ACTA CHEMICA SCANDINAVICA 8 (1954) 795—805

The Enzymatic Synthesis of Ureidosuccinic Acid in Rat Liver Mitochondria

PETER REICHARD

Biochemical Department, Karolinska Institutet, Stockholm, Sweden

It has been shown that the pyrimidine orotic acid (uracil-4-carboxylic acid) is rapidly synthesized *in vitro* in rat liver slices[1]. As orotic acid is the only free pyrimidine shown to be extensively utilized for nucleic acid synthesis in the rat and as the existing evidence points to orotic acid as either itself being a direct intermediate in the biogenesis of pyrimidines or very closely related to a direct intermediate, an investigation of the enzymatic synthesis of orotic acid in rat liver should prove very fruitful for the general problem of pyrimidine biogenesis. In a previous investigation [2] it was shown that aspartate, CO_2 and USA * were intermediates in orotic acid synthesis in rat liver slices. Of these substances USA had earlier been implicated in pyrimidine biogenesis by the work of Wright *et al.*[3,4], who demonstrated that the acid was an intermediate in pyrimidine synthesis in *Lactobacillus bulgaricus 09*.

Our earlier results with liver slices led us to the following formulation of the first step in the biogenesis of orotic acid [2]:

$$NH_3 \ (?) + CO_2 + \text{L-aspartate} \rightarrow \text{L-ureidosuccinate}$$

In the present paper the *de novo* synthesis of USA in rat liver mitochondria is demonstrated. Ammonia is shown to be one of the precursors in the synthesis. The mechanism of the reaction has been studied in isolated liver mitochondria and it is suggested that a glutamic acid derivative (*e. g.* UGA), ATP and Mg^{++} are required for the synthesis of USA. The reaction thus shows a great resemblance to the synthesis of citrulline from ornithine, CO_2 and ammonia, as studied by Grisolia and Cohen [5].

* The following abbrevations are used in the present paper: Ureidosuccinic acid: USA; Ureidoglutaric acid (= carbamyl glutamic acid): UGA; Adenosine triphosphate: ATP; Tris (hydroxymethyl) aminomethane : Tris.

EXPERIMENTAL

Preparation of rat liver mitochondria. The differential centrifugation method of Schneider and Hogeboom [6] with 0.25 *M* sucrose as homogenizing medium was used. "Versene" (ethylenediaminetetraacetic acid), 0.01 *M* at pH 7.8, was included in all isolation media, since Slater and Cleland [7] have demonstrated that the addition of this chelating agent increases the stability of the mitochondria.

Two rats were killed by decapitation, the livers immediately removed and chilled on ice. All the following operations were carried out at around 0°.. The livers were homogenized in a large Potter-Elvehjem apparatus with a plastic pestle in a volume of *ca.* 50 ml sucrose solution. The homogenate was diluted to 110 ml with the same medium. All following centrifugations were carried out in the head of the multispeed attachement of a refrigerated International centrifuge.

In a first centrifugation at 1 000 r.p.m. (100 g*) for 15 min. cellular debris, nuclei, etc. were removed. The supernatant from this run was centrifuged at 15 000 r.p.m. (18 000 g) for 15 minutes and the supernatant discarded. The sedimented mitochondria, which microscopically contained very few cell nuclei were gently suspended in sucrose and recentrifuged at 15 000 r.p.m. for 10 minutes. The sediment from this centrifugation was used for the experiments. It could be stored for several hours before use without evident impairment of its synthetic activity. Immediately before use the mitochondria were evenly suspended in isotonic KCl, giving a final volume of 10—15 ml.

Mitochondria prepared in this way were usually employed in about 10 experiments. Since the capacity of the mitochondria to synthesize USA varied some from one preparation to another, mitochondria from the same preparation were used for obtaining each curve in the graphs presented.

Incubation. All experiments were conducted in 200 ml Erlenmeyer flasks at 37° in an atmosphere of O_2:CO_2 (95 % : 5 %). Unless otherwise indicated incubation was carried out over a period of two hours in a total volume of 15 ml. About 4 mg of mitochondrial nitrogen were employed in each experiment.

