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URIC ACID METABOLISM IN THE TERRESTRIAL SNAIL,


tala lactea

by

Thomas W. Lee

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URIC ACID METABOLISM IN THE TERRESTRIAL SNAIL,

OTALA LACTEA
INTRODUCTION

The first purine (uric acid) was discovered as a component of human urinary calculi by Scheele and by Bergmann in 1776. By 1900, the structure of uric acid had been successfully elucidated by Emil Fischer, after much of the groundwork had been laid by others such as Wöhler, Liebig, and von Baeyer. Many of the methods first used for the study of uric acid by these and other early German researchers have been used again (for example, the now classical work of Sonne, Buchanan and Denuva in the late 1940's) to help discover the biological precursors of purines.

Early interest of physiologists in uric acid centered around its role as a nitrogenous excretory product. It had long been known that the ingestion of nitrogenous compounds, mainly proteins, ultimately resulted in the production of ammonia. Owing to the high toxicity of ammonia to organisms, it must be detoxicated or voided, or both. This fact had also been in the literature for a long time, as had the knowledge of the existence of three major nitrogenous end products, ammonia itself, urea and uric acid (or other purines), when Baldwin and Needham (1934) and Needham (1931, 1935) reported the first of their extensive investigations on nitrogen catabolism in invertebrates. The notion that availability of water to an organism in its habitual environment largely determines the type of nitrogenous end product gained general acceptance after the work of Needham (1935).
Thus, ammonia itself is generally excreted as such when the availability of water is essentially unlimited. The less toxic urea is the major nitrogenous end product in organisms with limited, but fairly liberal supplies of water. Uric acid, equally innocuous, is excreted in nearly solid form by animals that are severely limited in water supply.

Birds, terrestrial reptiles, some gastropods and many insects detoxify the ammonia produced by incorporating it primarily into uric acid. In these animals, uric acid results mainly from products of protein catabolism, while in other organisms (e.g., man) it is chiefly the special terminal product of purine metabolism.

Studies such as those in the 1930's led to the study of the biosynthesis of uric acid and other purines around 1950. Birds, which are uricotelic, were the animals of choice used by the biochemists for this study. Earlier studies, involving changes in the amount of uric acid excreted by animals that were given certain suspected precursor compounds, were felt to be unreliable. However, in the final analysis, many of these findings do agree with the presently known biosynthetic reactions.

Buchanan, and his co-workers, Sonne and Dellaqua, reported in a series of papers (1946, 1948) the incorporation of $^{13}C$ from certain small molecules into uric acid. Pigeons were injected with the $^{13}C$-labeled compounds and the uric acid was subsequently isolated from their excreta. This uric acid was then subjected to a selective
chemical degradation (a combination of methods used by German workers a century earlier) so that each of its carbon atoms was obtained separately. The fate of the C\textsuperscript{13} label was determined in this way. These investigators showed that C\textsuperscript{13}O\textsubscript{2} was incorporated into carbon 6 of uric acid, H\textsubscript{2}C\textsuperscript{13}O\textsubscript{2}H into carbon 2 and carbon 8, and the carboxyl carbon of glycine-\textsuperscript{1}-C\textsuperscript{13} primarily into carbon 4 of uric acid. Karlsson and Barker (1949) confirmed the previous work using C\textsuperscript{14}-labeled precursors. In addition, they found the methylene carbon of glycine to be incorporated chiefly into carbon 5 of uric acid also isolated from pigeon excreta.

Elwyn and Sprinson (1950) subsequently reported the incorporation of the \textit{\textbeta}-carbon (C\textsuperscript{14}-labeled) of serine into carbons 2 and 8 of uric acid in pigeons. Sakami (1948) had shown that the \textit{\textbeta}-carbon of serine contributes to the same "1-carbon" derivative as does formate.

Greenberg (1948, 1950) demonstrated the incorporation of C\textsuperscript{14}-labeled bicarbonate, formate and glycine into hypoxanthine by homogenates of pigeon liver.

Work on the source of the nitrogen atoms of uric acid was carried out with various N\textsuperscript{15}-labeled compounds. Shemin and Rittenberg (1947) found that glycine was incorporated as a unit into uric acid isolated from the urine of a human male, thus showing that nitrogen 7 arises from glycine. Buchanan, Sonne and Delluva (1948) demonstrated that this occurs in the pigeon as well. Work by Lagerkvist (1953), Sonne and collaborators (1952, 1953a,b, 1956) and Levenberg, Hartman
and Buchanan (1956) showed that aspartic acid is the donor of the nitrogen at position 1 of uric acid (and hypoxanthine) and that the amide nitrogen of glutamine is incorporated into positions 3 and 9 of these purines.

A summary of the findings on the origin of the atoms of the uric acid molecule is given in Figure 1.

Other investigations revealed that the same precursor compounds were used for the synthesis not only of uric acid and hypoxanthine, but also of adenine and guanine. This involved both free purines and nucleic acid-bound purines. Purine synthesis in a wide variety of organisms seemed to utilize the same precursors in a similar fashion; for example, in Torula, an "imperfect" fungus (Dimroth, Jaenicke, and Becker, 1952); the bacterium, Aerobacter aerogenes (Sutton and Werkman, 1953); in mammals, such as the rat (Sprinson, 1951); and in birds, as has already been discussed.

Flaks and Lukens (1963) presented a discussion of the enzymatic steps leading to the biosynthesis of inosinic acid, the first purine formed by the de novo synthetic pathway. The specific reactions involved in the synthesis of purines were elucidated by many different investigators over the past 15 years. Figure 2 is the scheme for the biosynthesis of inosinic acid as modified from Flaks and Lukens (1963). Most of the work was done using pigeons and chickens as the biological material.

The uricotelic invertebrates are less desirable organisms
Figure 1

The origin of the atoms of the uric acid molecule.
Figure 2

Pathway of inosinic acid biosynthesis (modified from Flaks and Lukens, 1963).
to use in studies such as these, mainly because of their small size. The enzyme-catalyzed reactions involved in purine synthesis are all quite difficult to study. Consequently, not much work has been done with organisms other than microorganisms and birds that were used in the original investigations.

Work by Wiener (1902) suggested that birds synthesize uric acid by condensing two molecules of urea with one molecule of a three-carbon dicarboxylic acid (tartronic acid) to form one molecule of that purine according to the scheme given in Figure 3. He reported that malonic acid and mesoxalic acid could replace tartronic acid in the reaction, but that the former must first be oxidized and the latter reduced to tartronic acid by the animal. Wolf (1933) and Baldwin (1935b) reported that snails synthesize uric acid by Wiener's scheme. It has been reported (McEnroe and Forgash, 1957; Desai and Kilby, 1958) that Leifert, in 1935, suggested that uric acid is synthesized in insects by the same reaction. She used larvae and pupae of the moth, *Antheraea pernyi*, whose fat bodies synthesized uric acid from sodium malonate and urea. In one case the synthetic rate was given as 1.464 mg of uric acid/hr/g of fat body (see Desai and Kilby, 1958). But, Brighenti and Colla (1940) showed that injection of urea into silkworms resulted in no increase of uric acid. Barnes and Schoenheimer (1943) showed that N\textsuperscript{15}-labeled ammonia, but not N\textsuperscript{15}-urea, was incorporated into uric acid in pigeons.

Even with evidence against urea serving as a direct
Figure 3

Wiener's (1902) scheme for the synthesis of uric acid in birds.
\[
\text{NH}_2 - \text{C} = \text{O} - \text{CH(OH)} - \text{C} = \text{O} - \text{NH}_2 + 2 \text{H}_2\text{O} \\
\text{(DIALURIC ACID)}
\]

\[
\text{NH}_2 - \text{C} = \text{O} - \text{NH}_2 \\
\text{(UREA)}
\]

\[
\text{COOH} - \text{CH(OH)} - \text{COOH} \\
\text{(TARTRONIC ACID)}
\]

\[
\text{NH}_2 - \text{C} = \text{O} - \text{NH} - \text{NH} - \text{C} = \text{O} - \text{NH}_2 \\
\text{(URIC ACID)}
\]

\[
2\text{H}_2\text{O} + 
\]

\[
\text{(UREA)}
\]
precursor of uric acid in invertebrates, the idea that Wiener's suggestion was correct for invertebrates persisted until very recently. Anderson and Patton (1955) reported that urea did not increase the uric acid when incubated with extracts of fat bodies isolated from larvae of both Prodenia eridania and Tenebrio molitor. When sodium malonate was used as a substrate, the formation of uric acid was inhibited. Thus, further doubt was cast on Leifert's work.

McEnroe and Forgash (1957) showed that female roaches, Periplaneta americana, incorporated HC\textsuperscript{14}OONa primarily into carbons 2 and 8 of uric acid. The labeled formate was injected into the animals and the uric acid was subsequently isolated from their fat bodies. This investigation not only disproved that Wiener's synthetic scheme operates in insects, but also showed the similarity between insects and other organisms previously studied.

Desai and Kilby (1958) attempted to repeat Leifert's observations with larvae (150 to 180 days old) of Calliphora, Schistocerca and Telea. No synthesis of uric acid occurred when sodium malonate and urea were incubated together with the insect tissues. They found 4-amino-5-imidazolecarboxamide stimulated uric acid synthesis when incubated with the same tissue from Calliphora and Schistocerca larvae.

Porembaska and Heller (1962) reported that Heller and Jezewska had shown, in 1959, that the moth used by Leifert (Antheraea pernyi) formed uric acid according to Buchanan's scheme.
Baldwin and Needham (1934) reported that nitrogen excretion in terrestrial gastropods had been studied as early as 1820 and that this work might have indicated the uricotelic nature of these animals. They reported that Marchal, in 1889, had shown that the snail kidney has a high content of uric acid, and that Cooke, in 1895, reported that the uric acid was in the form of spherical concretions in that organ. That uric acid was the primary nitrogenous compound excreted by *Helix pomatia* was shown by Hesse in 1910 (see Bricteaux-Grégoire and Florkin, 1962b).

Baldwin and Needham (1934) concluded that about three-fourths of the dry weight of the *Helix pomatia* kidney is uric acid. They found 660 mg of uric acid/g of kidney (dry), which was said to be equivalent to 131 mg of uric acid/g of fresh kidney. These authors later found 720 and 810 mg of uric acid/g of dry kidney. Needham (1935), in an extensive survey of molluscs, concluded that a uricotelic metabolism, as judged by uric acid content of nephridia, is an adaptation to terrestrial life in gastropods. Baldwin and Needham (1934) felt that final proof of uricotelism in terrestrial gastropods required the demonstration of the biosynthesis of that purine. They cited Wolf, who in 1933 incubated hepatopancreas brei with urea to show that uric acid was formed. However, because less than 1% yield was obtained under unphysiological conditions, they questioned the validity of Wolf's observations.

Baldwin and Needham (1934) reported an attempt to demonstrate
the similarity of uric acid synthesis in Helix to that in birds.
Schuler and Reindel (1933) had just shown that pigeon liver produced some substance that kidney tissue slices, but not liver, from the same animals could convert to uric acid. Edson, Krebs and Model (1936) reported that pigeon liver lacks xanthine oxidase and that the intermediate which accumulates is hypoxanthine. In addition, they showed that pigeon kidney does possess the necessary oxidizing enzyme, xanthine oxidase. Baldwin and Needham did not have the benefit of this information. Indeed, they stated (1934) that both pigeon liver and kidney possess xanthine oxidase, so that purine oxidation by kidney and not by liver would be ruled out of their assumptions. Their findings were as follows: (1) snail hepatopancreas produced some substance that pigeon kidney converted into uric acid and that (2) snail hepatopancreas, while it did synthesize uric acid, could not utilize the substance from pigeon liver to produce uric acid. They explained (1) by showing that it was a xanthine oxidase-catalyzed reaction, and that it did not lie on the direct route leading to the formation of uric acid. The second result was taken by the authors as proof that the synthesis of uric acid in Helix was not the same as it was in the pigeon. It is indeed unfortunate that this experiment failed. The extract of pigeon liver should have contained hypoxanthine, which then should have been converted into uric acid by the snail hepatopancreas, as will be seen later in this paper. In the same investigation, Baldwin and Needham demonstrated the presence of xanthine oxidase
(their work would not allow one to differentiate between oxidase and dehydrogenase activity) in Helix hepatopancreas. This, as the authors stated, was surprising in view of their results given above. Reflecting perhaps, Baldwin (1957) wrote, concerning uricogenesis in invertebrates:

The outlook in this field has been much prejudiced by the view, now abandoned in so far as it affects uricotelic vertebrates, that urea is an intermediary in the synthesis. The best attitude to adopt at the present time is one of ignorance.

Baldwin (1935a) used some of Needham's (1935) values for uric acid content of kidneys of various gastropods in combination with his own data for arginase levels in the hepatopancreas of the same animals. A double-logarithmic plot of nephridial uric acid content against arginase activity for the different species resulted in an increasing linear curve. Baldwin suggested that arginase may be related to uric acid synthesis in these animals.

