STUDIES ON THE BIOLOGICAL FORMATION OF GLUCOSAMINE IN VIVO*

II. ORIGIN OF THE NITROGEN ATOM

BY SIDNEY V. RIEDERT AND JOHN M. BUCHANAN‡

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania)

(Received for publication, January 28, 1958)

Although the carbon precursor of glucosamine has been studied in many organisms (1–5), only recently has work been done on establishing the precursor of the nitrogen atom. A preliminary report from this laboratory (6) showed that ammonia was an excellent source of this nitrogen. Recent work by Leloir and Cardini (1) has shown that, in a crude enzyme system from Neurospora crassa, the amide group of L-glutamine was the precursor of the nitrogen of glucosamine. This same observation has been made in experiments with rat liver (7) and streptococci (8). Ammonia itself can also be the source of this nitrogen atom in the synthesis of glucosamine 6-phosphate by the glucosamine-6-phosphate deaminase system prepared from Escherichia coli (9) and from rat and pig liver (10).

In this paper are presented the details of the previously reported findings, and data are presented showing that ammonia nitrogen is a precursor of the nitrogen atom of glucosamine isolated from the ovomucoid of the chicken egg.

Methods and Materials

The methods employed in the conditioning of hens of the New Hampshire Red variety and the isolation of the glucosamine from ovomucoid have been described (5). Samples of nitrogen for mass analysis were prepared from the various compounds by the method of Rittenberg (11).

The radioactive samples in the case of the glycine experiment were counted with a thin end window Geiger-Müller tube or in a gas flow counter with a 99.05 per cent helium-0.95 per cent isobutane mixture as the quenching gas. All samples were counted to a standard error of 5 per cent.

* Aided by grants from the National Cancer Institute, National Institutes of Health, United States Public Health Service, and the Damon Runyon Memorial Fund for Cancer Research, Inc.

† Predoctorate Fellow of the United States Public Health Service (1951–52). Present address, Department of Biochemistry, Yale University School of Medicine, New Haven, Connecticut.

‡ Present address, Division of Biochemistry, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts.
Uric acid was isolated according to the procedure of St. John and Johnson (12) and was characterized by its nitrogen content before degradation for mass analysis by one of three separate methods. In the first method glycine was obtained by hydrolysis of uric acid in concentrated HCl at 150° (13) and was isolated as the benzoyl derivative. The α-carbon and nitrogen atom of glycine are derived from C₆ and N₇ of uric acid. The carboxyl carbon, however, is derived equally from C₄ and C₅ (14). In the second method uric acid was oxidized with chlorine to give nitrogen atoms 7 and 9 as urea and alloxan (15, 16). This latter compound was converted to alloxantin which was isolated and subsequently oxidized with PbO₂ to urea. This urea consists of nitrogen atoms derived from N₁ and N₃ of uric acid. Finally uric acid was oxidized by the alkaline peroxide method of Brandenberger (17) to yield oxonic acid and ammonia which is derived equally from N₁ and N₇.

N¹⁵-Glycine was synthesized from N¹⁵-potassium phthalimide (Eastman Organic Chemicals) according to the procedure of Schoenheimer and Ratner (18). It was then mixed with 0.5 mc. of glycine-1-C¹⁴ (Tracerlab), dissolved in water, and recrystallized by adding 5 volumes of warm ethanol and by cooling to 0°. The product had a N¹⁵ concentration of 30.4 atom per cent excess and a specific activity of 0.78 μc. per mmole (end window counter). The C¹⁴:N¹⁵ ratio was 56,700 c.p.m. per mmole per atom per cent excess N¹⁵.

In each experiment the isotopic compound contained in a gelatin capsule was fed to a chicken weighing approximately 2 kilos. The animal had previously been conditioned to lay an egg on 3 consecutive days and then rest a day. In the experiment with N¹⁵H₂Cl, 200 mg. were fed every 8 hours for a total of nine feedings. 242 mg. of N¹⁵-glycine-1-C¹⁴ were fed in the same way. No untoward effects on the animals were noted when the above amounts of NH₄Cl or glycine were fed over the same period of time in a trial experiment. Fecal droppings were collected over a 12 hour period, and the pooled droppings were used for the isolation of uric acid.

