Studies on the Metabolism of Plasma Glycoproteins*

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Plasma glycoproteins were prepared by combination of methods: differential precipitation, electrophoresis, ion-exchange chromatography, and gel filtration. A plasma glycoprotein was found to turn over at a rate at least three times faster than albumin in the intact animal and in the isolated perfused liver. Rates of 5 mg glycoprotein synthesized per kg liver per minute were observed, which are of the same general magnitude as was obtained for the quantity of albumin synthesized. Glucosamine was incorporated into the protein more extensively than *N*-acetyl glucosamine and hexoses. Although the protein contained large amounts of dicarboxylic amino acids, free glutamic and aspartic acids were poorly utilized in the synthesis of this protein. When puromycin was added to the blood perfusing a liver which had been synthesizing protein for two hours, the rate of incorporation of amino acids into all plasma proteins was inhibited. Under these same conditions the incorporation of labeled carbohydrates into the glycoprotein was not markedly altered by puromycin; however, the incorporation of C^{14} from carbohydrates into albumin and total plasma protein was inhibited.

This paper is concerned with the rate of metabolism of glycoproteins in rabbits and rats and in isolated perfused rat livers. In recent years a great deal has been learned of the chemical nature of a number of carbohydrate-containing proteins (Johansen *et al.*, 1960; Schmid, 1950; Lee and Montgomery, 1962; Nuenke and Cunningham, 1961; Spiro, 1962; Eylar and Jeanloz, 1962; Winzler, 1955), and some data are available on the appearance of labels in this class of proteins (Boström *et al.*, 1958; Richmond, 1959) as well as on the association of labeled carbohydrates with protein precipitates of tissues and plasma (Kohn *et al.*, 1962; Spiro, 1959; Roseman, 1959).

EXPERIMENTAL

I. Materials

A. Animals.

Male Charles River rats weighing 250–350 g were used. The rabbits employed in these studies were 2 to 2.5 kg male New Zealand whites. The animals were maintained on standard Purina stock diets. All animals were fasted for 16–20 hours previous to the commencement of an experiment. The animals had access to water at all times and had food and water *ad libitum* after the administration of the tracer.

B. Tracers

Freshly prepared C^{14}-labeled compounds were obtained from New England Nuclear Corporation and from Schwarz Biochemicals.

C. Proteolytic Enzymes

1. Papain.—Two-times recrystallized papain was purchased from the Nutritional Biochemical Corporation. Mercuripapain was prepared and recrystallized by the procedure of Kimmel and Smith (1954). Digestion by this enzyme was carried out at a glycoprotein concentration of 0.1–0.3% by the procedure of Rosevear and Smith (1961).

2. Carboxypeptidase.—Three-times crystallized carboxypeptidase was purchased from Nutritional Biochemical Corporation. The enzyme was prepared and the digestion was performed by the method of Fraenkel-Conrat *et al.* (1955).

3. Pepsin, Chymotrypsin, and Trypsin.—Three-times recrystallized pepsin and chymotrypsin and twice recrystallized trypsin were bought from Nutritional Biochemical Corporation. The digestions were carried out for each of the enzymes either separately or in combinations by procedures similar to those used by Spiro (1962).

D. Other Enzymes

1. Neuraminidase.—Crystalline neuraminidase was kindly given by Dr. G. L. Ada. Other preparations were made from *Vibrio cholerae* extracts by the procedure of Ada and French (1959). Digestion at various enzyme and substrate concentrations were carried out at 37° in 0.1 M sodium acetate buffer, *p*H 5.8, containing 0.001 M $CaCl_2$.

2. Glucosidase and Hyaluronidase.—Beta-glucosidase, almond, was obtained from Worthington Biochemicals Corporation. The hyaluronidase preparations were obtained from Nutritional Biochemical Corporation. Digestions were carried out at 37° in 0.08 M sodium acetate buffer, *p*H 5.4, at an enzyme concentration of 30% of substrate for periods up to 24 hours.

3. L-Amino Acid Decarboxylases.—L-Glutamic acid, L-lysine, and L-tyrosine decarboxylases were purchased from Worthington Biochemical Corporation and Nutritional Biochemical Corporation.

II. Methods

A. Preparation of Glycoprotein

The glycoprotein fraction was prepared from veal, rat, and rabbit plasma by combinations of methods of differential precipitation, chromatography, and electrophoresis at 3–5°.

Plasma perchloric acid filtrates (Winzler, 1955) were used as the starting products for the preparation of the glycoprotein fraction in the initial studies and the perchloric acid–insoluble phase was used in the preparation of the other plasma proteins. One volume (usually 2 ml) of plasma was mixed with a volume of 0.15 M NaCl, and 4 volumes of 0.75 M perchloric acid were added slowly with stirring. The mixture was centrifuged, poured through Whatman No. 42 filter paper, and dialyzed against 0.15 M NaCl for 72 hours, then was dialyzed against H_2O for 24–36 hours. The volume of the dialyzing fluid was at least 100 times the volume of the protein solution and was changed four to six times daily in all instances. The dialyzed protein was centrifuged at 20,000 × *g* for 15 minutes

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and the supernatant fluid was freeze-dried. The initial perchloric acid precipitate was washed three times with 10 ml of 0.75 M perchloric acid, dialyzed in the same manner as the soluble fraction, freeze-dried, and used in assays of the total plasma protein. The solid glycoprotein (perchloric acid soluble) fraction was made into a 2–5% solution in buffer (Aransson and Grönwall, 1958) 0.25 M Tris, 0.011 M EDTA–0.028 M boric acid, final *p*H 9.0. The protein was equilibrated with the buffer by dialysis for 12 hours. The soluble protein was applied to an LKB 3340 column electrophoresis apparatus which was charged with the buffer and 150–250 mesh cellulose acetate powder, and was subjected to 900 v and a current of 40–50 ma for 14–16 hours. After the completion of electrophoresis, the protein was eluted with the same buffer. The effluent was monitored by an LKB Uvicord flow absorption cell (254 mμ), and 5-ml fractions were collected. These fractions were dialyzed exhaustively against H_2O, freeze-dried, subjected to gel filtration, and then assayed for radioactivity and protein.