In a second paper, Baldwin (1935b) tested the suggestion (Wolf, 1933; Baldwin, 1935a) that uric acid was synthesized by Helix pomatia by Wiener's (1902) scheme. Tissue slices of Helix hepatopancreas were incubated for 5 hr at 28°C with constant shaking. The incubation fluid was a Ringer's solution with galactose added. Some preparations contained both tartronic acid and urea, others only urea, while the controls contained neither substrate. One control was not incubated, and the uric acid of the tissue was determined directly. The other control preparation was incubated for 5 hr along with the experimentals. This control was used to measure the endogenous
synthesis of uric acid. The uric acid synthesized in excess of this second control by the experimental preparations was expressed as percent increase over that control. A typical set of values showed a 47.5% increase when both urea and tartronic acid were present, and 9.9% when only urea was present. But, Baldwin did not report the use of any control preparations that contained both urea and tartronic acid. This was unfortunate, for even Baldwin stated that Wiener (1902) had shown the reaction to occur non-enzymatically. Also, whether or not the presence of tartronic acid and urea would result in increased color under the conditions for uric acid determination was not given. The failure to include these controls makes their observations of little significance.

After the present investigation was begun, two papers by Bricteux-Grégoire and Florkin (1962a,b) appeared. Bricteux-Grégoire and Florkin reported the investigation of uric acid synthesis in *Helix pomatia*. The snails were injected with urea-$^{14}$C and the uric acid was isolated from the whole animals after 24 hr. Under these conditions, uric acid contained radioactivity. The fate of the urea-$^{14}$C incorporated into the carbon atoms of uric acid was determined after its selective degradation, and the results obtained were as follows:

<table>
<thead>
<tr>
<th>CARBONS</th>
<th>% OF ACTIVITY IN URIC ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 &amp; 8</td>
<td>0.3</td>
</tr>
<tr>
<td>6</td>
<td>62.</td>
</tr>
<tr>
<td>4 &amp; 5</td>
<td>23.</td>
</tr>
</tbody>
</table>
The ratio of activity in carbon 4 to carbon 5 was 133:31. Thus, if urea had been incorporated into uric acid as predicted by Wiener's (1902) scheme, the major portion of the activity should have been found in carbon atoms 2 and 8. Instead, the $^{14}C$ was incorporated mainly into carbon atom 6. The authors interpreted this finding as evidence that *Helix* synthesizes uric acid according to Buchanan's scheme for uric acid biosynthesis in birds as well as the other organisms studied. It was suggested that urea-$^{14}C$ was degraded by urease in the snail and the resulting $^{14}CO_2$ incorporated into uric acid. These observations ended the view held until their publication that uric acid is formed by Wiener's scheme in *Helix*.

Thiele (1963) questioned whether the free purines he found in whole *Helix pomatia* individuals arose metabolically or were ingested by the animals. In addition to uric acid, he found free xanthine and guanine.

The present investigation was undertaken to establish in greater detail the origin of the carbon atoms in the uric acid of *Otala lactea*. If uric acid were to be found in *Otala* kidney and excreta, then it could be reasonably assumed (Needham, 1935) that these snails are uricotelic. This was predicted from the knowledge that *Otala lactea* is primarily a desert-inhabiting gastropod (Cooke, Shipley and Reed, 1959), and is, at best, severely limited as regards the natural availability of water to it. If the above were found to be true, then the use of *Otala lactea* as the experimental animal would be
justified.

In view of all the evidence (excluding that given by Bricteux-Grégoire and Florkin, 1962a,b) that purine synthesis involves generally the same precursor compounds by similar mechanisms in a wide variety of organisms, it was postulated that a uricotelic gastropod would follow the same pattern. Rather than repeat Baldwin's (1935b) work, this study was undertaken to answer the general question given above; that is, does *Otalia lactea* follow Buchanan's scheme for the synthesis of uric acid? In the meantime, Bricteaux-Grégoire and Florkin (1962a,b) were found to have directly negated Baldwin's (1935b) work.
MATERIALS AND METHODS

Biological Materials

The snails used throughout the course of this study were *Otalora lactea* (Müller, 1774) as identified by reference to Pilsbry (1939). They were obtained in bulk lots from Salvatore Scozzaro Wholesale Groceries, Brooklyn, New York. During the early part of this investigation, snails were maintained in an aestivating state at 4º. It was found, however, that the survival of the snails was more favorable at room temperature (around 25º). The snails remained in a dormant state under these conditions.

Active snails were obtained by increasing the moisture of their environment. Lettuce and cabbage were used as the source of nutriment. A food source also tended to maintain the activity of the snails, provided the moisture was fairly high.

Domestic chickens, obtained from a local poultry house, were required as sources for control enzyme preparations in a number of cases.

Chemical Materials

All chemicals used were of the highest purity available. The sources of specific reagents will be given in the individual discussions of the methods employed.
General Methods

The contents of this section will be devoted to certain of
the methods employed most frequently throughout the course of this
study. Where methods were used only once or infrequently, their
descriptions may be found along with the results in the following
section.

The identification and determination of uric acid. The
method used for the identification of uric acid depended upon the
conditions of the particular experiment. Ultraviolet absorption
spectral analysis (Praetorius, 1948) was used where interference from
other substances was found to be low. This method could also be used
for determining the concentration of uric acid in the sample solutions,
knowing the molar extinction coefficient at the absorption maximum of
uric acid \((12.6 \times 10^3\) at 292 \(\text{m} \mu\) at pH 9.4, Praetorius, 1948). This
was also employed for determining the amounts of other purines
(xanthine and guanine) at their respective maxima.

A colorimetric method was found to be quite suitable for the
identification and determination of uric acid even in the presence of
many other components of tissues. The procedure was a slight modifi-
cation of that given by Fearon (1944). Uric acid forms a bright yellow
compound by reaction with 2,6-dichloroquinonechlooroimide in borate
buffer at pH 10. The procedure was modified for use with a Coleman,
Jr. colorimeter, the samples being read at 435 \(\text{m} \mu\) exactly 1 min. after
mixing in the chloroimide reagent, since there was a fairly rapid
decay of the colored complex with time (approximately 0.007 optical density units/min. for the first 20 min., which was found to be the region of linear decay). Reading the tubes this rapidly (rather than after 5 to 10 minutes, as suggested by Fearon) has several advantages: (1) sensitivity is maximal, being in the same order of magnitude as that found with measurement by ultraviolet absorption. Linearity was found over the range tested (0.5 to 50 µg of uric acid). (2) The only other known substances giving a yellow color with this reagent under the conditions employed are cysteine and reduced glutathione (Fearon, 1944), but these compounds do not react within the short time period used.

KCN, which was used as an inhibitor of uricase activity in certain cases, did not interfere with the determination of uric acid by either of the above procedures.

The chloroimide reagent was also used as a spray reagent for locating uric acid on paper chromatograms. While this use was devised and tested in the present study, a search of the literature revealed its description by Forrest, Hatfield and Lagowski (1961), who also showed that methyl derivatives of uric acid and ribosyl uric acid reacted with the chloroimide.

Paper chromatography was also used to identify and separate uric acid. Several solvent systems were employed. The following system (Jezewska, Gorzkowski and Heller, 1963a) was initially used for qualitative identification of uric acid and other kidney purines:
ascending chromatography on Whatman No. 3 filter paper first with 1-propanol:water, 3:1 by volume (Rabinowitz, 1956), then, in the same dimension, with acetone:1-butanol:water, 8:1:1 by volume (Dorough and Seaton, 1954). The samples to be chromatographed were dissolved in 0.1% Li₂CO₃ prior to spotting.

For subsequent separation of purines, where better resolution of the components was required, the following method was employed: the tissue was homogenized in water, and measured portions were thoroughly dissolved in saturated Li₂CO₃. The samples (50 to 100 μl portions) were then spotted and developed in the two-dimensional system of Carter (1950). This solvent system is 1-butanol saturated with a 10% aqueous solution of urea, followed by 5% Na₂HPO₄ saturated with 2-methyl-4-butanol under a layer of the same alcohol in the second dimension. Uric acid, guanine, xanthine and hypoxanthine all separated quite well from each other.

After separation, the compounds were visualized as spots under ultraviolet light. Where required, the various compounds were eluted from the paper with 0.1 N HCl (guanine or hypoxanthine) or saturated Li₂CO₃ (uric acid and xanthine). Partial identification and estimation of the purines was by ultraviolet absorption analysis in the Beckman DU spectrophotometer.

**Isolation and purification of uric acid.** While it was found to be possible to isolate and purify uric acid crystals from Otala tissues and excreta by Korn's (1957) combination of the methods of
St. John and Johnson (1931) and Fisher (1935), an easier and more successful means of accomplishing the same is the following modification of the procedure given by McEnroe and Forgash (1957): the tissue (or excreta) was homogenized in approximately 0.1 M NaOH, and concentrated NaOH solution was added dropwise until dissolution of concrements appeared to be complete. The uric acid was precipitated from solution upon addition of concentrated HCl. The liquid was evaporated to approximately the volume of HCl added with the aid of infrared lamps. This insured the complete precipitation of the uric acid. The precipitate was washed as outlined in the original method, after which it was redissolved in NaOH solution and reprecipitated 3 times, washing the precipitate each time. It was found to be practical to add known amounts of uric acid to the homogenates as carrier.

Li$_2$CO$_3$ is usually used for the dissolution of uric acid, because uric acid tends to be degraded more rapidly in NaOH. However, almost immediate acidification of the solutions with HCl and precipitation of the uric acid prevent degradation. The use of NaOH in lieu of Li$_2$CO$_3$ avoids the necessarily time-consuming step of heating the uric acid in Li$_2$CO$_3$ solution at 80° to yield complete dissolution as called for by the parent method.

The uric acid obtained from Otala nephridia and hepatopancreases was chromatographically pure, showing only one ultraviolet-absorbing spot (corresponding to uric acid), and was free of ninhydrin-positive material. Figure 4 shows the spectrum obtained for the uric acid.
Figure 4

Comparison of ultraviolet absorption spectra of uric acid isolated from *Oxala* nephridia before (curve 1) and after (curve 2) purification.
isolated by the method described as compared with the spectrum of the initial extract (before purification) of *Otalora* nephridia. The latter shows a shift to the left of the purified product, which conformed exactly to a standard solution of uric acid. When C\textsuperscript{14}-labeled uric acid was isolated by this method, constant specific activity (cpm/mg) was obtained.

*Incubation of snails with suspected precursor compounds.* All of the snails used for the injection of suspected precursors were fed on lettuce before and during the experiment. The precursors, NaH\textsubscript{14}O\textsubscript{3} (Vclk), H\textsubscript{14}OONa (CalBioChem), glycine-1-C\textsubscript{14} (New England Nuclear), glycine-2-C\textsubscript{14} (New England Nuclear), serine-3-C\textsubscript{14} (CalBioChem), 4-amino-5-imidazolecarboxamide hydrochloride-2-C\textsubscript{14} (CalBioChem) and hypoxanthine-2-C\textsubscript{14} (CalBioChem), were each prepared in 2.0 ml of autoclaved *Helix* saline (after Pantin, as given by Welsh and Smith, 1960) containing buffered penicillin G (Campbell and Bishop, 1963), streptomycin (Campbell and Bishop, 1963), 10 μcuries of suspected precursor, about 20 μmoles of HCOONa, 10 μmoles of glycine and 1 mg of casein hydrolysate. Each snail received two 0.1 ml injections daily for 3 consecutive days. The injections were made through a small hole drilled in the shell 2-1/2 turns from the apex, in the region of the hepatopancreas. The holes were sealed with paraffin after each injection. On the beginning of the fifth day from the first injection, the snails were sacrificed and the nephridia and hepatopancreases were pooled separately. The uric acid was isolated by the modified method of McEnroe and Forgash (1957).
The uric acid crystals obtained were plated and the radioactivity was determined using gas-flow counting apparatus. The uric acid was then degraded by procedures discussed below in order to determine the atom(s) into which the precursor was incorporated.

The incubation of Otala tissues directly with precursor compounds was carried out in much the same way as described above for the injection of these compounds, utilizing the same saline preparations which now bathe the tissue preparations. Specific details are given along with the presentation of the results of such experiments.

Selective chemical degradation of uric acid into component carbon atoms. After the C\textsuperscript{14}-labeled uric acid was isolated and purified, it was degraded so that each carbon atom could be obtained separately and quantitatively. The methods for accomplishing this were outlined by Buchanan, Sonne and Dellaqua (1948) and Sonne, Buchanan and Dellaqua (1948). The methods, as given by these authors, were used in the present work with only slight modifications. Figure 5 shows the scheme given by the authors as shown in Buchanan (1951) and modified by Brandenberger (1956) for obtaining carbons 4, 5 and 6 separately and carbons 2 and 8 mixed. Figure 6, from Buchanan (1951), shows the method used for separating carbons 2 and 8. The primary modification used in this laboratory was to determine (after Ratner, 1955) the amount of urea present for carbon 2 and for carbon 8, for the urea recovered containing carbon 8 was in excess of that from carbon 2. These two solutions were equalized as to urea content, by using an appropriate portion of
Degradation of uric acid by alkaline manganese dioxide oxidation (modified from Buchanan, 1951, and Brandenberger, 1956). DHP = β-diureido-β-hydroxypyruvic acid; TDP = α,α,β-trihydroxy-β-diureido-propionic acid; DCUI = 4,5-dihydroxy-4-carboxy-5-ureido-imidazolidone-2 (uroxanic acid).
Figure 6

Degradation of uric acid by acid perchlorate oxidation (after Buchanan, 1951).
the solution containing carbon 8. Thus, while the level of radioactivity was lower in both cases, any disproportion found indicated a differential incorporation of precursor carbon rather than unequal recovery of urea.

