ACTA CHEMICA SCANDINAVICA 7 (1953) 1207—1217

The Biogenesis of Orotic Acid in Liver Slices

PETER REICHARD and ULF LAGERKVIST

Biochemical Department, Karolinska Institutet, Stockholm, Sweden

The biological existence of orotic acid was first demonstrated by Biscaro and Belloni [1], who isolated this substance from cow's milk in 1905. Orotic acid is the only pyrimidine carboxylic acid known to exist in nature and its possible significance has at different times evoked speculations. The finding of Loring and Pierce [2] and Rogers [3] that uracil can be replaced by orotic acid as a growth stimulant for certain bacteria suggested a connection between this acid and nucleic acid pyrimidine biogenesis. Mitchell and coworkers [4,5] extensively studied the function of orotic acid in the pyrimidine metabolism of different mutants of *Neurospora*. From genetic evidence Mitchell, Houlahan and Nyc [5] came to the conclusion that orotic acid is formed as a byproduct in a side reaction during pyrimidine synthesis in *Neurospora* and thus is not a normal intermediate in pyrimidine biogenesis. Instead it was proposed that pyrimidines are formed from oxaloacetic acid via aminofumaric acid, and that ribosidation of an acyclic pyrimidine precursor precedes ringclosure [4].

Interest in the metabolism of orotic acid was greatly stimulated by the finding of Arvidson *et al.*[6] that 3-N^{15}-orotic acid is utilized by the rat for the synthesis of pentose nucleic acid (PNA) pyrimidines. Even though no degradation of the pyrimidines was carried out the low dilution of the isotope seemed to justify the conclusion that the whole molecule of orotic acid was incorporated. Confirmation of that has come from the finding in different laboratories that orotic acids labeled in either the 2 or 6 positions are equally well utilized for pyrimidine synthesis by a great variety of different organisms [7–9].

The incorporation of orotic acid into polynucleotide pyrimidines *in vitro* has been demonstrated by Weed *et al.*[10] and Reichard and Bergström [11]. Our interest in the *in vitro* system arose from the hope to be able to decide if orotic acid is a "normal" intermediate in pyrimidine biogenesis. Reichard has shown that the presence of a pool of orotic acid greatly diminishes the incorporation of isotope from $N^{15}H_4Cl$ into uridine in liver slices [12]. Furthermore it was found that the reisolated orotic acid at the end of the experiment contained a high label of N^{15}. These findings agreed with the hypothesis that orotic acid is a direct intermediate in pyrimidine biogenesis.

The finding of a *de novo* synthesis of orotic acid in liver slices which was about ten times more rapid than the simultaneous synthesis of PNA pyri-

midines, made the liver slice system a very valuable tool for further investigations of pyrimidine biogenesis. In the present investigation the synthesis of orotic acid from various labeled precursors has been investigated. It is thought that this synthesis reflects the more general pathways of pyrimidine biogenesis in the rat.

Two experimental approaches were chosen. The first one consisted of incubation of the suspected labeled precursor together with unlabeled orotic acid and liver slices. After incubation orotic acid was reisolated from the medium and the distribution of the isotope within the molecule determined after a partial degradation.

The second approach consisted of the incubation of the suspected nonlabeled precursor in the liver slices together with $N^{15}H_4Cl$ and nonlabeled orotic acid. The effect of the presence of the nonlabeled substance on the incorporation and distribution of N^{15} in orotic acid after incubation was studied.

By a combination of these two methods it was possible to demonstrate that aspartic acid was a precursor of N_3 plus C_4, C_5, C_6 and C_7 of orotic acid and CO_2 a precursor of C_2. Ureidosuccinic acid was an intermediate in the synthesis of orotic acid.

EXPERIMENTAL

Incubation. Slices from nonregenerating livers were used in the present investigation. The livers were obtained from young albino rats weighing between 150 and 200 g. Slices from three livers in 50 ml of a Krebs-Henseleit substrate [13] + 15 mg non-labeled orotic acid + 0.1 m*M* − 2 m*M* of the different labeled or non-labeled precursors were incubated in a 300 ml Erlenmeyer flask from 80 minutes to 8 hours. When more material was needed in one experiment the contents of several flasks were combined after incubation. Details of the procedure and of the isolation of orotic acid after incubation have been described earlier [12].

Synthesis of isotopic compounds

N^{15}-L-*aspartic acid* (33 % excess N^{15}) was synthesized according to the method of Wu and Rittenberg [14].