Determination of enzyme activity. No specific method for the determination of USA in the presence of a large excess of UGA was available. Therefore the experiments were carried out with isotopic precursors (N^{15}-L-aspartic acid and N^{15}-ammonium chloride), and the amount of USA formed was determined by the isotope dilution technique. 200 μmoles of non-labeled D,L-USA was added as carrier at the end of each experiment, the USA was reisolated by a chromatographic technique and recrystallized to constant isotope content. As the amount of N^{15}-precursors in the experiments was very large as compared to the pools of the same unlabeled substances in the mitochondria (assuming rapid and complete mixing between the added precursors and the mitochondrial aspartate and ammonia), and as the amount of formed L-USA was very small as compared to the amount of added carrier D,L-USA the following approximation is valid:

$$\mu\text{moles USA formed} = \frac{200 \cdot a}{b}$$

where a = xs N^{15} in reisolated USA, and b = xs N^{15} in the precursors. Both NH_4Cl and aspartic acid contained throughout the experiments 32 % excess N^{15}. Hence: μmoles USA formed = $6.25 \cdot a$.**

Reisolation of USA. At the end of the experiment the reaction was stopped by immersion of the flasks in a boiling water bath for 3 minutes. Two hundred μmoles D,L-USA, dissolved in 1 ml of 0.4 *N* NaOH, were added and the coagulated proteins were filtered off with the aid of some celite. The solution was brought to pH 10—11 with a few drops of

* This centrifugal force was considerably lower than what is generally used for removal of cell nuclei. However, already at 200 g great losses of mitochondria took place under the experimental conditions used.

** The calculation does not take into consideration the possibility of non labeled aspartate and ammonia being formed from glutamate in the experiments, where the latter acid was included in the medium. There is some evidence that a slight dilution of the isotopic precursors did take place through this mechanism. The amounts of USA obtained by the calculation represent thus minimum values.

2 *N* NaOH and added to the top of a Dowex 2 column (200—400 mesh Cl^- form, diam. 2 cm, length 10 cm). After all the solution had penetrated into the ion exchange resin the column was washed with 50 ml of water and then with 0.005 *N* HCl until the effluent became distinctly acid (*ca.* 300 ml). From that point on another 100 ml of 0.005 *N* HCl were run through the column and the effluent discarded. The solvent was changed to 0.04 *N* HCl and all the USA was eluted by 100 ml of this acid. If orotic acid had been included in the medium, it could be obtained from the column by further elution with 100 ml of 0.5 *N* HCl.

Two drops of Kjeldahl indicator were added to the effluent containing the USA, the solution was neutralized with *N* NaOH and evaporated to dryness *in vacuo*. The residue was dissolved in a total of 3 ml of water, filtered and transferred to a centrifuge tube. The solution was acidified with two drops of concentrated HCl, seeded with a very small crystal of USA, and crystallization allowed to take place in the refrigerator over night. The crystals were centrifuged, washed twice with 0.5 ml of ice cold water and recrystallized from 1 ml hot water. The crystals from this second crystallization had generally the same isotope content as their mother liquor and this isotope content as determined in the mass spectrometer was used for the calculation of enzyme activity.

The method was tested by addition of 1.0 and 2.0 μmoles of N^{15}-USA to heat coagulated mitochondria in the medium of Table 1. After reisolation in the way described the recovery of the USA was calculated from isotope analysis to be 96 and 103 % respectively.

The method outlined above permitted a relatively rapid determination of the different factors influencing the USA synthesizing capacity of mitochondria. Up to 15 ion exchange columns were run parallell and the rate of flow through them was accelerated by air pressure. At the conclusion of each run the columns were washed first with 250 ml of 4 *N* HCl and then with water to remove the excess HCl. They were then immediately ready for use in a new experiment.

D,L-*Ureidosuccinic acid* and L-*ureidoglutaric acid* (carbamyl glutamic acid) were synthesized according to Nyc and Mitchell [8]. The syntheses of N^{15}-L-*aspartic acid*, N^{15}-L-*ureidosuccinic acid* and C^{13}-L-*aspartic acid* have been described earlier [2].