The same glycoprotein fraction could be prepared by electrophoresis of whole plasma and subsequent differential precipitation of the globulin fraction. Eight ml of plasma was freeze-dried, then dissolved in 3 ml of the Tris-EDTA-borate buffer, *p*H 9, and subjected to electrophoresis using the above procedure. Albumin, α_1-, α_2-, and β-globulins were obtained. These fractions were dialyzed exhaustively against H_2O, then freeze-dried and assayed for radioactivity. Glycoproteins were prepared from the α_1-, α_2-, and β-globulin fractions by combinations of gel filtration and perchloric acid precipitation. The resulting α_1-glycoprotein fraction was similar to the glycoprotein fraction obtained from perchloric acid filtrates of plasma.

Some crude glycoprotein preparations were purified by electrophoresis on Whatman No. 3 MM filter paper. Less than 5 mg of protein in 5 μl was applied along a 4-cm line across a 2-in. strip of filter paper wetted with buffer–0.25 M Tris–0.011 M EDTA–0.028 M boric acid. After electrophoresis the paper was dried and a 2- to 4-mm-wide strip was cut down the center of the length of the paper. The protein was detected by staining this center strip with Buffalo Black (Richmond, 1959) or fuchsin (Björnesjö, 1955). The zones corresponding to the stained sections were cut out and eluted with three 4-ml portions of the buffer in a centrifuge tube. The salts were then removed by exhaustive dialysis. The contents of the bags were centrifuged, and the supernatant fluid was freeze-dried. The resulting glycoprotein fraction was then subjected to gel filtration or mixed-bed resin chromatography, in most cases, before analysis. Albumin was similarly prepared by electrophoresis of plasma on Whatman No. 3 MM filter paper.

Analytical paper electrophoresis of approximately 10 μg of protein was carried out on cellulose acetate paper (Kohn, 1957) in the same buffer as was employed for column electrophoresis, 0.05 M barbital, *p*H 8.6, 0.05 M phosphate, *p*H 5–8, and in 0.05 M acetate, *p*H 3.5–5.5. A cut was then made down the center of the entire length of the paper; one portion was stained with Buffalo Black and the other portion was stained with fuchsin.

Plasma proteins were also separated into approximate molecular sizes by gel filtration on Sephadex G-100, and G-200 in 0.5 M NaCl—0.1 M Tris, *p*H 8.0, and in 1 M NaCl—0.1 M Tris, *p*H 8.0, according to the method of Porath (1960).

B. *Ion Removal*

Glycoprotein preparations were subjected to gel filtration and/or mixed-bed resin. A 0.1–0.5% solution of the protein was passed through a mixed-bed resin column according to Dintzis (1952), and the elution by H_2O was followed by simultaneous measurements of the conductivity and ultraviolet absorption (254 mμ) of the effluent stream. The conductivity was determined with an LKB 5300B Conductolyzer equipped with a recorder and an LKB 5311 conductivity flow cell. The 254 mμ absorption was recorded by an LKB Uvicord flow cell and a recorder. The recorders were synchronized with a fraction collector; a signal indicator was fed into the recorder for each fraction. The effluent was collected in 2- or 5-ml fractions and each of these fractions was freeze-dried and analyzed.

Proteins were separated into approximate molecular sizes and ions were removed by gel filtration. Gel filtration was carried out on columns (75 × 2cm) of high molecular weight cross-linked dextran, Sephadex G-25 fine, G-50, G-75, and G-100, according to Porath (1960). The column was equilibrated with H_2O and 0.5–1.0 ml (1–100 mg) of protein in H_2O was applied to the top of the gel bed and washed through with H_2O. The effluent was monitored and processed in the same manner as that used with mixed-bed resins.

Partial protein hydrolyzates were centrifuged and the soluble phase was subjected to gel filtration. The resulting fractions were handled in the same way as those from mixed-bed resin columns. The low molecular weight components in these fractions were separated and identified by chromatography and assayed for radioactivity. Sephadex G-25, G-50, G-75 fine, and medium gels were used in specific instances.

C. *End Group Analysis*

Amino acid end-group analysis was carried out with the fluorodinitrobenzene method of Sanger (1945) as was described by Fraenkel-Conrat *et al.* (1955). The 2,4-dinitrophenyl (DNP) amino acid derivatives were subjected to two-dimensional chromatography in the toluene–1.5 M phosphate and in the tertiary amyl alcohol–phthalate systems on Whatman No. 1 filter paper and on thin-layer cellulose plates. The glycoprotein was subjected to the action of carboxypeptidase according to Fraenkel-Conrat *et al.* (1955), and the products were separated by differential precipitation and gel filtration.

D. *Partial Hydrolysis*

1. 0.04 N Acid Hydrolysis.—The protein at a concentration of 0.05–0.15% in a solution of 0.04 N HCl (low sulfate) or 0.04 N H_2SO_4 was heated in a water bath at 85° for 1 hour. The resulting clear solution was subjected to equilibrium dialysis or gel filtration for the removal of ions.

