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# CONTROL OF PYRIMIDINE BIOSYNTHESIS IN MAMMALIAN TISSUES

## II. GLUTAMINE-UTILIZING CARBAMOYL PHOSPHATE SYNTHETASE OF VARIOUS EXPERIMENTAL TUMORS: DISTRIBUTION, PURIFICATION AND CHARACTERIZATION

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SUMMARY

1. Yoshida ascites hepatomas, AH 13, AH 130, AH 601 and AH 7974, as well as Walker carcinosarcoma and Ehrlich ascites tumor, were investigated for the ability to synthesize carbamoyl-*P*. Supernatants of homogenates of all the experimental tumors were found to contain activities to synthesize carbamoyl-*P*.

2. The enzymes were labile in aqueous buffer solutions, but the inactivation was retarded in the presence of glycerol. The enzymes were active in the absence of *N*-acetyl-L-glutamate, and its addition did not affect the activities, indicating that these enzymes are distinct from the ammonia- and acetylglutamate-dependent carbamoyl-*P* synthetase (EC 2.7.2.5). The latter activity was also not detected in the particulate fractions of the tumors.

3. The levels of enzyme activities detected are greater than the assumed values of demands for carbamoyl-*P* as precursor of uracil in the tumor cells.

4. The enzymes of AH 130, AH 13 and Ehrlich ascites tumor were partially purified from the soluble supernatants in the presence of glycerol, dimethylsulfoxide and dithiothreitol as enzyme stabilizers. The AH 130 enzyme was purified about 750-fold.

5. The partially purified AH 130 enzyme utilized either L-glutamine or ammonia as carbamoyl nitrogen donor. The activity with glutamine was not separated from the activity with ammonia in the purification process, and Arrhenius plots of the two reactions indicated participation of one enzyme protein in these reactions. Evidence for glutamine as direct nitrogen donor was obtained by the stoichiometric formation

Abbreviation: HEPES, *N*-2-hydroxyethyl-piperazine-*N*'-ethanesulfonic acid. Carbamoyl-*P*, carbamoyl phosphate.

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of glutamate from glutamine that accompanied the synthesis of carbamoyl-*P*. The apparent $K_m$ values for L-glutamine and ammonia were $1.4 \cdot 10^{-5}$ and $3.6 \cdot 10^{-3}$ M, respectively, at pH 7.8. Properties of the partially purified enzymes of AH 13 and Ehrlich ascites tumor are essentially the same as those of the AH 130 enzyme.

6. $\gamma_2$-Globulin obtained from rabbit antiserum against the ammonia- and acetylglutamate-dependent carbamoyl-*P* synthetase of frog liver, which can cross-react with the same type of enzyme from rat liver, did not inhibit glutamine-utilizing enzyme of AH 130, indicating that this enzyme protein is immunologically distinct from the ammonia-dependent type of enzyme.

7. The enzymes of AH 130, AH 13 and Ehrlich ascites tumor were subject to the specific "feedback" inhibition by UTP among nine nucleotides so far tested. The feedback inhibition as well as low intracellular levels of the enzymes suggest that the glutamine-utilizing carbamoyl-*P* synthetase may play a key role in control of the orotic acid pathway in the tumor cells.

8. Another implication of the findings described above is that hepatomas AH 13 and AH 130, considered to be derived from parenchymal cells of liver, contained only the glutamine-utilizing carbamoyl-*P* synthetase but not the ammonia-dependent enzyme (liver type). The significance of the fact was discussed.

## INTRODUCTION

Carbamoyl-*P* is a common initial intermediate of the pyrimidine and arginine biosynthesis. Two types of carbamoyl-*P* synthetase have been described in normal mammalian tissues. One is ammonia- and acetylglutamate-dependent carbamoyl-*P* synthetase (EC 2.7.2.5) and is known to be localized principally in the liver[1]. The other is glutamine-utilizing carbamoyl-*P* synthetase and has been only recently demonstrated in many nonhepatic tissues of the mouse[2,3] and the liver of fetal[4] and adult[5] rat. The glutamine-utilizing enzyme is considered to be responsible for the synthesis of carbamoyl-*P* for pyrimidine biosynthesis, and it is suggested that the enzyme plays a key role in control of the biosynthesis[2].

On the other hand, information on the initial steps of pyrimidine biosynthesis in cancer cells has been so far limited. ONO *et al.*[6] described the presence of ammonia-dependent carbamoyl-*P* synthetase in minimal deviation hepatomas but failed to detect the same activity in other cancer cells. Recently HAGER AND JONES[7] demonstrated the glutamine-dependent synthesis of carbamoyl-*P* by the extracts of Ehrlich ascites tumor, but the detailed characterization of the activity was not reported. The present paper deals with the detection of glutamine-utilizing carbamoyl-*P* synthetase in four Yoshida ascites hepatomas, Walker carcinosarcoma and Ehrlich ascites tumor, and with its partial purification and characterization.

## EXPERIMENTAL PROCEDURE

### *Materials*

The chemicals were obtained as previously described[3]. Ascites hepatomas AH 13, AH 130, AH 601 and AH 7974 (about $1 \cdot 10^6$ cells per rat) were inoculated and grown for 3, 4, 7 and 8 days, respectively, in male rats of Donryu strain, 2–3 months old.

