The Biosynthesis of Glycoproteins

III. GLUCOSAMINE INTERMEDIATES IN PLASMA GLYCOPROTEIN SYNTHESIS IN LIVERS OF PUROMYCIN-TREATED RATS

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It was previously shown that the particulate fraction of liver was the site of incorporation of glucosamine-1-14C into rat plasma glycoproteins (1). In continuing these studies, we wished to determine whether this incorporation was dependent on the synthesis of protein de novo. Since puromycin inhibits protein biosynthesis (2-5), we have studied the effect of this drug on the incorporation of glucosamine into liver and plasma proteins in vivo.

Puromycin inhibited the incorporation of glucosamine-14C into liver and plasma proteins, and there was an accumulation of radioactive glucosamine derivatives in the acid-soluble fraction of the liver. The nature of these compounds has been elucidated.

EXPERIMENTAL PROCEDURE

Animals—Male albino rats of the Charles River strain, weighing 250 to 350 g, were fed ad libitum before use.

Materials—DL-glucosamine-1-14C (5.1 mc per mmole), DL-leucine-1-14C (1.8 mc per mmole), and puromycin were dissolved in 0.9% NaCl solution for injection. These materials, together with glucosamine, N-acetylglucosamine, UDP-N-acetylgalactosamine, alkaline phosphatase, and yeast hexokinase were commercial preparations.

Glucosamine 6-phosphate was prepared from glucosamine with N-acetylhexosaminidase (6). Nucleotides were removed from the reaction mixture by adsorption on charcoal, and the product was precipitated as the barium salt in 70% ethanol.

N-Acetylglucosamine 6-phosphate was prepared by acetylation with acetic anhydride (7). The product was purified by chromatography on Dowex 1-chloride resin, under the conditions described below. The material was eluted between 0.021 and 0.028 M hydrochloric acid.

The preparations of acetylglucosamine 6-phosphate and 1-phosphate were identical with samples of these materials provided by Dr. Saul Roseman, when compared by paper and ion exchange chromatography.

Treatment of Animals and Preparation of Liver Subcellular Fractions—Rats received intraperitoneal injections of 15 mg of puromycin 1 hour before and concurrently with injection of the isotopic material, then at subsequent hourly intervals. Either glucosamine-14C or DL-leucine-14C, 4 μc, was administered intraperitoneally. In some experiments, the conditions of puromycin administration differed from those cited above and are described in “Results.”

At selected times, the animals were killed by decapitation, the blood was drained into 10 ml of 0.9% NaCl solution containing 0.01 M EDTA, pH 7.0, the volume was measured, and the plasma was separated by centrifugation. The livers were quickly weighed and homogenized in 10 volumes of 0.25 M sucrose containing 0.02 M Tris-hydrochloric acid buffer at pH 7.4. The homogenates were centrifuged at 960 × g for 10 minutes in a Servall refrigerated centrifuge. The residues were again homogenized and centrifuged in the same manner. The combined supernatants were centrifuged at 78,000 × g for 45 minutes in the Spinco model L centrifuge. The sediments from this centrifugation were suspended in 10 ml of water to give the particulate fraction, and the supernatants were taken as the supernatant fraction. Protein was precipitated with 10% trichloroacetic acid as described previously (1).

Preparation of Liver Acid-soluble Extracts—In experiments in which the nature of the trichloroacetic acid-soluble intermediates were studied, rats were treated by injection with puromycin and isotope or with isotope only as indicated in the text, and were killed at suitable times after injection. The livers were quickly removed and homogenized in a Waring Blender with 5 volumes of chilled 10% trichloroacetic acid. The homogenates were centrifuged, and the residues were re-extracted with 5 volumes of 10% trichloroacetic acid. The supernatants were combined, extracted with ether to remove trichloroacetic acid, neutralized, and concentrated under vacuum.

In a number of experiments, the livers were homogenized in 5 volumes of 50% ethanol and heated to boiling. The suspensions were filtered after addition of Celite, 1 g/20 ml, and the filtrates were concentrated under vacuum. The results obtained with ethanol extraction were similar to those obtained with trichloroacetic acid.