RESULTS AND DISCUSSION

Feeding of N¹⁵H₂Cl—It has been shown that ammonia N is readily incorporated into uric acid (19, 20). More recent work by Sonne et al. (16), Shemin and Rittenberg (21), and Levenberg et al. (22) has established the precursors of each of these nitrogen atoms. This work has demonstrated that N₁ of uric acid is derived from aspartic acid, N₃ and N₅ from the amide nitrogen of glutamine, and N₇ from the glycine nitrogen. Therefore the uric acid excreted was used as a comparative index of the efficiency of the incorporation of N¹⁵H₂Cl into glucosamine.

Eggs were laid by the hen 26, 54, and 81 hours after the initial feeding
of \(^{15}N\)H\(_4\)Cl. After missing the next day two more eggs were laid. This latter cycle was repeated; a total of seven eggs from which glucosamine was isolated and analyzed for \(^{15}N\) concentration was obtained. The results of the experiment are shown in Fig. 1 where average \(^{15}N\) values of the uric acid are compared to the values for the \(^{15}N\) concentration of the glucosamine. The values for glucosamine are plotted for the estimated time of egg white deposition about the yolk of the egg. This egg white deposition, which occurs 3 to 4 hours after ovulation (23), is followed rapidly by the formation of a shell membrane, thus rendering the egg white metabolically inert with respect to the rest of the body. The shell is formed during the remainder of the time before the egg is laid. Since the dura-

![Fig. 1. Incorporation of \(^{15}N\) into uric acid and glucosamine after feeding \(^{15}N\)H\(_4\)Cl to a hen. X, the uric acid; O, glucosamine. The \(^{15}N\) concentration of the glucosamine is plotted at an approximated time of egg white formation.](image-url)
Nitrogen atom of glucosamine

collection period. This double feeding would tend to keep the N\textsuperscript{15} concentration of the uric acid precursors higher. At the cessation of feeding N\textsuperscript{15}H\textsubscript{4}Cl the N\textsuperscript{15} concentration of the uric acid dropped off precipitously, evidence indicating that the N\textsuperscript{15} concentration of the uric acid precursors diminished rapidly. This is to be expected, because uric acid is synthesized rapidly in the bird and then excreted; the isotopic concentration of the uric acid thus would reflect directly the isotopic concentration of its precursors. Contrarily, the N\textsuperscript{15} concentration of the glucosamine isolated after the cessation of feeding N\textsuperscript{15}H\textsubscript{4}Cl dropped off more slowly. Thus glucosamine after synthesis is utilized less rapidly in the bird than is uric acid. These data can be interpreted as meaning that the glucosamine used for ovomucoid

### Table I

<table>
<thead>
<tr>
<th>Nitrogen atom</th>
<th>N\textsuperscript{15} concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uric acid (total)</td>
<td>2.00</td>
</tr>
<tr>
<td>N\textsubscript{7}</td>
<td>0.68</td>
</tr>
<tr>
<td>N\textsubscript{1} + N\textsubscript{8}</td>
<td>2.03</td>
</tr>
<tr>
<td>N\textsubscript{7} + N\textsubscript{9}</td>
<td>1.87</td>
</tr>
<tr>
<td>N\textsubscript{1} + N\textsubscript{7}</td>
<td>0.85</td>
</tr>
<tr>
<td>N\textsubscript{1}</td>
<td>1.02</td>
</tr>
<tr>
<td>N\textsubscript{2}</td>
<td>3.04</td>
</tr>
<tr>
<td>N\textsubscript{8}</td>
<td>3.06</td>
</tr>
</tbody>
</table>

