Fatty Livers Induced by Orotic Acid

II. CHANGES IN NUCLEOTIDE METABOLISM*

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(Received for publication, February 11, 1963)

Rats fed a purified diet supplemented with orotic acid (1%) have gross alterations in their lipid metabolism that can be essentially nullified by further supplementation of the diet with adenine sulfate (0.25%), as described in the first paper of this series (1). Accompanying these changes are certain alterations in the metabolism of pyrimidine derivatives, which have been described in an earlier report (2). Other reports of alterations in the pools of pyrimidine nucleotides in animals fed orotic acid have appeared in preliminary form (3). Hurlbert and Potter (4) have studied the fate of tracer doses of orotate-6-C14 with emphasis on its role as a precursor of the pyrimidine-containing components of nucleic acids. The present report presents an analysis of the fate of dietary orotic acid and the levels of purine and pyrimidine nucleotides in the livers of rats fed various supplemented diets, as well as studies of the formation of nucleotides and the catabolism of orotate in liver slices and cell-free extracts.

EXPERIMENTAL PROCEDURE

Male Sprague-Dawley rats weighing 100 to 150 g were housed in suspended wire cages and given water and standard laboratory chow pellets ad libitum. Three days before each experiment a purified diet ad libitum was substituted for the laboratory chow. At the beginning of each experiment various supplements were added to the purified diet, as indicated.

Fate of Dietary Orotic Acid—Seven rats were given a liquid diet by gastric intubation. A mixture of 75% sucrose, 12% casein, 10% vitamin mixture, 2% corn oil, and 1% orotic acid-6-C14 (47 × 10^6 c.p.m. per mmole) was suspended in water at a concentration of 0.33 g per ml so that about 7% of the daily food intake could be administered in a volume of 2 ml. The rats were housed individually in metabolic cages, where they were offered water and a dry purified diet containing 1% unlabeled orotic acid ad libitum. The respiratory CO₂ was trapped in two series (1). Accompanying these changes are certain alterations in the metabolism of pyrimidine derivatives, which have been described in an earlier report (2). Other reports of alterations in the pools of pyrimidine nucleotides in animals fed orotic acid have appeared in preliminary form (3). Hurlbert and Potter (4) have studied the fate of tracer doses of orotate-6-C14 with emphasis on its role as a precursor of the pyrimidine-containing components of nucleic acids. The present report presents an analysis of the fate of dietary orotic acid and the levels of purine and pyrimidine nucleotides in the livers of rats fed various supplemented diets, as well as studies of the formation of nucleotides and the catabolism of orotate in liver slices and cell-free extracts.

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orotate for PP-ribose-P; this preparation could be stored for several weeks at -10° without loss of activity. Measurements of this competition were carried out in modified Erlenmeyer flasks (5), each of which contained, in a final volume of 1.5 ml, 0.2 ml of the particle-free preparation, orotate-C14O4H (0.15 μmole, 150,000 c.p.m.), 0.25 ml of phosphate buffer (pH 7.2), and a PP-ribose-P-generating system equivalent to one-half of the final concentration previously employed (6); different amounts of adenosine sulfate and additional PP-ribose-P were added. After incubation at 37° for 20 minutes, the reaction was stopped by the addition of 0.3 ml of 6 N perchloric acid. The C14O4 evolved during the reaction was trapped in the center well in 0.2 ml of 2 N NaOH, a 0.1-ml aliquot of which was assayed for radioactivity in a liquid scintillation counter. Conversely, experiments to determine the influence of orotic acid on the condensation of adenosine and PP-ribose-P employed similar incubation conditions, except that the labeled compound was adenosine-8-C14 (0.15 μmole, 300,000 c.p.m.) and the orotate (added in varying concentrations) was unlabeled. The supernatant fraction, obtained after stopping the reaction with 0.3 ml of 6 N perchloric acid, was adjusted to pH 9 with potassium hydroxide, centrifuged at 0-4°, and passed through a 5-ml column of Dowex 1-X4 (formate form, 100 to 200 mesh). The column was washed subsequently with 5 ml of water and eluted with three 10-ml portions of 0.1 N formic acid to remove adenine and nucleoside derivatives; adenine nucleotides were eluted with four 10-ml portions of 2 N ammonium formate. The eight fractions thus obtained were assayed for radioactivity, and the proportion of total radioactivity found in the four ammonium formate fractions was taken as a measure of condensation of adenine with PP-ribose-P.