Ehrlich ascites tumor (about $1 \cdot 10^6$ cells per mouse) was grown for 6 days in male mice of dd strain, 1.5–2 months old. The ascites cells and fluid were aspirated with a syringe, followed by washing the abdominal cavity twice with 5 ml of cold 0.15 M KCl containing 0.005 M potassium phosphate buffer (pH 7.4). The cells were collected by centrifugation at 3000 rev./min for 3 min. The packed cells were used as source of enzyme. Walker carcinosarcoma was grown for 9 days in male rats of Donryu strain, 2–3 months old. Rabbit antibody to the ammonia-dependent carbamoyl-*P* synthetase of the frog liver was prepared as described previously[5]. The ammonia-dependent carbamoyl-*P* synthetase of the rat liver was prepared as described by MARSHALL AND COHEN[8] and stored at 4° as a suspension in 3 M $(NH_4)_2SO_4$ solution containing 0.01 M Tris·HCl buffer (pH 7.5). Gel filtration on Sephadex G-25 was used to remove $(NH_4)_2SO_4$. Ornithine transcarbamoylase (carbamoyl phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) was prepared from bovine liver as described[3]. The specific activity of the preparation obtained was 3660 units/mg of protein.

*Methods*

Radioactivity was determined in a Beckman liquid scintillation spectrometer with 0.5-ml aqueous samples and 10 ml of a scintillator fluid which consisted of 10 vol. of toluene containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-2-(5-phenyloxazolyl)-benzene and 7 vol. of ethylene glycol monoethyl ether[9]. Other analytical methods used are essentially the same as described previously[3].

Glutamine-utilizing carbamoyl-*P* synthetase was assayed as described[3], with slight modifications in the composition of the assay mixture and incubation time. The reaction mixture (pH 7.8) used for assay of the AH 130 and AH 13 enzymes contained 50 $\mu$moles of the potassium salt of *N*-2-hydroxyethyl-piperazine-*N'*-ethanesulfonic acid (HEPES), 15 $\mu$moles of $KH^{14}CO_3$ (generally, $1.5 \cdot 10^7$ counts/min), 1 $\mu$mole of L-glutamine or 10 $\mu$moles of $NH_4Cl$, 10 $\mu$moles of ATP, 15 $\mu$moles of $MgCl_2$, 0.5 $\mu$mole of L-ornithine, 0.1 $\mu$mole of L-citrulline, 10 units of ornithine transcarbamoylase, 38 $\mu$l of glycerol, 56 $\mu$l of dimethylsulfoxide and enzyme preparation in a final volume of 0.75 ml. Final concentrations of glycerol and dimethylsulfoxide are 5% (v/v) and 7.5% (v/v), respectively. Concentrations of glycerol and dimethylsulfoxide are expressed in terms of per cent of volume in volume in this paper except where indicated. The pH of the reaction mixture used for assay of the Ehrlich ascites tumor enzyme was 7.3 and the final concentrations of glycerol and dimethylsulfoxide were 4.8 and 3.3%, respectively. Incubation was for 10 min at 37°. In studies of distribution of enzyme in tumors, a slightly different system was used (see the legend to Table I). For the determination of the enzyme activities of crude preparations, *i.e.* sonic extracts and $(NH_4)_2SO_4$ precipitates, the reaction product was purified on Dowex 50, prior to counting, essentially as described[3]. In elution of [$^{14}$C]citrulline from the column, 2 M ammonium hydroxyde was used in place of 10% pyridine. An unit of carbamoyl-*P* synthetase is defined as that amount of enzyme which produces 1 nmole of carbamoyl-*P* per min at 37° when L-glutamine is the nitrogen donor.

## RESULTS

*Distribution of carbamoyl-P synthetase in various experimental tumors*

In Table I are shown the results of determinations of carbamoyl-*P* synthetase

in the ascites hepatomas AH 13, AH 130, AH 601, AH 7974 and Walker carcinosarcoma. Activities were determined both in the presence and absence of acetylglutamate. Ammonia was used as amino group donor. Definite activities were detected in the soluble supernatants of all the tumors examined. As the cellular disruption was incomplete under the conditions employed, the activity of the supernatant obtained does not necessarily represent the intracellular levels of the carbamoyl-*P* synthesizing activity. Thus the results in Table I, expressed as activities (μmoles of carbamoyl-*P*

TABLE I

DISTRIBUTION OF CARBAMOYL-*P* SYNTHETASE IN EXPERIMENTAL TUMORS AND REQUIREMENT OF ACTIVITIES FOR *N*-ACETYL-L-GLUTAMATE

Fresh tumor cells or tissues were homogenized 5–10 min with 4 vol. of 0.25 M sucrose containing 1 mM ATP, 2 mM $MgSO_4$, 3 mM mercaptoethanol and 50 mM potassium phosphate (pH 7.5), followed by centrifugation at 20000 × *g* for 10 min at 0°. Glycerol was added to the supernatant so as to be 30% (w/v). The paticulate fractions were extracted twice each time with a volume of 0.1% cetyltrimethylammonium bromide equal to 2 times the original weight of the tissue. The supernatant and the extracts were assayed for enzyme activity. The reaction mixture (pH 7.3) contained 18 μmoles of potassium HEPES, 6 μmoles of ATP, 9 μmoles of $MgCl_2$, 0.3 μmole of L-ornithine, 15 units of ornithine transcarbamoylase, 30 μmoles of KCl, 15 μmoles of $KH^{14}CO_3$ ($3.8 \cdot 10^6$ counts/min), 8.3% (w/v) glycerol, 20 μmoles of ammonium acetate and enzyme in a final volume of 0.6 ml. Where indicated, 3 μmoles of *N*-acetyl-L-glutamate were added. Incubation was for 15 min at 37°. $^{14}C$-Citrulline synthesized was purified on Dowex 50 prior to counting.

| *Tumor* | *Acetyl-glutamate in assay system (mM)* | *Enzyme activity** | | |
|---|---|---|---|---|
| | | *Supernatant* | | *Extract of particulate fraction (μmole of citrulline/g per h)* |
| | | *nmoles of citrulline/min per mg protein* | *μmoles of citrulline/g per h*** | |
| Yoshida ascites hepatoma | | | | |
| AH 13 | 0 | 1.16 | 4.89 | 0.03 |
| | 5 | 0.97 | 4.07 | 0 |
| AH 130 | 0 | 0.15 | 0.63 | 0.01 |
| | 5 | 0.16 | 0.67 | 0.01 |
| AH 7974 | 0 | 0.34 | 1.43 | 0.08 |
| | 5 | 0.30 | 1.26 | 0.07 |
| AH 601 | 0 | 0.30 | 1.26 | 0 |
| | 5 | 0.30 | 1.26 | 0 |
| Walker carcinosarcoma | 0 | 0.22 | 0.93 | 0 |
| | 5 | 0.20 | 0.84 | 0 |

