosphate buffer it was possible, however, to remove acidic impurities from the enzyme and to obtain further purification.

Five g of the cellulose powder prepared according to Peterson and Sober [14] was washed first with 500 ml of M phosphate buffer, pH 7.4 in a chromatographic column, and then with three 20 ml portions of 0.01 M phosphate buffer, pH 7.4. The cellulose was then suspended in an equal volume of 0.01 M phosphate buffer (90 mg of dry cellulose per ml).

One ml of 0.1 M phosphate buffer, pH 7.2, and 28 ml of the cellulose suspension were added to the dialyzed solution (70 ml) from the second ammonium sulfate precipitation step. After 10 min the cellulose was filtered off by suction and washed twice with 10 ml of 0.01 M phosphate buffer.

Saturated ammonium sulfate (102 ml), pH 8.0, was added to the filtrate (102 ml) and the resulting precipitate was discarded after centrifugation. Solid ammonium sulfate (21 g) was added to the supernatant and precipitation allowed to take place overnight.

The precipitate was centrifuged and dissolved in the smallest possible amount of 0.05 M tris buffer, pH 7.4. It was dialyzed for 18 h against a total of 4 liters 0.03 M tris buffer, pH 8.5.

Zone electrophoresis. After the previous step the solution was deep red. Paper electrophoresis demonstrated that at pH 8.5 the enzyme could be

* In some preparations P_4 was also used for the further purification. In one preparation practically all activity was contained in P_2.

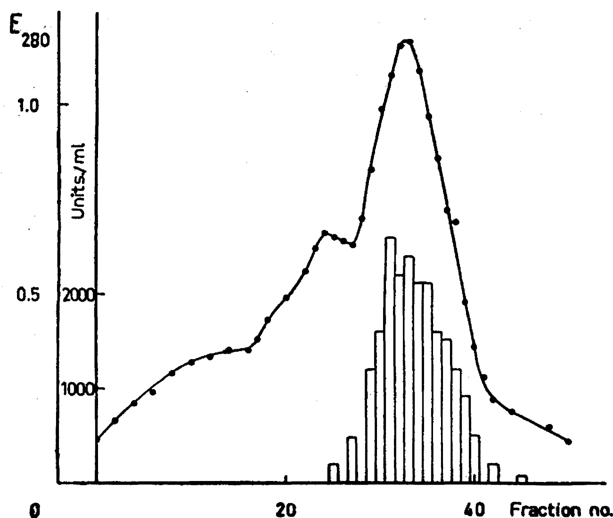

Fig. 3. Zone electrophoresis of ornithine carbamyl transferase. Continuous curve = E_{280}; stepped curve = enzyme activity.

separated from the coloured components. At this pH the enzyme moved very slowly towards the anode, while the coloured components moved faster in the same direction. Separation on the preparative scale was therefore carried out by electrophoresis on a cellulose column according to Porath [15].

The size of the cellulose column was 7.1 $cm^2 \times 52$ cm. Tris buffer (pH 8.5, 0.03 M) was used for the electrophoresis. The enzyme (240 mg of protein, volume = 5 ml) was applied to the top of the column. It was displaced 10 cm downward on the column by buffer. A current of 30 mA at a voltage of 5.5 V/cm was applied for 20 h. Ice water was circulated through the cooling jacket of the column during the electrophoresis.

The proteins were eluted from the column with buffer. Fifteen minute fractions (5—7 ml) were collected and analyzed for protein (light absorption at 280 mμ) and enzyme activity (Fig. 3). The enzyme started to appear after 160 ml. The first 39 ml containing enzyme were discarded, since the specific activity was lower than in the following fractions. Between 200 and 250 ml the specific activity of the protein was constant. These fractions were pooled and the volume of the solution concentrated by ultra filtration. During this process the filtration sac was suspended in 0.01 M tris buffer, pH 7.4, to avoid precipitation of salts on the outside of the membrane *.

