Role of Sugar Nucleotides in the Incorporation of Sugars into Glycoproteins*

Jonas E. Richmond

ABSTRACT: Sugar-nucleotide derivatives were labeled by the injection of ^{14}C-labeled sugars into intact animals or into isolated perfused livers. The peak activity of the label appeared first in the sugar phosphate, then in the sugar nucleotide, and still later in the sugar moiety of the glycoprotein. This suggests a precursor-product relationship. Glucosamine served as a precursor of glycoprotein *N*-acetylglucosamine as well as glycoprotein *N*-acetylneuraminic acid. Galactose was converted into galactose 1-phosphate, then galactose 1-phosphate was converted into uridine diphosphate galactose.

Uridine diphosphate galactose served as the galactose donor of the glycoprotein galactose. Similar results were obtained with either perfused livers or the intact animals.

Glucosamine has been shown to give rise to protein-bound glucosamine in a number of tissues (Spiro, 1959; Kohn *et al.*, 1962; Shetlar *et al.*, 1964). Molnar *et al.* (1964) have isolated labeled phosphorylated and sugar-nucleotide derivatives after the injection of glucosamine into animals, and have suggested that these products are intermediates in the biosynthesis of glycoproteins from glucosamine. From the brilliant work of Leloir (1964), Roseman (1959), and Del Giacco and Maley (1964), this seems to be a plausible suggestion.

Since it has been shown that intact animals (Richmond, 1963) and isolated perfused livers (Richmond, 1963; Richmond *et al.*, 1963; Sarcione, 1963) utilize [^{14}C]hexoses and [^{14}C]glucosamine in the synthesis of α_1 acid glycoprotein, these compounds were employed in further studies on the intermediates involved in glycoprotein biosynthesis. This report is concerned with an endeavor to study precursor-product relationships of the sugar intermediates in the biosynthesis of α_1 acid glycoprotein following pulse labeling of the sugars in isolated perfused rat livers and in intact rats.

Experimental

Materials

ATP,[1] ADP, UMP, UTP, GMP, GDP, GTP, CMP, CDP, CTP, UDP-*N*-acetylglucosamine, UDP-galactose, GDP-mannose, galactose 1-phosphate, galactose 6-phosphate, mannose 1-phosphate, mannose 6-phosphate, and glucose 1-phosphate were purchased from Sigma Chemical Co., St. Louis, Mo., and from California Corp. for Biochemical Research, Los Angeles.

Galactose oxidase was obtained from Worthington Biochemical Corp., Freehold, N.J., and purified by the procedure of Amaral *et al.* (1963). *N*-Acetylneuraminic acid of 95% purity was obtained from Sigma. Neuraminidase was obtained from Behringwerke, AG, Marburg, Germany, and purified by the method of Ada and French (1959). All other reagents were obtained from commercial sources.

[1-^{14}C]Glucosamine, [1-^{14}C]galactose, [1-^{14}C]mannose, [2-^{3}H]glycine, and L-[4,5-^{3}H]leucine were purchased from New England Nuclear Corp., Boston, Mass. Carrier-free radiophosphate was obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.

Perfused Livers. Livers from 180 to 200-g male Wistar rats were perfused by essentially the procedure of Miller *et al.* (1951) (Richmond, 1963). Triple-labeling techniques were used to obtain an independent assessment of the synthesis of the carbohydrate and protein moieties and aid in the isolation and identification of intermediates. After perfusing the liver for 15–20 minutes, approximately 100 μc (5–100 mc/mmole) of tritium-labeled amino acid, 0.5 mc of carrier-free $Na_2H^{32}PO_4$, and either 25 μc (2 mc/mmole) of [1-^{14}C]-glucosamine or 50 μc (5 mc/mmole) of [1-^{14}C]galactose were injected into the liver via the portal cannula. The specific activity of the tracer in the efflux blood of the liver for the first 45 seconds was arbitrarily used to measure the initial specific activity of the tracer (Richmond, 1963). After this 45-second interval, the liver was perfused with nonlabeled blood for 5 minutes to wash the label out of the liver; thus, the blood flowing from the superior vena cava was not recirculated to the liver until after the 5-minute sample was taken. Various

