The Enzymatic Steps of Pyrimidine Biosynthesis in the Unfertilized Frog Egg*

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ABSTRACT: The *de novo* synthesis of pyrimidines in the unfertilized frog egg has been investigated. The necessary enzyme systems are present in soluble extracts of both ovulated eggs from *Rana pipiens* and egg-ovary preparations from *Rana catesbeiana* for the synthesis of pyrimidines *via* the orotic acid pathway. The initial reaction in this pathway, the formation of carbamyl phosphate, is catalyzed by a glutamine-dependent carbamyl phosphate synthetase. Although either L-glutamine or ammonia is utilized as a nitrogen donor for this reaction, the enzyme has a much greater affinity for glutamine. Other properties of the enzyme are described.

Enzymes catalyzing the conversion of carbamyl phosphate into uridine nucleotides were demonstrated using radioactive substrates. The level of activity of aspartate transcarbamylase in the unfertilized frog egg is about 100 times that of carbamyl phosphate synthetase. Present data suggest that the formation of carbamyl phosphate is the rate-limiting step in the over-all pathway and that carbamyl phosphate synthetase may be a regulatory site for pyrimidine biosynthesis.

Many studies relating to the biosynthesis of DNA and RNA in early embryonic development of the amphibian have been reported. This work has been reviewed recently (Grant, 1965). The rapid replication of DNA initiated at fertilization, as well as the net formation of RNA that occurs at later developmental stages, has posed the question as to the source and nature of the precursors of these macromolecules. A pool of acid-soluble deoxyribonucleotides (Grant, 1958a,b; Kuriki and Okazaki, 1959) and of ribonucleotides (Finamore and Crouse, 1962; Warner and Finamore, 1962) has been demonstrated in the unfertilized frog egg and later in development. The changes in the amounts of the nucleotides following fertilization suggest their utilization for nucleic acid biosynthesis in the embryo. In addition, isotopic studies have indicated that smaller molecules are precursors for DNA and RNA formation. In the intact frog embryo, glycine and P_i (Kutsky, 1950; Grant, 1958a,b), as well as CO_2 (Cohen, 1954; Flickinger, 1954), are utilized for the formation of nucleotides and nucleic acids. During development, control of transcription and the associated synthesis and turnover of RNA involved in translation must involve a number of factors in order to ensure adequate regulation. However, the entire system in turn must depend upon the availability of ribonucleotides both for RNA synthesis and as precursors of deoxyribonucleotides used in the formation of DNA. Hence, recognition of possible regulatory mechanisms relating to the *de novo* synthesis of these nucleotides is of importance. However, little or no information is available with regard to the properties of the enzymes that are involved in the pathways or of the control mechanisms that must be functioning in the developing embryo.

The initial step in the *de novo* synthesis of pyrimidines is the formation of carbamyl phosphate. In animal systems, this compound may be formed by the action of two different enzymes. The first carbamyl phosphate synthetase to be characterized was the hepatic enzyme involved in urea biosynthesis (Cohen, 1966). It is a mitochondrial enzyme which utilizes ammonia as the nitrogen donor and requires *N*-acetylglutamate as a cofactor. The *de novo* synthesis of this enzyme has been demonstrated in amphibians at the onset of metamorphosis during which the ammonotelic tadpole becomes a ureotelic frog (Metzenberg *et al.*, 1961). The second type of carbamyl phosphate synthetase, which was found initially in mushrooms (Levenberg, 1962) and later in *Escherichia coli* (Pierard and Wiame, 1964; Pierard, 1966) and yeast (Lacroute *et al.*, 1965), has recently been demonstrated in animal tissues (Tatibana and Ito, 1967; Hager and Jones, 1967a,b; Maresch and Kalman, 1968) and the seedlings of higher plants (Kleczkowski and Reifer, 1967; O'Neal and Naylor, 1968). This enzyme utilizes glutamine as the preferred nitrogen donor, does not require *N*-acetylglutamate for activity, and is present in the cytosol. The activity of the enzyme from *E. coli* is inhibited by pyrimidine nucleotides and stimulated by purine nucleotides (Anderson and Meister, 1966). It has been reported that the enzyme from mouse spleen is subject to feedback inhibition by UTP (Tatibana and Ito, 1967). Present evidence is consistent with the fact that the glutamine-dependent carbamyl phosphate synthetase plays a primary role in providing carbamyl phosphate for pyrimidine biosynthesis and is a regulatory site for this pathway.

In view of the above, studies were initiated at the enzymatic level on the synthesis of pyrimidines by the orotic acid pathway in the unfertilized frog egg. This system was chosen in an effort to explore the systems and regulatory factors which exist and function in the primordial cell preceding fertilization, differentiation, and development.