A summary of the purification procedure is given in Table 1. The specific activities of an extract of whole liver acetone powder and of a 0° extract of

* The total recovery of enzyme and protein after zone electrophoresis was always low and never exceeded 60 %, probably because of adsorption of proteins to the ethanolyzed cellulose.

I am much indebted to Dr. J. Porath and Mr. J. Glomset for advice in connection with the zone electrophoresis prior to publication of Dr. Porath's paper [15].

Table 1. Purification of ornithine carbamyl transferase from rat liver.

	mg protein	Enzyme units × 10^6	Spec. activ.	Yield %
52° extract, liver resid. powder	31 500	3.44	110	100
First ammonium sulfate precip.	11 200	2.68	240	77
Alcohol fractionation	2 600	1.89	730	55
Second ammonium sulfate precip.	700	1.10	1 570	32
DEAE-cellulose treatment	310	0.67	2 160	19
Zone electrophoresis	68	0.21	3 050	6
0° extract, liver resid. powder			32	
0° extract, whole liver powder			11	

acetone powder from liver residue are also included in the table for comparison.

Properties of the enzyme. The concentrated enzyme solution (1—3 % protein) had a slightly yellowish colour. It was kept at 0° and lost little activity over a period of 4 months. After freezing in buffer solution some precipitation and denaturation sometimes occurred.

Because of the scarcity of the material no free electrophoresis was carried out. On paper electrophoresis between pH 5.0 and 8.5 the protein migrated as a single component. The isoelectric point was around 8.1. On ultra centrifugation * (protein concentration 0.4 % in 1 % NaCl and 0.01 M tris buffer, pH 7.4) two peaks were observed. The main component had a sedimentation constant ($s_{w,20}$) of 5.5 *S*, while a minor component amounting to *ca.* 5—8 % of the main peak had a sedimentation constant of *ca.* 8 *S*.

Properties of the transferase reaction

The *stoichiometry* of reaction (1) is demonstrated in Table 2. The slightly larger formation of P_i than citrulline is fully explained by the non-enzymic decomposition of CAP during the incubation.

Isolation of L*-citrulline.* In a large scale experiment 1 600 μmoles of L-ornithine, 2 000 μmoles of CAP and 5 000 units of transferase were incubated for 15 min at 37°. The solution was then put in a boiling water bath for 1 min

Table 2. Stoichiometry of transferase reaction.

	L-Ornithine	CAP	Citrulline	Phosphate
		μmoles		
Initial	40.0	40.7	0	2.1
Final	21.2	20.5	18.6	22.9
Δ	18.8	20.2	18.6	20.8

Incubation for 5 min at 37° with 60 units of transferase, vol. 1 ml.

Ornithine was determined by ion exchange chromatography. CAP was determined by phosphate analysis [12] (phosphate after 5 min at 100° minus P_i).

* I am indebted to civ.ing. A. Ehrenberg for performing the ultra centrifugation.

and thereafter poured through a Dowex-2-formate column (diam. 0.9, length 15 cm). The column was washed with 20 ml of water and the washing evaporated to a small volume *in vacuo*. $CuCl_2$ (800 μmoles) was added and the solution allowed to crystallize in the refrigerator overnight.

The crystals were filtered by suction and washed carefully with cold water. They were then suspended in 20 ml of boiling water and decomposed with H_2S for 2 h. The CuS was removed by filtration and the solution evaporated to dryness *in vacuo*. The residue was crystallized twice from 95 % alcohol. The product (145 mg) melted with decomposition at 232—235° (uncorr.). A mixed melting point with authentic citrulline gave the same value. It showed $[\alpha]_D^{25} = +24°$ in 6 N HCl as compared to a value of 26° for authentic L-citrulline.