Table 1. USA synthesis in different cell fractions.

Cell fraction	mg N	μmoles USA synthesized	μmoles USA synthesis/mg N	% recovery
Whole homogenate	156	19.6	0.126	100
"Cell nuclei"	51	3.1	0.061	16
Mitochondria	26	9.3	0.358	47
Cell sap + microsomes	68	*ca.* 0.2		
Mitochondria + cell sap + microsomes	94	9.7	0.103	49

The values are calculated for one rat liver. An aliquot from each fraction suspended in 2 ml sucrose was incubated in the following medium: 0.006 *M* N^{15}L-aspartate * and $N^{15}H_4Cl$, 0.012 *M* $NaHCO_3$, 0.015 *M* L-glutamate, 0.007 *M* L-UGA. 0.008 *M* $MgCl_2$ *, 0.001 *M* ATP *, 0.04 *M* phosphate buffer and 0.04 *M* tris buffer, pH 7.4 *. Gas = O_2:CO_2 (95 % : 5 %), volume = 15 ml, KCl to isotonicity *.

* All salts, unless otherwise indicated, were potassium salts. $MgCl_2$ was added only immediately before the start of the experiment. — Ba-ATP (purchased from the Sigma company) was suspended in ice cold water and dissolved with a minimum amount of 0.5 *N* HCl. A slight excess of Na_2SO_4 was added, the solution neutralized to pH 7 and centrifuged. — The buffers were chosen such as to give the desired pH after saturation with the gas used. — No consideration was taken of the osmotic pressure of phosphate in the calculation of isotonicity (K. W. Cleland, *Nature* **170** (1952) 497).

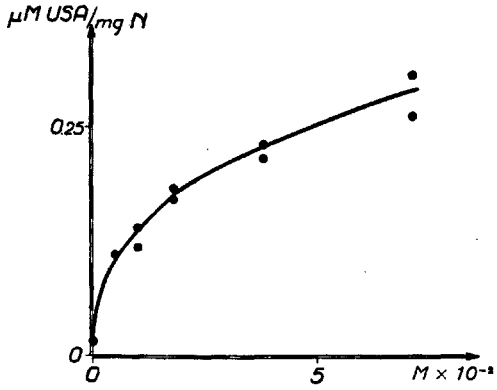

Fig. 1. USA synthesis in the presence of glutamate. Each flask contained aspartate, ammonium chloride, $NaHCO_3$, $MgCl_2$, ATP and buffers as in Table 1. L-*Glutamate as indicated on the abscissa.*

RESULTS

Localization of USA synthesis. Early experiments to demonstrate USA synthesis in cellfree systems using the same substrate as in the slice experiments did not meet with success. It was found, however, that whole homogenates either prepared with isotonic KCl or sucrose could synthesize USA quite rapidly, if glutamate in relatively high concentrations was included in the medium. When USA synthesis was studied in different fractions of a sucrose homogenate the results of Table 1 were obtained. In these experiments part of the glutamate was substituted by UGA to obtain optimal synthesis (see below).

It can be seen that the largest amount of USA formed per mg nitrogen was obtained in the mitochondrial fraction. The relatively high synthesis in the "nuclear" fraction is probably explained by inclusion of mitochondria and whole cells in this fraction. The experimental setup presumably did not allow ATP synthesis in the supernatant fraction and therefore the failure to synthesize USA in the reported experiment is not conclusive. However, only insignificant synthesis was obtained in the supernatant, also when 3-phosphoglyceric acid was added to provide ATP. Furthermore, very little stimulation was observed, when mitochondria were combined with supernatant. It was never possible to recover more than 75 % of the activity of the whole homogenate in the different cellular fractions. *Under the experimental conditions* of Table 1 no more than traces of orotic acid were synthesized in the mitochondria, a fact which might favourable influence the accumulation of USA in the present system *.