Experimental Procedure

Materials. [^{14}C]Sodium bicarbonate (20 mCi/mmole), DL-[carbamyl-^{14}C]carbamylaspartic acid (1.76 mCi/mmole), [6-^{14}C]4,5-dihydroorotic acid (3.7 mCi/mmole), and [6-^{14}C)orotic acid (4.6 mCi/mmole) were obtained from New England Nu-

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clear Corp. Uniformly labeled L-[^{14}C]aspartic acid (1.95 μCi/mg) was purchased from Schwarz BioResearch, Inc. [^{14}C]-Carbamyl phosphate (4.3×10^5 cpm/μmole), nonradioactive carbamyl phosphate, as the diammonium salts, and *O*-carbamyl-L-serine were kindly supplied by Dr. Margaret Marshall of this laboratory. Radioactive compounds were dissolved in and diluted with 0.05 M potassium phosphate buffer (pH 7.5) to give appropriate concentrations (see individual experiments). L-Glutamine, *N*-acetyl-L-glutamic acid, L-aspartate, 5-phosphoribosyl 1-pyrophosphate, uracil, uridine, and purine and pyrimidine nucleotides were obtained from Sigma Chemical Co. L-Ornithine was purchased from Cyclo Chemical Corp. Ammonium sulfate suspensions of purified ornithine transcarbamylase from *Streptococcus faecalis* and beef liver with specific activities of 3200 and 600 units per mg of protein, respectively, were gifts of Dr. Margaret Marshall of this laboratory. A unit is defined as the amount of enzyme capable of forming 1 μmole of citrulline/min at 37° (Marshall and Cohen, 1966). Before use, the enzyme preparations were freed of ammonium sulfate by dialysis overnight against 10,000 volumes of 0.05 M potassium phosphate buffer, pH 7.5, at 4°.

Gravid female frogs of *Rana pipiens* and *Rana catesbeiana* were purchased from the Lemberger Co., Oshkosh, Wis., or J. R. Schettle Biologicals, Stillwater, Minn.

Enzyme Preparations. Two types of eggs were used in these studies, namely, ovulated eggs from *R. pipiens* and ovarian eggs from *R. catesbeiana*. Gravid females of *R. pipiens* were induced to ovulate by the injection of a pituitary suspension and were obtained in this form from the commercial source. Eggs were stripped 24–48 hr later. The jelly coat was removed from the eggs prior to homogenization as described earlier (Adachi *et al.*, 1968). After removal of the jelly coat, the eggs were allowed to settle. A volume of settled eggs was homogenized in a glass homogenizer with an equal volume of cold homogenizing medium (described below). The homogenate was centrifuged at 105,000*g* for 30 min and the supernatant solution was recovered. All operations were carried out at 0–4°. In the studies with ovarian eggs, gravid females were killed by decapitation and the eggs plus ovary were removed. The tissue extracts were prepared exactly as outlined above except that a known weight of the egg-ovary tissue was homogenized with an equal volume of homogenizing medium. The enzyme preparation used in a given experiment is shown with the experimental results.

In the studies of carbamyl phosphate synthetase, the homogenizing medium was 0.2 M potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 5 mM ATP–magnesium chloride, and 5 mM β-mercaptoethanol. The supernatant solution (20 ml) was gel filtered through Sephadex G-25 (4×50 cm column) that had been equilibrated with 0.2 M potassium phosphate buffer (pH 7.5) containing 15% glycerol (v/v). The bulk of the protein, contained in 40 ml of eluate, was used in the initial assays of carbamyl phosphate synthetase (referred to as gel-filtered supernatant solution). In later experiments, the supernatant solution was dialyzed against 4 l. of 0.1 M potassium phosphate buffer (pH 7.5) containing 2.5 mM β-mercaptoethanol and 15% glycerol (v/v), overnight at 3° (referred to as dialyzed supernatant solution).

In the experiments carried out to demonstrate the subsequent steps in pyrimidine biosynthesis, the homogenizing medium was 0.05 M potassium phosphate buffer (pH 7.5). The supernatant solution obtained after homogenization and centrifugation was used without further treatment in the incubation system (referred to as tissue extract).

Assay for Carbamyl Phosphate Synthetase. The activity of carbamyl phosphate synthetase was measured by converting the radioactive carbamyl phosphate formed from [^{14}C]bicarbonate into acid-stable [^{14}C]citrulline in the presence of excess L-ornithine and ornithine transcarbamylase. Unless otherwise indicated, the standard assay system contained the following: ATP (50 μmoles), magnesium chloride (50 μmoles), [^{14}C]-$NaHCO_3$ (10 μmoles; specific activity used is given with individual experiments), L-ornithine (20 μmoles), ornithine transcarbamylase (25 units), potassium phosphate buffer (pH 7.5, 200 μmoles), nitrogen donor, either L-glutamine (20 μmoles) or ammonium chloride (100 μmoles), and enzyme preparation (1 ml) to give a final volume of 2 ml. The reaction mixture was incubated at 37° for 30 min. The reaction was stopped by the addition of 0.2 ml of 30% perchloric acid and the precipitated protein was removed by centrifugation. The supernatant solution was kept under a hood at room temperature for an additional 2 hr with occasional agitation to ensure the complete removal of radioactive carbon dioxide. (Control experiments with known amounts of [^{14}C]$NaHCO_3$ demonstrated that [^{14}C]CO_2 was completely removed under these conditions.) Aliquots (1.8 ml) of the deproteinized reaction mixtures were applied to a Dowex 50 (H^+) column (1×2 cm). The column was washed with 10 ml of water and then eluted with 0.5 M ammonium acetate; 5-ml fractions were collected. For the determination of radioactivity, 1.0-ml aliquots were mixed with 18 ml of dioxane scintillation fluid (Bray, 1960) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Since more than 90% of the radioactivity fixed in an acid-stable form was recovered after chromatography on Dowex 50 (H^+), in later experiments, 0.2-ml aliquots of the deproteinized reaction mixtures were counted directly and used as a measure of the activity of the enzyme.