The carbon atoms released during the degradation were evolved as CO$_2$, which was trapped in NaOH as BaCO$_3$ essentially as given in Comar (1955). Correction for self-absorption was made by comparison with standard curves.
RESULTS AND DISCUSSION

Nephridial and Excretal Purines

Before *Otala lactea* could be used as the experimental animal in this study of uric acid biosynthesis, it was necessary to show that this species of terrestrial gastropod is uricotelic. It was previously predicted that *Otala* should exhibit uricotelism. Because Jezewska, Gorzkowski and Heller (1963a) had reported that *Helix pomatia* nephridia and excreta contain not only uric acid, but xanthine, guanine and an unidentified purine as well, *Otala lactea* was examined for all of these purines. Coupling chromatographic and spectral analyses, xanthine and guanine were shown to occur in the kidneys of aestivating and feeding snails. Two unknown compounds were also demonstrated. Figures 7 and 8 show the acid and alkaline spectra obtained for these compounds after eluting them from the chromatograms. The spectra in Figure 7 are from the unknown with the least mobility, while those in Figure 8 are from the unknown with the greatest mobility in the chromatographic system used. The spectra for the first unknown (Figure 7) are of questionable value, because the amount of material was not sufficient to obtain good resolution. Neither compound was identified from its absorption spectra. Excreta obtained from snails that had just come out of aestivation possessed the same purines and unknown compounds. Neither of the unknown compounds reacted with diphenylamine (Schneider, 1957) or orcinol (Schneider, 1957), so the unknowns contained neither

(28)
Figure 7

Ultraviolet absorption spectra of unknown compound 1 isolated from Otala kidney.
Figure 8

Ultraviolet absorption spectra of unknown compound 2 isolated from Otala kidney.
deoxypentose nor pentose, respectively.

In order to estimate the relative concentrations of uric acid, xanthine and guanine in *Otala*, the kidney of an aestivating snail was extracted and chromatographed using Carter's (1950) solvent systems. The purines were eluted from the chromatogram and the amounts were determined with the aid of the Beckman DU spectrophotometer. The levels (in mg of purine/g of fresh kidney) obtained were as follows: uric acid, 144; xanthine, 30.9; and guanine, 16.8. This gave a percent distribution of 75.2:16.1:8.7 for uric acid:xanthine:guanine. This ratio compared favorably with that obtained by Jezewska, Gorzkowski and Heller (1963a) for aestivating *Helix pomatia*. These authors reported 76.8:13.4:9.8 as the ratio of the same purines.

From the foregoing results, it was evident that *Otala lactea* accumulates uric acid and other purines in its kidney and that these purines are excreted. In several cases, excretal concrements were followed over the course of their passage from the kidney to the outside of the animal. Their uric acid concentration was determined (about 30% uric acid, fresh weight), as given later in this paper. *Otala lactea* thus met the usual criteria for uricotelism (Needham, 1935), and was considered to be suitable as such for the study of uric acid biosynthesis.

Neither of the two unknown compounds gave an ultraviolet absorption spectrum similar to that given by Jezewska, Gorzkowski and Heller (1963a) for the unknown purine from *Helix*. In a later paper
(1963b), these authors concluded that the unknown purine in *Helix* was an ingested compound, since it accumulated only in the kidneys of feeding snails.

Jezewska, Gorzkowski and Heller (1963a,b) reported a change in the ratio of purines from both nephridia and excreta under different environmental conditions. Seasonal changes were also reported. The lack of solid excreta from *Otala* under extremely moist feeding conditions largely prevented this study. Liquid excreta appear to have been passed under such conditions, but this awaits further examination. Jezewska, Gorzkowski and Heller (1963a) reported that Delaunay, in 1927, had assumed that *Helix pomatia* eliminated liquid rather than solid excreta during moist feeding conditions. The former authors never observed the formation of liquid excreta by members of the same species.

The question of the interconversion of purines in *Otala* arose as a result of several recent publications. Jezewska, Gorzkowski and Heller (1963a) postulated that, when *Helix* undergoes aestivalion, there is a reversible amination of xanthine to form guanine. This was suggested primarily from their finding an increased guanine to xanthine ratio in the kidneys of these snails.

Secondly, Hayashi, in a series of publications (1960, 1961, 1962), found that the uricotelic silkworm, *Bombyx mori*, possesses xanthine dehydrogenase, but not xanthine oxidase. Preliminary evidence from work with *Otala* indicated the possible absence of xanthine oxidase in kidney, blood and hepatopancreas.
Portions of kidney homogenate, hepatopancreas homogenate and blood from aestivating snails were incubated in the presence of 25 μg of hypoxanthine. Both homogenization and incubation were done in 0.05 M phosphate buffer, pH 7.5 (Allard, DeLamirande and Cantero, 1957). The kidney and blood preparations were incubated, in a total volume of 3.0 ml, for 45 min. at room temperature in 1 cm quartz cuvettes. Controls which lacked either the tissue or the substrate were incubated simultaneously. Absorbancy measurements at 271.3 μμ (the hypoxanthine maximum) and at 292 μμ (the uric acid maximum) were made at 2 and 5 min. intervals. There was no decrease in absorbancy at the former wavelength or increase at the latter during this time. No xanthine oxidase was thus demonstrated under these conditions.

The hepatopancreas preparation, with its controls, was incubated in glass tubes for 0, 15, 30 and 60 min. as above. The reactions were stopped by placing the tubes in a boiling-water bath for 3 min. Uric acid was determined colorimetrically by the modified method of Fearon (1944). No increase in uric acid occurred under these conditions.

Despite these findings, hypoxanthine-2-C14 was shown to be readily converted into uric acid when the former was injected into specimens of Otala lactea. In addition, Baldwin and Needham (1934) showed that tissue slices of Helix hepatopancreas contained "xanthine oxidase" activity. The conditions of their experiments would not allow one to differentiate between an oxidase or a dehydrogenase enzyme,
however. While it is clear that *Helix* and *Otalpa* convert hypoxanthine and xanthine to uric acid, the chemical nature of this transition remains to be shown.

An experiment was designed to determine whether or not uric acid was readily convertible to xanthine, hypoxanthine or guanine by *Otalpa*. The sodium salt of uric acid-2-$^{14}C$, pH 7.0, was injected into a feeding snail over a two-day period. The total activity injected was about $2.16 \times 10^6$ cpn. At the beginning of the third day, the nephridium was removed and homogenized (1:19, weight:volume) in water. A 0.5 ml sample was rehomogenized in 4.5 ml of saturated Li$_2$CO$_3$ solution, and a 100 $\mu$l portion of this was subjected to chromatography on Whatman No. 1 filter paper. In addition, 100 $\mu$l of excreta homogenate (1:199, w:v, in saturated Li$_2$CO$_3$ solution) and a sample of the uric acid-2-$^{14}C$ used for injection were similarly treated. Activities of about 1700 cpn for kidney extract and 300 cpn for excreta extract were subjected to chromatography. The chromatograms were developed by the use of Carter's (1950) solvent systems. The chromatograms were exposed to Ansco non-screen x-ray film for 3 weeks. The radioautographs revealed the following: (1) the uric acid-2-$^{14}C$ used for injection contained several (6 spots other than that for uric acid) radioactive contaminants, none of which was xanthine or hypoxanthine. The radioactive spot at the origin may have contained guanine, but this was not determined. (2) The radioautograms of both kidney and excretal extracts showed only one radioactive spot, which corresponded exactly to that for uric acid in
both cases. The former extract gave a very dense spot, while the latter gave a rather light one.

There was no measurable conversion of uric acid-2-C\textsuperscript{14} to any other substance in the kidney extract. The level of activity in the excretal extract was too low to make any generalizations, but no conversion was observed there either. Whatever the radioactive contaminants of the uric acid preparation were, they were not found in the extracts of the snail kidney or excreta.

Uric acid probably does not undergo significant conversion to other purines in *Otala lactea*. This was taken as one further piece of evidence of the uricotelic behavior of this animal.

Uric Acid Content of *Otala* Tissues

From the work discussed in the preceding section it was shown that uric acid is the most abundant purine in *Otala* kidney. More precise measurements of uric acid in kidney and other tissues were developed whereby tissue extracts could be analyzed directly for this purine without prior separation by chromatography. Both colorimetric and spectrophotometric procedures were employed for this.

Table I is a compilation of average concentrations of uric acid in various parts and excreta of specimens of *Otala*. The numbers in parentheses represent the number of snails used in the determinations. The tissue from snails kept at 4° was homogenized directly in 0.1 M sodium borate/KCl buffer, pH 9.2. The tissue from snails kept at room
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Aestivating (cold)</th>
<th>Aestivating (room temp.)</th>
<th>Feeding (room temp.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>111.5 (6)</td>
<td>198.2 (11)</td>
<td>124.8 (19)</td>
</tr>
<tr>
<td>Hepatopancreas</td>
<td>0.624 (5)</td>
<td>2.827 (6)</td>
<td>2.922 (6)</td>
</tr>
<tr>
<td>Apical Lobe</td>
<td>0.376 (3)</td>
<td>---</td>
<td>4.059 (4)</td>
</tr>
<tr>
<td>Outer Lobe</td>
<td>0.420 (3)</td>
<td>---</td>
<td>2.276 (4)</td>
</tr>
<tr>
<td>Inner Lobe</td>
<td>0.435 (3)</td>
<td>---</td>
<td>2.153 (4)</td>
</tr>
<tr>
<td>&quot;Kidney&quot; Lobe</td>
<td>0.556 (3)</td>
<td>---</td>
<td>2.214 (4)</td>
</tr>
<tr>
<td>Hepatopancreatic Membrane</td>
<td>0.313 (3)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Blood</td>
<td>0.410 (4)</td>
<td>---</td>
<td>0.125 (2)</td>
</tr>
<tr>
<td>Heart</td>
<td>---</td>
<td>---</td>
<td>0.460 (4)</td>
</tr>
<tr>
<td>Reproductive System (excluding gonad)</td>
<td>---</td>
<td>---</td>
<td>0.232 (4)</td>
</tr>
<tr>
<td>Albumen Gland</td>
<td>0.301 (3)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Gonad</td>
<td>---</td>
<td>---</td>
<td>0.530 (4)</td>
</tr>
<tr>
<td>Foot Muscle</td>
<td>---</td>
<td>---</td>
<td>0.242 (4)</td>
</tr>
<tr>
<td>Excreta</td>
<td>---</td>
<td>---</td>
<td>330.0 (6)</td>
</tr>
</tbody>
</table>
temperature (r.t.) was homogenized in approximately 0.1 M NaOH and portions were diluted with borate/KCl buffer. The values obtained using these two different homogenizing fluids were normalized using a conversion factor obtained for kidney and hepatopancreas preparations. The lobes of the hepatopancreas were so designated as to correspond to their position relative to the digestive tract, i.e., apical to, outside the loop of and inside the loop of the digestive tract. The lobe designated "kidney" is adjacent to the kidney.

It is evident that the kidneys of these snails contained rather high concentrations of uric acid, and that some uric acid was present in all the tissues examined. The uric acid concentration in hepatopancreatic tissue was not as variable as it was in the kidneys. For example, in 13 feeding snails studied for kidney uric acid, the levels varied from 48 to 194 mg of uric acid/g of fresh tissue. This is to be expected, however, because the kidney is an organ of accumulation and elimination of this purine (see Needham, 1935, for Helix pomatia; present observations for Otala lactea).

While the values obtained using the different homogenizing media were made comparable, the snails used (i.e., those kept at 4°C against those at room temperature) were from different lots. Those snails taken from 4°C had been maintained at that temperature for several months before being used, whereas the ones maintained at room temperature were studied soon after their arrival from the distributor. The difference in values for uric acid concentration between the two
different aestivating groups may have resulted from their being members of different colonies, the difference in storage temperature or the difference in duration of inactivity, or any combination of these factors.

The values obtained for uric acid concentration of excreta were always higher than those for kidney. As can be seen in Table I, the kidneys of aestivating (room temperature) snails had a higher concentration of uric acid than did those of feeding snails. This may have been the result of some differential excretion or an increased water content of the kidneys of the feeding snails, or both. The average uric acid content of a single kidney was 33 mg for 11 aestivating snails and 26.2 mg for 19 feeding snails. Active, feeding snails eliminate uric acid, so the uric acid content of their kidneys would be expected to be lower than that of aestivating snails, as was the case. The average weight of the 11 kidneys from aestivating snails was 166 mg, while that of the 19 kidneys from feeding snails was 208 mg. The weight differences were probably a reflection of water content.