1,4-C^{13}-L-*aspartic acid* (35 % excess) was synthesized from $KC^{13}N$, *via* succinic and fumaric acids [15]. The fumaric acid was converted to aspartic acid according to Wu and Rittenberg, though an excess NH_4Cl was used in the synthesis (2 g NH_4Cl per 1.2 g fumaric acid).

2,3-C^{14}-L-*aspartic acid* (8 100 c/min) was synthesized from C^{14}-acetylene, *via* ethylene, dibromoethylene, succinic acid and fumaric acid [15,16].

N^{15}-L-*ureidosuccinic acid* was synthesized from N^{15}-L-aspartic acid by condensation with KCNO [17]. It was found that the L-isomer unlike the racemic form of ureidosuccinic acid did not crystallize from the reaction mixture after acidification. Because of that a procedure had to be worked out for its isolation. The alkaline solution from condensation of 0.8 g N^{15}-L-aspartic acid with 1.2 g KCNO was adjusted to pH 4 − 5 by addition of small amounts of Dowex-50(H^+-form, 200 − 400 mesh). After filtration the combined filtrate and washings were brought to pH 10 − 11 and chromatographed on Dowex-2(Cl-form, 200 − 500 mesh), column 10 × 4 cm. Elution was first carried out with 1 400 ml of 0.005 *N* HCl, followed by 0.05 *N* HCl. The ureidosuccinic acid was completely eluted by 600 ml of 0.05 *N* HCl. The ureidosuccinic acid was localized in the chromatogram by a modified ninhydrine reaction [18]. For this purpose 0.2 ml of each chromatographic fraction was transferred into a test tube and after addition of 0.5 ml *N* H_2SO_4 was kept in an oven at 100° over night. After exact neutralisation with *N* NaOH a buffered ninhydrin solution was added and a violet colour developed after 10 minutes at 100°, when ureidosuccinic acid was present.

The solvent was evaporated *in vacuo* from the combined fractions containing ureidosuccinic acid. Great care was taken to ensure low temperature during the procedure. The ureidosuccinic acid was obtained as an oil. It was dissolved in 10 ml of water. A hot saturated solution of barium hydroxide was added to pH 8—9, and the barium salt of the acid was precipitated by addition of 4 volumes of alcohol. After filtration it was dissolved in a small amount of warm water. Alcohol was added to incipient crystallization, and crystallization was completed in the ice box over night. A first crop of 660 mg of a white crystalline powder was obtained. By further addition of alcohol a second crop of 230 mg was obtained. Isotope analysis showed the presence of 16.7 atom per cent excess N^{15} (calc. 16.5). The substance contained 9.30 % N (calc. * 9.10). $[\alpha]_D^{25} = +24.1°$ (c = 3 % in water). The substance did not melt below 300° C.

Immediately before use in an experiment the barium salt was converted to the free acid by dropwise addition of 0.1 *N* sulfuric acid to a water solution used directly in the experiment.

Reisolation of ureidosuccinic acid

In some experiments incubation with $N^{15}H_4Cl$ was carried out in the presence of a non-labeled hypothetical precursor. It was sometimes desirable to investigate whether the added substance had received some isotope at the end of the experiment. This was done with D,L-ureidosuccinic acid which was reisolated by a carrier technique from the same ion exchange column (Dowex-2, Cl^--form) used for the reisolation of orotic acid [12]. The effluent preceding the orotic acid peak was collected in three fractions and each fraction was treated separately. After evaporation of the HCl *in vacuo* 100 mg of non-labeled D,L-ureidosuccinic acid was added to each fraction and the mixture dissolved in a small amount of hot water. On standing in the ice box ureidosuccinic acid crystallized. A small part was analyzed for N^{15}, the rest was recrystallized twice and analyzed each time for N^{15}. Isotopic excess was found in the ureidosuccinic acid of only the last fraction, obtained from the 100 ml which immediately preceded the orotic acid on the column.

Degradation of orotic acid

The partial degradation of orotic acid was carried out by a method similar to that used by Lagerkvist [19] for the degradation of uracil. Orotic acid was thus hydrogenated with chloroplatinic acid as catalyst. Between 10 and 30 mg of orotic acid was dissolved by warming in 10 ml of a 0.5 % freshly prepared solution of gum arabic. Then 0.3 ml of a 10 % solution of catalyst in water was added and reduction carried out at 80° in a hydrogen atmosphere (pressure = 3 atmospheres) for one day. After that an aliquot was removed and the light absorption at 280 mμ was determined after proper dilution. Unless the light absorption was below 5 % of the original, another 0.3 ml of 10 % chloroplatinic acid solution was added and the reduction repeated. If necessary the process was repeated again.

