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Aspects of Arginine Biosynthesis and Degradation in the Terrestrial Pulmonate, Otala lactea

by

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Aspects of Arginine Biosynthesis and Degradation in the Terrestrial Pulmonate, *Otala lactea*
INTRODUCTION

Arginine, which was first isolated from etiolated Lupin seedlings (Schulze & Steiger, 1886), plays a diverse role in cellular processes. It is, for example, a universal protein constituent, being especially abundant in the "arginine-rich" histones (Busch, 1965). The histones as a group have been implicated as key compounds in the sequential control of genetic transcription during differentiation (Bonner et al, 1968). Arginine is, at present, unique among the protein amino acids since there are at least two mechanisms for its incorporation into peptide bond in vitro. One of these, which is the classical system, depends upon the presence of ribosomes, magnesium ion, and an energy source such as guanosine triphosphate and is inhibited by puromycin (Nirenberg, 1963). The other system requires neither ribosomes nor magnesium ion and is not affected by puromycin (Soffer & Mendelssohn, 1966).

As a cellular metabolite, arginine is a key intermediate in the biosynthesis of urea by the ornithine-urea cycle (Krebs & Henseleit, 1932). In this role, it is involved in both nitrogen excretion and the maintenance of osmotic balance. Arginine is also involved as a muscle phosphagen in the energetics of muscle contraction. Its involvement is direct in invertebrates as phosphoarginine (Ennor & Morrison, 1958) and indirect in vertebrates in the biosynthesis of creatine (Walker, 1965). It may also play
an additional role in muscle biochemistry in certain invertebrates through the formation of octopine. The condensation of arginine with pyruvate to form octopine may function to reoxidize reduced pyridine nucleotides formed during glycolysis in a manner analogous to the formation of lactic acid in vertebrate muscle (Thoai, 1965).

Except in its role as a muscle phosphagen, the metabolism of arginine in invertebrates has received little attention. This is somewhat surprising since interest in the comparative biochemistry of arginine as a muscle phosphagen dates back to the study by J. Needham and his associates in 1932. It was not until 1965, however, that the biosynthesis of arginine in an invertebrate was shown to take place. Bishop & Campbell (1965) showed that protein arginine was synthesized de novo in the earthworm Lumbricus terrestris and that the arginine pathway was also utilized in this species for the synthesis of excretory urea. Evidence presented by Campbell (1965) suggests a similar situation in the land planarian. In contrast to the conclusions drawn by Baldwin some years ago and held by him to the present (Baldwin, 1967), these studies indicate that ureotelism has been exploited by the invertebrates, especially in their invasion of the land environment, in much the same way as it has in vertebrates (Cohen & Brown, 1960).

Much of the confusion attendant to arginine and urea biosynthesis in invertebrates stems from the observations by Baldwin & Needham (1934, 1935) on terrestrial snails and it is with arginine biosynthesis and degradation in
these organisms that this dissertation is concerned.

Terrestrial snails are known to store and excrete uric acid. The initial identification of uric acid in *Helix pomatia* is attributed to Jacobson in 1828 by Perrier (1889) although the metabolic origin of the uric acid was not known at this time. Weiner (1902) suggested that birds synthesized uric acid by condensing two molecules of urea with the C₃-dicarboxylic acid, tartromic acid.

The enzyme arginase which is responsible for the degradation of arginine to ornithine and urea was initially observed in mammalian liver by Kossel & Dakin (1904). Its distribution was extended to invertebrates when Clementi (1918) demonstrated its presence in snail hepatopancreas. This observation was a contradiction to his rule (Clementi, 1914) that uricotelic species do not possess arginase activity.

Delaunay (1927), in a survey of the end-products of nitrogen metabolism by invertebrates, reported *H. pomatia* to excrete 22 percent of its total excretory-nitrogen as urea. Delaunay's analyses were performed on the water in which the snails were immersed or on water extracts of the nephridium (kidney). Because of the insolubility of the purines, these methods would leave at least 80 percent of the nitrogen—-as purine-nitrogen—undetected. This was recognized by him as he makes reference to "water-soluble nitrogen substances".

The high percentage of excretory-urea reported by Delaunay (1927) in conjunction with the report of arginase activity in the hepatopancreas of *H. pomatia* (Clementi,
1918) prompted Baldwin & Needham (1934) to investigate the metabolic origin of urea in this snail. During this period, the ornithine-urea cycle had been formulated by Krebs & Henseleit (1932) as a cyclic process involving ornithine, ammonium and bicarbonate ions, citrulline, and arginine and the enzyme arginase. Baldwin & Needham (1934) concluded, however, that the ornithine-urea cycle did not function in \textit{H. pomatia} hepatopancreas since their studies with tissue slices of the hepatopancreas failed to demonstrate urea formation from ammonium and bicarbonate ions. They also failed to demonstrate a stimulation of urea formation by catalytic amounts of ornithine, a phenomenon which was critical in the formulation of the ornithine-urea cycle in mammalian liver. The fact that only added arginine gave rise to significant amounts of urea suggested to them that the major metabolic source of urea was from the action of arginase on dietary arginine.

A critical point of Baldwin and Needham's work on \textit{H. pomatia} was their failure to detect urease activity in the hepatopancreas which was the main tissue they used to study urea synthesis. Przylecki (1922) had observed urease activity in \textit{H. pomatia} and this observation was confirmed by Baldwin & Needham for kidney tissue only. It will be shown here that urease activity is present in all tissues of \textit{Otala} (=\textit{Helix}) \textit{lactea} and, because of the action of this enzyme, urea does not normally accumulate under the conditions used by Baldwin & Needham.

Wolf (1933) suggested that land snails synthesize
uric acid from urea according to the mechanism proposed by Weiner (1902). This suggestion and the high levels of arginase in *H. pomatia* led Baldwin (1935) to the same conclusion. It might be pointed out that the arginase activity measured in *H. pomatia* was equivalent to the amount found in mammalian liver. Leifert (1939) provided evidence for the synthesis of uric acid from urea by insects and this mechanism of uric acid synthesis was held for several years.

Until the early 1960's, terrestrial pulmonates occupied a unique position in comparative biochemistry. Being uricotelic, they were a contradiction to the "rule of Clementi" that arginase only occurs in the livers of ureotelic species. They also represented a contradiction to the "rule of Przylecki" that purinolytic enzymes do not occur in uricotelic species. In invertebrates, urease is generally considered to be part of the purinolytic pathway. The metabolic origin of uric acid was considered to be from urea which was derived from dietary arginine through the action of arginase.

Elegant work by Buchanan & Sonne (1946), Buchanan *et al.* (1948), and Sonne *et al.* (1948) demonstrated that the synthesis of uric acid in birds was by a pathway other than that described by Weiner (1902). Anderson & Patton (1955) first demonstrated that urea was not a precursor of uric acid in an invertebrate. The *de novo* synthesis of the purine ring was first shown for an invertebrate when it was found that insects utilize the same precursors for uric acid synthesis as do birds (McEnroe & Forgash, 1957; Desai &
Kilby, 1958; Heller & Jezewska, 1959). The mechanism of Weiner (1902) was finally ruled out for land snails when Bricteux-Gregoire & Florkin (1962 a,b) demonstrated that after $^{14}$C]urea was injected into H. *pomatia*, the majority of the $^{14}$C incorporated into uric acid was in carbon-6 of the purine ring. On the basis of Buchanan and co-worker's pathway, the incorporation of $^{14}$C into this position indicated that it came from carbon dioxide. The carbon-2 and carbon-8 positions would have been labeled had urea been incorporated by Weiner's (1902) scheme. A convincing demonstration of the pathway of uric acid synthesis in a land snail based on $^{14}$C precursor studies was provided by Lee & Campbell (1965) in *O. lactea*. The pathway is essentially identical to that in birds and insects. The theory that purine synthesis was used by land snails for nitrogen excretion was amplified by the demonstration of Jezewska *et al* (1963 a,b) that essentially all of the nitrogen present in the excreta and nephridium of H. *pomatia* could be accounted for as the purines uric acid, xanthine, and guanine.

Interest in the Krebs-Henseleit cycle in terrestrial snails was revived, however, by the demonstration in *O. lactea* of ornithine transcarbamylase activity, the enzyme necessary for the conversion of ornithine to citrulline (Linton & Campbell, 1962). The enzyme has also been demonstrated in H. *pomatia* (Foremska & Heller, 1962; Bricteux-Gregoire & Florkin, 1964) and in the fresh-water pulmonate *Lymnaea stagnalis* (Friedl & Bayne, 1964). The
second substrate necessary for the enzymatic conversion of ornithine to citrulline is carbamyl phosphate. It is supposedly synthesized in eucaryotic cells by carbamyl phosphate synthetase, an energy requiring enzyme which utilizes either ammonium and bicarbonate ions or bicarbonate and the amide group of glutamine. The presence of this enzyme activity has not as yet been demonstrated in land snails (Linton & Campbell, 1962; Bricteux-Gregoire & Florkin, 1964). Campbell & Bishop (1963) did, however, demonstrate the in vivo incorporation of $^{14}$C bicarbonate into the ureido- group of citrulline in O. lactea indicating that carbamyl phosphate or a similar compound was formed. $^{14}$C Bicarbonate was also incorporated into urea by O. lactea hepatopancreas. Similar results were obtained for H. pomatia by Bricteux-Gregoire & Florkin (1964) who isolated $^{14}$C urea after the injection of $^{14}$C bicarbonate into whole snails. Argininosuccinate lyase has also been demonstrated in O. lactea along with some evidence for the presence of argininosuccinate synthetase (Linton & Campbell, 1962).

On the basis of the evidence thus far presented, it might be expected that some land snails have a capacity for the synthesis of arginine. This should not be too surprising since arginine is a universal constituent of protein. Since land snails are uricotelic, it is also understandable that the levels of urea biosynthetic enzymes would be low so that there would be no competition between several nitrogen excretory mechanisms. Water conservation is probably extremely important to land snails
and the excretion of nitrogen wastes as purines would allow them to conserve water. Autotrophic organisms as well as many heterotrophic organisms still possess the capacity to degrade arginine to ornithine and urea and to further degrade urea to inorganic ions. The significance of this is not fully understood although the answer may be linked to the fact that ornithine, derived from the reaction, can be converted to energy-yielding compounds such as glutamate. In the discussion to follow it will be shown with some certainty that arginine is synthesized to the extent that it at least partially meets the organisms requirement for protein arginine. Further, it will be shown that excess arginine may play a role in nitrogenous waste excretion in estivating snails, being a precursor for ammonia that is excreted as such through the snail's shell. A physiological function for urease and its interrelationship with carbonic anhydrase for calcium carbonate deposition in shell is also proposed.
MATERIALS & METHODS

Animals:
Otala (=Helix) lactea were obtained from a commercial supplier (S. Scozzaro Wholesale Groceries, Brooklyn, New York). Helix aspersa were collected in the vicinity of Northridge, California, and were provided through the courtesy of Dr. Earl Segal, San Fernando Valley State College, Northridge, California. Both species were maintained for extended periods of time in the laboratory in a state of estivation. Feeding snails were kept in a moist terrarium and were fed lettuce.

Reagents:
General: Inorganic salts, acids, and bases were purchased from Fisher Scientific Company and were of the highest quality available. Most of the organic compounds were purchased from either the Fisher Scientific Company or the Sigma Chemical Company, St. Louis, Mo., with the following exceptions. N-Hydroxyurea was obtained from the Nutritional Biochemical Company, Cleveland, Ohio. Acetohydroxamic acid was synthesized by a modification of Fishbein's procedure (Fishbein et al., 1965) from acetic anhydride and hydroxylamine in alkaline solution. The hydroxamate was extracted from the reaction residue with ethyl acetate and was crystallized from this solvent. I am indebted to Mr. J.A. McDonald of this laboratory for this compound. Acetazolamide (DiamoxR, Lederle Laboratories Division, American Cyanamid Company) was kindly supplied.
by Dr. Selby B. Davis. Azaserine was supplied by the Southwest Cancer Chemotherapy Study Group Headquarters, M.D. Anderson Hospital, Houston, Texas. Cetyltrimethylammonium bromide was purchased from Eastman Organic Chemicals (Division of Eastman Kodak Company).

Enzymes: Lactic dehydrogenase, Type III, and urease, Type III, were obtained from the Sigma Chemical Company. Pyruvate kinase, A grade, was obtained from Calbiochem, Los Angles, California. Arginase, AR, was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. Argininosuccinase (argininosuccinate lyase) was purified from steer liver by the method of Havir et al (1965). The purification was carried through the heat-treatment step (Step 4).

Subsequent purification renders the enzyme less stable.

Radioisotopes: Both [12C] and [14C] argininosuccinate (barium salt) were prepared by the method of Ratner (1957). The [14C] argininosuccinate (assaying 2.49 umoles argininosuccinate per mg barium salt) had a specific activity of 36,200 dpm/umole and contained no more than 0.0002 umole [14C] arginine per umole argininosuccinate. [1,3-15N] Urea (30.5 atoms % 15N) was purchased from Volk Radiochemical Corporation, Skokie (Chicago), Illinois. [Guanidino-14C] L-Arginine was obtained from Nuclear-Chicago (G.D. Searle & Co.). [U-14C] L-Aspartic acid, [14C] urea, [ureido-14C] L-citrulline, sodium [14C] bicarbonate, [1-14C] glycine, [U-14C] DL-ornithine, and [5-14C] L-ornithine were obtained from New England Nuclear Corporation, Boston, Mass.
Analytical procedures:

1) **Ammonia**: Ammonia was estimated colorimetrically using a modified Koch & McMeekin; Nessler's reagent (Minari & Zilversmit, 1963). The color given with the reagent at 420 mu was compared to that given with the reagent and known ammonium sulfate standards. The ammonium ion content of tissue homogenates and blood was estimated following its liberation from solution by saturated potassium carbonate. The liberated ammonia was absorbed in 1N sulfuric acid in microdiffusion vials similar to the ones described by Seligson & Seligson (1951).

2) **Stable isotopes**: $^{15}$N determinations were carried out with a Consolidated Electrodynamics Corporation Type 21-103C mass spectrometer. Calculations of $^{15}$N atoms % excess were based on the mass 28 and 29 peaks. The necessary air corrections were applied in the final calculations. These determinations were performed by Mr. David Baugher of Petroleum Analytical Research Corporation of Houston, Texas.

3) **Urea**: Urea was measured either colorimetrically on deproteinized solutions through its reaction with 1-phenyl-1,2-propanedione-2-oxime by a modification of the Archibald method (1945) as described by Ratner (1955), or after treatment with urease. For the latter, ammonia was measured before and after treatment with urease.

4) **Arginine**: Arginine was determined as described by Wu (1959).

5) **Citrulline**: Citrulline was determined colorimetrically on deproteinized solutions through its reaction with 2,3-
butanedione-2-oxime (Archibald, 1944).

6) Protein: Protein estimations were made by the method of Lowry et al (1951) using the Folin phenol reagent.

7) Total Nitrogen: Total nitrogen determinations were carried out after the method of Lang (1958).

General procedures:

1) Surface sterilization of shells: When intact snails or snail shell was used, mucous, bacteria, and foreign particles were removed from the shell surface with 95% ethanol, pHisoHexR (Winthrop Laboratories Division, Sterling Drug, Inc.), or Zephiran chlorideR (Winthrop Laboratories). Ethanol was used mainly in experiments in which ammonia evolution from whole snails was measured. Trace amounts of ammonia were evolved from large quantities of pHisoHex. When pHisoHex was used, (generally in experiments with 14C precursors) the snails were scrubbed with a Nylon brush, rinsed with tap water and then allowed to air dry. Plate counts of the shell surface were made by pressing the shell against nutrient agar. No viable bacteria were detected when either of the three reagents was used to wash the shells. Washing with tap water alone generally reduced the viable bacteria from about 12/cm2 to 1/cm2 of shell surface.

2) Injection of substrates: Precursors of ammonia or carbon dioxide were injected into estivating snails through a hole in the shell. This hole was made with a high-speed drill and was located about a three-quarter turn from the aperture. Care was taken during drilling not to penetrate
the mantle tissue underlying the shell. The precursors were dissolved in glass-distilled water and no more than 0.1 ml was injected per snail. Terrestrial pulmonates normally undergo rather gross changes in the ionic compositions of their body fluids (Burton, 1964, 1965; Howes & Wells, 1934) and dilution of the blood by this amount was not considered significant. Following the injection of the substrate, the hole was sealed with a drop of melted paraffin (Gulfwax⁸, Gulf Oil Corporation). The snails withstood treatment of this kind remarkably well. The other route of injection was directly into the extended foot of crawling snails. This was used with actively feeding snails.

3) Estimation of evolved ammonia gas: Ammonia evolution was measured by placing estivating snails, whose shells were surface-sterilized, in glass chambers of approximately 350 cc volume. These chambers were provided with an inlet and outlet. No more than 3 snails were placed in an individual chamber. The chambers were flushed with compressed air which was first passed through a Matheson Model 450 Gas Purifier (Matheson Scientific Inc.) and then through 2 successive gas washing bottles containing 100-200 ml of 1N sulfuric acid. The flow rates through the chamber ranged from 10-40 cc/min. With flow rates in this range, there was no correlation between ammonia production and flow rate. The air exiting from the chambers was passed through 4 ml of 0.01N sulfuric acid via a fritted glass (coarse) bubbler. These traps gave quantitative recovery
of the ammonia produced by the snails. This was shown by initial experiments in which two successively traps were used on the chamber outlet. The ammonia, trapped as ammonium sulfate was determined by direct Nesslerization. Some (about half) of the estivating snails in the chambers awakened during the course of an experiment. They later sealed themselves to the chamber wall and returned to estivation. Locomotion by these snails involves the secretion of mucous from the foot and it was necessary to determine the contribution of this secretion to the observed ammonia production. The concentration of the ammonium ion in the mucous, produced artificially by stimulation with a glass rod, was about 0.23 umole/ml. This contributed little, if any, to the ammonia measured since removal of the snails from the chamber at any time during an experiment resulted in a sharp decline in ammonia production. It was found impractical to measure ammonia evolution from active snails. Fecal matter from active snails or bacterial action on the fecal matter produced large quantities of ammonia.

4) General Radioactivity determinations: Most radioactivity determinations were made by liquid scintillation spectrometry. Methanolic hydroxide of HyamineR (Rohm & Haas), 2,5-diphenyl- oxazole (PPO), and 1,4-bis-(4-methyl-5-phenyloxazolyl) benzene (dimethyl-POPOP) were purchased from the Packard Instrument Company, Inc., Downers Grove, Illinois. For radioactivity determinations upon compounds in aqueous solutions, an aliquot was mixed with enough Bray's solution (Bray, 1960) to bring the final volume to about 15 ml.
For compounds in organic solvents, an aliquot was mixed with a volume of a mixture containing 5 g PPO and 0.5 g dimethyl-POPOP per liter toluene sufficient to bring the final volume to about 15 ml. The efficiency of the liquid scintillation counting was determined by the channels-ratio method (Bruno & Christian, 1961). Several experiments were performed with precursors which were specifically labeled with $^{14}$C so that the end-product of their degradation was $^{14}$C carbon dioxide. These experiments were generally of two types.

*In vivo* experiments: The evolution of $^{14}$C carbon dioxide, formed from $^{14}$C-labeled precursors injected into whole snails, was measured by passing the air leaving the glass chambers through 6 ml of a monoethanolamine-methanol mixture (20:80, v/v; Baggiolini & Bickel, 1966). Five ml of this mixture was then mixed with 10 ml of the PPO:dimethyl-POPOP:toluene scintillation mixture.

