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Protein Arginine Biosynthesis in Mammalian and Amphibian Liver

by

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PROTEIN ARGinine BIOSYNTHESIS IN
MAMMALIAN AND AMPHIBIAN LIVER
INTRODUCTION

The amino acid L-arginine is a necessary protein constituent which is synthesized by some vertebrate and invertebrate animals by the pathway shown in Fig. 1. Those species in which this metabolic pathway is nonfunctional show a dietary requirement for L-arginine. In addition to arginine biosynthesis, the arginine pathway also serves as the main route for ammonia detoxication in ureotelic species. In the latter capacity, the pathway is known as the urea or ornithine-urea cycle. The relationship of the two functions of the arginine pathway, nutritional and excretory, is shown in Fig. 1.

Following the formulation of the ornithine-urea cycle by Krebs and Henseleit (1932), the majority of comparative studies have been directed toward the function of the pathway in ammonia detoxication and ureogenesis in animals. Ammonia is relatively toxic to most higher animals and, in general, only low concentrations are found in their body fluids. Bats appear to represent unusual exceptions to this generalization since they tolerate high concentrations of blood ammonia (Studier, 1966; Studier et al., 1967). The mechanism of ammonia toxicity is that a high concentration induces alkalosis to maintain relatively low concentrations of ammonia (Goldstein and Schooler, 1967). The maintenance of low concentrations of blood ammonia presumably does not present a problem to aquatic organisms with an abundant water supply since they can lose the ammonium ion by diffusion or possibly by an ion exchange mechanism (Maetz and Romeu, 1964). The invasion of the land by animals with a
Fig. 1. Arginine metabolism in the liver of ureotelic animals (after Campbell, 1970).
\[
\begin{align*}
\text{NH}_4^+ \text{ (GLUTAMINE)} + \text{HCO}_3^- + \text{ATP} & \xrightarrow{\text{Carbamyl phosphate synthetase}} \text{CARBAMYL PHOSPHATE} \\
\text{CARBAMYL PHOSPHATE} & \xrightarrow{\text{Ornithine transcarbamylase}} \text{CITRULLINE} \\
\text{CITRULLINE} & \xrightarrow{\text{Argininosuccinate synthetase}} \text{ARGININOSUCCINIC ACID} \\
\text{ARGININOSUCCINIC ACID} & \xrightarrow{\text{Argininosuccinate lyase}} \text{ARGININE} \\
\text{ARGININE} & \xrightarrow{\text{Arginase}} \text{ORNITHINE + UREA} \quad \text{or} \quad \text{ARGINYL-tRNA} \\
\text{ORNITHINE + UREA} & \rightarrow \text{EXCRETORY UREA} \\
\text{ARGINYL-tRNA} & \rightarrow \text{PROTEIN ARGinine}
\end{align*}
\]
concomitant restriction of water availability required that ammonia, which could not be lost through exchange with the environment, be converted to a less toxic compound such as urea. The synthesis of urea via the arginine pathway presumably required certain modifications rendering this pathway capable of functioning in a dual capacity. That the nutritional function is the more primitive function of the pathway is supported by its functioning in this capacity in procaryotic cells (Campbell, 1965). Protein arginine synthesis has been demonstrated in at least one group of uricotelic invertebrates (Campbell and Speeg, 1968) and in several ureotelic invertebrates (Bishop and Campbell, 1965; Campbell, 1965). The presence of the enzymes for protein arginine synthesis have been demonstrated in many vertebrates (Cohen and Brown, 1960).

Animals that cannot synthesize arginine require it in the diet for both protein and phosphagen synthesis. For instance, invertebrates phosphorylate arginine to form phosphoarginine (Ennor and Morrison, 1958), an energy store in invertebrate muscle, while in vertebrates a transamidination reaction occurs between arginine and glycine (Walker, 1965) as the first step in the synthesis of creatine phosphate, the energy store in muscle of vertebrates. Nutritional studies have indicated a need in many animals for dietary arginine. In the chick, citrulline but not ornithine can partially replace dietary arginine (Klose and Almquist, 1940). Studies by Tamir and Ratner (1963a,b) have shown that arginine cannot be synthesized de novo in avian liver or kidney although the enzymes necessary for the conversion of citrulline to arginine are present in the kidney. Likewise, nutritional studies on insects have also indicated that citrulline but not ornithine can partially replace
arginine in the diet of many insects; these include the red-banded leaf roller, *Argyrotaenia velutinana* (Rock, 1969); the fruit fly, *Drosophila melanogaster* (Hinton, 1965b); and the saw-tooth grain beetle, *Oryzaephilus surinamensis* (Davis, 1962). Ornithine transcarbamylase, one of the enzymes for citrulline synthesis appears to be low or absent in insects (Poremb ska and Mochnacka, 1964; Reddy and Campbell, 1967).

The specific objectives of this study are as follows:

1. To determine the ratio of arginine incorporated into protein from endogenous and exogenous sources in a growing mammal (white rat) and in a metamorphosing amphibian (tadpole and frog).

2. To determine if the pool(s) of arginine used for protein synthesis and nitrogen excretion are compartmented.

3. To establish the mechanism by which arginine is incorporated into protein in frog and rat liver.

Although mammals can synthesize arginine, they still show a dietary requirement for this amino acid for maximum growth. Borman *et al.* (1946) demonstrated that arginine stimulated growth when fed to rats. Young rats receiving an arginine supplement in the diet showed a 15% greater weight gain during a 28 day period than those whose diet was not supplemented with arginine. Rose *et al.* (1948) later concluded that arginine synthesis in the rat does not proceed at a rate sufficiently rapid to supply the arginine required for maximum growth. Similar data have recently been obtained for the rabbit (McWard *et al.*, 1967). Arginine is not required, however, in the growing rat for nitrogen balance even though it is essential for optimal growth. It is thus not classified as an essential or non-essential amino acid but appears to occupy an
intermediate position (Rose, 1938). It should be pointed out, however, that glutamate or proline can partially replace dietary arginine in augmenting growth (Rose et al., 1948).

Kennon and Cohen (1961) have assessed the metabolic basis of the arginine requirement for optimal growth in rats. According to their analysis, urinary excretion of urea amounts to 720 mg per day (Carr and Krantz, 1949) for an adult rat with a 12 g liver. From the levels of the ornithine-urea cycle enzymes (Greenstein et al., 1957) in the liver, calculations show that an adult rat with the same size liver can synthesize on the order of 710 mg urea in a day. These data led Kennan and Cohen to suggest that rat liver had very little reserve capacity for arginine synthesis. Obviously, this may explain the need for dietary arginine for maximum growth.

As early as 1930, Scull and Rose demonstrated that the rat could synthesize protein arginine. This was shown by adding known quantities of arginine to a synthetic diet. The subsequent increase in tissue arginine was found to be two or three times as large as could be accounted for by the total arginine in the diet. Subsequently, tracer studies by Delliwa and Wilson (1946), Swick et al. (1953), and Swick and Handa (1956) documented the ornithine-urea cycle as the source of protein arginine. However, the relationship of exogenously-supplied and endogenously-synthesized arginine to protein and urea synthesis has not yet been systematically investigated.

While the rat may serve as an example of ureotelic animals in which the ornithine-urea cycle serves both a nutritional and excretory function, the metamorphosing tadpole, on the other hand, demonstrates a switch from
only the nutritional function in the tadpole to the dual function in the adult. This is one of the significant characters (the switch from ammoniotelism to ureotelism) that allowed the invasion of the land by animals. The transition from ammoniotelism to ureotelism was first reported by Bialazewicz and Mincowna (1921) and Munro (1939). Thus the metamorphosing tadpole may serve as a model system for assessing some of the biochemical factors which preceded and thus permitted the invasion of land by aquatic animals.

Brown and Cohen (1958) have shown that activities of the enzymes of the ornithine-urea cycle may be correlated with the change from ammoniotelism to ureotelism in the tadpole. During metamorphosis the greatest increase in enzyme activities of the urea cycle occurs between stages XVII and XXIV. Little or no activity can be demonstrated in the argininosuccinate synthetase step prior to stage XVII and this step has been shown to be the rate limiting step in the overall biosynthetic process (Brown et al., 1959). The enzyme data has been correlated with production of $^{14}$C-urea from $^{14}$CO$_2$ in liver slices from tadpoles at different stages of metamorphosis (Brown, 1962).

One approach to the problem was to study the ratio at which exogenously supplied and endogenously synthesized arginine is incorporated into protein during different metamorphic stages in the tadpole and growth in the rat. In the tadpole the urea synthesizing ability remains low until about stage XX, while in the postnatal rat the capability for urea synthesis is lower than the adult until 14 days (15 gms) following birth (Kennan and Cohen, 1959; Räihä and Suihkonen, 1968). This implies that both the tadpole and young rat require larger supplements of arginine in
the diet at least until all the enzymes for arginine synthesis reach high values characteristic of the respective adult stages. Once the high values are reached it is possible that at least part of the requirement may be relieved.