Comparison of the values obtained for uric acid in the various tissues of Otala with those found by other investigators for other species of snails is difficult. Not only do the methods of extraction and determination of uric acid differ, but seasonal differences (Jezewska, Gorzkowski and Heller, 1963b) in the animals themselves make this of doubtful value. The following values are given for rough comparison. Baldwin and Needham (1934) reported Helix pomatia had 131 mg of uric acid/g of fresh kidney at the end of aestivation. This was
given as the equivalent of 660 mg of uric acid/g of dry kidney. Other values given were 720 and 810 mg of uric acid/g of dry kidney, or 143 and 161 mg of uric acid/g of fresh kidney. The average weight of these kidneys was given as 240 mg, so that each kidney averaged 34.8 mg of uric acid. Jezewska, Gorzkowski and Heller (1963a) found average values of 94.0 and 22.5 mg of uric acid for kidneys from aestivating and feeding snails, respectively. They also found 65.4 mg of uric acid in 100 mg of dry excreta, the first after aestivation. This later fell to an average of 42.8 mg of uric acid/100 mg of dry excreta during the feeding period. The present study revealed an average of 33 mg of uric acid/100 mg of fresh Otala excreta.

Needham (1935) surveyed a considerable number of molluscs for nephridial uric acid. This list is too extensive to review here. After Helix pomatia, the next highest nephridial uric acid concentration was in Bulimulus sporadicus (38.3 mg of uric acid/g of fresh kidney). Other terrestrial pulmonates had similar values, with some being as low as 2.93 mg of uric acid/g of fresh kidney.

Otala lactea nephridia and excreta contained about the same amounts of uric acid as had been found previously for Helix pomatia. The latter species was shown to possess considerably more uric acid than other terrestrial gastropods studied (Needham, 1935). Therefore, the use of Otala lactea in this study received further justification.

Of the other tissues of Otala examined for uric acid concentration, only the hepatopancreas has been studied in other species of
snails. Baldwin (1935b) reported finding between 0.1 and 1 mg of uric acid/g of fresh hepatopancreas from *Helix pomatia*. These values are roughly similar to those found here.

The concentration of uric acid in Otala blood was within the range of values known for a wide variety of animals (see, for example, Wilber, 1948). The membrane enveloping the hepatopancreas in Otala often contains white concrements, which were shown here not to be uric acid.

Baldwin (1935a) suggested that there is a direct correlation between nephridial uric acid concentration and the activity of arginase in the hepatopancreas of several species of terrestrial gastropods. This work led to his paper (1935b) dealing with the involvement of urea in the synthesis of uric acid by *Helix pomatia*. Arginase was then regarded as the primary link between urea and uric acid synthesis in these animals.

Lal and Saxena (1952) studied *Pila globosa*, an amphibious snail that develops through a cleidoic egg. Animals developing from cleidoic eggs are generally uricotelic (see Needham, 1931, 1935). These snails are active in fresh water during the rainy season and burrow in the mud to aestivate during the winter months. Lal and Saxena found the uric acid content of the nephridia to increase during aestivation to a maximum of about 145 mg/g dry weight and decrease while the animals were active in water to about 30 mg/g dry weight after 27 days. Yet, the arginase levels in the hepatopancreases of these snails showed
no appreciable differences while the uric acid levels changed. Thus, unless the snails were synthesizing uric acid at the same rate (this was not measured) under the different conditions, Baldwin's correlation between nephridial uric acid concentration and arginase levels in the hepatopancreas is questionable.

The correlation between nephridial uric acid levels and hepatopancreatic arginase levels in *Otala lactea* was studied. Fifteen snails were isolated in individual finger bowls and were placed on a diet of cabbage given *ad libitum* for 18 days. The intake of each snail was measured by difference in fresh cabbage weight every second day. The changes in weight of each snail were recorded as well. The finger bowls were covered so that the humidity would be roughly constant. The snails were maintained at room temperature under controlled conditions of light and dark, alternating 12 hr of each. At the end of the 18-day period, the snails were sacrificed, and the kidneys of 13 of them were removed, weighed and analysed for uric acid concentration and content. Dr. James W. Campbell assayed hepatopancreas tissue from each snail for arginase activity as a part of a separate experiment, using the procedures outlined in his (1961) publication.

Table II gives the values obtained for kidney weights, uric acid concentration and content of kidneys and arginase activity of hepatopancreases.

There was no correlation between uric acid in the kidney and arginase activity of hepatopancreases.
<table>
<thead>
<tr>
<th>Snail No.</th>
<th>Average Specific Activity of Arginase (µM/mg prot./hr)</th>
<th>Fresh Kidney Weight (g)</th>
<th>Uric Acid Conc. (mg/g)</th>
<th>Uric Acid Content (mg/kidn.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>171.9</td>
<td>0.367</td>
<td>163</td>
<td>59.8</td>
</tr>
<tr>
<td>2</td>
<td>113.5</td>
<td>0.224</td>
<td>69</td>
<td>15.5</td>
</tr>
<tr>
<td>3</td>
<td>145.2</td>
<td>0.250</td>
<td>127</td>
<td>31.8</td>
</tr>
<tr>
<td>4</td>
<td>74.8</td>
<td>0.228</td>
<td>80</td>
<td>18.3</td>
</tr>
<tr>
<td>5</td>
<td>254.2</td>
<td>0.210</td>
<td>48</td>
<td>10.1</td>
</tr>
<tr>
<td>6</td>
<td>197.0</td>
<td>0.295</td>
<td>134</td>
<td>39.5</td>
</tr>
<tr>
<td>7</td>
<td>81.0</td>
<td>0.190</td>
<td>117</td>
<td>22.2</td>
</tr>
<tr>
<td>8</td>
<td>155.9</td>
<td>0.202</td>
<td>116</td>
<td>23.5</td>
</tr>
<tr>
<td>9</td>
<td>101.4</td>
<td>0.202</td>
<td>132</td>
<td>26.7</td>
</tr>
<tr>
<td>10</td>
<td>49.8</td>
<td>0.274</td>
<td>119</td>
<td>32.6</td>
</tr>
<tr>
<td>11</td>
<td>350.4</td>
<td>0.250</td>
<td>138</td>
<td>34.5</td>
</tr>
<tr>
<td>12</td>
<td>331.4</td>
<td>0.356</td>
<td>194</td>
<td>69.0</td>
</tr>
<tr>
<td>13</td>
<td>151.2</td>
<td>0.197</td>
<td>65</td>
<td>12.8</td>
</tr>
</tbody>
</table>
There was no correlation between uric acid in the kidney and arginase activity of the hepatopancreas of the snails under the conditions of this study. The uric acid concentration and content of these kidneys were directly proportional to each other over a portion of the range, then tended toward a constant concentration of uric acid with increasing content. Figure 9 is a plot of this relationship. Further measurements along this curve would be difficult to make, for excretion of the kidney contents becomes limiting.

Degradation of Uric Acid by Otala Tissues

Although it was shown in the preceding experiments that uric acid is abundant in the tissues of Otala lactea, the possibility existed that this purine might be degraded by enzyme-catalyzed reactions. In order to test this possibility, two different types of experiments were employed. The first involved incubation of minced hepatopancreas with C\textsuperscript{14}-labeled uric acid and the subsequent measurement of radioactivity in the CO\textsubscript{2} fractions derived directly and from urea. Both CO\textsubscript{2} and urea are products formed in the oxidation of uric acid by many animals (see, for example, Prosser and Brown, 1961, Chapter 6). The second approach involved a search for uricase activity in various preparations of hepatopancreas and kidney, for only partial degradation of uric acid would not be evident using the preceding procedure.

From 0.5 to 1.0 g of Otala hepatopancreas minces were incubated with uric acid-2-C\textsuperscript{14} (Volk), sodium or lithium salt, in 2.5 ml of Helix
Figure 9

Plot of uric acid concentration against uric acid content of kidneys from 13 individuals of Otala.
saline containing antibiotics. The vessels, Warburg flasks, were shaken constantly at 30\(^\circ\) for 1, 2 and 3 hr. Heat-killed tissue and controls lacking tissue were also incubated for 3 hr. The center-well of each vessel contained 0.35 ml of CO\(_2\)-free 3.5 M NaOH. The reactions were stopped with 0.45 ml of 5 N H\(_2\)SO\(_4\), which was added by "tipping-in" from the side-arm of the flask. The vessels were shaken 1 hr beyond the addition of the H\(_2\)SO\(_4\) to insure complete evolution of the CO\(_2\) from the reaction mixture and its absorption in the NaOH solution in the center-well. The tissue and the incubation medium were centrifuged at 40,000 x g and the residue was washed with 1 N H\(_2\)SO\(_4\). The supernatant fluids were combined and the pH was adjusted to 7.0. The urea contained in this solution was degraded with jackbean urease (Sigma, Type II), and the CO\(_2\) evolved was trapped in NaOH solution, then plated on aluminum planchets as BaCO\(_3\). The radioactivity of both preparations was measured using a gas-flow counter, and is expressed as cpm/mg of BaCO\(_3\)/cm\(^2\), corrected for self-absorption, in Table III. The values were adjusted to represent the total activity resulting from incubation with 1 g of tissue.

From these results, it is fairly clear that there was no total degradation of lithium urate, and very little (1% or less in 3 hr), if any, of sodium urate. Uric acid labeled in the number 2 carbon would have to have been degraded as far as urea for it to have been detected. Hepatopancreas from Otaia lactea possesses urease (Linton and Campbell, 1962), so at least some of the urea produced would probably be converted
TABLE III

STUDY OF DEGRADATION OF URIC ACID-2-$^{14}$C
BY OTALA HEPATOPANCREAS MINCES

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity from Metabolic Carbon</th>
<th>Radioactivity from Urea Carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium urate:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat-killed tissue, 3 hr</td>
<td>2.6</td>
<td>14.5</td>
</tr>
<tr>
<td>No tissue, 3 hr</td>
<td>0.3</td>
<td>12.9</td>
</tr>
<tr>
<td>1 hr</td>
<td>5.0</td>
<td>7.6</td>
</tr>
<tr>
<td>2 hr</td>
<td>8.2</td>
<td>6.8</td>
</tr>
<tr>
<td>3 hr</td>
<td>6.7</td>
<td>7.0</td>
</tr>
<tr>
<td>Sodium urate:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat-killed tissue, 3 hr</td>
<td>0.6</td>
<td>26.3</td>
</tr>
<tr>
<td>No tissue, 3 hr</td>
<td>1.6</td>
<td>14.6</td>
</tr>
<tr>
<td>1 hr</td>
<td>33.2</td>
<td>33.1</td>
</tr>
<tr>
<td>2 hr</td>
<td>34.8</td>
<td>11.8</td>
</tr>
<tr>
<td>3 hr</td>
<td>39.2</td>
<td>29.7</td>
</tr>
</tbody>
</table>

The radioactivity is expressed as cpm/mg of BaCO$_3$/cm$^2$, corrected for self-absorption.
to CO₂ and NH₃.

The results of a search for a "uricase" activity in several preparations of Otala hepatopancreas lend support to the view that uric acid is not degraded to any appreciable extent. The methods used in this study are given below.

Hepatopancreatic tissue from Otala lactea was found to contain some substance(s) which prevented its use in direct assays for uricase activity by measuring decrease in absorbancy at 292 mp, after the method of London and Hudson (1956). The tissue preparation consisted of a 30,000 x g supernatant from a 1:9 dilution of a 600 x g supernatant of a 10% homogenate of the tissue in 0.1 M sodium borate/KCl buffer, pH 9.2.

A tissue extract was then prepared and assayed, at room temperature, according to London and Hudson's (1956) modification of Leone's (1953) method. Because the uricase should have been solubilized by this method, further centrifugation of the extract was employed for greater clarification. Thus, portions of 7,000 x g supernatant and pellet and 105,000 x g supernatant were assayed as well. None of the preparations was active in degrading the uric acid substrate.

In another experiment, Otala hepatopancreas was homogenized separately in each of two different buffers, 0.1 M sodium borate/KCl, pH 9.2, and 0.05 M potassium phosphate, pH 7.5. No exogenous substrate was added, because endogenous uric acid content is approximately 1 mg/g of tissue. Portions (0.2 ml) of 10% homogenates were added to 2.8 ml
of the same buffer systems, and incubated at 31° for 0, 1 and 2 hr. The reactions were stopped by heating the incubation mixtures for 3 min. in a boiling-water bath. The modified colorimetric method of Fearon (1944) was used to measure uric acid content. The uric acid content was nearly identical at 0, 1 and 2 hr of incubation for each of the two preparations, again indicating that no degradation of uric acid was mediated by this tissue under the conditions stated.

In a final attempt to demonstrate the presence of uricase activity in _Otala_ hepatopancreas, acetone powders of mitochondria were prepared by following the method outlined for beef liver by Mahler, Wakil and Bock (1953). The powders were then extracted so that the uricase could be solubilized from the pellet obtained after centrifuging at 105,000 x g according to the method of Mahler, Hübscher and Baum (1955). The tissue preparations, both before and after high-speed centrifugation, were assayed as before in the Beckman DU spectrophotometer. In this experiment, 1.0 ml of "enzyme" extract was used in a total volume of 3.0 ml, with 10 µg of uric acid substrate dissolved in 0.1 M sodium borate/KCl buffer, pH 9.2. There was no decrease in absorbancy at 292 m\u00b5 over that of the controls after 40 min. at room temperature. Therefore, uricase was not demonstrated under the conditions of this experiment.

