GALACTOSAMINE: A PRECURSOR OF GLYCOGEN GLUCOSAMINE*

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During a study of the metabolism of galactosamine-1-^{14}C in rat liver, it was found that, in addition to a number of acid-soluble products, considerable radioactivity was associated with the glycogen (Maley et al., 1966). To our surprise, the component that contained the radioactivity was neither glucose nor galactosamine, but glucosamine. Further studies revealed the glucosamine to be present as an integral part of the glycogen. The evidence supporting these findings and the mechanism by which glucosamine is incorporated into the glycogen will be presented in this report.

RESULTS AND DISCUSSION

Glycogen Labeling -- In contrast to our previous studies with glucosamine-1-^{14}C (DelGiacco and Maley, 1964), where little if any radioactivity was found in the glycogen, perfusion of rat liver with galactosamine-1-^{14}C led to the incorporation of radioactivity into the glycogen,[1] amounting to 10% of that in the liver acid-soluble fraction. The evidence indicating the radioactive compound to be glucosamine glycosidically linked to glucose was as follows:

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[1] Obtained by perfusing a liver for 3 hrs with 56 μmoles of D-galactosamine-1-^{14}C (New England Nuclear Corp.) (8.1 X 10^5 cpm/μmole) in an enriched perfusate. The glycogen (9 ml) was precipitated from the acid-soluble fraction and dialyzed in the cold against five changes of water (2 liters each) for 2 days. The radioactivity retained by the glycogen was 3.1 X 10^6 cpm.

1. Chromatography of the glycogen on a 90- x 1.2-cm column of Sephadex G-100 demonstrated the radioactivity to elute with the glycogen.

2. Extensive dialysis did not remove the radioactivity from the glycogen.

3. Nitrous acid, which cleaves 1,4-hexosaminidic linkages (Foster et al., 1953; Yosizawa, 1964) was found to solubilize most of the radioactivity.

4. Acid hydrolysis (4 N HCl at 100° for 12 hrs) verified the presence of hexosamine, since the radioactivity was retained by Dowex 50-H^+. Chromatography of the eluted hexosamine on a modified Gardell column (Pearson, 1963) and electrophoresis of the N-acetylated hexosamine in 1% borate provided evidence that the radioactive compound incorporated into the glycogen was glucosamine.

5. Treatment of the glycogen with α-amylase solubilized the radioactivity associated with it, as seen in the time versus solubility data presented in Table I. Similar results were obtained with β-amylase.

Table I

Solubilization of Glycogen Radioactivity with α-amylase

Minutes	cpm
0	807
2	1,590
5	3,620
10	4,600
60	20,800
300	26,600

The reaction mixtures contained 0.1 ml of glycogen (10.7 mg; 31,920 cpm); 0.1 ml of enzyme solution consisting of 0.02 M potassium phosphate, 0.02 M NaCl; and 0.36 μgm of α-amylase (Worthington Biochemicals Corp.). The incubation temperature was 25° and at the indicated times 0.4 ml of 95% ethanol was added. After centrifugation, a 0.2-ml aliquot was taken from the supernatant fraction for radioactivity determination.

Chromatography of the solubilized components revealed mainly two and possibly three radioactive products to be formed on α-amylase digestion of the glycogen (Fig. 1). None of these apparently coincided with the reducing sugars (glucose, maltose, and higher oligosaccharides). A somewhat similar finding was obtained with the product resulting from β-amylase treatment, for, as seen in Fig. 1, the radioactivity migrated more slowly than did maltose. The above results could be explained by the presence of a single glucosamine unit in the di-, tri-, and tetrasaccharides produced on enzymic digestion of the labeled glycogen, and are consistent with the observation that glucosamine moves more slowly than glucose in this chromatographic system (Fig. 1).

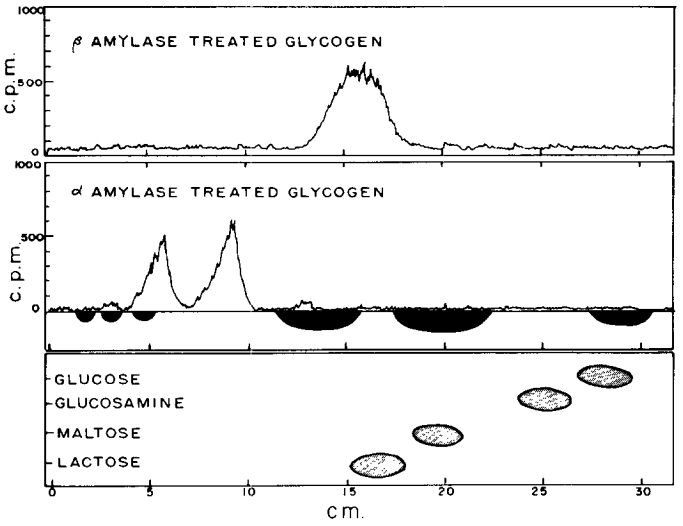

Figure 1. Descending chromatography (75% isopropanol-acetic acid (9:1)) of the alcohol-soluble fractions from α- and β-amylase-treated glycogen. The dark areas adjacent to the α-amylase-treated glycogen signify the reducing sugar regions, as do the shaded areas in the chromatogram of the known sugars. The radioactive regions were located with a Nuclear Chicago Corp. actigraph III, paper chromatogram scanner.