2. 0.05 N to 4 N H_2SO_4 Hydrolysis.—The protein was taken up in enough 0.05 N–4 N H_2SO_4 to obtain a protein concentration of 0.25–1.5 mg/ml. The glass tube was partially evacuated from a nitrogen atmosphere and sealed. The solution was heated at 102–105° for various times from 15 minutes to 20 hours. The release of products was followed by chromatography. Macromolecular components were separated from the smaller molecular components by gel filtration. Neuraminic acids, neutral sugars, amino acids, and hexosamine were separated by chromatography on a series of columns of Dowex 50 (H^+ form) and Dowex 1 (formate form) according to Boström *et al.* (1958). The amino acids and neutral sugars were determined by thin-layer and paper chromatography (Brenner, 1960). Hexosamine and neuraminic acids were purified on ion-exchange resins and then subjected to thin-layer chromatography as described below.

3. Complete Acid Hydrolysis for Amino Acids.—Samples of the glycoprotein which had been subjected

to mixed-bed resin chromatography were weighed out and hydrolyzed in glass-distilled, constant-boiling HCl for 25–72 hours according to Hirs *et al.* (1954). The amino acid composition was determined by quantitative paper (McMenamy *et al.*, 1957) and two dimensional thin layer (Brenner, 1960) chromatography.

Quantitative amino acid analyses were also carried out according to Spackman *et al.* (1958). Samples, approximately 3 mg, were hydrolyzed in glass-distilled constant-boiling HCl for 24–72 hours and the amino acid composition of each sample was determined according to Spackman *et al.* (1958) with a Spinco Model 120 amino acid analyzer.[1] Separate samples were oxidized with performic acid as described by Hirs (1956) and similarly analyzed. Tryptophan content was estimated by the procedure of Spies and Chambers (1949). The result obtained on the amino acid analyzer served as a secondary standard for the routine chromatographic methods.

4. Sialic Acids.—Sialic acid was determined by the Warren (1959) method before and after preliminary separation on ion-exchange columns.

5. Glucosamine.—Colorimetric assays of glucosamine were carried out by the Cessi method as described by Johansen *et al.*, (1960).

6. Neutral Sugars.—Hexoses were determined by the anthrone method (Roe, 1955).

7. Specific Radioactivity of Some Amino Acids.—A sample of 1 ml of plasma or approximately 0.3 g tissue homogenate was put in $^{8}/_{32}$ in. Visking casing and dialyzed against 5–10 ml of H_2O until equilibrium was reached. The dialyzate was used for the assay of amino acids while the bag contents were used for the isolation of albumin. The dialyzate was freeze-dried and the specific activities of 1-C^{14} or uniformly labeled L-amino acids were determined by measuring the specific radioactivity of the evolved CO_2 by the corresponding L-amino acid decarboxylases, glutamic, lysine, and tyrosine (Gale, 1955). The protein fraction in the Visking bag was dialyzed exhaustively and albumin isolated from the resulting supernatant.

The specific activities of the amino acids in the protein were determined after hydrolysis by the same method as used for the free amino acids.

8. Ultracentrifugation.[2]—A 0.5–1% solution of the deionized protein in 0.15 M NaCl was dialyzed against 0.15 M NaCl for 15 hours. This solution was then analyzed in the Spinco Model E ultracentrifuge at 25°. Sedimentation patterns were obtained at 0, 4, 12, 16, 32, 48, 64, 80, 96, and 112 minutes, respectively, after reaching 59,780 rpm.

E. Perfusion

Rat livers were perfused according to Miller *et al.* (1951). The blood glucose concentration was maintained between 1.5 and 3.0 mg/ml of blood by constant infusion of glucose. The amino acid mixture consisted of 5.0% L-arginine, 6.5% glycine, 2.5% L-histidine, 5.0% L-isoleucine, 11% L-leucine, 6.5% L-lysine, 3.7% L-methionine, 0.7% DL-methionine, 5.5% L-phenylalanine, 3.3% L-threonine, 0.6% L-tryptophan, 3.9% L-valine, 18.9% L-glutamic acid, 4.8% L-aspartic acid, 5.9% L-proline, 4.4% L-alanine, 4.4% L-serine, 2.1% L-cysteine·HCl, and 5.0% L-tyrosine. The amino acids were California Biochemical Corporation "cfp grade" and were adjusted to *p*H 7.5 with base. This mixture, 540 mg of the above amino acid mixture in 30 ml of solution, was infused into the circulating blood at a constant rate of 7.5 ml/hour. The blood pressure head was 10–12 cm and the flow rate was 20–40 ml/minute/7–10 g of liver. The solution containing the tracer was mixed with the blood in the reservoir after the commencement of the perfusion. The blood samples and liver biopsies were obtained at various time intervals, and the plasma was separated.

Labeled glycoprotein was obtained by injection of labeled precursors into animals or by perfusion of the liver with labeled precursors. The glycoprotein was purified from plasma. The labeled glycoprotein, 10.0–150 mg, was dissolved in 5 ml of a Ringer solution (8.6 g/liter NaCl, 0.33 g/liter $CaCl_2$ and 0.3 g/liter KCl) filtered through a bacteriological filter, and the supernatant was mixed with the circulating blood. A blood sample was taken immediately and at various time intervals after perfusing the protein; the glycoprotein was then isolated and assayed. All of the conditions for perfusion, including the amino acid mixture, were the same as those used when a labeled precursor was being perfused.

Blood flow usually decreased after 5–6 hours because of the formation of small clots even though 75,000 to 100,000 units of heparin were added to 75–100 ml of blood during its collection. Longer perfusion experiments were possible only when the decreased flow rate was not encountered or when the blood volume was renewed.

In the puromycin experiments control rates of incorporation of a label were obtained during the first two hours of perfusion in the absence of puromycin. Then approximately 15 mg of puromycin were dissolved in 2 ml of Ringer solution and mixed with the perfusing blood at the 2- and 3-hour time intervals. The rates of appearance of the label in proteins were observed in the presence of puromycin in the samples collected at the 3- and 4-hour time intervals. A marked puromycin effect was not very apparent after the 5-hour time interval upon giving puromycin at the 2- and 3-hour time intervals.