It was postulated that kidney tissue from these snails might possess a uricase activity, for it is in the kidney that uric acid accumulates before being excreted. After dilution of homogenized
kidney (all in 0.1 M sodium borate/KCl buffer, pH 9.2), 0.3 mg of tissue was introduced into the cuvettes for assay as usual. The kidney homogenate had to be diluted greatly so that its high endogenous uric acid would be measurable in the spectrophotometer. No exogenous uric acid was added. A KCl-inhibited control preparation was assayed simultaneously. There was no activity observed, even after one hr of incubation.

Baldwin and Needham (1934) examined slices of hepatopancreas, gut wall and kidney from Helix pomatia for their ability to degrade uric acid during a 3 or 4 hr incubation. They measured urea as the end-product of this reaction. The authors concluded that no enzymes were found in these tissues that degrade uric acid under the conditions employed. The presence of urease in Helix hepatopancreas was confirmed in the same publication, but no allowance was made for the presence of that enzyme in their work on uric acid degradation.

Truszkowski and Chajkinówna (1935) incubated an extract of whole specimens of Helix pomatia in 0.1% uric acid solution for 24 and 48 hr at 37°. Boiled extract was incubated similarly and served as a control. No uricase activity was demonstrated.

The present study was undertaken, as previously stated, to examine Otala for its ability to degrade uric acid, for the work done in the 1930's on Helix was felt to be insufficient. In conclusion, as Truszkowski and Chajkinówna (1935) had stated for Helix, Przylecki's Rule (uricotelic organisms do not possess uricase) was found to be valid for Otala lactea.
Synthesis of the Carbon Skeleton of Uric Acid by \textit{Otala}

\textbf{In vivo incorporation of suspected precursors.} As was mentioned previously, it was hypothesized that \textit{Otala lactea} synthesizes uric acid in the same general fashion as other organisms thus far studied. Once it was found that NaH$^{14}$O$_3$ was incorporated into uric acid by the snail, the remainder of the C$^{14}$-labeled precursor compounds were studied.

Following the methods outlined earlier, the precursor compounds were injected into feeding snails, the uric acid was recovered and purified both from the hepatopancreas and from the kidney, and the presence of radioactivity was established. In all cases but one, the uric acid was degraded to its component carbon atoms and the radioactivities of the latter were measured and compared.

Owing to the difficulty of accurately injecting known levels of radioactive materials into the snails (there is often an outflow of fluid at the site of injection), no attempt was made to determine the per cent incorporation of radioactivity in the uric acid isolated.

The first compound studied for its incorporation into uric acid was NaH$^{14}$O$_3$. The uric acid isolated from both kidney and hepatopancreas was radioactive, but that from the kidney was of a higher specific activity. Degradation of the uric acid into its component carbon atoms gave the results shown in Table IV. The results are also expressed as the per cent of the total of the component activities.

Data from Karlsson and Barker (1949) for pigeon and Bricteux-Grégoire
**TABLE IV**

DISTRIBUTION OF RADIOACTIVITY IN THE CARBON ATOMS OF URIC ACID ISOLATED FROM OTALA KIDNEY AFTER INJECTION OF THE SNAILS WITH SODIUM BICARBONATE-\( ^{14}C \)

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Average Radioactivity (cpm/mg BaCO(_3)/cm(^2))</th>
<th>Per Cent of Total for Otala</th>
<th>Per Cent of Total for Pigeon (K &amp; B, 1949)</th>
<th>Per Cent of Total for Helix (B-G &amp; F, 1962)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 &amp; 8</td>
<td>1.4</td>
<td>3.3</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>6</td>
<td>221.6</td>
<td>91.1</td>
<td>75.8</td>
<td>80.9</td>
</tr>
<tr>
<td>4</td>
<td>3.6</td>
<td>1.5</td>
<td>19.9</td>
<td>15.2</td>
</tr>
<tr>
<td>5</td>
<td>14.7</td>
<td>6.0</td>
<td>3.6</td>
<td>3.5</td>
</tr>
</tbody>
</table>
and Florkin (1962b) for *Helix pomatia* were recalculated for direct comparison with the results obtained for *Otala*. These values are also presented in Table IV.

The $^{14}$C-label from NaH$^{14}$O$_3$ is known to be incorporated primarily into carbon atom 6 of purines. The results with *Otala lactea* in this regard compared favorably with the expectation, that *Otala* would synthesize uric acid according to the general scheme found for other organisms.

*Otala*, as with the organisms studied by others, did not convert the bicarbonate into a form that could serve as an active "one-carbon" unit. This was evidenced by the very low incorporation of $^{14}$C-label into atoms 2 and 8 of the uric acid.

The relative incorporation of NaH$^{14}$O$_3$ into carbon atoms 4 and 5 was opposite to that given by Karlsson and Barker (1949) for pigeon and by Bricteux-Grégoire and Florkin (1962b) for *Helix*. This was also found for certain other precursors used in this work. It is most probable that these abnormally low levels of activity from carbon 4 resulted from an incomplete oxidation of the formic acid representing this unit from the original uric acid molecule (see Figure 5). The oxidation of formate by the procedure given by Buchanan, Sonne and Delluva (1948) is normally a slow one (about 1 to 4 hr). The use of glyoxylic acid-2-$^{14}$C of known activity would have been required to calibrate the extent of this oxidation. This was not done. This possibility of an incomplete recovery of carbon atom 4 of uric acid must be considered in the sequel.
The presence of radioactivity in carbon atoms 4 and 5 of uric acid can be accounted for by their having come directly from glycine. The difficulty lies in being able to show the route(s) by which $^{14}C\text{O}_2$ is incorporated into both of the carbon atoms of glycine in Otala. Neither formate nor CO$_2$ participate directly in the synthesis of glycine in other organisms.

The process of incorporation of $^{14}C\text{O}_2$ into alanine, aspartic acid and glutamic acid in the presence of hepatopancreatic tissue from Otala lactea was discussed by Awapara and Campbell (1964). One of the suggested intermediate compounds was pyruvic acid-1-C$^{14}$. It is possible that Otala possesses the necessary enzymes to convert pyruvic acid-1-C$^{14}$ ultimately into glycine-1-C$^{14}$ by reversal of the glycolytic sequence to form 3-phosphoglyceric acid-1-C$^{14}$. Then perhaps 3-phosphoglyceric acid-1-C$^{14}$ is converted to serine-1-C$^{14}$ by the pathway proposed by Sallach (1956). There is good evidence that serine is converted into glycine and formate in Otala, and the glycine formed would be labeled in the carboxyl carbon. This could then result in the labeling of carbon atom 4 of uric acid, as will be seen in the sequel.

Another possibility for the incorporation of $^{14}C\text{O}_2$ into glycine (both carbons) involves the metabolism of threonine. Abelson (1954) showed that E. coli synthesizes homoserine and threonine from oxaloacetate via aspartic acid. In the work of Awapara and Campbell (1964), Otala lactea was found to incorporate $^{14}C\text{O}_2$ into aspartic acid. The label could have been incorporated into both carboxyl carbons of that
amino acid. If Otala were to synthesize threonine from aspartic acid-1,4-C$^{14}$, then the threonine would be labeled in the same positions. Karasek and Greenberg (1957) reported that threonine can be cleaved to glycine and acetaldehyde in certain higher animals. The acetaldehyde may subsequently be converted to acetate. The resulting glycine would be labeled in the carboxyl carbon, and this could also account for the activity in carbon 4 of uric acid. The acetate, labeled in the methyl carbon, may then enter the citric acid cycle (Awapara and Campbell, 1964) to form oxaloacetate. The oxaloacetate would be labeled in carbon atoms 2 and 3. This could ultimately result in the formation of serine-2,3-C$^{14}$, and finally in the labeling of carbons 2 and 8 (from the $\beta$-carbon of serine) and carbon 5 (from the $\alpha$-carbon of glycine) of uric acid.

The foregoing is suggested as a possible means of explaining the presence of radioactivity in carbons 4 and 5 of uric acid when incubated with C$^{14}$O$_2$. It has a partial factual basis, but extensive study would be required to substantiate the suggestions.

HC$^{14}$OONa and serine-3-C$^{14}$, two other compounds suspected of being precursors of uric acid in Otala, were incorporated into uric acid when treated according to the procedures described before. Table V summarizes the results of this study.

From Table V it can be seen that the incorporation of HC$^{14}$OONa into uric acid in Otala conforms generally to the pattern found for its incorporation in the uric acid of pigeons (Karlsson and Barker, 1949) and Periplaneta americana larvae (McEnroe and Forgash, 1957). Most of
TABLE V

DISTRIBUTION OF RADIOACTIVITY IN THE CARBON ATOMS OF URIC ACID ISOLATED FROM OTALA KIDNEY AFTER INJECTION OF THE SNAILS WITH EITHER SODIUM FORMATE-C$^{14}$ OR SERINE-3-C$^{14}$

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Sodium Formate-C$^{14}$</th>
<th>Serine-3-C$^{14}$</th>
<th>Sodium Formate-C$^{14}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average Radioactivity (cpm/mg BaCO$_3$/cm$^2$)</td>
<td>Per Cent of Total for Otala</td>
<td>Average Radioactivity (cpm/mg BaCO$_3$/cm$^2$)</td>
</tr>
<tr>
<td>2 &amp; 8</td>
<td>1509.1</td>
<td>94.4</td>
<td>607.4</td>
</tr>
<tr>
<td>2</td>
<td>282.0</td>
<td>(51.0)</td>
<td>----</td>
</tr>
<tr>
<td>8</td>
<td>240.7</td>
<td>(43.4)</td>
<td>----</td>
</tr>
<tr>
<td>6</td>
<td>68.8</td>
<td>4.3</td>
<td>20.7</td>
</tr>
<tr>
<td>4 &amp; 5</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>4</td>
<td>3.3</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>17.2</td>
<td>1.1</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* From Karlsson and Barker (1949)
+ From McEnroe and Forgash (1957)
the radioactivity resided in carbon atoms 2 and 8, and was nearly equally divided between the two.

Elwyn and Sprinson (1950) and Sprinson (1951), working with pigeons and rats, showed that the β-carbon of serine contributed to the "formate" or "one-carbon" pool, which is readily incorporated into carbons 2 and 8 of purines in these animals. The same was found for Otala, where the C\textsuperscript{14}-labeled β-carbon of serine was incorporated chiefly into carbons 2 and 8 of uric acid.

The preceding authors also showed that serine is an equally good source of glycine, and that the latter amino acid may be utilized for purine synthesis. This serine-glycine interconversion was originally described by Shemin (1946) and by Sakami (1948). The demonstration that serine contributed to the "one-carbon" pool in Otala indicated that a serine-glycine reaction is operative in this snail as it is in many vertebrates studied.

Both serine-3-C\textsuperscript{14} and HC\textsuperscript{14}OONa contributed slightly to a CO\textsubscript{2} fraction as was evidenced by a low level of radioactivity in carbon 6 of uric acid. Nakada and co-workers (1955, 1958) demonstrated the following enzyme-catalyzed pathways in rat liver:

\[
\begin{array}{c}
\text{CH}_2\text{NH}_2\text{COOH} \leftrightarrow \text{CHO}_{\text{glyoxylic acid}} \rightarrow \text{HCOOH} + \text{CO}_2 \\
(\text{glycine}) \quad (\text{glyoxylic acid}) \quad (\text{formic acid})
\end{array}
\]
The operation of this or a similar system in *Otala* could explain the incorporation of label into carbon 6 of uric acid. Further evidence for the presence in *Otala* of the complete pathway for glycine (and formate) oxidation is given below.

Table VI records the incorporation of glycine-1-C\(^{14}\) into uric acid by *Otala lactea* in vivo. Again, the values obtained for this precursor in *Otala* are compared with those given by Karlsson and Barker (1949) for pigeons.

The radioactivity did not reside chiefly in carbon atom 4 of uric acid as was expected. Incomplete oxidation of the formate containing this carbon atom could account for the abnormally low levels that resulted. The majority of the activity was in carbon atoms 4 and 5, as shown by direct measurement of the radioactivity in the semicarbazone derivative of glyoxylic acid. The rapid oxidation of this semicarbazone giving carbon 5 of uric acid as CO\(_2\) was probably fairly complete, so the majority of the remaining activity should represent that from carbon atom 4 of uric acid. Values estimating relative recoveries of carbons 4 and 5 were calculated from figures given by Bricteux-Grégoire and Florkin (1962b). Based on these calculations, carbon 4 should have yielded about 1,650 cpm/mg BaCO\(_3\)/cm\(^2\) had its oxidation been complete. This is a more reasonable value in view of these and other findings.