* Enzyme was assayed with ammonia as nitrogen source.
** These values are calculated as described in the text.

formed per h) per g cells of tissue, are calculated from the activities (units) per mg protein, assuming that the protein content of the soluble fractions of the tumors is 70 mg/g cells or tissue. It is also shown that acetylglutamate did not affect the enzyme activities. To further confirm the negative results of acetylglutamate dependency, all the enzymes were fractionated with $(NH_4)_2SO_4$ (0–40%) and similar experiments were made on the partially purified preparations. There was also no dependency of the activities on acetylglutamate. Since the fractionation brought

about 2–4-fold purification of the enzymes and possibly could eliminate a major portion of endogenous acetylglutamate or related compounds, if present at all, it is obvious that these activities are distinguishable from the ammonia- and acetylglutamate-dependent carbamoyl-*P* synthetase (liver type).

Extracts of particulate fractions of the tumors were shown to be devoid of carbamoyl-*P*-synthesizing activity. These findings are consistent with previous results of other experimental cancer cells[6]. However, the data presented here indicate only that ammonia- and acetylglutamate-dependent carbamoyl-*P* synthetase was not present in the particulate fractions (as in the supernatants) and did not give definite answers as to the localization of the glutamine-utilizing enzyme, because the enzyme extraction with 0.1% cetyltrimethylammonium bromide, as used in these experiments, did not seem to permit the detection of the labile activity of the latter. A possibility of the presence of this activity in particulate fractions of various mammalian tissues has been previously suggested[3].

*Purification of enzyme from ascites hepatoma AH 130*

Carbamoyl-*P* synthetase of AH 130 was very labile in aqueous buffer solutions but was stable in the presence of glycerol, dimethylsulfoxide and dithiothreitol, as was the case for the mouse spleen carbamoyl-*P* synthetase[3]. Further stabilization of the AH 130 enzyme was obtained by the addition of substrates, L-glutamine *plus* $Mg^{2+}$–ATP, as described below. Fractionations with $(NH_4)_2SO_4$ and hydroxylapatite were performed by a slight modification of the method for spleen enzyme[3]; all steps were carried out at 0–4°. Buffer solutions containing dimethylsulfoxide were adjusted to the described pH values after the addition of the solvent[3].

*Centrifuged extract.* The packed cells (62.5 g wet weight) were suspended in 4 vol. (250 ml) of 30% glycerol solution (pH 7.3) containing 20 mM potassium phosphate buffer, 3 mM mercaptoethanol, 10 mM $MgCl_2$, 1 mM EDTA, 3 mM L-glutamine and 1 mM dithiothreitol. The cell suspension was subjected to sonic oscillation at 10 kcycles for 1 min and centrifuged at 100 000 × *g* for 30 min. Almost all the activity was recovered in the supernatant.

*$(NH_4)_2SO_4$ fractionation.* To the supernatant (258 ml) obtained in the previous step was added with stirring 0.6 vol. (154 ml) of a saturated $(NH_4)_2SO_4$ solution (pH 7.3) which contained 50 mM potassium phosphate and 1 mM EDTA. The suspension was stirred for additional 5 min and centrifuged at 10 000 × *g* for 10 min. The operation of this step was quickly performed to minimize loss of the activity.

*Chromatography on hydroxylapatite.* The precipitate obtained in the previous step was dissolved in 125 ml of 5% glycerol–30% dimethylsulfoxide solution (pH 7.3) which contained 20 mM Tris–HCl buffer, 3 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM dithiothreitol. Insoluble matter was removed by centrifugation at 10 000 × *g* for 10 min. The supernatant was placed on a hydroxylapatite column (62.5 ml) previously equilibrated with the 5% glycerol–30% dimethylsulfoxide solution described above. The enzyme was completely adsorbed and then eluted stepwise from the column each time with 125 ml of potassium phosphate buffer (pH 7.6) containing 5% glycerol, 30% dimethylsulfoxide, 3 mM mercaptoethanol, 1 mM EDTA and 1 mM dithiothreitol. About 50% of the enzyme activity applied to the column was obtained in the fractions eluted with phosphate concentrations of 0.075, 0.090 and 0.105 M. The three fractions were combined (375 ml) and concentrated to 23.8 ml with the

use of a small column of hydroxylapatite (11.9 ml), essentially as described for the spleen enzyme[3].

*Sucrose density gradients.* Sucrose gradients from 5 to 20% were prepared in tubes for the swinging bucket rotor SW-65 designed to fit the Model L4 Spinco centrifuge. The sucrose solution (pH 7.3) contained 5% glycerol, 30% dimethylsulfoxide, 20 mM Tris·HCl, 3 mM mercaptoethanol, 1 mM EDTA, 1 mM dithiothreitol, 3 mM ATP, 4.5 mM $MgCl_2$, 4 mM L-glutamine and 50 mM KCl. The eluate (0.6 ml) from the small column of hydroxylapatite in the previous step was layered on the sucrose gradient (4.6 ml) and centrifuged at 65 000 rev./min for 13 h. After the centrifugation, drops from a hole at the bottom of the tube were collected into 0.28-ml fractions. The enzyme activity was found to be separated from a major portion of protein and the enzyme activity formed a single peak. The fractions containing most of the activity were combined and concentrated as in the previous step, followed by dialysis for 3 h against 24 vol. of 2 mM potassium phosphate buffer (pH 7.0) containing 5% glycerol, 30% dimethylsulfoxide, 3 mM mercaptoethanol, 1 mM EDTA and 1 mM dithiothreitol. The addition of L-glutamine and $Mg^{2+}$ ATP as enzyme stabilizers to the sucrose gradients was indispensable to attain a maximal stabilization of enzyme as described below. The recovery of the enzyme activity in this step was about 40%.