Assay System and Column Chromatographic Procedures Used for Subsequent Enzymatic Reactions of Pyrimidine Biosynthesis. Experimental details are given with the individual experiments in the Results section. In general, a given radioactive substrate was dissolved in 1 ml of 0.05 M potassium phosphate buffer (pH 7.5) and incubated with 4 ml of the tissue extract (see above) at 37° for 1 hr. The reaction was stopped by the addition of 1 ml of 30% perchloric acid. The precipitated protein was removed by centrifugation and was washed twice by resuspending in 5 ml of distilled water. The supernatant solution and washings were combined and neutralized to pH 7.0 with 4 N KOH. After standing at 0° for 1 hr, insoluble potassium perchlorate was removed by centrifugation. The resulting supernatant solution was chromatographed on a column (2×25 cm) of Dowex 1 X8 (formate form, 200–400 mesh). The column was washed with 150 ml of water and then subjected to a stepwise elution with increasing concentrations of formic acid and ammonium formate. The elution solvents used in a given experiment are shown with the results. The eluate was collected in 10-ml samples with an automatic fraction collector. Radioactivity in each fraction was measured by counting 0.2-ml aliquots as described above. In control experiments, the pattern of elution of known radioactive compounds was established under the same conditions used for the separation of the products.

Paper Chromatography was used in the further identification of the products formed in a given experiment. For this

purpose, the fractions containing radioactivity and corresponding to a given peak were pooled and brought to dryness either by lyophilization or by evaporation under reduced pressure at 40° in a rotary evaporator. Eluates containing ammonium formate were freed of ammonium ions prior to concentration by chromatography on Dowex 50 (H^+). The radioactive residues were dissolved in a minimum of distilled water and subjected to ascending paper chromatography on Whatman No. 1 paper in the four following solvent systems: (A) ethanol–methanol–formic acid–water (6:6:3:5, v/v), (B) 1-butanol–acetic acid–water (80:16:40, v/v), (C) isopropyl alcohol–water (3:2, v/v), and (D) ethanol–1 M ammonium acetate (70:30, v/v). Known radioactive compounds were chromatographed simultaneously under the same conditions to obtain standard R_F values for comparative purposes. Following chromatography, radioactive spots were detected using a paper strip scanner (Nuclear-Chicago, Actigraph III).

Other Analytical Procedures. L-Citrulline was determined colorimetrically by the method of Archibald (Archibald, 1944). Protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Results

Carbamyl Phosphate Synthetase. A glutamine-dependent carbamyl phosphate synthetase, the enzyme catalyzing the initial reaction in the orotic acid pathway for pyrimidine biosynthesis, was observed in crude extracts of both unfertilized eggs of *R. pipiens* and egg-ovary preparations from *R. catesbeiana*. The occurrence of the enzyme in such preparations was demonstrated using an assay system in which the [^{14}C]carbamyl phosphate formed enzymatically from [^{14}C]$NaHCO_3$ was converted into [^{14}C]citrulline in the presence of excess ornithine transcarbamylase and L-ornithine. The acid-stable product formed under such conditions was identified as citrulline by the following criteria. The radioactive product was cochromatographed with nonradioactive L-citrulline on a column of Dowex 50 (H^+) (Figure 1). The radioactive product was retained on the resin; it was eluted with 4 N HCl and appeared as a single peak. The elution profile of the radioactive product coincided with that of L-citrulline as determined by colorimetric assay. In addition, the R_F values of the radioactive product on paper chromatography in four different solvent systems (see Experimental Procedure) were identical with those of authentic citrulline. The R_F values obtained in the respective solvent systems were (A) 0.67, (B) 0.13, (C) 0.63, and (D) 0.42. Similar results were obtained with enzyme preparations from egg-ovary of *R. catesbeiana*.

As is the case with the enzyme from mammalian tissues (Tatibana and Ito, 1967; Hager and Jones, 1967a,b), carbamyl phosphate synthetase from frog eggs is very unstable but can be stabilized by the addition of glycerol (Tatibana and Ito, 1967). Omission of the glycerol from the homogenizing medium results in a marked reduction in the activity of the enzyme.

Either L-glutamine or ammonia is utilized as a nitrogen donor in the standard assay system (Table I). However, when both glutamine and ammonia are included in the incubation system, there is no summation of the activity observed with either substrate alone. Addition of *N*-acetylglutamate, the cofactor required for the hepatic carbamyl phosphate synthetase involved in urea biosynthesis (Cohen, 1966), does not increase the amount of product formed. The incorporation of [^{14}C]bicarbonate into citrulline is dependent upon the presence of both the enzyme from frog eggs and ornithine transcarbamylase in the incubation system. Control experiments demonstrated that the amount of ornithine and ornithine transcarbamylase used in the standard assay system were in excess. When either ATP or Mg^{2+} is omitted from the incubation system, little or no synthesis is observed.