F. Radioactivity

Radioactivity was determined on 0.3- to 1.0-mg duplicates of the protein. The glycoprotein, approximately 0.3 mg, was weighed, then spread on stainless steel planchets with a surface area of 1.44 cm^2 with H_2O. The planchets were allowed to dry at room temperature. Albumin and total plasma protein, approximately 1 mg, were weighed on aluminum planchets, spread with H_2O or aqueous ethanol and allowed to dry. The radioactivity was measured in a gas-flow proportional counter (Richmond, 1959) and the counting rate was corrected to infinite thinness from self-absorption curves. The counting errors were less than ± 3% in all instances.

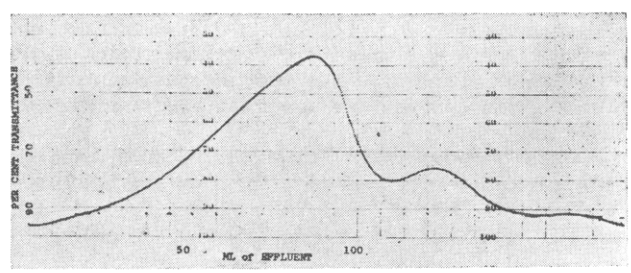

FIG. 1.—Ultraviolet absorption of the eluate from a cellulose acetate column after subjecting the perchloric acid–soluble fraction to 900 v, 45 ma for 12 hours in Tris-EDTA-borate buffer, *p*H 9.

[1] The author is very grateful to Dr. D. Elwyn, Michael Reese Hospital, Chicago, Illinois, for these analyses.

[2] The author is indebted to Dr. J. L. Oncley for these determinations.

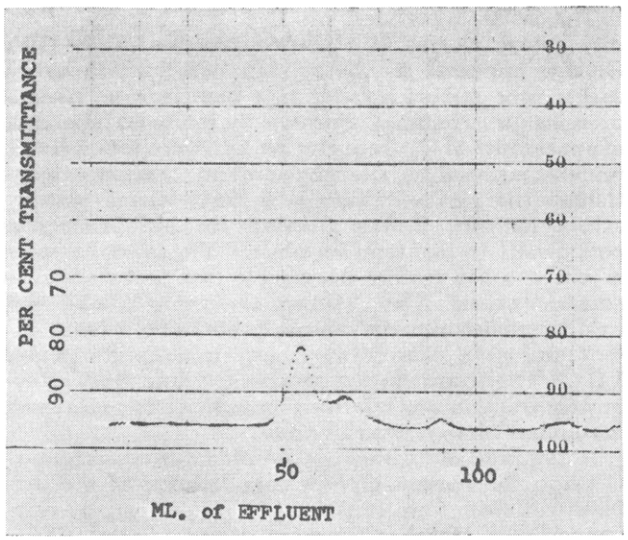

FIG. 2.—Ten mg of the rat glycoprotein fraction were dissolved in 1 ml H_2O and applied to a 2 × 40-cm Sephadex G-25 column. The column was eluted with H_2O and the effluent was monitored with an ultraviolet-absorption cell.

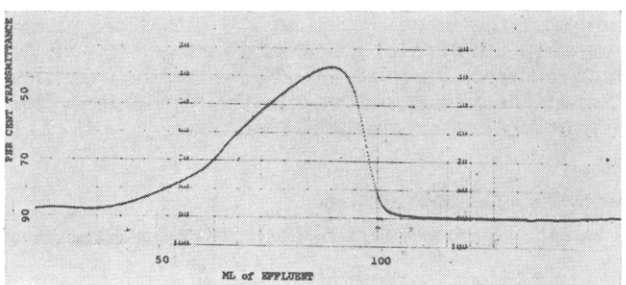

FIG. 3.—After the protein from the first peak of Figure 1 was again subjected to electrophoresis in the original system, only one zone was found.

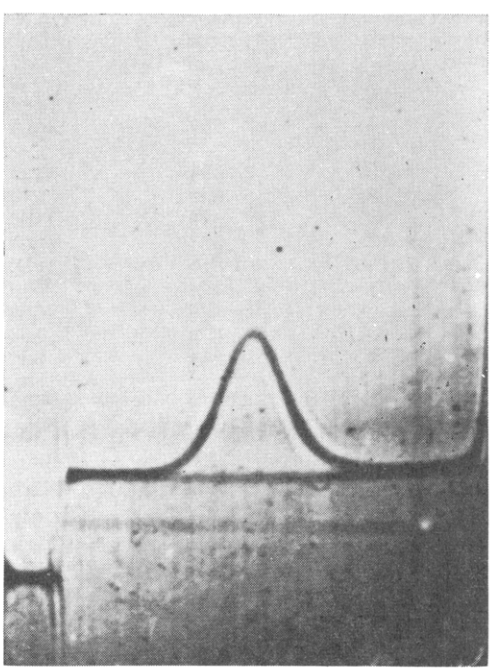

FIG. 4.—Sedimentation pattern of a 0.5% solution of the glycoprotein fraction in 0.15 M NaCl. This pattern was obtained in the Spinco Model E analytical centrifuge operated at 25° using the schlieren optical system. The photograph was taken at 112 minutes after the rotor reached 59,780 rpm. The direction of sedimentation is from left to right.