TABLE II

PURIFICATION OF CARBAMOYL-*P* SYNTHETASE FROM ASCITES HEPATOMA AH 130, AH 13 AND EHRLICH ASCITES TUMOR

The experimental details are given in the text. The carbamoyl nitrogen donor was 1 μmole of L-glutamine. The starting materials were 62.5, 3.3 and 8.5 g of the packed cells of AH 130, AH 13 and Ehrlich ascites tumor, respectively.

| *Fraction* | *AH 130* | | | *AH 13* | | *Ehrlich ascites tumor* | |
|---|---|---|---|---|---|---|---|
| | *Vol. (ml)* | *Total units* | *Specific activity** | *Total units* | *Specific activity** | *Total units* | *Specific activity** |
| Sonicated | 290 | 1413 | 0.06 | 60 | 0.32 | 98 | 0.18 |
| Centrifuged | 258 | 1443 | 0.13 | 63 | 0.67 | 85 | 0.40 |
| $(NH_4)_2SO_4$ | 143 | 820 | 0.40 | 43 | 3.00 | 56 | 1.90 |
| Hydroxylapatite | 375 | 450 | 7.70 | 16 | 6.80 | 29 | 2.80 |
| Sucrose density gradients** | 9 | 24 | 47 | | | | |

* Units per mg of protein.

** Only a part of the enzyme preparation at the hydroxylapatite step was submitted to this procedure.

Table II gives a summary of the purification procedure. The enzyme at the final step was purified about 750-fold from the sonic extract. The enzyme preparations were stored on dry ice. Experiments with the AH 130 enzyme reported in this paper were carried out with the preparations at this step of purification except where indicated.

*Purification of enzymes from ascites hepatoma AH 13 and Ehrlich ascites tumor*

As shown in Table II, the enzymes of AH 13 and Ehrlich ascites tumor were

partially purified from soluble supernatants of the sonic extracts through the step of chromatography on hydroxylapatite. The AH 13 enzyme was purified about 21-fold and the enzyme of Ehrlich ascites tumor, 15-fold. The preparations were stored on dry ice.

*Identification of reaction product*

Radioactive carbamoyl-*P* to be formed by the enzyme reaction was converted *in situ* to L-citrulline in the presence of excess amounts of L-ornithine and ornithine transcarbamoylase. The radioactive product was identified as L-citrulline by its behavior on paper electrophoresis and by its constant specific radioactivity through the steps of repeated recrystallization with the authentic L-citrulline. When the enzyme at the step of hydroxylapatite or of sucrose density gradients was used, more than 90% of the radioactivity fixed during the reaction was found in L-citrulline either with L-glutamine or ammonia as carbamoyl nitrogen donor.

*Properties of the AH 130 enzyme*

*Stability of the enzyme.* In the absence of specific stabilizers the enzyme was so labile either at 0 or 25° that almost all activity was lost in 1 h. A marked stabilization was obtained in buffers containing 5% glycerol, 30% dimethylsulfoxide and 1 mM dithiothreitol as was the case with the spleen enzyme[3]. When frozen on dry ice in the presence of the stabilizers, 80% of the original enzyme activity remained after 3 months. When kept at 0°, about 15% of activity was lost in 20 h. However, the inactivation at 0° was nearly completely prevented by the further addition of L-glutamine and $Mg^{2+}$-ATP as seen in Fig. 1. L-Glutamine alone seemed to promote inactivation of the enzyme.

*Requirements for enzymatic synthesis of citrulline.* When ATP, $Mg^{2+}$, L-glutamine, L-ornithine, or ornithine transcarbamoylase was omitted, essentially no synthesis of citrulline occurred, as shown in Table III. Dependency of the reaction on ornithine transcarbamoylase indicates that the direct product of the reaction is carbamoyl-*P*.

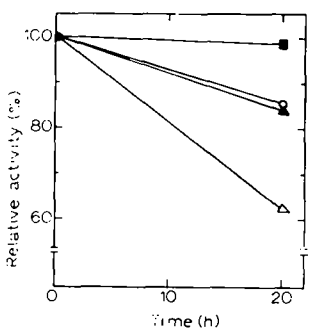



Fig. 1. Effect of substrates on stability of the enzyme. The enzyme preparation at the hydroxylapatite step was diluted to 3.8 units/ml and kept at 0°. The storage media (pH 7.6) contained as common ingredients 26 mM potassium phosphate, 5% glycerol, 30% dimethylsulfoxide, 1 mM dithiothreitol, 1 mM EDTA and 3 mM mercaptoethanol. Further additions were: none, (—○); 20 mM L-glutamine, (—△); 30 mM $MgCl_2$ and 20 mM ATP, ( -▲); and 20 mM L-glutamine, 30 mM $MgCl_2$ and 20 mM ATP, ( ■). The activity was determined on 0.02-ml aliquots, as described under *Methods*. This amount of enzyme could fix, at zero time, 767 counts/min of $H^{14}CO_3^-$ into citrulline. The carbamoyl nitrogen donor was 1 μmole of L-glutamine.