Requirement for glutamate or UGA. Fig. 1 demonstrates the requirement of the reaction for glutamate. It can be seen that the enzyme system is not yet saturated at a concentration of glutamate of 0.07 *M*. Succinate + UGA can substitute for glutamate in the reaction (Fig. 2, curve II). Saturation of the enzyme system is obtained around 0.02 *M* UGA and 0.015 *M* succinate. The

* U. Lagerkvist (unpublished) has found that *under the proper conditions* the synthesis of orotic acid from USA is localized in rat liver mitochondria.

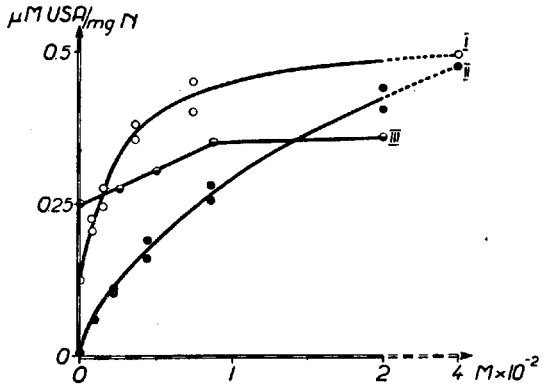

Fig. 2. UGA and USA synthesis. Medium: 0.02 M aspartate; ammonium chloride, $NaHCO_3$, $MgCl_2$, ATP and buffers as in Table 1.

Curve I: 0.015 M L-glutamate, L-UGA as indicated on the abscissa.
Curve II: 0.015 M succinate, L-UGA as indicated on the abscissa.
Curve III: 0.015 M succinate, 0.01 M L-UGA, L-glutamate as indicated on the abscissa.

function of glutamate in Fig. 1 seems to be a double one: partly it serves as a substrate for combustion, partly it serves as the source of a derivative that is necessary for the reaction. The first function can be fulfilled by succinate, the second one by UGA. UGA or succinate separately, however, even at concentrations of 0.1 *M* only permit small amounts of USA synthesis.

Curve I in Fig. 2 demonstrates the influence of varying concentrations of UGA in the presence of glutamate. It is interesting to compare curves I and II in Fig. 2. The same concentrations of glutamate and succinate respectively are used in the two curves. Curve I shows a steeper slope and earlier saturation of the enzyme with UGA than curve II. Curve III shows the slight stimulation of USA synthesis exerted by glutamate at optimal succinate and slightly suboptimal UGA concentrations. When both UGA and succinate

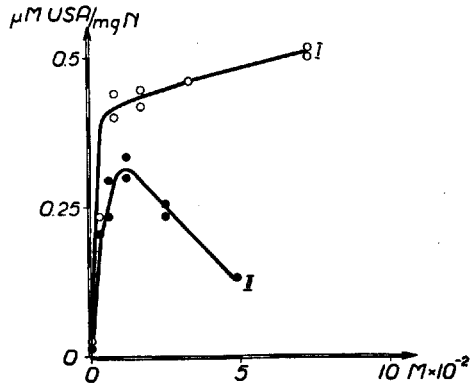

Fig. 3. Requirement of oxidizable substrate. Medium: 0.02 M aspartate; ammonium chloride, $NaHCO_3$, $MgCl_2$, ATP and buffers as in Table 1.

Curve I: 0.007 M L-UGA, L-glutamate as indicated on the abscissa.
Curve II: 0.02 M L-UGA, succinate as indicated on the abscissa.

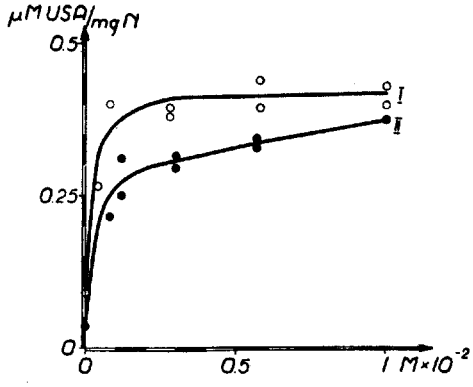

Fig. 4. Ammonia and USA synthesis. Medium: 0.02 M aspartate; $MgCl_2$, ATP and buffers as in Table 1.

Curve I: 0.015 M L-glutamate, 0.007 M L-UGA, $N^{14}H_4Cl$ as indicated on the abscissa.

