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*J Clin Invest.* 1959;**38**(5):798-809. <https://doi.org/10.1172/JCI103862>.

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# PYRIMIDINE METABOLISM IN MAN. I. THE BIOSYNTHESIS OF OROTIC ACID \* †

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(Submitted for publication November 12, 1958; accepted January 15, 1959)

Many studies of purine metabolism in man have been carried out. This is largely due to continued interest in the pathogenesis of gout and the ease of measuring serum and urinary uric acid. In contrast there have been few studies of pyrimidine metabolism in man. The major degradation products of pyrimidines are carbon dioxide and ammonia, which are lost in the general body pools. Only recently has a "pyrimidine disease," orotic aciduria, been described (1). The role of pyrimidine nucleotides in the formation of nucleic acids and in a number of coenzyme functions emphasizes the importance of quantitative data concerning pyrimidine metabolism in man. This report describes studies of the enzymatic steps in normal blood cells leading to the formation of the first pyrimidine product, orotic acid.

## EXPERIMENTAL PROCEDURE

*Preparation of reactants.* Carbamylphosphate (CAP) was synthesized and analyzed as described by Jones, Spector and Lipmann (2). *l*-Carbamylaspartate- $C^{14}$  (CAA- $C^{14}$ ) was prepared from *l*-aspartate and cyanate- $C^{14}$  (3) and measured by the Koritz and Cohen procedure (4). Cyanate- $C^{14}$  was synthesized by the fusion of potassium carbonate and urea- $C^{14}$  (5). *dl*-Dihydroorotate- $C^{14}$  (DHO- $C^{14}$ ) was synthesized by the catalytic hydrogenation of orotate- $C^{14}$  (labeled in carbon 6) using a rhodium-alumina catalyst (6) and measured as described by Yates and Pardee (7). Nonlabeled *l*-dihydroorotate was synthesized by the method of Miller, Gordon and Englehardt (8). *dl*-5-Carboxymethylhydantoin- $C^{14}$  (5-CMH- $C^{14}$ ) labeled in the carboxyl group was prepared from the above *dl*-DHO- $C^{14}$  (3) and measured as described by Lieberman and Kornberg (9). Nonlabeled 5-CMH was correspondingly prepared from carbamylaspartate. Bicarbonate- $C^{14}$  was prepared from barium carbonate- $C^{14}$  and analyzed as described by Zamecnik, Frantz, Loftfield and Stephenson (10). Uniformly labeled *l*-aspartate- $C^{14}$  was purchased

from Nuclear Chicago Corporation. Urea- $C^{14}$  and orotate- $C^{14}$  were purchased from New England Nuclear Corporation. In enzymatic assays, the above isotopic precursors were added in the following specific activities (infinite thinness, copper or glass planchettes, Nuclear Chicago gas flow counter with micromil window of approximately 32 per cent efficiency):

Labeled compound	Specific activity (cpm per $\mu$ M)
bicarbonate- $C^{14}$	$9.3 \times 10^5$
<i>l</i> -aspartate- $C^{14}$	$9.0 \times 10^4$
<i>l</i> -CAA- $C^{14}$	$2.6 \times 10^5$
<i>dl</i> -5CMH- $C^{14}$	$1.9 \times 10^5$
<i>dl</i> -DHO- $C^{14}$	$4.2 \times 10^5$

Triphosphopyridine nucleotide (TPN), diphosphopyridine nucleotide (DPN), and crystalline alcohol dehydrogenase (ADH) were purchased from the Sigma Chemical Company.

*Preparation of blood cells and incubation.* Studies of normal levels of each enzymatic activity were carried out on blood cells from laboratory personnel. Isolation of leukocytes was based on a method of Skoog and Beck (11). Approximately 25 ml. of venous blood was drawn into a siliconized<sup>1</sup> syringe containing 1 ml. of 5 per cent EDTA,<sup>2</sup> mixed quickly, and immediately added to a 60 ml. test tube containing 25 ml. of a 3 per cent dextran,<sup>3</sup> 3 per cent dextrose solution in saline at 0° C. The tube was covered with Parafilm and inverted three to four times to insure complete mixing.

1). *Leukocytes.* After one hour of sedimentation in an ice bath the supernatant suspension was transferred to a 50 ml. Lusteroid tube and spun in an International Refrigerated Centrifuge at 2° for seven minutes at  $150 \times G$ . Following removal of the supernatant, the sediment was resuspended in 10 ml. of ice-cold isotonic KCl containing 0.4 per cent dextrose. A second centrifugation was carried out at 2° for four minutes at  $200 \times G$ . The washing procedure was then repeated a second time. The leukocyte-erythrocyte sediment was then suspended in KCl-dextrose at a final leukocyte count of approximately 40,000 to 100,000 per mm.<sup>3</sup>. The leukocyte count was calculated as the average of eight determinations. The isolation procedure tended to concentrate polymorphonuclear cells at the expense of the less dense lymphocytes

\* This study was supported by a research grant from the Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service.

† A preliminary report of this work has been published in abstract form (J. clin. Invest. 1958, 37, 932).

<sup>1</sup> All glassware, pipettes, and Lusteroid tubes used in the isolation of intact leukocytes were siliconized with Siliclad, Clay-Adams, Inc., New York, N. Y.

<sup>2</sup> Disodium ethylenediaminetetraacetate in saline.

<sup>3</sup> Lot number HH-1, R. K. Laros Co., Bethlehem, Pa.

so that they constituted approximately 80 to 90 per cent of the total leukocytes. The number of contaminating erythrocytes was usually three to six times greater than the leukocytes. Because of the differences found in cellular enzymatic activities (see below) this degree of contamination was not a significant source of error. The leukocytes were disrupted by sonication or homogenization:

a). *Sonication*. The cellular suspension (2 to 4 ml. in a Lusteroid test tube) was subjected to sonication at maximal intensity for exactly 2.5 minutes using a Raytheon 10 kc. Oscillator (model DF 101). This uniform sonication procedure was sufficient to produce complete destruction of the leukocytes and their nuclei. Prolongation of the sonication time led to a progressive loss of enzymatic activity. Because of the uniformly complete cell destruction obtained, sonication was used in all enzyme preparations from leukocytes except in studies pertaining to intracellular enzyme location.

b). *Homogenization*. Due to the difficulty in obtaining complete or uniform destruction of leukocytes (or other free-floating nucleated cells such as Ehrlich ascites tumor cells) by homogenization, the following procedures were used. The leukocytes were suspended in cold distilled water in a 10 ml. homogenization tube (Potter-Elvehjem type, teflon plunger) and allowed to stand at 0° for five minutes to allow for osmotic damage. The contents were then quickly frozen in acetone-dry ice and thawed under cool tap water. An equal volume of 0.5 M sucrose was added to the damaged cells and vigorous homogenization was then carried out for two to three minutes at 0° C. Examination of the homogenate by phase microscopy or Wright's stained smear showed no undamaged cells, many nuclei with surrounding cytoplasmic or membranous shreds, and many naked nuclei.