TABLE III

REQUIREMENTS FOR ENZYMATIC SYNTHESIS OF CITRULLINE BY AH 130 ENZYME

The assay conditions are described under *Methods*. For each experiment 0.1 unit of the enzyme was used. The carbamoyl nitrogen donor was 1 μmole of L-glutamine.

| *System* | *Citrulline formed (counts/min)* |
|---|---|
| Complete | 949 |
| − Enzyme | 0 |
| − ATP | 33 |
| − $Mg^{2+}$ | 1 |
| − L-Glutamine | 4 |
| − L-Ornithine | 35 |
| − Ornithine transcarbamoylase | 0 |

The enzyme exhibits a high degree of specificity as to energy source: citrulline synthesis did not take place when ATP was replaced by ITP, GTP, CTP or UTP. The requirement for $Mg^{2+}$ is relatively specific. $Zn^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Ca^{2+}$ and $Fe^{2+}$ at $1 \cdot 10^{-3}$ M were completely ineffective. $Mn^{2+}$ was only 20% as effective as $Mg^{2+}$.

*Specificity for nitrogen donors and effect of N-acetyl-L-glutamate.* Carbamoyl-*P* synthetase activity was observed either when L-glutamine or ammonia was used as carbamoyl nitrogen donor as shown in Table IV. Both of these as such do serve as direct carbamoyl nitrogen donor: the enzyme preparations were found to be free from glutamine synthetase activity and from glutaminase activity. When L-[$^{14}$C]-glutamine was incubated with the enzyme in the standard assay mixture *minus* $Mg^{2+}$ (Table V), practically no production of [$^{14}$C]glutamate was observed, and when ammonia was the nitrogen donor, the addition of glutamate failed to enhance the rate of carbamoyl-*P* synthesis. D-Glutamine, L-asparagine and D-asparagine neither served as nitrogen source nor inhibited the reaction with L-glutamine or ammonia as nitrogen donor. The activity obtained with a saturating level of L-glutamine (1.3 mM) was about 1.3-fold as active as that obtained with ammonia (13.3 mM) and the activities were not additive. *N*-Acetyl-L-glutamate did not enhance the rate of reactions either with L-glutamine or ammonia. The results indicate that the ammonia-

TABLE IV

NITROGEN DONORS AND EFFECT OF *N*-ACETYL-L-GLUTAMATE

For each experiment 0.08 unit of the AH 130 enzyme was used. The following were added to the standard assay mixture *minus* nitrogen source: 1 μmole of L-glutamine, 10 μmoles of $NH_4Cl$, 10 μmoles of *N*-acetyl-L-glutamate, or 10 μmoles of L-glutamate.

| *Addition* | *Citrulline formed (counts/min)* |
|---|---|
| None | 3 |
| L-Glutamine | 812 |
| L-Glutamine + *N*-acetyl-L-glutamate | 804 |
| $NH_4Cl$ | 618 |
| $NH_4Cl$ + *N*-acetyl-L-glutamate | 594 |
| $NH_4Cl$ + L-glutamate | 680 |
| $NH_4Cl$ + L-glutamine | 774 |

TABLE V

STOICHIOMETRY OF GLUTAMATE FORMATION IN CARBAMOYL-*P* SYNTHESIS

Determinations of glutamate and citrulline formed were carried out in separate tubes. L-[$^{14}$C]-glutamine was used for the assay of glutamate formation. In Expt. 1, the complete reaction mixture (0.38 ml) contained 25 $\mu$moles of potassium HEPES (pH 7.5), 9.5 mg of glycerol, 28 mg of dimethylsulfoxide, 5 $\mu$moles of ATP, 7.5 $\mu$moles of $MgCl_2$, 0.25 $\mu$mole of L-ornithine, 10 units of ornithine transcarbamoylase, 0.3 unit of enzyme and 21.1 $\mu$moles of $NaH^{14}CO_3$ (5770 counts/min per nmole) *plus* 5.1 nmoles of L-glutamine or 21.1 $\mu$moles of $NaHCO_3$ *plus* 5.1 nmoles of L-[$^{14}$C]-glutamine (16370 counts/min per nmole). In Expt. 2, 0.1 unit of enzyme and 5.0 nmoles of L-glutamine or L-[$^{14}$C]glutamine were used. Reactions were carried out at 37° for 15 min and stopped by adding 15 $\mu$moles of EDTA (0.02 ml). For analysis of [$^{14}$C]glutamate formed, 2.0 $\mu$moles each of L-glutamate and L-glutamine were added to 0.05-ml aliquots and the mixture was submitted to paper electrophoresis. The chromatograms were cut into strips and counted as described under *Methods*. The amounts of glutamine used and glutamate formed were calculated from the radioactivities of the corresponding spots. Citrulline formed was determined as described under *Methods*.

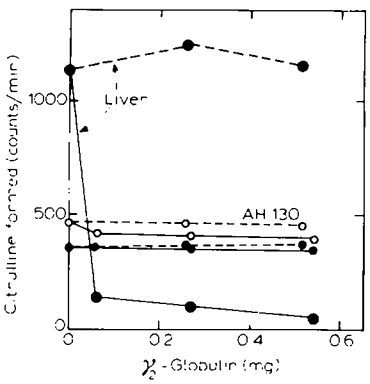
| *Expt.* | *System* | *Glutamine consumed (nmoles)* | *Glutamate formed (nmoles)* | *Citrulline formed (nmoles)* |
|---|---|---|---|---|
| 1 | Complete | 1.53 | 1.64 | 1.54 |
| | $-Mg^{2+}$ | 0.01 | 0.07 | 0.04 |
| 2 | Complete | 0.58 | 0.65 | 0.50 |
| | $-Mg^{2+}$ | 0.01 | 0.06 | 0 |

mediated reaction is not due to the coexistence of the ammonia-dependent carbamoyl-*P* synthetase (liver type).

*Stoichiometry of glutamate formation in carbamoyl-P synthesis.* Two sets of experiments were carried out and the results are shown in Table V. It is indicated that glutamate and citrulline were formed in an approximate molar ratio of 1:1. In the control tubes where $Mg^{2+}$ was omitted, only negligible formation of glutamate was observed.

