Increased Activity of Enzymes for *de Novo* Pyrimidine Biosynthesis after Orotic Acid Administration

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SUMMARY

Administration of a purified diet supplemented with 1% orotic acid is attended by a marked elevation in the specific activities of liver aspartate transcarbamylase (ATC), ornithine transcarbamylase, and dihydroorotase (DHOase) and in alterations in the composition of the acid-soluble nucleotide pool. Return of the rats to the control diet is followed by an immediate reinstatement of the normal acid-soluble nucleotide composition and by a slower return of the enzyme activities to basal values. The half-life of the return has been calculated as 2.5 and 0.5 days, for ATC and DHOase, respectively. Both ATC and DHOase activities are augmented in regenerating liver derived from rats fed either control or orotic acid diet. The studies suggest that high intracellular concentrations of pyrimidine nucleotides may not "repress" the enzymes involved in the *de novo* synthesis of pyrimidines in mammalian liver.

INTRODUCTION

In bacterial systems, pyrimidine biosynthesis is subject to regulatory mechanisms which may function in the control of cell growth (1). In this system, a feedback mechanism is operative in which the end-product of pyrimidine synthesis, cytidine triphosphate, regulates the activity of aspartate transcarbamylase, an enzyme which catalyzes the formation of carbamyl aspartate from L-aspartic acid and carbamyl phosphate. Yates and Pardee (1) have also shown that the *synthesis* of this enzyme is under metabolic control, i.e., repression. Subsequent studies have established the allosteric nature of the *Escherichia coli* aspartate transcarbamylase (2, 3). The enzyme from mammalian systems (4, 5) does not behave in a similar fashion; end-product inhibition is not manifested by cytidine triphosphate or by other pyrimidine derivatives at reasonable concentrations.

Dihydroorotase, an enzyme which catalyzes the cyclization of carbamyl aspartate to dihydroorotic acid appears also to be a locus for feedback inhibition of pyrimidine biosynthesis in *E. coli* (1, 6). Although end-product inhibition of dihydroorotase is demonstrable in mammalian systems (7, 8), it is not known whether this mechanism plays any physiological role in the latter.

The effective enzyme activity within a tissue may also be modulated by the *amount* of enzyme, i.e., synthesis. Perhaps the more important means for controlling pyrimidine biosynthesis *de novo* in mammalian systems is related to altering the actual levels of the enzymes. It was of interest then to determine whether the effective activities of both dihydroorotase and aspartate transcarbamylase could be regulated by alterations in the intracellular concentrations of pyrimidine nucleotides.

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Previous reports had indicated that administration of a purified diet supplemented with orotic acid leads to alterations in the metabolism of pyrimidine derivatives and to increases in the liver pools of pyrimidine nucleotides (9–11). The orotic acid-fed rat appeared to be ideally suited for ascertaining the effects of high levels of the pyrimidine nucleotides upon liver aspartate transcarbamylase and dihydroorotase activities. The results of the investigation reported herein indicate that the activities of both these enzymes are elevated rather than depressed by administration of the orotic acid-containing diet. The results also show that it is not possible to "repress" the augmentation in enzyme activities that occurs in regenerating liver by means of high levels of pyrimidine nucleotides.

MATERIALS AND METHODS

Animals. Male rats, approximately 100–130 g, purchased from the Cheek-Jones Co., Houston, Texas, were employed in this study. The rats were fed pellets of a purified diet obtained from the Nutritional Biochemicals Corporation, a vitamin B complex test diet (complete), for at least 10 days prior to the actual experimentation. They were then either continued on the same diet or were offered pellets of the vitamin B complex test diet containing 1% orotic acid. Both groups of rats ingested the same quantity of food daily.

In several experiments, the animals were partially hepatectomized according to the procedure of Higgins and Anderson (12). These rats had also been maintained on the purified diet (but without orotic acid).

Enzyme assays. Periodically, the rats were sacrificed by exsanguination, the livers were removed and washed in cold 0.25 M sucrose, and a 20% homogenate in 0.25 M sucrose was prepared at 4°. The homogenate was centrifuged at 100,000 *g* for 60 min and the middle third of the supernatant fraction which did not contain any visible fat nor was contaminated with loosely packed microsomes was removed and employed as the enzyme source. The protein concentration of the latter, as determined by the method of Lowry *et al.* (13), was approximately 15 mg/ml.