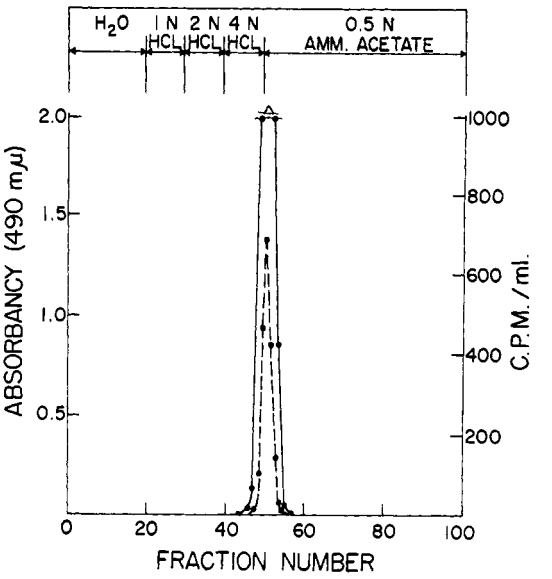

FIGURE 1: Identification of [^{14}C]citrulline as the product formed from [^{14}C]bicarbonate in the coupled carbamyl phosphate synthetase–ornithine transcarbamylase system. The standard assay system with glutamine as the nitrogen donor, incubation conditions, and method of enzyme preparation (gel-filtered supernatant solution from ovulated eggs of *R. pipiens*) are described in Experimental Procedure. Nonradioactive citrulline (20 mg) was added to pooled deproteinized reaction mixtures from 20 incubations and the solution was adjusted to pH 7 with 4 N KOH. After chilling, insoluble potassium perchlorate was removed by centrifugation and the resulting supernatant solution was chromatographed on a column (2 × 20 cm) of Dowex 50 (H^+). The elution solvents were as indicated at the top of the diagram. Fraction size was 10 ml. The broken lines represent radioactivity and the solid lines absorbance.

A significant synthesis of radioactive product was observed in the absence of any added nitrogen donor (Table I). Paper chromatography of aliquots of reaction mixtures established that the product formed under these conditions was citrulline; this result was consistent with the fact that no synthesis was observed in the absence of added ornithine transcarbamylase. The synthesis of citrulline observed in the absence of either added glutamine or ammonium chloride was shown to be due to the release of L-glutamine from the frog egg enzyme preparation during the course of the incubation as described below. L-Glutamine and other free amino acids have been demonstrated in unfertilized eggs of *R. pipiens* and other amphibia (*cf.* Deuchar, 1966). In view of the relatively low K_m reported for the glutamine-dependent carbamyl phosphate synthetase from mammalian sources (Tatibana and Ito, 1967; Hager and Jones, 1967a,b), it seemed possible that the observed synthesis of the product in the absence of any added nitrogen donor was due to small amounts of glutamine in the enzyme preparation following gel filtration. Analyses of pro-

TABLE I: Requirements of the Carbamyl Phosphate Synthetase Reaction.[a]

Incubation System	Nitrogen Donor	Total $^{14}CO_2$ Fixed[b] Expt 1[c]	Expt 2[c]
Complete	Glutamine	2855	3085
Complete	NH_4Cl	3070	2950
Complete	Glutamine + NH_4Cl		2815
Complete + *N*-acetyl-glutamate (0.1 mM)	NH_4Cl	2492	2265
Complete	None[d]	1793	1135
Complete − enzyme	Glutamine	165	35
Complete − ATP	Glutamine	120	40
Complete − Mg^{2+}	Glutamine	143	10
Complete − ornithine transcarbamylase		154	

[a] The standard assay system, incubation conditions, and method of enzyme preparation are described in Experimental Procedure. The specific activity of the [^{14}C]bicarbonate was 3.5×10^5 cpm/μmole. The enzyme preparations used were the gel-filtered supernatant fractions of ovulated eggs of *R. pipiens* (15.0 mg of protein) and of egg-ovary of *R. catesbeiana* (15.5 mg of protein) in expt 1 and 2, respectively. [b] Determined after adsorption to and elution from Dowex 50 (H^+ form). [c] Cpm/2-ml reaction mixture. [d] Control experiments, in which enzyme fractions were incubated under standard conditions in the absence of substrates and the ornithine transcarbamylase system, deproteinized at 0 time and at end of incubation, and aliquots of the supernatant solution analyzed with the Beckman-Spinco amino acid analyzer, demonstrated that glutamine was released from the enzyme preparation during the course of the incubation.

TABLE II: Effect of L-Glutamine or Ammonium Chloride Concentration on the Activity of Carbamyl Phosphate Synthetase.[a]

Nitrogen Source (mM)	Total $^{14}CO_2$ Fixed Expt 1[b]	Expt 2[b]
I. L-Glutamine		
0	1700	1515
0.01	2570	
0.05	3310	4320
0.1	3580	4345
0.5	3590	
1.0	3650	4466
5.0	3510	3935
10.0	3500	
II. Ammonium Chloride		
1.0	1800	1955
5.0	2130	2345
10.0	2410	2600
50.0	3350	4035

[a] The standard assay system with glutamine or ammonium chloride as the nitrogen donor in the concentrations indicated, incubation conditions, and method of enzyme preparation are described in Experimental Procedure. The specific activity of the [^{14}C]bicarbonate was 1.2×10^5 cpm/μmole. The enzyme preparations were the dialyzed supernatant fractions of ovulated eggs from *R. pipiens* (30.3 mg of protein) and of egg-ovary from *R. catesbeiana* (20.0 mg of protein) in expt 1 and 2, respectively. [b] Cpm/2-ml reaction mixture.

tein-free extracts of the enzyme preparation prior to gel filtration were carried out on the Beckman-Spinco amino acid analyzer and demonstrated that aspartic acid, glutamic acid, glutamine, and glycine were present in relatively large amounts. However, analyses of similar extracts prepared after gel filtration of the enzyme demonstrated that more than 95% of the total free amino acids were removed by this procedure. The possible release of amino acids from the enzyme preparation during the course of the incubation was then investigated. Enzyme preparations were incubated under the standard assay conditions in the absence of added substrates and the ornithine transcarbamylase system. Samples were deproteinized at zero time and at the end of the incubation period. Analysis of aliquots of protein-free extracts on the amino acid analyzer demonstrated that glutamine was released during the course of the incubation.

