Incorporation of Glucose-C^{14} into Plasma Glycoprotein Fractions by the Isolated Rat Liver*

EDWARD J. SARCIONE

From the Division of Medicine, Roswell Park Memorial Institute, New York State Department of Health, Buffalo 3, New York

Received June 25, 1962

Direct evidence that the liver is a site of incorporation of hexoses and hexosamine into plasma glycoprotein fractions has been obtained with the isolated perfused rat liver. When glucose-C^{14} was added to blood perfusing the liver, the specific activity of the galactose, mannose, and glucosamine of the plasma glycoproteins and the seromucoid fraction increased progressively. In contrast, there was only negligible change in the specific activity of plasma glycoprotein hexoses during a 6-hour period in control experiments without a liver in the circuit. The incorporation of C^{14} into the carbohydrate moieties of the seromucoid glycoprotein fraction was consistently more rapid than that into the remainder of the plasma glycoproteins. These data indicate that synthesis of the seromucoid fraction by the liver is considerably more rapid than that of the remaining plasma glycoproteins. Glucose-C^{14} incorporation into galactose and mannose of plasma glycoprotein fractions proceeds progressively and at approximately equal rates, and is more than twice as rapid as incorporation into glucosamine.

The question of the origin and biologic function of the serum glycoproteins has aroused much interest in recent years. It is well documented that the liver is the major site of plasma protein synthesis, except for γ-globulins (Miller and Bale, 1954; Miller *et al.*, 1954). However, only indirect evidence is available regarding the ability of the liver to incorporate carbohydrates into these plasma proteins.

In the present study, the isolated perfused rat liver was utilized to investigate the incorporation of glucose-C^{14} into the galactose, mannose, and glucosamine of plasma glycoprotein fractions, and to compare the relative rates of its incorporation into these fractions.

METHODS

Liver Perfusion.—The details of the perfusion technique have been described previously (Sokal *et al.*, 1958). Liver donors were 24-hour fasted male rats of the Sprague-Dawley strain, weighing 300–400 g. The perfusion mixture consisted of heparinized blood obtained from normal unfasted donor rats, diluted with $^1/_4$ its volume of Ringer's solution and containing 25,000 units of penicillin, 6.25 mg streptomycin sulfate, 2.5 mg polymyxin B sulfate, 220 mg of an amino acid mixture,[1] 57 mg of non-labeled glucose, and 18 mg of glucose-U-C^{14} (100 μc), in a total volume of 156.5 ml. The blood was oxygenated with a mixture of 95% oxygen and 5% CO_2, and the temperature was thermostatically controlled at 37°. All perfusions were continued for 6 hours. Control experiments, in which comparable amounts of blood and glucose-C^{14} were circulated without the presence of the liver in the circuit, were carried out simultaneously in a second apparatus under identical conditions.

Isolation of Perfusate Glycoproteins.—A 16-ml sample was removed just before the blood was placed in the perfusion apparatus and at 1, 2, 3, 4, and 6 hours after the start of the perfusion. The plasma was removed by centrifugation, and the bulk of the glycoproteins and the seromucoid fraction were isolated by addition of perchloric acid, followed by phosphotungstic acid, as described by Winzler (1955). The term "PCA fraction" refers to material precipitated from plasma by 0.6 N perchloric acid and represents the major portion of the plasma proteins, containing approximately 90% of the total protein-bound hexose in plasma. The "PTA fraction" represents the "seromucoid" fraction of plasma, which has been defined by Winzler as that material which is soluble in 0.6 N perchloric acid but insoluble in 1 M phosphotungstic acid.

Perchloric- and phosphotungstic acid–insoluble fractions were suspended in distilled water and dissolved by the addition of a minimal amount of NaOH, dialyzed against distilled water for 4 days, then lyophilized to dryness.

Isolation of Sugar Components.—Lyophilized glycoprotein samples (10 mg) were hydrolyzed for 3 hours with 2 N HCl in a boiling water bath, then evaporated to dryness in vacuo. The hydrolysates were dissolved in 0.1 N HCl and passed through a 0.6 × 18 cm column of Dowex-50 ion exchange resin (200–400 mesh, H+ form, X-8). The columns were washed with 20.0 ml distilled water, and the neutral hexose-containing

* This investigation was supported in part by research grant A-4961 from the Division of Arthritis and Metabolic Diseases of the National Institutes of Health. A preliminary report was presented at the Fifth International Congress of Biochemistry, Moscow, August, 1961.