*In vitro* experiments: $^{14}$C precursors were incubated with whole tissue or tissue homogenates in closed 25 ml reaction flasks provided with a polyethylene center well (Kontes Glass Company). The reactions were stopped by injecting 1 ml of 5N sulfuric acid through the rubber stoppers into the flasks. The $^{14}$C carbon dioxide liberated from the reaction mixture was trapped in 0.2 ml of 1M methanolic Hyamine hydroxide contained in the center well. The center well and its contents were transferred to 15 ml of the PPO:dimethyl-POPOP:toluene scintillation mixture.
5) **Protein Arginine, Aspartate, and Glutamate**

**Preparation of labeled protein amino acids**: Whole body protein was prepared by the modified method of Siekevitz (1952) as described by Campbell (1965). The extraction procedures were conveniently carried out in heavy-walled 40 ml centrifuge tubes which were capped with glass marbles during extractions at temperatures near or above the boiling point of the organic solvents. No more than 0.05% of the incorporated $[^{14}C]$ from $[^{14}C]L$-arginine was liberated from the isolated protein when it was treated with ninhydrin (Van Slyke *et al.*, 1941). Essentially 100% of the theoretical amount was liberated following acid hydrolysis indicating that the only $[^{14}C]$ amino acid present was in peptide bond. Small protein samples (5-10 mg) were hydrolyzed in sealed tubes (Moore & Stein, 1963). For larger samples (20-100 mg), from which arginine was chemically isolated, hydrolysis was for 20-24 hr under reflux in 50-100 volumes 6N hydrochloric acid rendered peroxide-free by distillation from stannous chloride. After hydrolysis, the excess hydrochloric acid was removed by evaporation under reduced pressure over sodium hydroxide at 40° in a rotating evaporator. The residue was twice re-dissolved in water and evaporated to dryness. The final residue was taken up in 10% (v/v) 2-propanol for paper chromatography or in 2.2N sodium citrate for ion exchange chromatography. Two to five mg of
protein were dissolved in 1 ml.

**Chromatography:** The protein amino acids were analyzed by the automated column procedure described by Moore et al. (1958) and Piez & Morris (1960) and by two-dimensional paper chromatography and autoradiography (Campbell, 1965). The latter methods were used for the final confirmation of the identity of the compounds and the incorporation of $[^{14}\text{C}]$ therein. Known compounds were co-chromatographed with both the extracts and individual compounds isolated by paper chromatography in both of the chromatography systems used. In addition to the ninhydrin reaction, specific color reactions were also used for individual amino acids (Campbell, 1965).

**Radioactivity:** For a simultaneous measurement of the incorporated radioactivity in the compounds during their separation on the automated column, a stream divider was used to divert half the column effluent through a flow monitor crystal scintillation detector with a recorder readout. Two methods were used to quantitate the amount of $[^{14}\text{C}]$ incorporated into each compound. In one, the total radioactivity present in a portion of the protein amino acid fraction was determined prior to analysis. This determination was made on the same volume of extract as that analyzed. The solution was dissolved in 15 ml Bray's solution and counted in a liquid scintillation spectrometer. Efficiency of counting was determined by the channels-ratio method. The total integrated area recorded by the crystal scintillation detector during separation
of the compounds on the automated column was determined as the sum of the individual recorded peaks of radioactivity of the sample. The amount of radioactivity present in each compound was calculated, as a percent of this total, from the individual peak areas. In the second method of quantitation a $^{14}C$ norleucine standard was co-chromatographed with the extract and the recorded area from this known amount of radioactivity was used to calculate the incorporated $^{14}C$ in the individual compounds. The two methods agreed within 10%. To determine the amount of incorporated $^{14}C$ in the amino acids separated by paper chromatography, they were located on the chromatogram by autoradiography, eluted from the paper with water, and counted in Bray’s solution.

Isolation and degradation of protein arginine: Labeled protein arginine was chemically isolated as the flavianate (Vickery, 1940) from 100 mg portions of whole body protein by the method described by Campbell (1965). The specific activity (dpm/umole) of the arginine was followed during the isolation procedure and, in the final calculations, corrections were made for recovery and for dilution due to the added carrier arginine.

The isolated arginine (13-19 mg) in 2 ml was transferred to a reaction vessel and the following components added, 300 umoles Tris-sulfate, pH 7.6; 5 umoles cobalt sulfate; 300 mg bovine arginase; and 300 mg urease. The reaction mixture was shaken at 38° for 4 hr and the reaction stopped by the injection of 1 ml of 3N sulfuric acid.
The evolved $[^{14}\text{C}]$ carbon dioxide (representing the guanidino-C of arginine) was trapped in Hyamine and counted as previously described. Less than 1 umole arginine remained after this treatment and, since its specific activity was known, the appropriate corrections were made. The precipitated protein was removed from the reaction mixture by centrifuging. Ornithine was isolated from the supernatant fluid with Dowex 50 (H$^+$) (Campbell, 1965). It was crystallized from 80% (v/v) ethanol and dissolved in water. A portion was used for its colorimetric determination (Chinard, 1952) and another portion was dissolved in Bray’s solution for counting.

6) Measurement of blood pH changes and evolution of gases from the blood: Blood was collected from the pericardial sinus of the snails with a mouth micropipet in the following manner. A small hole was drilled in the shell about two and one-half turns from the apex. A slightly larger hole was gently chipped in the shell, and the outermost membrane immediately under the shell and covering the underlying tissues was then carefully cut. Usually blood came forth through the cut and could be easily drained into a tube or drawn into a micropipet. Frequently more blood could be obtained by very gently massaging the tissues in the immediate area of the opening. Only bluish blue blood was collected. Contamination of the blood due to injury of the kidney, albumen gland or hepatopancreas was indicated by the appearance in the blood of a white to brownish liquid.
The blood was kept at 0° in a closed container until used. For pH measurements, the blood was placed in a closed jacketed-chamber maintained at constant temperature with circulating water. Most of the measurements were performed at 30°. The chamber was provided with an inlet and outlet for gas and was generally flushed with 100% nitrogen gas during experiments. The chamber was also provided with a combination microelectrode (Metrohm, Ltd.), and the pH was recorded through a Metrohm Model E300 pH meter coupled with a Sargent Model SR recorder. During recording, the blood was stirred continuously with a magnetic stirring bar. Carbon dioxide evolution from the blood was measured following the addition of known amounts of sodium [14C]-bicarbonate whose specific activity (dpm/umole) had been determined. This allowed a measurement of the rate of evolution of the [14C] carbon dioxide from the blood by scintillation counting, also the endogenous bicarbonate content of the blood could be calculated by isotope dilution. Following the addition of sodium [14C] bicarbonate, the nitrogen gas leaving the chamber was bubbled through 5 ml of 0.1N carbon dioxide-free sodium hydroxide to trap the evolved carbon dioxide. A 2.0 ml portion of the sodium hydroxide solution was acidified and the liberated carbon dioxide was measured manometrically in a Warburg apparatus. Duplicate 0.5 ml portions of the trap solution were used to determine the amount of radioactivity present. These 0.5 ml portions were transferred to closed reaction flasks, acidified with sulfuric acid and the [14C] carbon dioxide evolved was trapped in Hyamine and counted by liquid
scintillation counting as described previously. The specific activity (dpm/umole) of the $[^{14}C]$ carbon dioxide could thus be calculated and the dilution of its original specific activity by the endogenous bicarbonate in the blood gave a measure of the blood bicarbonate content. The ammonia evolved from the blood in vitro was measured by passing the gas leaving the chamber through 4 ml of 0.01N sulfuric acid. The ammonia, trapped as ammonium sulfate, was then determined by direct Nesslerization. The gas entering the chamber had been passed through a gas-washing bottle containing about 100 ml of 1N sulfuric acid thus insuring that the ammonia measured had indeed come from the blood.

7) Purines:

Preparation: The kidney of O.lactea was dissected intact, any ruptured kidneys being discarded. Each kidney was analyzed separately. The purines were extracted by macerating the kidney in saturated lithium carbonate. Complete solution of the purines was obtained by using 30 ml of saturated lithium carbonate per 100 mg total kidney weight. The initial extracting solution was then decanted and the tissue was twice re-extracted with small volumes of saturated lithium carbonate. The combined extracts were thoroughly mixed and filtered through glass wool. Duplicate 0.1 ml samples were used for total-N determinations. The filtered extracts were kept at 4°C until analyzed. Corrections were made for the
degradation of the purines during extraction and analysis by preparing a standard solution of the purines in saturated lithium carbonate at the same time the kidney extracts were made and subjecting them to complete analysis. The standard solutions contained the purines in approximately the same ratios as in the intact kidney. Recovery experiments were made on standards of from 10-100 ug uric acid, 5-25 ug xanthine, and 3-15 ug guanine.

**Isolation:** The three kidney purines were separated by ascending two-dimensional paper chromatography on Whatman 3MM paper (W. & R. Balston, Ltd.). One hundred microliters of the extract was used per chromatogram. The solvent for the first dimension was 0.35M dibasic sodium phosphate saturated with *iso*-amyl alcohol (Carter, 1950). This is a biphasic system and is prepared by shaking 100 ml of 0.35M dibasic sodium phosphate with 60 ml of *iso*-amyl alcohol in a separatory funnel. The layers are allowed to separate and both are drained into the solvent dish. The second dimension solvent was 60% (v/v) *n*-propyl alcohol. After drying, the purines were located on the chromatograms by uv-quenching. No hypoxanthine, which separates from the other purines in these solvent systems, was ever present in the kidney extracts of either active or estivating snails. Other unidentified uv-absorbing compounds were occasionally present in extracts from feeding snails but never from estivating
snails. Unknown uv-absorbing compounds have previously been reported in the kidneys of *H. pomatia* (Jezewska et al., 1963 a) and *O. lactea* (Lee & Campbell, 1965). Their sporadic occurrence in feeding snails is consistent with the suggestion by Jezewska et al. (1963 b) that they may be of dietary origin.

**Quantitation:** The areas of the chromatograms containing uric acid and xanthine were cut from the paper and placed in 10 and 5 ml, respectively, of saturated lithium carbonate contained in heavy-walled centrifuge tubes. The purines were eluted for 2 hr with shaking. Guanine was eluted with 5 ml of 0.1N hydrochloric acid. Equivalent areas of the chromatograms not containing purines were similarly treated with these solutions to serve as blank corrections. The eluates were clarified by centrifuging at room temperature. The amount of purine in each eluate was determined by the differential extinction technique of Vishhner & Chargaff (1948). The absorption maximum for uric acid in saturated lithium carbonate was determined to be at 295 μm and the $E_{\text{max}}$ was $13.43 \times 10^3$. For xanthine in saturated lithium carbonate, the maximum was at 280 μm and the $E_{\text{max}}$ was $11.52 \times 10^3$. For guanine in 0.1N hydrochloric acid, the maximum was at 249 μm and the $E_{\text{max}}$ was $11.52 \times 10^3$. The differences in extinction used were as follows: for uric acid, the difference in extinction between 295 and 310 μm;
for xanthine, between 280 and 310 μμ; and, for guanine between 249 and 290 μμ. For 1 ml of solution, the difference in absorbance per ug (Δ/μg) was 0.050 for uric acid, 0.056 for xanthine, and 0.049 for guanine.

Radioactivity measurements: The purines were eluted from the chromatograms as above and 1 ml was added to 15 ml Bray's solution for liquid scintillation counting. Quench corrections were made by the channels-ratio method.

8) Calcium carbonate deposition into shell:

In vivo experiments: Estivating *O.lactea* were surface-sterilized with pHisohex, rinsed with tap water and air dried. The [14C] precursors were injected through a hole in the shell as previously described. Twenty-four to twenty-nine hours after injection, the snails were killed and the shells dissected and pooled. Each group contained the shells from all the snails injected with a particular substrate; there were usually 3-6 snails per group. The pooled shells were washed on a Büchner funnel with 1 liter of glass-distilled water followed by 400 ml of 95% ethanol. The shells were then air dried overnight. The shells were weighed and then crushed with a mortar and pestle. They were ground to a very fine powder with either an electric mortar and pestle or an intermediate Wiley grinding mill. After grinding, the shells had the appearance and consistency of face powder. The fine powder was mixed by rotation with large glass
beads for 24 hr. Four 10 mg samples of each group were transferred to closed 25 ml reaction flasks fitted with polyethylene center wells containing 0.3 ml Hyamine. Two ml of 18N phosphoric acid was injected into the flasks to decompose the calcium carbonate. The flasks were shaken for 10 min. At the end of this period 2 ml of 5N sulfuric acid was added and the flasks were shaken for an additional 30 min. The center wells were then removed and placed in 15 ml PPO:dimethyl-POPOP:toluene solvent for liquid scintillation counting.

In vitro experiments: Estivating *O. lactea* were surface-sterilized with pHisoHex and a 1 cm² section of the shell adjacent to the aperture was marked off. The section was cut out with a saw attachment for a high-speed drill. The vascularized section of the mantle tissue (referred to as the lung, about 1.5 cm²) was then excised from the snail. The reaction mixture contained in a 2 ml volume: a 1 cm² piece of shell; the mantle tissue from one snail; pulmonate saline, pH 7.6 (Chiarandini, 1964; modified by the omission of sodium bicarbonate); 10 umoles glycylglycine, pH 7.6; 10 umoles calcium chloride dihydrate; and 5 umoles [¹⁴C] substrate. Incubation was for 5 hr at 25° with shaking in 25 ml reaction flasks capped with glass marbles. The reaction was begun by the addition of the substrate. All flasks were incubated 30 min before the addition of substrate. The reaction was
stopped by removal of the shell and mantle tissue. The mantle tissue was blotted dry on filter paper and weighed. The shell was washed in 500 ml of glass-distilled water followed by rinsing in 200 ml of 95% ethanol. The shell was then air dried overnight and weighed. The dry shell was ground in a mortar by hand to a very fine powder and mixed well. Ten mg samples were decomposed and counted as described in the in vivo experiments.

**Enzyme Systems:**

**General:** All assays were performed in duplicate. Assays in which the product formed was measured colorimetrically were executed in 12 ml conical centrifuge tubes. Assays in which $[^{14}\text{C}]$ precursors were used were executed in heavy-walled 25 ml reaction flasks fitted with removable polyethylene center wells containing Hyamine. Controls were run with all assays. These consisted in most cases of heat-inactivated extracts to correct for endogenous chromogenicity and non-enzymatic product formation. In other cases, 0 time incubations were used in which acid was added prior to the addition of the enzyme solution. The controls for the experiments with $[^{14}\text{C}]$ precursors usually were incubation mixtures from which the enzyme solution had been deleted.

**Preparation of tissue:** Tissues were dissected from estivating (except where noted) *O. lactea* or *H. aspersa* and were homogenized at $0^\circ-4^\circ$ in a TenBroek homogenizer. Ten percent homogenates (w/v) were prepared and these
were used as the enzyme solution or served as a starting point for further manipulations of the enzyme solution. The homogenizing solutions peculiar to each enzyme assay are given below for each enzyme.

**Ornithine Transcarbamylase:** The assays were carried out in a manner similar to that described by Linton & Campbell (1962). Tissues from *O. lactea* were homogenized in 4.5 vol of 0.1% (w/v) cetyltrimethylammonium bromide. The homogenate was centrifuged at 5000 x g and 4°C for 30 min. The supernatant fluid was decanted and saved. The residue was re-homogenized in an additional 4.5 vol of 0.1% cetyltrimethylammonium bromide and centrifuged. The two supernatant solutions were then pooled. Whole blood, which does not contain cellular components, was used directly in enzyme assays. The assay system contained in a 1 ml volume, in umoles: L-ornithine, pH 8.3, 10; glycylglycine, pH 8.3, 50; carbamyl phosphate (dilithium salt), 10; and 0.1 ml enzyme solution unless otherwise noted. The reaction was started by the addition of carbamyl phosphate. The reaction was allowed to proceed for 15 min at 25°C and was then stopped by the addition of 5 ml of 0.5M perchloric acid. After removal of the precipitated protein by centrifuging, the citrulline formed was measured colorimetrically.

**Argininosuccinate Synthetase:**

**Substrate preparation:** [Ureido-14C]L-citrulline undergoes radiolysis resulting in a [14C]urea contaminant. The labeled citrulline was therefore purified by chromatography
on a Dowex-50 (H⁺) resin by the method of Hall et al (1960). 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 a portion in Bray’s solution and using an additional portion for the colorimetric determination of citrulline.

Enzyme preparation: Tissues from feeding O. lactea or H. aspersa were homogenized in 0.1% (w/v) cetyltrimethylammonium bromide. The homogenate was centrifuged for 20 min at 4° and 4000 x g. The supernatant fluid was decanted and used as the enzyme solution. Whole blood was used as such. The rat liver enzyme solution was prepared in a manner similar to that used for the snail enzyme. Substantially less activity was found for both the snail and the rat when the tissue was homogenized in 5 mM potassium phosphate, pH 7.5, even when supplemented with 2 umoles each ATP and magnesium chloride per ml.

Contents of the reaction mixture: The assay system contained in a 2 ml volume, in umoles: L-aspartate, pH 8 (sodium salt), 10; [ureido-14C]L-citrulline, 200,000 dpm/umole, 5; ATP, pH 7 (sodium salt), 2; magnesium chloride, 2; potassium phosphate, pH 7.5, 100; phosphoenolpyruvate, 2.5; pyruvate kinase, 6.6 IU; argininosuccinase, 15 IU; arginase, 3.2 IU; urease, 4.6 IU; sodium bicarbonate, 10; and 1.0 ml enzyme
solution (or 0.1 ml of rat liver enzyme solution).

Modifications of this are noted. Initial experiments indicated that these amounts of argininosuccinase, arginase, and urease were in excess of that needed to completely degrade all of the argininosuccinate synthesized even when rat liver enzyme was used. Incubation was at 25° with shaking for various periods of time. The reaction was started by the addition of the enzyme solution and was stopped by injecting sulfuric acid into the flasks. [14C] Carbon dioxide was trapped and counted as previously described.

Accumulation of [14C] argininosuccinate formed from [14C] - citrulline and [14C] aspartate: The reaction mixture consisted of the following in a 2 ml volume, in umoles: ATP, 2; magnesium chloride, 2; potassium phosphate, pH 7.5, 100; phosphoenolpyruvate, 2.5; pyruvate kinase, 6.6 IU; argininosuccinate (potassium salt), 2.6; L-citrulline ([12C] or [Ureido-14C], 3 ucr/umole), 5; L-aspartate, pH 8, ([12C] or [U-14C], 0.5 ucr/umole), 10; and enzyme solution (small: 1.0 ml; rat: 0.1 ml). The reaction proceeded for 2 hr at 25° and was stopped by the addition of 2 ml of 10% (w/v) trichloroacetic acid. This was allowed to sit for 30 min and was then centrifuged at room temperature to sediment the denatured protein. The supernatant fluid was neutralized with sodium hydroxide and rinsed into a Dowex-1X8 column (25 cm, 200-400 mesh) in the acetate form as described by Ratner & Kunkemuller (1966). Elution was begun with 80 ml of 0.05N acetic acid. This
was followed by 160 ml of 0.1N acetic acid and then by
80 ml of 0.5N acetic acid. Five ml fractions were collected.
The column was regenerated with 3N acetic acid and reused
for each experimental reaction mixture. A portion of
each fraction was counted in Bray’s solution by liquid
scintillation spectrometry. Standard solutions were also
passed through the column to ascertain the respective
elution patterns for L-citrulline, L-aspartate, and
argininosuccinic acid. The fractions from the Anhydride I
region from the Dowex-1 column were taken to dryness at
50° under reduced pressure over sodium hydroxide in a
rotating evaporator. The residue was taken up in 1 ml
of glass-distilled water followed by two 1 ml washings.
The 3 portions were combined. The Anhydride I content
was estimated by passage through an Amberlite CG-120-
pyridine column. The Amberlite column was prepared as
described by Rochavansky and Ratner (1967) and by
Canfield (1963). Aspartate and citrulline emerged
from the column with 90 ml of 0.1N pyridine-acetate
buffer and on changing to 0.3N pyridine-acetate buffer
the anhydride of argininosuccinic acid emerged from the
column between 60 and 110 ml. The pyridine-acetate was
removed under reduced pressure at 50°. The residue was
then taken up in 1 ml of glass-distilled water and the
radioactivity of each fraction was determined.

**Argininosuccinase (lyase):** Tissues from *O.lactea* were
homogenized in 10 mM potassium phosphate, pH 7.5.
Whole blood was used as such. The reaction mixture
contained in a 1 ml volume, in umoles: [14C]arginino-
succinate (potassium salt, labeled in the guanidino-C,
36,200 dpm/umole), 5; potassium phosphate, pH 7.5, 50;
arginase, 1 IU; urease, 11 IU; and 0.2 ml of snail
homogenate except where noted. The reaction was started
by the addition of the [14C]argininosuccinate and ran
for various times at 25°. The reaction was stopped by
injecting sulfuric acid into the flasks. The evolved
[14C] carbon dioxide was trapped and counted as described
previously.