When the light absorption at 280 mμ had reached a low enough value, the solution was centrifuged and the precipitate washed twice with a few ml of warm water. The combined supernatants were evaporated to dryness *in vacuo*. The residue was transferred to a bombtube with a total of 5 ml of concentrated HCl and hydrolyzed at 100° for 24 hours. After that the bomb tube was opened and the contents were centrifuged. The precipitate was twice washed with a few ml of warm water. The combined supernatants were evaporated *in vacuo*. The residue was dissolved in a few ml of water and transferred to the top of a Dowex-50 column (H^+-form, 200—400 mesh, 15 × 0.9 cm). Elution was carried out with 0.5 *N* HCl, the products were localized by treatment with ninhydrin as described by Moore and Stein [18]. Two peaks were observed in this way. The position of the first one corresponded to aspartic acid, the second one to ammonia. When the substance appearing in the first peak was mixed with 5 mg of aspartic acid and rechromatographed on a starch column with propanol — 0.5 *N* HCl, 2 : 1, as described by Moore and Stein [20] one single peak corresponding to aspartic acid was obtained. Furthermore, the same sub-

* Consideration was taken to the presence of N^{15} in the compound.

stance could be transformed to its crystalline copper salt immediately after Dowex-50 chromatography (see below). The evidence that this substance is aspartic acid seems us to be conclusive. The identity of the second substance with ammonia was established in the following way: After removal of solvent, the substance was dissolved in a small amount of water and distilled into 0.01 *N* HCl in a Kjeldahl apparatus after addition of borate buffer (pH = 10.5). All nitrogen could be distilled in this way. When alkaline hypobromite was added to the solution for mass spectrometer analysis gaseous nitrogen was obtained showing the original presence of ammonia.

The aspartic acid from the first peak was purified as its copper salt. For this purpose the solvent from the combined fractions was carefully removed by repeated evaporation *in vacuo*. The dry residue was transferred to a small centrifuge tube with a minimum amount of water (1—1.5 ml), and neutralized to pH 7—8 by addition of a few drops of *N* NaOH. A saturated solution of $CuSO_4$ (0.4 ml) was added. The pH of the solution was adjusted to 5—6 by dropwise addition of 0.2 *N* NaOH. Crystallization was allowed to take place in the ice box for 2—3 days. The crystals were then centrifuged and washed twice with 0.5 ml of ice cold water. The yield of aspartic acid after crystallization was about 40 % calculated on orotic acid. When the degradation was carried out in a N^{15} experiment the copper aspartate and the ammonia from the second peak were directly analyzed for N^{15}.

When isotopic carbon was used the copper aspartate was further degraded with ninhydrin. The copper aspartate was suspended in 5 ml hot water and treated with H_2S for 15 min. The CuS was centrifuged and the precipitate washed twice with a few ml hot water. The combined supernatants were transferred to a small flask and 100 mg citrate buffer pH 2.5 was added. A stream of nitrogen was passed through the solution for 5 minutes while it was warmed on a boiling water bath. After cooling 100 mg of ninhydrin was added and the solution was heated to 100° C for 15 min. During this periode nitrogen was passed through the solution and the CO_2 collected as $BaCO_3$.

The reliability of the method was tested by the degradation of 3-N^{15}-orotic acid. The results are summarized in Table 1 and show that the nitrogen of the copper aspartate was representative of N_3 of the orotic acid. Furthermore this must mean that the carbon dioxide obtained by ninhydrin decarboxylation of the copper aspartate represents C_6+C_7 of orotic acid. The results in Table 1 also show that ammonia is not derived to any significant amount from N_3. We do not feel that ammonia accurately represents N_1 during our experimental conditions, as no special care was taken to avoid the introduction of ammonia from the air, gum arabic, *etc.* However, the discrepancy between the isotope values obtained as ammonia and those calculated by difference are comparatively small. Therefore both values are given in the tables.

A diagram of the degradation is given in Fig. 1.