Measurements of the catabolism of the ring structure of orotate were carried out in modified Erlenmeyer flasks containing orotate-2-C14 (0.1 μmole, 192,000 c.p.m.). In the experiments with liver slices, each flask contained, in addition, 400 to 500 mg of slices (prepared freehand), 1.5 ml of a modified Krebs III buffer (7), and appropriate supplements in a final volume of 1.65 ml. In the experiments with cell-free preparations, each flask contained, in addition to the labeled substrate, 0.5 ml of 0.5 M Tris buffer (pH 7.2), 0.5 ml of the cell-free preparation, 1.5 μmoles of PP-ribose-P (Mg2+ salt), and 0.2 μmole of NADPH in a final volume of 1.55 ml. Cell-free extracts were freed from low molecular weight compounds by passage at 4° through a column of Sephadex (G-25, medium mesh, 1 × 24 cm) and elution with 0.05 M Tris buffer, pH 7.4. This fractionation yielded a preparation that contained 100% of the original protein content. After precipitation of the protein, the preparation exhibited less than 5% of the original absorption at 260 mμ; this finding indicated that it accounted for less than 5% of the absorbed isotope. It is apparent that animals given a diet supplemented with orotic acid for 10 days instead of 1 day metabolize a greater proportion of the labeled orotate to C14O2; however, the amount of radioactivity found in the liver was similar to that in the urine of both groups of animals.

After an intraperitoneal tracer dose of orotate-6-C14, only 41% of the absorbed radioactive material was metabolized to C14O2 in 24 hours, but the amounts recovered from the liver and the urine were still equal (Table I). Under these experimental conditions, the carcass contained significant radioactivity, and the determination of the oxidized pyridine nucleotides, 1 part of liver was homogenized in 25 parts of 0.01 M H2SO4-0.1 M Na2SO4 and heated at 60° for 45 minutes, whereas in the determination of the reduced pyridine nucleotides, 1 part of liver was homogenized in 25 parts of 0.02 N NaOH containing 8 mg of cysteine per 100 ml; the homogenate was heated at 60° for 10 minutes. The alkaline extract was cooled and neutralized by the addition of 0.2 N Tris buffer (pH 6.5). Both extracts were then centrifuged at 20,000 × g for 30 minutes, and the supernatant solutions were assayed for pyridine nucleotides by the fluorometric method described for tissue extracts by Lowry, Roberts, and Kapphan (9). The fluorescence was measured on a Turner fluorimeter with the use of a Corning No. 7-00 primary filter and a combination of Kodak Wratten No. 8 and No. 75 secondary filters. These filters correspond to a peak excitation wavelength of 360 μμ and a peak fluorescence wavelength of 490 μμ.

**RESULTS**

Fate of Orotate in Vivo—Twenty-four hours after the intragastric administration of orotic acid-6-C14, the fecal material contained from 2 to 59% of the total radioactivity recovered; the variation is probably attributable to variable absorption of orotate from the gut. It was more meaningful, therefore, to express the rate of absorption of radioactive orotate as the percentage of the total radioactivity absorbed (Table I). The radioactivity of the carcase (including all organs except the liver) is not included in these results, since preliminary determinations on two animals indicated that it accounted for less than 5% of the absorbed isotope.