Another problem is to establish whether or not there is a common pool of arginine for both protein and urea synthesis. The only known compartmentation of the arginine pathway in animals is the two pools of carbamyl phosphate which are formed by two distinct carbamyl phosphate synthetases. One enzyme is associated with the mitochondrion, utilizes ammonium ions, and is stabilized by N-acetyl-L-glutamate while the cytosol form utilizes L-glutamine and is not affected by N-acetyl-L-glutamate. The mitochondrial form has been shown to be involved primarily in arginine and urea synthesis while the carbamyl phosphate formed in the cytosol appears to be a precursor to the pyrimidines (Hager and Jones, 1967). Recently Natale and Tremblay (1969) have demonstrated that the carbamyl phosphate formed intramitochondrially may be available for pyrimidine synthesis and they concluded that the carbamyl phosphate formed intramitochondrially may serve as a major source for pyrimidine biosynthesis.

The findings of Natale and Tremblay, however, do not entirely rule out compartmentation of the two pools of carbamyl phosphate. Even if the glutamine enzyme is not present in adult rat liver, Hager and Jones (1967) and Kerson and Appel (1968) have found low activity of the ammonium ion dependent carbamyl phosphate synthetase in the supernatant fraction of homogenates from adult rat liver. It should be pointed out that during purification of E. coli carbamyl phosphate synthetase the affinity for
glutamine may be lost and that for the ammonium ion retained (Kalman, Duffield, Bryzozowski, 1965). However, if there are two carbamyl phosphate synthetases it is possible that "channeling" (see Davis, 1967) may occur. Thus the carbamyl phosphate formed intramitochondrially may be converted immediately to citrulline and would become unavailable for pyrimidine biosynthesis. This view has recently been supported by Iue and Kaplan (1969) who have found a multienzyme complex consisting of aspartate transcarbamylase and carbamyl phosphate synthetase activities from *S. cerevisiae*. Thus there remains the possibility that the pools of carbamyl phosphate are compartmented for pyrimidine and urea synthesis.

Another consideration is that arginine may be incorporated into protein by two mechanisms. The best known mechanism is the one in which a peptide bond is formed and this requires: (1) a species of t-RNA, (2) amino acyl synthetase enzyme, (3) ribosomes, (4) Mg$^{++}$, and (5) GTP. The other mechanism, the arginine transfer reaction, requires only arginyl-tRNA, an enzyme, and a nonenzymatic protein (Soffer, 1968). The required enzyme, arginyl transfer ribonucleic acid-protein transferase, has recently been purified and its properties studied (Soffer, 1970). Protein synthesis in which a peptide bond is formed is puromycin sensitive while the arginine transfer reaction is not. The arginine transfer reaction occurs in sheep thyroid and in the cytoplasm of rabbit liver cells (Soffer, 1969). It appears to be an addition reaction since the incorporated arginine contains a free α-amino group.
MATERIALS AND METHODS

A. Animals

White rats (Rattus rattus) were obtained from Holtzman Co., Madison, Wisconsin. Following shipment of the initial stock, a breeding colony was maintained in the animal facilities at Rice University. Rats were fed routinely on a diet of Purina Rat Chow (crude protein content 23%) ad libitum. Unless otherwise noted, rats were not allowed to feed 48 hours prior to experimentation. Rana catesbeiana frogs and tadpoles were obtained from the Lemberger Co., Oshkosh, Wisconsin. Frogs were maintained at 4°C in plastic dishpans with about one inch of water in a cold room. Frogs were not fed while in captivity. Tadpoles were kept at 20°C in plastic dishpans in about one inch of oxygenated water. Three or four tadpoles were placed in each dishpan and were fed boiled lettuce. During thyroxine treatment the tadpoles were immersed in a 10^-8 M solution (Zarrow, Yochim, and McCarthy, 1964). Tadpoles were staged according to Taylor and Kollros (1946).

B. Reagents

General: Inorganic salts, acids, bases, and organic compounds were of the highest quality available and were purchased from Fisher Scientific Co., Houston, Texas; Sigma Chemical Co., St. Louis, Missouri; or Mallinckrodt Chemical Co., St. Louis, Missouri. Solubilizer was obtained from Amersham-Searle, Des Plaines, Illinois; Puromycin dihydrochloride from Nutritional Biochemical Co., Cleveland, Ohio; and Thyroxine (3, 3', 5, 5'-tetraiodothyroxine) from Sigma Chemical Co., St. Louis, Missouri.
Enzymes: Urease, type III, was purchased from Sigma Chemical Co. and kept as a 0.5% solution in 50% glycerol with 1 mM ethylenediaminetetraacetic acid.

Isotopically labeled compounds: [Guanidino-$^{14}$C]L-arginine and [ureido-$^{14}$C]L-citrulline were obtained from New England Nuclear Corp., Boston, Massachusetts. A urea-$^{14}$C contaminant was always present in the [ureido-$^{14}$C]citrulline from radiolysis. The labeled citrulline was purified by column chromatography on Dowex-50 (200-400 mesh) H$^+$ resin by the method of Hall et al. (1960) with the following modifications. The column bed was 1 x 14 cm, the mixer contained 200 ml glass distilled water, and the reservoir contained 200 ml 4.2 N HCl. The fractions containing the citrulline were pooled and dried under reduced pressure over sodium hydroxide at 40° in a rotating evaporator. The residue was taken up in glass distilled water. The specific activity was determined by counting an aliquot in Bray's solution and another aliquot was used for the colorimetric determination (Archibald, 1944).

C. Analytical procedures

1. Arginine: arginine was determined by the Sakaguchi reaction as described by Wu (1959).

2. Citrulline: citrulline was determined through its reaction with 2, 3-butanedione-2-oxime (Archibald, 1944).
D. Experimental procedures

1. Preparation of thin liver slices: Livers were removed from animals following a sharp blow to the head followed by cervical dislocation in rats or pithing in frogs and tadpoles. The livers were immediately placed in a beaker of cool saline (0.9% NaCl) and the liver lobes separated. Cubes of liver were cut from the lobes and placed in another beaker containing physiological saline. Liver slices were cut from each cube with a Stadie-Riggs microtome (Stadie and Riggs, 1944). The first and last slice from each cube were discarded. The liver slices were then placed in a petri dish containing physiological saline and remained there until being weighed. Thin liver slices were approximately 0.1 mm thick and weighed 0.15 to 0.20 grams. In the case of tadpoles and some of the smaller rats, thin liver slices were made with a razor blade.

2. Incubation of thin liver slices: White rat thin tissue slices were incubated in Kreb's Ringer Phosphate solution (Cohen, 1957). Frog and tadpole tissue slices were incubated in amphibian ringer solution (Cavanaugh, 1954). The salines were supplemented with blood concentrations of D-glucose and the L-amino acids histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Concentration of salines, amino acids, and glucose are listed in Table 1. For studies on the incorporation of exogenously supplied arginine for protein synthesis, [guanidino-14C]L-arginine was added to a final concentration of 1 mM. [Ureido-14C]L-citrulline concentration, in those experiments where incorporation of
Table 1. Components of incubation mixtures for thin liver slices from rats and tadpoles and frogs

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat</td>
</tr>
<tr>
<td>Saline:</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>120</td>
</tr>
<tr>
<td>KCl</td>
<td>4.8</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.6</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1.2</td>
</tr>
<tr>
<td>Na₂H₂PO₄</td>
<td>--</td>
</tr>
<tr>
<td>Na₂H₂PO₄/Na₂HPO₄</td>
<td>15.6</td>
</tr>
<tr>
<td>Final pH</td>
<td>7.4</td>
</tr>
<tr>
<td>L-Amino Acids:</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>0.06</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.10</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.20</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.39</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.06</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.08</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.11</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.08</td>
</tr>
<tr>
<td>Valine</td>
<td>0.22</td>
</tr>
<tr>
<td>D-Glucose:</td>
<td>760</td>
</tr>
</tbody>
</table>
endogenously synthesized arginine into protein was studied, was 0.2 mM.
Final volume of all flasks was 5.0 ml. Rat tissue slices were incubated at 37° and tadpole or frog tissue slices at 25° in a met abolite shaker at 150 RPM. After the addition of the tissue slices the reaction was allowed to proceed for a specified period of time and was subsequently stopped by the addition of 5 ml of 0.5 M perchloric acid. The liver slices were then homogenized at 0-4° in the acidified incubation medium with a TenBroeck homogenizer. The homogenate was centrifuged and the supernatant fluid poured into a beaker and the protein washed three more times with 2 ml 0.5 M perchloric acid. The supernatant fluid from these washes was added to the original supernatant fluid.