2). *Erythrocytes*. An aliquot of the original venous blood was used for determination of red cell indexes, where quantitative data were required. The erythrocyte count was taken as the average of eight determinations. Hemoglobin was determined in duplicate photometrically. The original erythrocyte sediment was resuspended in 25 ml. of the cold dextran-dextrose solution and allowed to stand for an additional 30 minutes at 6° C. The supernatant suspension and the superficial layer of erythrocytes were removed by suction. The cells were then washed twice by suspension in cold isotonic KCl-glucose and centrifugation (400 × G, five minutes at 2° C.). An aliquot from the erythrocyte sediment was suspended in isotonic KCl-glucose in a concentration of approximately three million RBC's per mm.<sup>3</sup>, and duplicate hemoglobin determinations were carried out. Using this value and the previously determined red cell indexes, the actual erythrocyte count was calculated. This indirect determination of the erythrocyte count was necessary because of a variable residual agglutination of the cells following exposure to dextran. The red cell suspensions, virtually free of leukocyte contamination, were then frozen in acetone-dry ice, thawed under a cold water tap and used immediately.

3). *Incubation*. Incubations were carried out in a final volume of 1 ml. in stoppered 10 ml. Erlenmeyer flasks with shaking at 37° C. in a water bath. In the study of soluble aspartate carbamyltransferase from erythrocyte incubations were carried out in 12 ml. centrifuge tubes.

*Analytic methods*. Each determination of an enzymatic step involved the incorporation of an isotopic precursor of high specific activity into a product which was then isolated and determined by the carrier technique.

1). *Carbamylaspartate (CAA)*. The assays of carbamylphosphate synthetase, aspartate carbamyltransferase, 5-carboxymethylhydantoinase and dihydroorotase (see Figure 1) all led to the incorporation of C<sup>14</sup> into CAA. The assay of dihydroorotase, carried out in conjunction with the assay of dihydroorotic dehydrogenase is described in the next section. For the other enzymes, the flasks were chilled after incubation, and 10 μM of nonlabeled CAA carrier was added. Protein was precipitated with 0.8 N perchloric acid (PCA), centrifuged and the sediment washed with 0.4 N PCA. The combined supernatant solutions were neutralized with concentrated KOH (phenol red) and allowed to stand in an ice bath. The solution was then decanted from the KClO<sub>4</sub> onto a Dowex-1-formate ion exchange column (1 × 12 cm.). Elution was carried out with the 0.05 M sodium formate adjusted to pH 3.2 with formic acid (9). The elution peak, located by spot tests for the ureide group (12), generally began at 23 to 26 column volumes of eluate and was complete at 28 to 33 volumes. Because of this constancy as many as 12 chromatographic separations could be conveniently run in parallel with individual eluate collections only at the time of the anticipated elution. All of the C<sup>14</sup> precursors were effectively eliminated by this procedure: aspartate, two to six column volumes; DHO, four to nine column volumes; 5-CMH, eight to fifteen column volumes. [With aspartate-C<sup>14</sup> as a precursor, the neutralized PCA supernatant was first passed through a Dowex 50-H column (1 × 6 cm.), to remove the bulk of the isotope.] The eluate containing CAA was neutralized with KOH and added to a second Dowex-1-formate column (1 × 5 cm.). The column was washed, blown dry and elution was carried out with successive 2.0 ml. aliquots of 2 N formic acid. Again the elution pattern was determined by spot test, with the peak concentration generally appearing after 8 to 10 ml. This second chromatographic procedure allowed: a) rapid concentration of the large volume of dilute CAA solution from the first chromatography (approximately 50 ml.), b) replacement of sodium formate with volatile formic acid prior to plating at infinite thinness, and c) only incidentally some further chromatographic purification. Samples from two successive tubes of eluate were analyzed for CAA content and radioactivity to allow duplicate calculations of specific activity. CAA concentration was determined by the method of Koritz and Cohen (4), modified for a final volume of 3.0 ml. in 2.0 N formic acid, and read in a Beckman model DU spectrophotometer at 550 μμ. Radioactivity was determined on 0.5 ml. aliquots of the eluate (containing 0.3 to 0.6 μM CAA).

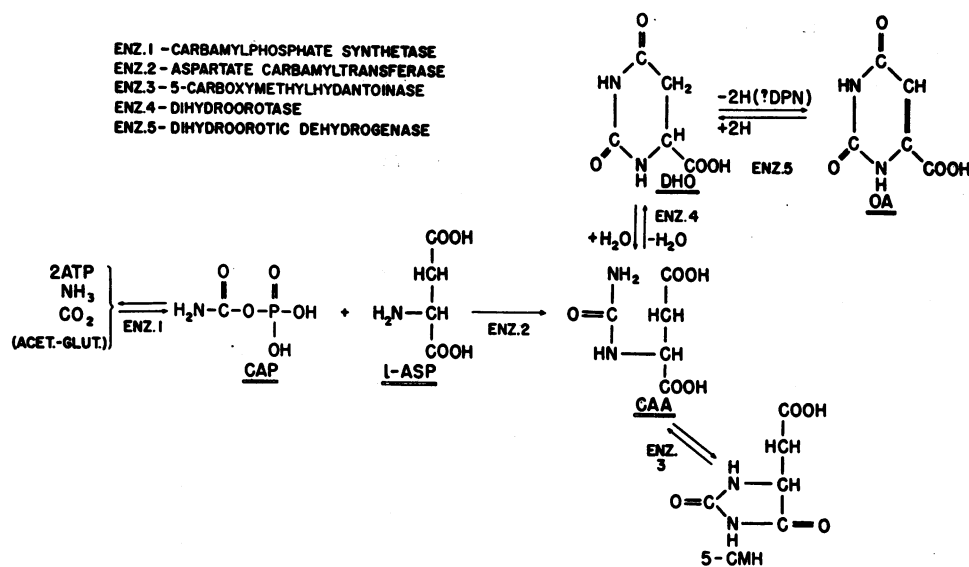


FIG. 1. PATHWAY OF OROTIC ACID BIOSYNTHESIS STUDIED IN HUMAN BLOOD CELLS

Abbreviations include: acet.-glut., acetylglutamate; CAP, carbamylphosphate; l-asp., l-aspartate; CAA, carbamylaspartate; 5-CMH, 5-carboxymethylhydantoin; DHO, dihydroorotate; OA, orotate. Enzymes 1 and 3 were not demonstrated in leukocytes or erythrocytes.