[1] Amino acids HeLa dried (Difco).

effluent was collected and lyophilized to dryness for paper chromatography. The glucosamine was eluted from the column with 0.3 N HCl according to the method of Gardell (1953). The acid effluents were analyzed for glucosamine by the Boas (1953) modification of the Elson and Morgan method, and those containing glucosamine were combined and evaporated to dryness in vacuo for paper chromatography.

Hexoses.—Lyophilized samples of the neutral effluent were dissolved in distilled water and chromatographed on Whatman No. 1 paper developed in ethyl acetate–pyridine–H_2O (10:4:3). A mannose-galactose standard (25 μg of each) was chromatographed simultaneously on each paper. The hexoses on the dried papers were visualized by dipping in aniline phthalate in water-saturated butanol as described by Partridge (1949). The papers were air dried and heated at 100° for 7 minutes. Galactose and mannose were separated efficiently in this system at amounts ranging from 10 to 100 μg of either hexose in the neutral hexose fractions. No ninhydrin-positive material was detected.

Glucosamine.—Lyophilized samples of the acid eluate were dissolved in distilled water and chromatographed on Whatman No. 1 paper developed in ethyl acetate–pyridine–water–glacial acetic acid (5:5:3:1) as described by Fisher and Nebel (1955). A glucosamine standard (50 μg) was chromatographed simultaneously. The dried papers were heated at 105° for 10 minutes and examined for fluorescence with a long-wavelength ultraviolet lamp (Mineralite).

Radioactivity Measurements.—A 1.25-in. diameter circle was drawn around each stained hexose or fluorescent glucosamine spot; this area was cut from the paper and counted on 1.25-in. aluminum planchets in a gas flow counter (Nuclear-Chicago). The samples were counted sufficiently long to reduce the counting error to 5%.

Quantitative Determination of Sugars.—After counting, the neutral sugars on the paper chromatograms were quantitated by cutting the papers into small pieces and extracting with 0.7 N HCl in 80% ethanol as described by Wilson (1959). The optical density of the eluted material was read at 390 mμ. Glucosamine was quantitated on paper chromatograms by the method of Moore and Stein (1948).

Results

A total of three pairs of liver perfusions and their respective controls (blood circulated in the apparatus, without the presence of a liver) were carried out. In two of the experiments, the incorporation of C^{14} into plasma PCA- and PTA-insoluble fractions was similar; data from one of these perfusions and its control are presented. In the third experiment, the results were qualitatively similar, but the amount of C^{14} incorporation into each fraction was approximately one-half that observed in experiments 1 and 2. In experiment 3, the perfusing blood glucose values increased during the first hour to approximately double the initial concentration, in contrast to a slight decline in blood glucose values observed in the other experiments. This rise in blood glucose is best explained by the hypothesis that the perfused liver was not depleted of glycogen, despite the 24-hour fast, and that hepatic glycogenolysis during the early phase of this perfusion released unlabeled glucose into the blood, thus lowering the specific activity of the C^{14}-glucose in the system.

The time courses of incorporation of glucose-C^{14} into the galactose of the PCA- and PTA-insoluble plasma glycoprotein fractions, with and without a liver in the blood circuit (Liver and Control), are shown in Figure 1. Galactose radioactivity at zero time was negligible in all fractions, indicating satisfactory separation from C^{14}-glucose. No glucose was identified on chromatograms of either PCA or PTA fractions at any interval studied. There was a delay of 2 hours before appreciable C^{14} incorporation into the galactose of the PCA fraction of liver perfusates took place; this was followed by a progressive increase in specific activity during the remaining 4 hours of perfusion. The incorporation of C^{14} into the galactose of the PTA fraction of liver perfusates, on the other hand, occurred throughout the entire 6-hour period, and the specific activity of this fraction was greater at all time intervals than that in the PCA fraction. These results are in sharp contrast with the slight incorporation of C^{14} into galactose of PCA and