Dependence on substrate concentration. The Lineweaver-Burk plots in Fig. 4 demonstrate the dependence of the reaction rate on the concentrations of CAP and ornithine, respectively. No inhibition was observed at substrate concentrations up to 0.05 M. When DL-ornithine was used but the substrate concentration was calculated only from the amount of L-ornithine present (triangles in Fig. 2) identical results were obtained as with pure L-ornithine. This demonstrates that (a) only the L-isomer was participating in the reaction, (b) D-citrulline did not influence the reaction rate when present in equimolar concentration with the L-isomer.

From the two curves Michaelis constants of 1.4×10^{-3} and 4.1×10^{-4} can be calculated for L-ornithine and CAP, respectively.

The *pH optimum* (7.3) of the reaction is demonstrated by Fig. 5.

Specificity of the reaction. The following amino acids (60 μmoles of L-isomer, 120 μmoles of DL-isomer) were incubated with 12 μmoles of CAP, 200 μmoles of tris buffer, pH 7.4, and 90 units of transferase: L-α-alanine, β-alanine, DL-α-aminoadipic acid, L-arginine, L-asparagine, L-aspartic acid, L-citrulline, L-cysteic acid, L-glutamic acid, L-glutamine, glycine, L-histidine, DL-homoserine, L-hydroxylysine, L-*iso*leucine, L-leucine, L-lysine, L-methionine, L-norleucine, L-phenylalanine, L-serine, L-threonine, L-tryptophane, L-tyrosine and L-valine.

None of these substances had the ability to bring about the enzymic dephosphorylation of CAP *.

Inhibition of transferase activity. The effect of several different enzyme inhibitors is demonstrated in Table 3. The inhibition by phosphate and arsenate is explained by the fact that phosphate is one of the products of the reaction. Inhibition was also observed with the SH-inhibitors iodosobenzoate and *p*-chloromercuribenzoate and could be partially reversed by glutathione. Of interest was also the observation that both substrates had the capacity to protect the enzyme to some degree from inhibition. Thus the inhibition was higher if CMBA (10^{-7} M) was added to the buffered enzyme solution before either of the two substrates than if one substrate was added before the inhibitor.

* J. M. Lowenstein and P. P. Cohen (*J. Biol. Chem.* **220** (1956) 227) have found that a low speed supernatant solution of rat liver was able to form a small amount of homocitrulline from lysine + CAP. Our experimental conditions would not allow a demonstration of enzyme activity with lysine which amounts to less than 0.5 % of the activity with ornithine.

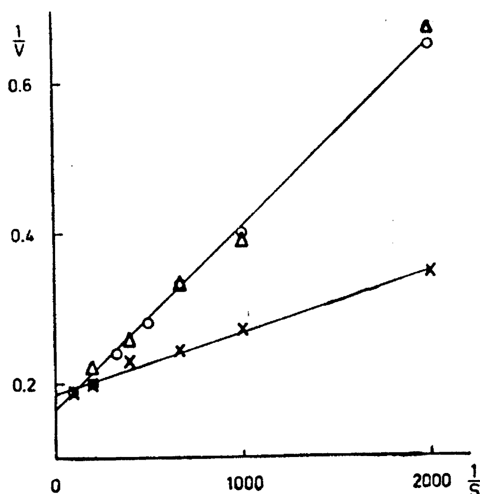

Fig. 4. Dependence of transferase action on CAP (×), L-ornithine (O) and DL-ornithine (△). In the last case the substrate concentration was calculated from the amount L-ornithine in the DL-isomer. Standard conditions except for substance under investigation. Citrulline determination.

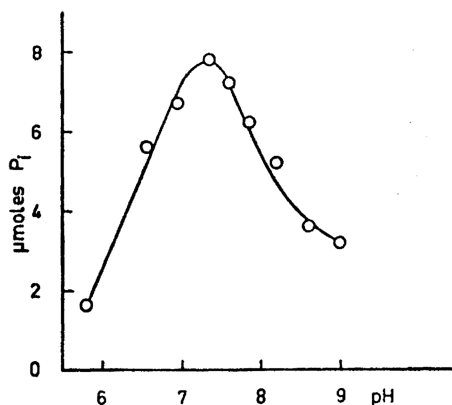

Fig. 5. pH curve of transferase action. Standard conditions but with tris and imidazol buffers at pH values indicated. Phosphate determination.