Ion Exchange Chromatography—Columns (1.2 × 16 cm) were
prepared from washed Dowex 1-X4-chloride resin (200 to 400 mesh). The concentrated, neutralized extracts were applied directly to columns; in some cases, the extracts from two livers were chromatographed on the same column. A two-step linear gradient was used for elution: the first gradient consisted of 300 ml of water and 300 ml of 0.05 N hydrochloric acid; the second was 300 ml of 0.05 N hydrochloric acid and 300 ml of 0.5 N hydrochloric acid. The flow rates of columns were 30 ml per hour; 10-ml fractions were collected; columns were operated at 3°C.

Paper Chromatography—The solvents used were: Solvent A, 95% ethanol-1 M ammonium acetate, pH 7.0 (7.5:3, v/v); Solvent B, ethyl acetate-pyridine-water (10:4.3, v/v); Solvent C, 1-butanol-pyridine-water (6:4:3, v/v); Solvent D, ethyl acetate-acetic acid-water (3:1:3, v/v); Solvent E, isobutyric acid-1 M ammonium hydroxide-0.1 M EDTA (100:60:16, v/v).

Nucleotides were located by ultraviolet illumination from a Mineralight lamp. Phosphorus-containing compounds were detected by the method of Burrows, Grylls, and Harrison (9), acetylated hexosamines by the method of Cardini and Leloir (10), sugars by alkaline silver nitrate (11), and sialic acids by Ehrlich's reagent (12).

Chemical Determinations—Hexosamines were determined by the Boas method (13) with glucosamine as standard. N-Acetylated hexosamines were determined by the procedure described by Reissig, Strominger, and Leloir (14) with N-acetylgalactosamine as standard. Sialic acids were determined by the thiobarbituric acid method (15), with N-acetylgalactosamine acid as a standard. Phosphate was determined by the method of King (16), and protein according to Gornall, Bardawill, and David (17).

**TABLE I**

**Effect of puromycin on incorporation of glucosamine-1-14C into proteins of plasma and of liver**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Time</th>
<th>Protein specific activity</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>d.p.m./mg</td>
<td>Puromycin- treated</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>290</td>
<td>85</td>
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<tr>
<td></td>
<td>60</td>
<td>1830</td>
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<td>120</td>
<td>4180</td>
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<td></td>
<td>180</td>
<td>4090</td>
<td>100</td>
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<tr>
<td>Liver particulate fraction</td>
<td>30</td>
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<td></td>
<td>60</td>
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<td>200</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>1020</td>
<td>200</td>
</tr>
<tr>
<td>Liver supernatant fraction</td>
<td>30</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>00</td>
<td>200</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>120</td>
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<td>40</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>250</td>
<td>40</td>
</tr>
</tbody>
</table>

Throughout the paper, N-acetylated hexosamines are referred to as N-acetylated hexosamines, but it is recognized that other acyl groups may be present.

Column Chromatographic Analysis of Hexosamines—Glucosamine was separated from galactosamine and mannosamine by automated chromatography on Dowex 50 resin (18). In this system, galactosamine and mannosamine emerged as a single peak. The radioactivity of the effluent was determined with a Packard continuous flow counter.

Determination of Radioactivity—The method of preparing and counting samples was previously described (1). In these experiments, a toluene-methyl Cellosolve scintillation system was used (19). For each sample, the efficiency of counting was determined by again counting each sample after the addition of 14C-benzoic acid as an internal standard, and results are expressed as disintegrations per minute. Radioactive areas on paper chromatograms were detected by placing strips of chromatography paper directly into counting vials.

Detection of N-Acetylmannosamine with N-Acetylmannuronic Acid Aldolase—According to the procedure published by Comb and Roseman (20), N-acetylmannosamine can be identified by using N-acetylmannuronic acid aldolase. This enzyme specifically catalyzes the reversible formation of NAN from pyruvic acid and N-acetylmannosamine.