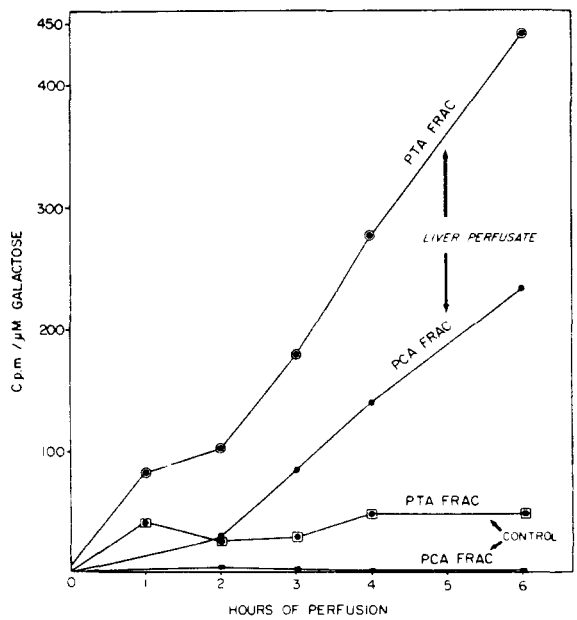

FIG. 1.—Time courses of incorporation of glucose-C^{14} into galactose of the perchloric acid (PCA) and phosphotungstic acid (PTA) insoluble fractions of plasma obtained from liver perfusates and control blood circulated without the presence of the liver.

PTA fractions of control plasma (no liver in the circuit); the specific activity of these fractions remained relatively constant during this 6-hour period. The control data indicate that whole blood, *per se*, cannot affect any appreciable synthesis of plasma glycoprotein galactose.

As is seen in Figure 2, the incorporation of glucose-C^{14} into mannose of plasma glycoprotein fractions follows a similar pattern, with evidence of a 2-hour lag period for incorporation into mannose of the PCA fraction of liver perfusate, followed by a progressive increase during the remaining 4-hour period. Again, the incorporation of C^{14} into the PTA mannose was more rapid than into the PCA fraction, and the specific activity of the PTA mannose increased progressively during the entire perfusion period. The specific activity of mannose in the glycoprotein fractions of control plasma showed little or no change during a 6-hour interval. A comparison of the rates of incorporation of C^{14} into mannose with those into galactose in these experiments indicated that they were approximately equal, with a small but consistent difference in favor of galactose. However, since only three experiments were performed, it is not certain whether this difference is significant.

In Figure 3, it is seen that incorporation of C^{14} into glucosamine of the plasma PCA fraction of liver perfusates was very slow during the perfusion period, differing from the control only at the 6-hour point. Since the radioactivity was so low, no conclusion regarding a time lag can be made. Incorporation into glucosamine of the plasma PTA fraction of liver perfusates proceeded steadily, however, and was significantly different from the control at all intervals studied. A comparison of these data with those in the previous figures indicates that incorporation of glucose-C^{14} into glucosamine of the PTA fraction proceeded at less than half the rate of that into galactose or mannose. Control plasma glycoprotein fractions contained only traces of glucosamine-C^{14} at all time periods.

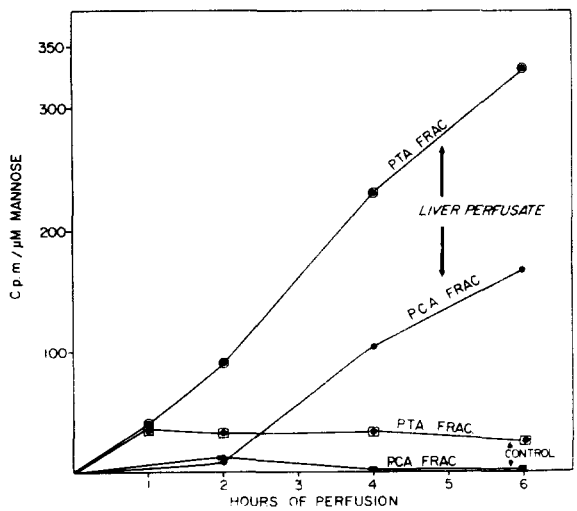

FIG. 2.—Time courses of incorporation of glucose-C^{14} into mannose of the perchloric acid (PCA) and phosphotungstic acid (PTA) insoluble fractions of plasma obtained from liver perfusates and control blood circulated without the presence of the liver.