Aspartate transcarbamylase activity in the extract was assayed as described previously (4). The components of the assay mixture included: DL-aspartic acid-4-^{14}C 1.3 mM (0.1 μC); dilithium salt of carbamyl phosphate, 5 mM; supernatant fraction, 0.2 ml (approximately 3 mg protein); 0.2 M Tris buffer, pH 9.2, up to 1.5 ml. After a 15-min incubation at 37°, the assay tubes were placed in an ice bath and the reaction was stopped by the addition of 0.2 ml of 4 N $HClO_4$. The denatured protein was removed by centrifugation, phenol red was added to the extract, and the latter was neutralized with 4 N KOH. After 5 min at 4°, the $KClO_4$ was removed by centrifugation. The neutralized extract was placed on a column (0.6 × 5 cm) of Dowex 50, H^+, X8, 200–400 mesh. The eluate and water washings which contained the carbamyl aspartate-^{14}C were collected and the radioactivity was determined by liquid scintillation counting in Bray's phosphor solvent (14). The efficiency under these conditions was 70%. Control tubes were run in which either the carbamyl phosphate or enzyme had been omitted. In the early studies, aspartate transcarbamylase was assayed by an additional method utilizing carbamyl phosphate-^{14}C as the labeled precursor (4). The results with both assays were comparable, and in subsequent investigations the assay employing the labeled aspartic acid was employed.

Dihydroorotase activity in the 100,000 *g* supernatant fraction was assayed as described by Bresnick and Blatchford (15) in the following assay system: 100,000 *g* supernatant fraction, 0.5 ml; carbamylaspartic acid, neutralized, 5 mM; 0.1 M phosphate buffer, pH 6.5, to 3.0 ml. Suitable controls were run in which carbamyl aspartate was not present in the assay mixture. After an incubation period at 37° for 30 min, the tubes were immersed in an ice bath, 0.2 ml of 4 N $HClO_4$ was added, and the denatured protein was removed by centrifugation. The dihydroorotic acid produced in the reaction was measured by the decrease in absorbance at 240 mμ which

the pyrimidine undergoes in the presence of alkali. To 1.4 ml of deproteinized extract was added in a cuvette 1.4 ml of 1.5 N NaOH. The cuvette was quickly shaken and the absorbance at 240 mμ was recorded by using a Beckman DB spectrophotometer with a Sargent recorder. The blank cuvette contained 1.4 ml of the incubated control (no carbamyl aspartate). Dihydroorotase activity was expressed as ΔA_{240}[2] per 4 minutes.

Ornithine transcarbamylase activity was determined by the method of Brown and Cohen (16) employing carbamyl phosphate-^{14}C as the labeled precursor. The carbamyl phosphate-^{14}C had been prepared from potassium cyanate-^{14}C as described by Spector *et al.* (17) and had a specific activity of 28 μC/mmole. The enzyme source was a 4000 *g* supernatant fraction (15 min) of a 10% homogenate prepared in 0.1% CTAB.

Isolation of acid-soluble and DNA fractions. Segments of liver were quick-frozen in dry ice-acetone and were stored until analyzed. The frozen liver was weighed and a 20% homogenate was prepared in 0.6 N $HClO_4$ at 4°. An aliquot of the homogenate was centrifuged in the cold, the extract was saved, and the precipitate was resuspended in cold 0.3 N $HClO_4$. The procedure was repeated. The combined extracts represented the *acid-soluble fraction.* The precipitate was defatted with 70% ethanol, 95% ethanol, and ethanol-ether (3:1). The nucleic acids were extracted from the defatted precipitate by heating the latter in 10% trichloroacetic acid at 90° for 30 min. The suspension was cooled, centrifuged and the precipitate was resuspended in 5% trichloroacetic acid. The latter suspension was centrifuged and the extracts were combined to yield the *nucleic acid fraction.* The DNA content of this fraction was ascertained by the Burton modification (18) of the Dische method.

The acid-soluble fraction was boiled for 1 hr to convert the purine nucleotides to purines and the pyrimidine nucleoside di- and triphosphates to monophosphates. The acid-soluble fraction was neutralized in the cold with 4 N KOH, and the precipitate of $KClO_4$ was removed by centrifugation. The absorbance of a sample of this extract was determined at 260, 280, and 320 mμ. One milliliter of the neutralized extract was passed through a column (0.5 × 5 cm) of Dowex 50, H^+, X8, 200–400 mesh, the eluate was collected, and the column was washed twice with 2 ml of H_2O. The purines were absorbed and remained on the Dowex 50. The combined eluate and washings represented the total acid-soluble pyrimidine nucleotide fraction. Its absorbance at 260, 280, and 320 mμ was determined.