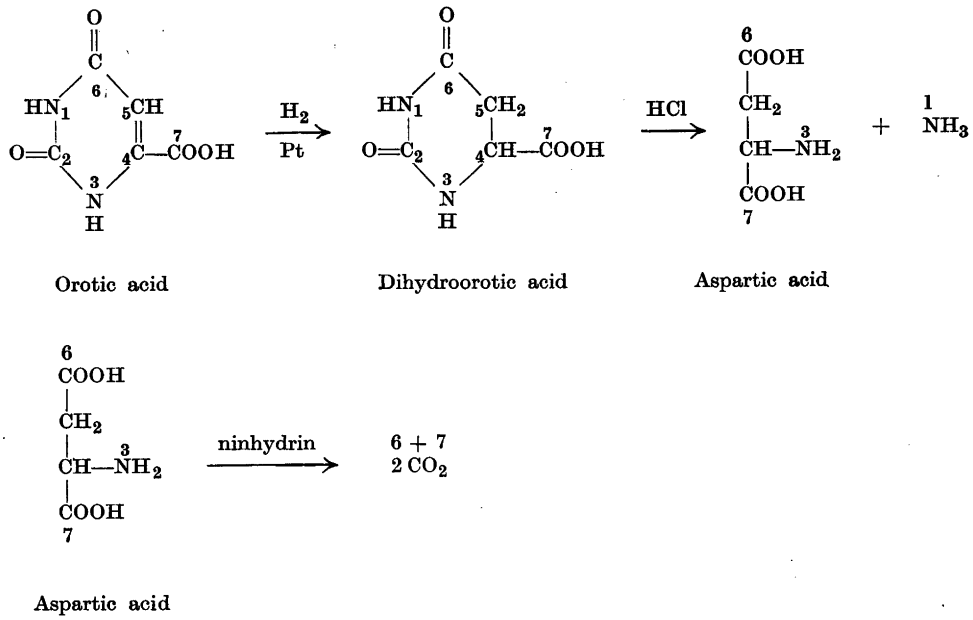
C_2 of orotic acid was obtained by the following degradation. 15—25 mg of orotic acid was dissolved in 3 ml of water by dropwise addition of 0.5 *N* NaOH and warming. A 5 % solution of $KMnO_4$ was added dropwise until the permanganate colour persisted [1]. Precipitation of MnO_2 was facilitated by addition of a few drops of *N* H_2SO_4. The remain-

Table 1. Degradation of 3-N^{15}-*orotic acid.*

Exp. number	Orotic acid	atom % excess N^{15} in N_1^a	N_1^b	N_3
16 [c]	0.415	0.03	0.00	0.83
17 [d]	0.415	0.03		0.74

[a] Determined as ammonia.
[b] Calculated by difference between orotic acid and N_3.
[c] The aspartic acid was crystallized as its copper salt.
[d] The aspartic acid was used directly after chromatography on Dowex-50.

Fig. 1. Degradation of orotic acid.



ing $KMnO_4$ was destroyed with H_2O_2 and the solution was centrifuged. The precipitate was washed twice with 2 ml of warm water. One ml of 5 *N* NaOH was added to the combined supernatants and the solution hydrolyzed at 100° for 15 minutes. One drop of phenol-red was added and the solution was acidified with 5 *N* sulfuric acid. A stream of nitrogen was passed through the solution, which was warmed to 100° C. After cooling 5 *N* NaOH was added until a slight pink colour developed. One ml urease solution [21] was introduced and the mixture kept at 38—42° for 30 minutes. The solution was then acidified with 5 *N* H_2SO_4, placed in a boiling water bath and the CO_2 driven over into a barium hydroxide solution with nitrogen gas. The yield calc. as $BaCO_3$ was 55—60 % of the theory.

Isotope measurements

N^{15} and C^{13} were determined as N_2 and CO_2 respectively in the mass spectrometer. C^{14} was measured as $BaCO_3$ at infinite thickness.

RESULTS

Ammonia as precursor

It has earlier been found that ammonia is a very effective precursor of orotic acid [12]. It was thought of interest to determine the distribution of isotope in orotic acid using this precursor. Slices from three livers were incubated in 50 ml Krebs-Henseleit substrate with 15 mg orotic acid and 1 m*M* of $N^{15}H_4Cl$. The results of the subsequent degradation of the orotic acids from the different experiments are summarized in Table 2.

Table 2. Incubation of liver slices + orotic acid with $N^{15}H_4Cl$ (32 % excess) and subsequent isolation and degradation of orotic acid.