The mean protein-bound hexose concentrations in zero-time plasma glycoprotein fractions were: total plasma hexose, 127 mg/100 ml; hexose-protein ratio, 1.9 g/100 g; seromucoid hexose, 14.1 mg/100 ml; and seromucoid hexosamine, 9.0 mg/100 ml. The mean galactose-mannose ratios in the PCA- and PTA-insoluble fractions of plasma were 1.0:0.99 and 1.0:1.08 respectively.

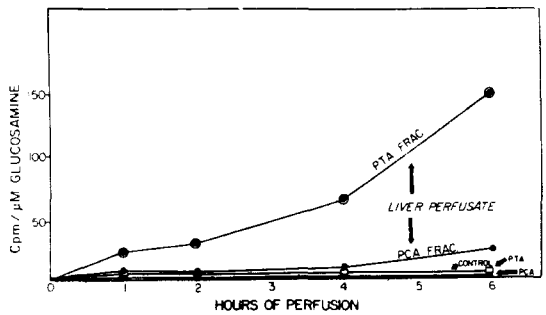

FIG. 3.—Time courses of incorporation of glucose-C^{14} into glucosamine of the perchloric acid (PCA) and phosphotungstic acid (PTA) insoluble fractions of plasma obtained from liver perfusates and control blood circulated without the presence of the liver.

Bile.—The total radioactivity in bile increased progressively during the 6-hour perfusion, reaching a total of 1.3% of the zero-time blood radioactivity at the end of 6 hours. Addition of PCA and PTA to bile resulted in no precipitation with PCA and a small amount of precipitate with PTA. Only glucose could be identified in 1, 2, and 3 hour PTA fractions of bile; it was radioactive. In the 4- and 6-hour samples, galactose was also identified and contained C^{14}, with specific activity of 200 and 271 cpm/μmole, respectively.

DISCUSSION

Considerable indirect evidence has been cited to indicate that the liver is an important site of origin of the serum glycoproteins. In many instances, this has consisted of demonstration of decreased levels of serum glycoproteins in liver disease (Greenspan and Dreiling, 1953; Greenspan *et al.*, 1952) or after destruction of the liver (Nettelbladt and Sundblad, 1959; Werner, 1949). Unfortunately, this type of indirect evidence suffers from the unavoidable defect that any diminution in synthesis of precursor serum proteins or hexose constituents, following destruction of the liver, would be expected to produce a concomitant decrease in serum glycoprotein levels, even if the liver were not the site of attachment of hexoses to proteins. Thus, attention must be focused on the actual site of attachment of hexoses

to protein, as well as on the origin of the individual precursors. Spiro (1959) and Shetlar (1961) have presented arguments based on specific activity time curves for liver and serum glucosamine values *in vivo* which strongly suggest that the liver is the site of synthesis of the protein-bound serum glucosamine.

In the present experiments, the progressive increase in the specific activity of C^{14} incorporated into galactose, mannose, and glucosamine of plasma glycoprotein fractions obtained from liver perfusates, as contrasted with their negligible change in specific activity in the control experiments without a liver in the circuit, provides direct evidence that the liver was the site of attachment of the carbohydrates in these glycoprotein fractions. Further, plasma glucose has been shown to be a precursor of the protein-bound galactose, mannose, and glucosamine.

Clinical interest in the seromucoid (PTA) fraction has been stimulated by numerous reports that this glycoprotein fraction of serum is elevated in patients with many diverse pathologic conditions, including malignancy, tuberculosis, rheumatoid arthritis, chronic infections, and collagen diseases (Greenspan, 1955; Heiskell *et al.*, 1961; Story, 1959). The major component of this fraction has been identified as orosomucoid in human serum (Weimer *et al.*, 1950) and serum of several animal species, including the rat (Weimer and Winzler, 1955). In the present experiments, the incorporation of glucose-C^{14} into the carbohydrate moieties of the seromucoid fraction of liver perfusates was consistently more rapid than its incorporation into the hexoses of the other plasma glycoproteins. This indicates that the rate of hepatic synthesis of the seromucoid fraction of plasma is considerably more rapid than that of other plasma glycoproteins, a conclusion which is in agreement with observations utilizing turnover studies in humans (Weisman *et al.*, 1961), guinea pigs (Boström *et al.*, 1958), and rabbits (Richmond, 1959) *in vivo*. The different rates of synthesis by the liver of the seromucoid fraction and the remainder of the serum glycoproteins offers a possible explanation for reports that levels of total serum glycoproteins and of seromucoid can vary independently. In diffuse parenchymatous liver disease, the seromucoid levels may decline in spite of a concomitant rise in total protein-bound hexose (Greenspan *et al.*, 1951). In many pathologic conditions, including cancer and tuberculosis, the seromucoid level is elevated to a greater degree than is the total protein-bound hexose (Greenspan, 1955). The seromucoid and the other glycoproteins may have different sites of synthesis within the liver, which may respond to various pathologic processes in a different manner. It is equally conceivable, however, that the liver is only responsible for the normal endogenous levels of the circulating plasma glycoprotein and that, under certain pathologic conditions, areas of tissue destruction or inflammation may be the source of the elevated serum glycoprotein levels observed.