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distribution of radioactivity 24 hours after administration of orotate-6-C14</td>
</tr>
<tr>
<td>The radioactivity of each fraction was measured as described in 'Experimental Procedure.' Percentages are expressed on the basis of the total amount of radioactivity absorbed; figures within parentheses show ranges.</td>
</tr>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>After 1 day on orotic acid diet</td>
</tr>
<tr>
<td>CO2</td>
</tr>
<tr>
<td>Urine</td>
</tr>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Carcass</td>
</tr>
</tbody>
</table>

* Mean values from four rats.
* Mean values from three rats.
* Mean values from two rats.
* An asterisk indicates that the data were not included in the calculation (see "Results").
Fatty Livers Induced by Orotic Acid. II

Vol. 238, No. 7

OROTIC ACID + ADENINE DIET

VOLUME OF ELUATE IN ML

FIG. 1. Representative ion exchange chromatograms of the acid-soluble fraction of liver from rats fed a purified diet, a purified diet plus 1% orotic acid, or a purified diet plus 1% orotic acid and 0.25% adenine sulfate. Each diet was fed for 10 days. For the details of the fractionation, see "Experimental Procedure." The peak marked A corresponds to UMP, B to UDP-N-acetylglucosamine, C to UDP-glucose, D to AMP, E to UDP, F to ADP, and G to ATP.

FIG. 2. The changes in acid-soluble nucleotides from livers of rats fed the indicated diets. Each point is the average value for two animals and represents the sum of the total nucleotides containing either uracil or adenine, as calculated from the areas under the peaks identified in the same manner as in Fig. 1.

Fig. 1 presents representative profiles of ultraviolet-absorbing material obtained by ion exchange chromatography of the acid-soluble fraction of the livers of rats that had received the indicated diets. Peak A was identified as UMP by electrophoresis, paper chromatography, hydrolysis to uracil, and the ultraviolet spectrum. The subsequent peaks were identified as nucleotides of uracil and adenine by their absorption at 260 and 280 nm, and by comparing their elution volumes with those of standards. Thus, B corresponds to UDP-N-acetylglucosamine, C to UDP-glucose, D to AMP, E to UDP, F to ADP, and G to ATP. In further support of the identification of the peaks, a tracer dose of orotate-6-C14 was injected intraperitoneally into an animal that previously had been fed the diet supplemented with 1% orotic acid for 24 hours. Four hours after the injection the animal was killed, and the acid-soluble fraction of the liver was analyzed by ion exchange chromatography; the peaks of radioactivity coincided with the peaks identified as uracil nucleotides. It is apparent from Fig. 1 that the amounts of the uracil nucleotides were elevated and that those of the adenine nucleotides were decreased in animals fed orotic acid. This alteration was partially prevented, however, if the orotic acid diet was also supplemented with adenine sulfate, 0.25%.

A summary of the changes in these nucleotides as a function of time is given in Fig. 2. Within 1 day, the sum of uracil nucleotides, identified above, reached a level of almost 4 times that of controls, while the sum of the adenine nucleotides was lowered by 50%. These altered levels were maintained for at least 10 days, and in the case of UMP, elevated levels were present after the animal had been on the diet containing 1% orotic acid for 1 year; with 0.25% adenine sulfate present in the diet, as well as 1% orotic acid, however, these changes were minimized. It also was observed that, in animals fed a diet with 0.25% adenine sulfate as the only supplement, the nucleotide levels in the liver were essentially equal to those of controls.

Studies of Nucleotide Metabolism in Liver—The competition between orotate and adenine for PP-ribose-P was assessed in soluble, cell-free extracts. The conversion of orotate to orotidylate and its subsequent decarboxylation to UMP were followed by measuring the C1402 evolved from orotate-C14OOH (Fig. 3). Adenine inhibited this sequence of reactions only in the presence of limiting amounts of added PP-ribose-P, apparently by competing with orotate for PP-ribose-P, since an excess of PP-ribose-P nullified the inhibitory action of adenine. Although, in the analogous reaction (Fig. 4), orotate caused only minor inhibition of the conversion of adenine to adenine nucleotides, this inhibi-

A sample of urine from a rat given orotate-6-C14 by intubation was passed through a charcoal column, and the adsorbed radioactivity was eluted with 50% ethanol containing 2% NH2OH. Of the radioactivity of the urine, 70% was recovered in the eluate. This material was then identified as orotate by paper chromatography in butanol-acetic acid and by its ultraviolet spectrum; other C14-containing compounds were not found.