Exp. number	Hours incubation	Atom per cent excess N^{15} in			
		Orotic acid	N_1^a	N_1^b	N_3
1.	1.33	0.67	0.69	0.69	0.65
2.	4	1.71	1.64	1.89	1.53
3.[c]	4	0.82			

[a] Determined as ammonia.
[b] Calculated by difference between orotic acid and N_3.
[c] No orotic acid was added at the beginning of the experiment. Instead 15 mg carrier orotic acid was added before the isolation procedure.

In experiment No. 1 the slices were first incubated with 15 mg orotic acid without $N^{15}H_4Cl$ for 80 minutes at 37°, after which time the isotope was added. This was done in order to get conditions comparable with experiment No. 7.

The degradations after both 80 minutes and 4 hours show an almost equal distribution of the isotope between N_1 and N_3. The distribution of the N^{15} between these two positions was not influenced by the presence of 1 m*M* of non-labeled L-glutamine in a 6 hours experiment.

Aspartic acid as precursor

N^{15}-aspartic acid, 1,4-C^{13}-aspartic acid and 2,3-C^{14}-aspartic acid were used as precursors.

One m*M* N^{15}-*aspartic acid* (33 atom per cent excess) and slices from three livers were incubated in 50 ml Krebs-Henseleit substrate with 15 mg orotic acid for 2 and 8 hours (Table 3).

Table 3. Incubation of liver slices + orotic acid with N^{15}-aspartic acid (33 % excess) and subsequent isolation and degradation of orotic acid.

Exp. number	Hours incubation	Atom per cent excess N^{15} in			
		Orotic acid	N_1^a	N_1^b	N_3
4.	2		0.012		0.106
5.[c]	2	0.093	0.030	0.011	0.175
6.[c]	8	0.638	0.523	0.491	0.785

[a] Determined as ammonia.
[b] Calculated by difference between orotic acid and N_3.
[c] 1.5 m*M* NH_4Cl was added to the substrate.

Both after 2 and 8 hours more isotope was incorporated into position 3 than 1. After 8 hours, however, the difference was much smaller than in the

short time experiments. The presence of non-labeled ammonium chloride did not affect the distribution of N^{15}.

The influence of the presence of non-labeled aspartic acid on the incorporation of isotope from $N^{15}H_4Cl$ is demonstrated by Table 4. Slices from 3 livers were incubated with 15 mg orotic acid and 1.5 m*M* non-labeled aspartic acid in 50 ml Krebs-Henseleit substrate for 80 minutes. After that time 1 m*M* $N^{15}H_4Cl$ + 1.5 m*M* L-aspartic acid were added and incubation allowed to proceed for another 80 minutes. For comparison an identical experiment was performed without aspartic acid.

Table 4. Influence of L-*aspartic acid on the incorporation of* $N^{15}H_4Cl$ *into orotic acid in rat liver slices.*

Exp. number	Isotopic compound	Non-isotopic compound	Atom per cent excess N^{15} in Orotic acid	N_1^a	N_1^b	N_3
1.	$N^{15}H_4Cl$	none	0.67	0.69	0.69	0.65
7.	»	1.5 m*M* L-aspartic acid	0.28	0.56	0.49	0.07

[a] Determined as ammonia.
[b] Calculated by difference between orotic acid and N_3.

The results indicate that the incorporation of isotope into position 3 of orotic acid is considerably diluted in the experiment with non-labeled aspartate.

The experiments with *carbon labeled aspartic acid* were conducted with 0.5 m*M* labeled aspartate + 1.5 m*M* NH_4Cl + 15 mg orotic acid per three rat livers. Incubation was carried out during 8 hours. The orotic acids from three and four experiments respectively were pooled and degraded, with the results shown in Table 5.

Table 5. 1,4-C^{13}-L-*aspartic acid (atom per cent excess = 18) and* 2,3-C^{14}-L-*aspartic acid (8 100 counts/min.) as precursors of orotic acid.*

Exp. number	Isotopic precursor	Amount isotope [a] in Orotic acid	C_2	$C_6 + C_7$	$C_4 + C_5$
8.	C^{13}-aspartic acid	0.25	0.04	0.53	0.04 [b]
9.	C^{14}-aspartic acid	129	2	56	265 [b]

[a] Atom per cent excess in exp. 8, counts/min. in exp. 9.
[b] Calculated by difference between orotic acid, C_2 and $C_6 + C_7$.