RESULTS

Glycoprotein preparations of constant composition were obtained from veal, rabbit, and rat plasma by a number of methods. Figure 1 is an ultraviolet absorption recording of the effluent stream of the glycoprotein fraction prepared by removing most of the plasma proteins with perchloric acid and then subjecting the resulting soluble protein to electrophoresis on a cellulose acetate column. Two major protein fractions were eluted from the column. The faster moving impurities were allowed to run off the column. Essentially the same results were obtained when the soluble protein was subjected to paper electrophoresis. The crude perchloric acid–soluble fraction could also be fractionated into at least two fractions by gel filtration (Figure 2) which gave the same type of pattern as electrophoresis. Even subjecting the crude protein to mixed-bed resin chromatography resulted in a slight purification of the glycoprotein fraction due to the retention of globulins which are insoluble at very low ionic strength. These fractions, which were separated by gel filtration, were not further resolved into subfractions when they were again subjected to electrophoresis (Figure 3) or to gel filtration. The major glycoprotein fraction, the one reported in this paper, had a mobility similar to an α_1-globulin when analyzed by paper electrophoresis using Buffalo Black as stain. In contrast to the results obtained with Buffalo Black, occasionally the metachromasia of the fuchsin-positive components gave some slight indications of heterogeneity. Ultracentrifugal analysis yielded a reasonably homogeneous protein (Figure 4).

The composition of this protein was similar to that reported by Eylar and Jeanloz (1962) for human serum α_1-glycoprotein (Table I); however many of the properties of this glycoprotein were species specific. The crude rabbit glycoprotein exhibited a greater degree of heterogeneity and a slower mobility during electrophoresis than rat or bovine preparations. Different methods of preparation yielded a product of remarkably constant composition and similar properties.

The amino acid composition of this protein is shown in Table II. This protein had a high content of the dicarboxylic amino acids and isoelucine.

Small but significant amounts of L-leucine were incorporated into the glycoprotein 10 minutes after the administration of C^{14}-L-leucine to rabbits (Table III). Appreciable amounts of the isotope were present in the glycoprotein fraction during the first 20 to 30 minutes, at a time when the incorporation of isotope into albumin or total plasma proteins was small. Maximum labeling of the glycoprotein occurred earlier and the degree of labeling was greater than that found for albumin. The slope of the radioactivity decay curve of the glycoprotein is greater than that of total plasma proteins or albumin. The leucine content of the glycoprotein

TABLE I

CHEMICAL COMPOSITION OF THE GLYCOPROTEIN FRACTION

N-Acetylneuraminic acid	10.0
Galactose	6.6
Mannose	6.5
Fucose	0.6
Hexosamine	15.0
Nitrogen[a]	12.0
Sulfur[a]	1.07
Ash[a]	0

[a] These microanalyses were carried out by Dr. M. Manser, Basel, Switzerland.

TABLE II

AMINO ACID COMPOSITION OF VEAL PLASMA GLYCOPROTEIN

Value Amino Acid	g/100 g Dry Protein 23-Hour[a] Hydrolysate	71-Hour[a] Hydrolysate	Average or Extrapolated Value
Aspartic acid	5.08 ± 0.03	5.03 + 0.00	5.06 ± 0.02
Proline	2.09 ± 0.07	2.06 ± 0.07	2.08 ± 0.07
Threonine	2.64 ± 0.00	2.35 ± 0.07	2.78 ± 0.05[b]
Serine	2.41 ± 0.00	1.65 ± 0.06	2.78 ± 0.06[b]
Glutamic acid	8.88 ± 0.04	8.74 ± 0.06	8.81 ± 0.05
Glycine	1.33 ± 0.04	1.34 ± 0.01	1.34 ± 0.03
Alanine	2.90 ± 0.04	2.90 ± 0.04	2.90 ± 0.04
Valine	2.64 ± 0.04	2.70 ± 0.02	2.70 ± 0.04[c]
Methionine	0.87 ± 0.03	0.84 ± 0.00	0.86 ± 0.02
Isoleucine	3.12 ± 0.08	3.34 ± 0.01	3.34 ± 0.04[c]
Leucine	3.63 ± 0.01	3.70 ± 0.01	3.67 ± 0.01
Tyrosine	2.88 ± 0.04	2.63 ± 0.02	3.01 ± 0.03[b]
Phenyl-alanine	3.45 ± 0.08	3.47 ± 0.10	3.46 ± 0.08
Lysine	4.92 ± 0.07	4.98 ± 0.01	4.95 ± 0.05
Histidine	1.52 ± 0.03	1.50 ± 0.02	1.51 ± 0.03
Arginine	3.03 ± 0.07	2.98 ± 0.02	3.01 ± 0.04
Tryptophan			1.8 ± 0.1[d]
Half cystine	1.05 ± 0.12	0.95 ± 0.07	1.34 ± 0.10[e]
NH_3	1.50 ± 0.02	1.90 ± 0.01	0.78 ± 0.01[b]

[a] Averages and deviation from the mean of three samples analyzed. [b] Value extrapolated to time zero. [c] Value of the 71-hour hydrolysate. [d] Estimated on intact protein by the method of Spies and Chambers (1949). [e] Estimated as cysteic acid.

The isolated perfused rat liver was capable of incorporating glycine into plasma proteins (Table VI). Glycine appeared in the glycoprotein fraction at an earlier time and at a faster rate than in total plasma proteins or albumin. Puromycin inhibited the rate of appearance of C^{14} from glycine-1-C^{14} into total plasma proteins as well as the glycoprotein fraction. Even though the perfused liver is a fairly closed system, except for bile, it was necessary to add puromycin periodically to maintain its effect. The effect of puromycin was not readily discernible two hours after its administration. The C^{14} from uniformly labeled glucose was fixed into the plasma proteins to a lesser extent and at a slower rate than amino acids (Table VII). The incorporation of the C^{14} into the glycoprotein fraction was relatively insensitive to puromycin in comparison to plasma albumin.

Initial rates of fixation of the C^{14} from glucosamine-1-C^{14} into the plasma glycoprotein fraction of the perfused rat liver were considerably faster than was observed with total plasma proteins (Table VIII). Radioactivity appeared in the glycoprotein in a shorter time interval than was observed with glucose and glycine. A greater fraction of the total C^{14} was associated with the sialic acid (0.04 N H_2SO_4 labile) initially than at later time intervals (Table IX). Puromycin inhibited the incorporation of C^{14} into total plasma proteins; however, at a dose of 15 mg puromycin per hour no detectable effect was observed on the incorporation of C^{14} of glucosamine into the glycoprotein fraction under these experimental conditions.