Incorporation of radioactive orotic acid into RNA. Orotic acid-6-^{14}C, 4 μC, was injected intraperitoneally into male rats, and the rats were sacrificed 45 min later. The livers were removed, the acid-soluble extract was prepared, and the pyrimidines were concentrated as described above. The RNA was isolated and quantitated by the method of Fleck and Begg (19). The radioactivity was determined by liquid scintillation counting.

Chemicals. DL-Aspartic acid-4-^{14}C (2.5 μC/μmole) was obtained from Volk Radiochemicals; potassium cyanate-^{14}C (5 μC/μmole) and orotic acid-6-^{14}C (5 μC/μmole) were purchased from New England Nuclear Corporation.

RESULTS

One day after the ingestion of the purified diet containing 1% orotic acid, the activities of aspartate transcarbamylase and dihydroorotase increased by 70 and 175%, respectively (Fig. 1); at 2 days, by 115 and 200%. Enzyme activities gradually reached a plateau after 2 days and no further increase was evident even after 2 weeks on the diet containing the pyrimidine.

In confirmation of previous reports (9–11), the composition of the acid-soluble fraction is grossly affected by the adminis-

[2] ΔA_{240}, decrease in absorbance at 240 mμ; A_{260}, absorbance at 260 mμ; DHO, dihydroorotic acid; ATC, aspartate transcarbamylase; OTC, ornithine transcarbamylase; DHOase, dihydroorotase; DNA, deoxyribonucleic acid; CTAB, cetyltrimethylammonium bromide; RNA, ribonucleic acid; RNA-P, ribonucleic acid phosphorus.

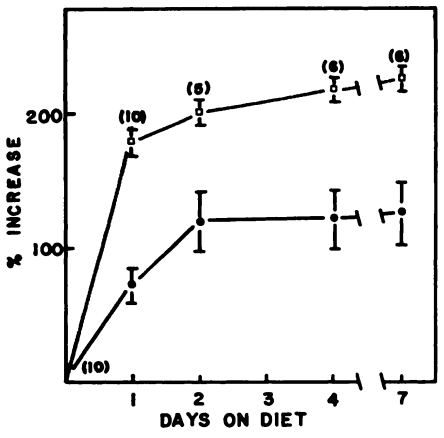

FIG. 1. *ATC and DHOase activities in liver of rats fed orotic acid diet*

Male rats were maintained on vitamin B complex test diet (complete) for 10 days and then transferred to a diet supplemented with 1% orotic acid. The specific activities of ATC (●——●) and of DHOase (□ - - - - □) were determined as described in the text. At each point are presented the mean ± standard error and in parentheses, the number of rats. The percent increase in specific activity of ATC and DHOase is presented on the ordinate. The specific activity of liver ATC of these rats in terms of dpm product/mg protein was 1800 ± 150; that of liver DHOase, in terms of ΔA_{240}/4 min/g protein, was 9.0 ± 0.4.

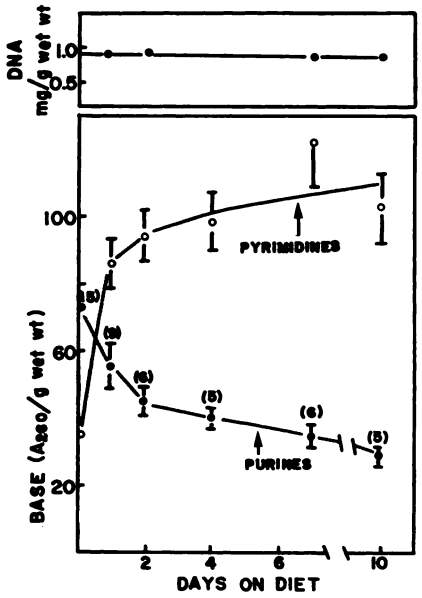

FIG. 2. *Alterations in the composition of the acid-soluble fraction of liver of rats fed orotic acid*

See Legend to Fig. 1. The concentrations of acid-soluble pyrimidines and purines were ascertained as described in the text.

tration of the orotic acid diet (Fig. 2). The concentration of pyrimidines in terms of A_{260}/g wet weight of liver, is augmented by a factor of 3 at 1 day after ingestion of the diet, and gradually plateaued at this elevated level by 2–3 days. The purines, on the other hand, were depressed gradually, reaching a minimum value by 2–3 days. The latter was approximately 50% of the value present in liver under control diet conditions. The concentration of DNA in the liver was not altered by these dietary conditions (upper chart of Fig. 2).