The results clearly indicate that the carboxyl groups of aspartic acid were utilized for the synthesis of $C_6 + C_7$ of orotic acid, while the methylene carbons donate their isotope mainly to $C_4 + C_5$. This together with the results obtained with N^{15}-aspartate indicates that the whole molecule of this amino acid was utilized for the synthesis of $N_3 + C_4 - C_7$ of the orotic acid.

Bicarbonate as precursor

From the results of Heinrich and Wilson [22] and of Lagerkvist [23], which showed that CO_2 by the rat was specifically utilized for the synthesis of the ureidocarbon from uracil, it was highly probable that a corresponding relation between bicarbonate and C_2 of orotic acid should exist. In an experiment 2 m*M* $NaHC^{13}O_3$ (atom per cent excess 36) + 1 m*M* of NH_4Cl + 0.5 m*M* L-aspartic acid + 15 mg orotic acid were incubated with slices from 3 rat livers for 6 hours. The isotope content of the orotic acid and that of C_2 of the orotic acid were determined. (Table 6.)

Table 6. $NaHC^{13}O_3$ (atom per cent excess = 36) as precursor of orotic acid.

Exp. number	Isotopic precursor	Atom per cent excess C^{13} in		
		Orotic acid	C_2	C_4, C_5, C_6 and C_7
10	$NaHC^{13}O_3$	0.146	0.534	0.049 [a]

[a] Calculated by difference between orotic acid and C_2.

Ureidosuccinic acid as precursor

The work of Wright [24,25] and coworkers has demonstrated that this acid is an intermediate in pyrimidine biogenesis in *Lactobacillus bulgaricus* 09. Weed and Wilson * have shown that ureidosuccinic acid is utilized for pyrimidine synthesis in liver slices. Because of this an investigation was started to find out, if ureidosuccinic acid was an intermediate in the present system. It was thought to be of advantage to test the L-isomer, especially since in an earlier work only the D,L-isomer had been used. Two types of experiment were carried out. First the N^{15}-L-ureidosuccinic acid was incubated with orotic acid and liver slices for 4 and 7 hours respectively. Second, $N^{15}H_4Cl$ was incubated together with orotic acid and liver slices in the presence of non-labeled D,L-ureidosuccinic acid. In both cases the incorporation of isotope into the orotic acid was determined with the results of Table 7.

Clearly ureidosuccinic acid was very effectively utilized for the synthesis of orotic acid. That the isotope incorporation did not take place after degradation of the acid was shown by the N^{15} values for N_1 and N_3; practically all isotope was located in N_3, as would be expected after direct ringclosure of the ureidosuccinic acid. The dilution experiment furthermore showed that the presence of non-labeled ureidosuccinic acid diminished the incorporation of $N^{15}H_3$ into orotic acid and the isotope content of the ureidosuccinic acid at the end of the experiment demonstrated a *de novo* synthesis of this acid. All this speaks in favour of L-ureidosuccinic acid as a normal intermediate in the biogenesis of orotic acid in liver slices.

* Personal communication from Dr. Wilson.

Table 7. Ureidosuccinic acid as precursor of orotic acid in rat liver slices.

Exp. number	Isotopic [a] precursor	None-isotopic addition	Hours incubation	Atom per cent excess N^{15} in Orotic acid	N_1	N_3
11	0.1 m*M* N^{15}-DL-ureido succinic acid	none	4	0.75		
12	0.2 m*M* N^{15}-DL-ureido succinic acid	none	7	2.70	0.10	5.76
2	1 m*M* $N^{15}H_4Cl$	none	4	1.71		
13	1 m*M* $N^{15}H_4Cl$	0.1 m*M* D,L-ureido succinic acid [b]	4	0.29		

[a] The amounts are given per slices from 3 rat livers. Atom per cent excess N^{15} : $N^{15}H_4Cl$ = 32, N^{15}-*L*-ureido succinic acid = 16.7.

[b] The ureido succinic acid was reisolated after the experiment by addition of 100 mg D,L-ureido succinic acid as carrier and subsequent crystallization. It contained 0.082 per cent excess N^{15}.

DISCUSSION

Some evidence has earlier been obtained by Lagerkvist, Reichard and Ehrensvärd [26] that the carbon chain of aspartic acid was used for the synthesis of the polynucleotide pyrimidines in rat liver slices. N^{15}-aspartic acid, however, donated very little of its isotope to uracil. In view of our present results we think that the earlier finding of the non-utilization of the N^{15} of aspartic acid probably arose from the rapid transamination of the aspartic acid in liver slices as compared to the slow synthesis of polynucleotide pyrimidines. Furthermore a relative permeability barrier has been demonstrated in liver cells for aspartic acid [27], which again would tend to give an erroneous low incorporation of the isotope. The synthesis of orotic acid in liver slices, however, proceeds at a much higher rate than that of polynucleotide pyrimidines, and it was therefore possible to demonstrate the specific incorporation of isotope from N^{15}-aspartic acid into position 3 of the orotic acid. Thus we do not believe that our present results show a difference between the biogenesis of polynucleotide uracil and orotic acid respectively.