This demonstration is similar to results obtained earlier with aspartate carbamyl transferase. In this case the presence of both substrates was necessary for protection from CMBA.

A tentative interpretation for both transferases is that SH-groups are involved during the enzyme reaction and that both substrates are bound to these groups during the reaction.

Reversibility and equilibrium of transferase reaction. The equilibrium of the reaction greatly favoured citrulline synthesis but reversibility could be readily demonstrated and the equilibrium position measured from the back reaction with the aid of isotopic compounds.

The qualitative formation of radioactive CAP from $^{32}P_i$ and L-citrulline in the presence of transferase could be demonstrated by paper chromatography and ion exchange chromatography [5].

The quantitative measurement of the amount of CAP formed under different experimental conditions was carried out by using citrulline-carbamyl-^{14}C as substrate. Radioactive CAP was formed only in the presence of both enzyme and phosphate. CAP was decomposed with acid and the radioactive CO_2 formed was measured as described in the experimental part for the back reaction. Time curves of CAP formation with different amounts of substrates and large amounts of enzyme are demonstrated in Fig. 6.

All curves show at first a very rapid formation of CAP followed by a much slower linear increase. The curves are interpreted in the following way: The

Table 3. Inhibition of transferase reaction.

	Conc. of inhibitor (M)	% inhibition
Phosphate, pH 7.4	0.1	51
Arsenate, pH 7.4	0.1	12
EDTA	0.01	0
NaCN	0.01	0
NaCl	1	20
$(NH_4)_2SO_4$	1	50
Semicarbazide	0.01.	0
Phenylhydrazine	0.01	0
Arsenite	0.01	0
Iodoacetate	0.01	0
Iodosobenzoate	0.001	46
CMBA	5×10^{-7}	82
CMBA + glutathione (10^{-4} M)	5×10^{-7}	45
CMBA	10^{-7}	56
CAP + CMBA	10^{-7}	15
Ornithine + CMBA	10^{-7}	15
CAP + ornithine + CMBA	10^{-7}	13

Incubation under standard conditions with 5 units of transferase. In the last three experiments the indicated substrates were added to the enzyme before the inhibitor. In all other experiments the inhibitor was first added to the buffered enzyme solution (0°) and CAP + ornithine was added afterwards in a small volume.

rapid phase represents the attainment of the equilibrium. The formed CAP undergoes, however, a slow non-enzymic decomposition and disappears from the reaction. This in turn pulls the reaction towards more formation of CAP which results in the second slow phase of the curve. By extrapolation to zero time a correction can be obtained for this non-enzymic decomposition.

The equilibrium constant

$$K_{eq} = \frac{[\text{citrulline}]\ [\text{phosphate}]}{[\text{ornithine}]\ [\text{CAP}]}$$

was calculated from the three experiments of Fig. 6 and values of 89 000, 103 000 and 114 000 were obtained, giving an average value of 10.2×10^4. The reasonable conformity between the three values is good support for the correctness of the applied method.

Identity of citrulline phosphorylase and ornithine carbamyl transferase. Krebs *et al.*[8] described an enzyme from mammalian liver which formed CO_2, NH_3 and ornithine from citrulline in the presence of phosphate or arsenate. These authors could not demonstrate the formation of a phosphorylated intermediate. It seemed nevertheless rather probable that their enzyme was identical with ornithine carbamyl transferase and that their inability to demonstrate the formation of carbamyl phosphate was dependent on the equilibrium position of the reaction and on the fact that carbamyl phosphate at that time had not been described in the literature.