The N\textsuperscript{15} concentration of the various nitrogen atoms of uric acid was calculated as follows: N\textsubscript{1} = 2(N\textsubscript{1} + N\textsubscript{7}) - N\textsubscript{7}, N\textsubscript{2} = 2(N\textsubscript{1} + N\textsubscript{8}) - N\textsubscript{1}, N\textsubscript{9} = 2(N\textsubscript{7} + N\textsubscript{9}) - N\textsubscript{7}.

synthesis is either in equilibrium with the general body stores of glucosamine or at least with a pool which is used for ovomucoid synthesis. If the glucosamine were synthesized for immediate use in ovomucoid synthesis, its isotopic concentration would be expected to have diminished as rapidly as did that of the uric acid after cessation of the administration of the N\textsuperscript{15}H\textsubscript{4}Cl.

Since the data indicated that a precursor of uric acid may well be a precursor of the nitrogen of glucosamine, the uric acid collected at the point at which maximal incorporation of N\textsuperscript{15} into glucosamine occurred was degraded. The results of this degradation are shown in Table I. The value of the glucosamine isolated at this point was 1.83 atom per cent excess N\textsuperscript{15}. The low values obtained for atoms 1 and 7 of the uric acid would presumably exclude aspartic acid and glycine as precursors for the nitrogen of the glucosamine. Although the values for atoms 3 and 9 of the uric acid are high compared to that for glucosamine, they are close enough
to the glucosamine value to be consistent with the point of view that all three nitrogen atoms share a common nitrogen precursor. This is especially true, since the exact time at which the ovomucoid was synthesized prior to incorporation into the egg is not known and the value of the uric acid is an average of this period. Thus glucosamine may have been synthesized at a time when the value of the isotopic concentration of ammonia incorporated into atoms 3 and 9 of the uric acid was somewhat lower than in the sample actually degraded. Since the amide nitrogen of glutamine is the actual precursor of atoms 3 and 9 of uric acid, these results would imply that the amide group of glutamine is also involved as the direct precursor of the nitrogen atom of glucosamine. This is consistent with the known fact that glutamine may be readily synthesized in animal tissues from glutamic acid and ammonia (24). Although these data cannot determine whether ammonia or glutamine is the actual donor of the amino group of glucosamine, there is no reason to assume that the mechanism of the reaction is any different from that suggested by enzymatic experiments (1, 7, 8).

Feeding of $N^{15}$-Glycine-$1-C^{14}$—Although it has been shown that the carbon skeleton of glucose is used intact for the synthesis of glucosamine in the hen, the similarity of the location of the amino group in the 2 position of amino acids and glucosamine suggested the possibility that glucosamine might also be formed by a condensation of an amino acid and a carbon fragment. Such a reaction can be visualized as a condensation of glycine with a tetrose. To test such a hypothesis $N^{15}$-glycine-$1-C^{14}$ was fed to a hen, and the glucosamine was isolated as before. Since glycine is incorporated intact into uric acid, the uric acid could again serve as an index of the isotopic concentration of the glycine available intracellularly for synthetic purposes. If glycine per se is an actual precursor of glucosamine, the $C^{14}:N^{15}$ ratio should be the same in the glycine isolated from the uric acid as that in the glucosamine.

Eggs were laid 29, 74, 102, 146, 175, and 218 hours after the beginning of the feedings. The data obtained are given in Table II. The ratios of $C^{14}:N^{15}$ in the glycine isolated from three samples of uric acid were 38,900, 38,700, and 33,300 c.p.m. per mmole per atom per cent excess $N^{15}$ compared to the initial ratio of 56,700 present in the glycine administered, while ratios in the glucosamine isolated at comparable times were 1330 and 1090. These data indicate that glycine was not used intact for glucosamine synthesis and that the nitrogen of glycine was used more readily than was its carbon chain.