3. Radioactivity determinations: All radioactivity determinations were made by liquid scintillation spectrometry. For compounds in aqueous solutions, 10 ml Bray's solution (Bray, 1960) was used. For compounds in organic solvents, a mixture of 5 g of 2,5-diphenylloxazole (PPO) and 0.5 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) per liter toluene were used. Methanolic hydroxide of Hyamine\textsuperscript{R} (Rohm and Haas), PPO, and dimethyl POPOP were purchased from Packard Instrument Co. Ind., Downers Grove, Illinois. The efficiency of counting was determined by the channels ratio method (Bruno and Christian, 1961).

Determination of radioactive urea: The acidified supernatant fluid was neutralized with 5.0 N KOH and the precipitated salt, potassium perchlorate, was removed by centrifugation at 2,000 RPM
in an International Centrifuge. A portion of the supernatant fluid was then pipetted into a 25 ml reaction flask which also contained 100 μmoles pH 7.4 Tris-Cl buffer. After addition of urease the flasks were capped with rubber stoppers containing a polyethylene center well and incubated at 30° for thirty minutes in a shaking water bath. The reactions were stopped by injecting 1 ml of 5 N sulfuric acid through the rubber stoppers into the flasks. The \(^{14}\text{CO}_2\) liberated from the reaction mixture was trapped in 0.2 ml of 1 M methanolic Hyamine hydroxide contained in the center well. The center well and its contents were then transferred to 10 ml of the PPO:dimethyl POPOP:toluene scintillation mixture.

Determination of radioactive protein arginine: The homogenized protein was prepared by the modified method of Siekevitz (1952) as described by Campbell (1965). After the final ether wash the protein was allowed to air dry. The remaining protein pellet was weighed and approximately 10 mg of the pellet was transferred to a scintillation vial. The protein was moistened with water and 0.5 ml of NCS solubilizer was added to the vial. The vials were then placed in a shaker bath. When dissolution was complete, 10 ml of PPO:dimethyl POPOP:toluene scintillator was added to the vial and the radioactivity determined.

4. Protein arginine determinations: Ten to fifteen mg of washed protein were placed in an ignition tube with 200 ml 6 N HCl for hydrolysis. The samples were hydrolyzed in vacuo for 24 hr at 115° after which the hydrolysates were filtered and the filtrates were dried under
infrared lamps. The residue was dissolved in water and again taken to dryness. The final residue was brought to volume in either water for chemical arginine determination or in pH 2.2 sodium citrate buffer at a concentration of 6 mg original protein weight per ml for ion exchange chromatography. Arginine was separated from the hydrolysates using Beckman custom resin PA-35. The method used was as described by Benson and Patterson (1965) except that a 5 cm column of resin was employed at 55°C. A fraction collector was used to collect the column effluent. The ninhydrin reaction (Lee and Takahashi, 1966) was used to standardize the column and show that arginine separated from other basic amino acids; the Sakaguchi reaction (Wu, 1959) was used routinely to determine arginine in the fractions collected from the column. A portion of these fractions was counted in Bray's solution to determine the ¹⁴C incorporated in protein arginine.
RESULTS AND DISCUSSION

A. Standardization

Before experiments could be performed with tissue slices, it was necessary to determine the concentration of isotopic arginine and citrulline to be used in the incubation medium. Standardization was done with thin tissue slices from rat liver since this species was most readily available. The smallest amounts still giving linear responses were the substrate concentrations for the rest of the experiments. When 1 µmole (0.2 mM) [ureido-14C]L-citrulline was the substrate it was found that both urea production and the incorporation of radioactive arginine into protein proceeded in a linear fashion (Fig. 2). The specific radioactivity of the citrulline was 6, 225, 600 and a total of 201, 275 disintegrations per minute were converted to product compounds (urea and protein arginine). Therefore approximately 3% of the citrulline added to the incubation mixture was metabolized.

The concentration of [guanidino-14C]L-arginine to be used in the incubation medium presented a more difficult problem since the tissue enzyme activity of arginase is at least two-fold greater than that of argininosuccinate synthetase in rat liver (Schimke, 1962a). Incubations were done with 1 µmole and 5 µmoles of the radioactive arginine per 5 ml incubation media (0.2 mM or 1.0 mM). As shown in Fig. 3, incubating tissue slices with 1 µmole arginine resulted in no further labeling of protein after 30 minutes. When 5 µmoles isotopic arginine were used in the incubation media, radioactive arginine incorporated into
Fig. 2. Urea synthesis and arginine incorporation into protein from [ureido-14C]L-citrulline in a 150 g rat

The reaction mixture contained Kreb's Ringer Phosphate, 9 amino acids, and D-glucose in the concentrations listed in Table 1 plus 1 μmole radioactive citrulline. Final volume was 5 ml. Approximately 150 mg of thin liver slices were added to each flask. Closed circles indicate mm arginine incorporated/10 mg protein; open circles indicate μmoles urea/gram tissue.
Fig. 3. Incorporation of arginine into protein from [guanidino-$^{14}$C]$\text{-}$arginine precursor

The reaction mixture contained Kreb's Ringer Phosphate, 9 amino acids, and D-glucose in the concentrations listed in Table 1 plus 1 $\mu$ mole (open circles) or 5 $\mu$ moles (closed circles) radioactive arginine. Final volume was 5 ml. Approximately 150 mg of thin liver slices were added to each flask.
protein was linear throughout a 60-minute time period (Fig. 3). Because linearity was demonstrated with 5 μmoles radioactive arginine over a 60 minute time interval, this value was chosen for further substrate incubations.

Excess amino acids for protein synthesis were provided by the addition of the essential amino acids and glucose from which the other amino acids could be formed. Kreb's Ringer Phosphate was chosen as the saline solution for the rat and amphibian ringer solution for the frog. Concentrations of physiological salines, amino acids, and glucose at normal blood levels of the rat and frog are listed in Table 1 (Dittmer, 1961).

The choice of arginine as a substrate for protein synthesis is advantageous since hydrolysis of guanidino-labeled arginine by arginase yields only urea which is metabolically inert in vertebrate liver. Thus, there is no effective reutilization of the labeled carbon. Swick and Handa (1956) estimated that, at most, 2% of the urea derived from the guanidino-carbon of arginine is reutilized in protein synthesis. In this study, at least 86% of the radioactivity present in solubilized protein was in arginine. To demonstrate radioactivity in the protein arginine, the arginine was isolated from protein hydrolysates by column chromatography as previously described. In the separation of the protein amino acids by column chromatography the arginine peak and the radioactivity peak coincided. The fractions containing the arginine peak were pooled and acidified with an equal volume of 10% trichloroacetic acid. Arginine was then isolated using a column of Dowex 50 (H⁺) as described by Campbell and Speeg (1968). The final column eluate was
evaporated to dryness and the residue dissolved in NCS solubilizer. Ten ml of toluene scintillator were added and the radioactivity was determined by scintillation spectrometry. Fig. 4A shows an elution profile of the Sakaguchi-positive and radioactive fractions when \[\text{guanido-}^{14}\text{C}\]L-arginine was the substrate. The amount of radioactivity in arginine demonstrated by this method was 86.6% of that found in the solubilized protein sample. Fig. 4B shows an elution profile of the Sakaguchi-positive and radioactive fractions when \[\text{ureido-}^{14}\text{C}\]L-citrulline was the substrate. In this instance 96.6% of the radioactivity was demonstrable in arginine. Table 2 gives the actual figures and calculations for these determinations.

B. Growth and nutrition

In this study, weight gain was the criterion used as a means of determining maturity in rats. Miller (1969) points out that weight gain has been the most commonly used measure of increase in the analysis of growth patterns. A typical growth curve of white rats used in this study is shown in Fig. 5. While the total body weight appears to increase as a smooth progression, other components of the body are changing in relation to each other. The period of growth marked by accretion occurs from birth until approximately 120 grams or 45 days and during this period the protein content of the major tissues increases from 12% to 24% (Spray and Widdowson, 1950).