Samples thought to contain radioactive N-acetylmannosamine were treated with a mixed bed resin before use to remove salts and sialic acid. The solutions were then incubated at 37°C for 6 hours in the presence of 50 μmoles of sodium pyruvate, 5 μmoles of carrier N-acetylmannosamine, and the purified NAN-aldolase in a total volume of 3.5 ml of 0.01 M Tris buffer, pH 7.4. The reactions were stopped at 100°C, and the supernatants from centrifugation were applied to Dowex 1-chloride columns; these were eluted with the first gradient described above. The fractions containing NAN were pooled and concentrated for measurement of sialic acid and radioactivity.

**RESULTS**

**Effect of Puromycin on Incorporation of Glucosamine-14C and Leucine-14C into Proteins of Liver and Plasma**

The effect of puromycin on the incorporation of glucosamine into liver and plasma proteins is shown in Table I. Incorporation into these proteins was inhibited by 71 to 95% during the 3-hour experimental period. This inhibition was comparable to or somewhat less than inhibition of leucine incorporation, 90 to 100% (Table II).

Table III shows that radioactive glucosamine derivatives rapidly accumulated in the acid-soluble fraction of the livers of both control and test animals. After 1 hour, this radioactivity decreased in the controls but remained unchanged in the puromycin-treated animals.

The inhibition of glucosamine incorporation by puromycin could possibly have resulted from a large increase in the acid-soluble glucosamine precursor pool in the livers of puromycin-treated animals. This could cause a diminution of the specific activity of glucosamine which would be manifested as an apparent decrease in incorporation even though the rate of glucosamine incorporation was not altered. Measurement of the total acid-soluble glucosamine pool in the livers of control and puromycinated animals showed that this decrease was not significant.

1 The abbreviation used is: NAN, N-acetylmannosaminic acid.
cin-treated rats by the Boas method (13) gave values of 0.45 ± 0.03 μmole and 0.56 ± 0.05 μmole per g of liver, respectively. In addition, measurement of the specific activities of the UDP-N-acetylglucosamine pools (see below) in both groups gave 438,000 d.p.m. per μmole in control animals and 410,000 d.p.m. per μmole in puromycin-treated animals. Thus, the large effect of puromycin on incorporation cannot be due to an alteration of the size or of the specific activity of the acid-soluble glucosamine pool.

Nature of Acid-soluble Radioactive Substances Found in Livers from Rats Treated by Injection with Puromycin and Glucosamine

The results above established that puromycin inhibits the incorporation of glucosamine into protein and causes an accumulation of acid-soluble radioactivity in the liver. We have investigated the nature of these acid-soluble substances to obtain information about possible intermediates involved in the incorporation of glucosamine into protein. McGarrahan and Maley (21) had previously investigated acid-soluble intermediates appearing in rat liver a short time after glucosamine-14C injection, and their studies served as a guide in the work reported here.

Rats were given puromycin and glucosamine as described, and trichloroacetic acid extracts of the livers were prepared 30 minutes after glucosamine injection. These extracts were chromatographed on Dowex 1-chloride resin and the radioactivity of the column effluent was measured. Fig. 1 shows the results of a typical experiment in which four radioactive peaks emerged from the column. Peak I appeared in the neutral region, Peak II was eluted at 0.005 to 0.011 N acid concentration, Peak III at 0.015 to 0.025 N, and Peak IV at 0.11 to 0.18 N acid concentration. These four peaks contained all the radioactivity applied to the column. The fractions in each peak were pooled, neutralized with dilute ammonium hydroxide, and dried under vacuum for analysis.

Composition of Fraction I—This fraction contained glycogen which could be removed by precipitation with 50% ethanol. No radioactivity was associated with the precipitate after washing with 50% ethanol. The ethanolic solution was dried, and the residue was dissolved in water. Since it was likely that free glucosamine would occur in this fraction, the solution was applied to a short column of Dowex 50-IIx which was eluted first with water and then with 2 N hydrochloric acid. In most experiments, approximately 50% of the radioactivity was eluted with water and the rest with 2 N hydrochloric acid. The acid eluate was

TABLE III
Effect of puromycin on total acid-soluble radioactivity in plasma and liver after intraperitoneal injection of glucosamine-14C or leucine-1-14C

Conditions were as for Table I. Radioactivities of the acid-soluble fractions of plasma and of the liver supernatant fractions were determined as described in the text. The radioactivity of the liver is expressed as total activity per 11.7 g of liver, and that of plasma is calculated from a plasma volume of 3.5% of the body weight.