Calculations were based on a total of 5,000 counts per sample using the previously described gas flow counter.

2). *Orotic acid (OA) and CAA from dihydroorotate- $C^{14}$  (DHO- $C^{14}$ )*. The oxidation of DHO- $C^{14}$  to OA and its hydrolysis to CAA (see Figure 1) were studied simultaneously in the same flask. After incubation 5  $\mu\text{M}$  non-labeled OA carrier and 30  $\mu\text{M}$  CAA carrier were added and proteins were precipitated and washed with PCA as described above. The combined supernatants were passed through a charcoal column (1  $\times$  3 cm. Nuchar Grade C, 40 to 60 mesh) which was then washed with six volumes of water. The charcoal column retained the OA and much of the precursor DHO- $C^{14}$ . The charcoal-column eluate and wash were combined for the isolation of CAA.

a). *CAA*. The solution containing CAA was then neutralized with KOH with extreme care to pH 7.4 to 7.6 with rapid magnetic stirring and constant monitoring of pH. This extra care was demanded because of the chemical lability of DHO in alkaline solutions (7) causing a nonenzymatic hydrolysis of DHO- $C^{14}$  to CAA- $C^{14}$  to occur at higher pH values (see below). Further purifications and analysis of the CAA were carried out as previously described.

b). *OA*. The charcoal column was washed with 200 ml. water after which the OA and residual DHO- $C^{14}$  were eluted with 12 ml. of concentrated  $\text{NH}_3$ -ethanol- $\text{H}_2\text{O}$  (1:2:2 by volume) onto a Dowex 2-Cl column (1  $\times$  10 cm.). The column was washed with 10 column volumes of 0.03 N HCl to elute DHO- $C^{14}$ . OA was then eluted with 10 ml. of 2 N HCl. The eluate was alkalinized with 6 ml. of 5 N KOH and allowed to stand for 10 minutes to

insure conversion of any contaminating DHO- $C^{14}$  to CAA- $C^{14}$  for ease in separation from OA in the subsequent procedures. After being acidified with concentrated HCl the solution was passed through a second charcoal column (1  $\times$  3 cm.), which was washed and eluted as described above into a tapered centrifuge tube. The eluate was taken to dryness under an air jet in a boiling water bath. The residual OA was taken up in hot water and further purified by ascending chromatography on Whatman No. 3 paper with propanol-concentrated formic acid-water (7:1:2) (13). The OA band ( $R_f$  approximately 0.40), located by ultraviolet light, was eluted with hot water. Aliquots were taken for counting, as described for CAA, and for measurement by ultraviolet absorption at 280  $m\mu$  in 0.1 N HCl. This extensive purification procedure (two charcoal absorptions, one ion exchange chromatography, one paper chromatography) was used because of the low rates of synthesis studied and the consequent low tolerance for  $C^{14}$  contamination by the isotopic precursor of high specific activity.

3) *Calculations*. The amount of product synthesized was calculated from the standard carrier formula:

$$\mu\text{M synthesized} = \frac{\mu\text{M carrier added} \times \text{specific activity of isolate (cpm per } \mu\text{M)}}{\text{specific activity of precursor (cpm per } \mu\text{M)}}$$

When rates of synthesis were to be compared, the results were expressed as millimicromoles ( $m\mu\text{M}$ ) of product formed per  $10^8$  WBC or per  $10^8$  RBC.

## RESULTS

*Absence of detectable CAA and OA in plasma and blood cells*

The carrier technique for recovery and analysis of a product is based on the assumption that the amount of the product in the reaction flask is negligible in comparison with the amount of the added carrier. By the isotope dilution method, therefore, an attempt was made to determine the normal concentrations of CAA and OA in hemic cells. Three  $\mu\text{M}$  OA- $\text{C}^{14}$  (4,200 cpm per  $\mu\text{M}$ ) and 5  $\mu\text{M}$  of CAA- $\text{C}^{14}$  (4,500 cpm per  $\mu\text{M}$ ) were added to  $8 \times 10^{10}$  RBC,  $8 \times 10^7$  WBC, and 10 ml. spun plasma. The recovered OA- $\text{C}^{14}$  and CAA- $\text{C}^{14}$  had no change in specific activities. It is estimated that this method would have allowed the detection of 0.3  $\mu\text{M}$  OA and 0.5  $\mu\text{M}$  CAA. "Product dilution" cannot therefore be a significant source of error in the carrier technique employed.

*Effect of an internal carrier*

In these crude cell preparations the presence of two or more sequential enzymes in the pyrimidine synthetic pathway introduces a source of error, in that during incubation the product of the reaction under study may serve as a substrate for the following enzymatic step. To a large degree this is negated by the choice of experimental conditions (pH, time and so forth) to give biochemical advantage to the preferred reaction and by the low concentration of the product formed. Several attempts were made, however, to determine whether the use of an "internal carrier" during the reaction would serve as a trap and thereby increase the calculated yield of the product. In brief, it was found that a carrier trap during incubation decreased rather than increased the calculated yield. Presumably the tendency toward reversal of the reaction outweighed any trapping function. The carriers were therefore added at the end of incubation in all subsequent experiments.