Turnover Rate—At the cessation of the feeding of the isotopic compounds, the decay rate of glucosamine could be determined by the measurement of the isotopic concentration of glucosamine isolated from successive eggs.
TABLE II
Isotopic Concentration of Glucosamine, Uric Acid, and Glycine (Derived from Uric Acid) after Feeding $N_{15}^{15}$Glycine-1-$C^{14}$ to Hen

<table>
<thead>
<tr>
<th>Elapsed time after initial feeding</th>
<th>$N_{15}^{15}$ (atom per cent excess)</th>
<th>$C^{14}$ (c.p.m. per mmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucosamine*</td>
<td>Glycine</td>
</tr>
<tr>
<td>hrs.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.028</td>
<td>0.51</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>0.382</td>
<td>2.29</td>
</tr>
<tr>
<td>56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>2.09</td>
<td>1.16</td>
</tr>
</tbody>
</table>

Feeding of isotope was stopped

<table>
<thead>
<tr>
<th></th>
<th>Glucosamine*</th>
<th>Glycine</th>
<th>Uric acid</th>
<th>Glucosamine*</th>
<th>Glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>1.34</td>
<td>0.73</td>
<td>523 (3000)</td>
<td>44,600</td>
<td></td>
</tr>
<tr>
<td>84</td>
<td>0.478</td>
<td></td>
<td>180 (1160)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>0.210</td>
<td></td>
<td>110 (570)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>157</td>
<td>0.160</td>
<td></td>
<td>70 (407)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>0.116</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The glucosamine isotope concentration is recorded at the approximate time of egg white formation.
† The values in parentheses were obtained with a gas flow counter and used for the determination of the half life of the glucosamine. All other counts were obtained with a thin end window Geiger tube.

![Graph](https://example.com/graph.png)

Fig. 2. Decay curves of glucosamine after feeding $N_{15}^{15}$-glycine-1-$C^{14}$ and $N_{15}^{15}H_5Cl$. The (○) plot was obtained from the $N_{15}^{15}$-glycine experiment and the (●) plot from feeding $N_{15}^{15}H_5Cl$. The (△) plot was obtained from the glycine-1-$C^{14}$ experiment. The time is plotted as that approximated for egg white formation.
This decay rate followed first order kinetics. This was true for both the N$_{15}$ and the C$_{14}$ experiments. The method of least squares was used to obtain the slope of the semilog plot of isotopic concentration versus time (Fig. 2). This value was used to calculate the velocity constant, and it, in turn, was used to determine the half life values. In the experiment in which N$_{15}$H$_4$Cl was fed, a value of 1.8 days was obtained, while a value of 2.3 days was obtained when N$_{15}$-glycine was used. The specific activity of the glucosamine-C$_{14}$ determined with a gas flow counter permitted the calculation of the half life of the carbon chain of glucosamine. This value was found to be 1.6 days. The agreement of the values obtained for both nitrogen and carbon of glucosamine indicates that the entire molecule of glucosamine used for ovomucoid synthesis has a half life of about 1.6 to 2.3 days.

The authors would like to express their appreciation to Dr. Sidney Weinhouse and the Houdry Process Corporation, Marcus Hook, Pennsylvania, for the N$_{16}$ analyses, and to Dr. H. Brandenberger for the alkaline peroxide degradation of the uric acid.

**SUMMARY**

The origin of the nitrogen atom of the glucosamine isolated from the ovomucoid of the egg white has been investigated by the feeding of N$_{15}$ compounds. It has been shown that fed N$_{15}$H$_4$Cl was as readily incorporated into the glucosamine as into the uric acid. The correspondence of the concentration of N$_{15}$ in the glucosamine and in positions 3 and 9 of uric acid after administration of N$_{16}$-labeled ammonium salts suggests that either glutamine or ammonia may be an intermediate in the synthesis of glucosamine in the bird.

N$_{15}$-Glycine-1-C$_{14}$ was fed to a hen. Although the nitrogen of the fed glycine could be utilized for glucosamine synthesis to some extent, its carbon was not.

The decay curves obtained after cessation of feeding N$_{15}$ and C$_{14}$ followed first order kinetics and permitted the calculation of the half life of the glucosamine. A value of 1.6 to 2.3 days was found.

**BIBLIOGRAPHY**

NITROGEN ATOM OF GLUCOSAMINE