TABLE III

DISTRIBUTION OF RADIOACTIVITY BETWEEN PLASMA FRACTIONS

Effect of the dialyzing media on the C^{14} associated with plasma and the glycoprotein fraction after the administration of 1.1×10^8 cpm of 1-C^{14}-L-leucine intravenously to a 2.4-kg rabbit.

Time	Dialysis against H_2O for 96 Hours: Glycoprotein (cpm/mg)	Dialysis against H_2O for 96 Hours: Albumin (cpm/mg)	Dialysis against 0.15 M NaCl for 72 Hours[a]: Total Plasma Protein (cpm/mg)	Dialysis against 0.15 M NaCl for 72 Hours[a]: Glycoprotein (cpm/mg)	Dialysis against 0.02 M Leucine for 72 Hours[a]: Total Plasma Protein (cpm/mg)	Dialysis against 0.02 M Leucine for 72 Hours[a]: Glycoprotein (cpm/mg)
Minutes						
10	4	0	1	6	0	6
30	655	83	277	826	220	588
60	1259	282	488	1447	399	1106
Hours						
1.5	1239	342	544	1639	594	1126
2	1339	366	671	1658	623	1226
4	1299	376	658	1456	579	1102
6	1228	381	572	1377	531	1067
8	1048	373	526	1349	515	1025
12	759	362	431	861	474	753
24	629	342	401	649	386	656
34	584	320	421	606	374	564
48	515	290	434	580	348	544

[a] These fractions were dialyzed against H_2O for 24 hours to remove the solids after dialysis against NaCl and leucine.

fraction is the same order of magnitude as that of albumin.

Table IV illustrates the results obtained following the administration of 1-C^{14}-glycine to a rabbit. The label appeared in the glycoprotein after a slightly longer time interval and the slope of the curve was less than was determined with leucine. The incorporation of glycine into total plasma proteins was very similar to that observed with leucine. Dialysis against salt resulted in the loss of more solids bound to the protein and consequently gave a product of greater specific activity.

Uniformly labeled L-aspartic acid exhibited a longer lag period and smaller degree of labeling than was observed with other L-amino acids (Table V).

N-acetyl-1-C^{14}-glucosamine was fixed into the plasma proteins of the perfused liver at a slower rate than that determined with glucosamine (Table X). There was less labeling of the glycoprotein and even a smaller proportion of the label was found in total plasma protein than was measured with glucosamine.

After a brief lag period, galactose-1-C^{14} was incorporated into the glycoprotein and into total plasma proteins at a rate greater than was measured for glucose (Table XI). Mannose gave a pattern similar to galactose.

Some experiments were carried out by adding the purified labeled glycoprotein fraction to the perfusing blood and then determining the rate of disappearance of the label from the glycoprotein fraction in the cir-

TABLE IV

DISTRIBUTION OF RADIOCARBON BETWEEN PLASMA FRACTIONS

Incorporation of C^{14} into plasma proteins and the glycoprotein fraction after the administration of 1×10^8 cpm of 1-C^{14} glycine intravenously to a 2.3-kg rabbit.

Time	Dialysis against H_2O for 96 Hours		Dialysis against 0.15 M NaCl for 72 Hours.[a]		Dialysis against 0.05 M Glycine for 72 Hours[a]	
	Total Plasma Proteins (cpm/mg)	Glycoprotein (cpm/mg)	Total Plasma Protein (cpm/mg)	Glycoprotein (cpm/mg)	Total Plasma Protein (cpm/mg)	Glycoprotein (cpm/mg)
Minutes						
1 to 2	0	6	0	3	0	0
4 to 6	2	5	0	3	0	3
9 to 12	0	5	0	3	2	4
19 to 21	0	6	0	4	4	8
29 to 32	8	24	8	35	14	22
60	67	175	69	268	63	166
Hours						
2	149	341	148	417	142	372
4	211	382	214	488	206	339
6	233	392	222	516	207	424
10	219	391	226	503	207	385
24	163	327	182	368	160	313
34	135	261	137	277	136	260
48	118	232	118	229	116	215

[a] These fractions were dialyzed against H_2O for 24 hours to remove the solids after dialysis against NaCl and glycine.

TABLE V

INCORPORATION OF C^{14} INTO PLASMA PROTEINS AFTER THE ADMINISTRATION OF 0.9×10^8 CPM OF UNIFORMLY LABELED C^{14}-L-ASPARTIC ACID INTRAVENOUSLY TO A 2.5-kg RABBIT

Time	Glycoprotein (cpm/mg)	Total Plasma Protein (cpm/mg)
Minutes		
2	4	0
15	3	2
30	45	18
60	103	50
Hours		
1.5	125	65
2	150	77
4	173	92
6	171	96
10	169	93

TABLE VI

ASSOCIATION OF C^{14} FROM 8 μc OF GLYCINE-1-C^{14}[a] WITH THE PLASMA OF THE ISOLATED PERFUSED RAT LIVER

Time	Glycoprotein Fraction (cpm/mg)	Albumin (cpm/mg)	Total Plasma (cpm/mg)
Minutes			
15	20	0	0
30	44	0	0
Hours			
1	106	30	24
2	252	46	113
	17 mg puromycin		
3	300	46	120
	17 mg puromycin		
4	352	47	130
5	560	49	142

[a] A total of 25 mg glycine and 23 mg of L-serine was infused into 75 ml of perfusate.