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[1] Abbreviations used in this work: ATP, UTP, GTP, and CTP, the 5′-triphosphates of adenosine, uridine, guanosine, and cytidine, respectively; ADP, UDP, GDP, and CDP, the 5′-diphosphates of adenosine, uridine, guanosine, and cytidine, respectively; UMP, GMP, and CMP, the 5′-monophosphates of uridine, guanosine, and cytidine, respectively.

lobes of the liver were tied off just prior to biopsy at specific time intervals. The excised sample was immediately frozen and stored in liquid N_2 until processed. Pooled liver biopsies from five to seven livers (2–12 g) were used in each experiment. The liver donors for each experiment were litter mates. The blood donors were 400–450-g, healthy, highly inbred members of the same colony. Labeled phosphate was employed as an analytical aid in the isolation of sugar intermediates. Tritium-labeled amino acids were used to obtain an independent assessment of the synthesis of the peptide moiety from the mean specific activity of the amino acid over a given time interval and the specific activity of the amino acid residue in the protein (Richmond, 1963).

Intact Rats. Male Wistar rats weighing 200–250 g were lightly anesthetized with ether and the abdomen cavity was opened. Ligaments around the liver were cut and the lobes of the liver were freed. Loose ligatures were placed around various liver lobes. [1-^{14}C]Glucosamine (35 μc; 5 mc/mmole) were injected intraportally. All of the other experimental procedures for the intact rat experiments were the same as for the perfused liver.

Methods

Methods for the isolation, degradation, and the analysis of the degradative products of the glycoprotein have been reported (Richmond, 1963). All operations concerned with the fractionation of these components were carried out at 3° or below. The pooled liver samples were extracted three times in a blender with approximately 4 volumes of 0.25 M perchloric acid. An α_1 glycoprotein was isolated from an aliquot of the perchloric acid–soluble phase (Richmond, 1963). The resulting supernatant fluid was neutralized with $KHCO_3$, centrifuged, and freeze-dried. Approximately 10% of the residue was employed for the isolation and analysis of free sugars and sugar phosphates. The remainder of the residue was taken up in approximately 0.05 volume of H_2O and centrifuged, and the supernatant fluid was treated with charcoal-Celite (1:1) according to Lin and Hassid (1964). The charcoal-Celite was washed three times with 0.01 M EDTA, *p*H 7.0, then washed twice with H_2O and eluted by dialysis at −20° against three changes of 0.1% NH_3 in 50% aqueous ethanol. The dialysate was freeze-dried and taken up in 0.5–2.0 ml of H_2O. After centrifugation, an aliquot was subjected to analytical paper- and thin-layer chromatography to obtain partial identification of the components. Ethyl acetate–pyridine–H_2O (2:1:2), butanol-ethanol-H_2O (5:1:4), butanol–acetic acid–H_2O (4:1:5), and isoamyl acetate–acetic acid–H_2O (3:3:1) were used as solvent systems. The remainder of the solution was subjected to ion-exchange chromatography (Urivetzky *et al.*, 1964). The effluent from the column was monitored by a LKB Unicord and by a Packard Tri-Carb scintillation counter. All fractions were neutralized with NH_3 and freeze-dried. This residue was taken up in 0.1–0.5 ml of H_2O and subjected to paper- and thin-layer chromatography using the solvent systems of Roseman *et al.* (1961) and Kornfeld *et al.* (1964). If substantial amounts of salt were present, as in the fractions eluted with a salt gradient, the fractions were treated with charcoal-Celite, washed, and eluted as indicated. Further identification and characterization of the isolated components were accomplished by the procedures of Del Giacco and Maley (1964), and the properties of the isolated compounds were compared and found to be the same as authentic compounds in the following solvent systems.

Galactose was identified by the fact that its R_F was the same as the known sugar in ethyl acetate–pyridine–H_2O (2:1:2), butanol-ethanol-H_2O (5:1:4), and butanol–acetic acid–H_2O (4:1:5). Galactose and galactose derivatives having an unsubstituted C-6 position were further characterized by the oxidation of the C-6 position to a carbonyl group with galactose oxidase. The reaction products were identified by chromatography (Taylor *et al.*, 1964) and the resulting dialdehydes were then converted to the corresponding uronic acid and characterized (Rosen and Horecker, 1963).