About 90% of the radioactivity in the acid-soluble fraction of the liver was associated with uracil nucleotides, and a small amount was present in derivatives of cytosine. Despite the high dietary intake of orotate, this compound could not be detected in the acid-soluble extract either by radioactivity or by ultraviolet absorption. The nature of the radioactive compounds in the feces, the carcass, and the acid-insoluble fraction of the liver was not determined.

Chromatography of Acid-soluble Nucleotides in Liver—Fig. 1 presents representative profiles of ultraviolet-absorbing material obtained by ion exchange chromatography of the acid-soluble fraction of the livers of rats that had received the indicated diets. Peak A was identified as UMP by electrophoresis, paper chromatography, hydrolysis to uracil, and the ultraviolet spectrum. The subsequent peaks were identified as nucleotides of uracil and adenine by their absorption at 260 and 280 nm, and by comparing their elution volumes with those of standards. Thus, B corresponds to UDP-N-acetylglucosamine, C to UDP-glucose, D to AMP, E to UDP, F to ADP, and G to ATP. In further support of the identification of the peaks, a tracer dose of orotate-6-C14 was injected intraperitoneally into an animal that previously had been fed the diet supplemented with 1% orotic acid for 24 hours. Four hours after the injection the animal was killed, and the acid-soluble fraction of the liver was analyzed by ion exchange chromatography; the peaks of radioactivity coincided with the peaks identified as uracil nucleotides. It is apparent from Fig. 1 that the amounts of the uracil nucleotides were elevated and that those of the adenine nucleotides were decreased in animals fed orotic acid. This alteration was partially prevented, however, if the orotic acid diet was also supplemented with adenine sulfate, 0.25%.

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this fraction was accounted for, therefore, in the calculation of percentages presented in the table.

In the intubation experiment, the average total recovery of radioactivity was 71% and in the tracer experiment, 73%; these values are similar to those reported previously (4).
tion also was prevented by an excess of PP-ribose-P. In agreement with this explanation, AMP (1 \times 10^{-4} \text{ M}) did not inhibit the formation of orotidylate from orotate, and UMP (6 \times 10^{-3} \text{ M}) had no effect on the formation of adenine nucleotides from adenine.

Earlier studies had indicated an 80% decrease in the catabolism of orotate in liver slices obtained from rats fed for 10 days a diet supplemented with 1% orotic acid (2); in fact, the same decrease was observed with liver slices from animals that had ingested the diet for only 1 day (Table II). Two possibilities exist for the catabolism of the ring structure of orotate. The first implicates a reversal of the pathway for biosynthesis of pyrimidines \textit{de novo}, with the intermediate formation of L-dihydro-orotate and carbamyl-L-aspartate (10, 11), whereas the second involves the prior anabolism of orotate to UMP, with subsequent breakdown to uracil; the latter, in turn, is catabolized through dihydrouracil and carbamyl-\beta-alanine to NH_{3}, CO_{2}, and \beta-alanine (12). Several lines of evidence strongly suggest that, in rat liver, orotate is catabolized primarily via the latter pathway; thus, orotate was catabolized to CO_{2} at a rate of 24.4 mmoles per hour per g of liver slices (Table II), whereas the corresponding rate of catabolism of carbamyl-L-aspartate was only 4.9 mmoles per hour per g. Similarly, in homogenates of liver, in which the influence of cellular permeability was eliminated, carbamyl-L-aspartate also was catabolized to CO_{2} at a slower rate than was orotate. These observations are not consistent with a primary

**Fig. 3.** Percentage inhibition, \textit{in vitro}, by adenine of the conversion of orotate to UMP at different concentrations of PP-ribose-P (PRPP) and orotate. The incubation medium contained: orotate-C^{14}OH, concentrations of adenine sulfate (expressed on the graph as molar ratios relative to the concentration of orotate), particle-free preparation of rat liver, phosphate buffer (pH 7.2), and PP-ribose-P-generating system; additional PP-ribose-P was included as indicated on the abscissa. The uninhibited conversion of orotate to adenine nucleotides proceeded at rates of 48, 75, and 93 mmoles per hour at concentrations of added PP-ribose-P of 0.0, 0.1, and 1.0 \mu mole per ml, respectively. After incubation at 37° for 20 minutes, adenine and adenosine in the medium were separated from phosphorylated derivatives. For further details of the incubation and separation, see "Experimental Procedure."