*Carbamylphosphate synthetase*

Carbamylphosphate is a labile intermediate which is difficult to purify and analyze in the microquantities anticipated in these cell preparations. The presence of aspartate carbamyltrans-

ferase having been demonstrated (see below), attempts were made to detect the presence of CAP-synthetase in hemic cells by coupling the first two reactions shown in Figure 1. In practice the assay depended upon the incorporation of bicarbonate- $\text{C}^{14}$  into the ureide carbon of CAA. Conditions similar to those shown to be optimal in rat liver (14) were used in this study with erythrocyte hemolysates and leukocyte sonicates and homogenates. The appearance of  $\text{C}^{14}$  in CAA could not be demonstrated despite the sensitivity of this system, which would have detected the synthesis of 1  $\mu\text{M}$  of CAA from bicarbonate- $\text{C}^{14}$ . This enzymatic step, even if detectable, would not lend itself to reliable quantitative analysis.

*5-Carboxymethylhydantoinase (5-CMH'ase)*

This enzyme, which catalyzes the reversible ring closure of CAA to the corresponding hydantoin (see Figure 1), has been described only in *Zymobacterium oroticum* and was found to be absent in rat liver (9). An attempt was made to demonstrate the presence or absence of this enzyme in human blood cells. In two hours of incubation of 0.002 M *dl*-5-CMH- $\text{C}^{14}$  ( $1.9 \times 10^6$  cpm per  $\mu\text{M}$ ) at pH 8.2 (Tris buffer) with enzyme preparations from leukocytes (*ca.*  $1.6 \times 10^8$  cells), erythrocytes (*ca.*  $3 \times 10^9$  cells), and rat liver homogenate (*ca.* 1.5 mg. N) no synthesis of CAA could be demonstrated.

*Aspartate carbamyltransferase*

Aspartate carbamyltransferase, previously studied in rat liver (15) and bacterial systems (16), irreversibly catalyzes the carbamylation of 1-aspartate by carbamyl phosphate to yield 1-carbamyl-aspartate, as shown in Figure 1. This reaction also occurs spontaneously in the absence of the specific enzyme so that appropriate nonenzymatic controls must be carried out. Aspartate carbamyltransferase was found to be present in circulating leukocytes and erythrocytes. In hemolysates of erythrocytes the enzyme was largely soluble (in the  $100,000 \times G$  supernatant). In leukocyte homogenates the activity was confined to the particulate fractions (Table I), with almost half of the activity appearing in the larger cell fragments and nuclei separated at  $600 \times G$ . More complete fragmentation of the cell particles by sonica-

TABLE I  
Location of enzymatic activities in preparations of leukocytes and erythrocytes

Source of enzyme	Aspartate carbamyltransferase CAA formed*	Dihydroorotase CAA formed	Dihydroorotic dehydrogenase OA formed†
	$m\mu M$	$m\mu M$	$m\mu M$
I. Leukocyte homogenate			
a. Whole homogenate	133	106	5.5
b. 600 × G supernat.	71	54	0
c. 100,000 × G supernat.	1	5	0
II. Leukocyte sonicate			
a. Whole sonicate	370	119	22
b. 600 × G supernat.	294	80	0
c. 100,000 × G supernat.	124	62	0
III. Erythrocyte hemolysate			
a. Whole hemolysate	195	99	0
b. 100,000 × G supernat.	170	84	0

\* CAA, carbamylaspartate.

† OA, orotic acid.

tion brought 30 to 40 per cent of the enzymatic activity into solution.

Studies on the characteristics of this enzyme system in man were carried out on erythrocyte hemolysates. These results are summarized by the series of graphs presented in Figure 2. The substrate concentration curves illustrate the high concentration of aspartate (0.01 M) and CAP (0.005 M) necessary for enzyme saturation. The pH curve demonstrated optimal enzymatic synthesis at pH 9.2 to 9.3, with no significant change in nonenzymatic synthesis over the range of pH tested (8.0 to 10.0). Enzymatic activity of aspartate carbamyltransferase varied directly with the number of erythrocytes employed and was linear with time over the first hour of incubation. The increasing rate of nonenzymatic CAA synthesis with time seemed anomalous. It was anticipated that the diminishing concentration of CAP during incubation would result in a corresponding diminution in nonenzymatic synthesis. Based on these studies, standard conditions for assay of aspartate carbamyltransferase were established as: 30 minutes incubation, pH 9.0 (Tris buffer 0.2 M); concentration of substrates: aspartate, 0.015 M; CAP, 0.010 M. With these conditions the nonenzymatic control synthesis of CAA was repeatedly found to be 12 to 14  $m\mu M$ .

### Dihydroorotase

Dihydroorotase was studied by hydrolysis of DHO rather than by the ring closure of CAA, allowing a simultaneous determination of dihydroorotase and dihydroorotic dehydrogenase from precursor DHO-C<sup>14</sup> (see Figure 1). Table I illustrates that dihydroorotase was found in circulating human leukocytes and erythrocytes. In erythrocytes the enzyme was almost completely soluble, but in leukocytes (as with aspartate carbamyltransferase) only a trace of enzymatic activity was brought into solution by homogenization. Approximately 50 per cent of the activity was solubilized by sonication. Table II demonstrates the constancy of CAA synthesis from DHO-C<sup>14</sup> in a number of variable conditions, which were mostly chosen for study of their possible effect on simultaneous OA synthesis. Mg<sup>++</sup> has been reported to stimulate the hydrolysis of dihydrouracil but not of dihydrothymine by rat liver hydroxypyrimidine hydrase, seemingly an analogous enzyme (17). Mg<sup>++</sup> was without effect on dihydroorotase in man.

Some of the biochemical characteristics of dihydroorotase are presented in the graphs of Figure 3. The substrate concentration curve shows enzyme saturation to be attained at approximately

TABLE II  
Effect of varying conditions on dihydroorotase activity (CAA formed) and dihydroorotic dehydrogenase activity (OA formed) in a leukocyte sonicate \*

Incubation conditions	CAA formed	OA formed
	$m\mu M$	$m\mu M$
Control	79	33
+ 5 × 10 <sup>-3</sup> M DPN	75	26
+ 5 × 10 <sup>-3</sup> M TPN	80	23
+ 1 × 10 <sup>-3</sup> M Mg <sup>++</sup>	74	16
+ 5 × 10 <sup>-3</sup> M EDTA	74	29
+ 5 × 10 <sup>-3</sup> M cysteine	61	36
+ N <sub>2</sub> gas phase		27
+ N <sub>2</sub> gas phase, 5 × 10 <sup>-3</sup> M DPN, 3 × 10 <sup>-2</sup> nicotinamide, ADH†		24
Preincubation control	51	31
DPN <sup>ase</sup> preincubation‡	56	26

\* Abbreviations are as follows: CAA, carbamylaspartate; OA, orotic acid; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate; and ADH, alcohol dehydrogenase.