Arginase: These assays were performed in a manner
similar to that described by Linton & Campbell (1962).
Tissues from O. lactea were homogenized in 0.1% (w/v)
cetyltrimethylammonium bromide. Whole blood was used
as such. The reaction mixture contained in a 1 ml volume,
in umoles: L-arginine, pH 9.5 (sodium salt), 85; glycine,
ph 9.5 (sodium salt), 50; manganese chloride, 0.5; and
0.1 ml of enzyme solution. Incubation was for 15 min
at 25°. The reaction was started by the addition of the
enzyme solution and stopped by the addition of 5 ml of
0.5M perchloric acid. Denatured protein was sedimented
by centrifuging and the amount of urea formed was
measured colorimetrically. An error is introduced because
the snail enzyme solution contains a small amount of urease
activity. If the incubation time is short, the error is
quite small, however, and accurate measurements may be
achieved due to the fact that the activity of the arginase
is far greater than the urease activity.

**Urease:** Tissues of *O. lactea* were homogenized in 5 mM disodium ethylenediaminetetraacetic acid. The homogenate was centrifuged for 30 min at 4°C and 12,100 x g. The supernatant fluid was decanted and used as the enzyme solution. Whole blood was used as such. The reaction mixture contained in a 1 ml volume, in umoles: $[^{14}C]$ urea, 100,000 dpm/umole, 5; Tris-maleate, pH 8.8, 50; and 1.0 ml enzyme solution. Incubations were for various times at 25°C. The reaction was started by the addition of the enzyme solution and was stopped by injecting sulfuric acid into each flask. The evolved $[^{14}C]$ carbon dioxide was trapped and counted as previously described.

**ε-Ornithine Transaminase:** These assays were carried out in a manner similar to that described by Peraino & Pitot (1963). Enzyme activity was measured as either glutamic acid or glutamic acid-γ-semialdehyde formation. For the former, the enzyme source was a homogenate of *O. lactea* hepatopancreas in 0.05M potassium phosphate, pH 7.4. This was dialyzed for 12 hr against the same buffer. The reaction mixture consisted of the following in a 2.1 ml volume, in umoles: L-ornithine (sodium salt), pH 7.4, 40; α-ketoglutarate (sodium salt), pH 7.4, 40; pyridoxal-5'-phosphate, 0.08; and potassium phosphate, pH 7.4, 50. Incubation was anaerobic ($N_2$) at 30°C. Glutamic acid was determined as described by Awapara & Campbell (1964). For the measurement of glutamic acid-γ-semialdehyde
formation, the reaction mixture contained in moles in a 3 ml volume: L-ornithine, pH 7.4, 300; \( \alpha \)-ketoglutarate, pH 7.4, 60; and potassium phosphate, pH 7.4, 750. The enzyme source was a homogenate of \textit{O. lactea} hepatopancreas in 0.1M potassium phosphate, pH 7.4, containing 0.01M mercaptoethanol. Incubation was in air at 30\(^\circ\). Glutamic acid-\( \gamma \)-semialdehyde was determined as described by Peraino & Pitot (1963). Dependence upon the presence of L-ornithine, \( \alpha \)-ketoglutarate, pyridoxal phosphate and enzyme solution was demonstrated.

\textbf{Carbonic Anhydrase:} This enzyme was assayed in two ways:

\textbf{Manometrically:} Carbonic anhydrase was assayed in extracts of \textit{O. lactea} lung and hepatopancreas and in whole untreated blood. The lung and hepatopancreas extracts were prepared by homogenizing the tissue in glass-distilled water. The manometric method employed was that of Waygood (1955). Glass-distilled water was used in place of the enzyme solution to serve as a measure of the uncatalyzed reaction.

\textbf{Colorimetrically:} Hydration of carbon dioxide was measured by the method of Rickli \textit{et al} (1964) and Wilbur and Anderson (1948) with bromothymol blue as indicator. The method measures the time required to shift the pH from 8.2 to 6.3. The enzyme solutions consisted of either \textit{O. lactea} lung or hepatopancreas homogenized in glass-distilled water. The homogenate was then centrifuged at 48,000 x g for 20 min at 4\(^\circ\). The activity was in the supernatant fluid. The reaction mixture contained 2 ml
of 0.025M Veronal buffer, pH 8.2, containing bromothymol blue (1 mg/100 ml) at 2°. The enzyme solution, 1 ml of appropriate concentration, was added and equilibrated at 2°. Two ml of a cold, saturated carbon dioxide solution was injected by means of a syringe into the buffer. The time was recorded from the moment of injection of the carbon dioxide solution to the color change of the indicator from blue to greenish yellow. The assays were carried out in a constant temperature water bath at 2°. Conditions were chosen such that the uncatalyzed reaction took approximately 100 sec and the catalyzed reaction took about 50 sec.
RESULTS AND DISCUSSION

Arginine Synthesis

Precursor Studies: The pathway for the biosynthesis of arginine from ornithine and carbon dioxide is depicted in Figure 1. To test whole O. lactea for the capacity to synthesize arginine by this pathway, the following substrates were injected: sodium $^{14}$C bicarbonate (19.2 uc/umole), $^{14}$C urea (5.6 uc/umole), $^{U-14}$C L-ornithine (235 uc/umole), $^{5-14}$C DL-ornithine (9.6 uc/umole), ureido-$^{14}$C L-citrulline (1.1 uc/umole), and guanidino-$^{14}$C L-arginine (15.7 uc/umole). These substrates were injected on the basis of 10 uc each per g tissue. With DL-ornithine, only the L-form was considered. The tissue wt was estimated from the total wt for the injections. This estimate was only approximate since shell was found to account for from 57 to 64% of the total wt at the end of the experiment. After 24 hr, arginine was isolated from the whole body protein as the flavianate derivative and the incorporation from the specifically labeled precursors into the guanidino-C and carbons 1-5 determined. The results are presented in Table 1.

The incorporation obtained was consistent with the operation of the arginine biosynthetic pathway as it is known in other species (Figure 1). $^{14}$C bicarbonate and ureido-$^{14}$C citrulline gave rise to the guanidino-C of
Figure 1

Pathway of *de novo* urea biosynthesis
\[ \text{NH}_4^+ + \text{HCO}_3^- + 2 \text{ATP} \rightarrow \text{CARBAMYL PHOSPHATE} \]

\[ \text{CARBAMYL PHOSPHATE} \xrightarrow{\text{Ornithine transcarbamylase}} \text{CITRULLINE} \]

\[ \text{CITRULLINE} \xrightarrow{\text{ASPARTATE ATP}} \text{arginosuccinate} \]

\[ \text{ARGINOSUCCINATE} \xrightarrow{\text{Arginosuccinase}} \text{FUMARATE} \]

\[ \text{FUMARATE} \xrightarrow{\text{Arginase}} \text{ARGININE} \]

\[ \text{ARGININE} \xrightarrow{\text{Arginase}} \text{UREA} \]
Table 1—Synthesis by *O. lactea* of whole body protein arginine from \([^{14}\text{C}]\) precursors

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Incorporation*</th>
<th>Distribution of (^{14}\text{C}) in arginine as % of total</th>
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<tbody>
<tr>
<td></td>
<td>DPM in Protein</td>
<td>DPM in Protein Arginine</td>
</tr>
<tr>
<td>NaH ([^{14}\text{C}]) O₃</td>
<td>878,500</td>
<td>50,567</td>
</tr>
<tr>
<td>([^{14}\text{C}]) Urea</td>
<td>354,300</td>
<td>43,268</td>
</tr>
<tr>
<td>([U-^{14}\text{C}]) Ornithine</td>
<td>5,811,900</td>
<td>3,145,996</td>
</tr>
<tr>
<td>([5-^{14}\text{C}]) Ornithine</td>
<td>5,054,400</td>
<td>1,736,124</td>
</tr>
<tr>
<td>([\text{Ureido-}^{14}\text{C}])-Citru lline</td>
<td>1,251,000</td>
<td>1,068,190</td>
</tr>
<tr>
<td>([\text{Guanidino-}^{14}\text{C}])-Arginine</td>
<td>3,356,400</td>
<td>2,815,382</td>
</tr>
</tbody>
</table>

* The incorporation into protein is expressed as DPM/100 mg protein. For arginine, it is expressed as the DPM present in the arginine representing 100 mg protein. The arginine content was \(5.12 \pm 0.09\%\) (standard error of the mean, 6 determinations) of the weight of the protein. The protein content of the tissue was \(7.88 \pm 0.48\%\) (6).
the protein arginine. \(^{14}\text{C}\text{Urea was also incorporated into this carbon. The incorporation of the urea-C is presumed to take place after an initial degradation of the \(^{14}\text{C}\text{urea to }^{14}\text{C}\text{bicarbonate by urease. Both[U-}^{14}\text{C}-\text{ and [5-}^{15}\text{C}]ornithine contributed almost exclusively to carbons 1-5 of the arginine molecule. [Guanidino-}^{14}\text{C}\text{Arginine, used to check the isolation methods, was also incorporated into protein. Its labeling, following incorporation into protein, remained almost exclusively in the guanidino-C.}

An attempt was made to determine whether or not the requirement for protein arginine could be met by de novo synthesis in \text{O.lactea}. Each of two snails was injected with 40 uc (6 umoles) of sodium \(^{14}\text{C}\text{bicarbonate. The total wt of the snails was 19.4 g and the tissue wt was 7.4 g. Twelve hr after injection, the whole body free and protein amino acids were isolated and their specific activities (dpm/umole) were determined. Portions of the free amino acid fractions equivalent to 0.5 g tissue and of the protein hydrolysate representing 2 mg protein were analyzed. The results are represented in Table 2.}

It can be seen that \(^{14}\text{C}\text{bicarbonate was incorporated into both the free and protein arginine. The data obtained with this substrate were considered qualitative in nature because of the difficulty in estimating the actual specific activity of the injected \(^{14}\text{C}\text{bicarbonate. The bicarbonate content of \text{O.lactea} body fluids (blood or hemolymph) was analyzed by isotope dilution of known sodium \(^{14}\text{C}\text{bicarbonate. The specific activity of the diluted \(^{14}\text{C}\text{bicarbonate}}

Table 2—Synthesis of whole body amino acids from $[^14\text{C}]$ bicarbonate by *O. lactea*

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Specific Activity (DPM/umole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free</td>
</tr>
<tr>
<td>Arginine</td>
<td>15,771 (1.64)*</td>
</tr>
<tr>
<td>Aspartate</td>
<td>33,529 (0.57)</td>
</tr>
<tr>
<td>Glutamate</td>
<td>34,443 (1.58)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent umole/g tissue.
resulting from its addition to a known volume of body fluid was determined manometrically and by liquid scintillation counting. The values found varied from 12 to 31 umoles/ml. with different groups of snails. It is thus not possible to predict with any precision the endogenous levels of bicarbonate. The continuous formation and evolution of bicarbonate as metabolic carbon dioxide during the incubation period would also complicate the estimation of the specific activity of the $^{14}$C bicarbonate actually metabolized by the snails.

Although the rate of arginine synthesis in vivo could not be estimated from the amount of $^{14}$C bicarbonate incorporated into arginine, an indirect estimate of the quantitative importance of the synthesis could be obtained by a comparison of the final specific activity of the protein arginine with the specific activities of the protein glutamate and aspartate. This comparison was predicated on the work of Tamir & Ratner (1963 b) and Roberts et al (1963). With the chick, a species which requires dietary arginine because of its inability to synthesize this amino acid, Tamir & Ratner found that the specific activity of the protein arginine was negligible when compared with that of either the protein glutamate or aspartate following the prolonged administration of sodium $^{14}$C carbonate to whole animals. Roberts et al on the other hand, found that the specific activity of protein arginine was approximately twice that of protein
glutamate or protein aspartate in *E. coli*, a microorganism known to be capable of de novo arginine synthesis and which does not require dietary arginine for growth. In *O. lactea* the final specific activity of the protein arginine was also approximately twice that of the protein glutamate and aspartate. These latter two compounds are known to be synthesized by carbon dioxide fixation and transamination reactions in *O. lactea* (Awapara & Campbell, 1964). If the specific activities of the protein arginine, glutamate, and aspartate are corrected for the mole percentage composition of the protein of each of these amino acids, they are approximately equal. On a mole percentage basis, the glutamate and aspartate contents of the protein of *O. lactea* are 12.9 and 10.6%, respectively, or roughly twice that of arginine which is 5.3%. [*14C*]Glutamate or [*14C*]-aspartate incorporated into protein would be diluted by their [*12C*] analogs twice as much as would be [*14C*] arginine. If the specific activities of all three amino acids were equal prior to incorporation into protein, the final specific activity of the arginine after incorporation would be twice that of the glutamate and aspartate because of this dilution. Within the limits of the analytical methods, these are essentially the results obtained. Because of the differences in the metabolism of glutamate and aspartate on the one hand and arginine on the other, these results would be difficult to interpret in themselves. By comparison with the results obtained by Tamir & Ratner
(1963) with the chick and Roberts et al. (1963) with E. coli, they imply that the rate of arginine synthesis in vivo may be sufficient to satisfy the requirements for this amino acid by the protein-synthesizing system. The fact that even a partial requirement for protein arginine is being satisfied in the snail by biosynthesis also implies that the arginine pathway is functioning in its primitive nutritional capacity (Campbell, 1965).

In whole animal experiments, the contribution by each tissue to the overall metabolic pattern obtained is not known. Since only one or a few tissues might be involved in arginine synthesis, I examined the incorporation of $^{14}\text{C}$ bicarbonate into the free and protein arginine in several tissues of O. lactea. A total of 200 uc (1.1 umoles) of sodium $^{14}\text{C}$ bicarbonate was injected into 9 snails whose wt totaled 82.8 g (including shell). Twelve hr later, the individual tissues were dissected, pooled and the specific activities of the free and protein arginine determined. These data are presented in Table 3. Incorporation of $^{14}\text{C}$ bicarbonate into free arginine was detected only in lung and foot tissues. Protein arginine was labeled in all tissues including hemolymph. Further data from this type of experiment can be obtained and treated as in Table 4. Here again, most, if not all, tissues appear to be capable of arginine synthesis. An alternate explanation is that arginine is synthesized by one or a few tissues and is transported in the blood to
Table 3—Synthesis in vivo of free arginine and protein arginine, aspartate, and glutamate from $[^{14}C]$bicarbonate by *O. lactea* tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific Activity (DPM/umole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free Arg</td>
</tr>
<tr>
<td>Blood</td>
<td>--*</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>--</td>
</tr>
<tr>
<td>Kidney</td>
<td>--</td>
</tr>
<tr>
<td>Lung</td>
<td>971</td>
</tr>
<tr>
<td>Foot</td>
<td>2438</td>
</tr>
<tr>
<td>Reproductive tract</td>
<td>--</td>
</tr>
<tr>
<td>Albumen gland</td>
<td>--</td>
</tr>
</tbody>
</table>

*-- No radioactivity detected.*
Table 4—Incorporation in vivo of $^{14}$C bicarbonate into the protein arginine of *O. lactea* tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Incorporation*</th>
<th>Distribution of $^{14}$C</th>
<th>Guanidino-C</th>
<th>Carbons 1-5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPM in Protein</td>
<td>DPM in Arginine</td>
<td>of total</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>52,000</td>
<td>6,714</td>
<td>80.88</td>
<td>13.64</td>
</tr>
<tr>
<td>Shell</td>
<td>9,500</td>
<td>952</td>
<td>76.53</td>
<td>13.73</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>428,300</td>
<td>24,770</td>
<td>87.99</td>
<td>8.99</td>
</tr>
<tr>
<td>Kidney</td>
<td>399,000</td>
<td>6,262</td>
<td>81.69</td>
<td>11.72</td>
</tr>
<tr>
<td>Lung</td>
<td>116,500</td>
<td>15,909</td>
<td>87.46</td>
<td>6.83</td>
</tr>
<tr>
<td>Foot</td>
<td>172,000</td>
<td>32,681</td>
<td>87.10</td>
<td>6.84</td>
</tr>
<tr>
<td>Reproductive tract</td>
<td>240,000</td>
<td>12,000</td>
<td>62.89</td>
<td>10.73</td>
</tr>
<tr>
<td>Albumen gland</td>
<td>103,800</td>
<td>19,367</td>
<td>62.98</td>
<td>14.69</td>
</tr>
</tbody>
</table>

* The incorporation into protein is expressed as DPM/100 mg. For arginine, it is expressed as the DPM present in the arginine representing 100 mg protein. The arginine content, on a per wt basis as determined with the Sakaguchi reaction, was as follows: Blood protein, 4.44%; Shell protein, 1.10%; Hepatopancreas protein, 5.18%; Kidney protein, 1.53%; Lung protein, 3.93%; Foot protein, 5.54%; Reproductive tract protein, 5.44%; and Albumen gland protein, 2.86%.
the rest. Free $^{14}$Carginine was not detected in the blood but this might be due to a rapid utilization of any arginine appearing in the blood by the tissues. The data in Table 4 also show that most of the $^{14}$C bicarbonate was incorporated into the guanidino-C of the protein arginine in all of the tissues. Incorporation into shell protein was detected in this experiment because a larger amount of protein was analyzed (50 mg as opposed to 2 mg in Table 3). Once again, the final specific activity of the protein arginine in most of the tissues was equal to or higher than that of the protein glutamate or protein aspartate. Since these precursor studies indicated that arginine was being synthesized by the pathway depicted in Figure 1, experiments were undertaken to demonstrate the enzyme activities responsible for the conversion of ornithine and carbon dioxide into arginine and to determine the extent to which these enzymes are distributed in the tissues of the snail.

Enzyme Studies

Carbamyl phosphate synthesis: The enzymatic synthesis of carbamyl phosphate has not been demonstrated in O.lactea or any other terrestrial pulmonate. Based on the precursor studies already mentioned it seems reasonable to assume that there is some mechanism available for forming from bicarbonate a substrate which can be condensed with ornithine by ornithine transcarbamylase to form citrulline. Three types of enzymes are known which can perform this required reaction. These are:
1) Carbamyl kinase

\[
\text{ATP} + \text{NH}_2\text{COOH} + \text{Mg}^{2+} \xrightarrow{} \text{Carbamyl phosphate} + \text{ADP} \quad \text{down arrow} \quad \text{NH}_4^+ + \text{HCO}_3^-
\]

2) Vertebrate-type carbamyl phosphate synthetase

\[
2\text{ATP} + \text{NH}_4^+ + \text{HCO}_3^- \xrightarrow{\text{N-acetyl glutamate}} \frac{\text{Mg}^{2+}}{\text{CAP} + 2\text{ADP} + \text{P}_i}
\]

3) Bacterial-type carbamyl phosphate synthetase

\[
\text{Glutamine} \quad \xrightarrow{\text{Mg}^{2+}} \quad \frac{\text{NH}_4^+}{\text{HCO}_3^- + 2\text{ATP}} \xrightarrow{} \text{CAP} + 2\text{ADP} + \text{P}_i
\]

\(+ \text{glutamate if glutamine is used as donor}\)

Each of these groups of enzymes has special control mechanisms related to the pathway in which it is found.

Experiments in this laboratory over the past several years indicate that none of these enzymes can be demonstrated in \(0.\text{lactea}\) hepatopancreas tissue with the currently available tissue extraction and assay procedures. The possibility that an intermediate other than carbamyl phosphate is formed from bicarbonate and condensed with ornithine to form citrulline therefore cannot be ruled out at this time.