Table:<br>\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Fraction} & \text{Time} & \text{Glucosamine-14C} & \text{DL-Leucine-1-14C} \\
\hline
\text{Plasma} & \text{min} & \text{d.p.m.} \times 10^3 & \text{d.p.m.} \times 10^3 \\
\hline
\text{Liver} & \text{min} & \text{d.p.m.} \times 10^3 & \text{d.p.m.} \times 10^3 \\
\hline
\end{array}
\]

FIG. 1. The chromatographic separation of radioactive acid-soluble substances from the livers of rats treated by injection with glucosamine-14C and puromycin. A rat was given an injection of 15 mg of puromycin 1 hour before and at the time of injection of 4 μC of glucosamine-14C. After 30 minutes, the animal was decapitated, and an acid extract of the liver was prepared as described in the text. The material was applied to a column of Dowex 1-chloride (1.2 X 16 cm) and eluted with two linear gradients; the first was 300 ml of water and 300 ml of 0.05 N hydrochloric acid, the second was 300 ml of 0.05 N hydrochloric acid and 300 ml of 0.5 N hydrochloric acid. The flow rate of column was 30 ml per hour, and 10-ml fractions were collected. The column was operated at 3°.
analyzed for hexosamines by column chromatography (18), and all the radioactivity emerged as free glucosamine.

The radioactivity of the water eluate from the Dowex 50 column was due to several compounds. A major portion of the radioactivity (40 to 60%) was associated with a substance behaving like N-acetylhexosamine on paper chromatography in Solvents A and B. In addition, the eluate gave a positive test for N-acetylhexosamine (14). Analysis of the free hexosamines (18) after decylation in 2 N hydrochloric acid, for 2 hours, at 100°, showed that glucosamine contained about 96% of the radioactivity while the rest was associated with the galactosamine-mannosamine fraction. Experiments with NAN aldolase indicated that approximately 4% of the total hexosamine radioactivity was due to N-acetylmannosamine.

When the water eluate was treated with alkaline phosphatase under conditions suitable for the hydrolysis of glucosamine 6-phosphate, a part of the radioactivity subsequently behaved as free hexosamine, it was retarded by a Dowex 50 column, and could be eluted with 2 N hydrochloric acid (18). This experiment indicated that hexosamine 6-phosphate was present in this fraction as was shown by McGarrahan and Maley (21).

Sialic acid was also found in the water eluate from the Dowex 50 column. Recromatography of the water eluate on Dowex 1 chloride gave two radioactive peaks, the major part was eluted with water while the remainder was eluted with acid at the position where sialic acid was expected. The latter radioactive material was concentrated and was found to give the color for sialic acid in the thiobarbituric acid test (15).

Thus, Fraction I contained free glucosamine, N-acetylglucosamine, N-acetylmannosamine, hexosamine 6-phosphate, and sialic acid.

**Composition of Fraction II**—This fraction, not comparable with any of the compounds reported by McGarrahan and Maley (21), was identified as free sialic acid. It emerged from the column in the position expected for sialic acid, and when Fraction II was cochromatographed on Dowex 1 with added NAN, the radioactivity was eluted in a pattern identical with that for the added NAN. A small part of the NAN and of the radioactivity emerged in the neutral region, this was probably caused by the presence of salt and explained the finding that sialic acid was also present in Fraction I. Fraction II gave a color with the thiobarbituric acid method (15), and the absorption spectrum was identical with that for NAN. In addition, the radioactive material showed RF values identical with those of NAN on paper chromatography in Solvents A and B.