† Flasks evacuated and flushed with N<sub>2</sub> three times. ADH, approximately 60,000 units.

‡ Preincubation of the enzyme was carried out for 20 minutes at 37° C. with and without a *Neurospora* DPN<sup>ase</sup> prior to the addition of precursor DHO-C<sup>14</sup>.

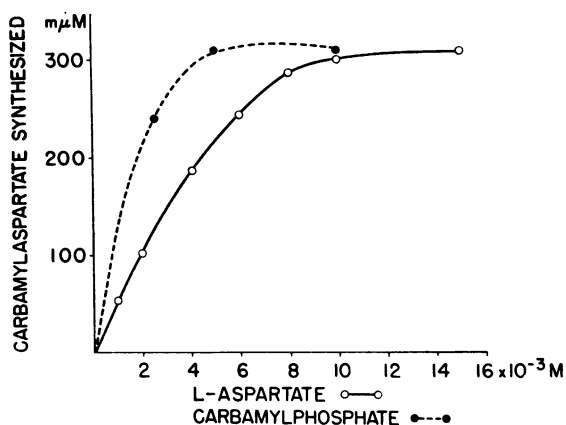


FIG. 2a. SUBSTRATE CONCENTRATION CURVE OF ASPARTATE CARBAMYLTRANSFERASE

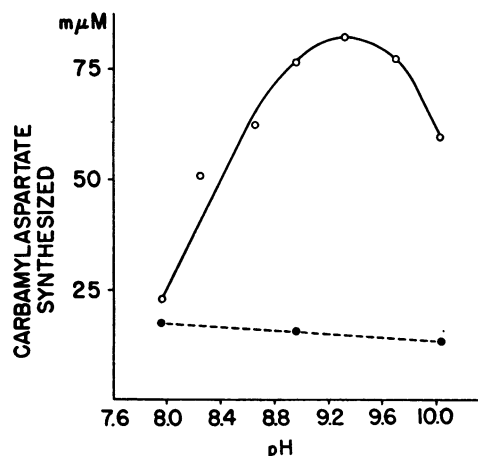


FIG. 2b. pH CURVE OF ASPARTATE CARBAMYLTRANSFERASE  
Enzymatic synthesis, ○—○; nonenzymatic synthesis, ●---●. Enzymatic synthesis has been corrected for nonenzymatic synthesis.

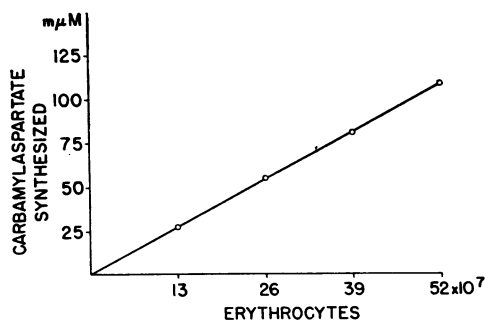


FIG. 2c. ENZYME CONCENTRATION CURVE OF ASPARTATE CARBAMYLTRANSFERASE

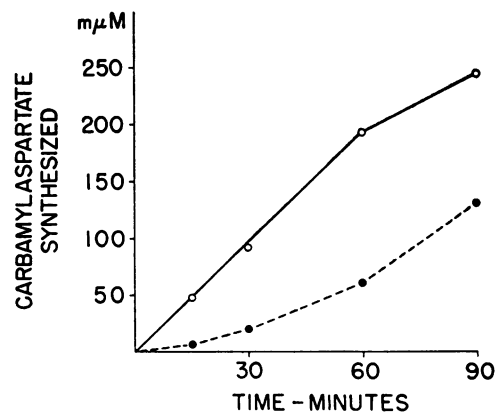


FIG. 2d. TIME RELATIONSHIP OF CAA SYNTHESIS BY ASPARTATE CARBAMYLTRANSFERASE  
Enzymatic synthesis, ○—○; nonenzymatic synthesis, ●---●. Enzymatic synthesis has been corrected for nonenzymatic synthesis.

$1 \times 10^{-3}$  M DHO. The optimal pH for enzymatic synthesis was 8.2. At pH's greater than 9, nonenzymatic hydrolysis of the hydroypyrimidine introduces a significant source of error. Enzymatic synthesis was proportional to enzyme concentration (number of leukocytes) over the range studied, and was linear with time over the first three hours of incubation. Based on these studies and those of dihydroorotic dehydrogenase to be described, the following standard conditions were established for the assay of dihydroorotase: 120 minutes incubation, pH 8.2 (Tris buffer 0.25 M), DHO-C<sup>14</sup>  $10^{-3}$  M. At pH 8.2 nonenzymatic hy-

drolisis of DHO-C<sup>14</sup> was not a source of error (less than one mμM during 120 minutes incubation).

#### *Dihydroorotic dehydrogenase*

DHO dehydrogenase was first demonstrated as a DPN requiring enzyme in *Zymobacterium oroticum* by Lieberman and Kornberg (18). DHO dehydrogenase was found in human leukocytes, but not in erythrocytes (Table I). In contrast, nucleated avian erythrocytes did contain this enzymatic activity. A number of attempts to solubilize the enzyme were unsuccessful (sonica-

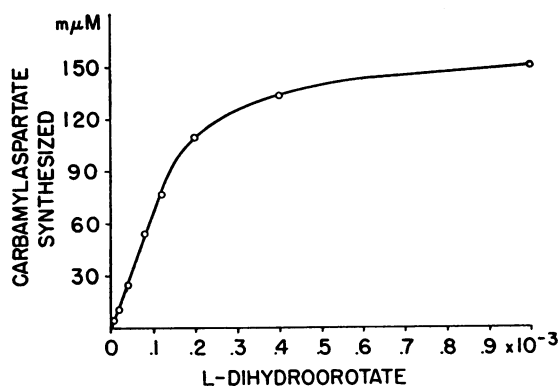


FIG. 3a. SUBSTRATE CONCENTRATION CURVE OF DIHYDROOROTASE

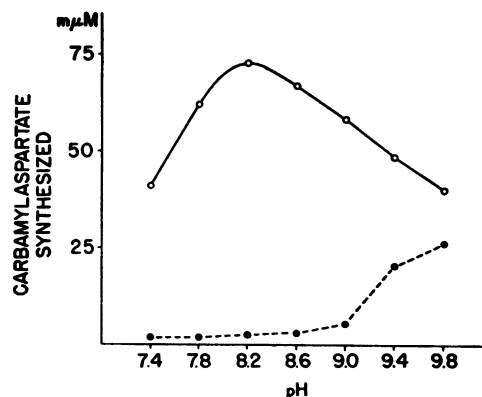


FIG. 3b. pH CURVE OF DIHYDROOROTASE

Enzymatic hydrolysis, ○—○; nonenzymatic hydrolysis, ●---●. Enzymatic synthesis has been corrected for nonenzymatic synthesis.