**Ornithine Transcarbamylase (EC 2.1.3.3)**

\[
\text{Ornithine} + \text{CAP} \xrightarrow{} \text{citrulline} + \text{P}_i
\]

Ornithine transcarbamylase activity was measured in various tissues of \(0.\text{lactea}\) and the data are presented in Table 5. It can be seen that all the tissues assayed possessed enzyme activity. The tissue activity found here for hepatopancreas
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific Activity (umole citrulline formed/mg protein per hr)</th>
<th>Tissue Activity (umole citrulline formed/g tissue per hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatopancreas</td>
<td>(6)* 2.06**</td>
<td>206.4**</td>
</tr>
<tr>
<td>Kidney</td>
<td>(15) 1.65</td>
<td>52.8</td>
</tr>
<tr>
<td>Lung</td>
<td>(9) 5.08</td>
<td>213.6</td>
</tr>
<tr>
<td>Foot</td>
<td>(6) 7.72</td>
<td>280.8</td>
</tr>
<tr>
<td>Reproductive tract</td>
<td>(6) 3.45</td>
<td>184.8</td>
</tr>
<tr>
<td>Columellar muscle</td>
<td>(6) 2.70</td>
<td>103.2</td>
</tr>
<tr>
<td>Albumen gland</td>
<td>(6) 0.72</td>
<td>139.2</td>
</tr>
<tr>
<td>Heart</td>
<td>(15) 11.88</td>
<td>417.6</td>
</tr>
<tr>
<td>Blood</td>
<td>(6) 0.17</td>
<td>2.9/ml</td>
</tr>
</tbody>
</table>

* Number of snails pooled for enzyme solution.
** The values given for specific activity and tissue activity have been corrected for a heat-inactivated control.
agrees very well with that found by Linton & Campbell (1962) of 217 umoles citrulline formed/g tissue per hr. The purpose for re-assaying this enzyme was to ascertain its distribution in the other tissues of the snail. It is present in all of the nine tissues assayed with the possible exception of blood. The activity found for blood is about the minimal amount detectable by this assay procedure. It has been suggested that this enzyme is mainly restricted to tissues of endodermal origin in vertebrates (Jones et al. 1961). The distribution of ornithine transcarbamylase in O. lactea indicates that this not true for this organism. The presence of this enzyme activity for condensing ornithine and carbamyl phosphate is an encouragement in the attempt to demonstrate carbamyl phosphate synthetase or something similar since the tendency in evolution is to delete unneeded or unused enzymes.

Argininosuccinate Synthetase (EC 6.3.4.5)

\[
\text{L-Citrulline} + \text{L-aspartate} + \text{ATP} \xrightarrow{\text{Mg}^{2+}} \text{argininosuccinate} + \text{AMP} + \text{PP}_i
\]

This enzyme activity had not previously been conclusively demonstrated in O. lactea although Linton & Campbell (1962), using an unspecific assay system had presented some evidence for its presence. The assay system used here contained \([\text{ureido}^{14}\text{C}]\) L-citrulline, ATP, L-aspartate and excesses of argininosuccinate lyase, arginase, and urease. The \([^{14}\text{C}]\) -carbon dioxide produced was trapped and counted by liquid scintillation spectrometry. The effect of incubation time
and enzyme concentration upon enzyme activity was a linear function. These data are presented in Figure 2A and 2B. Due to the extremely low enzyme activity present, a clearer definition of the requirements of the enzyme was sought. As can be seen from the data presented in Table 6, there is a definite dependence upon the presence of citrulline, ATP, magnesium ion, and enzyme. There is also some dependence upon the presence of L-aspartate as can be seen by the inhibition obtained in the presence of analogs of L-aspartate. It should be pointed out that the equivalent of 100 mg of tissue was used in each assay, therefore the endogenous levels of L-aspartate could be rather high. Guanosine triphosphate, inosine triphosphate, uridine triphosphate, and cytidine triphosphate could not be substituted for adenosine triphosphate. Due to the endogenous activities of argininosuccinate lyase, arginase, and urease, deletion of the exogenous enzymes did not reduce the activity to background. The tissue distribution of the argininosuccinate synthetase activity was determined and these data are presented in Table 7. As with ornithine transcarbamylase, all of the tissues tested had some activity. Once again, blood has the least activity.

Data from an experiment in which snail extract and an extract of rat liver, known to be capable of argininosuccinate synthesis from citrulline and aspartate (Ratner, 1962; Bohen & Brown, 1960), were mixed are presented in Table 8. It can be seen that the rat enzyme was approximately 75% inhibited by 1 ml of the snail enzyme solution. Passage
Figure 2

A. Effect of incubation time upon argininosuccinate synthetase activity in *O. lactea*

B. Effect of enzyme concentration upon argininosuccinate synthetase activity in *O. lactea*

Assay procedure described in Methods section
Table 6—Argininosuccinate synthetase activity in O. lactea hepatopancreas

<table>
<thead>
<tr>
<th>Assay system modification</th>
<th>Molecules argininosuccinate formed measured as [14C]O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete with heat-</td>
<td>5.20*</td>
</tr>
<tr>
<td>inactivated enzyme</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>16.80</td>
</tr>
<tr>
<td>Minus citrulline</td>
<td>0</td>
</tr>
<tr>
<td>Minus L-aspartate</td>
<td>12.90</td>
</tr>
<tr>
<td>Plus D-aspartate</td>
<td>16.40</td>
</tr>
<tr>
<td>Plus α-methyl aspartate</td>
<td>15.00</td>
</tr>
<tr>
<td>Plus β-methyl aspartate</td>
<td>15.90</td>
</tr>
<tr>
<td>Minus magnesium chloride</td>
<td>9.40</td>
</tr>
<tr>
<td>Minus PEP &amp; pyruvate kinase</td>
<td>15.60</td>
</tr>
<tr>
<td>Minus argininosuccinate lyase</td>
<td>8.80</td>
</tr>
<tr>
<td>Minus arginase</td>
<td>15.00</td>
</tr>
<tr>
<td>Minus urease</td>
<td>16.30</td>
</tr>
<tr>
<td>Minus ATP</td>
<td>7.80</td>
</tr>
<tr>
<td>Minus ATP, plus UTP</td>
<td>8.70</td>
</tr>
<tr>
<td>Minus ATP, plus CTP</td>
<td>9.50</td>
</tr>
<tr>
<td>Minus ATP, plus GTP</td>
<td>8.10</td>
</tr>
<tr>
<td>Minus ATP, plus ITP</td>
<td>8.70</td>
</tr>
<tr>
<td>Complete</td>
<td>12.60**</td>
</tr>
<tr>
<td>Minus L-asp, plus D-asp</td>
<td>9.40** 25.4% inhibition</td>
</tr>
<tr>
<td>Minus L-asp, plus α-methyl asp</td>
<td>7.90**      37.3% inhibition</td>
</tr>
<tr>
<td>Minus L-asp, plus β-methyl asp</td>
<td>7.60**      39.7% inhibition</td>
</tr>
</tbody>
</table>

The complete assay system contained in a 2 ml volume, in umoles: L-aspartate, 10; [ureido-14C] L-citrulline, 200,000 dpm/umole, 5; ATP, 2; magnesium chloride, 2; potassium phosphate, pH 7.5, 100; phosphoenolpyruvate, 2.5; pyruvate kinase, 6.6 IU; argininosuccinate lyase, 15 IU; arginase, 3.2 IU; urease, 4.6 IU; sodium bicarbonate, 10; and 1 ml of enzyme solution. Additions were in the following amounts, in umoles, to the reaction system: D-aspartate, 10; α-methyl aspartate, 10; β-methyl aspartate, 10; UTP, 2; CTP, 2; GTP, 2; ITP, 2. All systems were incubated for 60 min at 25° with shaking.

* The [ureido-14C] citrulline contained a small [14C] urea contaminant. These values have not been corrected for this contamination.

**Corrected for heat-inactivated control.
Table 7—Argininosuccinate synthetase activity in the tissues of *O.lactea*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific Activity (mumoles formed/mg protein per hour)</th>
<th>Tissue Activity (mumoles formed/g tissue per hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatopancreas</td>
<td>2.50*</td>
<td>126.0*</td>
</tr>
<tr>
<td>Foot</td>
<td>5.30</td>
<td>113.0</td>
</tr>
<tr>
<td>Lung</td>
<td>5.00</td>
<td>106.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>25.00</td>
<td>100.0</td>
</tr>
<tr>
<td>Albumen gland</td>
<td>0.70</td>
<td>91.0</td>
</tr>
<tr>
<td>Reproductive tract</td>
<td>3.10</td>
<td>72.0</td>
</tr>
<tr>
<td>Blood</td>
<td>9.90</td>
<td>26.0/ml</td>
</tr>
</tbody>
</table>

*O.lactea*, estiv., hepatopancreas

*O.vermiculata*, estiv., hepatopancreas

*H.aspersa*, feeding, hepatopancreas

* The values given for specific activity and tissue activity have been corrected for a heat-inactivated control.
<table>
<thead>
<tr>
<th>Assay Time (min)</th>
<th>Enzyme Source</th>
<th>Theoretical from mixture</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Snail</td>
<td>Snail(G-25) Rat Mixture (Rat + Snail)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>3.90</td>
<td>105.8</td>
<td>30.5</td>
</tr>
<tr>
<td>40</td>
<td>5.70</td>
<td>234.3</td>
<td>46.9</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>2.40</td>
<td>105.8</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
<td>2.20</td>
<td>234.3</td>
</tr>
</tbody>
</table>

The snail enzyme was prepared from reproductive tract (similar inhibition was also obtained with hepatopancreas) in the manner described in the Methods section. One-half of the preparation was passed through Sephadex G-25 which had been equilibrated with 0.1% cetyltrimethylammonium bromide and the protein fraction (absorbance at 280 μm) was collected by elution with 0.1% cetyltrimethylammonium bromide. The incubations were at 25° with shaking for the times indicated. One-tenth ml of rat liver enzyme solution (preparation described in Methods section) was used here after it had been found that this amount gave measurable activity and activity was linear with time. For the mixture, both 1.0 ml snail enzyme solution and 0.1 ml rat enzyme solution were used. One ml (equal to 100 mg tissue) of the snail enzyme solution was used. The values presented have been corrected for the heat-inactivated control. All values are the average of duplicates.
of the snail enzyme solution through Sephadex G-25 to remove small molecular weight compounds (<5000) actually increased the inhibition. It is assumed from this that the inhibitor of the rat enzyme has a molecular weight greater than 5000. Although the snail supernatant fluid inhibits the rat enzyme, this does not prove that the snail enzyme itself is inhibited.

The use of this assay procedure for argininosuccinate synthetase is, in itself, a specific one since it indicates that the product of the reaction is acted upon by argininosuccinate lyase to give arginine. Nevertheless, because of the low activity and the failure of the deletion experiments to reduce the activity to 0, it seemed necessary to show the direct formation of argininosuccinate as a reaction product. For this, the snail enzyme was incubated with either \(^{12}\text{C}\) citrulline and \(^{14}\text{C}\) aspartate or with \(^{12}\text{C}\) aspartate and \(^{14}\text{C}\) citrulline and the \(^{14}\text{C}\)-labeled product was isolated by ion exchange chromatography. Column chromatography on Dowex-1 separates citrulline, aspartate, and argininosuccinate as shown in Figures 3A, 3B, and 3C. The \(^{14}\text{C}\) aspartate and \(^{14}\text{C}\) citrulline contained no \(^{14}\text{C}\) contaminate which was eluted from the column in the position of argininosuccinate. Figure 3D presents the results obtained when the enzyme reaction product was subjected to ion exchange chromatography. It can be seen that from the reaction mixture containing the rat enzyme plus \(^{14}\text{C}\) citrulline and \(^{14}\text{C}\) aspartate a
Figure 3

A. Elution pattern of citrulline from Dowex-1-acetate

B. Elution pattern of aspartate from Dowex-1-acetate
Figure 3

C. Elution pattern of argininosuccinate from Dowex-1-acetate

D. Elution pattern of biosynthetic argininosuccinate from Dowex -1- acetate

- Rat enzyme with $[^{14}C]$ aspartate and $[^{14}C]$ citrulline
- Snail enzyme with $[^{14}C]$ citrulline
- Snail enzyme with $[^{14}C]$ aspartate
- Heat-inactivated snail enzyme with $[^{14}C]$ aspartate and $[^{14}C]$ citrulline

Assay procedure as described in Methods section
$[^{14}C]$-labeled peak was obtained in the same position as that of the standard $[^{14}C]$ argininosuccinate. The same was found for the snail incubation mixtures whether incubated with $[^{14}C]$ aspartate or $[^{14}C]$ citrulline. However, no $[^{14}C]$-peak was evident from the incubation mixture containing heat-inactivated snail enzyme in the presence of $[^{14}C]$-aspartate and $[^{14}C]$ citrulline, indicating that the peak was dependent upon native enzyme activity. Argininosuccinate may exist in three forms under various conditions (Westall, 1960; Ratner & Kunkemueller, 1966). Two of the three forms are probably ring compounds. The exact structures of the ring forms are still in doubt, however. Anhydride I displays the same chromatographic behavior on Dowex-1 as does citrulline under the conditions used here. It was therefore necessary to further analyze the initial "citrulline-peak" (Figure 3A) of the experimental situations for the presence of Anhydride I. This was done by chromatography on Amberlite CG-120 (pyridine$^+$ form). These results are presented in Table 9. It can be seen that no Anhydride I was formed. The incubation mixture containing the rat enzyme which synthesized the most argininosuccinate had very little radioactivity in the fraction which should have contained the Anhydride I. Also, from the snail enzyme incubations, the control containing heat-inactivated enzyme contained the most radioactivity indicating that no Anhydride I was formed. Even though an absolute dependence upon the presence of L-aspartate could not be demonstrated in the data of Table 6, it seems obvious that L-aspartate
Table 9—Synthesis and accumulation of argininosuccinate
by *H. aspersa*: the contribution of Anhydride I
to the total

<table>
<thead>
<tr>
<th>Enzyme Source and Substrates</th>
<th>DPM in front peak from Dowex column</th>
<th>DPM in Fraction 1 from Amberlite column</th>
<th>DPM in Fraction 2 from Amberlite column</th>
<th>% recovery of DPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail, [(^{14}\text{C})] aspartate</td>
<td>501,215</td>
<td>318,620</td>
<td>1,200</td>
<td>64</td>
</tr>
<tr>
<td>Snail, [(^{14}\text{C})] citrulline</td>
<td>31,275,385</td>
<td>24,844,000</td>
<td>20,160</td>
<td>80</td>
</tr>
<tr>
<td>Heat-inactivated Snail, [(^{14}\text{C})] aspartate and [(^{14}\text{C})] citrulline</td>
<td>32,257,279</td>
<td>11,363,600</td>
<td>31,670</td>
<td>35</td>
</tr>
<tr>
<td>Rat, [(^{14}\text{C})] aspartate and [(^{14}\text{C})] citrulline</td>
<td>30,357,329</td>
<td>25,000,000</td>
<td>14,112</td>
<td>82</td>
</tr>
<tr>
<td>[(^{14}\text{C})] citrulline*</td>
<td>-</td>
<td>426,250</td>
<td>36,600</td>
<td>100</td>
</tr>
<tr>
<td>Anhydride I**</td>
<td>-</td>
<td>1,500</td>
<td>14,691</td>
<td>-</td>
</tr>
</tbody>
</table>

* Known to have a [\(^{14}\text{C}\)] urea contaminant.
** Anhydride I was prepared from [\(^{14}\text{C}\)] argininosuccinate by making it 0.25M with respect to sulfuric acid and heating in a boiling water-bath for 1 hr.
is one of the substrates for the enzyme since a $^{14}$C-argininosuccinate peak was present after incubation of the snail enzyme with $^{14}$C-L-aspartate and unlabeled citrulline.

**Argininosuccinate Lyase** (EC 4.3.2.1)

\[
\text{L-Argininosuccinic acid} \rightarrow \text{L-arginine + fumarate}
\]

The activity of this enzyme in tissue extracts is usually determined by measuring the formation of urea from argininosuccinate in the presence of an excess of added arginase. This has been found convenient because argininosuccinate lyase activity is usually found in tissue extracts with measureable endogenous arginase activity and because the reaction products arginine and fumarate are inhibitory to the lyase activity (Ratner *et al.*, 1953 a). The assay system used here contained $^{14}$C-argininosuccinate and excesses of arginase and urease. This was done because of the simple and rapid methods available for measurement of $^{14}$C-carbon dioxide formation and because the tissue extracts tested possessed endogenous arginase and urease activities. Maximal argininosuccinate lyase activity, measured as $^{14}$C-carbon dioxide formation from guanidino-$^{14}$C-argininosuccinate was dependent upon the snail enzyme solution, added arginase and added urease. In all assays, the results were corrected for the possible non-enzymatic breakdown of $^{14}$C-argininosuccinate and the trace amounts of guanidino-$^{14}$C-arginine present in the substrate. The effect of enzyme concentration and incubation time upon
the formation of $[^{14}C]$ carbon dioxide from $[^{14}C]$ argininosuccinate is presented in Figures 4A and 4B. Several tissues of *O. lactea* were assayed for argininosuccinate lyase activity and, again, activity was found in all eight of the tissues tested. Blood had no measureable activity as can be seen in Table 10. Table 11 presents data indicating that the *O. lactea* enzyme is also inhibited by fumarate accumulation as has been described for the vertebrate enzyme. The snail enzyme was actually slightly more inhibited than the beef enzyme in the only case tested. Table 12 presents data on the effects of ammonium ion concentration on argininosuccinate lyase activity which indicates that at high molarities (0.15M or greater) the snail enzyme behaves in a manner similar to the beef enzyme. No inhibition of the snail enzyme was apparent at molarities below 0.1.

The demonstration of ornithine transcarbamylase, argininosuccinate synthetase, and argininosuccinate lyase in all of the tissues of *O. lactea*, with the possible exception of blood, and the demonstration that specifically $[^{14}C]$ labeled precursors gave rise to arginine labeled in the expected positions indicate that arginine is normally synthesized by this land snail. It seems possible that this synthesis may take place in most of the tissues in contrast to vertebrates where the synthesis is mainly restricted to the liver. Also, the fact that the specific activity of the protein arginine was equal to or greater than the specific activities of protein aspartate and protein
Figure 4

A. Effect of enzyme concentration upon argininosuccinate lyase activity in *O. lactea*
   Assay procedure in Methods section

B. Effect of incubation time upon argininosuccinate lyase activity in *O. lactea*
   Assay procedure in Methods section
Table 10—Argininosuccinate lyase activity in tissues of *O. lactea*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific Activity (umoles/mg protein per hr)</th>
<th>Tissue Activity (umoles/g tissue per hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatopancreas</td>
<td>0.058*</td>
<td>8.40*</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.082</td>
<td>16.80</td>
</tr>
<tr>
<td>Lung</td>
<td>0.061</td>
<td>7.80</td>
</tr>
<tr>
<td>Foot</td>
<td>0.066</td>
<td>7.50</td>
</tr>
<tr>
<td>Reproductive tract</td>
<td>0.061</td>
<td>9.60</td>
</tr>
<tr>
<td>Columellar muscle</td>
<td>0.046</td>
<td>8.10</td>
</tr>
<tr>
<td>Albumen gland</td>
<td>0.052</td>
<td>10.30</td>
</tr>
<tr>
<td>Heart</td>
<td>0.081</td>
<td>10.60</td>
</tr>
<tr>
<td>Blood</td>
<td>trace?</td>
<td>trace?</td>
</tr>
</tbody>
</table>

* The values given for specific activity and tissue activity have been corrected for a non-enzymatic control. The assay conditions are as described in the Methods section.
<table>
<thead>
<tr>
<th></th>
<th>Fumarate* added (umoles)</th>
<th>argininosuccinate fumarate</th>
<th>% inhibition</th>
<th>[^{14}C]O(_2) formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail Enzyme</td>
<td>0</td>
<td>--</td>
<td>0.090</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>0.088</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>2</td>
<td>0.083</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td>0.068</td>
<td>24.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.5</td>
<td>0.056</td>
<td>37.8</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.2</td>
<td>0.030</td>
<td>66.7</td>
</tr>
<tr>
<td>Beef Enzyme</td>
<td>0</td>
<td>--</td>
<td>3.678</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0.2</td>
<td>1.677</td>
<td>54.4</td>
</tr>
</tbody>
</table>

Incubations were for 30 min at 25\(^\circ\). The enzyme solution was 0.2 ml of a 10\% homogenate. The reaction mixture and manipulations were the same as those described in the Methods section.