The interrelationship between the marked change in the composition of the liver acid-soluble fraction and of several of the enzymes in the pathway for *de novo* pyrimidine biosynthesis is indicated in Fig. 3. Within 1 day after return to the control diet, the acid-soluble pyrimidine concentration had returned to the normal basal level and the purine concentration had increased to almost control values (upper Fig. 3). ATC and DHOase activities, however, returned slowly to the lower basal control values (lower Fig. 3). The specific activities of ATC and DHOase reached the control values by 4 and 2 days, respectively, after return to regular diet. The $t_{1/2}$ for the return has been calculated and is 2.5 and 0.5 days for ATC and DHOase, respectively.

The permanency of the alterations in the specific activity of these enzymes was investigated in another manner. In previous communications, Handschumacher and colleagues (20, 21) had reported the nullification by 0.25% adenine sulfate of the gross alterations induced by dietary orotic acid. The effects of the dietary purine upon the orotic acid-induced changes in the enzymatic activities are demonstrated in Table 1. Inclusion of adenine sulfate in the diet not only nullified the orotic acid-induced augmentation in acid-soluble pyrimidines and depression of acid-soluble purines, but helped reestablish the basal control activities of ATC and DHOase. All values were

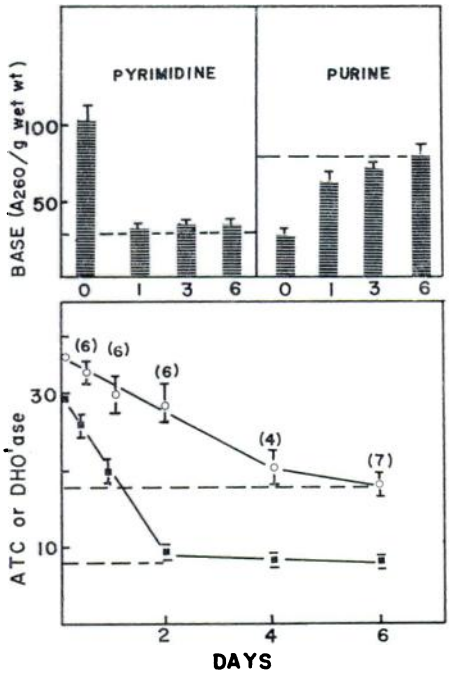

FIG. 3. *Changes in enzyme activity and in acid-soluble composition after return to control diet*

Male rats were placed on orotic acid diet for 10 days after an equilibration period on control diet. The rats were then transferred to control diet (no orotic acid) for the days indicated on the abscissa. Enzyme activity and the composition of the acid-soluble fraction were determined as described in the text. The control values (on control diet) are indicated by the dashed lines. At each point are presented the mean ± standard error. On the ordinate are given the specific activities of ATC as dpm × 10^{-2}/mg protein (○——○) and DHOase as ΔA_{240}/4 min/g protein (■——■).

returned to control levels by 3 days of the orotic acid-adenine diet. Inclusion of adenine sulfate in the diet alone exerted little effect upon the specific activities of ATC and DHOase.

The effect of the orotic acid regimen upon the incorporation of radioactive orotic acid into liver RNA is indicated in Table 2. The total incorporation of the isotope into acid-soluble pyrimidines per gram of liver was independent of the dietary regimen although the specific activity of the pyrimidine pool was decidedly less in orotic acid-fed rats. Consequently, the specific activity of the RNA in the latter was also lower.

Effect of Orotic Acid Diet upon Pyrimidine Biosynthesis in Regenerating Liver

ATC activity is markedly elevated in regenerating liver (22, 23). The present study was designed to ascertain whether the concentration of intracellular pyrimidine nucleotides plays any role in the modulation of ATC activity in the regenerative process and, in particular, whether the augmentation which takes place subsequent to partial hepatectomy could be prevented by high concentrations of these substances. The results are graphically presented in Figs. 4 and 5. In these studies,