The effectiveness of glutamine and ammonia as nitrogen donors for carbamyl phosphate synthetase activity was compared (Table II). The enzyme has a much greater affinity for glutamine than for ammonia. Under the experimental conditions used, maximal activity is observed with 0.05 mM glutamine whereas 50 mM ammonium chloride is required for equivalent activity. In view of the demonstrated release of glutamine from the enzyme preparation during the incubation period, no exact data for this substrate can be established for the enzyme until further purification is achieved. However, the relatively low levels of glutamine required indicate that it is the preferred substrate, in agreement with previous work on this enzyme from other animal sources (Tatibana and Ito, 1967; Hager and Jones, 1967a,b), and also support the conclusion that the activity observed in the absence of any added nitrogen donor is due to the small amount of glutamine released from the enzyme preparation.

It has been demonstrated that carbamyl phosphate synthetase from Ehrlich ascites carcinoma is inhibited by glutamine analogs (Hager and Jones, 1967a); one of the most effective compounds was *O*-carbamyl-L-serine. The effect of this analog on the frog enzyme was investigated using glutamine as the nitrogen donor and inhibition was observed (Table III). The reduction in activity in the presence of *O*-carbamyl-L-serine observed in the experiments without added nitrogen donor is additional evidence that the activity observed under these conditions is due to the release of glutamine from the enzyme during the course of the incubation. This is also the probable explanation for the fact that only 60% inhibition was observed with the inhibitor at a concentration of 5 mM whereas an 80% inhibition was observed with the enzyme

TABLE III: Effect of *O*-Carbamyl-L-serine on Activity of Carbamyl Phosphate Synthetase.[a]

Incubation System	*O*-Carbamyl-L-serine (mM)	Total $^{14}CO_2$ Fixed (cpm/2-ml reaction mixture)	% Act.
Complete	0	2990	100
Complete	5	1208	40
Complete	10	843	28
Complete — nitrogen donor	0	1835	61
Complete — nitrogen donor	5	432	14
Complete — nitrogen donor	10	329	11

[a] The standard assay system with glutamine (0.1 mM) as the nitrogen donor, incubation conditions, and method of enzyme preparation are described in Experimental Procedure. The specific activity of the [^{14}C]bicarbonate was 1.2×10^5 cpm/μmole. Enzyme preparation was the dialyzed supernatant fraction from ovulated eggs of *R. pipiens* (30.5 mg of protein).

TABLE IV: Effect of Uridine Nucleotides on Activity of Carbamyl Phosphate Synthetase.[a]

Nucleotide Added[b]	Total $^{14}CO_2$ Fixed (cpm/2-ml reaction mixture)	% Act.
None	3000	100
UTP	1066	35
UDP	933	31
UMP	975	32
UTP + UMP	1040	34

[a] The standard assay system containing ATP (25 mM) and glutamine (5 mM) as the nitrogen donor, incubation conditions, and method of enzyme preparation are described in Experimental Procedure. The specific activity of the [^{14}C]-bicarbonate was 1.2×10^5 cpm/μmole. The enzyme preparation was the dialyzed supernatant fraction of ovulated eggs from *R. pipiens* (21.0 mg of protein). [b] The Mg^{2+}–uridine nucleotides were added at a final concentration of 10 mM. In the assay system containing both UTP and UMP, the final concentration of each was 5 mM.

from Ehrlich ascites carcinoma under the same conditions (Hager and Jones, 1967a).

On the basis of the above results, it is clear that the unfertilized frog egg contains a glutamine-dependent carbamyl phosphate synthetase whose properties are similar to those reported for the mammalian enzyme (Tatibana and Ito, 1967; Hager and Jones, 1967a,b). Since evidence has been presented that this enzyme is a regulatory site in the pathway for pyrimidine biosynthesis in bacterial (Anderson and Meister, 1966) and mammalian (Tatibana and Ito, 1967) systems, it is of particular interest from the standpoint of development to determine if the activity of the enzyme from frog eggs is subject to such regulation. Purification of the enzyme is required to permit definitive studies relating to this point. However, preliminary studies with the crude enzyme suggest that uridine nucleotides (Table IV) do inhibit the reaction, although at relatively high concentrations.

In view of the fact that extracts of unfertilized frog eggs contain an active carbamyl phosphate synthetase, studies were carried out to determine if the other enzymes of the orotic acid pathway are present in these preparations as well. The studies reported below were carried out with enzyme preparations from both ovulated eggs of *R. pipiens* and egg-ovary preparations from *R. catesbeiana*. Since similar results were obtained with both sources of the enzymes, only the studies utilizing extracts from ovulated eggs of *R. pipiens* are reported below.