* Adjusted to pH 7.5 with KOH.
<table>
<thead>
<tr>
<th>umole salt added</th>
<th>Final molarity</th>
<th>umoles $[^{14}C]O_2$ formed</th>
<th>% inhibition (compared to $K_2SO_4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$(NH_4)_2SO_4$</td>
<td>$K_2SO_4$</td>
</tr>
<tr>
<td>Snail Enz. 0</td>
<td>--</td>
<td>0.093</td>
<td>0.102</td>
</tr>
<tr>
<td>10</td>
<td>0.01</td>
<td>0.065</td>
<td>0.086</td>
</tr>
<tr>
<td>50</td>
<td>0.05</td>
<td>0.078</td>
<td>0.078</td>
</tr>
<tr>
<td>100</td>
<td>0.10</td>
<td>0.079</td>
<td>0.068</td>
</tr>
<tr>
<td>150</td>
<td>0.15</td>
<td>0.058</td>
<td>0.081</td>
</tr>
<tr>
<td>Beef Enz. 150</td>
<td>0.15</td>
<td>2.830</td>
<td>3.678</td>
</tr>
</tbody>
</table>

Incubations were for 30 min at 25°. The enzyme solution was 0.2 ml of a 10% homogenate. The reaction mixture and manipulations were the same as those described in the Methods section.
glutamate, amino acids not normally required in the diets of animals, indicates that biosynthetic arginine is used, at least in part, to meet the requirements of protein synthesis.

**Arginine Degradation**

**Enzyme Studies**

There are a number of pathways by which arginine may be degraded. One degradative mechanism is the arginine dihydrolase pathway found in some bacteria (Cohen & Brown, 1960) and several Mycoplasmas (Schimke & Barile, 1966) possessing carbamate kinase. This pathway is concerned with the production of energy by means of the following reactions:

1) Arginine + H$_2$O $\xrightarrow{\text{arginine desiminase}}$ citrulline + NH$_3$

2) Citrulline + P$_1$ $\xrightarrow{\text{OTC}}$ ornithine + carbamyl phosphate

3) Carbamyl phosphate + ADP $\xrightarrow{\text{carbamate kinase}}$ ATP + CO$_2$ + NH$_3$

This system has not been detected in any higher organisms. Another degradative pathway involves the oxidation of L-arginine by the following reactions:

1) Arginine $\xrightarrow{\text{O}_2}$ $\gamma$-guanidobutyramide + CO$_2$

2) $\gamma$-Guanidobutyramide $\xrightarrow{\text{H}_2\text{O}}$ $\gamma$-guanidobutyrate + NH$_3$

3) $\gamma$-Guanidobutyrate $\xrightarrow{\text{H}_2\text{O}}$ $\gamma$-aminobutyrate + urea
This pathway was first formulated by Thoai (1965) for *Streptomyces griseus*. Thoai *et al* (1957) demonstrated arginine oxygenase (Reaction 1) in the snail *L. stagnalis* and Baret *et al* (1965) have shown γ-guanidobutyrate-ureohydrolase (Reaction 3) to be present in several molluscs including *H. pomatia*, *H. aspersa*, and *O. lactea*. Extracts of *O. lactea* hepatopancreas hydrolyze γ-guanidobutyrate to urea at a rate 0.33 times that of L-arginine at pH 9.5 and 0.22 times at pH 7.5. This degradative system may thus be present in *O. lactea*. The third catabolic pathway of arginine which must be considered involves the enzymes arginase, urease and ω-ornithine transaminase which catalyze the following reactions:

1) L-Arginine + H₂O → L-ornithine + urea
2) Urea + H₂O → 2NH₃ + CO₂
3) L-Ornithine + α-ketoglutarate → Glutamic-γ-semialdehyde + glutamic acid

The complete catabolic sequence as depicted above is known with certainty in only a few species such as the bread mold, *Neurospora crassa* (Davis, 1967; Fowden, 1965); the Jack bean, *Canavalia ensiformis* (Fowden, 1965); and the bacterium *Bacillus licheniformis* (Ramaley & Bernlohr, 1966). It is probable that many other species also possess the complete pathway. Several organisms use arginase to supply ornithine by degrading arginine. The ornithine serves as a precursor for either proline or glutamate via the ornithine transaminase reaction. This is the case for Chang's liver cells.
(Eliasson & Strecker, 1966; Strecker & Eliasson, 1966) which lack ornithine transcarbamylase. The same situation is found in sporulating B. licheniformis (Ramaley & Bernlohr, 1966). Protein is catabolized to the constituent amino acids. Arginine is catabolized further to ornithine which is then converted to proline or glutamate. The proline or glutamate may subsequently be converted to a citric acid cycle intermediate. The ultimate result is therefore, energy production. There are also a number of species, primarily ureotelic vertebrates, in which urea is the primary product formed. This urea is either excreted as such as the major nitrogenous waste or retained to serve in osmotic regulation. Although arginase is found in some microorganisms, urease seems to be the one of the two enzymes which is considered common among microorganisms. Evidence follows which indicates that the complete system, i.e. arginase, urease, and ornithine transaminase, is present in O. lactea.

Arginase (EC 3.5.3.1)

\[
\text{L-arginine} \xrightarrow{\text{H}_2\text{O}} ^{\text{Mn}^{2+}} \text{L-ornithine} + \text{urea}
\]

This enzyme has been measured and characterized in O. lactea by Linton & Campbell (1962) and by Campbell (1966). The tissue distribution of the enzyme was ascertained and is presented in Table 13. As can be seen, all tissues possessed activity although the levels in the blood and columnellar muscle were near the lower limit of detection. It is interesting that foot tissue, which has the highest arginase
Table 13—Arginase activity in the tissues of *O. lactea*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific Activity (umole/mg protein per hr)</th>
<th>Tissue Activity (umole/g tissue per hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatopancreas</td>
<td>19.80*</td>
<td>2455.2*</td>
</tr>
<tr>
<td>Kidney</td>
<td>12.10</td>
<td>1018.8</td>
</tr>
<tr>
<td>Lung</td>
<td>18.32</td>
<td>1425.6</td>
</tr>
<tr>
<td>Foot</td>
<td>144.00</td>
<td>6048.0</td>
</tr>
<tr>
<td>Reproductive tract</td>
<td>9.00</td>
<td>990.0</td>
</tr>
<tr>
<td>Columellar muscle</td>
<td>0.17</td>
<td>19.2</td>
</tr>
<tr>
<td>Albumen gland</td>
<td>3.86</td>
<td>738.0</td>
</tr>
<tr>
<td>Heart</td>
<td>12.06</td>
<td>943.2</td>
</tr>
<tr>
<td>Blood</td>
<td>&lt;0.11</td>
<td>&lt;14.4/ml</td>
</tr>
</tbody>
</table>

Incubations were at $25^\circ$. The reaction mixture and manipulations were as described in the Methods section. * The values given for specific activity and tissue activity have been corrected for a heat-inactivated control.
activity measured in vitro, should be one of the two tissues in which free $^{14}\text{C}$ arginine was found after incubation with $^{14}\text{C}$ bicarbonate (Table 3).

**Urease** (EC 3.5.1.5)

$$\text{Urea} \xrightarrow{\text{H}_{2}\text{O}} 2\text{NH}_{3} + \text{CO}_{2}$$

This enzyme has been previously reported in *H. pomatia* (Heidermanns & Kirchner-Kühn, 1952) and *O. lactea* (Linton & Campbell, 1962). The snail enzyme can be distinguished from the plant enzyme and most bacterial enzymes because of its alkaline pH optimum at pH 8.8-9.0. Table 14 presents the tissue distribution of urease in *O. lactea*. While all tissues had activity, the value for blood was near the lower limit of detection. To further demonstrate the presence of the snail urease, bacteria possessing urease activity were isolated from sterile-water homogenates of whole snails. Only three such bacteria were found. These were identified by Miss Betty W. Patterson as *Proteus vulgaris*, *Staphylococcus aureus*, and *Corynebacterium* sp. Each of these species was grown in triptcose soy broth and harvested by centrifuging. The cells were washed twice with 5 mM ethylenediaminetetraacetate (disodium salt) and then homogenized with glass beads in 9 volumes of 5 mM ethylenediaminetetraacetate. Optimal conditions of substrate and enzyme concentration and reaction time were determined in preliminary experiments. The effect of pH upon enzyme activity was then determined with the extract of each species using Tris-maleate buffer. A comparison of the pH optima of *O. lactea* and *Staph. aureus*
Table 14--Urease activity in the tissues of *O. lactea*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Specific Activity (umole/mg protein per hr)</th>
<th>Tissue Activity (umole/g tissue per hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatopancreas</td>
<td>0.32*</td>
<td>20.8*</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.47</td>
<td>12.4</td>
</tr>
<tr>
<td>Lung</td>
<td>0.40</td>
<td>17.6</td>
</tr>
<tr>
<td>Foot</td>
<td>0.34</td>
<td>10.8</td>
</tr>
<tr>
<td>Reproductive tract</td>
<td>0.33</td>
<td>16.0</td>
</tr>
<tr>
<td>Columellar muscle</td>
<td>0.03</td>
<td>1.2</td>
</tr>
<tr>
<td>Albumen gland</td>
<td>0.04</td>
<td>4.4</td>
</tr>
<tr>
<td>Heart</td>
<td>0.07</td>
<td>2.4</td>
</tr>
<tr>
<td>Blood</td>
<td>&lt; 0.003</td>
<td>&lt; 0.04/ml</td>
</tr>
</tbody>
</table>

Incubations were at 25°. The reaction mixture and manipulations were as described in the Methods section. * The values given for specific activity and tissue activity have been corrected for a non-enzymatic control.
ureases is presented in Figure 5. These two enzymes can
be distinguished by their respective pH optimum alone or
in mixture. The pH-activity curve for the mixture is
qualitatively identical to the theoretical curve calculated
from the individual activities. There was, however, less-
than-theoretical recovery of the two activities in the
mixture. This could possibly have been due, among other
things, to proteolytic activity in the bacterial homogenate.
The pH-activity curve obtained for Corynebacterium sp was
similar to that depicted for Staph. aureus and showed
optimal enzyme activity near pH 6.5. The pH-activity curve
for Proteus vulgaris showed a broad optimum around pH 7.5.
Hase & Kobashi (1967) reported that Proteus vulgaris OX19
had a broad urease pH optimum between pH 7.7-8.9 in Tris-
sulfate buffer. Their methods, unfortunately, are not
fully described. Another possibility for the discrepancy
may be that, as pointed out by Guo & Liu (1965), the various
members of the Proteus genus are not readily distinguishable.
Our P. vulgaris was identified primarily on biochemical
reactions and the possibility that there may be differences
between strains or mutants, if indeed it is the correct
species, must be kept in mind.

\[ \text{-Ornithine Transaminase (EC 2.6.1.13)} \]

\[ L-\text{Ornithine} + \alpha-\text{ketoglutarate} \rightarrow \text{glutamic-}\gamma-\text{semialdehyde} + L-\text{glutamate} \]

Ornithine does not take part in many metabolic inter-
conversions. When \( \delta \)-ornithine transaminase is present,
pH optima of *O. lactea* and *Staph. aureus* ureases

For the individual pH-activity curves, the amount of protein in the snail extract was 2.48 mg and in the bacterial extract, 0.38 mg; for the combined pH-activity curve, the enzyme source contained 1.24 mg snail protein and 0.19 mg bacterial protein. The theoretical curve for the mixed ureases was calculated from the individual activities at each pH.
the ornithine is a precursor of proline and glutamate.
Either of these compounds has the potential for energy
production because they are precursors of α-ketoglutarate,
an intermediate in the citric acid cycle. As can be seen
in Table 15 activity was found in 0. lactea hepatopancreas
when either the formation of glutamate or glutamic-γ-
semialdehyde was measured.

Precursor Studies

In studying the degradation of ornithine it is necessary
to demonstrate not only that there is a potential for
arginine degradation embodied in the presence of arginase
and urease but that this potential is actually expressed.
The degradative pathway has been studied in whole tissues
in vitro and in intact snails in vivo.

Degradation of [guanidino-\(^{14}\)C]L-arginine and \([^{14}\text{C}]\)urea by whole tissues in vitro

Both arginase and urease in 0. lactea tissues function
to degrade exogenous arginine and urea to bicarbonate. This
is shown by the data in Table 16. The rate of urea breakdown
is only slightly higher than that of arginine in lung and
hepatopancreas. This would indicate that urease is the
rate-limiting step in arginine degradation in these tissues.
It would also indicate that the great excess of arginase
activity measured in vitro as compared with urease in both
tissues is functional. However, foot tissue differs from
lung and hepatopancreas in degrading urea almost twice as
fast as arginine by the combined arginase-urease system
<table>
<thead>
<tr>
<th>Modification of Assay Conditions</th>
<th>Enzyme (ml)</th>
<th>Incubation Time (min)</th>
<th>Product Formed (umole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>As glutamate formation:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.0</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>None</td>
<td>1.0</td>
<td>30</td>
<td>1.89</td>
</tr>
<tr>
<td>None</td>
<td>1.0</td>
<td>60</td>
<td>3.49</td>
</tr>
<tr>
<td>None</td>
<td>1.0</td>
<td>60</td>
<td>2.56</td>
</tr>
<tr>
<td>Minus ornithine</td>
<td>1.0</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>Minus α-ketoglutarate</td>
<td>1.0</td>
<td>60</td>
<td>0.21</td>
</tr>
<tr>
<td>Minus enzyme</td>
<td>0</td>
<td>60</td>
<td>0.17</td>
</tr>
<tr>
<td>Minus pyridoxal phosphate</td>
<td>1.0</td>
<td>60</td>
<td>2.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>As glutamic acid-γ-semialdehyde formation:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.6</td>
<td>30</td>
<td>0.60</td>
</tr>
<tr>
<td>None</td>
<td>0.6</td>
<td>15</td>
<td>0.30</td>
</tr>
<tr>
<td>None</td>
<td>0.3</td>
<td>30</td>
<td>0.31</td>
</tr>
<tr>
<td>Plus 0.25 umole pyridoxal phosphate</td>
<td>0.6</td>
<td>30</td>
<td>0.71</td>
</tr>
<tr>
<td>Minus ornithine</td>
<td>0.6</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>Minus α-ketoglutarate</td>
<td>0.6</td>
<td>30</td>
<td>0.27</td>
</tr>
<tr>
<td>Minus enzyme</td>
<td>0</td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 16--Rate of degradation of $[^{14}C]$ urea and $[^{14}C]$ guanidinoarginine by \textit{O. lactea} tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Substrate</th>
<th>Rate of degradation as \text{umole}[^{14}C]O_2/100 \text{ mg tissue per hour}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$[^{14}C]$ urea</td>
<td>$[^{14}C]$ guanidinoarginine</td>
</tr>
<tr>
<td>Lung:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) feeding</td>
<td>0.83 ± 0.14</td>
<td>0.79 ± 0.10</td>
</tr>
<tr>
<td>b) estivating (20 mo)</td>
<td>0.64 ± 0.15</td>
<td>0.57 ± 0.05</td>
</tr>
<tr>
<td>Hepatopancreas:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) feeding</td>
<td>0.41 ± 0.05</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>b) estivating (2 mo)</td>
<td>0.71 ± 0.08</td>
<td>0.50 ± 0.07</td>
</tr>
<tr>
<td>c) estivating (10 mo)</td>
<td>0.30 ± 0.04</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>Foot:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) feeding</td>
<td>0.30 ± 0.04</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>b) estivating (10 mo)</td>
<td>0.32 ± 0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Albumen gland:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) feeding</td>
<td>0.20 ± 0.05</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>b) estivating (10 mo)</td>
<td>0.43 ± 0.02</td>
<td>0.16 ± 0.05</td>
</tr>
</tbody>
</table>

Fifty or 100 mg of each tissue, pooled from several individuals, were incubated at 20, 40, and 60 min intervals with either 5 umoles of $[^{14}C]$ urea or $[^{14}C]$ guanidinoarginine in 2 ml Chiarandini's saline (Chiarandini, 1964; modified by omitting NaHCO$_3$ and adding 5 mM glycylglycine. The final pH was 7.6). The reaction was stopped with 1 ml of 5N H$_2$SO$_4$ and the $[^{14}C]$O$_2$ liberated was trapped and counted as described in the Methods section. The values given represent the average ± the standard deviation (18 determinations for each value).
even though foot contains a higher arginase activity than lung or hepatopancreas. The same situation is found in albumen gland from 10-month estivating snails although here it might be expected since there is much less arginase activity than in lung or hepatopancreas. This indicates that the level of urease activity may not be the controlling factor in the rate of arginine degradation in these tissues. Moreover, the relative rates of urea degradation in the tissues from 10-month estivating snails did not correlate with the levels of activity found in homogenates. The reason for this is not apparent.

Degradation of [guanidino-\(^{14}\text{C}\)]L-arginine and [\(^{14}\text{C}\)]urea by whole snails in vivo

Degradation of labeled arginine and labeled urea was also found to occur in whole snails. As seen in Figure 6, this degradation could be determined by the formation and volatilization of [\(^{14}\text{C}\)]carbon dioxide after the injection of [guanidino-\(^{14}\text{C}\)]L-arginine or [\(^{14}\text{C}\)]urea through a hole in the shell. The rate of degradation of urea was faster than that of arginine as predicted by the whole tissue experiments. It should be noted that the percent of the [\(^{14}\text{C}\)] from each [\(^{14}\text{C}\)] substrate injected, such as [\(^{14}\text{C}\)] urea, that is evolved as metabolic [\(^{14}\text{C}\)] carbon dioxide varies a great deal. This variation may be partly due to the site of injection. Because the snails are quite mobile when withdrawn into their shells, it was difficult to inject them uniformly. By paraffin-coating various parts of the snail after injection of [\(^{14}\text{C}\)] urea it was possible to determine the route (s) of
Figure 6

Metabolism of $[^{14}C]$urea and $[^{14}C]$-guanidino-arginine to $[^{14}C]O_2$ by *O. lactea*

Thirty individuals were each injected with 5 umoles each substrate. The vertical bars represent the standard deviation from the mean.
exit of $[^{14}C]$ carbon dioxide. The data of Table 17 indicate
the primary route of exit is the aperture. The $[^{14}C]$ carbon
dioxide probably arises from the lung surface since it
is through the aperture that the lung communicates with the
exterior. A significant percentage of the $[^{14}C]$ carbon dioxide
exits through the shell indicating that the shell is not
an impermeable barrier to gases.