White rats were fasted 48 hours prior to experimentation which results in approximately 22 to 26% loss in total body weight (Table 3A). The relationship of the liver to total body weight in different weight
Fig. 4A. Elution profile of Sakaguchi-positive fractions and radioactive fractions from a protein hydrolysate of a rat

The incubation conditions of the liver slices were the same as previously described with 5 μmoles of radioactive arginine. The arginine was isolated from a 5 cm column with PA-35 resin as described in the text.
Fig. 4B. Elution profile of Sakaguchi-positive fractions and radioactive fractions from a protein hydrolysate of a rat

The incubation conditions of the liver slices were the same as previously described with 1 μmole radioactive citrulline. The arginine was isolated from a 5 cm column with PA-35 resin as described in the text.
Table 2. Recovery of radioactive arginine from protein hydrolysate

<table>
<thead>
<tr>
<th></th>
<th>Arginine &amp; DPM in treated</th>
<th>Arginine &amp; DPM in treated</th>
<th>Recovery of DPM &amp; arginine from treated protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>3995/10</td>
<td>96.6%</td>
<td>7496/10 10%</td>
<td>68.7%</td>
</tr>
<tr>
<td>3996/10</td>
<td>96.4%</td>
<td>7497/10 10%</td>
<td>68.4%</td>
</tr>
</tbody>
</table>

Note: Recovery values are approximate and may vary slightly.
Fig. 5. Growth curve for the white rat

Each point represents an average of approximately 12-16 animals. After 100 g body weight each point represents ≥ 3 animals. Experimental animals never varied from means by more than 3% for the first 15 days and not more than 2% for the rest of the growth studies.
Table 3A. Weight loss in rats following 48 hour fast

<table>
<thead>
<tr>
<th>Original weight (Grams)</th>
<th>Weight after 48 hour fast (Grams)</th>
<th>Weight loss after fast (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>31</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td>41</td>
<td>32</td>
<td>22</td>
</tr>
<tr>
<td>51</td>
<td>34</td>
<td>34</td>
</tr>
<tr>
<td>107</td>
<td>81</td>
<td>24</td>
</tr>
<tr>
<td>114</td>
<td>114</td>
<td>22</td>
</tr>
<tr>
<td>196</td>
<td>196</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 3B. Relationship of liver weight to total body weight following 48 hour fast

<table>
<thead>
<tr>
<th>Weight after 48 hour fast (Grams)</th>
<th>Liver weight after 48 hour fast (Grams)</th>
<th>% Liver weight of body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5*</td>
<td>0.274</td>
<td>3.7</td>
</tr>
<tr>
<td>15</td>
<td>0.48</td>
<td>3.2</td>
</tr>
<tr>
<td>23</td>
<td>0.83</td>
<td>3.6</td>
</tr>
<tr>
<td>32</td>
<td>1.15</td>
<td>3.6</td>
</tr>
<tr>
<td>34</td>
<td>0.9</td>
<td>2.6</td>
</tr>
<tr>
<td>81</td>
<td>4.1</td>
<td>4.7</td>
</tr>
<tr>
<td>114</td>
<td>4.9</td>
<td>4.2</td>
</tr>
<tr>
<td>196</td>
<td>8.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* This group of animals not starved prior to experimentation.
groups after a 48 hour fast is shown in Table 3B. The liver appears to be an extremely labile organ supplying the rest of the body with amino acids during short periods of fasting. Addis (1936) has shown that the liver loses about 25% of its protein during a 48 hour fast and, more recently, Gan and Jeffay (1967) have shown that during a 48 hour fast the liver loses about 30% of its weight. The total body weight loss during a 48 hour fast was found to be 16%. During 5 more days of fasting, the liver loses only an additional 10% of its weight. Gan and Jeffay (1967) have also determined that, during the 48 hour fasting period, as much as 90% of the liver free amino acids are derived from protein degradation. A major fraction of the liver free amino acids enter the plasma to supply amino acids to other tissues. This loss of free amino acids by the liver decreases to about 50% after the third day of fasting. Thus, during a 48 hour fast, the liver appears to supply the bulk of amino acids for the rest of the body tissue.

After 48 hours of fasting the cytosol form of phosphoenolpyruvate carboxykinase activity increases about three-fold and this is indicative of gluconeogenesis (Lardy et al., 1964; Utter et al., 1964). Phosphoenolpyruvate carboxykinase has been implicated as an indicator of gluconeogenesis since it was discovered the phosphoenolpyruvate could be formed from oxalacetate which suggested that the pyruvate kinase step, which is not reversible, may be by-passed for glucose formation from amino acids (Utter and Kurahashi, 1954). The rats used in this study were starved 48 hours prior to experimentation and thus they were in the gluconeogenic state and this has an effect on the parameters measured here. Schimke (1962b) has shown that, over a
4 day fast, urea excretion increases from 418 mg/day to 965 mg/day with a concomitant increase in the activity of all urea cycle enzymes. On the other hand, the rate of protein synthesis is retarded in starved animals (Munro, 1968). In the present work, one age groups of animals (7.5 g) were not starved prior to experimentation. However, since the milk of the rat is low in carbohydrate and high in protein (Dymsza et al., 1964), the gluconeogenic amino acids are the major precursors of glucose in the suckling rat. This has been confirmed by Ballard and Hanson (1967) who observed a 25-fold increase in the activity of the cytosol form of phosphoenolpyruvate carboxykinase between term fetuses and 2 day old animals. They also found that the gluconeogenic activity of the liver increases up to 5 days before birth and declines to adult levels.

On the other hand, it is harder to ascertain the physiological state of the adult frog and the tadpole used in this study since they show metabolic variation with season as well as diet (Brown, 1964). Figure 6 shows the relationship between liver and body weight of a group of tadpoles induced to metamorphose from November to December, 1969. There appears to be a slight decrease in body weight between stages XX and XXIV. This is due to the loss of some body parts (such as the tail) during the transition from the aquatic to the amphibous form. The losses in total weight represent the loss of such body parts as the muscular swimming tail and gills. Part of this reduction in body parts is due to the interruption of food intake since the tadpole is changing from a strictly herbivorous to a carnivorous diet.
Fig. 6. Tadpole whole body and liver weights at various stages during metamorphosis

Body weight is indicated by circles and liver weight by triangle.
Frogs are seasonal, hibernating in winter and becoming active during the warmer summer months. During the winter months, the frog does not feed and it has been reported that amphibians may sustain starvation for one year and possibly longer (Rose, 1966). It was noted that frogs received during the summer and early fall had a large, red liver while those received or maintained in the laboratory during the winter months had a smaller, darker appearing liver. The darkened appearance of the liver of wintering animals is presumably due to a high concentration of melanin (Helmy and Hack, 1964).

The physiological state of frogs received in the laboratory from the supplier was not known since they may undergo long periods of starvation without obvious ill effects. Even though frogs may become gluconeogenic, their metabolism is quite different from that of mammals since blood glucose levels are not necessarily maintained within a particular range. Wright (1959) reported that 28% of a group of bullfrogs (Rana catesbeiana) had glucose levels below the limit of detection although these individuals showed no signs of hypoglycemic convulsions. Wright also found that the dose of alloxan used to produce diabetes in the rat was without effect in the frog.

C. Protein arginine and urea synthesis during growth in rats

Growth is an increase in size due to synthesis of body constituents. Net synthesis is a result of anabolic processes of the body predominating over catabolic processes: if the rate of synthesis and degradation of body materials proceeds at the same rate there is no increase in size and no growth. Based on studies with the rat Enesco and Leblond (1962) have divided tissue growth into three phases: the first phase of growth
is marked by hyperplasia and lasts until the rat weighs approximately 25 g; the next growth phase, from 25 g to 120 g, is marked by both hyperplasia and hypertrophy; and, in the final phase, from 120 g till death, hypertrophy is the predominant process.

In this study, liver tissue slices from rats were incubated in substrates which served as a source of arginine for protein synthesis. These substrates represented either an endogenous source, [ureido-\(^{14}\)C]L-citrulline, or an exogenous source, [guanidino-\(^{14}\)C]L-arginine. The tissue slices were incubated with either of these substrates for 20, 40, and 60 minutes. The data obtained in experiments with different age group rats are shown in Table 4. A plot of these data (Figs. 7 and 8) illustrates the decrease in the incorporation of arginine, derived from both an exogenous and an endogenous source, into protein with age.

Arginine incorporation into protein was greater in younger animals than in older ones whether derived from an exogenous or endogenous source. Miller (1969) has reported that protein synthesis in the newborn or early postpartum rat is relatively low and suggests that this depressed protein synthetic rate is due to the traumatic shift from the nutrients supplied by the mother through the placenta to the alimentation of the nutrients of the mother's milk. This period of depressed protein synthesis occurs, according to Miller, between days 1 and 11. It may be noted in Figs. 8 and 9 that no depressed period of protein synthesis, as evidenced by the incorporation of arginine, was detected in the present study. The newborn pups (7.5 g), which fall within the time interval studied by Miller, did not show a depressed protein synthetic rate. An explanation for this discrepancy may be the different experimental techniques employed. Miller's
Table 4. Incorporation of [guanidino-\(^{14}\)C]L-arginine into protein from exogenous and endogenous sources

<table>
<thead>
<tr>
<th>Total body weight (Grams)</th>
<th>Exogenous arginine ([guanidino-(^{14})C]L-arginine)</th>
<th>Endogenous arginine ([ureido-(^{14})C]L-citrulline)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incorporation (cpm/10 mg protein)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>7.5</td>
<td>1.3</td>
<td>3.2</td>
</tr>
<tr>
<td>20</td>
<td>.34</td>
<td>.72</td>
</tr>
<tr>
<td>30</td>
<td>.24</td>
<td>.70</td>
</tr>
<tr>
<td>40</td>
<td>.21</td>
<td>.36</td>
</tr>
<tr>
<td>50</td>
<td>.11</td>
<td>.20</td>
</tr>
<tr>
<td>100</td>
<td>.27</td>
<td>.37</td>
</tr>
<tr>
<td>150</td>
<td>.09</td>
<td>.15</td>
</tr>
<tr>
<td>250</td>
<td>.08</td>
<td>.10</td>
</tr>
</tbody>
</table>

Incubations were performed as described in Materials and Methods.
Fig. 7. Incorporation of arginine into protein from $^{14}$C-guanidino]L-arginine at different weights of the white rat
Fig. 8. Incorporation of arginine into protein from $^{14}$C-ureido-L-citrulline at different weights of the white rat
studies were conducted in vivo and, at birth, the only nutrients available to the pups would be through suckling. In the experimental approach used here, excess amino acids were supplied to the liver slices and this may stimulate protein synthesis. Munro's (1968) observation of an increase in protein synthesis following a meal is consistent with this explanation. Furthermore, Hanking and Roberts (1965) have shown that if a high level of phenylalanine is present this stimulates protein synthesis. Thus, these data would seem to indicate that the capacity for an increased protein synthetic rate is present in the young rat if the amino acids necessary for protein synthesis are present.