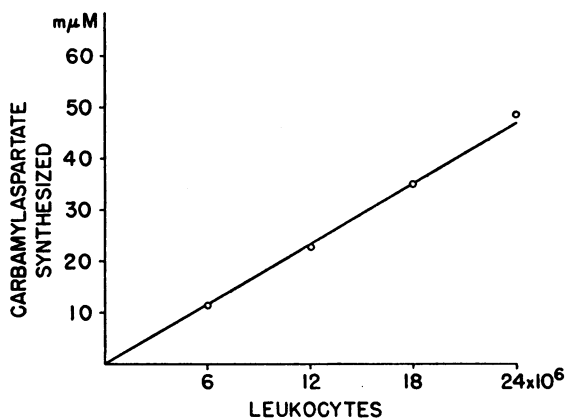


FIG. 3c. ENZYME CONCENTRATION CURVE OF DIHYDROOROTASE

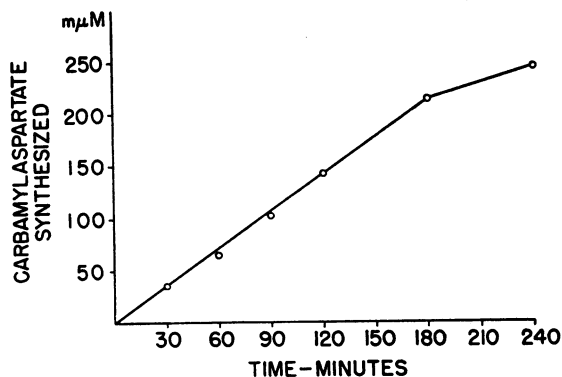


FIG. 3d. TIME RELATIONSHIP OF CAA SYNTHESIS BY DIHYDROOROTASE

tion, freeze-thawing in various buffers, lyophilizing, digitonin extraction), the activity always being sedimented with the nuclei and larger particles at  $600 \times G$ . A variety of factors were studied for possible effect on the oxidation of DHO-C<sup>14</sup> to orotic acid in this system (Table II). With the exception of a slight inhibition by Mg<sup>++</sup>, no constant effects were obtained. A particular effort was made to demonstrate a requirement for DPN, but such a requirement (reported for bacterial systems) could not be shown. Negative studies included: 1) Stimulation of activity: addition of  $10^{-3}$  to  $10^{-2}$  M DPN to a dialyzed leukocyte sonicate, use of a DPN regenerating system (alcohol dehydrogenase and acetaldehyde 0.025 M), addition of DPN and the regenerating system to the incubation carried out under nitrogen to diminish

any effect of DPN oxidase in the control flask (18), preparation of sonicates or homogenates in nicotinamide (0.03 M), and finally the use of all these conditions simultaneously. 2) Reduction of activity: On two occasions pre-incubation of the leukocyte sonicate for 20 minutes with a diphosphopyridine nucleotidase (DPNase) prepared from *Neurospora crassa*<sup>4</sup> failed to cause a significant reduction of subsequent orotic acid synthesis from DHO-C<sup>14</sup>. TPN was also tested, having been shown to be the cofactor for dihydroorotic dehydrogenase from *Corynebacterium* (19), but again no effect could be demonstrated.

Studies on some characteristics of DHO dehydrogenase are summarized in Figure 4. The sub-

<sup>4</sup> We are grateful to Dr. M. N. Swartz for the preparation and purification of this enzyme.