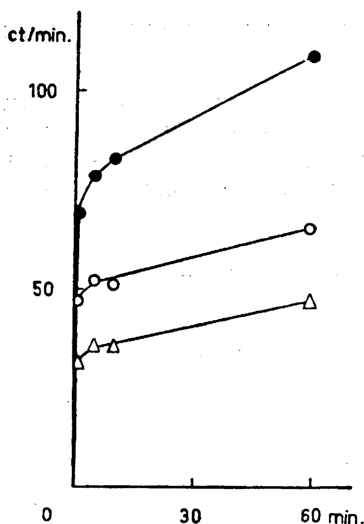

Fig. 6. Formation of ¹⁴CAP from citrulline-carbamyl-¹⁴C (see text). Experimental conditions (for one point): 40 units of transferase, vol. 1 ml, temperature 37° in all experiments. *Upper curve:* 94 μmoles of phosphate (pH 7.4), 28 μmoles of citrulline. *Middle curve:* 47 μmoles of phosphate, 28 μmoles of citrulline. *Lower curve:* 47 μmoles of phosphate, 14 μmoles of citrulline.

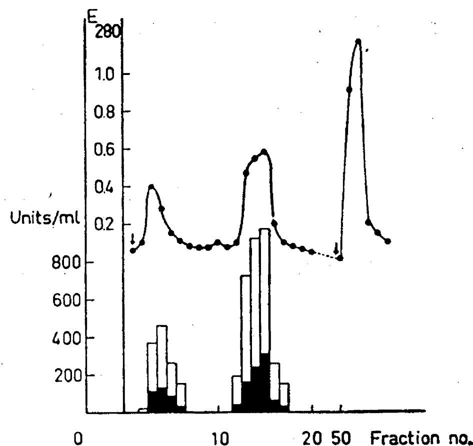

Fig. 7. Identity of citrulline phosphorylase with ornithine carbamyl transferase. 20 mg of transferase (specific activity = 1 500) was adsorbed to a column (0.61 cm² × 14 cm) of DEAE cellulose which had been washed with 0.02 M phosphate buffer, pH 7.4. Elution (4 ml fractions / h) was started with 0.01 M phosphate, pH 7.2, at the first arrow, changed to M KH_2PO_4 at the second arrow. Each fraction was analyzed for protein (E_{280}, continuous curve) transferase activity (upper stepped curve) and arsenolysis of citrulline (shaded stepped curve).

The arsenolysis of citrulline could be readily demonstrated with the pure transferase preparation. It was also found that the ratio between the rate of arsenolysis of citrulline and the rate of citrulline formation from ornithine and CAP was the same in a crude liver extract and with the enzyme after final purification. Further proof was obtained when the enzyme was chromatographed on a column of DEAE cellulose after the second ammonium sulfate precipitation step. In each chromatographic fraction the amount of protein, citrulline formation from ornithine, and arsenolysis of citrulline was determined (Fig. 7). It is evident that the rates of the two enzyme reactions follow each other closely in all fractions.

It should be mentioned that the two peaks with enzyme activity in Fig. 7 do not represent two transferases. The first peak merely represents a break through of non adsorbed protein. The total amount of protein and of enzyme in this peak depended on the load of the column.

Coupling of ornithine carbamyl transferase with carbamyl transferase

In previous investigations we were interested in the formation of carbamyl aspartate from citrulline [7]. We found that CAP could be formed from citrulline and ATP in the presence of acetyl glutamate (reaction 2 below). CA-aspartate could then be formed from CAP + aspartate through the action of aspartate carbamyl transferase (3):

$$\text{Citrulline} + \text{ATP} \xrightarrow[\text{glutamate}]{\text{acetyl}} \text{CAP} + \text{ornithine} + (\text{ADP}) \quad (2)$$

$$\text{CAP} + \text{aspartate} \longrightarrow \text{CA-aspartate} + \text{phosphate} \quad (3)$$