Formation of [^{14}C]*Carbamylaspartate from Either* [^{14}C]*Carbamyl Phosphate or* [^{14}C]*Aspartate.* The formation of radioactive carbamylaspartate from [^{14}C]carbamyl phosphate and L-aspartate was demonstrated after incubation of these substrates with cell-free extracts of unfertilized eggs from *R. pipiens* (Figure 2). The principal product formed was eluted from the Dowex 1-formate column in fractions 52–58 and, on the basis of radioactivity, represents a conversion of 4.7 μmoles of the carbamyl phosphate added originally. It was identified as carbamylaspartate on the basis of its elution characteristics from the column, which were identical with those of authentic DL-[carbamyl-^{14}C]carbamylaspartate chromatographed under the same conditions, and on the basis of identical R_F values for the radioactive product and the authentic compound on paper chromatography in the four different solvent systems (see Experimental Procedure). The R_F values obtained were (A) 0.80, (B) 0.43, (C) 0.34, and (D) 0.13, respectively.

To confirm these results, similar experiments were carried out using radioactive L-aspartate and nonisotopic carbamyl phosphate as substrates. Of the total aspartic acid added (20 μmoles), 27%, or 5.4 μmoles, was converted into a radioactive product which was identified as carbamylaspartate by the same criteria used in the previous experiments.

For comparative purposes, the activity of aspartate transcarbamylase in extracts of ovulated eggs from *R. pipiens* was measured under the conditions outlined by Hager and Jones (1967b). The specific activity of the enzyme was found to be 0.23 μmole of product formed per mg of protein per hr. It is clear from the above studies that the unfertilized frog egg contains an active aspartate transcarbamylase.

The feedback inhibition of aspartate transcarbamylase by UTP and CTP in bacterial systems is well established (Gerhardt and Pardee, 1962); however, the mammalian enzyme appears to be unaffected by these compounds (Curci and Donachie, 1964; Bresnick and Mossé, 1966). Addition of millimolar concentrations of CTP to the incubation systems in the present studies did not lead to any decrease in the total amount of radioactivity incorporated into carbamylaspartate.

Utilization of [^{14}C]*Carbamylaspartate and* [6-^{14}C]*Dihydro-*

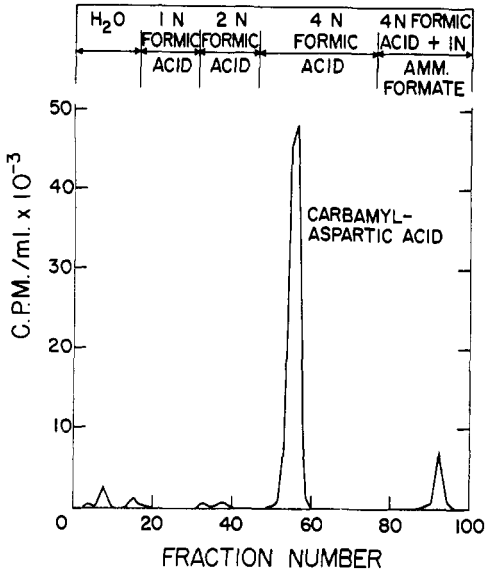

FIGURE 2: Carbamylaspartate formation from [^{14}C]carbamyl phosphate and L-aspartate in cell-free extracts of ovulated eggs of *R. pipiens*. The reaction mixture contained 23 μmoles of [^{14}C]carbamyl phosphate (3.6×10^5 cpm/μmole), 100 μmoles of L-aspartate, and 4 ml of enzyme preparation; incubation was carried out at 37° for 1 hr. Other details are described in Experimental Procedure. The elution solvents were as indicated at the top of the diagram. Fraction size was 10 ml.

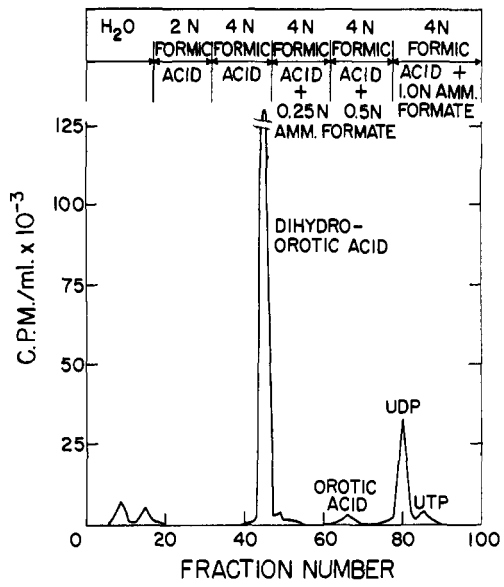

FIGURE 3: Formation of orotic acid and uridine nucleotides from [6-^{14}C]dihydroorotic acid in cell-free extracts of ovulated eggs from *R. pipiens*. The reaction mixture contained 1.0 μmole of [6-^{14}C]-dihydroorotic acid, 3.6×10^6 cpm/μmole, and 4 ml of enzyme preparation; incubation was carried out at 37° for 1 hr. Other details are described in Experimental Procedure. The elution solvents were as indicated at the top of the diagram. Fraction size was 10 ml.