Curve II: 0.015 M succinate, 0.02 M L-UGA, $N^{14}H_4Cl$ as indicated on the abscissa.

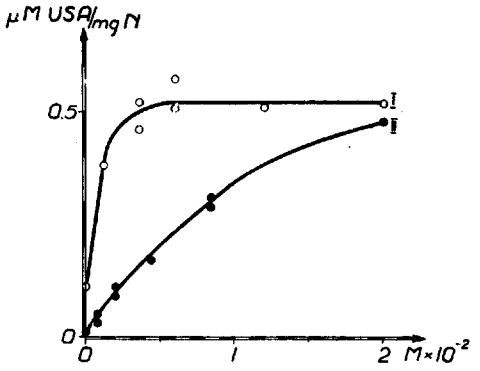

Fig. 5. Aspartate and USA synthesis. Ammonium chloride, $NaHCO_3$, $MgCl_2$, ATP and buffers as in Table 1.

Curve I: 0.015 M L-glutamate, 0.007 M L-UGA and N^{14}-aspartate as indicated on the abscissa.

Curve II: 0.015 M succinate, 0.02 M L-UGA and N^{14}-aspartate as indicated on the abscissa.

concentrations were optimal, glutamate no longer showed this stimulating effect.

Fig. 3 shows the influence of varying concentrations of glutamate (curve I) and succinate (curve II) at constant UGA concentration. The apparent inhibiting effect of larger concentrations of succinate cannot be explained without further experiments.

Influence of ammonia and aspartate. The dependence of USA synthesis on the simultaneous presence of ammonia and aspartate in the medium is demonstrated by Figs. 4 and 5. In these experiments only one of the two precursors contained N^{15}. The concentration of the non isotopic precursor varied in the presence of a constant amount of the labeled precursor. Isotope incorporation into USA did thus in the aspartate experiments (Fig. 5) only take place from $N^{15}H_4Cl$ and in the ammonia experiments (Fig. 4) from N^{15}-aspartate. In the degradation experiments related below it was demonstrated that each of the precursors donated its nitrogen almost exclusively to only one of the two nitrogens of the USA. In the calculation of the amount USA formed the isotopic excess observed in the reisolated USA was therefore multiplied with 12.5 instead of 6.25.

Curves I and II in Figs. 4 and 5 represent experiments in which glutamate and succinate respectively were used as oxidizeable substrates. In Fig. 5 curve I saturation of the enzyme with aspartate apparently occurs at much lower concentrations than in curve II. One explanation for this might be that the presence of glutamate inhibits the disappearance of aspartate by transamination reactions.

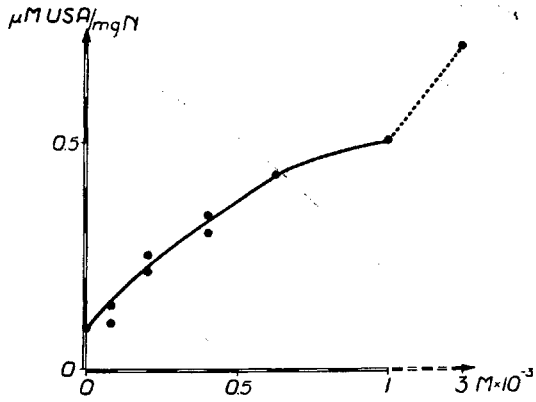

Fig. 6. ATP and USA synthesis. Aspartate, ammonium chloride, $NaHCO_3$, $MgCl_2$, glutamate, UGA and buffers as in Table 1. ATP as on the abscissa.

ATP and Mg^{++}. The demonstrated requirement of an oxidizable substrate is best explained on the basis of an ATP dependence of the reaction studied. A small amount of ATP (which can be replaced by ADP or AMP) was always included in each experiment and the influence of varying concentrations of ATP is demonstrated by Fig. 6. The ATP concentration (0.001 *M*) chosen in all the experiments, where ATP was not varied, was not optimal. The influence of Mg^{++} is demonstrated by Fig. 7. At this point it cannot be decided with certainty whether Mg^{++} was necessary for the regeneration of ATP or whether it directly participated in the synthesis of USA.