Urea synthesis

An attempt was made to study the actual synthesis of
urea in O.lactea. This was done by incubating whole
tissue in the presence of sodium $[^{14}C]$ bicarbonate, $[^{14}C]$ guanidino-
$[^{14}C]$ arginine, or $[^{14}C]$ ureido-$[^{14}C]$ citrulline with and without the
urease inhibitor, acetohydroxamic acid. In these experiments
the amount of metabolic $[^{14}C]$ carbon dioxide produced, except
in the case of $[^{14}C]$ bicarbonate, was determined. The $[^{14}C]$-
urea and $[^{14}C]$ arginine present was determined enzymatically
with urease or urease and arginase. The $[^{14}C]$ carbon dioxide
evolved by these enzymes was determined. These results
are presented in Table 18. It is evident that free pool
arginine was synthesized from both $[^{14}C]$ bicarbonate and
$[^{14}C]$ citrulline in lung and hepatopancreas tissue (Column D).
It is also clear that the arginine synthesized was being
degraded as evidenced by the fact that $[^{14}C]$ urea was present
and $[^{14}C]$ carbon dioxide was produced. The urease inhibitor
acetohydroxamic acid worked as expected in lung tissue
giving 140% $[^{14}C]$ urea from $[^{14}C]$ bicarbonate, 180% $[^{14}C]$ urea
from $[^{14}C]$ citrulline and 170% $[^{14}C]$ urea from exogenous
Table 17--Effect of paraffin-coating of estivating *O. lactea* on $^{14}$C$O_2$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of snails</th>
<th>% Injected $^{14}$C urea evolved as $^{14}$C$O_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 hr</td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>7.7</td>
</tr>
<tr>
<td>Entire snail</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>coated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epiphragm coated</td>
<td>6</td>
<td>3.7</td>
</tr>
<tr>
<td>Shell coated</td>
<td>6</td>
<td>11.8</td>
</tr>
</tbody>
</table>

Each snail was injected with 5 umoles $^{14}$C urea. Three snails were used per chamber and the evolved $^{14}$C$O_2$ was trapped and counted as described in the Methods section. The snails were paraffin-coated as indicated.
Table 18--Synthesis and degradation of urea from endogenous and exogenous arginine by O.lactea tissues

<table>
<thead>
<tr>
<th>Substrate</th>
<th>mmoles/g lung/hr</th>
<th>mmoles/g hepatopancreas/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[14C]O₂</td>
<td>B Urea</td>
</tr>
<tr>
<td>[14C] Bicarbonate:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Experimental</td>
<td>ND*</td>
<td>0.99</td>
</tr>
<tr>
<td>2) Experimental plus AHA**</td>
<td>ND</td>
<td>1.39</td>
</tr>
<tr>
<td>[Ureido-14C] Citrulline:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Heat-killed</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2) Experimental</td>
<td>18.67</td>
<td>62.67</td>
</tr>
<tr>
<td>3) Experimental plus AHA</td>
<td>0</td>
<td>114.67</td>
</tr>
<tr>
<td>[Guanidino-14C] Arginine: ***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Heat-killed</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2) Experimental</td>
<td>9965</td>
<td>14,574</td>
</tr>
<tr>
<td>3) Experimental plus AHA</td>
<td>0</td>
<td>25,337</td>
</tr>
</tbody>
</table>

* ND= Not determined
**AHA: = acetohydroxamic acid, flasks containing inhibitor were incubated with the tissue for 15 min at 25° before the addition of substrate. Ten umoles of AHA were used in each of the flasks with inhibitor.
***All values were corrected for the endogenous arginine in the free pool-hepatopancreas:
0.4 umole/g tissue; lung: 0.05 umole/g tissue.
Fifty or 100 mg of each tissue, pooled from several individuals, were incubated for 90 min at 25° with one of the following: NaH14CO3, 25 uc/2.6 umoles; [ureido-14C] citrulline, 7.46 uc/4.8 umole; or [guanidino-14C] arginine, 4 uc/2 umoles in 2 ml Chiarandini's saline (Chiarandini, 1964; modified by omitting NaHCO3 and adding 5 mM glycylglycine. The final pH was 7.6) The reaction was stopped with 1 ml of 5N H2SO4 and the CO2 liberated was trapped and counted. Each value represents the average of duplicates.
[guanidino-14C] arginine. There was a 280% increase in [14C] urea in hepatopancreas from exogenous [guanidino-14C] arginine. The inhibitor did not, however, produce the expected results from endogenously synthesized arginine (from either [14C] bicarbonate or [ureido-14C] citrulline) in hepatopancreas. The explanation for this is not obvious at this time. The data in Column B indicate once again that urease is the rate-limiting step in arginine degradation in lung and hepatopancreas tissue.

It is possible to calculate a rate of arginine synthesis from citrulling in the whole tissues used in this experiment. The rate for whole lung tissue is 0.574 umole/g tissue per hr. The rate for whole hepatopancreas is 0.221 umole/g tissue per hr. It is somewhat surprising that in both tissues the rate measured here is faster than the rate of argininosuccinate synthesis in vitro (lung: 0.106 umole argininosuccinate synthesized/g tissue per hr; Hepatopancreas: 0.126 umole argininosuccinate synthesized/g tissue/per hr).

Table 18 also indicates that extremely small amounts of arginine and urea are formed. Such small amounts can be measured only with [14C]-labeled precursors. It is not surprising then that Baldwin & Needham (1934) failed to detect urea formation from ammonium and bicarbonate ions by H. pomatia hepatopancreas. They were limited in three ways: 1) very little urea is synthesized by the tissue in vitro, 2) their methods of detection were crude, and 3) the small amount of urea formed under their conditions was probably degraded by the tissue urease.
As stated, the ornithine formed by the arginase reaction could either be recycled for arginine synthesis or possibly be converted to proline or glutamate. We might now turn our attention to the products of the urease reaction. The carbon dioxide formed may either leave the snail or remain as a part of the carbon dioxide-bicarbonate pool. It may now be asked what happens to the ammonia formed from the urease reaction.

**Ammonia Formation and Evolution**

Formation of ammonia is a characteristic of most organisms but the excretion of ammonia, as the ammonium ion, as a primary nitrogenous waste is characteristic mainly of aquatic organisms. The route of ammonium ion elimination is often extrarenal. Some organisms which are not strictly aquatic but which live in moist environments also excrete appreciable quantities of the ammonium ion. Some terrestrial organisms retain the ability to excrete ammonia but it must be voided as the ammonium ion with a rather large quantity of water. This function is performed by renal mechanisms in the formation of the urine. There are only a few reports of extrarenal excretion of ammonia by terrestrial inhabitants indicating that it is not a widely distributed physiological mechanism. Dresel & Moyle (1950) reported that terrestrial isopods eliminate 10-30% of their ammonia-N in the gaseous form, whether this was evolved directly from the body surfaces
or from the excreta is not clear in their report. Clark (1953) has reported that black snake embryos also excrete gaseous ammonia. The guano bat (Studier, 1966) apparently also can void gaseous ammonia extrarenally. The observation that *O. lactea* could produce ammonia gas was therefore of interest because it might represent a significant extrarenal mechanism for voiding nitrogenous wastes in a terrestrial organism considered to be extremely committed to uricotelic nitrogen excretion although capable of urea synthesis and degradation.

The production of ammonia gas by *O. lactea* was initially identified by the odor of the snails and by the observation that litmus paper placed in a closed chamber with estivating snails gave an alkaline reaction. After trapping in dilute sulfuric acid, the ammonia produced by the snails was identified by its reaction with Nessler's reagent and p-nitrobensenediazonium chloride (Feigl, 1954). These two reagents are generally considered specific for ammonia and other ammonia derivatives tested with them under the same conditions did not give the characteristic color reaction. These derivatives included methylamine, ethylamine, diethylamine, phenylamine, ethanolamine, and hydroxylamine.

The formation of ammonia by estivating *O. lactea* and *H. aspersa* over a 42 hr period is shown in Figure 7. There was considerable individual variation in the amount of ammonia produced which was not correlated with several wt
Figure 7

Ammonia production by estivating *O. lactea* and *H. aspersa*

Each point represents the average ammonia produced by 5 individual *O. lactea* or 6 individual *H. aspersa*. The vertical bars represent standard deviation from the mean; only representative standard deviations are given for *O. lactea*. Measurements were made at room temperature (22°-24°). The tissue wt was determined at the end of the experiment.
parameters such as total wt (including shell), tissue wt (minus shell), dry tissue wt, or shell wt. The total and tissue wt are quite variable in themselves because of the extensive fluctuations in the water contents of these snails (Howes & Wells, 1934; Burton, 1964, 1965). An average rate of ammonia production was calculated for 50 individual *O. lactea* whose ammonia evolution was monitored for 25-35 hr periods. This rate was $0.024 \pm 0.002$ (standard error of the mean) umole ammonia/g tissue per hr. This corresponds to about 0.1 umole/hr in a 11-12 g snail. For 10 individual *H. aspersa*, also monitored for 25-35 hr periods, the rate was $0.013 \pm 0.002$ umole ammonia/g tissue per hr. This corresponds to about 0.06 umole/hr for an 8-9 g snail. These average rates of ammonia formation would account for the elimination of about 9.6 mg ammonia-N/kg tissue per day by *O. lactea* and 5.5 mg ammonia-N/kg tissue per day by *H. aspersa*. Obviously these rates are subject to a great deal of variation since nitrogen excretion itself is a variable physiological trait. This amount of ammonia-N does, however, seem to be significant when compared with the amount of ammonia-N eliminated by other molluscs (Potts, 1967). Exact figures on the total-N excreted by terrestrial molluscs are not available. Essentially all of the data for gaseous ammonia excretion by *O. lactea* and *H. aspersa* were obtained from estivating snails. Data could not be obtained for active snails except for short initial periods because the feces voided by these snails is a rich source of ammonia and bacterial contamination. The mucous
secreted during movement by these snails also contains ammonia. Estivating snails, however, do not excrete and normally seal themselves off from the environment during estivation by secreting an epiphramg over the opening of the shell—the shell aperture. It is through the shell aperture that the respiratory chamber or lung communicates with the external environment. It was initially assumed that ammonia was evolved from the lung as it is in mammals (Jacquez et al., 1959; Robin et al., 1959). However, the data shown in Table 19 indicate that the major surface from which ammonia is evolved is the shell. When compared with uncoated controls, paraffin-coating the shells decreased the ammonia evolution by over 90%. A much smaller percentage of ammonia exited through the epiphramg than through the shell. This was quite different from carbon dioxide evolution (Table 17).

Sources of Ammonia

Since paraffin-coating prohibited the escape of ammonia, this technique was used to approach the question of whether or not the ammonia formed by whole snails originated from within their tissues and, if so, what was its metabolic presursor(s). As shown in Figure 8, there was some increase in the ammonium ion concentration of both the blood and the lung when snails were prevented from volatilizing ammonia. The increase in the ammonium ion content of lung tissue suggests that the ammonia evolved from the shell surface originates, at least in part, from within the tissues of the snail. The urea content of the blood and lung also
Table 19—Effect of paraffin-coating on ammonia volatilization from *O. lactea*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of snails</th>
<th>umole NH₃/snail</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3</td>
<td>1.40</td>
</tr>
<tr>
<td>Epiphragm coated</td>
<td>6</td>
<td>0.80</td>
</tr>
<tr>
<td>Shell coated</td>
<td>6</td>
<td>0.12</td>
</tr>
<tr>
<td>Epiphragm and shell coated</td>
<td>3</td>
<td>0.09</td>
</tr>
</tbody>
</table>

These experiments were performed by Dr. L.G. Swartz. Individual estivating snails were coated with paraffin as indicated. The amount of NH₃ formed was determined for an 11 hr period.
Figure 8

Effect of paraffin-coating of *O. lactea* shell on the blood and lung tissue contents of NH$_4^+$, urea and arginine.

Each point represents the average of duplicate analyses on the pooled tissues from 10 snails. The maximum deviation of the duplicate analyses was 2%.
increased in the paraffin-coated snails while the arginine content of the two tissues remained constant. The concentrations of these compounds were determined because they represent potential sources of ammonia due to the presence of arginase and urease activities. As has been shown, these enzymes function to degrade arginine and urea \textit{in vivo} in \textit{O. lactea}. Whether the ammonia produced from these sources was evolved extrarenally was tested by injecting estivating snails with arginine and urea. The data of Figure 9 demonstrate that volatilization of gaseous ammonia was increased by urea and arginine after a lag period. Injection of glutamic acid produced slightly higher ammonia production than injection of water into control snails. In all experiments except one, there was a lag period of approximately 72 hr. In the one exception, the response was almost immediate (2-3 hr after injection). The normal lag period was never observed in the evolution of \[^{14}C\text{]}\text{carbon dioxide formed from either}[^{14}C\text{]}\text{urea or}[^{14}C\text{]}\text{guanidino-}^{14}\text{C}\text{] arginine by whole snails (Figure 6). The lag period in ammonia volatilization from these precursors, in conjunction with the observation that ammonia appears to exit mainly through the shell, suggests that the shell may function as a "trap" for ammonia. Thus, any ammonia or ammonium ion produced from metabolic sources would first be delayed in the shell before it was lost from the shell surface. If this were true, the observed variation in ammonia production by individual snails might be related to the degree of "saturation" of the shell with ammonia or ammonium ion. There is little free water present in
Figure 9

Effect of injected L-arginine, urea and L-glutamate on ammonia production by O. lactea

Ten umoles of each substrate were injected at the time indicated by the arrow into each of 6 snails. The total ammonia produced by the 6 snails was measured.
shell as determined by wt differences after drying overnight at 100°. The form of ammonia in the shell, whether actually ammonia or ammonium ion thus remains an intriguing problem. Additional evidence for an "ammonia trap" function of the shell came by comparing the evolution of ammonia from the shell alone with that from whole snails. Shell from snails whose combined wt totaled 49.9 g was compared with intact estivating snails weighing 47.6 g. The evolution of ammonia from both was monitored for 700 hr. Approximately 250 hr were required to leach the ammonia from the shell. The intact snails continued to evolve ammonia throughout the observation period. Also shells from a number of O. lactea were dissolved in acid and the ammonium ion content determined to be about 1 umole ammonia-N/g shell. As shown in Table 20, the direct origin of the evolved ammonia from endogenous urea could be shown by injecting \( [1,3-^{15}N] \) urea into whole estivating snails and measuring the isotope enrichment of the evolved ammonia. In keeping with the results obtained when the effect of injected urea on the quantitative production of ammonia was studied (Figure 8), there was also a lag period of about the same magnitude in the isotope enrichment of the evolved ammonia. The ammonia produced during the first 72 hr period with Group I in Table 20 contained only 0.64 atoms % excess \( [^{15}N] \) whereas that produced during the next 44 hr contained 6.95 atoms % excess \( [^{15}N] \). The results with Group II again confirm the movement of ammonia through the shell. In this group, the apertures were sealed with paraffin and the only route of exit for
Table 20—Formation of $^{15}$N$_3$H$_3$ from $[1,3$-$^{15}$N]urea by G. lactea

<table>
<thead>
<tr>
<th>Conditions</th>
<th>NH$_3$ collection period (hr)</th>
<th>Isotope content of evolved NH$_3$</th>
<th>Atoms % 15N</th>
<th>Atoms % 15N excess</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) snails in estiv.</td>
<td>0-72</td>
<td>1.01</td>
<td>1.00</td>
<td>0.64</td>
</tr>
<tr>
<td>all epiphragms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intact.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) same snails as in (a) except that 7 of the 9 have epiphragms intact</td>
<td>72-116</td>
<td>7.32</td>
<td>6.95</td>
<td></td>
</tr>
<tr>
<td><strong>Group II:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snails in estivation, aperture in shell sealed by paraffin-coating epiphragm</td>
<td>0-96</td>
<td>9.46</td>
<td>9.09</td>
<td></td>
</tr>
<tr>
<td>100% N$_2$</td>
<td></td>
<td></td>
<td>0.37</td>
<td>0</td>
</tr>
</tbody>
</table>

Each snail was injected at hour 0 with 10 umoles $[1,3$-$^{15}$N]urea (determined to contain 32.47 atoms % 15N). The evolved $^{15}$N$_3$H$_3$ was trapped in 0.01N H$_2$SO$_4$ and oxidized to $^{15}$N$_2$ with alkaline hypobromite (San Pietro, 1957) for mass spectrometry. Nine snails were used in Group I and 3 snails were used in Group II.
ammonia was through the shell. Further evidence of a negative nature also indicates that urea is the principle source, although probably not the only source, of volatilized ammonia. No glutaminase activity could be measured in several tissues by several methods. Also when homogenates of lung tissue were incubated with one of the following: urea, glutamine, glutamate, aspartate, serine, glycine, and alanine only urea stimulated ammonia formation. The bacterial contribution can never be completely ruled out in experiments of this type but since the shells were surface-sterilized, the snail had a urease distinguishable from the bacterial enzyme and substrates injected into the snail stimulated ammonia production it was felt that if any bacteria were contributing to the ammonia production it was the ones embodied within the snail and could be considered as symbionts.

**Blood Chemistry and Ammonia Evolution**

Although ammonia is quite diffusible (Robin et al, 1959), its formation at physiological pH's from ammonium ion is quite small since the pK_a is 9.26. Thus, any mechanism for shifting the pH of body fluids from which ammonia is evolved toward the alkaline region would greatly facilitate the deprotonization of ammonium ion to form ammonia. The subsequent increase in the P_{NH_3} of the body fluids would increase its rate of diffusion. The previous observation on the alkaline nature of the blood of terrestrial snails
and the marked sensitivity of the blood pH to \( P_{CO_2} \) (Spoek et al, 1964) were therefore of considerable interest in connection with the possible physiological adaptation made by these snails for evolving ammonia.

The pH of freshly-collected \( Q. lactea \) blood is around 7.4-7.6. This changes \textit{in vitro} to pH 8.6 or higher and during this change, carbon dioxide is lost from the blood. This is shown in Figure 10. For the measurements shown in Figure 10, the chamber in which the blood was contained was flushed constantly with 100% nitrogen; in other experiments, the rate of change of pH was the same as that shown when either air or 100% oxygen was substituted for 100% nitrogen or when blood was allowed to stand open to the air. However, when carbon dioxide was used, the pH change was reversed and the blood became acid. The decrease in the hydrogen ion concentration could also be followed by automatically titrating the blood with 0.077N hydrochloric acid through a Metrohm Model E 373 Impulsomat to maintain the initial pH (7.4-7.6). The alkalization of the blood was negligible when it was overlayed in the chamber with 0.2-0.3 cm of mineral oil. This indicated that the pH change was actually due to the loss of carbon dioxide and not to proton uptake by an endogenous reaction. The bicarbonate content of \( Q. lactea \) blood, determined by isotope dilution, ranged from 12-31 umoles/ml. The bicarbonate contents reported for other terrestrial snails fall within this range.
Loss of carbon dioxide from *O. lactea* blood during alkalinization *in vitro*.

To 6.5 ml blood, pooled from several snails, was added 0.75 umole NaH[¹⁴C]O₃ (10 uc/umole). The blood was maintained at 30° in a chamber which was flushed constantly with N₂. The [¹⁴C]O₂ evolved was trapped from the exiting N₂ and analyzed for total CO₂ and [¹⁴C]O₂ as described in the text. The average specific activity of the [¹⁴C]O₂ lost from the blood was $2.2 \times 10^5$ dpm/umole. From this dilution of the original specific activity of the added NaH[¹⁴C]O₃, the endogenous HCO₃⁻ content of the blood was calculated to be 11.5 umoles/ml.
(De Jorge et al, 1965; Duval & Portier, 1927; Trams et al, 1965). Because of the equilibrium existing between bicarbonate, carbonic acid, carbon dioxide and carbonate, measurements made by isotope dilution probably reflect more accurately the total carbon dioxide content of the blood. For the data shown in Figure 10, the total carbon dioxide content was calculated to be 12 umoles/ml at pH 7.4. The PCO₂ can be estimated to be approximately 19.1 mm Hg at this pH from the equation:

$$\text{pH} = pK_a + \log \frac{\text{total CO}_2 - \alpha \text{PCO}_2}{\alpha \text{PCO}_2}$$

The value used for $\alpha$ was that for human plasma at 37° (0.0301). At pH 8.5, the total carbon dioxide was 10.5 umoles/ml and the estimated PCO₂ was 1.3 mm Hg. A comparison of these estimated PCO₂'s for O.lactea blood at the two pH's with the pH's found by Spoek et al (1964) for H.pomatia blood at known PCO₂'s shows very good agreement between the behavior of the bloods from the two species and emphasizes the marked dependence of the pH of these bloods on PCO₂.

Although carbonic anhydrase rarely occurs in invertebrate bloods (Van Goor, 1947) and is absent from H.pomatia blood (Spoek et al, 1964), it was considered necessary to establish this for O.lactea blood since considerable carbon dioxide was evolved from the blood in vitro. No carbonic anhydrase could be detected in O.lactea blood using a standard manometric procedure.

In addition, the following observations indicated that
even low levels of the enzyme, which might not be detected
easily, were not present. First of all, the rate of loss
of carbon dioxide from the blood at several pH's was similar
to the rate of loss from phosphate buffer (Figure 11). This
is not in itself, conclusive evidence since phosphate buffers
may have small catalytic effects on the dehydration of
carbonic acid (Roughton & Booth, 1938; Maren, 1967).
Secondly, the Q₁₀ for the pH change of O. lactea blood from
7.5–8.0 was 6.92 between 5° and 15°; 1.28 between 15° and 25°;
and 1.21 between 25° and 35°. The pH change in O. lactea
blood is taken to represent the loss of carbon dioxide and
somewhat similar Q₁₀'s have been reported for the non-
 enzymatic liberation of carbon dioxide from solution
(Roughton, 1948). Finally, acetazolamide in a final
concentration of 3 x 10⁻⁴M had no effect on the rate of
change of blood pH. It is concluded that carbonic anhydrase
is absent from this blood and this point is an important
one in any formulation of a possible mechanism for ammonia
volatilization as will be discussed later. On the other
hand, both lung and hepatopancreas tissues showed measurable
carbonic anhydrase activity. The activity in the lung
was about twice that in the hepatopancreas. Both were
completely inhibited by acetazolamide at 6 x 10⁻⁴M
concentration.