*Immunological distinction of the AH 130 carbamoyl-P synthetase and the ammonia-dependent carbamoyl-P synthetase of the rat liver.* It was known that rabbit antibody to the frog liver carbamoyl-*P* synthetase can cross-react with the ammonia-dependent carbamoyl-*P* synthetase of the rat liver and inhibits its activity[8]. This antibody did not affect the activity of the glutamine-utilizing carbamoyl-*P* synthetase of the rat liver, as described previously[5]. The data given in Fig. 2 show that the same rabbit antibody ($\gamma_2$-globulin), which was shown to inhibit the ammonia-dependent enzyme from rat liver, did not inhibit the AH 130 enzyme either when L-glutamine or ammonia was the nitrogen donor. When the rabbit antibody was added to a mixture of the AH 130 enzyme and the ammonia-dependent enzyme of rat liver, only the glutamine-utilizing activity due to the former remained untitrated. Serum $\gamma_2$-globulin obtained from nonimmunized rabbits did not affect either AH 130 or the ammonia-dependent enzyme. The results indicated that the AH 130 enzyme is immunologically distinct from the ammonia-dependent enzyme of the rat liver.

*Effect of the concentrations of L-glutamine and ammonia on the rate of carbamoyl-P synthesis.* Effect of varying concentrations of L-glutamine and ammonia on the reaction rate was examined at pH 7.8, with $13.3 \cdot 10^{-3}$ M ATP, $20 \cdot 10^{-3}$ M $Mg^{2+}$, and $20 \cdot 10^{-3}$ $HCO_3^-$. Linear double reciprocal plots were obtained for both substrates, and



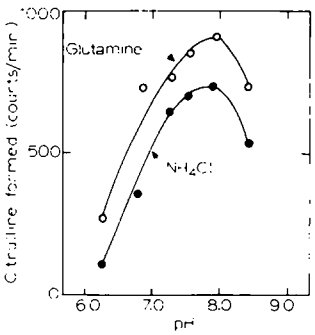



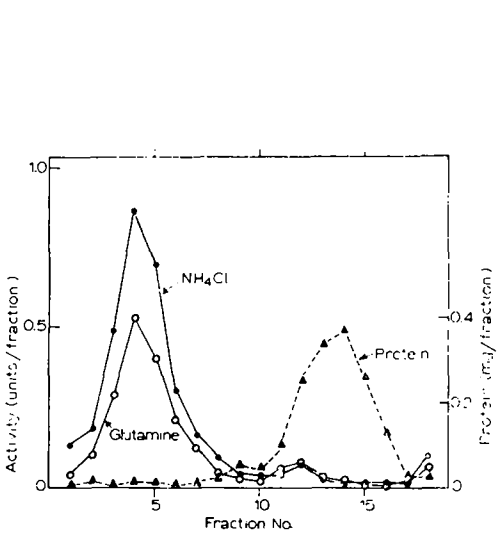

Fig. 2. Effects of the rabbit antibody against frog liver carbamoyl-*P* synthetase on the AH 130 carbamoyl-*P* synthetase and the ammonia-dependent carbamoyl-*P* synthetase of rat liver. Various amounts of $\gamma_2$-globulin were incubated for 5 min at 37° with the AH 130 enzyme (2.8 μg of protein) or with the ammonia-dependent liver enzyme (1.4 μg of protein) in 0.15 ml of 130 mM Tris·HCl buffer (pH 7.5) containing 1.7% glycerol, 10% dimethylsulfoxide, 33 mM KCl, 100 mM NaCl and 200 μg of bovine serum albumin. The tubes were then kept at 0° for 25 min. The whole mixture of each tube was assayed for carbamoyl-*P* synthetase activity either with 1.3 mM L-glutamine (○) or 13 mM $NH_4Cl$ (●) as nitrogen donor. $\gamma_2$-Globulin used was that from rabbit antisera against the frog liver carbamoyl-*P* synthetase (·····) or that from nonimmunized rabbit sera (— - —).

Fig. 3. Activity of carbamoyl-*P* synthetase as a function of pH. Potassium HEPES buffer was used for all determinations. Each tube contained 0.09 unit of enzyme. The carbamoyl nitrogen donor was 1 μmole of L-glutamine or 10 μmoles of $NH_4Cl$ as indicated. The incubation conditions are described under *Methods*.

the apparent $K_m$ values calculated are $1.4 \cdot 10^{-5}$ M and $3.6 \cdot 10^{-3}$ M for L-glutamine and ammonia, respectively. The pH optima of the two reactions lie between 7.7 and 8.0 as seen in Fig. 3. The glutamine-mediated activity is higher than the ammonia-mediated activity within the pH range tested.

*Ultracentrifugal analysis.* The enzyme preparations at all steps of purity were active either with L-glutamine or ammonia, and the activities were not separated in the process of the enzyme purification. The ratio of the two activities remained essentially constant. However, when the enzyme at the hydroxylapatite step was submitted to sucrose density gradient centrifugation in the absence of glutamine as enzyme stabilizer, a preferential decrease in the glutamine-mediated activity was observed. The recovery of the activity was only about 15%, whereas the recovery of the ammonia-mediated activity was about 40% (Fig. 4). The activity ratio decreased from 1.4 to 0.6. Each activity formed a single peak and both peaks were coincident with each other. When glutamine was included in the sucrose density gradient as in the usual procedure, there was no differential decrease of the glutamine-mediated activity. As evidence indicates that activities with glutamine and ammonia are catalyzed by a single enzyme protein (see above and below), the results could best be explained by assuming that only the binding site of the enzyme for glutamine was modified or damaged during ultracentrifugation while that for ammonia remained intact.