A comparison of the relative rates of incorporation of glucose-C^{14} into hexose components of both the PCA and the PTA glycoprotein fractions of plasma revealed that, with the exception of the initial 2-hour lag period for incorporation into the PCA-insoluble fraction, galactose and mannose incorporation proceeded rapidly and at approximately equal rates during the 6-hour perfusion. Incorporation into glucosamine was much slower. These data are in agreement with those of Boström *et al.* (1958), who found that the specific activity of the neutral sugar fraction of the serum α_1-glycoprotein was about twice that of the glucosamine and sialic acids after the administration of glucose-C^{14} to guinea pigs *in vivo*. Kohn *et al.* (1962) have also observed that D-glucose serves as a poor precursor of protein-bound C^{14}-glucosamine in the rat *in vivo*. These data indicate that the conversion of glucose-C^{14} to glucosamine and its subsequent incorporation into serum glycoproteins by the liver occurs at a much slower rate than that for galactose and mannose.

Acknowledgment

The extremely competent technical assistance of Miss Marion Leahy is gratefully acknowledged.

References

Boas, N. F. (1953), *J. Biol. Chem.*, *204*, 553.
Boström, H., Roden, L., and Yamashina, I. (1958), *J. Biol. Chem. 230*, 381.
Fisher, F. G., and Nebel, H. J. (1955), *Z. physiol. Chem. 302*, 10.
Gardell, S. (1953), *Acta Chem. Scand. 7*, 207.
Greenspan, E. M. (1955), *Advances in Int. Med. 7*, 101.
Greenspan, E. M., and Dreiling, D. A. (1953), *Arch. Int. Med. 91*, 474.
Greenspan, E. M., Lehman, I., Graff, M. M., and Schoenbach, E. B. (1951), *Cancer 4*, 972.
Greenspan, E. M., Tepper, B., Terry, L. L., and Schoenbach, E. B. (1952), *J. Lab. & Clin. Med. 39*, 44.
Heiskell, C. L., Carpenter, C. M., Weimer, H. E., and Nakagawa, S. (1961), *Ann. N. Y. Acad. Sci. 94*, 183.
Kohn, P. Winzler, R., and Hoffmann, R. (1962), *J. Biol. Chem. 237*, 304.
Miller, L. L., and Bale, W. F. (1954), *J. Exp. Med. 99*, 125.
Miller, L. L., Bly, C. G., and Bale, W. F. (1954), *J. Exp. Med. 99*, 133.
Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.
Nettelbladt, E., and Sundblad, L. S. (1959), *Proc. Soc. Exp. Biol. & Med. 102*, 225.
Partridge, S. M. (1949), *Nature 164*, 443.
Richmond, J. E. (1959), *J. Biol. Chem. 234*, 2713.
Shetlar, M. R. (1961), *Ann. N. Y. Acad. Sci. 94*, 44.
Sokal, J. E., Miller, L. L., and Sarcione, E. J. (1958), *Am. J. Physiol. 195*, 295.
Spiro, R. G. (1959), *J. Biol. Chem. 234*, 742.