Glucosamine and *N*-acetylglucosamine were identified by chromatography using isopropyl alcohol–H_2O (4:1), *n*-butyl alcohol–acetic acid–H_2O (50:12:25), or 1-butanol-pyridine-H_2O (6:4:3), and by electrophoresis in 1% borate. *N*-Acetylglucosamine 6-phosphate was identified by the procedure of Del Giacco and Maley (1964). UDP-*N*-acetylglucosamine was identified by chromatography in ethanol–1 M ammonium acetate, *p*H 3.8 (70:30), in ethanol–1 M ammonium acetate, *p*H 7.5 (70:30), and by hydrolysis in 0.1 N HCl at 100° for 10 minutes followed by deionization and electrophoresis in 1% borate.

N-Acetylneuraminic acid was identified by chromatography in ethanol–ammonium acetate, *p*H 7.5 (5:2), *n*-butyl alcohol–propyl alcohol–HCl (1:2:1), and *sec*-butyl alcohol–acetic acid–H_2O (4:1:5). *N*-Acetylneuraminic acid was determined by the colorimetric method of Warren (1959).

An attempt was made to isolate acid-labile fractions by homogenizing the frozen pooled liver in six volumes of ice water, whereupon ethanol was added to make the mixture 80% with respect to ethanol. The homogenate was then centrifuged at 20,000 × *g* for 30 minutes, freeze-dried, taken up in 0.2 volume H_2O, and treated with charcoal-Celite as before. The charcoal eluate was then analyzed as described.

The quantity and identity of material in spots on chromatograms were determined by spectral analysis of the base after elution from the paper (Lin and Hassid, 1964) and/or by phosphorus analysis (Hanes and Isherwood, 1949). Galactose and galactose derivatives containing a free hydroxyl group in the C-6 position were quantitated by the galactose oxidase method (Taylor *et al.*, 1964; Blumenfeld *et al.*, 1963).

Differential counting of each of the three isotopes were carried out in a Packard Tri-Carb liquid scintillation spectrometer in the usual scintillation fluids (Robinson *et al.*, 1964; Kornfeld *et al.*, 1964). Two sets of operating values were used for each sample: (1) channel I gain 11%, window AB 100–1000; channel

TABLE I: Relative Specific Activities of Some Amino Sugar Derivatives in Pooled Liver Biopsies after the Passage of 25 μc of [1-^{14}C]Glucosamine through Perfused Rat Livers.

Time (min)	Free Glucosamine	Liver *N*-Acetylglucosamine 6-Phosphate	Liver UDP-*N*-acetylglucosamine	Glycoprotein *N*-Acetylglucosamine	Glycoprotein *N*-Acetylneuraminic Acid
0–0.75	100				
5	48	6.2	0.8	0.07	
10	30	14.3	2.1	0.16	0.03
15	21	13.8	2.8	0.33	0.06
20	12	9.8	2.5	0.68	0.12
30	6	7.2	2.3	0.84	0.14

II gain 0.9%, window CD 100–1000 for ^{14}C and ^{32}P, respectively; and (2) channel I gain 70%, window AB 100–1000; channel II gain 10%, window CD 300–1000 for ^{3}H and ^{14}C, respectively. Assay of standards indicated quantitative determination of each of the isotopes with the above-mentioned operating values. However, from a practical point of view, it was necessary only to distinguish ^{3}H from ^{14}C radioactivity in the glycoprotein since no ^{32}P was present in the glycoprotein, and to distinguish ^{32}P from ^{14}C in purified sugar intermediates since they did not contain any ^{3}H activity. Spots were cut from the paper or the cellulose was dislodged from thin-layer plates, mixed with the scintillation liquid, and assayed for radioactivity. The radioactivity determined on spots directly agreed with the values obtained on eluted fractions following freeze-drying. The efficiency of the counting system was determined by adding successive small increments of standard [^{14}C]benzoic acid to the spots and noting the efficiency.