TABLE VII

ASSOCIATION OF C^{14} FROM 12 μc OF D-GLUCOSE-C^{14}[a] WITH THE PLASMA PROTEINS OF THE ISOLATED PERFUSED RAT LIVER

Time	Glycoprotein Fraction (cpm/mg)	Albumin (cpm/mg)	Total Plasma Protein (cpm/mg)
Minutes			
15	10	0	0
30	21	0	3
Hours			
1	36	2	4
2	64	18	29
	17 mg puromycin		
3	149	18	30
	15 mg puromycin		
4	161	20	33
5	203	28	33

[a] The C^{14}-D-glucose was mixed with 50 mg of D-glucose in 75 ml of blood.

TABLE VIII

INCORPORATION OF C^{14} FROM D-GLUCOSAMINE-1-C^{14}[a] WITH THE PLASMA PROTEINS OF THE ISOLATED PERFUSED RAT LIVER

Time	Glycoprotein Fraction (cpm/mg)	Total Plasma Protein (cpm/mg)
Minutes		
15	182	7
30	372	61
Hours		
1	994	328
2	3,726	1,228
3	9,626	2,861
4	11,844	3,590
5	14,828	5,480

[a] A total of 3 mg (28 μc) of D-glucosamine-1-C^{14} and 57 mg of D-glucose were added to 90 ml of blood.

TABLE IX

EFFECT OF PUROMYCIN ON THE INCORPORATION OF C^{14} FROM D-GLUCOSAMINE-1-C^{14}[a] INTO PLASMA PROTEINS OF THE ISOLATED PERFUSED RAT LIVER

Time	Glycoprotein Fraction (cpm/mg)	Glycoprotein Fraction after 0.04 N H_2SO_4 (cpm/mg)	Total Plasma Protein (cpm/mg)
Minutes			
5	136	89	5
15	149	95	5
30	195	102	41
Hours			
1	442	298	116
2	960	888	554
	15 mg puromycin		
3	2445	1907	861
	15 mg puromycin		
4	3470	2547	916

[a] A total of 2.4 mg (22 μc) of D-glucosamine-1-C^{14} and 57 mg of glucose were present in 90 ml of perfusing fluid.

TABLE X

ASSOCIATION OF C^{14} FROM *N*-ACETYL-1-C^{14} D-GLUCOSAMINE[a] WITH PLASMA PROTEINS OF THE ISOLATED PERFUSED RAT LIVER

Time	Glycoprotein (cpm/mg)	Total Plasma Protein (cpm/mg)
Minutes		
15	20	2
30	29	3
Hours		
1	45	6
2	161	48
3	289	64
4	508	105
5	865	134

[a] A total of 5 mg (30 μc) of *N*-acetyl-1-C^{14} D-glucosamine and 65 mg of D-glucose were added to the 110 ml of blood.

culating blood. Table XII shows the results obtained after perfusing a rat liver with a glycoprotein preparation which was labeled using D-glucosamine-1-C^{14}. The glycoprotein was diluted in the liver with a volume of protein equal to approximately the same mass as is present in 20 ml of blood or about the blood volume of the liver donor. The protein disappeared at a slightly slower rate (not always linearly with time) than the rate of formation from labeled precursors; approximately 60% of it disappeared in 5 hours. Very little of the label was found in total plasma proteins. No significant amount of radioactivity was found in albumin during this period. Some 5–10% of the C^{14} was present in the evolved CO_2.

DISCUSSION

Incorporation rates of a label are a reliable measure of the turnover of a given metabolite when an accurate measurement of the specific activity of the immediate precursor is obtainable and the time required for the secretion or release of the metabolite is known. In the intact animal, it is not possible to have detailed data on all of the factors involved in protein synthesis. Therefore, in this study, relative rates of synthesis of albumin and the glycoprotein were obtained in the

TABLE XI

INCORPORATION OF C^{14} FROM D-GALACTOSE-1-C^{14}[a] INTO THE PLASMA PROTEINS OF THE ISOLATED PERFUSED RAT LIVER

Time	Glycoprotein (cpm/mg)	Total Plasma Protein (cpm/mg)
Minutes		
15	9	4
30	219	102
Hours		
1	431	200
2	519	242
3	555	253
4	623	234
5	680	279

[a] 3.6 mg (25 μc) of D-galactose-1-C^{14} and 60 mg of D-glucose were added to 100 ml of the perfusate.

TABLE XII

RATE OF DISAPPEARANCE OF C^{14} FROM THE GLYCOPROTEIN FRACTION (112 mg) CONTAINING 1800 cpm/mg FROM D-GLUCOSAMINE-1-C^{14} PERFUSED THROUGH A RAT LIVER IN 60 ml OF BLOOD

Time	Glycoprotein Fraction (cpm/mg)	Total Plasma Protein (cpm/mg)
Minutes		
15	812	14
30	750	10
60	739	11
Hours		
2	692	14
3	640	9
4	596	10
5	502	10

intact animal and in the perfused rat liver. Rates of protein synthesis were calculated for the rat liver perfused with essentially constant specific activity of precursor.

The amount of glycoprotein synthesized during 2 hours of perfusion was calculated from the data in Table 6. The mean specific activity of glycine was found to be 2×10^5 cpm/mg. Thus a glycoprotein counting rate of 252 cpm/mg corresponds to 1.3×10^{-3} mg glycine; or, making use of the glycine content of this protein (Table II), then 0.09 mg glycoprotein is synthesized per mg glycoprotein present in the perfusion fluid. If the amount synthesized in 75 ml of blood is compared to the fraction that would have been synthesized in the *in vivo* blood volume (22 ml) of the donor liver, then the equivalent of 0.27 mg glycoprotein is formed per mg glycoprotein during this 2-hour period. This calculated value is 15–30% too great because 15–30% of the label in the protein was present in components other than glycine, i.e., cysteine, serine, etc. (Richmond *et al.*, 1963). Thus an amount equal to 0.2 mg is formed per mg of glycoprotein present by a 7- to 8-g liver in a volume equal to its blood volume. Similar calculations for other amino acid precursors, based on the specific activity of the amino acid in the protein, gave values for an 8-g liver of 0.3–0.8 mg of newly formed glycoprotein per mg glycoprotein during 5 hours of perfusion; or, approximately 5 mg of this protein was synthesized per kg liver per minute. This quantity is not markedly different from the total amount of albumin synthesized, despite the much

smaller quantity of glycoprotein in plasma (Richmond *et al.*, 1963). The observed turnover time of 10–12 hours for the glycoprotein is three to five times faster than the observed rate of turnover of albumin. Calculations based on the initial rates of appearance of label in the glycoprotein and albumin yield an even greater relative rate for the glycoprotein fraction. The data from the intact rat and rabbit gave over-all rates similar to those observed in the perfused liver.