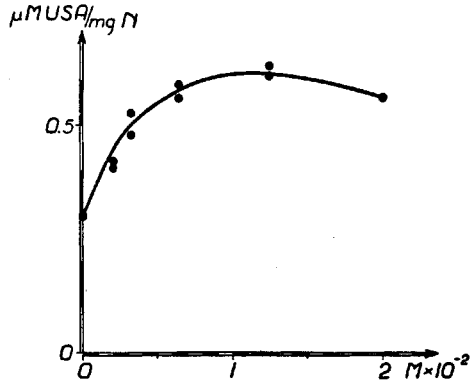

Fig. 7. $MgCl_2$ and USA synthesis. Aspartate, ammonium chloride, $NaHCO_3$, ATP, glutamate, UGA and buffers as in Table 1. $MgCl_2$ as on the abscissa.

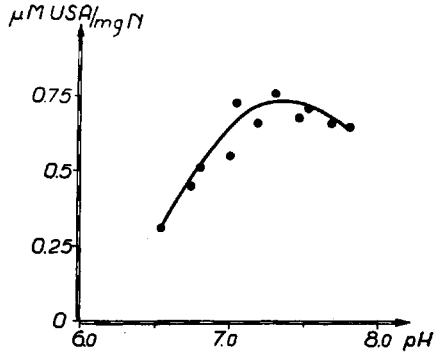

Fig. 8. Influence of pH. Aspartate, ammonium chloride, $NaHCO_3$, $MgCl_2$, ATP, glutamate and UGA as in Table 1. 0.04 M phosphate and tris buffers at varying pH. The pH was measured at the end of the experiment.

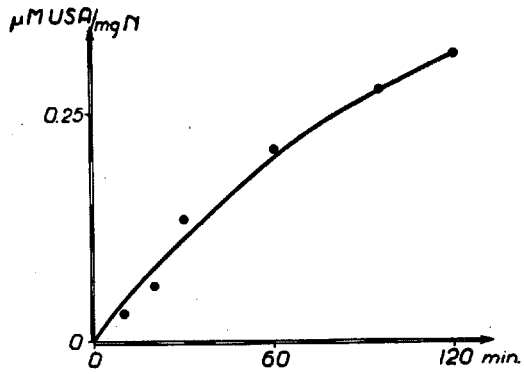

Fig. 9. Time curve of USA synthesis. Substrate as in Table 1.

pH optimum. The pH curve of Fig. 8 shows a rather broad maximum around pH 7.4. It must be kept in mind that the curve represents the pH optimum of a sum of several different reactions (*e. g.* oxidation of glutamate and USA synthesis).

The *time curve* of Fig. 9 demonstrates a linear reaction rate for 1—1.5 hours, after which time a slight reduction in the rate occurred.

Fig. 10 shows that a straight line is obtained when USA synthesis is plotted against increasing *amounts of mitochondria.*

Degradation of USA. The present system did not allow stoichiometric studies. Instead the direct transformation of aspartate and ammonia into USA was demonstrated by degradation of USA after administration of each of the isotopic precursors.

In each experiment mitochondria from one liver were incubated in 75 ml of the media specified in Table 2. After two hours 0.8 mmoles carrier USA was added, and the USA was reisolated in the way outlined earlier. After two recrystallizations a small part of the USA was analyzed for isotope, while the

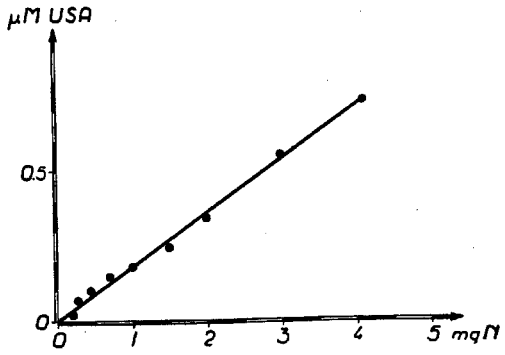

Fig. 10. USA synthesis with different amounts of enzyme. Substrate as in Table 1.