The equilibrium of reaction (3) is very favourable for CA-aspartate formation [5]. This together with the formation of CAP by the reversal of the citrulline forming reaction as demonstrated above made it very likely that an alternative formation of CA-aspartate from citrulline might take place through the participation of the two carbamyl transferases:

$$\text{Citrulline} + P_i \longrightarrow \text{CAP} + \text{ornithine} \quad (4)$$
$$\text{CAP} + \text{aspartate} \longrightarrow \text{CA-aspartate} + P_i \quad (3)$$

To test this possibility the following experiment was carried out: Two μmoles of citrulline-carbamyl-^{14}C (130 000 ct/min/μmole) + 100 μmoles of L-aspartate + 500 μmoles of phosphate, pH 7.4, were incubated with 800 units of ornithine carbamyl transferase and 250 units of aspartate carbamyl transferase for 15 min at 37°. The reaction was stopped by the addition of 0.2 ml of 4.4 M PCA and, after neutralization with KOH, CA-aspartate (0.52 μmoles calc. from total ^{14}C) was obtained by ion exchange chromatography *.

In a corresponding experiment with ^{14}CA-aspartate + ornithine + phosphate only trace amounts of radioactivity in the citrulline fractions could be demonstrated **.

It is thus clear that two enzymic ways exist for the transfer of the carbamyl group of citrulline to aspartate. Both involve the intermediate formation of CAP and the formation of CA-aspartate from CAP + aspartate. The relative importance of the two pathways is dependent on the availability of ATP and phosphate in the cell. Both reactions are strongly inhibited by

* The fractions containing CA-aspartate were pooled and evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml of hot water and 100 μmoles of carrier non-labeled CA-aspartate was added. CA-aspartate was recrystallized and its specific activity determined. From this value and the specific activity in the citrulline-carbamyl-^{14}C the amount of CA-aspartate formed during the enzyme experiment could be calculated by the isotope dilution method (*cf.* Ref[3]). A value of 0.46 μmoles was obtained.

** It has not been possible to determine the equilibrium constant for aspartate carbamyl transferase, since only very small amounts of $^{14}CO_2$ were formed from ^{14}CA-aspartate + phosphate. The absence of inhibition by phosphate or arsenate [5] and the fact that practically no citrulline could be obtained from ^{14}CA-aspartate in the presence of phosphate by the combined action of the two carbamyl transferases indicate that the equilibrium position is still more favourable for the synthesis of the carbamylamino acid than in the case of ornithine carbamyl transferase.

Table 4. Isotope exchange experiments with $^{32}P_i$.

	Reisolated P_i		Reisolated CAP	
	μmoles	ct/min/μmole	μmoles	ct/min/μmole
Complete	61.0	0.62×10^6	9.8	3.61×10^3
No ornithine	44.7	0.90×10^6	21.3	1.94×10^3
No enzyme	45.8	0.89×10^6	20.7	1.89×10^3
No enzyme and ornithine	44.7	0.98×10^6	20.5	1.78×10^3

Complete experiment: 40 μmoles of CAP and $^{32}P_i$ (1.02×10^6 ct/μmole), 100 μmoles of DL-ornithine and 15 units of enzyme. Incubation 15 min at 37°, vol. 1 ml.

ornithine and are of minor significance as compared to the *de novo* formation of the carbamyl group of CAP from CO_2, NH_3 and ATP in the presence of acetyl glutamate [1].

We have also tested the ability of the purified ornithine carbamyl transferase to form CAP from citrulline + ATP in the presence of acetylglutamate and absence of phosphate [7] (reaction (2) above). No such activity could be demonstrated.

The mechanism of the reaction

Enzymic citrulline formation might be imagined to involve the intermediate formation of a carbamyl enzyme:

$$\text{CAP} + \text{Enzyme} \rightleftharpoons \text{CA-Enzyme} + P_i \quad (5)$$

$$\text{CA-Enzyme} + \text{L-Ornithine} \rightleftharpoons \text{L-Citrulline} + \text{Enzyme} \quad (6)$$

This possibility was tested by isotope exchange experiments. The reversible reaction (5) was excluded by the absence of enzymic exchange of isotope between $^{32}P_i$ and CAP (Table 4). In the presence of ornithine, however, such an exchange did take place. This again demonstrates the reversibility of the over all reaction (1).