*Arrhenius plots for the reaction.* The two activities with glutamine and ammonia were determined at different temperatures and Arrhenius plots were made (Fig. 5). Either when L-glutamine or ammonia was used as nitrogen donor, the slope of



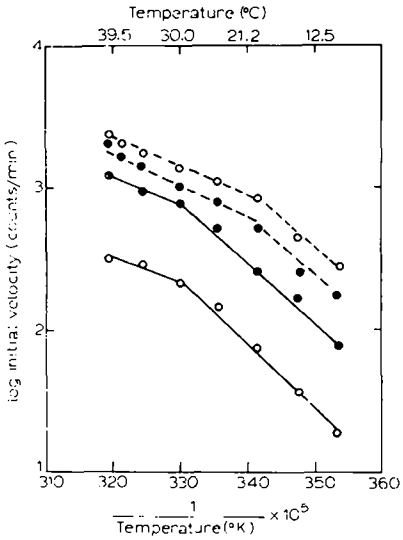




Fig. 4. Ultracentrifugal pattern of the AH 130 carbamoyl-*P* synthetase in the absence of L-glutamine. Enzyme at the hydroxylapatite step was submitted to ultracentrifugal analysis in a sucrose density gradient, in the same manner as described for enzyme purification except that L-glutamine was omitted from the gradient. The enzyme activities were determined either with 1 μmole of L-glutamine or 10 μmoles of $NH_4Cl$ as nitrogen donor. Sedimentation direction is from right to left.

Fig. 5. Effect of temperature on the AH 130 carbamoyl-*P* synthetase activity. Experiments were carried out with the enzyme (25 μg of protein) at the hydroxylapatite step (——) and also with the "modified" preparation (1 μg of protein) obtained by ultracentrifugation in the absence of glutamine (- - -). The standard reaction mixtures without enzyme were kept at the indicated temperatures for 5 min and then the reaction was started by the addition of enzyme. After 10 min incubation the reaction was terminated by the addition of 30 μmoles of EDTA. The nitrogen donor was 1 μmole of L-glutamine (○) or 10 μmoles of $NH_4Cl$ (●).

Arrhenius plots had a discontinuity at 20° and the bend was downwards. Activation energies for the upper and lower limbs are 9300 and 18 200 calories, respectively. There was no difference between the activation energies of the two reactions. The "modified" enzyme preparation obtained by ultracentrifugation in the absence of L-glutamine, as shown in Fig. 4, gives a discontinuity of slope with a transition temperature at 30° for both the reactions (Fig. 5). There was no noticeable difference between the activation energies of the reactions with L-glutamine and those with ammonia. The results support that these two reactions are catalyzed by the same enzyme[10].

*Properties of the AH 13 and Ehrlich ascites tumor enzymes*

Properties of the partially purified enzymes of AH 13 and Ehrlich ascites tumor are not distinguishable from those of the AH 130 enzyme. The enzymes of AH 13 and Ehrlich ascites tumor are also labile and stabilized by the addition of glycerol, dimethylsulfoxide and dithiothreitol. Both enzymes are active either with L-glutamine or ammonia and the two activities are not separated in the purification process. *N*-Acetyl-L-glutamate is not required. The apparent $K_m$ values of the AH 13 enzyme for L-glutamine and ammonia are $2.5 \cdot 10^{-5}$ and $5 \cdot 10^{-3}$ M, and the apparent $K_m$ values

TABLE VI

EFFECT OF NUCLEOTIDES ON THE RATE OF CARBAMOYL-*P* SYNTHESIS

The reaction mixture (pH 7.8) used for the AH 130 and AH 13 enzyme contained 50 $\mu$moles of potassium HEPES, 15 $\mu$moles of $KH^{14}CO_3$ ($1.5 \cdot 10^7$ counts/min), 1 $\mu$mole of L-glutamine, 5 $\mu$moles of ATP, 7.5 $\mu$moles of $MgCl_2$, 0.5 $\mu$mole of L-ornithine, 0.1 $\mu$mole of L-citrulline, 10 units of ornithine transcarbamoylase, 38 $\mu$l of glycerol, 50 $\mu$l of dimethylsulfoxide, 2.5 $\mu$moles of nucleotides and enzyme in final volume of 0.75 ml. The reaction mixture used for Ehrlich ascites tumor enzyme had a lower pH of 7.3 and contained a smaller amount of dimethylsulfoxide (25 $\mu$l). The specific activity of $KH^{14}CO_3$ was lower ($5 \cdot 10^6$ counts/min for 15 $\mu$moles of bicarbonate). The amount of enzyme protein used were 2.1, 48.5 and 29.0 $\mu$g for the AH 130, AH 13 and Ehrlich ascites tumor enzymes, respectively. Incubation was for 10 min at 37°. The fixation of $H^{14}CO_3^-$ into citrulline in the absence of nucleotides were 990, 3324 and 272 counts/min for the AH 130, AH 13 and Ehrlich ascites tumor enzymes, respectively.

| *Nucleotide* | *Relative activity (%)* | | |
|---|---|---|---|
| | *AH 130 enzyme* | *AH 13 enzyme* | *Ehrlich ascites tumor enzyme* |
| None | 100 | 100 | 100 |
| UMP | 104 | 84 | 95 |
| UDP | 114 | 115 | 44 |
| UTP | 22 | 30 | 14 |
| CMP | 88 | | 110 |
| CDP | 88 | | 102 |
| CTP | 73 | | 88 |
| TTP | 103 | | 87 |
| GTP | 85 | | 94 |
| ITP | 86 | | 95 |

of the Ehrlich ascites tumor enzyme for these substrates are $1.0 \cdot 10^{-5}$ and $5 \cdot 10^{-3}$ M, respectively.

*Effects of nucleotides and nucleosides on the rate of carbamoyl-P synthesis*

The reactions of carbamoyl-*P* synthesis catalyzed by the AH 130, AH 13 and Ehrlich ascites tumor enzymes were inhibited by UTP, as shown in Table VI, but to a much lesser extent, if at all, by other nucleotides and nucleosides. The concentration of UTP that inhibited the AH 130 enzyme by 50% was about $3.5 \cdot 10^{-3}$ M under the conditions used. A detailed mechanism of the inhibition remains to be elucidated.