**Fig. 4.** Percentage inhibition, \textit{in vitro}, by orotate of the conversion of adenine to adenine nucleotides at different concentrations of PP-ribose-P (PRPP) and orotate. The incubation medium contained adenine-8-\textsuperscript{14}N, concentrations of orotate (expressed on the graph as molar ratios relative to the concentration of adenine), phosphate buffer (pH 7.2), and PP-ribose-P-generating system; additional PP-ribose-P was included as indicated on the abscissa. The uninhibited conversion of adenine to adenine nucleotides proceeded at rates of 60, 165, and 315 mmoles per hour at concentrations of added PP-ribose-P of 0.0, 0.1, and 1.0 \mu mole per ml, respectively. After incubation at 37° for 20 minutes, adenine and adenosine in the medium were separated from phosphorylated derivatives. For further details of the incubation and separation, see "Experimental Procedure."

**Table II**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Additions, deletions, or modifications</th>
<th>Diet before assay</th>
<th>Control plus 0.1 % 5-Azaorotic acid for 1 day</th>
<th>CO_{2} formed in tissue or equivalent fraction ( \mu \text{mole/hr/g} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slices</td>
<td>None(^{a})</td>
<td>Control</td>
<td>24.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Cell-free fraction</td>
<td>None(^{b})</td>
<td>344.0</td>
<td>125.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ 5-Azaorotate ( (5 \times 10^{-4} \text{ M}) )</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ azaUMP(^{d}) (6.7 \times 10^{-4} \text{ M})</td>
<td>354.0</td>
<td>324.0</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Each value is the average of four to eight determinations.  
\(^{b}\) The assay system is presented in detail in the text.  
\(^{c}\) The assay system contained an aliquot of the cell-free fraction, buffer, PP-ribose-P, NADPH, and labeled substrate, as described in the text.  
\(^{d}\) 6-Azaoridine 5'-phosphate.
catabolism of orotate via dihydro-orotate and carbamyl-L-aspartate. Strong support for the alternate hypothesis is provided by the following experiments. 5-Azaorotate, a potent inhibitor of the conversion of orotate to orotidylate and UMP (13), decreased the catabolism of orotate by 93% in liver slices (Table II). In addition, in the soluble fraction from liver, PP-ribose-P, a substrate required for the conversion of orotate to orotidylate, was a necessary requirement for the catabolism of orotate to CO₂. Its absence from the assay mixture resulted in an essentially complete loss of activity (Table II). Furthermore, even in the presence of PP-ribose-P, 6-azauridine 5'-phosphate, a potent inhibitor of the decarboxylation of orotidylate (14, 15), completely blocked the catabolism of ring-labeled orotate (Table II). These last three observations lead to the conclusion that orotate must first be anabolized to UMP, before its degradation to CO₂ and β-alanine via the pathway for the degradation of uracil.

The prior conversion of orotate to UMP before degradation to CO₂ complicates an interpretation of the 80% decrease in the catabolism of orotate by livers of animals fed the pyrimidine in their diet. The observed decrease in the release of C⁴O₂⁻ from orotate-2-C⁴ is only in the large pools of unlabeled uracil nucleotides that arise following the ingestion of orotic acid. After supplementation of the diet with orotic acid, cell-free supernatant fractions of liver showed the same relative decrease in orotate catabolism as that seen in liver slices. For this reason, these soluble fractions afforded a system for eliminating the influence of soluble pools. In Table II are shown the results of an experiment in which the high speed supernatant fraction from livers of control rats and of rats fed 1% orotic acid were subjected to fractionation on a Sephadex column to remove the acid-soluble pyrimidine derivatives. Since this treatment eliminated the difference in the catabolic activity between control and experimental animals, it is concluded that the 70 to 80% decrease in the catabolism of orotate seen without fractionation on Sephadex can be attributed to dilution of labeled substrate in nonlabeled pools.