It seems likely that protein synthesis in the liver would occur at a greater rate than in the rest of the body since this organ doubles in size in relation to the total body weight from birth to 25 grams; at birth, the liver represents 4.4% of the total body weight and at 25 grams 7.8% (Miller, 1969). In embryonic rat, the liver doubles its weight every 24 hours during the latter stages of pregnancy (Kafer and Pollack, 1961) and, during this period, protein synthesis is much higher than in adult rats (Burston and Pollack, 1961). Since the liver is increasing rapidly in relation to other body parts and protein content is increasing from approximately 12 to 24% it appears that a high rate of protein synthesis would be necessary in this organ during this time.

Protein turnover is another important factor to consider in a growing organ. Miller (1969) found that the turnover rate of liver proteins was low at birth and increased until about 19 days of age. In contrast, Schreir et al. (1960) reported that protein turnover decreased with age. Because there is both an increase in liver size and in its protein content
an initial slower rate of protein turnover would be predicted along with a higher rate of protein synthesis in the young animal to account for its increased size and protein content. However, the possibility that the turnover rate in young and older animals is the same but that the rates of synthesis of body constituents proceeds at an increased rate cannot be discounted. The initial increased incorporation of arginine into protein found here is consistent with increased synthesis of protein.

The citrulline concentration of the incubation mixture was 0.2 mM and the arginine concentration was 1.0 mM. In serum from normal, fed rats the plasma concentrations of citrulline and arginine are 0.08 mM and 0.2 mM respectively, representing a normal ratio of arginine to citrulline (Mallette et al., 1969). In the incubation media, the concentration of arginine used was five times that used for citrulline. The absolute concentration of arginine used was five times the normal physiological level and for citrulline, 2.5 times the normal physiological level. As shown in Figs. 7 and 8, arginine of exogenous origin ([guanidino-$^{14}$C]L-arginine) was incorporated into protein to a greater extent than was arginine of endogenous origin ([ureido-$^{14}$C]L-citrulline) at all stages except the 250 gram rat. This may be due to the different concentrations of the two sources of arginine used since the velocity of an enzyme reaction depends upon substrate concentration. Table 5 presents the ratio of arginine incorporated into protein from both endogenous and exogenous sources. The general decrease in the ratio (nmole arginine incorporated exogenous source/nmole arginine incorporated endogenous source) with age indicates that a greater quantity of arginine
Table 5. Ratio of exogenous to endogenous arginine incorporated into rat liver protein

<table>
<thead>
<tr>
<th>Weight of rats (grams)</th>
<th>Ratio: $\frac{\text{nmole arginine incorporated from [guanidine-^{14}C]L-arginine}}{\text{nmole arginine incorporated from [ureido-^{14}C]L-citrulline}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>2.8</td>
</tr>
<tr>
<td>20</td>
<td>1.8</td>
</tr>
<tr>
<td>30</td>
<td>1.6</td>
</tr>
<tr>
<td>40</td>
<td>1.7</td>
</tr>
<tr>
<td>50</td>
<td>2.0</td>
</tr>
<tr>
<td>100</td>
<td>1.6</td>
</tr>
<tr>
<td>150</td>
<td>1.2</td>
</tr>
<tr>
<td>250</td>
<td>0.8</td>
</tr>
</tbody>
</table>
from an exogenous source is incorporated in the young animal than in an older animal. In fact, there does not appear to be any difference in this ratio between animals of 30 to 100 g. The older animals (150 and 250 g) show lower ratios than do younger animals and, in the older animals, the ratio approaches unity. Because the ratio approaches unity, it would appear that the synthesis of arginine in the young animal is not sufficient to supply its needs for protein synthesis whereas in the older animals sufficient arginine may be synthesized for the maintenance of liver protein. Mainwaring (1969) has shown that \[^{14}\text{C}]\text{phenylalanine} incorporation into protein differs with age in mice. If the rate of protein synthesis declines with senescence, then it is possible that a point may be reached at which the ornithine-urea cycle could supply enough arginine for the nutritional needs of the liver in rats. This appears to be the case when the animal approaches 250 g.

The apparent higher rates of arginine incorporation into protein by young animals was a direct reflection of the protein synthetic rate at that stage since there was no difference in the arginine content of young and old animals. As shown in Table 6, the protein composition, with respect to arginine content, remains the same during growth.

The hydrolysis of arginine from both exogenous and endogenous sources was also studied during growth of the rat. As shown in Fig. 9, there was a considerable amount of variation in the conversion of endogenous arginine to urea with no definite pattern apparent which could be correlated with developmental stage. Kennan and Cohen (1959) first demonstrated that urea synthesis in the fetal rat is highly dependent upon the mother and that the rate-limiting enzymatic step in urea synthesis, the argininosuccinate synthetase system, does not become detectable by the usual assay
Table 6. Arginine contents of liver protein from rats of various weights

<table>
<thead>
<tr>
<th>Animal weight (Grams)</th>
<th>Arginine content of protein (μmole arginine/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>0.35</td>
</tr>
<tr>
<td>20</td>
<td>0.36</td>
</tr>
<tr>
<td>30</td>
<td>0.36</td>
</tr>
<tr>
<td>40</td>
<td>0.35</td>
</tr>
<tr>
<td>50</td>
<td>0.35</td>
</tr>
<tr>
<td>100</td>
<td>0.35</td>
</tr>
<tr>
<td>150</td>
<td>0.35</td>
</tr>
<tr>
<td>250</td>
<td>0.36</td>
</tr>
</tbody>
</table>

20 mg protein was hydrolyzed in 6 N HCl at 115° for 24 hr. The hydrolyzate was then evaporated to dryness with infra-red lamps and brought to a total volume of 5 ml with glass distilled water. The Sakaguchi reaction was then done on an aliquot of hydrolyzate.
Fig. 9. Urea production from $[^{14}\text{C}]$L-citrulline at different weights of the white rat.
procedure until late in pregnancy. Kennan and Cohen state that urea is synthesized at significant rates in late fetal life and that the argininosuccinate synthetase system increases rapidly during the first 8 hours following birth. Subsequent studies by Räihä and Sulikonen (1968) have demonstrated a rapid increase in activity of all enzymes of the urea cycle in the postnatal rat. The increase in argininosuccinate synthetase is the most pronounced and this enzyme undergoes a secondary increase after 10 days. Räihä and Kretchmer (1965) found that unless optimal substrate concentrations (i.e., concentrations of substrate high enough to detect differences in activities of the enzymes) are used in the enzyme assays in vitro, the differences between the different stages may be minimized and adult levels of urea output are reached by the postnatal rat in 24 hours. In the study by Räihä and Kretchmer, it was also shown that starvation has no effect on urea production in postnatal rats. This indicates that the urea cycle is possibly operating at maximal capacity at this time. Kretchmer et al. (1966) found that the incorporation of $^{13}CO_2$ into urea attains adult values in liver slices after approximately 24 hours after birth and then does not change. The fact that no differences in urea synthesis could be shown between postnatal and older rats is probably not due to "limited penetration" of liver slices by citrulline since Bronk and Fisher (1956) found that citrulline equilibrated with the external medium after approximately 15 minutes.