**Composition of Fraction III**—This fraction corresponded with N-acetylglucosamine 6-phosphate found by McGarrahan and Maley (21). Synthetic N-acetylglucosamine 6-phosphate and N-acetylglucosamine 1-phosphate emerged from the column together in the same position as Fraction III. N-Acetylglucosamine 6-phosphate and Fraction III were cochromatographed, and the radioactivity emerged with the standard. Fraction III gave the color reaction for acetohexosamines (14), and the radioactivity moved as N-acetylglucosamine 6-phosphate on paper chromatography with Solvents A, B, and E.

The relative amounts of N-acetylhexosamine 1- and 6-phosphates in this fraction were examined by hydrolyzing the fraction in 1 N hydrochloric acid at 100° for 10 minutes. Under these conditions, hydrolysis of the 1-phosphate is complete, whereas that of the 6-phosphate is negligible. To test for hydrolysis, the hydrolysate was applied to a short Dowex 1 chloride column, and approximately 10% of the radioactivity was not retarded; this was assumed to be acetylglucosamine released during hydrolysis. Thus, Fraction III was presumably composed of 90% N-acetylhexosamine 6-phosphate and 10% N-acetylhexosamine 1-phosphate.

The identity of the hexosamines in this fraction was investigated by removing the phosphate with alkaline phosphatase and deacetyting with acid. The hydrolysate was analyzed for hexosamines (18); 14% of the radioactivity emerged with the acidic amino acids, 76% with glucosamine and, 10% with the galactosaminomannosamine peak. From control experiments in which N-acetylglucosamine 6-phosphate was hydrolyzed with alkaline phosphatase, approximately 15% of the substrate remained unhydrolyzed; therefore, the first peak was assumed to be derived from unhydrolyzed N-acetylglucosamine 6-phosphate. The presence of mannosamine was investigated by dephosphorylation with alkaline phosphatase followed by reaction with NAN-aldolase. The results indicated that approximately 4% of the radioactivity was due to N-acetylmannosamine.

**Composition of Fraction IV**—This material was eluted from the column in the region of the nucleotide sugars. McGarrahan and Maley (21) reported the presence of UDP-N-acetylglucosamine, and the results cited here support this analysis. The radioactive material was purified by charcoal adsorption (22) and by paper chromatography in Solvent A. This latter step separated the radioactive material from contaminating nucleotide sugars, and the radioactivity was quantitatively associated with a band migrating as UDP-N-acetylglucosamine. The purified material was homogeneous on high voltage electrophoresis at pH 3.7 and on paper chromatography in Solvents A, B, C, and D. In all these systems, it behaved as UDP-N-acetylglucosamine. Spectral analysis showed the presence of uridine, and chemical analysis gave a ratio of uridine to phosphate to acetylhexosamine of 1:1.9:0.8. After acid hydrolysis in 2 N hydrochloric acid for 2 hours at 100° and paper chromatography in Solvent B, the material showed the same pattern of reducing substances as did a hydrolysate of authentic UDP-N-acetylglucosamine. After hydrolysis in 1 N hydrochloric acid for 10 minutes at 100° and removal of UMP on a Dowex 1 chloride column, the material was inert in the orcinol test for hexoses (23), indicating the absence of nucleotide-bound hexoses.

Fraction IV was hydrolyzed in 2 N hydrochloric acid for 3 hours at 100°, then analyzed for hexosamines (18). Glucosamine accounted for 80% of the radioactivity and galactosamine for the remainder.

**Variation with Time of Glucosamine Intermediates in Liver**

The variation with time of the distribution of radioactivity in the various peaks was investigated in control animals, in puromycin-pretreated animals, and in animals that had received glucosamine and puromycin simultaneously. In all cases, Fraction IV increased with time and contained 70% to 90% of the radioactivity (Table IV). Fraction III was lower in the controls than in the puromycin-treated animals and did not alter appreciably during the experiment. Fraction I decreased while Fraction II increased with time. The puromycin-pretreated animals had less radioactivity in Fraction II than the controls, or animals treated by simultaneous injection with puromycin and glucosamine.
Variation with time of radioactivity in fractions from acid extracts of livers from rats treated by injection with glucosamine-\(^{14}\)C and puromycin

Rats were treated intraperitoneally with 15 mg of puromycin 1 hour before and at the time of injection of 4 \(\mu\)C of glucosamine-\(^{14}\)C, a second group received puromycin simultaneously with the isotope, and the control group received glucosamine alone. At indicated time intervals after the administration of isotope, the animals were killed, and acid extracts of livers were prepared as described in the text. The chromatographic separation of the compounds was performed as shown in Fig. 1.