Table 2. Degradation of USA after incubation with $N^{15}H_4Cl$ (32 % excess N^{15}) and N^{15}-1,4-C^{13}-L-aspartate (31.9 % N^{15} and 16.1 % C^{13}).

Precursor	Atom % excess N^{15} in			Atom % C^{13} in		N^{15} / C^{13} in	
	USA	carbamino-N	aspartate-N	USA	aspartate from USA	precursor	aspartate from USA
Ammonium chloride	0.293	0.525	0.058				
Aspartate	0.265	0.039	0.498	0.233	0.284	1.92	1.76

The same medium was used as in Table 1 with the exception that in the experiment with labeled NH_4Cl nonlabeled aspartate was used, and in the experiment with labeled aspartate the ammonium chloride contained no isotope. The degradations are described in the text, the carbamino group is represented by the isotope value of ammonia, the aspartate moiety of USA by the isotope values of the Cu-aspartate.

rest was hydrolyzed in 5 ml concentrated HCl at 100° for 24 hours. In this way the carbamino nitrogen of USA was obtained as ammonia, while the part of the molecule that corresponds to aspartic acid was obtained as aspartic acid. The subsequent separation and isolation of the derived ammonia and aspartic acid involved the same steps as described earlier in the corresponding degradation of orotic acid [2]. After purification, ammonia and copper aspartate were analyzed for isotope.

Table 2 presents the results of experiments with $N^{15}H_4Cl$ and N^{15}-1,4-C^{13}-L-aspartate respectively. It can be seen that the N^{15} from ammonia is incorporated into the carbamino nitrogen of USA, while that of aspartate forms the other nitrogen atom of the acid. Furthermore, in the aspartate experiment the N^{15}/C^{13} ratios in the precursor and in the aspartate derived by degradation from USA are almost the same, indicating that aspartic acid is utilized as an intact molecule.

DISCUSSION

From the data in Table 1 it can be calculated that the rat liver homogenate obtained from one animal synthesized USA at the rate of ca 200 μmoles per day. One can furthermore very approximately calculate that one rat liver contains about 100 μmoles of nucleic acid pyrimidines. The USA synthesis of the liver should therefore suffice to renew all the nucleic acid pyrimidines of the liver in 12 hours. The estimations of the "half life times" of nucleic acids in rat liver have arrived at values of around one week. The amount of USA synthesis demonstrated in Table 1 is thus far more than sufficient to provide for all pyrimidine synthesis in rat liver, even if one assumes that part of the USA is diverted to acid soluble pyrimidine derivatives with a high turnover rate.

The present experiments confirm the earlier finding [2] that aspartic acid is involved in orotic acid biogenesis, probably *via* USA. They furthermore establish that ammonia is directly involved in USA synthesis and thus is the

precursor of N_1 of orotic acid. In liver slices we had found that the isotope from N^{15}-ammonia even in short time experiments is almost evenly distributed between N_1 and N_3 of orotic acid, which made us reluctant to accept ammonia as a direct precursor of N_1. We can now explain these earlier results by the probability that N^{15}-ammonia in liver slices gives rise very rapidly to N^{15}-aspartate through amination of a dicarboxylic acid. The present interpretation also fits excellently with earlier results from *in vivo* experiments with rat liver obtained by Lagerkvist [9], who could demonstrate that the incorporation of isotope from N^{15}-ammonia into position 1 of polynucleotide uracil was between 3—4 times greater than that into position 3.

As to the mechanism of the reaction the similarity between USA synthesis and citrulline synthesis is apparent. For the latter case Grisolia and Cohen [5] have demonstrated that the fixation of ammonia and carbon dioxide proceeds in two steps: in the first step a labile phosphorylated derivative ("compound X") is formed from UGA, ammonia, CO_2 and ATP in the presence of Mg^{++}. The second reaction involves the formation of citrulline from ornithine + compound X, while UGA is regenerated. In recent experiments Grisolia and Cohen [10] have demonstrated that instead of UGA, formylglutamic, acetylglutamic, chloroacetylglutamic or propionylglutamic acids can be utilized in citrulline synthesis.