In starved animals Schimke (1962b) reported that arginase activity increases dramatically. Because of Schimke's data it was necessary to establish the initial velocity of urea formation by liver slices. The incubation media was the same as described in the Materials and Methods
section except that the specific radioactivity of the arginine was decreased to 110,000 dpm/μmole. As shown in Fig. 10 no differences in the initial rates of urea formation from exogenous arginine in 20, 30, 100, or 250 g rats were detected. Räihä and Suikkonen (1968) found that arginase activity reached adult levels in 20 days after birth (approximately 30 g). It is again possible that no differences were detected because of the low substrate concentration. However, the hydrolysis of arginine to urea at the same rate by all age groups indicates that the incorporation of arginine into protein, which declines with age, was a function of age and was not due to a concentration effect. Even though the arginase hydrolyses very little arginine after 16 minutes, the incorporation of exogenous arginine into protein continues at a somewhat linear rate. This is due to the difference in the two enzymes competing for arginine as a substrate. The reported $K_m$ for arginase is between 5.54 mM and 9.6 mM (Campbell, 1966) while the $K_m$ of arginyl-tRNA synthetase for arginine is 0.001 mM (Ikegami and Griffen, 1969). Thus arginase would not operate as efficiently at low substrate concentrations as does the arginyl-tRNA synthetase enzyme.

D. Protein arginine and urea synthesis in the frog and during metamorphosis in the tadpole

Metamorphosis is defined by Cohen (1966) as, "a sequence of post-embryonic changes in morphology and function which permit larva to adapt to an environment different from its natal environment". Many changes occur in both chemistry and morphology during metamorphosis from tadpole to frog. These changes include a transition from porphyrin to rhodopsin
Fig. 10. Hydrolysis of arginine by different weight groups of rats

Incubation conditions were the same as described in Materials and Methods. Open circles show a time course of hydrolysis of arginine by a 20 gm rat; closed circles show a time course of hydrolysis by 30, 100, and 250 gm rats.
as the primary visual pigment, an increase in hemoglobin concentration, and the reduction of the length of the intestine from the long (herbivorous type) to the shorter (carnivorous type) form of the frog (Brown, 1964). One of the biochemical transitions which has been studied in great detail is the change in the mode of nitrogen excretion from ammonia in the tadpole to urea in the frog (Brown, 1962; Brown and Cohen, 1958; Brown, Brown, and Cohen, 1959; Underhay and Baldwin, 1955). This phenomenon has been studied only in relation to the synthesis of urea for excretion. The nutritional aspect of the ornithine-urea pathway, that of protein arginine synthesis, has not been examined.

The pattern of arginine incorporation into protein from exogenous and endogenous sources, respectively, during metamorphosis of the tadpole are shown in Figs. 11 and 12. When either an exogenous or endogenous source of arginine is supplied as substrate, there is an increase in protein synthesis from stage X to XVII; there is then a decreased incorporation into protein between stages XX and XXIV. Some stages show a rather large standard error. Some of the variation (note stage X in Fig. 12) is due to individual differences in the time response to thyroxine (Bennett et al., 1969). Thyroxine also induces protein synthesis in liver in metamorphosing tadpoles (Bennett et al., 1969). Bennett et al. also have found that ornithine transcarbamylase activity is stimulated by the addition of thyroxine and remains at an elevated level which is approximately two-fold that of the controls. Shambaugh et al. (1969) have shown recently that another urea cycle enzyme, carbamyl phosphate synthetase, is affected by thyroxine in several ways. Using liver cubes, Shambaugh et al. found the effect in vitro of thyroxine on carbamyl
Fig. 11. Incorporation of arginine into liver protein from \textit{[guanidine-^{14}C]}\-L-arginine during metamorphosis in \textit{R. catesbeiana} tadpoles

Incubation conditions are described in Materials and Methods.
nm arginine incorporated/10mg protein/hr

Stage Number

IV
III
II
I
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Z

0.0
0.2
0.3
0.4
0.5
0.6
Fig. 12. Incorporation of arginine into liver protein from [ureido-$^{14}$C]L-citrulline during metamorphosis in R. catesbeiana tadpoles

Incubation conditions are described in Materials and Methods.
phosphate synthetase to include stimulation of synthesis, conversion of precursors, and enhancement of the rate of enzyme degradation. Consequently, the extent to which the increase in protein synthesis, i.e. that which occurs between stages X and XX (Figs. 11 and 12) may be attributable to the effect of thyroxine remains unknown. The decreased protein synthesis from stage XX to stage XXIV-XXV is probably due to the effects of starvation. Tadpoles are switching from a herbivorous type diet to a carnivorous one between stages XX and XXV and consequently do not feed during this time. Ashley et al. (1968) have also shown a decrease in urea synthesis between stages XXIV and XXV in *R. catesbeiana* tadpoles. They too attribute this phenomena to starvation. Brown and Caston (1962) found an increase in protein synthesis when they studied Shumway stages 7-25 (embryonic stages) in *R. pipiens*. Degroot and Cohen (1962) studied amino acid activating enzymes in the tadpole and adult frog of *R. catesbeiana* and found there was greater activity in the adult frog. In their study, they did not compare the activities of the activating enzymes in different metamorphic stages of the tadpole nor did they indicate which stage of tadpole was compared to the adult.

As shown in Table 7, there is also no change in the arginine content of liver proteins during metamorphosis nor are there differences between the protein arginine contents of any metamorphic stage and the adult frog. This indicates that the increase in incorporation of arginine into protein is a measure of the rate of protein synthesis and not a reflection of a change in content of arginine in the liver tissue. Nakagawa et al. (1964) studied differences in tissue amino acids between tadpoles (stage not indicated) and frogs and found no significant
Table 7. Arginine contents of liver protein from tadpoles and frog

<table>
<thead>
<tr>
<th>Stage</th>
<th>Arginine content of protein (μmole arginine/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>0.21</td>
</tr>
<tr>
<td>XIV</td>
<td>0.21</td>
</tr>
<tr>
<td>XVII</td>
<td>0.21</td>
</tr>
<tr>
<td>XX</td>
<td>0.21</td>
</tr>
<tr>
<td>XXIV</td>
<td>0.21</td>
</tr>
<tr>
<td>adult frog</td>
<td>0.21</td>
</tr>
</tbody>
</table>

10 mg protein was hydrolyzed in 6 N HCl at 115°C for 24 hr. The hydrolyzate was then evaporated to dryness with infra-red lamps and brought to a total volume of 5 ml with glass-distilled water. The Sakaguchi reaction was then done on an aliquot of hydrolyzate.
differences. This in agreement with the hypothesis of Block and Weiss (1956) that the amino acid content of each organ or species is relatively the same.

As shown by the data in Figs. 11 and 12, the incorporation of arginine into protein from an exogenous source is about 5 to 6 times greater than is the incorporation from an endogenous source. Since the tadpole is ammoniotelic, the incorporation of arginine into protein from an endogenous source till approximately stage XX indicates that the arginine pathway is functioning in its primitive role (see Campbell, 1965). The formation of arginine by this pathway must be for nutritional purposes since the tadpole synthesizes very little urea (Brown, 1962). The formation of ornithine for arginine synthesis de novo poses a question. Nakagawa et al. (1964) found that glucose-U-\(^{14}\)C injected into tadpoles and frogs rapidly labeled glutamic acid but not arginine.

Arginine incorporation into protein in 4 male frogs is shown in Table 8. These values are subject to seasonal variation and thus reflects the state of protein synthesis only in late summer (August, 1969). It may be noted that, in the adult frog, the incorporation of arginine from an exogenous source is about 2 to 3 times that from an endogenous source. Fig. 13 gives the data for urea synthesis from citrulline in the same group of frogs.

The production of urea by tadpole liver slices from an endogenous arginine source during metamorphosis is shown in Fig. 14. Urea formation increases up until stage XX as described by Brown and Cohen (1958) but, at stage XXIV, there is a drop in urea synthesis. This may be due to starvation which occurs between stages XX and XXV as has been noted by Ashley et al. (1968).
Table 8. Protein arginine synthesis in four male adult *R. catesbeiana* frogs

<table>
<thead>
<tr>
<th>Frog weight (Grams)</th>
<th>Liver weight (Grams)</th>
<th>n mole arg/10 mg protein/hr [guanidino-¹⁴C]L-arginine</th>
<th>n mole arg/10 mg protein/hr [ureido-¹⁴C]L-citrulline</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.45</td>
<td>4.85</td>
<td>0.75</td>
<td>0.27</td>
</tr>
<tr>
<td>2.48</td>
<td>3.95</td>
<td>0.98</td>
<td>0.31</td>
</tr>
<tr>
<td>3.87</td>
<td>4.95</td>
<td>0.86</td>
<td>0.36</td>
</tr>
<tr>
<td>3.87</td>
<td>4.80</td>
<td>0.76</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Incubation conditions were as described in the Materials and Methods. The mean and the standard error for the arginine incorporated from [guanidino-¹⁴C]L-arginine was 0.84 ± .045 and that for arginine incorporated from [ureido-¹⁴C]L-citrulline was 0.30 ± .020.
Fig. 13. Urea synthesis by four male adult R. catesbeiana from [ureido-
$^{14}$C]L-citrulline
Fig. 14. Urea synthesis from citrulline by thin
liver slices of *R. catesbeiana* tadpoles

Incubations were performed as described in Materials
and Methods.
E. Puromycin inhibition of protein arginine synthesis in liver thin tissue slices

Soffer (1968) and Soffer and Horinishi (1969) have shown that sheep thyroid and rabbit liver cytoplasm contain a soluble enzyme which catalyzes the transfer of arginine onto certain acceptor proteins. This system does not involve the synthesis of protein de novo since there is no requirement for ribosomes, Mg++, or GTP nor is the system sensitive to puromycin. Kaji et al. (1963) described a system somewhat similar to that of Soffer's; however, the latter system was pyrromycin-sensitive. Kaji et al. suggested that the soluble incorporating system in rat liver was closely related to the conventional particle-supernatant protein synthesizing system. In the system described by Soffer, albumin appears to be the most active acceptor of those proteins which have been examined (Soffer and Horinishi, 1969). It appears that arginine is transferred by this system to aspartic acid, the amino-terminal amino acid of albumin.