### Table IV

<table>
<thead>
<tr>
<th>Time</th>
<th>Fraction 1</th>
<th>Fraction 2</th>
<th>Fraction 3</th>
<th>Fraction 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d.p.m.</td>
<td>% of dose</td>
<td>d.p.m.</td>
<td>% of dose</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>56.1</td>
<td>7.1</td>
<td>6.4</td>
<td>0.81</td>
</tr>
<tr>
<td>10</td>
<td>20.6</td>
<td>2.6</td>
<td>11.2</td>
<td>1.41</td>
</tr>
<tr>
<td>30</td>
<td>0.99</td>
<td>12.4</td>
<td>1.57</td>
<td>3.9</td>
</tr>
<tr>
<td>Puromycin-treated</td>
<td>61.5</td>
<td>7.8</td>
<td>1.7</td>
<td>0.21</td>
</tr>
<tr>
<td>10</td>
<td>38.4</td>
<td>4.8</td>
<td>1.2</td>
<td>0.15</td>
</tr>
<tr>
<td>20</td>
<td>16.6</td>
<td>2.1</td>
<td>2.9</td>
<td>0.37</td>
</tr>
<tr>
<td>30</td>
<td>22.8</td>
<td>2.8</td>
<td>6.2</td>
<td>0.65</td>
</tr>
<tr>
<td>Puromycin simultaneously with glucosamine-(^{14})C</td>
<td>14.7</td>
<td>1.8</td>
<td>33.6</td>
<td>4.2</td>
</tr>
<tr>
<td>60</td>
<td>8.42</td>
<td>1.06</td>
<td>34</td>
<td>4.3</td>
</tr>
</tbody>
</table>

The results suggest that in the liver injected glucosamine is rapidly converted to UDP-N-acetylhexosamine and slowly to sialic acid.

### Determination of Size of Pool of UDP-N-Acetylhexosamine

Since isotopically labeled UDP-N-acetylhexosamine of known specific activity was available from these experiments, it was possible to determine the amount of UDP-N-acetylhexosamine in the livers of normal rats by isotope dilution. Two rats were killed, and trichloroacetic acid extracts were prepared from the livers. A known amount, 0.56 \(\mu\) mole, of UDP-N-acetylhexosamine, with a specific activity of 0.143 \(\mu\)C per \(\mu\) mole, was added to each trichloroacetic acid homogenate at the time of homogenization and the UDP-N-acetylhexosamine was isolated from each liver as described. The dilution of specific activities were 11.5-fold and 9.1-fold corresponding to UDP-N-acetylhexosamine pools of 0.47 \(\mu\) mole per g of wet weight and 0.39 \(\mu\) mole per g of wet weight, respectively.

### Discussion

Although a general scheme has been proposed for the biosynthesis of simple proteins (24), the biosynthesis of conjugated proteins presents additional problems. In the instance of glycoprotein synthesis, we can pose several questions. At what site in the cell are carbohydrates added to the polypeptide? Are the oligosaccharide units completed before attachment to the polypeptide or are they formed on the polypeptide by addition of individual monosaccharides? What controls the specificity of structure of the oligosaccharide and the site of its insertion in the polypeptide?

In an earlier paper (1), we reported that the liver particulate fraction, probably the microsomes, was the site of incorporation of glucosamine into plasma protein. Since this incorporation of glucosamine into proteins might represent an interchange between existing glycoproteins and glucosamine or might reflect the synthesis de novo of glycoproteins, we have investigated the effect of puromycin on this incorporation. It was found to be markedly inhibited by puromycin while the accumulation of acid-soluble glucosamine derivatives in the liver was not affected. Since puromycin inhibits protein biosynthesis (2-5), it is tempting to assume that the incorporation of glucosamine is dependent on polypeptide synthesis de novo. However, it is possible that puromycin interferes with the synthesis of glycoproteins either by direct inhibition of the enzymes involved in glucosamine incorporation or by inhibiting the biosynthesis of these enzymes if they turn over rapidly. Further work is required to distinguish among these possibilities.