Pyridine Nucleotide Changes—Since the level of adenine nucleotides was decreased in the livers of animals fed the diet containing orotic acid, the effect of this change on the amounts of pyridine nucleotides was determined (Fig. 5). No significant change in the amounts of either NAD or NADH was caused by feeding the diet supplemented with orotic acid for 5 days; however, a significant decrease, of 37% and 49%, respectively, in both NADP and NADPH was observed.

DISCUSSION

Any consideration of the effects of dietary orotic acid on the nucleotide pools of rats must account for the fate of this normal pyrimidine precursor. Since the average daily intake was approximately 100 mg, an amount that approaches the entire pyrimidine content of the body, the metabolism and excretion of this material must be very extensive to prevent accumulation in the tissues. Only 5 to 20% of the absorbed, ring-labeled orotate can be accounted for in the urine, where it is found exclusively in the form of the unchanged parent compound. That only relatively small amounts of orotate are excreted in the urine is a fortunate circumstance, because the solubility of orotate is very low and larger amounts almost certainly would have caused crystalluria and impairment of renal function (16), as has been observed in certain patients who excrete up to 8 g of orotate daily after the administration of 6-azauridine (17).

Although there was no evidence of renal damage in the experimental animals, this result does not exclude the possibility that some of the metabolic effects observed may have resulted from a competition between orotate and other organic acids, normally either excreted or reabsorbed by the renal tubular epithelium, as has been demonstrated in the chicken (18).

The remaining 80 to 95% of the absorbed, ring-labeled orotate was metabolized, chiefly to C⁴O₂⁻ (64 to 90%), indicating that the catabolism of the pyrimidine ring had been extensive. The balance of the radioactivity was found primarily in the liver in the form of nucleotides of uracil and, to a small extent, of cytosine. Significant amounts of other radioactive metabolites could not be detected. It should be emphasized that in spite of the large dietary intake of orotic acid, this compound was not found in the liver.

Since the amount of UMP formed from the dietary orotic acid far exceeded the requirements of the organism for pyrimidines, the degradation of a large proportion of the compound to CO₂ was not unexpected. That this catabolism proceeds through the formation of uracil nucleotides is indicated by (a) the relatively poor breakdown of carbamyl-L-aspartate even in extracts of liver, (b) the dependence of the catabolism in soluble extracts on PP-ribose-P, and (c) its blockade by 5-azaorotate and 6-azauridine 5'-phosphate, which, respectively, inhibit orotidylate pyrophosphorylase (13) and decarboxylase (14, 15).

Previously reported data (2) on the decrease in the catabolism of orotate-2-C⁴ in vitro had been interpreted as attributable to a reduction in the activity of enzymes involved in the catabolic pathway. However, the small, but statistically significant, increase in catabolism of orotate in the intact animal after 10 days on the supplemented diet was not consistent with this observation. The finding that orotate first must be converted to UMP before its breakdown to CO₂ offers a ready explanation for the above mentioned decrease in catabolism in vitro. Thus, the radioactive UMP formed would be diluted in the large pre-existing pools of nucleotides present in rats fed a diet containing 1% orotic acid.

Perhaps the most significant change in the nucleotide metabolism of the livers was the accumulation of large amounts of UMP but also of the higher phosphates and coenzyme derivatives of uridine. That this increased synthesis of uracil
nucleotides may be responsible for the reduction in the amount of adenine nucleotides is suggested by the experiments in vitro that indicate a small but definite inhibition of the formation of adenine nucleotides by orotate, if PP-ribose-P is present in limiting amounts. In these experiments, however, adenine competed more effectively for PP-ribose-P than did orotate. In agreement with this concept is the fact that, with regard to changes in nucleotide levels, adenine can prevent and reverse the effect of 4 times larger amounts of orotic acid in the diet of intact animals. The mechanisms controlling the size of the uracil nucleotide pools in the liver seem unable to cope with large amounts of dietary orotic acid; but, despite the extensive metabolic changes, the rats appear to be healthy and do not show any lessening of life expectancy or reproductive capacity.