Studies on the β-amylase Product (β-A.P.) -- To determine if the above assumption was correct, a sample of glucosamine-labeled glycogen was exhaustively digested with β-amylase and passed through a column of Dowex 50-H^+, which retained almost all of the radioactivity. The column was then eluted

with 35 ml of water, 20 ml of 0.05 N HCl, and 20 ml of 0.5 N HCl. More than 90% of the radioactivity was eluted by the last eluent. Acid hydrolysis of the β-A.P. (4 N HCl at 100° for 17 hrs) yielded two reducing spots on paper chromatography in isopropanol-H_2O (4:1), one coinciding with glucose and the other coinciding with both glucosamine and the radioactivity. Analysis of the mixture after N-acetylation for, N-acetylglucosamine (Reissig et al., 1955), and for glucose by the coupled hexokinase-glucose-6-P dehydrogenase assay revealed these hexoses to be present in a 1:1 ratio (Fig. 2). If the β-A.P. is assumed to be a disaccharide composed of glucose and glucosamine, glycosidically linked α (1--->4), two sequences are possible: 0-2-amino-2-deoxy-α-D-glucopyranosyl-(1--->4)-D-glucopyranose and 0-α-D-glucopyranosyl-(1--->4)-2-amino-2-deoxy-D-glucopyranose. Both disaccharides were recently prepared and characterized by Wolfrom et al. (1964a and b). Comparison of the β-A.P. with the Rg's in n-butanol-ethanol-water (4:1:5) reported for these disaccharides (0.27 and 0.32, respectively) revealed an Rg of 0.15-0.25 for the β-A.P. However, N-acetylation (Roseman and Daffner, 1956) altered the Rg to 0.45, which compared favorably with an Rg of 0.47 for 0-2-acetamido-2-deoxy-α-D-glucopyranosyl-(1--->4)-D-glucopyranose (Wolfrom et al., 1964a). Assay for N-acetylglucosamine (Reissig et al., 1955) yielded only 15-20% of the color anticipated for free N-acetylglucosamine, which agrees favorably with the results obtained by Wolfrom et al. (1964a) for the glucosyl-N-acetylglucosaminide. Nitrous acid treatment of the β-A.P. yielded mainly anhydromannose and glucose. The latter was estimated by the coupled hexokinase-glucose-6-P dehydrogenase and the former by the indole assay (Dische and Borenfreund, 1950), as well as by electrophoresis in 1% borate, in which anhydromannose migrates to the cathode. The yield of free glucose, however, was only 70-80% of the theoretical amount expected if the β-A.P. was entirely 0-2-amino-2-deoxy-α-D-glucopyranosyl-(1---->4)-D-glucopyranose. The residual 20-30% was probably associated with 0-α-D-glucopyranosyl-(1---->4)-2-amino-2-deoxy-D-glucopyranose, a compound that should be resistant to nitrous acid cleavage.

If this assumption is valid, sodium borohydride treatment of the N-acetylated β-A.P. followed by acid hydrolysis and re-N-acetylation should yield radioactive N-acetyl-2-amino-2-deoxy-D-glucitol, in addition to the major product, N-acetylglucosamine. Fig. 2 presents the results of such a study. As seen,