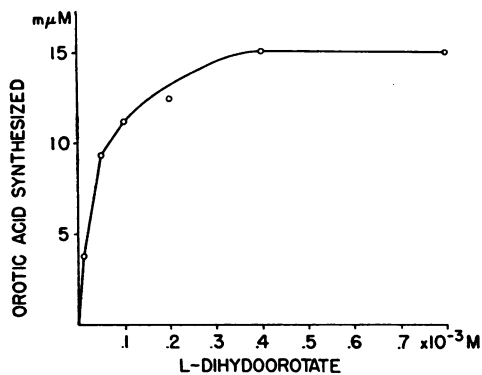


FIG. 4a. SUBSTRATE CONCENTRATION CURVE OF DIHYDRO-OROTIC DEHYDROGENASE

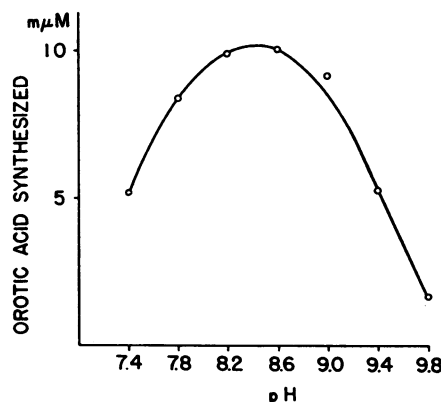


FIG. 4b. pH CURVE OF DIHYDROOROTIC DEHYDROGENASE

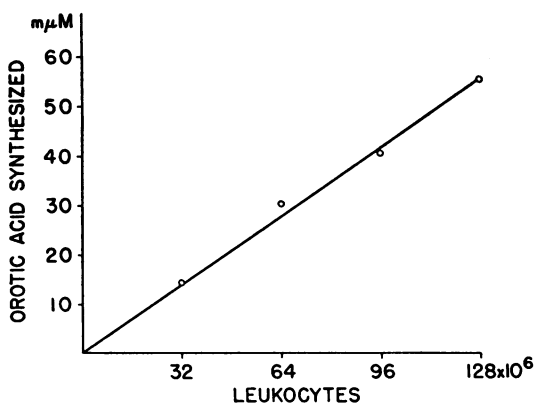


FIG. 4c. ENZYME CONCENTRATION CURVE OF DIHYDRO-OROTIC DEHYDROGENASE

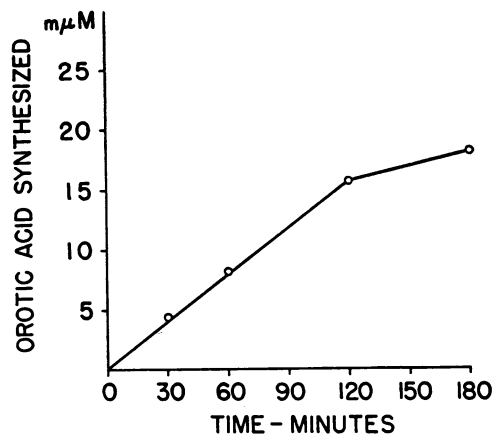


FIG. 4d. TIME RELATIONSHIP OF OA SYNTHESIS BY DIHYDROOROTIC DEHYDROGENASE

strate concentration curve demonstrated maximal enzymatic activity at DHO-C<sup>14</sup>  $5 \times 10^{-4}$  M. Optimal synthesis occurred at pH 8.4 to 8.6. The formation of orotic acid was directly related to the enzyme concentration (number of sonicated leukocytes) and to the time of incubation for the first 120 minutes. The standard conditions which were subsequently used for the assay of DHO dehydrogenase have been described for dihydroorotase above.

*Normal levels of enzymatic activity*

A study of the normal levels of these enzymatic activities in mature erythrocytes and leukocytes was carried out with 18 determinations on 12 normal subjects, summarized in Figure 5. Comparative studies with avian erythrocytes illustrate the presence of dihydroorotic dehydrogenase in

nucleated erythrocytes, in contrast to the absence of this activity in human erythrocytes. The data in Figure 5 also illustrate that circulating human leukocytes are roughly equivalent to rat liver in aspartate carbamyltransferase and dihydroorotase activities but contain only 2.5 per cent of its dihydroorotic dehydrogenase activity.

DISCUSSION

In this study the biosynthesis of orotic acid in man has been shown to follow the same pathway as that demonstrated in bacteria (9, 18) and in rat liver (20, 21). The single exception is that carbamylphosphate synthetase could not be demonstrated in leukocytes or erythrocytes. CAP was readily shown to be a substrate for the succeeding reaction, the formation of CAA. It is probable that the method employed, that of attempting to

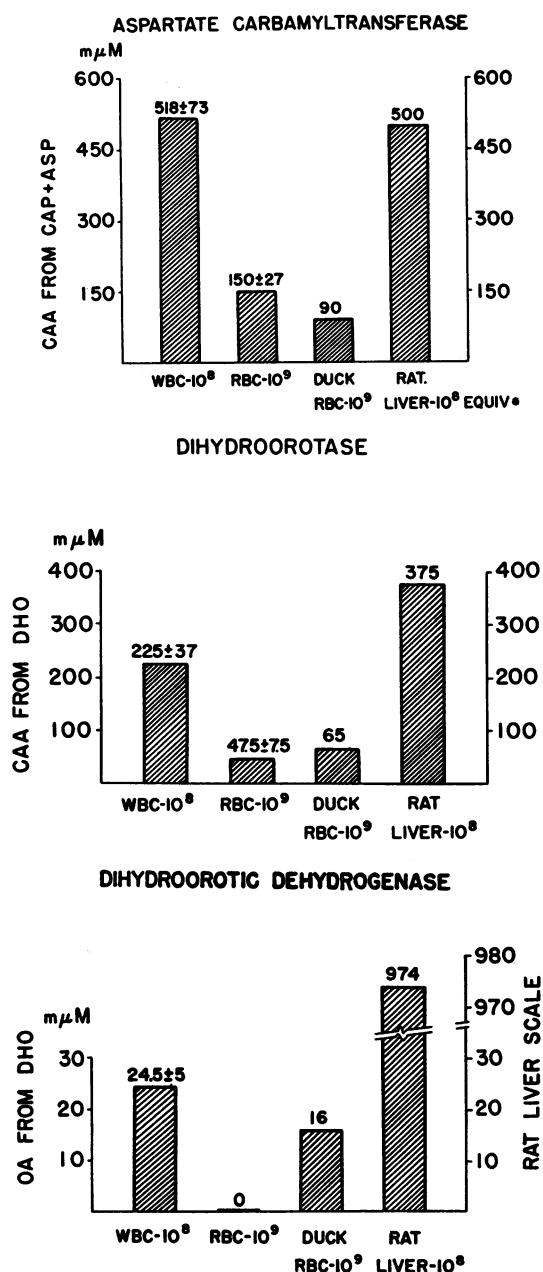


FIG. 5. NORMAL LEVELS OF ENZYMIC ACTIVITIES IN HUMAN BLOOD CELLS COMPARED WITH DUCK ERYTHROCYTES AND RAT LIVER HOMOGENATE (OF EQUIVALENT N CONTENT AS 10<sup>8</sup> WBC)

"trap" the synthesized CAP as CAA in the presence of aspartate and aspartate carbamyltransferase, was too insensitive to detect the minute amounts of this labile product formed. In mammalian tissues other than the liver, carbamylphosphate synthetase either could not be detected (blood

and muscle) or was present at only about one per cent the concentration in the liver (22), where its activity is greatly enhanced by a function in the urea cycle. In these studies, no 5-carboxymethylhydantoinase activity was found in hemic cells or in rat liver. The function of this enzyme, previously demonstrated only in a bacterial system (9), has remained obscure.