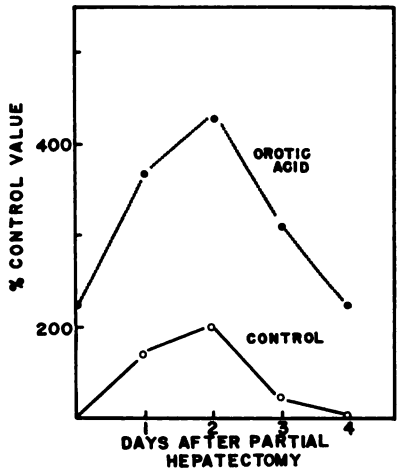

FIG. 4. *Effect of orotic acid diet upon ATC activity in regenerating liver*

Male rats were maintained on control diet and then either kept on this diet or placed on the orotic acid-containing diet for 10 days. The rats were partially hepatectomized and the liver ATC activity was determined as described in the text. Each point represents the average of 6–10 animals. On the ordinate the ATC activity is presented as percent of control value, i.e., specific activity of liver ATC from control diet-fed rats, 1800 ± 150 dpm/mg protein.

a 100% increase in liver ATC activity was noted 2 days after partial hepatectomy (Fig. 4) in rats both on control and orotic acid-supplemented diets. At 4 days after partial hepatectomy, ATC activity had just

TABLE 1

ATC and DHOase activities with different diets

Male rats were fed vitamin B complex test diet (complete) for 10 days before the start of the experiment (control diet). The rats were then switched to the purified diet containing 1% orotic acid for 10 more days. At this time, a group of animals were transferred to the purified diet containing 1% orotic acid and 0.25% adenine sulfate. The methodology is described in the text. The specific activity of ATC is in terms of dpm of product per milligram of protein; the specific activity of DHOase is ΔA_{240}/g protein/4 min.

Diet	Acid-soluble		Specific activity	
	Pyrimidines (A_{260}/g wet wt.)	Purines (A_{260}/g wet wt.)	ATC	DHOase
Control (10)[a]	34.4 ± 2.8[b]	73.2 ± 5.9	1800 ± 150	9.0 ± 0.4
Orotic acid, 10 days (6)	102 ± 10	28.3 ± 2.0	3950 ± 300	29.2 ± 1.0
Orotic acid, 10 days; orotic acid-adenine, 1 day (6)	58.6 ± 3.5	50.5 ± 2.5	3220 ± 200	20.8 ± 2.0
Orotic acid, 10 days; orotic acid-adenine, 2 days (6)	45.4 ± 3.0	75.1 ± 4.2	2510 ± 90	14.3 ± 1.5
Orotic acid-adenine 3 days (6)	35.6 ± 3.0	74.1 ± 5.5	1820 ± 130	10.0 ± 0.6

[a] Number of rats.

[b] Mean ± standard error.

about returned to basal level for each group.

The specific activity of liver DHOase was markedly elevated in regenerating liver, reaching a maximal value at 2 days after partial hepatectomy (Fig. 5). In the rats fed control diet, this value was 280% of the basal level. The magnitude of the increase in rats fed the diet supplemented with orotic acid was not as great although the basal level itself was 320% of the value observed in the livers of control diet fed rats.

ATC activity, as presented above, is elevated in the livers of orotic acid-fed rats and in regenerating liver. It was of interest to ascertain whether other transcarbamylases would undergo similar changes. Toward this end, OTC was employed as the model. The data are offered in Figs. 6 and 7.

The specific activity of liver OTC was also significantly elevated in orotic acid-fed rats (Fig. 6). Regeneration, however, resulted in a decrease in enzyme activity by day 2 after partial hepatectomy (Fig. 7) in rats fed control diet. In those animals receiving the diet supplemented with 1% orotic acid, the OTC activity declined immediately after partial hepatectomy.

TABLE 2

Incorporation of labeled orotic acid into acid-soluble pyrimidines and RNA in orotic acid fed rats

Male rats were fed either the vitamin B complex test diet (control diet), or control diet supplemented with 1% orotic acid for 4 days. Orotic acid-6-^{14}C (5 μC/μmole), 4 μC, was injected intraperitoneally into the rats; the livers were removed 45 min later and processed for acid-soluble pyrimidines and RNA as described in the text.

Diet	Acid-soluble pyrimidines		RNA, dpm/μg RNA-P
	dpm/A_{260}	dpm/mg liver	
Control (4)[a]	11,500 ± 1200[b]	414	70.0 ± 4.0
Orotic acid (4)	4,630 ± 500	417	37.5 ± 1.6

[a] Number of rats.

[b] Mean ± standard error.