From the incorporation of label into carbon atom 6 of uric acid, it is likely that a decarboxylation occurred, probably by way of the sequence outlined by Nakada and co-workers (1955, 1958). Some of
TABLE VI

DISTRIBUTION OF RADIOACTIVITY IN THE CARBON ATOMS OF URIC ACID ISOLATED FROM OTALA KIDNEY AFTER INJECTION OF THE SNAILS WITH GLYCINE-1-C\(^{14}\)

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Average Radioactivity (cpm/mg BaCO(_3)/cm(^2))</th>
<th>Per Cent of Total for Otala</th>
<th>Per Cent of Total for pigeon (K &amp; B, 1949)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 &amp; 8</td>
<td>1.3</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>65.9</td>
<td>13.0</td>
<td>2.2</td>
</tr>
<tr>
<td>4 &amp; 5*</td>
<td>2548.5</td>
<td>(97.4)</td>
<td>----</td>
</tr>
<tr>
<td>4</td>
<td>113.4</td>
<td>22.3</td>
<td>87.7</td>
</tr>
<tr>
<td>5</td>
<td>328.0</td>
<td>64.5</td>
<td>10.1</td>
</tr>
</tbody>
</table>

* Carbon atoms 4 & 5, combined, expressed as cpm/mg of glyoxylic acid semicarbazone/cm\(^2\).
the $^{14}\text{C}_2$O$_2$ was ultimately incorporated into carbon 5 of uric acid by whatever route(s) might be available to it.

The possibility of contamination of glycine-1-$^{14}\text{C}$ with glycine-2-$^{14}\text{C}$ was considered, but it was concluded that this was unlikely. From Table VI it can be seen that there was very little incorporation of $^{14}\text{C}$-label into carbons 2 and 8 of uric acid. As can be seen in Table VII, glycine-2-$^{14}\text{C}$ contributed heavily to carbons 2 and 8.

The incubation of glycine-2-$^{14}\text{C}$ with Otala lactea in vivo gave more of an indication that glycine is used in the synthesis of uric acid. Table VII summarizes the results obtained with this amino acid. The values are very closely allied with those obtained by Karlsson and Barker (1949) for pigeon, especially when the values for carbons 4 and 5 are shifted relative to each other to account for the incomplete recovery of carbon 4.

Synthesis of uric acid in Otala was again shown to resemble that in other organisms studied. Incorporation of radioactivity from glycine-2-$^{14}\text{C}$ was chiefly into carbon 5 of uric acid as expected. As Karlsson and Barker (1949) had shown for uric acid synthesis in pigeon, and Sprinson (1951) had confirmed for purine synthesis in pigeons and rats, glycine-2-$^{14}\text{C}$ contributed greatly to carbons 2 and 8 of uric acid in Otala. Nakada and co-workers (1955, 1958) showed how the methylene carbon of glycine was converted to formate in rat liver. Thus, the present findings for Otala and those of Nakada and co-workers for rat liver support each other. Karlsson and Barker (1949) first suggested
<table>
<thead>
<tr>
<th>Carbon</th>
<th>Average Radioactivity (cpm/mg BaCO₃/cm²)</th>
<th>Per Cent of Total for Otala</th>
<th>Per Cent of Total for pigeon (K &amp; B, 1949)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 &amp; 8</td>
<td>434.6</td>
<td>30.6</td>
<td>30.9</td>
</tr>
<tr>
<td>2</td>
<td>97.0</td>
<td>(13.0)</td>
<td>----</td>
</tr>
<tr>
<td>8</td>
<td>131.7</td>
<td>(17.6)</td>
<td>----</td>
</tr>
<tr>
<td>6</td>
<td>39.0</td>
<td>2.7</td>
<td>1.9</td>
</tr>
<tr>
<td>4</td>
<td>9.3</td>
<td>0.7</td>
<td>15.2</td>
</tr>
<tr>
<td>5</td>
<td>938.7</td>
<td>66.0</td>
<td>52.0</td>
</tr>
</tbody>
</table>
that the methylene carbon of glycine is converted to formic acid on the basis of their results (see Table VII).

The presence of activity in carbon atom 6 of uric acid indicated that some of the HC\textsuperscript{14}OONa formed was further oxidized to C\textsuperscript{14}O\textsubscript{2} or that there was some contamination with glycine-1-C\textsuperscript{14}, which was decarboxylated as before, or a combination of both. In addition, either or both of these possibilities were probably responsible for the incorporation of C\textsuperscript{14}O\textsubscript{2} into carbon 4 of uric acid via some unknown route(s).

The incorporation of both glycine-1-C\textsuperscript{14} and glycine-2-C\textsuperscript{14} into uric acid in Otala is similar to that in other organisms studied. Shemin and Rittenberg (1947) showed that glycine-N\textsuperscript{15} administered to human males resulted in the labeling of nitrogen 7 of uric acid with N\textsuperscript{15}. These workers correctly suggested that glycine is incorporated as a unit into uric acid in pigeons and man. It appears reasonably safe to conclude that this is also the case for uric acid synthesis in Otala lactea.

To this point it has been established that Otala lactea utilizes the same precursor compounds to form uric acid in vivo as had previously been shown for a wide variety of organisms. The involvement of 4-amino-5-imidazolecarboxamide ribotide as a direct precursor in the synthesis of purines in several organisms has been shown as a result of several investigations (see Reichard, 1955, for a review of these works). It was further reported that free 4-amino-5-imidazolecarboxamide could be converted to the ribotide form by most
tissues, and subsequently incorporated into uric acid and other purines. The free carboxamide, C\textsuperscript{14}\textsuperscript{-}labeled in the number 2 position, was injected into specimens of \textit{Otala lactea}. Table VIII summarizes the results obtained from the degradation of the uric acid isolated and purified from the kidneys of these snails.

\textit{4-Amino-5-imidazolecarboxamide hydrochloride-2-C\textsuperscript{14}} was incorporated into uric acid by \textit{Otala}. The radioactivity was localized primarily in carbon 8 of uric acid, and this conforms exactly to the expected fate of the label based on previous work on other organisms. Whether the compound was used as administered or first converted to a phosphorylated ribose derivative before ring closure to form the purine ring was not established. The narrowly localized radioactivity indicated that the 4-amino-5-imidazolecarboxamide was incorporated as a unit into uric acid.

\textit{Hypoxanthine-2-C\textsuperscript{14}}, a normal intermediate in the synthesis of uric acid was injected into specimens of \textit{Otala} as were the other suspected precursors. The uric acid was isolated and purified, and was found to possess considerable radioactivity. It is worth noting that a control preparation of unlabeled uric acid was isolated and purified in the presence of 1 \textmu{}curie of hypoxanthine. After purification was completed, the uric acid was essentially freed (2.9 cpm/mg/cm\textsuperscript{2}) of radioactivity. It was thus shown that uric acid could be isolated in the presence of hypoxanthine and be free from contamination by the latter purine.
TABLE VIII
DISTRIBUTION OF RADIOACTIVITY IN THE CARBON ATOMS
OF URIC ACID ISOLATED FROM OTALA KIDNEY AFTER INJECTION
OF THE SNAILS WITH
4-AMINO-5-IMIDAZOLECARBOXAMIDE HYDROCHLORIDE-2-C\textsuperscript{14}

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Average Radioactivity (cpm/mg BaCO\textsubscript{3}/cm\textsuperscript{2})</th>
<th>Per Cent of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 &amp; 8</td>
<td>2355.0</td>
<td>99.8</td>
</tr>
<tr>
<td>2</td>
<td>13.8</td>
<td>(1.9)</td>
</tr>
<tr>
<td>8</td>
<td>705.7</td>
<td>(97.9)</td>
</tr>
<tr>
<td>6</td>
<td>4.6</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(63)
The uric acid-C\textsuperscript{14} obtained from the snails injected with hypoxanthine-2-C\textsuperscript{14} was not subjected to chemical degradation, because of the excellent results obtained with 4-amino-5-imidazolecarboxamide hydrochloride-2-C\textsuperscript{14}.

It is evident that the carbon skeleton, and probably nitrogen atom 7 as well, of uric acid is synthesized from the same precursor compounds by \textit{Otala lactea} as it is in the other organisms studied thus far. Furthermore, the incorporation of the C\textsuperscript{14}-labeled carboxamide into uric acid indicates that the pathway of the synthesis is generally the same. Conversion of hypoxanthine to uric acid further substantiated this suggestion.

\textbf{Incorporation of HC\textsuperscript{14}OONa in vitro.} It was postulated that hepatopancreatic tissue is a site of synthesis of uric acid in \textit{Otala} (see Baldwin, 1935b). An experiment was designed to test this possibility, using minces of that tissue. One g of minced tissue was incubated for 6 hr at 30° in the presence of HC\textsuperscript{14}OONa. The fluid medium was \textit{Helix} saline (3.0 ml) containing the same concentrations of components as used during the \textit{in vivo} precursor studies. Ten \textmu curies of HC\textsuperscript{14}OONa was added in a 0.2 ml volume. The uric acid isolated from the incubation mixture yielded a corrected value of about 17,500 cpm/70 mg of uric acid, the amount of carrier added before isolation. The hepatopancreas contains about 1 mg of uric acid, but this was not included in the calculations. The uric acid was then subjected to selective chemical degradation, whereupon 81.4\% of the label was found
to reside in carbons 2 and 8. 17.6% of the radioactivity was in carbon 6, with the remainder in carbon atoms 4 and 5.

The values given above for conversion in vitro may be compared with those in vivo by reference to Table V. While it was shown that hepatopancreas from Otala was capable of incorporating Hc\textsuperscript{14}OONa into uric acid in the expected positions, it was evident that there was more C\textsuperscript{14}O\textsubscript{2} available for incorporation into carbon atom 6 of uric acid than in the in vivo preparation. This may have resulted from less utilization of C\textsuperscript{14}O\textsubscript{2} in other reactions, for carbon atoms 4 and 5 were no more labeled in vitro than in vivo. Limited availability of other substances required for "formate" metabolism may have been involved, but this is only a suggestion.

The use of a sterile medium containing antibiotics should have been sufficient to rule out bacterial synthesis of uric acid. In addition, Strasdin and Whitaker (1963) reported that the hepatopancreas of Helix pomatia is virtually sterile itself.

A second set of experiments, utilizing the conditions just outlined, was carried out in order to determine how the incorporation of radioactivity into uric acid varied with time. Measurements of radioactivity were made on uric acid isolated from different preparations at zero time, 0.25, 0.50, 1, 2, 4 and 6 hr. A linear increase in the incorporation of radioactivity was obtained for the first 2 hr, leveling off between 2 and 4 hr of incubation. Tissue samples killed by heating at 100\degree for 5 min were incubated for 1 and 6 hr, and the
levels of activity, which were nearly background, were taken as controls for non-enzymatic activity. By knowing the activity and quantity of formate used in the incubation, and that recovered in uric acid, the rate of uric acid synthesis was calculated to be approximately 0.01 μmoles of uric acid/g of tissue/hr, which is almost 1.7 μg of uric acid synthesized/g of fresh tissue/hr. Assuming 30 mg of uric acid present in an Otala kidney, and that the snail possesses a 250 mg hepatopancreas, it would take about 3,000 days for a complete turnover of the snail's kidney uric acid, when the calculated rate of synthesis found in vitro is also taken to be valid. This rate of synthesis of uric acid does not appear to be realistic. This becomes more questionable when it is considered that practically all of the nitrogen excreted by Helix pomatia is in purine compounds (Jezewska, Gorzkowski and Heller, 1963a,b).

Homogenates of Otala hepatopancreas incubated with HC\(^{14}\)OONa under similar conditions showed no incorporation of the label into uric acid after 6 hr. This may have been due to an increased hydrolysis of ATP by an ATPase. Neither exogenous ATP nor an ATP-generating system were employed. As was shown in Figure 2, several reactions of purine synthesis require ATP. ATPase activity is present in homogenates of Otala hepatopancreas.

Azaserine is a drug which has been found to inhibit purine synthesis in a variety of organisms by competing with glutamine (Skipper, Bennett and Schabel, 1954; Hartman, Levenberg and Buchanan,
1955). This drug blocks the incorporation of the amide nitrogen of glutamine into nitrogen atoms 3 and 9 of the purine ring. Azaserine was used in an attempt to block the synthesis of uric acid by a preparation of Otala hepatopancreas. A successful inhibition of incorporation of HCl4OONa into uric acid in the presence of azaserine would implicate glutamine as the donor of at least one nitrogen atom to the uric acid molecule. It would also be one further similarity of uric acid synthesis in this animal to that in others that have been studied.

Whiteley (1960) reported that formation of N-10-formyltetrahydrofolic acid and other active derivatives occurred primarily in the gonads and gametes of several marine molluscs, with little or none being formed in the digestive gland, or hepatopancreas. Because Otala may possess a similar distribution of formate-activating enzymes, the tissue preparation for the following experiment consisted of minces of hepatopancreas (apical lobes) and gonads. Tissue mince (0.5 g) was incubated in 3.0 ml of Helix saline as before, except that casein hydrolysate was excluded so that no exogenous glutamine might be added. Glutamine was shown to reverse the inhibitory action of azaserine upon purine synthesis (Bennett, Schabel and Skipper, 1956). Ten μcuries of HCl4OONa was added (in 0.2 ml) at the end of 10 min. preincubation of the tissue, one preparation with, and one without, azaserine (1 mg/ml of saline). The incubations lasted 1 hr at 30° with constant shaking. The uric acid was isolated, purified, and the radioactivity was measured.
Table IX shows the incorporation of HC\textsuperscript{14}OONa into uric acid under the conditions of the experiment. The radioactivity is given in cpm/70 mg of uric acid.