The formation of USA also involves a fixation of ammonia and CO_2, which proceeds with the aid of ATP and a glutamic acid derivative. UGA can act as this glutamic acid derivative, though relatively high concentrations are needed to saturate the enzyme. It is of interest to note that considerably smaller amounts of UGA are sufficient, if the oxidation of glutamate is used as an energy source than if succinate is used (curves I and II in Fig. 2). Furthermore relatively small amounts of glutamate still slightly stimulated USA synthesis in the presence of optimal amounts of succinate and slightly suboptimal amounts of UGA (curve III, Fig. 2). This might indicate that a glutamic acid derivative that differs from UGA is more directly involved in the synthesis. Matters are, however, complicated by the demonstrated inhibition of USA synthesis by succinate at higher concentrations, and it is hoped that experiments with soluble enzymes now in progress will answer this question.

The following over all mechanism for the synthesis of USA is thus suggested:

$$NH_3 + CO_2 + \text{L-aspartate} \xrightarrow[\text{ATP, } Mg^{++}]{\text{L-UGA or other glutamate derivative}} \text{L-ureidosuccinate}$$

It should be pointed out in this connection that the above mechanism has been suggested by Wright [11] already in 1951 in a review article, though at that time no experimental evidence for the reaction existed.

Another theory that has been brought forward at different times [12–14] is that arginosuccinic acid or a compound derived from it might be involved in pyrimidine biogenesis. This acid can be enzymatically synthesized from citrulline + aspartic acid in rat liver. If it were an intermediate in the synthesis of USA from aspartic acid this would explain the fixation of NH_3 and CO_2 during

the reaction. In experiments, where N^{15}-citrulline was incubated in liver slices together with orotic acid under the conditions described earlier [2], it was found, however, that no N^{15}-orotic acid was derived from citrulline. This experiment tends to exclude arginosuccinate as an intermediate in orotic acid synthesis at least in *rat* liver slices.

SUMMARY

The synthesis of ureidosuccinic acid from aspartic acid, ammonia and CO_2 was studied in rat liver mitochondria. Under the conditions chosen it was found that the presence of glutamic acid or ureidoglutaric acid + succinic acid was necessary for the reaction, which also depended upon ATP and Mg^{++}. The resemblance between ureidosuccinic acid synthesis and citrulline synthesis is pointed out.

This study has been supported by grants from *Statens Medicinska Forskningsråd* and from *Harald och Greta Jeanssons Stiftelse*.

REFERENCES

1. Reichard, P. *J. Biol. Chem.* **197** (1952) 391.
2. Reichard, P. and Lagerkvist, U. *Acta Chem. Scand.* **7** (1953) 1207.
3. Wright, L. D., Huff, J. W., Skeggs, H. R., Valentik, K. A. and Bosshardt, D. K. *J. Am. Chem. Soc.* **73** (1950) 2312.
4. Wright, L. D., Miller, C. S., Skeggs, H. R., Huff, J. W., Weed, L. L. and Wilson, D. W. *J. Am. Chem. Soc.* **73** (1951) 1898.
5. Grisolia, S. and Cohen, P. P. *J. Biol. Chem.* **198** (1952) 561.
6. Schneider, W. C. and Hogeboom, G. H. *J. Biol. Chem.* **183** (1950) 123.
7. Slater, E. C. and Cleland, K. W. *Biochem. J. (London)* **55** (1953) 566.
8. Nyc, J. F. and Mitchell, H. K. *J. Am. Chem. Soc.* **69** (1947) 1382.
9. Lagerkvist, U. *Arkiv Kemi* **5** (1953) 569.
10. Grisolia, S. and Cohen, P. P. *J. Biol. Chem.* **204** (1953) 753.
11. Wright, L. D. *Vitamins and Hormones* **10** (1951) 131.
12. Davison, D. C. and Elliott, W. H. *Nature* **169** 313 (1952).
13. Walker, J. B. and Myers, J. *J. Biol. Chem.* **203** (1953) 143.
14. Ratner, S., Anslow Jr. W. P. and Petrack, B. *J. Biol. Chem.* **204** (1953) 115.

Received February 1, 1954.