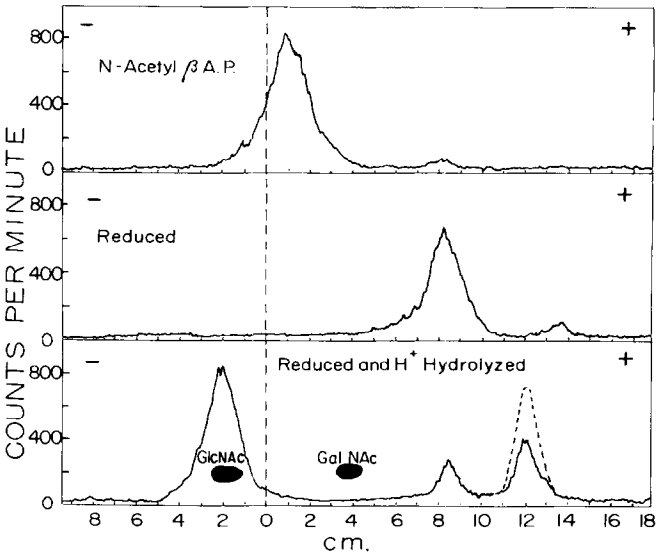

Figure 2. One-percent borate electrophoresis (4 hr at 17 v/cm, 10°) of N-acetylated β-A.P. before (upper strip) and after (middle strip) reduction with sodium borohydride (2.5 mg to about 0.25 μmole of disaccharide in 1 ml). The latter was also hydrolyzed in 4 N HCl for 3 hrs at 100°, followed by re-N-acetylation (lower strip). The peak of radioactivity (dashed line) indicates the migration distance of a known sample of N-acetyl-2-amino-2-deoxy-D-glucitol-1-^{14}C. Known samples of N-acetyl-D-glucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) were also included.

acid hydrolysis of the N-acetylated-reduced β-A.P. yielded mainly N-acetylglucosamine, a small amount of unhydrolyzed disaccharide, and a product that migrates exactly as a known sample of N-acetyl-2-amino-2-deoxyglucitol. These experiments would thus indicate that the β-A.P. is a mixture of the two above-mentioned disaccharides. Attempts at more definite methods of analysis were limited by the quantity of disaccharide available from each perfused liver, which was of the order of 2-3 μmoles.

Mechanism of Glucosamine Incorporation into Glycogen -- One of the acid-soluble products resulting from the metabolism of galactosamine-1-^{14}C by rat

Table II

Transfer of Glucosamine from UDP-Hexosamine to Glycogen

Reactions	cpm
Complete system	4,600
" " plus 0.025 μmole UDPG	2,236
" " plus 0.25 μmole UDPG	460

The reaction mixture contained the following components in μmoles: UDP-hexosamine, 1.80 x 10^6 cpm/μmole (galactosamine-glucosamine, 78:22), 0.015; glycine (pH 8.7), 12.5; glucose-6-P, 0.83; glycogen, 0.67 mg; UDPG as indicated; protein from partially purified glycogen synthetase (Friedman and Larner, 1963), 0.1 mg.; final volume, 0.16 ml. After incubation at 37° for 5 min, the reaction was stopped with 1 ml of 0.6 N perchloric acid. Glycogen (4 mg) was added, followed by 3 ml of ethanol. The precipitation procedure was repeated three times, the final precipitate being dissolved in 2 ml of water and aliquots taken for scintillation counting.

liver was shown by us (Maley et al., in press) to be a mixture of UDP-galactosamine and UDP-glucosamine, with the former nucleotide predominating. It has since been established that the UDP-galactosamine arises by two enzymic reactions: UTP + galactosamine and UDPG + galactosamine-1-P. As shown previously (Maley and Maley, 1959), UDP-glucosamine is a product of the UDPGal-4-epimerase reaction.

On the assumption that the UDP-glucosamine is reactive in the glycogen synthetase system (Leloir and Goldemberg, 1960), an explanation would be provided for the incorporation of glucosamine into glycogen. To test this contention, glycogen synthetase was partially purified from rat muscle as described by Friedman and Larner (1963) and assayed for transglucosylase activity with C^{14}-labeled UDP-hexosamine. The UDP-hexosamine was purified from a rat liver that had been perfused with galactosamine-1-^{14}C and consisted of a mixture of UDP-galactosamine and UDP-glucosamine in a ratio of 78:22. As seen in Table II, radioactivity was incorporated into glycogen in the in vitro system. Extensive dialysis of the labeled glycogen, followed by acid hydrolysis and paper chromatography revealed the radioactivity to be associated with glucosamine. The inhibition of incorporation by UDPG suggests that the same enzyme is capa-

ble of utilizing both nucleotides as substrates.

The incorporation of glucosamine into glycogen raises questions pertinent to the metabolic significance of the reaction. Since glucosamine-1-P cannot substitute for glucose-1-P in the phosphorylase reaction, glycogenolysis may be impaired if glucosamine units are interspersed in the glycogen. A glycogen storage defect may thus arise, but whether such cases will actually be discovered is currently in the realm of speculation and experimentation.

SUMMARY

Galactosamine-1-^{14}C was found to be incorporated into rat liver glycogen as glucosamine following perfusion or intraportal injection. The glucosamine was shown to be glycosidically linked to glucose by isolation of a disaccharide containing glucose and glucosamine after β-amylase treatment of the glycogen. The incorporation appears to be mediated by UDP-glucosamine, which substitutes for UDPG in the glycogen synthetase reaction.

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