orotic Acid as Precursors for Pyrimidine Biosynthesis. Since it has been demonstrated that carbamylaspartate is an intermediate in pyrimidine biosynthesis (Reichard and Lagerkvist, 1953) and is converted into orotic acid *via* dihydroorotic acid (Lieberman and Kornberg, 1954; Cooper *et al.*, 1955), tracer experiments were carried out to determine if the enzymes involved in these conversions are present in the unfertilized frog egg. Both radioactive carbamylaspartate and dihydroorotic acid were investigated. In these studies, radioactive products were identified on the basis of: (a) the same elution characteristics of authentic compounds and unknowns from Dowex 1-formate columns, and (b) identical R_F values of known and unknown compounds on paper chromatography in four different solvent systems. Incubation of 2.8 μmoles of DL-[carbamyl-^{14}C]carbamylaspartate (3.2×10^6 cpm/μmole) with 4 ml of frog egg extract at 37° for 1 hr followed by chromatography of the deproteinized reaction mixture on Dowex 1-formate columns led to the isolation of two radioactive products. These were identified as dihydroorotic acid and UDP. D-Carbamylaspartate has been shown to be inactive as an orotic acid precursor (Lieberman and Kornberg, 1954). The radioactivity recovered as dihydroorotic acid and as UDP indicated a conversion of 4% of the available L-carbamylaspartate into each of the products. Similar studies were carried out with [6-^{14}C]dihydroorotic acid (Figure 3). In these studies also, UDP was the principal product formed (10% conversion of substrate) and only a small amount of orotic acid (<1% conversion of substrate) was detected. A greater accumulation of the next immediate product, *i.e.*, dihydroorotic acid from carbamylaspartate and orotic acid from dihydroorotic acid, had been expected in these experiments than was observed. However, it should be pointed out that the extracts used in these experiments were not dialyzed. Unfertilized eggs of *R. pipiens* have been shown to contain ATP, as well as other nucleotides, which in the presence of the appropriate kinases, could account for the formation of UDP that was observed. These results indicate that both dihydroorotase and dihydroorotic dehydrogenase are present in unfertilized eggs and that they are active at relatively low substrate levels. The accumulation of radioactivity in uridine nucleotides suggests the presence of active orotidine 5'-phosphate pyrophosphorylase and orotidylic decarboxylase in the extracts as well. This was investigated as outlined below.

Formation of Uridine Nucleotides from [6-^{14}C]Orotic Acid and 5'-Phosphoribosyl 1-Pyrophosphate. It has been demonstrated that orotic acid is converted into UMP *via* OMP[1] in a reaction utilizing 5'-PRPP (Cooper *et al.*, 1955). These reactions were investigated using radioactive orotic acid as the substrate (Figure 4). Product identification was based on the criteria used in earlier experiments, *i.e.*, column and paper chromatography. Incubation of orotic acid and 5'-PRPP with the frog egg extract did not lead to a detectable accumulation of OMP. Radioactive products obtained under these experimental conditions (broken lines, Figure 4) were identified as UMP (3%), UDP (13.4%), and UTP (40.7%). It has been reported that in rat (Blair *et al.*, 1960) and tadpole (Lindsay *et al.*, 1964) liver preparations OMP production from orotic acid is increased in the presence of UMP, presumably due to the inhibition of orotidylic decarboxylase by this compound. When nonradioactive UMP was included in the reaction mixture (solid lines, Figure 4), the amount of radioactive orotic acid converted into UTP was decreased to 12.7% and that recovered as UMP was increased to 11.8%. In addition, there

[1] Abbreviations used are: OMP, orotidine 5'-phosphate; 5'-PRPP, 5'-phosphoribosyl 1-pyrophosphate.

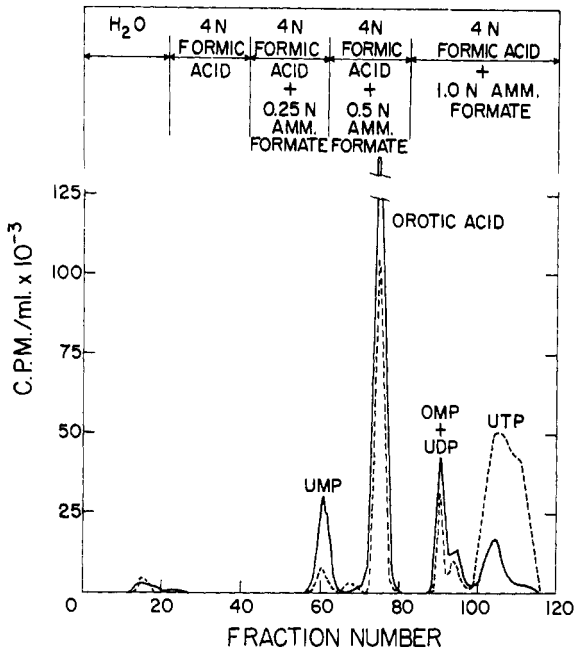

FIGURE 4: Formation of pyrimidine nucleotides from [6-^{14}C]orotic acid and 5′-phosphoribosyl 1-pyrophosphate in cell-free extracts of ovulated eggs of *R. pipiens* in the presence (solid lines) and absence (broken lines) of nonradioactive UMP. The reaction mixture contained, 1.0 μmole of [6-^{14}C]orotic acid, 9.0 × 10^6 cpm/μmole, 10 μmoles of 5′-phosphoribosyl pyrophosphate, 10 μmoles of UMP as indicated, and 4 ml of enzyme preparation; incubation was carried out at 37° for 1 hr. Other details are described in Experimental Procedure. The elution solvents were as indicated at the top of the figure. Fraction size was 10 ml.

was an increase in the total radioactivity in the fractions from the Dowex 1-formate column which usually contained only UDP (fractions 87–98; Figure 4). These fractions were pooled, concentrated, and subjected to paper chromatography as described in Experimental Procedure. Following paper chromatography, a second radioactive product in addition to UDP was detected. This product was identified as OMP on the basis of the following results. Aliquots of the concentrated pooled fractions were hydrolyzed in 6 N HCl at 100° for 6 hr and then subjected to paper chromatography. Radioactive orotic acid, uracil, and uridine were identified on the basis of their R_F values in the four different solvent systems.