Because the incorporation of arginine observed here could have been by the soluble, puromycin-insensitive system, incubations with puromycin were performed with each species. These incubations were carried out as previously described except that 0.3 mM puromycin was included in the incubation medium. The results obtained with liver slices from the rat, frog, and tadpole are shown in Table 9. All three systems examined were puromycin-sensitive. Puromycin, which is similar in structure to amino acyl-tRNA, inhibits protein synthesis by causing release of the incomplete chain from the ribosomes; therefore, it does not give 100% inhibition of protein synthesis. Some protein synthesis still occurs in the
Table 9. Puromycin inhibition of protein arginine synthesis in thin liver slices of rat, frog, and tadpole

<table>
<thead>
<tr>
<th>Animal</th>
<th>Weight (Grams) or Stage</th>
<th>nmoles incorporated/10 mg protein/hr</th>
<th>[guanidino-¹⁴C]L-arginine</th>
<th>[ureido-¹⁴C]L-citrulline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control + Puromycin</td>
<td>Control + Puromycin</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>150</td>
<td>0.17 0.04 (76)*</td>
<td>0.19 0.058 (70)</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>20</td>
<td>1.4 0.36 (61)</td>
<td>0.80 0.26 (68)</td>
<td></td>
</tr>
<tr>
<td>Frog</td>
<td>387</td>
<td>0.76 0.093 (88)</td>
<td>0.25 0.027 (90)</td>
<td></td>
</tr>
<tr>
<td>Tadpole</td>
<td>X</td>
<td>2.3 0.23 (90)</td>
<td>0.40 0.029 (93)</td>
<td></td>
</tr>
</tbody>
</table>

Incubations were performed as described in the Materials and Methods and in the text of this section.

* Denotes % inhibition.
presence of puromycin although there is a rapid breakdown of polysomes. As shown in Table 9, a minimum of 70% inhibition was obtained with puromycin in the present work. Most of the observed incorporation may thus be attributable to a puromycin-sensitive system. In the rat 0.3 mM puromycin inhibits protein synthesis by 70-77% while in both the tadpole and the frog, puromycin inhibits protein synthesis by approximately 90% (see Table 9).

F. Isotope dilution studies

The results obtained with liver slices from both the rat and frog show that arginine of both endogenous (i.e., that formed from citruline) and exogenous origin (i.e., that supplied directly to the tissue) are incorporated into protein by a puromycin-sensitive system. Arginine from both sources is also converted to urea. Although the physical association of enzymes into a multienzyme complex which catalyzes a complete sequence of metabolic reactions has been established for several metabolic pathways (see, e.g., Lynen, 1967; Wagner et al., 1967), the cellular localization of the enzymes of the ornithine-urea cycle indicates that this is not the case with this pathway. In the rat and the frog, the citrulline-forming system (carbamyl phosphate synthetase and ornithine transcarbamylase) is intramitochondrial (Brown and Cohen, 1959; Soberón et al., 1966) whereas the remainder of the pathway (argininosuccinate synthetase, argininosuccinate lyase and arginase in the case of urea synthesis and arginyl tRNA synthetase in the case of protein synthesis) is located in the cytosol (Brown and Cohen, 1959; Soberón et al., 1966). Although it has been suggested that there is some organization of the ornithine-urea cycle (Soberón et al., 1966), evidence for
this seems to be lacking (Palacios et al., 1970). Furthermore, Caffredo et al. (1966) have found that the basal pool of endogenous L-arginine is not available for catabolism in *Neurospora crassa* but when the pool is increased by the addition of L-citrulline it becomes available for catabolism. In *Escherichia coli*, Sercarz and Gorini (1964) have suggested that separate pools of endogenous and exogenous arginine exist since they found that endogenous arginine was utilized more efficiently for cell growth than was exogenous arginine. More recently, however, Tabor and Tabor (1969) have found that both endogenous and exogenous arginine contributed equally to protein synthesis. Tabor and Tabor added $^3$H-L-arginine and $^{14}$C-L-citrulline to an incubation mixture with *E. coli* and found that the $^3$H:$^{14}$C ratio in the arginine in protein was very close to that expected from the isotope mixture added. In vertebrates, the current interpretation of the extramitochondrial metabolism of arginine is as depicted in Fig. 1. A common pool of arginine exists for both urea and protein arginine synthesis. To test this, isotope dilution experiments were performed to determine if the endogenous pool could be diluted by exogenous arginine. The rationale for this experiment is that if there is a preferential use of endogenously synthesized arginine either for protein synthesis or urea formation it would probably be the former since protein arginine synthesis is considered to be the more primitive of the two functions (Campbell, 1965).

The overall scheme for the metabolism of arginine in the liver is shown in Fig. 15A. If [ureido-$^{14}$C]L-citrulline is used as the substrate, it is converted to argininosuccinic acid which is in turn acted upon by argininosuccinic acid lyase to form arginine. The [guanidino-$^{14}$C]L-arginine
Fig. 15A. Overall scheme for isotope dilution experiments

Fig. 15B. Experimental scheme for isotope dilution experiments
Effect of exogenously added arginine on the incorporation of endogenously synthesized protein arginine:

\[ {^{14}C}\text{CIT substrate} \]

\[ \text{added} \]

\[ \text{Time} \quad 0 \quad \rightarrow \quad 15 \text{ min} \quad \rightarrow \quad 30 \text{ min} \quad \rightarrow \quad 45 \text{ min} \quad \rightarrow \quad 60 \text{ min} \]

Fig. 15B.
formed endogenously then becomes available for either hydrolysis to urea or protein synthesis. When $^{12}\text{C}-\text{L-arginine}$ is added to the incubation mixture this increases the $^{12}\text{C}-\text{L-arginine}$ to $[^{14}\text{C}]\text{L-arginine}$ ratio and consequently, if the free pool of arginine is a common one, this should result in a dilution of the isotopic amount of arginine incorporated into protein when compared to controls. As the amount of non-radioactive arginine added to the incubation mixture is increased, the greater the dilution of the protein arginine. The experimental design used is shown in Fig. 15B. $[^{14}\text{C}]\text{L-citrulline}$ was added at 0 time followed by 3 pulses of $^{12}\text{C}$ arginine at 15, 30, and 45 minutes. The amount of arginine added at the three times was 1, 5, 10, 20, and 30 μmoles. Glass distilled water was added to controls. The $[^{12}\text{C}]\text{arginine}$ pulses were added to keep the non-isotopic arginine:$[^{14}\text{C}]\text{L-arginine}$ ratio high since the arginase activity is high. After the addition of all substrates the volume of each flask was 5.0 ml.

The results obtained in the dilution experiments are shown in Table 10. Qualitatively, in the rat, frog, and tadpole there is a dilution of the endogenously synthesized protein which is incorporated into protein by exogenously added arginine. The failure of exogenously added arginine to dilute endogenously synthesized protein arginine in direct proportion to its concentration, especially at the higher concentrations of arginine added, indicates that any interpretation of these results must be made with caution. An attempt was made to overcome the complication of the high levels of arginase activity (which rapidly hydrolyzes the added exogenous arginine) in these two species. The added arginine was pulsed at three time intervals during the
Table 10. Dilution of protein \([\text{guanidino}^{14}\text{C}]{\text{L-arginine}}\) from an endogenous precursor by a non-isotopic exogenous precursor

<table>
<thead>
<tr>
<th></th>
<th>Rat (150 g)</th>
<th>Frog (482 g)</th>
<th>Tadpole (stage X)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>Group 1</td>
</tr>
<tr>
<td>Control</td>
<td>0.110 (0)*</td>
<td>0.160 (0)</td>
<td>0.80 (0)</td>
</tr>
<tr>
<td>+ 3.0 (\mu)moles arginine</td>
<td>0.079 (28)</td>
<td>0.39 (51)</td>
<td>0.25 (48)</td>
</tr>
<tr>
<td>+ 15.0 (\mu)moles arginine</td>
<td>0.064 (42)</td>
<td>0.20 (75)</td>
<td>0.23 (52)</td>
</tr>
<tr>
<td>+ 30.0 (\mu)moles arginine</td>
<td>0.119 (26)</td>
<td>0.19 (76)</td>
<td>0.35 (37)</td>
</tr>
<tr>
<td>+ 60.0 (\mu)moles arginine</td>
<td>0.099 (40)</td>
<td>0.18 (77)</td>
<td>0.25 (48)</td>
</tr>
<tr>
<td>+ 90.0 (\mu)moles arginine</td>
<td>0.076 (53)</td>
<td>0.18 (77)</td>
<td>0.23 (52)</td>
</tr>
</tbody>
</table>

* % dilution.