After the glycoprotein was labeled with a given precursor, the protein was degraded by enzymatic and chemical methods to ascertain if the whole peptide chain was labeled (the entire protein is being synthesized), or if the precursor was bound in nonpeptide linkages, or if it was fixed predominantly in end groups. These studies showed that there were no indications of heterogeneous labeling or binding of the precursor after the 2-hour time interval. Similar results were obtained upon enzymatic and chemical degradation of the carbohydrate moiety before and after the removal of the major portion of the amino acids with papain and with other enzymes.

Amino sugar derivatives such as neuraminic acids and *N*-acetyl glucosamine make up 25% of the mass of the glycoprotein, a far greater proportion of the protein than any other residue; therefore the degree of labeling can be expected to be greater than was observed with amino acids if the dilutions are similar. Initial rates for the appearance of labeled glucosamine in the glycoprotein were faster and the amount of label present was greater than that found for amino acids. Glucosamine was converted to neuraminic acid residues at a slightly faster rate initially than to glycoprotein *N*-acetyl glucosamine. *N*-acetyl glucosamine was a poorer precursor of macromolecular *N*-acetyl glucosamine than glucosamine, which is in agreement with the findings of Kohn *et al.* (1962). Glucosamine and *N*-acetyl glucosamine did not appear to be metabolized to simple hexoses to a very great extent. Less CO_2 was produced from the amino sugars than was derived from hexoses. The degree of labeling of the glycoprotein was greater for galactose than for mannose, and the least for glucose.

The total amount of C^{14}-labeled glutamic and aspartic acids in the glycoprotein and the rate of appearance of these dicarboxylic amino acids in the glycoprotein were less than was observed with any of the other amino acids although the plasma level and the specific activity decreased more slowly in the intact animal than was observed with other amino acids. A number of other investigators have made similar observations in studying other plasma proteins (Anker, 1960). The glycoprotein is rich in these dicarboxylic amino acids.

References

Ada, G. L., and French, E. L. (1959), *Nature 183*, 1740.

Anker, H. S. (1960), *in* Putnam, F. W., ed., The Plasma Proteins, Vol. II, New York, Academic, p. 267.

Aransson, T., and Grönwall, A. (1958), *Scand. J. Clin. Lab. Invest. 10*, 348.

Björnesjö, K. B. (1955), *Scand. J. Clin. Lab. Invest. 7*, 153.

Boström, H., Roden, L., and Yamashina, I. (1958), *J. Biol. Chem. 230*, 381.

Brenner, M. (1960), *Experientia, 17*, 145.

Dintzis, H. M. (1952), Ph.D. thesis, Harvard University.

Eylar, E. H., and Jeanloz, R. W. (1962), *J. Biol. Chem. 237*, 622.

Fraenkel-Conrat, J., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Anal. 2*, 359.

Gale, E. F. (1957), *Methods Biochem. Anal. 4*, 285.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

Hirs, C. H. W., Stein, W. H., and Moore, S. (1954), *J. Biol. Chem. 211*, 941.

Johansen, P. G., Marshall, R. D., and Neuberger, A. (1960), *Biochem. J. 77*, 239.

Kimmel, J. R., and Smith, E. L. (1954), *J. Biol. Chem. 207*, 515.

Kohn, J. (1957), *Biochem. J. 65*, 9.

Kohn, P., Winzler, R. J., and Hoffmann, R. C. (1962), *J. Biol. Chem. 237*, 304.

Lee, Y. C., and Montgomery, R. (1962), *Arch. Biochem. Biophys. 97*, 9.

McMenamy, R. H., Lund, C. C., and Oncley, J. L. (1957), *J. Clin. Invest. 36*, 1672.

Miller, L. L., Bly, C. G., Watson, M. L., and Bale, W. F. (1951), *J. Exp. Med. 94*, 431.

Nuenke, R. H., and Cunningham, L. W. (1961), *J. Biol. Chem. 236*, 2452.

Porath, J. (1960), *Biochim. Biophys. Acta 29*, 193.

Richmond, J. E. (1959), *J. Biol. Chem. 234*, 2713.

Richmond, J. E., Shoemaker, W. C., and Elwyn, D. (1963), *Am. J. Physiol. 205* (in press).

Roe, J. H. (1955), *J. Biol. Chem. 212*, 335.

Roseman, S. (1959), *Ann. Rev. Biochem. 28*, 545.

Rosevear, J. W., and Smith, E. L. (1961), *J. Biol. Chem. 236*, 425.

Sanger, F. (1945), *Biochem. J. 39*, 507.

Schmid, K. (1950), *J. Am. Chem. Soc. 72*, 2816.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 21*, 1249.

Spiro, R. G. (1959), *J. Biol. Chem. 234*, 742.

Spiro, R. G. (1962), *J. Biol. Chem. 237*, 646.

Warren, L. (1959), *J. Biol. Chem. 234*, 1971.

Winzler, R. J. (1955), *Methods Biochem. Anal. 2*, 279.