When ornithine-^{14}C was incubated together with nonlabeled citrulline and enzyme no enzymic isotope exchange took place in the absence of phosphate

Table 5. Isotope exchange experiment with ornithine-^{14}C.

	^{14}C (ct/min) in	
	Citrulline	Ornithine
Phosphate buffer	6 000	42 000
Tris buffer	0	40 500

Experimental conditions: 2 μmoles of DL-ornithine-2-^{14}C (6.1×10^5 ct/μmole), 1 μmole of L-citrulline, 100 μmoles of phosphate buffer or 5 μmoles of tris buffer, pH 7.4, 80 units of enzyme. Vol. 0.14 ml, incubation 15 min at 37°.

Inactivation with 0.02 ml 4.4 M PCA. After centrifugation aliquots were directly subjected to paper electrophoresis (2 h, 5 V/cm) in 0.1 M ammonium acetate buffer, pH 5.0 (see experimental part). The areas corresponding to citrulline and orn thine were eluted with 10 ml of water and counted.

(Table 5) while one eighth of the isotope was transferred from ornithine to citrulline in the presence of phosphate.

As in the case of aspartate carbamyl transferase the isotope exchange experiments speak against the formation of a carbamylated enzyme. The mechanism for both carbamyl transferases seems to involve instead a single displacement [16] reaction during which both CAP and ornithine are attached to the enzyme. The carbon-oxygen bond of CAP is then broken simultaneously with the formation of the new carbon-nitrogen bond.

The participation of SH groups during the reaction is indicated from inhibition experiments for both carbamyl transferases. The partial protection from inhibition by both CAP and ornithine tentatively indicates that these substances are bound to SH groups of the enzyme during the displacement reaction.

REFERENCES

1. Grisolia, S. and Cohen, P. P. *J. Biol. Chem.* **198** (1952) 561.
2. Jones, M. E., Spector, L. and Lipmann, F. *J. Am. Chem. Soc.* **77** (1955) 819.
3. Reichard, P. *Acta Chem. Scand.* **8** (1954) 795.
4. Reichard, P. and Hanshoff, G. *Acta Chem. Scand.* **9** (1955) 519.
5. Reichard, P. and Hanshoff, G. *Acta Chem. Scand.* **10** (1956) 548.
6. Schulman, M. P. and Badger, S. J. *Federation Proc.* **13** (1954) 292.
7. Smith, L. H. Jr. and Reichard, P. *Acta Chem. Scand.* **10** (1956) 1024.
8. Krebs, H. A., Eggleston, L. V. and Knivett, V. A. *Biochem. J. London* **59** (1955) 185.
9. Smith, L. H. Jr. *J. Am. Chem. Soc.* **77** (1955) 6691.
10. Nyc, J. F. and Mitchell, H. K. *J. Am. Chem. Soc.* **69** (1947) 1382.
11. Bücher, T. *Biochim. et Biophys Acta* **1** (1947) 292.
12. Lowry, O. H. and Lopez, J. A. *J. Biol. Chem.* **162** (1946) 421.
13. Koritz, S. B. and Cohen, P. P. *J. Biol. Chem.* **209** (1954) 145.
14. Peterson, E. A. and Sober, H. A. *J. Am. Chem. Soc.* **78** (1956) 751.
15. Porath, J. *Biochim. et Biophys Acta* **22** (1956) 151.
16. Koshland, D. E. Jr. in Mc Elroy, W. D. and Glass, B. *The Mechanism of Enzyme Action,* John Hopkins Press, Baltimore 1954, p. 608.

Received November 26, 1956.