The finding that CO_2 was incorporated specifically into position 2 of orotic acid shows the similarity between the synthetic pathway for this substance and for uracil in this respect.

The origin of N_1 is not clear. In earlier experiments *in vivo* Lagerkvist [28] has found that N^{15}-ammonia in rat liver is preferentially incorporated into this position of uracil. In the present system no preferential incorporation of the same precursor could be demonstrated. This is maybe not so surprising in view of the rapid transamination reactions in liver slices. If anything it might indicate that ammonium ions are not the immediate precursor of N_1. The amido group of glutamine is probably not involved as indicated by the fact that the presence of non-labeled glutamine together with $N^{15}H_4Cl$ did not affect the incorporation of N^{15}. This experiment, however, is not considered quite

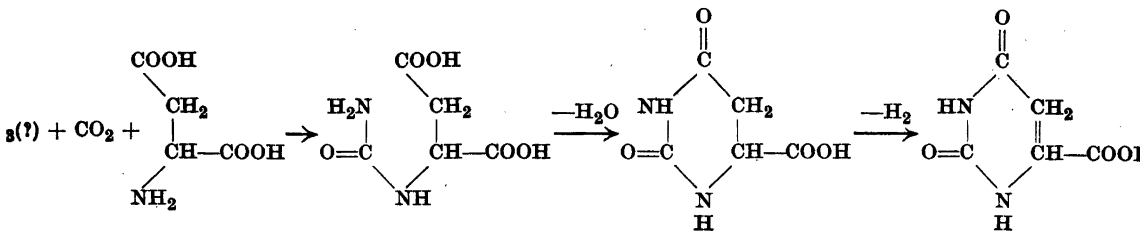
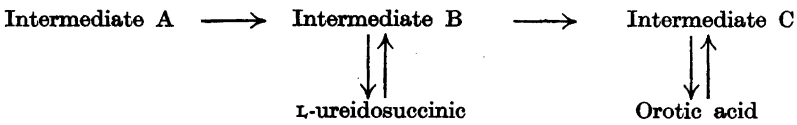


Fig. 2. Biogenesis of orotic acid in rat liver slices.

conclusive and more experiments — if possible in soluble enzyme systems — are needed to answer the question of the origin of N_1 of orotic acid.

Our experiments with L-ureidosuccinic acid are thought to indicate that this substance is an intermediate in the synthesis of orotic acid from aspartic acid. Another possible explanation is given below:



At the present time we cannot see any experimental evidence for this complicated explanation and prefer the more straightforward scheme represented by Fig. 2.

The transformation of ureidosuccinic acid to orotic acid requires at least two steps: ringclosure, and the introduction of a double bond. Depending on which of these reactions occurs first, either dihydroorotic acid or ureidofumaric acid might be an intermediate. Lieberman and Kornberg [29] have recently demonstrated that in an extract from an anaerobic bacterium the synthesis of orotic acid from ureidosuccinic acid proceeds *via* dihydroorotic acid. Spicer *et al.*[30] had earlier reported that dihydroorotic acid was not an intermediate in the formation of orotic acid from ureidosuccinic acid in *Lactobacillus bulgaricus 09*. In their investigation dihydroorotic acid was synthesized according to Bachstez and Cavallini [31]. However, it has recently been shown that dihydroorotic acid prepared in another way was as effective as orotic acid in promoting growth of *L. bulgaricus 09*.* We therefore consider it probable that dihydroorotic acid is an intermediate in orotic acid synthesis also in rat liver slices.

A summary of the synthetic reactions for the biogenesis of orotic acid as visualized by us is given in Fig. 2.

We wish to acknowledge the valuable technical assistance of Mr. K. Avots, Mr. J. Grabosz and Miss T. Koziorowska.

* Personal communication from Dr. L. D. Wright. This dihydroorotic acid was either prepared enzymatically (Lieberman and Kornberg) or by a new chemical synthesis.