Aspartate carbamyltransferase was present as a soluble enzyme in erythrocytes and in the particulate fractions of leukocytes. Similarly, it has been reported to be located in the particulate fractions of rat liver (14). Most studies of this enzyme have been carried out in bacterial systems (16). The enzyme in man resembles the enzyme from *E. coli* in its requirement for high concentrations of l-aspartate ( $10^{-2}$  M) and of CAP ( $5 \times 10^{-3}$  M) for enzyme saturation, although in the bacterial system an even higher concentration of l-aspartate ( $7.5 \times 10^{-2}$  M) is required. The pH optimum in man was found to be 9.0 as opposed to 7.5 in *E. coli*. It is not surprising that such differences are found. A major qualitative difference in bacterial and mammalian carbamylation reactions exists in the requirement of the mammalian system for a substituted glutamate (preferably acetylglutamate) in the synthesis of CAP (23). The formation of CAA from CAP and l-aspartate, if at all reversible, has an equilibrium far in the direction of CAA synthesis, the direction therefore chosen for the present assay procedure. Four other reactions involving CAA have been described: the previously discussed reversible ring closure to 5-CMH (9), irreversible decarboxylation to yield carbamyl  $\beta$ -alanine (24), irreversible decarbamylation to aspartate, carbon dioxide and ammonia (25), and reversible ring closure to dihydroorotate (Figure 1) by dihydroorotase.

Dihydroorotase has not been previously studied as an independent enzyme in a mammalian system, although dihydroorotate was shown to be an intermediate in orotic acid synthesis by rat liver (20). The original description of this enzyme (9) did not include data (pH curve, enzyme concentration curve) which would allow comparison with the present studies in man. Hypopyrimidine hydrase, a seemingly closely analogous enzyme which hydrolyzes dihydrouracil and dihydrothymine to their respective carbamyl compounds (17), also demonstrates pH optima for opening

of the hydroypyrimidine ring in the alkaline range (dihydrouracil, pH 10.0; dihydrothymine, pH 8.5; this study, dihydroorotic, pH 8.2). This might be anticipated from the lability of this class of compounds in alkaline solutions.

Dihydroorotic dehydrogenase activity was present in leukocytic nuclei and in nucleated avian erythrocytes, but not in adult human erythrocytes. Although this enzyme has been extensively studied in a bacterial system (18, 26), extension of this work to mammalian tissue has been largely limited to the two-step conversion of precursor carbamylaspartate to orotic acid (21). This study failed to demonstrate a requirement for DPN or TPN, previously reported for bacterial systems (18, 19). Evidence has recently been presented that dihydroorotic dehydrogenase is a flavoprotein from which DPN is a secondary but not obligate electron acceptor (26, 27). Oxygen and methylene blue can serve similarly as electron acceptors from the reduced flavin-enzyme complex. Oxygen was probably the electron acceptor in these leukocyte preparations although a trace contamination of DPN in the insoluble particulate system might allow for the extremely low rates of synthesis measured.

The current studies were undertaken largely to develop enzyme assay techniques to be used in the investigation of pyrimidine metabolism in disease. Figure 5 illustrates that aspartate carbamyltransferase, dihydroorotase and dihydroorotic dehydrogenase can be determined in circulating blood cells with acceptable reproducibility of normal enzyme activities. This reproducibility has been found despite the presence of many potential sources of error in these techniques: inaccuracy of cell counts, standardization of sonication, heterogeneity of the leukocyte population, repeated chromatographic procedures, radioactivity counting errors and colorimetric tests of the isolated carrier. A potentially large source of error in the quantitative application of these techniques to disease lies in the heterogeneity of the leukocyte population. The values which are given here were determined from cell mixtures containing approximately 80 to 90 per cent adult polymorphonuclear cells. Comparable studies on mature lymphocytes and on the effect of cell age on enzymatic activity have not yet been carried out.

Leukocyte aspartate carbamyltransferase and dihydroorotase activities were comparable to those found in an equivalent amount of rat liver sonicate (based on nitrogen content). In contrast, leukocyte dihydroorotic dehydrogenase activity was only 0.025 of that found in rat liver. The reason for this marked discrepancy in the oxidative step is not apparent from the studies at hand. The complete absence of dihydroorotic dehydrogenase activity in adult human erythrocytes and its presence in nucleated avian erythrocytes suggest that the enzyme or an essential unidentified cofactor is lost from the cell with the loss of its nucleus during the maturation sequence unique in the erythrocyte. There is a resulting block of pyrimidine synthesis in the mature erythrocyte at the stage of orotic acid formation. The adult erythrocyte is unique in that it contains no nucleic acids requiring pyrimidine synthesis for regenerative purposes. The present data indicate that pyrimidine nucleotide cofactors, which are found in the erythrocyte (28), cannot be renewed by *de novo* synthesis in the adult cell. The possibility of a "salvage synthesis" of pyrimidine nucleotides from uracil (29) has not been excluded, however. Erythrocyte aspartate carbamyltransferase and dihydroorotase, necessary during intramedullary nucleated life, seem to be vestigial in the mature circulating cell.

The techniques utilized in this study measure the maximal activity of an enzyme (presumably a direct function of its concentration) under optimal conditions after destruction of the cell. These *in vitro* studies cannot fully reflect the *in vivo* control of pyrimidine synthesis, of which the regulation of enzyme concentration is but one part. Additional data are required concerning the steady state concentration of each intermediate and the possible function of feedback control (30) in the intact cell. It is apparent that biochemical studies carried out on normal blood cells can be compared with similar studies on the abnormal cells of a variety of hematological disorders. Of equal interest is the use of blood cells as "representative cells" to reflect the enzymatic defects of inborn errors of metabolism, successfully illustrated, for example, in galactosemia (28). The application of these methods for measuring certain aspects of pyrimidine metabolism to the investigation of disease remains to be demonstrated.

## SUMMARY

1. Three enzymes leading to the formation of orotic acid—*aspartate carbamyltransferase*, *dihydroorotase* and *dihydroorotic dehydrogenase*—were found to be present in circulating human leukocytes. *Aspartate carbamyltransferase* and *dihydroorotase* were also present in erythrocytes. The intracellular locations and some of the biochemical characteristics of these enzymes were determined.

2. Micro assay procedures were developed which gave reproducible results for normal enzymatic activities of *aspartate carbamyltransferase*, *dihydroorotase* and *dihydroorotic dehydrogenase* in sonicates of mature leukocytes and hemolysates of mature erythrocytes.

3. *Carbamylphosphate synthetase* and *5-carboxymethylhydantoinase* could not be detected in these preparations.

4. Free *carbamylaspartate* and *orotate* could not be measured by isotope dilution techniques in plasma, erythrocytes, or leukocytes.

5. The methods described allow a first description in man of qualitative and quantitative aspects of pyrimidine metabolism.

## ACKNOWLEDGMENT

The authors wish to express their appreciation to Dr. Paul Zamecnik for his advice and review of this work.

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