As shown in Figure 12, ammonia added to O. lactea
blood in the ionic form is slowly volatilized during the
change of pH. This was also found to be true for the
Figure 11

Rate of loss of carbon dioxide from *O. lactea* blood and phosphate buffers as a function of pH

To 6 ml of 0.5 M potassium phosphate buffer (prepared CO₂-free) were added 150.75 umoles NaH[¹⁴C]O₃ (147,260 dpm/umole) and to 6 ml of *O. lactea* blood was added 0.75 umole NaH[¹⁴C]O₃. Three separate 6 ml blood samples were used at the different pH's. The specific activity of the H[¹⁴C]O₃⁻ in *O. lactea* blood was 147,260 dpm/umole at pH 7.45, 118,843 dpm/umole at pH 8.03 and 195,422 dpm/umole at pH 8.61. The chamber was maintained at 30° and was flushed with N₂.
Figure 12

Release of ammonia from *O.lactea* blood during alkalization *in vitro*

Five umoles NH$_4$HCO$_3$ were added to 6 ml blood. The chamber was maintained at 30º and was flushed with N$_2$. 
endogenous ammonium ion of freshly-collected blood. There is little or no increase in the endogenous ammonium ion content of the blood when it is allowed to stand, which indicates that its formation from endogenous precursors in the blood is low or non-existent. There may, however, be a very low level of urease present in the blood (Table 14) and, since urea is also normally present in small amounts, a potential for endogenous ammonium ion formation exists.

In both Figure 10 and 12, there is a sharp change in the pH of the blood after it reaches about 8.7-8.8. This drop is very reproducible and occurred in all blood samples. Concomitant with this drop, the blood becomes opaque because of the formation of a white precipitate. This precipitation phenomenon has also been noted by Spoek et al (1964) in H. pomatia blood. They found it necessary to filter out this precipitate before the blood could be used in studies on the respiratory function of hemocyanin. The precipitate in Q. lactea blood was shown here to contain calcium carbonate as follows. Ten ml of blood, pooled from several individuals, was allowed to become alkaline (pH 8.6-8.9). The white precipitate was collected by centrifuging and was washed several times with glass-distilled water made slightly alkaline (pH 9) with sodium hydroxide. It was then washed with 95% ethanol, ether, and finally dried overnight at 120°. A total of 3.2 mg was collected from the 10 ml blood sample. Treat-
ment of the precipitate with acid resulted in the evolution of gas bubbles. The calcium content of this precipitate, determined by a fluorometric method (Wallach & Steck, 1963), was 31.4% vs 40% theoretical for pure calcium carbonate.

There are several lines of evidence which indicate that physiological adaptations have been made by these snails expressly for the purpose of evolving ammonia gas. The most obvious is the potential for the blood to become alkaline. A mechanism can be formulated for the function of this pH change *in vivo* for ridding the blood of ammonium ion. The blood entering the lung contains ammonium ion, bicarbonate ion and carbon dioxide. Because of the action of carbonic anhydrase in the lung tissue, the $P_{CO_2}$ of this tissue would always be low relative to the $P_{CO_2}$ of the blood. Carbon dioxide would thus diffuse rapidly from the blood into the tissue down this pressure gradient. The loss of carbon dioxide from the blood would cause it to become alkaline by an uptake of protons. This results when the equilibrium,

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons H_2O + CO_2,$$

is shifted to the right by the mass action effect of removing carbon dioxide. The initial pH change is fast but slows down considerably as expected because as the pH increases, the equilibrium of the above equation tends to shift back toward the left. Since carbon dioxide has been removed, the pH cannot decrease. The increased alkalinity of the blood would then facilitate the deprotonization of the ammonium ion to increase the $P_{NH_3}$
of the blood. This would result in a pressure differential between the blood and tissue with respect to ammonia and ammonia would move down this pressure gradient into the tissues. In the lung tissue, excess hydrogen ions are created by the action of carbonic anhydrase and these would serve to "trap" the diffusing ammonia as ammonium ion. Such a "diffusion-trapping" mechanism, which is identical in theory to that proposed for the mammalian kidney, would account for the ammonium ion concentration difference between blood and lung. The ammonium ion content of the blood never normally exceeds about 0.2 umole/ml while lung ammonium ion content is from 3.5-3.9 umoles/g tissue. Although there is an apparent "up hill" gradient with respect to ammonium ion, the pH differences between the two tissues accounts for a reversed concentration gradient with respect to ammonia which is the diffusing species. The blood which leaves the lung will become less alkaline as metabolic carbon dioxide from the inner tissues is collected and the equilibrium restored.

The unique behavior of the hemocyanin of these terrestrial snails, which was heretofore not clearly understood (Spoek et al., 1964), also provides indirect support for the operation in vivo of the above exchange mechanism. An examination of Manwell's figure for the oxygen dissociation curve of H. pomatia hemocyanin (Froser & Brown, 1961) and the data of Spoek et al (1964) reveals a sigmoid function of this pigment only between pH 7.6 and 9.0. In this pH range, the pigment exhibits a normal Bohr effect. The maximum affinity
of the hemocyanin for oxygen at pH 9 suggests that it may actually load in the lung at similar pH's. Such pH's, which may be attained by the blood in vitro, would be required in the above "diffusion-trapping" mechanism to drive ammonia from the blood into the lung tissue. The data of Spoek et al (1964) also indicate that the pH of H. pomatia arterial blood is generally pH 7.65 or above. We found slightly lower pH's for O. lactea blood which was withdrawn from near the pericardial sinus and not directly from the heart. Whatever the mechanism employed for ridding the blood of ammonia, it appears to be a relatively efficient one. While the ammonium ion content of O. lactea blood (a maximum of 0.2 umole/ml) is higher than that of human blood (0.06 umole/ml, Calkins, 1956), it is lower than that of several other molluscs (Potts, 1967) and does not approach the levels tolerated by certain mammals (Studier, 1966).

Although the above mechanism for the exchange of ammonia between blood and lung tissues seems consistent with the normal blood chemistry of these snails and the tissue location of carbonic anhydrase, the apparent "one-way exit" for the ammonia through the shell remains unexplained. The volatilization of ammonia from the lung or mantle surfaces, or both, through the extrapallial space--the space or fluid between the mantle and lung surfaces and the shell--into the shell and then through the shell could be due to a mass action effect. The concentration of ammonium ion in the lung is relatively high and, in paraffin-coated snails, there is a fairly marked buildup of ammonium ion in this
tissue (Figure 8). A similar cyclic build-up may normally take place until a critical concentration of ammonium ion is reached. Such a build-up might account for the long lag period required for the injected precursors of ammonia to cause a stimulation in ammonia release.

Purine Biosynthesis and the Significance of Ammonia in Total Nitrogen Excretion

There are at least two major aspects of gastropod physiology in which extrarenal gaseous ammonia excretion might be significant. The first and most obvious aspect is nitrogen excretion. Terrestrial pulmonates like H. pomatia, H. aspersa, and O. lactea are considered to be strictly uricotelic. This follows from the work of Jezewska et al (1963 a) on the composition of kidney contents and excreta contents, and also from the work of Lee & Campbell (1965) who demonstrated that O. lactea could synthesize the purine ring by the same pathway known for other species capable of the de novo synthesis of purine. Analysis of kidney contents or excreta contents would not detect the extrarenal excretion.

Although the basic pathway for purine biosynthesis is known, its rate of operation in vivo in its excretory capacity is not. There are, in fact, few reliable quantitative data available for the total-N excreted by land snails either as purine-N or in some other form. An attempt was made, therefore, to determine the rate of purine synthesis in active and estivating snails.
Estimation of Purine Synthesis in Estivating Snails

The accumulation of purines during estivation was used to estimate the average rate of synthesis of the kidney purines in vivo. A series of 60 estivating snails was used. One group of 15 was analyzed at the start (month 0, Figure 13). The three remaining groups of 15 each were then analyzed at monthly intervals. The individual purines (uric acid, xanthine, and guanine) were determined in each snail. These data are presented in Table 21. The mean total purine contents at each monthly interval are plotted in Figure 13. The slope of the line resulting from such a plot was taken to represent the rate of synthesis of the purines deposited in the kidneys during estivation. Using the linear part of the line, the initial 60 day period, there was an average increase of about 10.7 μmoles purine/g total wt. The average daily rate was thus 0.18 μmole/g total wt. Snails weighing 5-6 g were used so this represents the synthesis of about 1 μmole purine per snail per day.

It is also possible to make the same estimation for H. pomatia from the data presented by Jezewska et al (1963b) by making one assumption. This assumption is the total wt of the snails used. Jezewska et al (1963a) reported an average kidney wt of 240 mg for H. pomatia. Since this is the same kidney wt as that reported by Baldwin & Needham (1934) for H. pomatia they used, it seems reasonable to assume that the average total wt of the snails used by the two groups were similar. Baldwin & Needham (1934) use an average total wt of 20 g for H. pomatia. Jezewska et al
<table>
<thead>
<tr>
<th>Date of analysis</th>
<th>Uric acid</th>
<th>Xanthine</th>
<th>Guanine</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>April 20</td>
<td>49.8 ± 3.1*</td>
<td>11.1 ± 1.2</td>
<td>7.3 ± 0.7</td>
<td>68.2 ± 4.2</td>
</tr>
<tr>
<td>May 20</td>
<td>52.4 ± 4.0</td>
<td>11.9 ± 1.3</td>
<td>9.3 ± 0.9</td>
<td>73.6 ± 6.0</td>
</tr>
<tr>
<td>June 20</td>
<td>54.0 ± 5.7</td>
<td>12.3 ± 1.4</td>
<td>12.6 ± 1.1</td>
<td>78.9 ± 8.0</td>
</tr>
<tr>
<td>July 25</td>
<td>54.5 ± 5.2</td>
<td>12.1 ± 1.6</td>
<td>12.1 ± 1.6</td>
<td>78.7 ± 8.0</td>
</tr>
</tbody>
</table>

* Standard error of the mean for 15 individuals.
Figure 13

Rate of accumulation of purines during estivation

At month 0, the kidney purines were analyzed in one group of 15 estivating snails. Additional groups of 15 snails each from the same population were analyzed thereafter at monthly intervals. The thin vertical line represents the standard deviation from the mean and the thick vertical bars the standard error of the mean. The average levels for active snails (51 individuals) and for estivating snails (6 months or longer in estivation) were taken from other experiments.
(1963b) show an increase of 541 umoles purine/snail from the end of the feeding period (X; October) to the end of the estivating period (III-IV; last of March). This represents an increase of 27 umoles/g total wt for a 20 g snail. Since this increase requires roughly 180 days, the estimated rate is about 0.15 umole purine/g total wt per day. There is thus very good agreement between the estimated rates of purine synthesis and deposition into the kidney during estivation by the two species even if the assumed total wt of H. pomatia is in error by several percent. Another way to show the similarity in the rates of purine synthesis by the two species is to consider the time required to reach the maximum level of purines found in estivating snails. For O. lactea, this can be shown by extrapolating the line in Figure 13 for estivating snails back to the mean level of purines in active snails. The approximate time involved in going from the active to the estivating level is 6 months. A similar amount of time is required by H. pomatia.

**Estimation of Purine Synthesis in Active Snails**

A more direct estimation of the rate of synthesis of the purines deposited in the kidneys of active snails could be made by measuring the rate of incorporation in vivo of [1-14C]glycine. Glycine has previously been shown to be a precursor of carbons 4 and 5 and nitrogen 7 of the purine ring in both H. pomatia (Jezewska et al, 1964b) and O. lactea (Lee & Campbell, 1965). As shown in Figure 4, there is
Figure 14

Incorporation of [1-14C]glycine into O. lactea kidney purines

Each point represents the average incorporation for 3 snails. At 0 time, each snail received 5 μc [1-14C]glycine (0.88 umole) injected directly into the foot. The final specific activity of the glycine was corrected for the average endogenous tissue content of free glycine which was found to be 0.26 umole per g tissue.
a fairly rapid incorporation of this precursor in vitro into the kidney purines. To estimate the potential capacity for purine biosynthesis it is best to make the calculations from initial rates. With this in mind, experiments were conducted in a manner similar to those depicted in Figure 14 except that shorter incorporation times were used. Converted to a 24 hr basis, the rate observed after 2 hr is 0.68 umole purine synthesized per g total wt. This is approximately four times the rate estimated for estivating snails.

**Evaluation of Total-N Excretory Capacity**

From the observed rates of synthesis in vivo of the kidney purines, an estimate can be made of the excretory capacity of *O. lactea* for purine-N. About 0.18 umole purine was accumulated in estivating snails/g total wt per day. Of this, 38.7% was uric acid, 11.6% was xanthine, and 49.9% was guanine. This corresponds to the excretion, a term used here to refer to the deposition of the purines in the kidney, of about 9.7 ug N or 0.69 uatoms N/g total wt per day.

The highest rate observed for active snails was about four-times that for estivating snails or 0.68 umole purine synthesized/g total wt per day. Of this, 79.7% was uric acid, 8.7% was xanthine, and 11.6% was guanine. This corresponds to the excretion of about 39.1 ug N or 2.79 uatoms N/g total wt per day.

From the above calculations and from those concerning ammonia evolution, it is possible to make a rough estimate of the contribution of the ammonia-N to the snail’s overall
nitrogen excretory capacity. This estimation is based on the assumption that purine-N plus ammonia-N represents most, if not all, of the total-N excreted by *O.lactea*. The observed rate of ammonia production by estivating snails was 0.024 umole ammonia/g tissue per hr. This corresponds to approximately 0.29 umole ammonia/g total wt per day. Considering that the tissue accounts for about 50% or so of the total wt. As previously pointed out, the rate of ammonia production is quite variable and subject to marked fluctuations in individual snails. This rate of ammonia formation would then correspond to about 4 ug N/g total wt per day. This represents 29% of the total-N (Purine-N + Ammonia-N).

It is difficult to measure with any confidence the amount of ammonia produced by active snails for reasons already stated. The best estimate is that the rate is about one-half that of estivating snails. This rate would be equivalent to 2 ug ammonia-N/g total wt per day. Ammonia-N thus represents no more than about 5% of the total-N excreted by active snails.

It may be concluded that, while the formation and volatilization of ammonia gas by these snails may reflect an important underlying physiological process, they do not appear to represent the major excretory mechanism in either active or estivating snails. During estivation when economy with respect to energy-rich compounds and water might be anticipated, the elimination of ammonia-N does,
however, appear to be more significant in terms of total-N eliminated than it is in active snails.

Formation of Calcium Carbonate for Deposition in Shell

The other major aspect of gastropod physiology which could conceivably be affected by extrarenal gaseous ammonia evolution is calcification of the shell. There is a considerable body of information available concerning the conditions necessary for the precipitation of calcium carbonate from a solution. However, there is a paucity of information to explain how living organisms precipitate calcium carbonate and incorporate it into extremely well organized structures.

Inorganic Reaction:

What is known about precipitation of calcium carbonate in seawater is summarized in Reaction 1 (Krauskopf, 1967).

\[
\begin{align*}
\text{CaCO}_3(s) + \text{H}_2\text{CO}_3 & \rightleftharpoons \text{Ca}^{2+} + 2\text{HCO}_3^- \\
& \quad \downarrow \\
& \quad \text{H}_2\text{O} + \text{CO}_2
\end{align*}
\]

A high \( \text{PCO}_2 \) causes the reaction to shift toward the right, dissolving calcium carbonate. An increase in temperature causes precipitation of calcium carbonate by lowering the \( \text{PCO}_2 \). The situation may be summarized by Reaction 1 only because the \( \text{pH} \) of the seawater in which these events take place is about 8.1.
(2) \[ \text{H}_2\text{O} + \text{CO}_2 \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^- \rightleftharpoons \text{H}^+ + \text{CO}_3^{2-} \]

(3) \[ \text{Ca}^{2+} + \text{CO}_3^{2-} \rightleftharpoons \text{CaCO}_3(s) \]

Reaction 1 does not represent the actual events which occur whereas Reactions 2 and 3 do. At pH 8 most of the carbon dioxide is in the form of bicarbonate and, in seawater, there is essentially an infinite supply of this anion. When carbon dioxide is removed, protons are taken up as is an equivalent amount of bicarbonate. There is still a large supply of bicarbonate remaining and in the absence of protons, that remaining tends to dissociate forming carbonate which precipitates as calcium carbonate when the limit of solubility is reached. When carbon dioxide is added, protons are formed and combine with carbonate to form bicarbonate. Calcium carbonate therefore dissolves to maintain the equilibrium. It is extremely important to remember that hydration of carbon dioxide produces protons. It should be obvious that at pH 7 or below, no calcium carbonate could be precipitated regardless of the \( \text{CO}_2 \). In these reactions it is always the carbonate concentration which determines how much calcium carbonate will be precipitated. Calcium carbonate has a very low solubility but under most circumstances there is extremely little carbonate present.

Reaction 1 has been used to explain calcium carbonate deposition by certain photosynthetic marine algae (Lewin, 1962). They supposedly precipitate calcium carbonate by removing carbon dioxide for photosynthesis and thereby
shift the equilibrium of Reaction 1 towards the left. Lewin (1962), however, points out that this theory raises more questions than it answers. For instance, why are not all photosynthetic marine algae covered with calcium carbonate? Why does the distribution of photosynthetic tissue not correlate with the presence of calcium carbonate in these algae which do precipitate calcium carbonate? Why do not seaweeds and other marine plants precipitate calcium carbonate? Other questions are also raised by this theory.

Inorganic Reaction Facilitated by the Dehydration Reaction of Carbonic Anhydrase

This hypothesis of deposition of calcium carbonate incorporates the inorganic reaction and the enzyme carbonic anhydrase. This enzyme is known to be present in the two cases to be mentioned and is postulated to increase the rate of dehydration of carbonic acid such that carbon dioxide may be removed more rapidly.

Goreau (1959) has proposed essentially the same set of circumstances which occur in seawater for calcium carbonate deposition by corals.

\[
(4) \quad \text{Ca}^{2+} + 2\text{HCO}_3^- \rightleftharpoons \text{Ca(HCO}_3)_2 \rightleftharpoons \text{CaCO}_3 + \text{H}_2\text{CO}_3
\]

\[
\text{removed by photo-synthetic algae} \quad \underline{\text{H}_2\text{O} + \text{CO}_2}
\]

Carbonic anhydrase in the coral tissue would facilitate the dehydration of carbonic acid and the symbiotic photosynthetic "zoanthellae" would remove the carbon dioxide
formed. Indeed the data which Goreau (1959) presents might point to an explanation of this type. He found that when corals were kept in the dark, calcium carbonate deposition decreased markedly. The same thing happened when corals were "cured" of their symbionts. Acetazolamide added to the seawater medium also reduced calcium carbonate deposition markedly. Here again, the interpretation may be questioned. All of the deposition experiments were conducted with $^{45}$Ca. The transport of calcium in the dark was not studied nor was the effect of acetazolamide upon the transport of calcium. As was stated before, it is the concentration of carbonate which controls the precipitation of calcium carbonate when calcium is in abundance. It is essential to know, therefore, all the factors which enter into the formation of carbonate in corals in the light and dark. Carbonic anhydrase in all cases investigated appears to work physiologically in the hydration of carbon dioxide rather than the dehydration of carbonic acid. Once again, Reaction 4 assumes alkaline conditions and an abundance of bicarbonate neither of which has been shown for coral tissue. One might also ask why organisms such as the sea anemones which can also be parasitized by photosynthetic algae do not precipitate calcium carbonate.

Gutowska & Mitchell (1945) also invoke the inorganic reaction and carbonic anhydrase for calcium carbonate deposition in chicken eggs. They propose:
Blood \hspace{1cm} \text{Shell Gland} \hspace{1cm} \text{Lumen}

\[
\begin{align*}
2\text{HCO}_3^- & \rightarrow 2\text{HCO}_3^- \rightarrow \text{H}_2\text{CO}_3 + \text{CO}_3^{2-} \rightarrow \text{egg shell} \\
\text{CA} & \downarrow \\
\text{H}_2\text{O} + \text{CO}_2 & \\
\text{CO}_2 & \hspace{1cm} \text{hydrated}
\end{align*}
\]

This scheme is based largely on the presence of carbonic anhydrase in the shell gland and the fact that administration of sulphanilamide to laying hens results in poorly calcified eggs. The hypothesis has, however, been roundly criticized by Diamantstein & Schluns (1964) and more recently by Hodges & Lorcher (1967). The latter authors could not find the necessary decrease in blood bicarbonate during eggshell formation. However, the methods used by these authors were inadequate to demonstrate conclusively what they wished to show. Again one might ask whether carbonic anhydrase functions to hydrate carbon dioxide or to dehydrate carbonic acid.