Richmond (25) has reported that puromycin inhibits the incorporation of glycine into glycoprotein in a perfused rat liver system but does not inhibit the incorporation of glucose or glucosamine. The author takes the initial rate of incorporation of the isotope as a control and examines any changes apparent in the rate of incorporation after addition of puromycin. This method of analysis, however, may be misleading, since a decrease in the rate of incorporation may reflect a decrease of specific activity in the precursor pool, even in the absence of puromycin. In view of the lack of appropriate control experiments, it seems as if his conclusions are not adequately supported.

Since there was a prolonged accumulation of acid-soluble radioactivity in the livers of puromycin-treated rats we studied the nature of these radioactive compounds. If oligosaccharides are completely or partially formed before addition to the polypeptide or are they formed on the polypeptide by addition of individual monosaccharides? What controls the specificity of structure of the oligosaccharide and the site of its insertion in the polypeptide?
peptide, a possibility suggested by Roseman (26), these might be found in the acid-soluble fraction. We were, however, unable to find such compounds in the livers of control rats or in rats which had had puromycin either prior to isotope administration or simultaneously with the isotope. The failure to observe any acid-soluble radioactive oligosaccharides may be explained in several ways, there may not be such intermediates, they may be present in amounts too small to detect by the procedures used or they may be labile and so be destroyed during the isolation. The present experiments do not permit us to distinguish among these possibilities. In any case, it seems that UDP-N-acetylglucosamine is a stable intermediate in the incorporation pathway of glucosamine.

It is still possible to argue that preformed oligosaccharides may be involved in the biosynthesis of glycoproteins but that they are present in the liver, even in the presence of inhibitors, in amounts too small to detect. More experiments are required before this possibility can be dismissed.

McGarrahan and Maley (21) have previously characterized intermediates appearing in liver after glucosamine administration. Our results closely parallel and extend their findings. In Fig. 2 the pathways of glucosamine metabolism taken from the literature (21, 26) are shown. The compounds identified in this study are underlined, and the results agree well with this scheme. We have shown that glucosamine is rapidly converted to UDP-N-acetylglucosamine which in turn is partly converted to UDP-N-acetylgalactosamine or sialic acid. The presence of UDP-N-acetylgalactosamine in liver has been known for some time (27), and our results are in agreement with the previous finding (28) that this is a mixture of UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine.

Studies by Roseman (26) and by Warren and Felsenfeld (29) suggest that sialic acid is formed from UDP-acetylgalactosamine via acetylmannosamine and acetylmannosamine 6-phosphate. Our finding that labeled mannose occurred in the acetylmannosamine and acetylhexosamine phosphate fractions is in agreement with this pathway. However, N-acetylmannosamine could also have been produced from sialic acid by the action of NAN-aldolase.

Although Roseman (26) has reported that sialic acid occurs as CMP-sialic acid, there was no evidence of this compound in these experiments. However, the conditions of isolation and chromatography employed in the current studies could have caused its hydrolysis.

SUMMARY

1. Puromycin has been found to inhibit markedly the incorporation of glucosamine-1-14C into rat liver and plasma proteins in vivo. At the same time, there was a prolonged accumulation of acid-soluble radioactivity in the liver.

2. The nature of the acid-soluble glucosamine intermediates was investigated. These intermediates were separated by ion exchange chromatography and were identified as glucosamine, N-acetylglucosamine, sialic acid, hexosamine 6-phosphate, N-acetylgalactosamine 6-phosphate and 1-phosphate, and UDP-N-acetylgalactosamine. Radioactive UDP-N-acetylgalactosamine, N-acetylmannosamine, and N-acetylmannosamine phosphate were also found.

3. The variation with time after glucosamine injection of the radioactivity of some of these compounds was investigated.

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