Results

[1-^{14}C]Glucosamine was rapidly metabolized in perfused rat livers and in intact animals. Table I shows that [1-^{14}C]glucosamine was taken up from the blood of the perfused liver at a rapid rate. The relative specific activities of the hexosamine derivatives indicated that liver glucosamine gave rise to *N*-acetylglucosamine 6-phosphate, and this product gave rise to UDP-*N*-acetylglucosamine. Although a small amount of glucosamine 6-phosphate was isolated in most experiments, the small sample and the present techniques did not allow us to make a significant distinction between the relative specific activities of glucosamine 6-phosphate and *N*-acetylglucosamine 6-phosphate. UDP-*N*-acetylglucosamine had the characteristics of being a precursor of glycoprotein *N*-acetylneuraminic acid.

The hexoses were not labeled to any marked extent from [1-^{14}C]glucosamine. Even though free *N*-acetylneuraminic acid was labeled after 5–10 minutes, free *N*-acetylneuraminic acid in perchloric acid extracts of liver did not appear to be a direct precursor of glycoprotein *N*-acetylneuraminic acid. The specific activity of *N*-acetylneuraminic acid was lower when isolated from acid extracts than when isolated directly from either the soluble phase of homogenates or a heat-denatured homogenate or an ethanol extract of the liver homogenate.

Perchloric acid liver extracts had a relative specific activity of free *N*-acetylneuraminic acid, at 10-, 15-, 20-, and 30-minute time intervals, of 0.02, 0.04, 0.09, and 0.08, respectively. These values are less than those of the glycoprotein (Table I) and are approximately the same as was obtained for the perchloric acid–insoluble liver following hydrolysis with 0.05 N HCl at 90° for 30 minutes. Qualitatively similar results were obtained in intact rats (Table II) as were observed in perfused livers.

TABLE II: Relative Specific Activities of Sugar Intermediates after Intraportal Injection of 35 μc of [1-^{14}C]-Glucosamine into 250-g Rats.

Time (min)	Plasma Glucosamine	Liver UDP-*N*-acetylglucosamine	Glycoprotein *N*-Acetylglucosamine
0–0.5	100		
2	80	0.5	0.06
5	64	2.5	0.10
10	48	6.8	0.33
15	31	11.2	0.58
20	18	9.8	0.81
30	7	5.6	0.82

[1-^{14}C]Galactose was a good precursor of glycoprotein galactose. Table III indicates that galactose was phosphorylated, then this galactose 1-phosphate was converted to UDP-galactose, and that UDP-galactose is the source of the galactose for the glycoprotein.

TABLE III: Relative Specific Activities of Some Sugar Intermediates in Pooled Liver Biopsies after the Passage of 50 μc of [1-^{14}C]Galactose through Perfused Rat Livers.

Time (min)	Liver Galactose	Liver Galactose 1-Phosphate	Liver UDP-galactose	Glycoprotein Galactose
0–0.75	100			
5	52	12.1	3.1	0.06
10	39	25.5	8.3	0.12
15	18	18.4	9.7	0.37
20	10	11.3	8.1	0.68
30	4	8.1	7.3	0.74

[1-^{14}C]Galactose gave rise to labeling of the amino sugar derivatives and to some extent mannose; however, the degree of labeling was less than was observed with glucose.

From the liver specific activities of the ^{14}C-labeled sugar, ^{3}H-labeled amino acid, and the specific activities of these residues in the protein it was possible to obtain an independent measurement of the rates of synthesis of the carbohydrate and protein moieties. These results indicated that approximately the same quantity of protein was formed for any corresponding set of time intervals for each experiment. The rate of labeling of the peptide was slower initially than the rate of labeling of the sugar component.

Discussion

The data obtained on glucosamine metabolism in these experiments are, in general, consistent with the pathways of glucosamine metabolism observed by Roseman (1962), McGarrahan and Maley (1962), Del Giacco and Maley (1964), and Molnar *et al.* (1964). The small degree of dilution of glucosamine, *N*-acetylglucosamine, and *N*-acetylglucosamine 6-phosphate suggests a small pool of these compounds in the liver as observed by McGarrahan and Maley (1962). The pattern of the specific activity of the free glucosamine with respect to time is to some extent a function of the experimental conditions. The size of the labeled free glucosamine pool is kept small by one-shot pulse labeling followed by extensive washing out with nonlabeled blood and by employing a large perfusion volume in comparison to the rat blood volume.