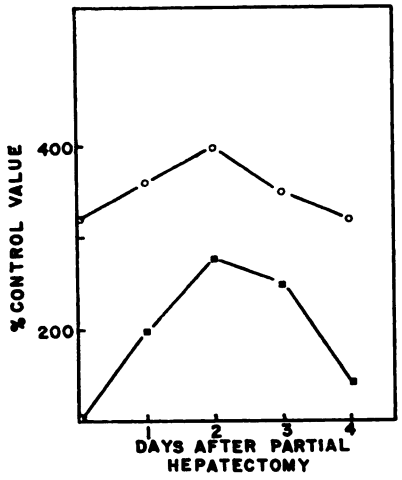

FIG. 5. *Effect of orotic acid diet upon DHOase activity in regenerating liver*

See Legend to Fig. 4. The specific activity of DHOase in the liver of control diet-fed rats in terms of $\Delta A_{240}/4$ min/g protein was 9.0 ± 0.4. ■——■ *Control diet;* ○——○ *orotic acid-containing diet.*

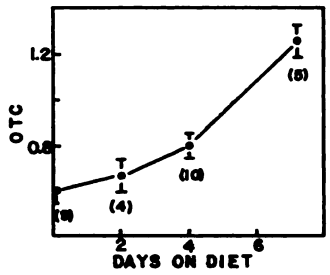

FIG. 6. *OTC activity in liver of orotic acid-fed rats*

Male rats were maintained on control diet for 10 days and then placed on diet supplemented with 1% orotic acid. The specific activity of OTC (ordinate), dpm product/mg protein, was determined as described in the text. At each point, the mean ± standard is presented as well as the number of rats (in parentheses).

DISCUSSION

The specific activities of aspartate transcarbamylase and dihydroorotase are markedly *elevated* after the administration to rats of a purified diet supplemented with 1% orotic acid. At first glance, the elevation in enzyme activities appears to correlate with the alterations in the composition of the acid-soluble fraction. The return of rats to the normal control diet results in immediate depression in the concentration of the acid-soluble pyrimidines and elevation of the purines. This immediate effect upon the concentrations of these substances is not followed by an immediate return of the elevated enzyme levels to basal values. Only after 4 days of control diet has the specific activity of aspartate transcarbamylase reached its basal value; by the end of 2 days, dihydroorotase has returned to control values. The $t_{1/2}$ for the decline in aspartate transcarbamylase specific activity has been calculated to be 48 hr, a value which agrees very well with the calculation made for the half-life of the messenger RNA responsible for the elaboration of this enzyme (22). In the latter studies, the half-life was calculated to be in excess of 24 hr.

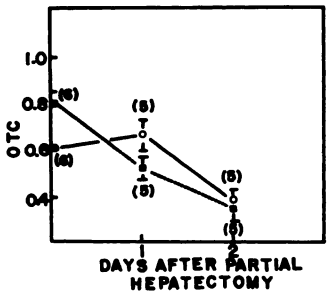

FIG. 7. *OTC activity in regenerating liver*

See Legend to Fig. 6. ○——○ Rats on control diet; ■----■ rats on orotic acid diet.

The addition of 0.25% adenine sulfate to the orotic acid-supplemented diet also results in a return of the acid-soluble composition to basal levels within a few days. The specific activities of aspartate transcarbamylase and dihydroorotase do not reach control levels until the third day on the latter diet.

The elevation in the activity of aspartate transcarbamylase in regenerating liver has been reported previously (22, 23). Additionally, the overall conversion of carbamyl aspartate to orotic acid, involving both dihydroorotase and dihydroorotic acid dihydrogenase, has been shown to increase in regenerating liver (24). In the present studies, dihydroorotase activity rises significantly after partial hepatectomy, reach-

ing a maximum 2 days after the operation. In unpublished observations, dihydroorotic acid dihydrogenase activity underwent no change after partial hepatectomy. The administration of orotic acid-containing diet resulted in a substantial increase in the concentration of the pyrimidine nucleotides in the liver.[3] The elevated levels of pyrimidine nucleotides were unable to prevent the increase in the specific activity of either aspartate transcarbamylase or dihydroorotase occurring in regenerating liver.

These studies indicate another difference between the aspartate transcarbamylase of the bacterial and the mammalian liver systems. In a previous report (4), the properties of ATC in these systems were compared and it was shown that the liver enzyme behaved more like the bacterial monomer than the tetramer, and accordingly, the former was not susceptible to end-product inhibition. In the present investigation, the inability to "repress" ATC and DHOase by elevated intracellular concentrations of pyrimidine nucleotides has been reported in either control or regenerating liver. In contrast, *E. coli* ATC synthesis is markedly sensitive to the concentration of pyrimidines in the medium (25).

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[3] This pyrimidine nucleotide fraction consisted largely of uridine nucleotides.