As Hodges & Lorcher (1967) point out, it is rather unlikely that carbonate could be secreted from the shell gland to the lumen as such. Further comments on the deposition of calcium carbonate by the laying hen will be made later.

**Inorganic Reaction Facilitated by the Hydration Reaction of Carbonic Anhydrase**

There have been a considerable number of studies on calcium carbonate deposition in molluscs and these are summarized by Wilbur (1960, 1964). It is suggested for *Crassostrea virginica* that the carbonate of the shell is derived from a carbon dioxide-bicarbonate pool within the mantle which is a part of a larger pool of the body. This
larger pool derives carbon dioxide-bicarbonate from 1) the medium, 2) urea via urease, 3) the Krebs citric acid cycle. The difficulty of carbonate formation is apparently realized and two schemes are proposed for its formation. A) Carbonate can be formed from bicarbonate in the presence of hydroxyl ions (why the hydroxyl ions are present or where they came from is not discussed). B) Carbonate may be formed from bicarbonate by the removal of carbon dioxide (the inorganic reaction). Wilbur was one of the first to suggest that carbonic anhydrase accelerates shell formation by accelerating bicarbonate formation. It is then suggested that when bicarbonate is in excess the removal of carbon dioxide speeds conversion of bicarbonate to carbonate. With regard to this last point, Wilbur suggests that carbon dioxide is fixed into succinate which in turn is converted to oxaloacetate. The oxaloacetate is decarboxylated in mantle tissue and the carbon dioxide formed would be converted to carbonate with catalysis by carbonic anhydrase. Carbon dioxide could be removed by its fixation into succinate. This hypothesis became untenable when it was shown that this organism lacked the propionyl CoA carboxylase activity necessary for succinate formation by a carboxylation reaction (Simpson & Awapara, 1964). Wilbur further states that acetazolamide decreases calcium carbonate deposition indicating that carbonic anhydrase might function in the deposition. The following equation given by Wilbur sums up the present state of knowledge (or lack thereof) as far as it has been definitely extended to molluscs:
Hodges & Lorch (1967) have approached the problem of calcium carbonate deposition around the chicken egg keeping in mind the actual inorganic chemistry involved and all the products of the carbonic anhydrase reaction. They indicate that from the information available, there are at least two possible mechanisms which can be put forth to explain egg shell formation.

A) Blood

\[
\begin{align*}
\text{HCO}_3^- & \quad \rightarrow \quad \text{HCO}_3^- \\
& \quad \rightarrow \quad \text{HCO}_3^- \quad \rightarrow \quad \text{H}^+ + \text{CO}_3^{2-}
\end{align*}
\]

This possibility relies on the removal of bicarbonate from the blood by the shell gland and apparently does not account for the presence of carbonic anhydrase in the shell gland. This possibility also does nothing to account for removal of the proton formed during formation of carbonate.

B) Blood

\[
\begin{align*}
\text{CO}_2 + \text{H}_2\text{O} & \quad \rightarrow \quad \text{H}_2\text{CO}_3 \\
& \quad \downarrow \quad \text{CA} \\
\text{H}_2\text{CO}_3 & \quad \rightarrow \quad \text{H}^+ + \text{HCO}_3^- \\
& \quad \rightarrow \quad \text{HCO}_3^- \quad \rightarrow \quad \text{H}^+ + \text{CO}_3^{2-}
\end{align*}
\]

As Hodges & Lorch (1967) point out, these theories still do
not explain how the bicarbonate ions in the lumen of the shell gland are deposited as calcium carbonate because when carbonate is formed a proton is also produced. Nothing is known of how or why this transformation occurs in the lumen or what is the fate of the proton.

The first mechanism (A) alone is not satisfactory nor is the second (B) by itself. However, the two together, although this case is not considered by Hodges & Lorch, provide a starting point for understanding how calcium carbonate might be precipitated. Hodges & Lorch, apparently without realizing it, actually provide evidence that blood bicarbonate is a source of shell carbonate. The blood bicarbonate, however, does not account for all of the carbonate deposited and therefore the shell gland may be the additional source of shell carbonate. The carbonic anhydrase in the shell gland would function to hydrate carbon dioxide and keep bicarbonate as such. Other aspects are obviously involved. Among these are the synthesis of the protein matrix upon which calcium carbonate is precipitated and metabolic aspects which influence the pH in the region of calcification. That pH is very important became apparent when Hunt & Simkiss (1967) showed that a decrease in pH even with an increase in bicarbonate content resulted in decalcified eggs.

There are several reasons for thinking that ammonia volatilization in land snails may be significant to calcium carbonate deposition. First of all, ammonia exits primarily through the shell whereas carbon dioxide exits primarily
through the aperture. Ammonia is a good candidate for causing the alkaline conditions essential for calcium carbonate precipitation. Secondly, the urease and carbonic anhydrase that we consider to be necessary for ammonia formation and transport are located among other places in the mantle tissue. This tissue must play an essential role in calcium carbonate deposition. A simple scheme might be proposed from the information already known for land snails to precipitate calcium carbonate as follows:

\[
\begin{align*}
\text{CO}_2 + \text{H}_2\text{O} & \rightleftharpoons \text{H}_2\text{CO}_3 \\
\text{Urea} & \rightarrow \text{CO}_2 + 2\text{NH}_3
\end{align*}
\]

\[
\begin{align*}
\text{1)NH}_3 & \rightarrow \text{NH}_4^+ \\
\text{H}^+ + \text{CO}_3^{2-} & \rightarrow \text{Ca}^{2+} \\
\text{H}_2\text{O} & \rightarrow \text{NH}_4^+ + \text{OH}^-
\end{align*}
\]

This could be simplified to the following:

\[
\text{NH}_3 + \text{Ca}^{2+} + \text{HCO}_3^- \rightleftharpoons \text{CaCO}_3 + \text{NH}_4^+
\]

This similar to the scheme proposed by Berner (1968) for calcium carbonate concretions formed by decay of organic matter. This scheme does not, of course, account for all parameters and is probably simpler than the actual case. It does, however, present the most plausible mechanism to date for calcium carbonate precipitation. Any ammonia producing system would function as well as the urea-urease system found in *O. lactea* and therefore the scheme if correct may be widespread.

A direct test of this hypothesis has not yet been
formulated with *O. lactea*. The snails used were probably not particularly concerned with calcium carbonate deposition as would be a young actively growing snail. It has not been possible to obtain young snails for experimentation. It could be shown (Table 22), however, that the urea-\(C\) and guanidino-\(C\) of arginine could both serve as precursors for shell carbonate as could bicarbonate-\(C\). Table 22 also demonstrates that little success was achieved with the urease inhibitor acetohydroxamic acid. A preliminary experiment suggests that acetazolamide might decrease calcium carbonate precipitation. Both urease and carbonic anhydrase were 95% inhibited as determined on tissue homogenates of lung and hepatopancreas after sacrificing the snails. These experiments must be considered qualitative because the specific activity of the substrates in the whole snail was not known. Variation in the endogenous content of the substrates might cause, to some degree, the extreme variability observed. It was quite frustrating that no critical control could be used to rule out the exchange phenomenon. An attempt was made, however, to assess the exchange phenomenon by removing the soft tissues of several snails and boiling the whole shell to denature the shell protein. Five umoles (in 0.1 ml) of sodium \(^{14}C\) bicarbonate was then placed in each shell and the shell placed in a closed beaker. After 24 hr the shells were emptied and washed as usual. The \(^{14}C\)-carbonate found in the shell was 1.28%. This apparent
Table 22—Deposition of CaCO₃ into shell of _O.lactea_

<table>
<thead>
<tr>
<th>Substrate</th>
<th>% injected substrate recovered as Ca[^14C]O₃ in shell after 25 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH[^14C]O₃</td>
<td>4.50 ± 2.45* (21)**</td>
</tr>
<tr>
<td>NaH[^14C]O₃ plus Acetohydroxamate</td>
<td>6.61 ± 2.84 (5)</td>
</tr>
<tr>
<td>NaH[^14C]O₃ plus Acetazolamide</td>
<td>2.93 (1)</td>
</tr>
<tr>
<td>[^14C]Urea</td>
<td>2.83 ± 1.07 (17)</td>
</tr>
<tr>
<td>[^14C]Urea plus Acetohydroxamate</td>
<td>4.74 ± 2.08 (5)</td>
</tr>
<tr>
<td>[^14C]Urea plus Acetazolamide</td>
<td>1.37 (1)</td>
</tr>
<tr>
<td>[guanidino-[^14C]arginine]</td>
<td>1.84 ± 0.97 (6)</td>
</tr>
</tbody>
</table>

* Standard deviation  
**Number of groups analyzed; 3 snails per group.  
Each snail was injected with 5 umoles substrate at 0 time. The inhibitors were injected as follows: acetohydroxamate, 10 umoles/snail/injection at 72, 48, and 24 hr before and at the time of injection of substrate; acetazolamide, 0.68 umole/snail/injection at 72, 48, and 24 hr before and at the time of injection of substrate. Twenty-five hrs after injection of substrate the snails were sacrificed and the shell analyzed for CaCO₃ as described in the Methods section. In those groups to which inhibitors had been given the hepatopancreas and lung tissues were pooled and assayed for urease (radioactively) and carbonic anhydrase (colorimetrically) as described in the Methods section.
Exchange may be compared with 4.50% incorporation in living snails (Table 22).

In vitro experiments were also performed to simplify the physiological parameters involved and to better assess the necessity, if any, for the enzyme systems. Table 23 presents data for the experiments in which $[^{14}\text{C}]$ urea was used as the substrate. It can be seen that urease is necessary to hydrolyze the urea and can be inhibited by both hydroxyurea and acetohydroxamate. The carbonic anhydrase activity also appears to be essential for calcium carbonate deposition as evidenced by the marked decrease in calcium carbonate deposition when acetazolamide is added to the reaction mixture. The effect of acetazolamide upon transport of bicarbonate from the lung tissue was not determined, however, and this remains as a possibility.

Boiling the shell apparently injures the protein matrix necessary for calcium carbonate deposition. Table 24 presents the data for the experiments in which $[^{14}\text{C}]$-bicarbonate was used as the substrate for calcium carbonate deposition. Addition of urease inhibitors has some inhibitory effect upon calcium carbonate deposition even when exogenous urea is not present. The urease may control the amount of ammonia available to take part in calcification. The reason for the decrease in calcium carbonate deposition when acetazolamide is added to the reaction mixture is not understood with certainty. Boiling the shell again lowers calcium carbonate deposition either by disrupting the protein matrix or possibly by destroying carbonic anhydrase activity in
<table>
<thead>
<tr>
<th>Modification of Reaction Mixture</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total DPM deposited as $[^{14}C]O_3^{2-}$ (% of &quot;complete reaction mixture&quot;)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>5254 (100)</td>
<td>32,931 (100)</td>
<td>9890 (100)</td>
<td>5108 (100)</td>
</tr>
<tr>
<td>Minus lung</td>
<td>141 ( 3 )</td>
<td>305 ( 1 )</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Plus hydroxyurea</td>
<td>360 ( 7 )</td>
<td>519 ( 2 )</td>
<td>692 ( 7 )</td>
<td>165 ( 3 )</td>
</tr>
<tr>
<td>Minus lung, plus hydroxyurea</td>
<td>120 ( 2 )</td>
<td>287 ( 1 )</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Boiled shell</td>
<td>-</td>
<td>-</td>
<td>8055 (24)</td>
<td>1051 (11)</td>
</tr>
<tr>
<td>Boiled shell, plus hydroxyurea</td>
<td>-</td>
<td>-</td>
<td>3553 (11)</td>
<td>-</td>
</tr>
<tr>
<td>Plus acetazolamide</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>948 (10)</td>
</tr>
<tr>
<td>Minus lung, plus urease</td>
<td>-</td>
<td>-</td>
<td>2870 (29)</td>
<td>-</td>
</tr>
<tr>
<td>Plus acetohydroxamic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The complete reaction mixture and the manipulations are described in the Methods section. Only that carbonate actually deposited on the 1 cm² piece of shell was considered. The $[^{14}C]$urea had a specific activity of 115,000 dpm/umole. Additions to the complete reaction mixture were in the following quantities: hydroxyurea, 25 mM; acetazolamide, $7.9 \times 10^{-4}$M; urease, 50 mg Sigma Type III; acetohydroxamate, 5 mM. The boiled shell was heated in a boiling water-bath for 30 min and then cooled to room temperature before being added to the reaction mixture.
Table 24—*In vitro* deposition of Ca\(^{14}C\)O\(_3\) by *O.lactea* from H\(^{14}C\)O\(_3\)

<table>
<thead>
<tr>
<th>Modification of Reaction Mixture</th>
<th>A</th>
<th>Experiment Number</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>5130 (100)</td>
<td>-</td>
<td>-</td>
<td>6600 (100)*</td>
</tr>
<tr>
<td>Plus urea</td>
<td>-</td>
<td>5471 (100)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Plus hydroxyurea</td>
<td>2504 (49)</td>
<td>-</td>
<td>-</td>
<td>3640 (55)</td>
</tr>
<tr>
<td>Plus acetohydroxamate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4346 (66)</td>
</tr>
<tr>
<td>Plus acetohydroxamate, plus urea</td>
<td>-</td>
<td>5306 (97)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Plus hydroxyurea, plus urea</td>
<td>3003 (59)</td>
<td>3533 (65)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Plus acetzolamide</td>
<td>1711 (33)</td>
<td>-</td>
<td>-</td>
<td>4465 (68)</td>
</tr>
<tr>
<td>Plus acetzolamide, plus urea</td>
<td>2062 (40)</td>
<td>2672 (49)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Minus lung</td>
<td>4660 (91)</td>
<td>4551 (83)</td>
<td>-</td>
<td>5767 (87)</td>
</tr>
<tr>
<td>Minus lung, plus urea</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Minus lung, plus acetzolamide</td>
<td>2977 (58)</td>
<td>3077 (56)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Calculated value for DPM
The complete reaction mixture and the manipulations are described in the Methods section. Only that carbonate actually deposited on the 1 cm\(^2\) piece of shell was considered. The H\(^{14}CO_3\) had a specific activity of 67,000 dpm/umole. Additions to the complete reaction mixture were in the following quantities: hydroxyurea, 25 mM; acetzolamide, 7.9 x 10\(^{-4}\)M; urea, 5 mM; acetohydroxamate, 5 mM. The boiled shell was heated in a boiling water-bath for 30 min and then cooled to room temperature before being added to the reaction mixture.
the shell. This last possibility seems tenable since
an experimental without lung tissue but containing acet-
azolamide decreased calcium carbonate deposition. This
might also explain why removal of lung tissue fails
to decrease calcium carbonate significantly. This fact
serves to obscure the function, if any, of the urease.

These preliminary experiments do not, therefore, pro-
vide any definite proof for the mechanism of calcification
involving ammonia suggested here. Nevertheless, it is
felt that the volatilization of ammonia through the shell,
the presence of urease and carbonic anhydrase in the mantle,
the fact that this snail has the capacity to synthesize
urea and degrade it in the mantle, and the inhibitions
found in vitro suggest the mechanism may function. It
should be remembered, also, that urease and carbonic
anhydrase may work at a distance because all that is really
necessary for the mechanism to function is the presence
of calcium, ammonia, and bicarbonate (of which there are
considerable free pools) in the vicinity of the outer
surface of the mantle and the inner surface of the shell.
The mechanism proposed deals mainly with the formation
of the carbonate ion and does nothing to explain where it
is formed or how it is transported, if indeed it is
transported. Neither does it deal with the interaction
of calcium carbonate and the organic matrix in the actual
calcification process.
SUMMARY & CONCLUSIONS

The present study demonstrates that the terrestrial pulmonate, *O. lactea* is capable of synthesizing arginine *de novo*. On the basis of precursor studies, bicarbonate gave rise to the guanidino-\( \text{C} \) of arginine and ornithine gave rise to carbons 1-5. Citrulline was also found to be a precursor of arginine. Three of the four enzymes necessary for the biosynthesis of arginine have been found in *O. lactea*. Only carbamyl phosphate synthetase remains to be demonstrated. The various enzymes are distributed throughout the snail: hepatopancreas, kidney, lung, foot, reproductive tract, columnellar muscle, albumen gland, and heart all possessed the three enzymes demonstrated. There thus appears to be little differentiation between the various tissues with respect to arginine biosynthesis. Biosynthetic arginine may be a significant source of the arginine necessary for protein synthesis by the snail. That biosynthetic arginine was incorporated into the protein fraction of all the tissues analyzed permits one to speculate that each tissue incorporates its own biosynthetic arginine into the proteins synthesized within the tissue. This independence between tissues with respect to arginine remains to be shown conclusively, however, as does its significance.

The fact that this uricotelic snail can synthesize arginine places it in a unique position. Most other uricotelec organisms apparently have lost the ability to synthesize
arginine de novo. Thus arginine is a dietary requirement in birds, insects and perhaps reptiles. The fact that *O. lactea* can synthesize both arginine and uric acid may demonstrate that the invasion of land by primitive organisms did not require the loss of one pathway or the other but possibly selection of either urea or uric acid as the principal nitrogen waste. This selection may have been influenced by availability of water for nitrogen excretion. It should be remembered that mammals, although ureotelic, retain the ability to synthesize and excrete uric acid.

*O. lactea* is capable of degrading both endogenously synthesized arginine as well as exogenously added arginine. The degradative pathway consists of arginase, urease, and ornithine transaminase. All of the tissues of *O. lactea* mentioned above have the ability to degrade arginine although columellar muscle has very little arginase activity and heart, albumen gland, and columellar muscle contain very low urease activity. It was also shown that urea was synthesized de novo and subsequently degraded. This is an important point because it demonstrates that some of the arginine synthesized is also degraded, i.e., the arginine synthesized is not necessarily the end-product of the pathway although arginine as such is probably used for a number of metabolic purposes. It also demonstrates that arginase can degrade endogenous as well as exogenously supplied arginine.
*O. lactea* has also been shown to volatilize gaseous ammonia through the shell. One source of the ammonia seems to be urea. This was shown by injecting $[1,3^{-15}N]_\text{urea}$ into snails and collecting the $[15N]_\text{ammonia}$ produced. Injected urea and arginine stimulated ammonia production by the snails. This extrarenal mechanism can account for 29% of the excreted nitrogen (assuming that ammonia and purines are the only end-products) in estivating snails. This was calculated from an estimation of the rate of purine excretion in estivating snails. The percentage of ammonia excreted of the total nitrogen wastes is much smaller in active snails than in estivating snails. *O. lactea* is therefore quite uricotelic.

Several factors have led us to speculate that there is at least one other physiological function for the continual synthesis and degradation of arginine in addition to furnishing arginine for protein synthesis and urea for nitrogen excretion (excreted as ammonia gas after degradation by urease). This physiological function is to provide the conditions necessary for the precipitation of calcium carbonate for deposition onto an organic matrix for shell formation. The factors leading us to this conclusion are as follows. Ammonia exits almost exclusively through the shell whereas carbon dioxide exits through the aperture. The pH seems to be the principal factor concerned with the formation of the carbonate necessary for precipitation of calcium carbonate; ammonia could provide the alkaline
conditions required for the formation of carbonate from bicarbonate. Carbonic anhydrase, an enzyme which may function to provide the bicarbonate ions is localized in the tissues and is not found in the blood. Moreover, the blood chemistry found for this snail indicates that the blood may transport ammonia from the inner tissues to the mantle tissue for subsequent volatilization. In vitro studies on model systems suggest that inhibition of urease or carbonic anhydrase, or both, decreases calcium carbonate deposition. Carbonic anhydrase would furnish bicarbonate ions and the ammonia from the urease reaction would provide the alkaline conditions necessary for carbonate formation. Calcium carbonate has an extremely low solubility and if calcium is not limiting, the more carbonate formed, the more calcium carbonate precipitated. This scheme for calcium carbonate precipitation while not proven is more plausible than others which have been proposed previously.
LITERATURE CITED


Roughton F.J.W. & V.H. Booth (1938). The catalytic effect of buffers on the reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ *Biochem. J.* 32, 2049-2069.