It can be seen that azaserine, in a concentration of 6 mg/g of \textit{Otala} hepatopancreas-gonad mince, almost completely inhibited the incorporation of HC\textsuperscript{14}OONa into uric acid. This finding opened the way to a study of the intermediates found when azaserine was used to inhibit the synthesis of uric acid.

Thus, more features common to biosynthesis of uric acid in \textit{Otala} and other organisms have been revealed by this experiment. While the amide nitrogen of glutamine now seems to be involved in the synthesis of uric acid in \textit{Otala}, the position(s) into which incorporation occurred were not shown. Some of the experiments that will be discussed below were designed to test the incorporation of nitrogen donated from glutamine into positions 3 and 9 of uric acid.

The inclusion of gonadal tissue seemed to increase the overall rate of uric acid synthesis in the foregoing experiment. Here, again assuming 30 mg of uric acid/kidney and a 250 mg hepatopancreas-gonad complex, it would take 948 days for complete turnover of uric acid in the kidney based on the synthetic rate found in this experiment. While this value is about 3 times greater than that found earlier, where no gonadal tissue was included, it still seems to be too low to be the true synthetic rate. No direct studies on \textit{Otala}, or any other terrestrial gastropod, are available to support this idea. Snails have
<table>
<thead>
<tr>
<th>Sample</th>
<th>Corrected cpm/70 mg Uric Acid</th>
<th>μmoles Uric Acid Synthesized/g Fresh Tissue/hr</th>
<th>μgrams Uric Acid Synthesized/g Fresh Tissue/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-killed tissue, minus azaserine</td>
<td>41.2</td>
<td>-----</td>
<td>----</td>
</tr>
<tr>
<td>Azaserine (Parke, Davis &amp; Co.), 1 mg/ml saline</td>
<td>98.5</td>
<td>-----</td>
<td>----</td>
</tr>
<tr>
<td>Minus azaserine</td>
<td>9735</td>
<td>0.031</td>
<td>5.27</td>
</tr>
</tbody>
</table>
been observed to eliminate considerable quantities of excreta, and yet still have a concrement-laden kidney. The fact that those concrements are largely purines has led the author to believe that a faster synthetic rate than given above must exist. If Hesse's (see Bricteaux-Grégoire and Florkin, 1962b) finding that Helix excreted 3.85 mg of nitrogen/kg/hr can be applied to Otala, then it can be calculated that a 10 g specimen of Otala might excrete around 2.5 mg of purine/day. This would certainly necessitate a higher synthetic rate for uric acid in Otala than the in vitro studies indicated.

Synthesis of Uric Acid via Phosphorylated Ribose Derivatives

While the foregoing studies revealed that Otala synthesizes uric acid from the same precursors as initially postulated, it remained to be shown whether or not synthesis proceeds via phosphorylated ribose derivatives, as is generally the case. Two methods of attack were employed in an attempt to answer this question. The first sought to demonstrate the accumulation of formylglycinamide ribotide and formylglycinamide riboside when preparations of Otala tissue were blocked by azaserine as Tomisek, Kelly and Skipper (1956) had demonstrated in E. coli. The second was an attempt to demonstrate the presence of the first enzyme, phosphoribosylpyrophosphate amidotransferase, in the sequence known for purine synthesis in several other organisms.

Accumulation of intermediates after azaserine treatment.

Having found that azaserine inhibited the incorporation of HCl4OONa
into uric acid at a level of 6 mg/g of *Otalapia* hepatopancreas-gonad tissue, the experiment was repeated in part. This time two flasks containing tissue were used, one with and the other without azaserine in the saline as before. After 10 min. of preincubation, 20 μcuries of HCl14OONa (in 0.2 ml) was added to each flask. The incubation was made for 15 min. at 30° with constant shaking. Following essentially the method of Tomisek, Kelly and Skipper (1956), the reactions were stopped by homogenization of the tissue and medium in 4 volumes of absolute ethanol. The resulting mixtures were placed in a water bath at 100° for 5 min., cooled, and centrifuged at 25,000 x g for 5 min. The supernatant was concentrated to about one-fifth its original volume with the aid of a flash evaporator. After recentrifugation, the supernatant was lyophilized and the resulting residue taken up in 0.5 ml of water. Portions (100 μl) of each were subjected to chromatography on Whatman No. 1 filter paper. The chromatograms were developed using the solvent system of Benson *et al.* (1950). This system is water-saturated phenol in the first dimension, followed by butanol:propionic acid:water in the second dimension. The air-dried chromatograms were mounted on Ansco non-screen x-ray film for radioautography. The chromatograms were exposed to the x-ray film for about 3 weeks, after which time the radioautograms were developed by standard techniques. The chromatograms and radioautograms were then compared with those obtained by Tomisek, Kelly and Skipper (1956) under similar conditions for *E. coli*.

Both extracts contained many radioactive compounds, so there
was an active metabolism of HC\textsuperscript{14}OONa. The azaserine-inhibited preparation formed fewer radioactive compounds and less radioactivity was present in certain others. While the control preparation showed high levels of radioactivity in what were apparently hypoxanthine, guanine and inosine, no radioactivity was present in these compounds in the azaserine-inhibited preparation. This finding strongly suggests that these purines are also formed in a manner similar to uric acid. The latter purine was not located on the chromatograms.

The azaserine-inhibited preparation did not, however, appear to have accumulated either radioactive formylglycinamide ribotide or formylglycinamide riboside. Further, no other labeled compounds accumulated in the azaserine-treated preparations. Several alternative explanations for this finding are evident. One possibility is that the azaserine strongly inhibited the synthesis of phosphoribosylamine, and that little or none of the latter compound or glycinamide ribotide were available for the synthesis of C\textsuperscript{14}-formylglycinamide ribotide, which should have accumulated if the azaserine blocked the next step utilizing glutamine (see Figure 2). A second possibility is that glutamine is not the nitrogen-donor for the reaction leading to the synthesis of formylglycinamidine ribotide, and, thus, would not be subjected to inhibition by azaserine. In this event, the inhibition would have to occur at the initial step, i.e., the synthesis of phosphoribosylamine. The third possibility is that uric acid synthesis does not proceed by the way of phosphorylated ribose derivatives, so that these compounds
would not be expected to accumulate in the presence of azaserine. But, if this is the case, some other compounds should have accumulated, for azaserine does inhibit uric acid synthesis under the conditions employed. Combinations of these explanations might also be possible.

In summary, azaserine blocked synthesis of uric acid (and, apparently, other purines as well) by Otala hepatopancreas-gonad minces. The inhibition presumably involves the substitution of azaserine for glutamine at a reactive site (Levenberg, Melnick and Buchanan, 1957) at least once during the synthetic sequence. Whether or not uric acid is synthesized via phosphorylated ribose derivatives remains to be established.

**Phosphoribosylpyrophosphate amidotransferase activity in Otala.** Phosphoribosylpyrophosphate amidotransferase (PATase) (Goldthwait, 1956) catalyzes the reaction that transfers the amide-group of L-glutamine to the 1-position of 5-phosphoribosyl-1-pyrophosphate (PRPP) to yield 5-phosphoribosylamine (PRA) plus L-glutamic acid and inorganic pyrophosphate according to the reaction shown in Figure 2. The reaction requires magnesium ions and has a pH optimum of about 8. This reaction is the first one unique to the synthetic pathway for purines, and is reportedly (Wyngaarden and Ashton, 1959) the step wherein the rate of purine synthesis is controlled. Thus, demonstration of the presence of PATase in Otala tissue extracts would be quite valuable, both as further evidence for the synthesis of uric acid via phosphorylated ribose derivatives and as the enzyme most desirable to
study for comparative purposes.

The method for the preparation and assay of PATase activity from Otala hepatopancreas was essentially that given by Flaks and Lukens (1963) for chicken liver. Tissue extracts from chicken liver (as controls) and Otala hepatopancreas were prepared by homogenization in a Servall blender in 2 volumes of 0.01 M ammonium citrate/0.003 M EDTA buffer at pH 5.3 for 1 min. at high speed. All steps were carried out at 0 to 4°C. The suspension was readjusted to pH 5.3 immediately after homogenization, then centrifuged at 20,000 x g for 30 min. The resulting supernatant was used directly in the assay. In some cases the supernatant was further treated by adjusting the pH to 7.0 with K_3PO_4 solution, then heating slowly to 60°C in a preheated water bath. The preparation was subsequently cooled in an ice bath, recentrifuged, and this supernatant used as the enzyme preparation.

The incubation system was exactly as given by Flaks and Lukens (1963): 10 µmoles of L-glutamine (Sigma), approximately 2 µmoles of PRPP (Sigma), 20 µmoles of Tris-(hydroxymethyl)-aminomethane-chloride buffer at pH 8.0, 5 µmoles of MgCl_2, 10 µmoles of NaF and tissue extract ("enzyme") in a total volume of 0.6 ml. The enzyme preparations were incubated for 30 min. at 30°C in some cases, for 15 min. at 38°C in others. Mixtures complete in all components except L-glutamine served as controls for activity, but those lacking PRPP or "enzyme" were also employed. The inorganic pyrophosphate was isolated as its manganese salt according to the procedure of Kornberg (1950) as
modified by Flaks and Lukens (1963). The pyrophosphate isolated was hydrolyzed to orthophosphate with $H_2SO_4$ and subsequently measured according to the method of Fiske and SubbaRow as given by Leloir and Cardini (1957). The recovery of pyrophosphate was corrected to 100% by comparison with a standard curve.

Fluoride was included in the incubation mixture to inhibit inorganic pyrophosphatase activity, because inorganic pyrophosphate was the product desired. Otala extracts prepared as given above were found to contain inorganic pyrophosphatase activity that operated under the conditions used for determining PATase activity. The addition of NaF to the incubation mixtures completely inhibited this undesired phosphatase.

Three separate preparations of Otala hepatopancreas and one of chicken liver, none of which was carried through the heat step, failed to reveal any PATase activity. These assays were of 30 min. duration at 30°. The PRPP (Sigma, Lot # 123B-0310) was purchased as the magnesium salt and was converted to the sodium salt according to the procedure of Flaks (1963b). Failure to obtain any activity with the preparation of chicken liver, for which this method was designed, suggested that the PRPP might not have been "active."

A different enzyme preparation for PATase activity was then tested, unfortunately with the unreactive PRPP as substrate. Acetone powder preparations of chicken liver and Otala hepatopancreas were extracted with 0.05 M potassium glycinate at pH 9.1, dialysed and
lyophilized essentially according to the procedure outlined by Goldthwait and Greenberg (1955). Incubation of extracts and assay for PATase activity were the same as before. Neither chicken liver nor Otala hepatopancreas demonstrated measurable PATase activity. This method for preparation of PATase was not repeated with the "active" PRPP.

The PRPP used in the experiments described thus far was subjected to certain analytical procedures. A pentose to phosphate ratio of about 1 to 2.8 was found. This was close to the theoretical ratio of 1 to 3. Pentose was measured by the orcinol method as given by Schneider (1957) using adenosine monophosphate as a standard. Phosphate was measured by the method of Fiske and SubbaRow, as before. The ratio of acid-labile phosphate to total phosphate was 2.12 to 3, where the theoretical value was 2 to 3. This indicated the presence of intact pyrophosphate in about the proper ratio. However, direct measurement of inorganic pyrophosphate, using the method of Flynn, Jones and Lipmann (1954), indicated that slightly greater than 40% of the pyrophosphate was inorganic and therefore not part of PRPP.

The same batch of PRPP was then tested in the presence of a second enzyme system, orotidine-5'-phosphate pyrophosphorylase. This enzyme was prepared from brewer's yeast and assayed according to the procedure of Flaks (1963a). No evidence of the expected enzymatic activity was found in 3 different yeast preparations, so it was quite likely that the PRPP preparation was not suitable for enzymatic
reactions.

Another attempt to circumvent the necessity of utilizing PRPP in order to demonstrate the initial steps in purine synthesis was made in the following manner: Lyophilized extracts (0.05 M K₂HPO₄) of acetone powders of chicken liver and Otala hepatopancreas were prepared according to the method of Goldthwait and Greenberg (1955). This time, the production of glycinamide ribotide was measured. This involved incubating glycine-1-C¹⁴ with L-glutamine, ATP, ribose-5-phosphate, 3-phosphoglyceric acid, MgCl₂ and the enzyme extract. After incubating for 60 min. at 30°, the reaction was stopped by adding trichloroacetic acid. The radioactivity remaining after decarboxylation with ninhydrin reagent was taken to be primarily from glycinamide ribotide, where adequate controls were employed. In a second experiment, L-asparagine and NH₄Cl were tested in place of L-glutamine for their potency as nitrogen-donors with Otala extract. Table X is a summary of the results obtained, where the numerical values represent averages of duplicate mixtures in cpm corrected for background.