Incubation conditions are described in the text.
incubations but the rapid hydrolysis of exogenous arginine may still remain a factor.

The "pool" of free arginine in the rat is very low, being 1.6 nmole/g (Schimke, 1963). The $K_m$ of arginase is high (minimum of 2.5 mM) relative to that of arginyl tRNA synthetase (0.0013 mM; Ikegami and Griffen, 1969). Thus, at the level of the arginine concentration of the free pool where arginase is very inefficient, one might still expect dilution to be proportional to concentration if the pool were completely accessible to exogenous arginine. In the rat, however, the amount of endogenously synthesized arginine incorporated into protein is decreased only 50% in the presence of an endogenous arginine (as citrulline)/exogenous arginine ratio of 0.011. This might be interpreted as indicating that endogenously synthesized arginine is, in fact, more accessible to the protein-synthesizing system than is exogenous arginine. The results obtained with the frog also supports this interpretation. In the tadpole, there is only a 40% decrease in the incorporation of endogenously synthesized arginine at the highest level of exogenous arginine added, whereas in the adult frog there is a 75% decrease. In the adult frog, the pathway is functioning for both urea and arginine synthesis while in the tadpole it is presumably functioning only for protein arginine synthesis. It is interesting, in this instance, that the exogenous arginine diluted the incorporation of radioactive arginine into protein more in the frog than in the tadpole. However, since the rat, frog, and tadpole incorporate exogenous arginine into protein, any quantitative differences may be due to other aspects of regulation not considered here.
The kinetics of the two enzymes competing for the free pool arginine, arginase and arginyl-tRNA synthetase, may be the most important consideration in vivo. The conversion of citrulline to arginine through the rate-limiting step, the argininosuccinate synthetase reaction, constantly provides low levels of arginine which can be efficiently activated for protein synthesis by the arginyl-tRNA synthetase enzyme. The rate-limiting step may be important not only in regulating the size of the free pool of arginine but also in regulating the rate of arginine incorporation into protein since argininosuccinic acid is a competitive inhibitor of arginyl-tRNA synthetase. (Nazario, 1967). Thus, both the level of arginase and argininosuccinic acid synthetase are important in regulating the amount of arginine available for protein synthesis.

As shown in Table 11, exogenously added arginine has very little effect on the amount of $^{14}$C citrulline converted to urea by either the rat or adult frog. These results indicate that exogenous arginine does not affect the conversion of citrulline to arginine by the adult rat and frog. Although the variation with tadpoles is great due to their variable response to thyroxine (Bennett et al., 1969), exogenous arginine appears to decrease the formation of urea from endogenous arginine. Since arginase activity is low at stage X (Brown, 1964) the exogenously added arginine may increase the pool size so that the capacity of the arginase to hydrolyze arginine is exceeded.
Table 11. Dilution of $^{14}$Curea formed from an endogenous precursor by an exogenous precursor

<table>
<thead>
<tr>
<th></th>
<th>Rat (150 g)</th>
<th>Frog (420 g)</th>
<th>Tadpole (stage X)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>Group 1</td>
</tr>
<tr>
<td>Control</td>
<td>250</td>
<td>160</td>
<td>116.7</td>
</tr>
<tr>
<td>+ 3.0 μmoles arginine</td>
<td>230</td>
<td>114.8</td>
<td>6.2</td>
</tr>
<tr>
<td>+ 15.0 μmoles arginine</td>
<td>240</td>
<td>102.3</td>
<td>4.0</td>
</tr>
<tr>
<td>+ 30.0 μmoles arginine</td>
<td>150</td>
<td>98.7</td>
<td>4.1</td>
</tr>
<tr>
<td>+ 60.0 μmoles arginine</td>
<td>150</td>
<td>97.2</td>
<td>3.7</td>
</tr>
<tr>
<td>+ 90.0 μmoles arginine</td>
<td>130</td>
<td></td>
<td>3.9</td>
</tr>
</tbody>
</table>

Incubation conditions are described in the text.
SUMMARY AND CONCLUSIONS

Several aspects of protein arginine synthesis were studied in thin tissue slices of liver from the growing rat, the metamorphosing tadpole, and the frog. Both an exogenous ([guanidino-$^{14}$C]$L$-arginine) and endogenous ([ureido-$^{14}$C]$L$-citrulline) source of the amino acid $L$-arginine were used. In the growing rat it was found that the exogenously-supplied source of arginine was incorporated into protein at a greater rate than that which was endogenously-synthesized. When either an exogenous or endogenous source of arginine was used, it was found that arginine was incorporated into protein at a greater rate in young rats than older rats. In the young rat (7.5 g) it appears that on the order of 3 nmol of arginine are incorporated into liver protein from an exogenous source to every 1 from an endogenous source. However, in an older rat (250 g) the ratio of exogenous arginine incorporated into liver protein to endogenous was 0.8. This may indicate that the liver in young rats requires arginine from the diet for protein synthesis to proceed at an optimal rate, while older rats may be able to synthesize sufficient quantities. Urea synthesis from citrulline during growth showed a large degree of variability.

On the other hand, the enzymes of the arginine pathway in the metamorphosing tadpole are at very low levels and begin to increase in activity at about stage XVII. The tadpole can synthesize protein arginine at all metamorphic stages examined (X, XIV, XVII, XX, XXIV, and XXV). When exogenous ([guanidino-$^{14}$C]$L$-arginine) arginine was supplied, 5 to 7 times
more arginine was incorporated into liver protein than from an endogenous source ([ureido-¹⁴C]L-citrulline). The ratio was constant from stages X to XXV. Incorporation of arginine into protein showed a slight increase from stages X to XX and then a decrease from XX to XXV. Likewise, urea synthesis was low in stages X and XIV and was markedly increased at stages XVII and XX and thereafter decreases slightly at stages XXIV and XXV. The "drop" in protein arginine and urea synthesis was believed to be due to the effects of starvation between stages XX and XXV. Since the tadpole is ammoniotelic during the early stages of metamorphosis (X-XV), the arginine pathway appears to be functioning in its primitive capacity, that of supplying arginine for protein synthesis. However, during the transition from an aquatic to an amphibious environment the activity of the urea cycle enzymes increases and the pathway begins to function in a dual capacity, i.e., nutritional and excretory.

Soffer (1968; 1970) has shown that arginine can be bound to protein by a transferase enzyme. This system requires only arginyl-tRNA, the transferase enzyme, and a non-enzymatic protein. This system is not sensitive to puromycin. The possibility existed, therefore, that the arginine incorporated into protein was by this system. It was found, however, that in the tadpole (stage X), the frog, and in two weight groups (20 and 150 gms) of rats the protein synthesis was puromycin sensitive in liver slices. In both weight groups of rats, puromycin inhibited protein synthesis by approximately 75% and in the tadpole and frog by approximately 90%. Protein arginine synthesis appears to take place by the conventional particle-supernatant protein synthesizing system in the animals studied.
Since the synthesis of arginine for protein synthesis appears to be the primitive function of the pathway, the capacity of exogenous arginine to dilute the incorporation of endogenously-synthesized arginine into protein was studied by isotope dilution. In the rat (150 g), frog (420 g), and the tadpole (stage X) it was found that $^{12}$C-arginine diluted the protein specific activity when [ureido-$^{14}$C]L-citrulline was the source of [guanidino-$^{14}$C]L-arginine. No subsequent dilution could be shown at the higher amounts of $^{12}$C-arginine added to the incubation mixture. This is possibly due to the action of arginase on the $^{12}$C-arginine added. Radioactive arginine is being formed from [ureido-$^{14}$C]L-citrulline at a steady rate while the $^{12}$C-arginine is being rapidly hydrolyzed by arginase. Only in the stage X tadpole could a dilution of [$^{14}$C]urea be demonstrated. This is explained by the action of arginase. Arginase activity is at greater levels in the rat and frog than in the tadpole, thus any $^{12}$C-arginine added to an incubation mixture, within certain limits, would be hydrolyzed by arginase. However, in the tadpole any $^{12}$C-arginine added to the incubation mixture increases the ratio of the radioactive to non-radioactive arginine and due to the lower arginase activity a dilution effect is shown on the production of $^{14}$C-urea.
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