## DISCUSSION

This paper demonstrated that all the experimental tumor cells so far as tested, *i.e.* Yoshida ascites hepatomas AH 130, AH 13, AH 601 and AH 7974 as well as Walker carcinosarcoma and Ehrlich ascites tumor, are provided with an activity for synthesis of carbamoyl-*P*. The observations strongly suggest the possible operation of the orotic acid pathway in these cells. The results concerning Ehrlich ascites tumor are consistent with those previously reported by HAGER AND JONES[7]. The carbamoyl-*P* synthetases of these tumors were extremely labile but stabilized by glycerol, dimethylsulfoxide, dithiothreitol and L-glutamine *plus* $Mg^{2+}$ ATP, which made it possible to partially purify the enzymes of AH 130, AH 13 and Ehrlich ascites tumor. The purified enzymes utilized either L-glutamine or ammonia as direct carbamoyl

nitrogen donor. However, in view of the marked difference in the apparent $K_m$ values and the possible difference in the relative tissue concentrations of the two substrates, glutamine, rather than ammonia, appears to have been the physiological substrate. Properties of the partially purified enzymes of AH 130, AH 13 and Ehrlich ascites tumor were essentially the same as those of the glutamine-utilizing carbamoyl-*P* synthetase of the hematopoietic mouse spleen[3], and the liver of fetal[4] and adult[5] rat. It is worth mentioning that there was no marked difference in properties between the enzymes of the cancer cells and those of normal mammalian tissues.

The observed rates of carbamoyl-*P* synthesis by the extracts of experimental tumor cells ranged from 0.20 to 1.2 nmoles/min per mg of protein (Table I). The maximal activity of the cells to synthesize carbamoyl-*P* was then calculated to be 0.6 4.9 μmoles/h per g cells. Whether these activities were enough to supply sufficient amounts of carbamoyl-*P* for synthesis of all uracil that the tissues demand poses an important problem. Our preliminary experiments showed that uracil molecules present as uridine nucleotides in hematopoietic mouse spleen were consumed at a rate of 0.10 0.15 μmole/h per g tissue[11]. The rate was estimated from the rate of disappearance of radioactivity from nucleotide uracil previously labeled by $^{14}CO_2$ administration and thus represents the total rate at which the uracil is consumed by conversion to other pyrimidines or to nucleic acids and by degradation *via* free uracil. Since the hematopoietic mouse spleen is considered one of the most rapidly growing tissues, whether normal or malignant, it is reasonable to assume that the demands for uracil of the experimental tumors are not too far from that estimated for the spleen and thus can be covered by the activities for carbamoyl-*P* synthesis observed in the tumors. This reasoning, however, is not meant to indicate that all uracil molecules utilized *in vivo* by the tumors are synthesized endogenously by themselves. The relative importance of endogenous synthesis and exogenous supply of pyrimidines of tumor tissues remains to be elucidated.

The presence of glutamine-utilizing carbamoyl-*P* synthetase in experimental tumor cells adds to the list of the known wide distribution of the enzyme in nature. Of particular interest here is that Yoshida hepatomas AH 130 and AH 13, known to be derived from parenchymal cells of liver, contain only this type of enzyme, whereas liver cells are rich in the different type of enzyme, *i.e.* ammonia- and acetylglutamate-dependent carbamoyl-*P* synthetase. Properties of the AH 130 and AH 13 enzymes are distinct from the known properties of the latter enzyme and, in addition, the two types of enzymes are immunologically distinguished. Thus the conversion of the ammonia enzyme to the glutamine enzyme in the process of carcinogenesis is not plausible. Since the presence of two carbamoyl-*P* synthetases in the normal liver tissue (probably in parenchymal cells) is shown[5], the possibility is that in the process of development of the hepatomas only the ammonia enzyme was deleted while the glutamine enzyme remained.

The carbamoyl-*P* synthetases of tumors are subject to "feedback" inhibition by UTP, as far as tested (Table VI). In addition, the enzyme activities are so low as to limit the rate of pyrimidine biosynthesis (Table I). These observations suggest that the enzymes may play a key role in control of the orotic acid pathway in the tumor cells. In fact, the step of carbamoyl-*P* synthesis was suggested to be a rate-limiting step in intact cells of Ehrlich ascites tumor[12].

When the AH 130 carbamoyl-*P* synthetase was subjected to the ultracentrifu-

gation in sucrose gradients containing no glutamine, a differential decrease of the glutamine-dependent activity, in comparison with the ammonia-dependent activity, was observed (Fig. 4). There was no such decrease, however, when the enzyme in the same sucrose gradient was allowed to stand for 20 h at 0°. It is known that enzymes responsible for some amidation reactions can utilize both glutamine and ammonia and sometimes lose only the ability to utilize glutamine during purification processes[13]. Xanthosine 5′-phosphate aminase prepared from *Aerobacter aerogenes*[14] and carbamoyl-*P* synthetase prepared from *Escherichia coli*[15] can be cited as examples. The explanation is that the site of the enzyme to bind glutamine might be relatively unstable and easily destroyed. The centrifugation of the AH 130 enzyme achieved a considerable purification, but at the same time this might have eliminated some high molecular component(s) possessing a stabilizing action towards the enzyme, and thus caused a structural modification of the enzyme protein with accompanying decrease in the glutamine-dependent activity. The shift in the transition temperature of Arrhenius plots (Fig. 5) affords strong evidence for a structural modification. KLEE[16] described a similar observation that the primer-independent polynucleotide phosphorylase prepared from *Micrococcus lysodeikticus* was converted to the primer-dependent enzyme after centrifugation for 10 days and suggested, based on analysis by electrophoresis, a structural modification of the enzyme.

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