For this reason, supplementing the diet with orotic acid offers a relatively physiological means for altering the precursor pools for RNA and DNA biosynthesis in vivo and may be used to study the assembly of these macromolecules in the intact animal or to alter the action of antimetabolites that affect the biosynthesis or utilization of nucleotides.

Rajalakshmi, Sarma, and Sarma (3) have shown that, in rats fed 1% orotic acid for 4 weeks, the total pyridine nucleotides per g of liver are decreased by 40%. The studies reported here show that a 23% decrease in the total amount of pyridine nucleotides occurred after 5 days on the orotic acid-containing diet. But a further analysis of the amounts of individual pyridine nucleotides indicates that the levels of neither NAD nor NADP change significantly, whereas the levels of both NADP and NADPH decrease; in addition, the ratio of oxidized to reduced forms of these cofactors was not altered. Since there is extensive biosynthesis of neutral lipid, which would involve an increased utilization of reduced pyridine nucleotides, these data imply an adequate compensatory mechanism for maintaining the normal ratio. The fact that the increased biosynthesis of lipid could occur in the face of the decreased content of NADPH might be explained by the data of Abraham, Matthes, and Chai- koff (19), who showed that the generation of NADPH normally is not a rate-limiting factor for the biosynthesis of fatty acids. The decreased content of NADP may be the result of a decreased phosphorylation of NAD because of decreased amounts of ad- nine-containing nucleotide cofactors.

At the present time it is not possible to relate the alterations in nucleotide metabolism to the ability of dietary supplementa- tion with orotic acid to spare, and in certain cases to replace, vitamin B<sub>12</sub> in animals (20) and in man (21). It may be associated, however, with a mass action effect on certain enzymatic reactions that involve uracil nucleotides, such as those responsible for the formation of thymine derivatives. Another possibility would be that the extensive changes in the structure of the liver may cause the release of small amounts of vitamin B<sub>12</sub> or its derivatives, which subsequently relieve the deficiency in more critical areas, such as the bone marrow and the central nervous system.

**SUMMARY**

When rats are fed a purified diet containing 1% orotic acid, which causes extensive infiltration of the liver with triglycerides, most of the absorbed orotate is first converted to uridine 5'-phosphate in the liver; subsequently, the pyrimidine ring is degraded, presumably by the enzymes in the liver that degrade uracil. This over-all catabolic reaction requires ribosylpyrophosphate 5-phosphate and is blocked by 5-azauracil and 6-azauridine 5'-phosphate. Although the primary excretion product in the urine was unchanged orotate, this compound could not be detected in the liver. Analysis of the acid-soluble nucleotides in the liver indicated, however, a 4-fold increase of uracil nucleotides associated with a 50% decrease in adenine nucleotides, including nicotinamide adenine dinucleotide phosphate and its reduced form. These changes were largely nullified if the diet also was supplemented with 0.25% adenine sulfate. Enzymatic studies indicated that adenine effectively competes with orotate for limiting amounts of ribosylpyrophosphate 5-phosphate in cell-free extracts, and that the apparent decrease in the rate of catabolism of radioactive orotate by slices or extracts of livers from animals fed orotic acid is caused by dilution of the radioactivity in the large pools of uracil nucleotides.

**Acknowledgment**—The authors gratefully acknowledge the capable technical assistance of Mrs. M. Cecrasta.

**Addendum**—While this manuscript was in preparation, Marchetti, Puddu, and Calderara (22) reported studies on acid-soluble nucleotides in livers of rats fed different amounts of orotic acid for 4 weeks. With a supplement to the diet of 1% orotic acid, these authors noted a doubling of the uracil nucleotides, as opposed to the 4-fold increase observed in the present studies. This difference may be attributable to the different periods of time that the two sets of animals were fed the experimental diets.

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