SUMMARY

The biogenesis of orotic acid from different precursors in rat liver slices has been investigated. Using N^{15}-L-aspartic acid, 1,4-C^{13}-L-aspartic acid and 2,3-C^{14}-L-aspartic acid it was found that N_3 plus C_4, C_5, C_6 and C_7 of orotic acid were derived from aspartic acid.

CO_2 was preferentially incorporated into position 2 of orotic acid.

N^{15}-L-ureidosuccinic acid was prepared as a crystalline barium salt from N^{15}-L-aspartic acid. It was found to be transformed to orotic acid in liver slices. The presence of non-labeled D,L-ureidosuccinic acid considerably diluted the incorporation of isotope from $N^{15}H_4Cl$ into orotic acid. At the same time a *de novo* synthesis of ureidosuccinic acid could be demonstrated.

REFERENCES

1. Biscaro, G., and Belloni, E. *Ann. Soc. chim. Milano* **11**, Nos. 1, 2 (1905); *Chem. Zentr.* 1905 II, 64.
2. Loring, H. S., and Pierce, J. G. *J. Biol. Chem.* **153** (1944) 61.
3. Rogers, H. J. *Nature* **153** (1944) 251.
4. Mitchell, H. K., and Houlahan, M. B. *Federation Proc.* **6** (1947) 506.
5. Mitchell, H. K., Houlahan, M. B., and Nyc, J. F. *J. Biol. Chem.* **172** (1948) 525.
6. Arvidson, H., Eliasson, N. A., Hammarsten, E., Reichard, P., v. Ubisch, H., and Bergström, S. *J. Biol. Chem.* **179** (1949) 169.
7. Weed, L. L., and Wilson, D. W. *J. Biol. Chem.* **189** (1951) 435.
8. Hurlbert, R. B., and Potter, V. R. *J. Biol. Chem.* **195** (1952) 257.
9. Edmonds, M., Delluva, A. M., and Wilson, D. W. *J. Biol. Chem.* **197** (1952) 251.
10. Weed, L. L., Edmonds, M., and Wilson, D. W. *Proc. Soc. Exptl. Biol. Med.* **75** (1950) 192.
11. Reichard, P., and Bergström, S. *Acta Chem. Scand.* **5** (1951) 190.
12. Reichard, P. *J. Biol. Chem.* **197** (1952) 391.
13. Krebs, H. A., and Henseleit, K. *Hoppe-Seylers Z. physiol. Chem.* **210** (1932) 33.
14. Wu, H., and Rittenberg, D. *J. Biol. Chem.* **179** (1949) 847.
15. Nystrom, R. F., Loo, Y. H., and Leak, J. C. *J. Am. Chem. Soc.* **74** (1952) 3434.
16. Kushner, M., and Weinhouse, S. *J. Am. Chem. Soc.* **71** (1949) 3558.
17. Nyc, J. F., and Mitchell, H. K. *J. Am. Chem. Soc.* **69** (1947) 1382.
18. Moore, S., and Stein, W. H. *J. Biol. Chem.* **176** (1948) 367.
19. Lagerkvist, U. *Acta Chem. Scand.* **7** (1953) 114.
20. Moore, S., and Stein, W. H. *J. Biol. Chem.* **178** (1949) 53.
21. Sumner, J. B., and Sisler, E. B. *Arch. Biochem.* **4** (1944) 207.
22. Heinrich, M. R., and Wilson, D. W. *J. Biol. Chem.* **186** (1950) 447.
23. Lagerkvist, U. *Acta Chem. Scand.* **4** (1950) 1151.
24. Wright, L. D., Huff, J. W., Skeggs, H. R., Valentik, K. A., and Bosshardt, D. K. *J. Am. Chem. Soc.* **72** (1950) 2312.
25. 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.
26. Lagerkvist, U., Reichard, P., and Ehrensvärd, G. *Acta Chem. Scand.* **5** (1951) 1212.
27. Kamin, H., and Handler, P. *J. Biol. Chem.* **188** (1951) 193.
28. Lagerkvist, U. *Arkiv Kemi* **5** (1953) 569.
29. Lieberman, I., and Kornberg, A. *Federation Proc.* **12** (1953) 239.
30. Spicer, D. S., Liebert, K. V., Wright, L. D., and Huff, J. W. *Proc. Soc. Exptl. Biol. Med.* **79** (1952) 587.
31. Bachstez, M., and Cavallini, G. *Ber.* **66** (1933) 681.

Received July 1, 1953.