Discussion

The results of the present investigation demonstrate that enzyme systems for the *de novo* synthesis of uridine nucleotides are present in soluble extracts of the unfertilized frog egg. On the basis of the requirements established for the formation of carbamyl phosphate in this system, the initial reaction in this pathway is catalyzed by a glutamine-dependent carbamyl phosphate synthetase. The properties of this enzyme are similar to those of the enzyme from other animal sources (Tatibana and Ito, 1967; Hager and Jones, 1967a,b).

It has been reported that the formation of carbamyl phosphate is the rate-limiting step in the over-all pathway for pyrimidine biosynthesis in mouse spleen (Tatibana and Ito, 1967) and in Ehrlich ascites carcinoma (Hager and Jones, 1967a). A comparison of the levels of activity of the glutamine-dependent carbamyl phosphate synthetase with those of the enzyme catalyzing the next reaction in the sequence, aspartate transcarbamylase, supports this conclusion. The reported level of activity of the latter enzyme exceeds that of the former 100 times in mouse spleen (Tatibana and Ito, 1967) and some 800-fold in Ehrlich ascites carcinoma (Hager and Jones, 1967a) and in avian and fetal rat liver (Hager and Jones, 1967b). The present studies indicate that the formation of carbamyl phosphate is also the rate-limiting step in pyrimidine biosynthesis in the unfertilized frog egg. The specific activity of aspartate transcarbamylase was found to be 0.23 μmole of carbamylaspartate formed per mg of protein per hr. In comparison, the specific activity of carbamyl phosphate synthetase (see expt 1, Table II) was much less, 0.002 μmole of product/mg of protein per hr. No attempt was made to measure the levels of activity of the enzymes catalyzing the subsequent reactions of the orotic acid pathway. However, the fact that uridine nucleotides were the chief radioactive products formed in the experiments with both isotopic carbamylaspartate and dihydroorotic acid at a final concentration of 0.2 μM suggests that the level of activity of the enzymes involved are not rate limiting for pyrimidine biosynthesis.

During development, the frog egg represents essentially a "closed system" as regards metabolic requirements from its environment. If the egg is provided with oxygen and an aqueous salts medium, it will develop into a young tadpole and its requirements during this period are provided by a rearrangement of stored materials. Hence, the regulation of its metabolism by alterations in the activity of enzymes is of great importance. Therefore, it is of particular interest from the developmental viewpoint to determine whether the activity of the glutamine-dependent carbamyl phosphate synthetase is subject to end-product control and thus plays a role in determining the availability of ribonucleotides used for the synthesis of nucleic acids which occurs during early embryonic development. Preliminary studies with the soluble extract from the unfertilized frog egg indicate that uridine nucleotides at millimolar concentrations in the standard assay system inhibit the activity of carbamyl phosphate synthetase. Definitive studies relating to this point must await the purification of the enzyme which is currently in progress.

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Transfer Ribonucleic Acid from *Mycoplasma**

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ABSTRACT: Transfer ribonucleic acid from three strains of *Mycoplasma* (*Mycoplasma laidlawii* B, *Mycoplasma gallisepticum* A 5969, and *Mycoplasma sp.* (Kid)) was studied. These transfer ribonucleic acids have sedimentation coefficients similar to that of *Escherichia coli* transfer ribonucleic acid as judged by cosedimentation of *E. coli* and *Mycoplasma* seryl transfer ribonucleic acid. *Mycoplasma* transfer ribonucleic acids contain minor nucleosides in lower amounts than are found in *E. coli* transfer ribonucleic acid. All three *Mycoplasma* strains investigated contain *N*-formylmethionyl transfer ribonucleic acid, which can be formed with formyltetrahydrofolate as formyl donor by both homologous or *E. coli* enzymes.

Chromatography of *M. laidlawii* B transfer ribonucleic acid on benzoylated DEAE-cellulose cleanly separates formylmethionyl transfer ribonucleic acid and methionyl transfer ribonucleic acid. In a transfer ribonucleic acid dependent cell-free amino acid incorporating system from *E. coli* directed by polyuridylic or f2 ribonucleic acid, *Mycoplasma* transfer ribonucleic acids stimulate polypeptide formation.

The smallest free-living cells that have been investigated are pleuropneumonia-like organisms (*Mycoplasma*) (Morowitz, 1966; Klieneberger-Nobel, 1962). *Mycoplasma* cells are much smaller than bacteria and possess a genome about one-quarter as large as *Escherichia coli*. In view of the small size of these organisms, it was of interest to examine more closely their tRNA to see whether it would exhibit special characteristics. The *Mycoplasma* strains used for the study were *Mycoplasma gallisepticum* and *Mycoplasma laidlawii* on which a large amount of biochemical work has been done, and *Mycoplasma sp.* (Kid) whose DNA has an unusually low content of G and C (Bode, 1966).

The present work shows that *Mycoplasma* tRNA has physical properties similar to *E. coli* tRNA. Minor nucleosides are constituents of *Mycoplasma* tRNA; however, they are present in lower amounts than in *E. coli* tRNA (see also Hall *et al.*, 1967). The three *Mycoplasma* strains investigated contain fMet-tRNA. *Mycoplasma* tRNA stimulates polypeptide formation in a tRNA-dependent amino acid incorporating system directed by poly U or f2 RNA.

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