In the formation of *N*-acetylglucosamine 6-phosphate, it is not clear if glucosamine is first phosphorylated and then acetylated or vice versa. *N*-Acetylglucosamine 6-phosphate is then converted into UDP-*N*-acetylglucosamine, which seems to be the donor of glycoprotein *N*-acetylglucosamine. This observation is in agreement with proposed schemes that UDP-*N*-acetylglucosamine is the source of glycoprotein *N*-acetylglucosamine (Leloir, 1964; Del Giacco and Maley, 1964; Molnar *et al.*, 1964; Roseman, 1959), although there is a slight deviation from the classical precursor-product relationship (Zilversmit *et al.*, 1948). The departure of the specific activity of the sugar-nucleotide derivatives from a strictly theoretical precursor-product relationship is similar to the findings of Robinson *et al.* (1964) on the incorporation of glucosamine into the total hexosamine pool of the liver. This suggests metabolic heterogeneity of the hexosamines as pointed out by Robinson *et al.* (1964); however, since UDP-glucosamine is undoubtedly the precursor of glucosamine in a large number of macromolecules (glycoproteins, polysaccharides, and the like) with different rates of turnover and reutilization of the amino sugars, it is conceivable that these factors and the location of enzymes within the cell might contribute to the observed relationship. Similarly, the above-mentioned factors and the possibility of alternate routes for the production of UDP-galactose (Leloir, 1964) might give rise to a heterogeneous metabolic pool of active galactose within different sites of the cell or different cells. The presence of a relatively large pool of existing α_1 acid glycoprotein in the system complicates an interpretation of the data.

Our data do not conclusively identify the hexosamine derivative which is the precursor of *N*-acetylneuraminic acid; however, Roseman (1962) and Warren and Felsenfeld (1962) have shown the precursor to be UDP-*N*-acetylglucosamine, which is consistent with our results. It is not clear if the *N*-acetylneuraminic acid in acid-soluble liver extracts is an obligatory precursor for α_1 acid glycoprotein. We found that the relative specific activity of the *N*-acetylneuraminic acid in acid-soluble extracts paralleled that liberated by neuraminidase from liver or that liberated from liver by 0.05 N HCl at 90° for 30 minutes more closely than that found in the glycoprotein. Our data, as well as those of Shetlar *et al.* (1964), indicate that hydrolysis of labile macromolecular *N*-acetylneuraminic acid is a major source of this compound in the so-called acid-soluble tissue fraction. Since Roseman (1962) has shown that CMP-*N*-acetylneuraminic acid is the precursor of glycoprotein *N*-acetylneuraminic acid, it is not clear whether free *N*-acetylneuraminic acid is an obligatory intermediate, inasmuch as it is possible to obtain the active donor from UDP-*N*-acetylglucosamine by a series of reactions not involving free *N*-acetylneuraminic acid (Warren and Felsenfeld, 1962).

The incorporation of the hexoses into the glycoprotein follows the known routes (Leloir, 1964; Roseman, 1959) for the incorporation of sugars in polysaccharides. Since these sugars can be derived from reversible reactions of intermediary metabolism, the labeling of all of the sugar derivatives to some extent is not unexpected. Qualitatively similar results were obtained for galactose and mannose. We were unable to demonstrate the presence of acid-labile obligatory intermediates in water or ethanol extracts of liver, nor were we successful in isolating di-, tri-, or oligosaccharides free or in association with nucleotides.

Although Molnar *et al.* (1964) called for some caution before concluding that our data (Richmond, 1963) using puromycin indicated that the synthesis of the carbohydrate moiety of the glycoprotein occurred subsequent to the biosynthesis of the peptide, the fact that the liver 2 hours after puromycin administration had a rate of synthesis equal to the untreated controls strongly suggests the validity of our initial interpretation. Furthermore, our data with pulse triple-labeling techniques at time intervals of 0 to 10 minutes indicated that the peptide of the glycoprotein was labeled at a slower rate initially than the carbohydrate, thus showing, as has been done by others (Sarcione, 1963; Richmond, 1963), that the sugars are added later in the series of biosynthetic reactions than the amino acids.

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