Story, Z. (1959), *Ergeb. Physiol. biol. Chem. u. Exp. Pharmakol. 50*, 174.
Weimer, H. E., Mehl, J. W. and Winzler, R. J. (1950), *J. Biol. Chem. 185*, 561.
Weimer, H. E., and Winzler, R. J. (1955), *Proc. Soc. Exp. Biol. & Med. 90*, 458.
Weisman, S., Goldsmith, B., Winzler, R., and Lepper, M. H. (1961), *J. Lab. & Clin. Med. 57*, 7.
Werner, I. (1949), *Acta Physiol. Scand. 19*, 27.
Wilson, C. M. (1959), *Anal. Chem. 31*, 1199.
Winzler, R. J. (1955), *Methods of Biochemical Analysis 2*, 279.

Dextransucrase, an Induced Enzyme from *Leuconostoc mesenteroides*

W. Brock Neely and Julie Nott

From the Biochemical Research Laboratories, The Dow Chemical Company, Midland, Michigan

Received January 26, 1962

Sucrose has been shown to induce the formation of dextransucrase in the organism *Leuconostoc mesenteroides*. Furthermore, the results imply that the fructofuranose portion of the sucrose molecule is the trigger which causes induction to take place. No other inducer for dextransucrase has been found.

The study of the ability of microorganisms to adapt to a new environment is an important and fascinating area of research. One example of this ability is the ease with which microorganisms evolve new enzyme system(s) to act on foreign substrates. This phenomenon is commonly known as induction, and many excellent review articles have been written; for example see Pollock (1959) or Dixon and Webb (1958). This facet of adaption provides a convenient method for following the formation of a catalytically active protein. Unfortunately, in studying this phenomenon it is difficult to separate *in vivo* synthesis of enzyme from simple conversion of inactive protein to active protein, and these two possibilities should always be considered.

The action of the enzyme dextransucrase from *Leuconostoc mesenteroides* on the substrate sucrose has been studied by a number of investigators (for a review see Neely, 1960). The reaction catalyzed by this enzyme is shown in equation (1). One of the characteristics of dextransucrase is specificity for sucrose. No other sugar has been

$$n \text{ sucrose} + \underset{\text{Acceptor}}{\text{HOR}} \longrightarrow \underset{\text{Dextran}}{(\text{glucose})_n\text{-OR}} + n \text{ fructose} \quad (1)$$

shown to act as a donor of glucosyl groups for the formation of the polymer dextran. Since the enzyme was specific for sucrose, we began to wonder if its biosynthesis was also dependent on the sucrose molecule. The only work of this nature was done by Karstrom (1938). He stated that the enzyme system from *L. mesenteroides* for dealing with sucrose was constitutive and not adaptive. However, he grew the organism at 37° and carried out his experiments with the washed cells at 45°. Since Neely (1959) and Tsuchiya *et al.* (1955) have shown that dextransucrase is quite unstable at this high temperature, it appears unlikely that Karstrom was dealing with this particular enzyme. We decided to reinvestigate the nature of the enzyme elaborated by *L. mesenteroides* in an effort to learn more about its biosynthesis.

Experimental

Enzyme Production and Assay.—The medium for the *Leuconostoc mesenteroides* NRRL-B512F was similar to the one described by Tsuchiya *et al.* (1952). The culture was maintained on a 2% sucrose medium and transferred every other day to fresh medium. Since the enzyme dextransucrase is exocellular, centrifugation at 8500 rpm (5500 × *g*) of the whole culture after adjustment of the *p*H to 5.2 gave a crude cell-free extract. The enzyme assay was based on the measurement of free reducing sugar when 1 ml of appropriately diluted extract was incubated with 1 ml of 100 mM sucrose at 25° for ½ hour. The dextransucrase unit (D.S.U.) as used in this paper was the amount of enzyme which would convert 1 mg of sucrose to dextran in one hour (releasing 0.52 mg of fructose) under the above-described conditions. Reducing sugars were measured by the Somogyi method (1945), with the colorimetric technique of Nelson (1944), incorporated.

Preparation of Washed Cells.—A 24-hour culture containing 2% sucrose was centrifuged at 8500 rpm (5500 × *g*) for ½ hour. The supernatant was discarded. The cells were dispersed in half their original volume with 0.05 M phosphate buffer, *p*H 6.5. They were recentrifuged. The washing and centrifugation were repeated three times and the cells were finally stored in the phosphate buffer. This preparation was fairly unstable and had to be used within 24 hours.

Fructose Measurement.—Samples from the cul-