While these experiments showed fairly clearly that the extract of chicken liver contained the necessary enzymes to synthesize glycinamide ribotide, the activity of the Otala extract is a puzzle. In the latter, neither glutamine nor asparagine served as nitrogen-donor compounds, but NH₄Cl might have.

PATase preparations from chicken liver and Otala hepatopancreas were made, incubated and assayed as stated previously,
<table>
<thead>
<tr>
<th>Source of &quot;Enzymes&quot;</th>
<th>Alterations</th>
<th>Minus &quot;Enzyme&quot; Deficient Value</th>
<th>Heat-Killed and N-donor-deficient values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otala hepatopancreas</td>
<td>none</td>
<td>295</td>
<td>0</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>heat-killed</td>
<td>292</td>
<td>26</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>minus ATP</td>
<td>354</td>
<td>23</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>minus R-5-P</td>
<td>233</td>
<td>36</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>minus &quot;enzyme&quot;</td>
<td>236</td>
<td>67</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>heat-killed</td>
<td>269</td>
<td>36</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>minus ATP</td>
<td>837</td>
<td>508</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>minus R-5-P</td>
<td>306</td>
<td>37</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>minus &quot;enzyme&quot;</td>
<td>392</td>
<td>23</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>minus NH2Cl</td>
<td>355</td>
<td>42</td>
</tr>
<tr>
<td>Chicken liver</td>
<td>none</td>
<td>449</td>
<td>0</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>heat-killed</td>
<td>373</td>
<td>76</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>minus ATP</td>
<td>640</td>
<td>234</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>minus R-5-P</td>
<td>373</td>
<td>60</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>minus &quot;enzyme&quot;</td>
<td>640</td>
<td>377</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>minus NH2Cl</td>
<td>406</td>
<td>93</td>
</tr>
</tbody>
</table>

* There was less glycine-1-C14 incubated with this chicken liver preparation than with the others, so these values are probably relatively lower than the others.
according to the procedure outlined by Flaks and Lukens (1963). A new preparation of PRPP (Sigma, Lot # 14B-1470) was used, this time directly as the magnesium salt. An analysis of this batch of PRPP by the Sigma Company revealed it to be 65 to 75% pure based on enzymatic assay. Inorganic orthophosphate and ribose diphosphate were stated to be impurities in the preparation.

In some cases, the L-glutamine was replaced by an equal quantity of either L-asparagine or NH₄Cl. Mixtures deficient in these nitrogen-donor compounds were the control preparations used in the calculations, as before. The preparations that lacked either "enzyme" or PRPP again served as indices of contamination of PRPP with inorganic pyrophosphate and tissue levels of inorganic pyrophosphate, respectively.

The first assay was carried out using an extract of an acetone powder (prepared according to Goldthwait and Greenberg, 1955) of chicken liver as the enzyme preparation. The homogenate of the acetone powder was made so that 1 g of the original fresh tissue was in 2 volumes of buffer. The incubation was carried out at 30° for 30 min. The enzyme preparation (0.2 ml, equivalent to about 66.7 mg of fresh tissue) catalyzed the formation of 0.42 μmole of inorganic pyrophosphate. Thus, about 12.6 μmoles of PRA were synthesized/g of fresh tissue/hr.

The second experiment utilized both an unheated and a heated (60°) extract of fresh chicken liver, where both contained about
66.7 mg of fresh tissue/0.2 ml. Here, the incubation was made at 38° for 16.5 min. The unheated preparation yielded 1.03 μmoles of inorganic pyrophosphate, while the heated one gave 1.08 μmoles of the same. Thus, the former enzyme preparation catalyzed the synthesis of about 56.2 μmoles of PRA/g of fresh tissue/hr, and the latter about 59 μmoles of PRA/g of fresh tissue/hr.

In an experiment identical to the preceding, except that both unheated and heated extracts were of Otala hepatopancreas, no enzyme-catalyzed formation of inorganic pyrophosphate resulted. Hence, no PRA was synthesized.

Next, chicken and Otala extracts were assayed simultaneously. The incubation, with unheated enzyme extracts, was carried out at 38° for 15 min. There was no activity with the preparation from Otala hepatopancreas, but chicken liver extract catalyzed the formation of 1.08 μmoles of inorganic pyrophosphate, or 64.8 μmoles of PRA/g of fresh tissue/hr.

It was postulated that Otala hepatopancreas might contain some substance that is inhibitory to the PAtase activity from chicken liver. Unheated extracts from chicken liver and Otala hepatopancreas were again prepared. Chicken liver extract was incubated separately, with Otala extract and with boiled Otala extract, each with a glutamine-deficient control. As before, 66.7 mg of fresh tissue were used from both sources. The results are presented in Table XI.

Therefore, Otala hepatopancreas extract did contain some
TABLE XI

INHIBITION OF PAPSE ACTIVITY FROM CHICKEN LIVER
BY SIMILAR EXTRACTS OF OTALA HEPATOPANCREAS

<table>
<thead>
<tr>
<th>System</th>
<th>µmoles Inorganic pyrophosphate formed</th>
<th>µmoles PRA Synthesized/g Tissue/hr</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken liver extract</td>
<td>0.59</td>
<td>35.4</td>
<td>0</td>
</tr>
<tr>
<td>Chicken liver extract + Otala hepatopancreas extract</td>
<td>0.03</td>
<td>1.8</td>
<td>95</td>
</tr>
<tr>
<td>Chicken liver extract + boiled Otala hepatopancreas extract</td>
<td>0.22</td>
<td>13.2</td>
<td>63</td>
</tr>
</tbody>
</table>
substance, possibly heat-stable, that inhibited the PATase activity of chicken liver extracts.

The search for PATase activity in *Otala* hepatopancreas was stopped at this point. The methods that were employed during this work were shown to be valid for extracts of chicken liver. The chicken liver preparations served as controls for the entire assay. Flaks and Lukens (1963) reported that their enzyme preparation from chicken liver yielded 13,600 μmoles of inorganic pyrophosphate/2 kg of fresh tissue/15 min. at 37° for unheated preparations. The heated (60°) extracts gave 18,000 units of the same. The values obtained in this study for chicken liver enzyme averaged 26,000 μmoles of inorganic pyrophosphate synthesized/2 kg of fresh tissue/15 min. at 38° for unheated preparations, while the heated (60°) one gave 29,500 units. Thus, the values compared favorably with each other.

The presence of some substance in *Otala* hepatopancreas that inhibited the chicken enzyme makes it impossible to conclude that *Otala* hepatopancreas lacks PATase activity. PATase may be present in this snail, but normally separated from inhibitory substances. Known natural inhibitors of PATase activity in chicken liver include 4-amino-5-imidazolecarboxamide ribotide, inosine monophosphate, adenosine monophosphate, guanosine monophosphate, adenosine diphosphate, guanosine diphosphate and adenosine triphosphate (Wyngaarden and Ashton, 1959). Wyngaarden and Ashton found the inhibition to be competitive and suggested a feedback control role for this enzyme. Dialysis of *Otala* extracts may yield fruitful results in a future study of this problem.
CONCLUSIONS

Terrestrial gastropods were the first uricotelic animals in which the biosynthesis of purines was studied extensively. It was nearly 30 years ago that Baldwin (1935b) concluded that uric acid was synthesized in Helix by the condensation of 2 units of urea plus 1 of tartronic acid. The interest in the problem centered around the nitrogenous excretory products of animals, and the relation of excretion to environment. Twenty-seven years after Baldwin's publication, Bricteux-Grégoire and Florkin (1962a,b) demonstrated that uric acid was not synthesized in the manner given previously for Helix, but that it probably follows the scheme outlined by Buchanan and his co-workers in the late 1940's. The present study has shown that the synthesis of uric acid in Otala lactea, a terrestrial gastropod, is indeed by Buchanan's scheme.

Different species of animals inhabiting similar habitats are often found to have similar adaptations that favor survival under those conditions. The similarity of many of those adaptations among different species is often superficial. So, while uricotelism may be a physiological adaptation to an environment that is limited in water supply, the mode of synthesis of this excretory product does not have to be the same in all uricotelic animals. This view is reasonable and has been justified in many similar cases, but the reverse has also been
proved valid in others. If the present findings for *Otalopoma* can be extended to other uricotelic gastropods, then it can now be said that all major groups of uricotelic animals synthesize uric acid in a similar fashion.

In the introductory section it was stated that purines, including uric acid, have been found to be synthesized according to Buchanan's scheme in all organisms studied. These organisms include bacteria, plants, vertebrates and invertebrates. The importance of purine compounds extends beyond their role as excretory products. Indeed, purines are components of nucleic acids and other compounds that are basic to the existence of all organisms. It would seem reasonable to assume that the general pattern of purine biosynthesis was established very early in the evolution of organisms. Then it would follow that uric acid is synthesized in the same manner in uricotelic (and other) organisms. Work by Oró and Kimball (1961, 1962) has shown that purines can be synthesized non-enzymatically under possible primitive earth conditions from hydrogen cyanide and aqueous ammonia. Glycine, formamide, glycinamide and 4-amino-5-imidazolecarboxamide were among the other compounds formed. This work suggests the basic origins of purines in the primitive abiotic world.

After having shown that *Otalopoma lactea* met the criteria (Needham, 1935) for uricotelism, it was shown that the carbon skeleton of this purine was formed according to Buchanan's scheme. Carbon atom 6 resulted from CO$_2$; carbon 4 from the carboxyl carbon of glycine; carbon
atom 5 from the methylene carbon of glycine; and carbons 2 and 8 from the "formate" pool. It appears that glycine was incorporated as a unit, so nitrogen atom 7 is probably derived from glycine too. The finding that azaserine inhibited the synthesis of uric acid implies that at least one nitrogen atom of the latter arises from the amide nitrogen of glutamine. The lack of accumulation of radioactive intermediates, especially formylglycinamide ribotide, in the azaserine-inhibited preparation suggests that some difference may exist between Otala and other organisms for purine synthesis. Whether or not purine synthesis in Otala proceeds via phosphorylated ribose derivatives remains to be shown. The very precise incorporation of 4-amino-5-imidazolecarboxamide hydrochloride-2-C\textsuperscript{14} into carbon 8 of uric acid suggests that the general mode of purine synthesis is the same in Otala as in other organisms studied.

In the preliminary work with Otala, where it was necessary to establish uricotelism for this snail, certain secondary observations were made. The findings of Jezewska, Gorzkowski and Heller (1963a,b), that Helix pomatia accumulates and excretes xanthine and guanine along with uric acid, were confirmed for Otala lactea. The ratios of these purines to each other were almost identical in aestivating specimens of Helix and Otala. The uric acid content of Otala kidney, excreta and hepatopancreas were shown to be roughly as high as for Helix pomatia. The unknown compounds found in Otala nephridia and excreta should be identified and studied further, for some interesting biochemical
relationships might result.

Even though Jezewska, Gorzkowski and Heller (1963a) stated that *Helix* passes only solid excreta, observations made on *Otala* indicate that the latter snail may eliminate a liquid urine under very moist feeding conditions. A study of the composition of such excreta might prove interesting, for the relatively insoluble uric acid could not be the major component.

Baldwin's (1935a) observation of a direct correlation between hepatopancreatic arginase activity and nephridial uric acid concentration in different species of gastropods led him to study uric acid synthesis in *Helix* (1935b) using Wiener's (1902) scheme as a basis. Baldwin reported no attempt to determine the existence of such a correlation in several specimens of the same species. The present study revealed no such correlation among several individuals of *Otala lactea*.

Truszkowski and Chajkinówna (1935) stated that *Helix pomatia* follows the rule that uricotelic animals do not possess uricase (Pryzlecki's Rule). *Otala lactea* appears to be void of uricase activity as well. Further, there doesn't appear to be any conversion of uric acid back to precursor purines in *Otala*, so that uric acid is really a terminal nitrogenous product in these animals, and does not represent a "nitrogen bank."

The mechanism of conversion of other purines to uric acid in *Otala* should be investigated. Preliminary evidence indicates that a xanthine oxidase system is not involved. Hayashi's (1960, 1961, 1962)
demonstration of a xanthine dehydrogenase enzyme in silkworms suggests that such a system might exist in Otala, for hypoxanthine was converted to uric acid in that snail.

During the course of this study, the existence of certain metabolic reactions other than those directly involved in the biosynthesis of uric acid were strongly suggested. One of these was the conversion of serine to glycine, as evidenced by the contribution of the β-carbon of serine to the "one-carbon" pool itself. Formate, the β-carbon of serine and the methylene carbon of glycine all appear to be involved in these reactions. The activity of the various formate-activating enzymes, and their localization within Otala, would indeed be a valuable investigation. The possible existence of a third pathway, involving the oxidation of glycine to CO₂, was also demonstrated. Clues to the possible existence of other pathways were also found, but extensive work would be required to support them.

Phosphoribosylpyrophosphate amidotransferase (PATase), the first enzyme unique to the synthetic pathway for purines, has not yet been demonstrated in Otala. The hepatopancreas of these snails contains some substance that is inhibitory to PATase isolated from chicken liver, so further work is also needed in this regard.

Thus, the mode of synthesis of uric acid in the animals first studied has been demonstrated last in those same animals.
LITERATURE CITED


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