A Plasma Protein Fractionation Procedure for Use in Studies of Protein Metabolism

ITS APPLICATION TO THE MEASUREMENT OF SYNTHESIS RATES OF TWO α_1 -GLYCOPROTEINS AND OTHER SERUM PROTEINS IN THE RAT

By J. HO, K. N. JEEJEEBHROY and R. H. PAINTER

*Departments of Medicine and Biochemistry, University of Toronto,
Toronto, Ontario, Canada M5S 1A8*

(Received 29 January 1974)

A two-step method for the separation of five different plasma proteins on a preparative scale, which is capable of being extended to allow the separation of other plasma proteins, is described. The proteins separated were fibrinogen, two α_1 -glycoproteins, albumin and transferrin. The α_1 -glycoproteins were characterized in terms of electrophoretic mobility, ultracentrifugal and immunological characteristics. By using this method, it was shown that a single sample of plasma could be fractionated to yield purified proteins in sufficient quantity to simultaneously measure the synthesis of the two α_1 -glycoproteins, albumin and transferrin in the rat with McFarlane's technique (McFarlane, 1963; Reeve *et al.*, 1963; McFarlane *et al.*, 1965).

Most methods available for studying the metabolism of plasma proteins *in vivo* involve measuring the distribution and degradation of exogenously radioactive-iodine-labelled proteins (Rothschild *et al.*, 1958; Picou & Waterlow, 1962; Jeejeebhoy, 1964; Jeejeebhoy *et al.*, 1965; Laster *et al.*, 1966). These methods require a metabolically steady state lasting several days for satisfactory interpretation of the results and even then allow only indirect measurement of the synthesis rate. Early and acute changes in synthesis cannot be measured by these methods. McFarlane has described a method for direct measurement of the synthesis rate of liver-made plasma proteins over short periods of time (McFarlane, 1963; Reeve *et al.*, 1963; McFarlane *et al.*, 1965; Koj & McFarlane, 1968). The method is based on the observation that both [^{14}C]urea and the [6- ^{14}C]arginine of liver-made plasma proteins are products of a common precursor, hepatic arginine, formed endogenously via the urea cycle by 'pulse labelling' the arginine pool with [^{14}C]CO₂. This method has been validated by other workers (Reeve *et al.*, 1963; Wochner *et al.*, 1968), and used to study the effects of dietary protein deprivation (Kirsch *et al.*, 1968; Rothschild *et al.*, 1968), hormones (Sellers *et al.*, 1969) and disease (Wochner *et al.*, 1968; Tavill *et al.*, 1968) on rates of albumin synthesis, and of toxins, the stagnant loop syndrome and gastrointestinal protein loss, on fibrinogen synthesis (Koj & McFarlane, 1968; Wochner *et al.*, 1968; Jones *et al.*, 1968). Jeejeebhoy *et al.* (1969) have used the method to observe the effect of malabsorption in man, and (1970) the effect

of bovine growth hormone in rats on the synthesis of both albumin and fibrogen. Jeejeebhoy *et al.* (1972*a,b*, 1973) with this method observed that the synthesis rates of three rat plasma proteins, albumin, transferrin and fibrogen, are affected concurrently but independently by a number of conditions, namely ethanol, hormones and starvation. Knowing of these differences and in the interest of elucidating the mechanisms by which protein synthesis is governed, it seems of considerable interest to extend these studies to other proteins of liver origin. This requires the development of a simple unified inclusive fractionation scheme which allows the separation of a number of rat plasma proteins. For synthesis studies, it is essential that the fractionation scheme be simple and capable of repeated application, that it should yield homogeneous preparations of the proteins, and that it should allow a number of proteins to be recovered from a single plasma sample for reasons of economy. The scheme described below is based on a two-step process and embodies these features. It yielded pure albumin, fibrinogen and transferrin in addition to two α_1 -glycoproteins, and further development should permit the separation of others. Since pure proteins have to be obtained for synthesis measurements, these two glycoproteins were characterized in detail to establish their purity. The synthesis rates of these glycoproteins, hitherto unknown, were determined and compared with those of albumin and transferrin in the same animal.

Materials and Methods

Materials

$\text{Na}_2^{14}\text{CO}_3$ (specific radioactivity 50mCi/mmol) and NCS were obtained from Amersham/Searle, Don Mills, Ont., Canada, and Na^{125}I in NaOH was obtained from Atomic Energy of Canada Ltd., Chalk River, Ont. ^{125}I -labelled proteins were prepared by the iodine monochloride method of McFarlane (1964), by using the proteins separated as described below.

Antiserum to rat serum proteins prepared in rabbits was purchased from Behringwerke A.G., Marburg/Lahn, Germany.

Arginase and urease (type III) were purchased from Sigma Chemical Corp., St. Louis, Mo., U.S.A.

Plasma proteins for ^{125}I -labelling were obtained, by methods described below, from the plasma of male Wistar rats (Hi Oak Ranch, Richmond Hill, Ont., Canada) fed *ad lib.* on Purina rat chow. Synthesis rates of albumin, transferrin and two α_1 -glycoproteins were determined in the same stock of male Wistar rats (220–240g) fed on a 40%-protein diet over the previous 4 days.

General methods

The sequence of events with the animals was similar to that used previously (Jeejeebhoy *et al.*, 1970, 1972a). Rats were individually caged and only those eating well and gaining weight steadily were used for synthesis measurements. One batch of ten rats was used for the experiment. Blood was withdrawn, under ether anaesthesia, from the retro-orbital plexus at 10min, 1h, 2h and 3h after intraperitoneal injection of $30\mu\text{Ci}$ of $\text{Na}_2^{14}\text{CO}_3$ and intravenous injection of $5\mu\text{Ci}$ of ^{125}I -labelled albumin or transferrin into the dorsal vein of the penis. At the 5th hour blood was obtained from the inferior vena cava under direct vision with a heparinized syringe and dispensed into centrifuge tubes. In all cases, blood was centrifuged for 15min at 500g and the plasma removed with a Pasteur pipette and diluted with an equal volume of 0.09M-trisodium citrate solution. This mixture was centrifuged at 100000g in an I.E.C. model B-60 ultracentrifuge for 20h at 4°C. The supernatant lipoprotein layer was removed carefully with a Pasteur pipette. The clear gummy pellet of remaining plasma proteins was resuspended and redissolved in the infranant plasma. The reason for this preliminary step is that it was found to be helpful in the later phases of gel filtration.

In addition to the above-mentioned ^{125}I -labelled albumin and transferrin, injected intravenously at the time of administering the $\text{Na}_2^{14}\text{CO}_3$, two biologically screened ^{125}I -labelled rat α_1 -glycoproteins, screened by methods described below, were also injected intravenously into two further batches

of Wistar rats (not given $\text{Na}_2^{14}\text{CO}_3$) and blood samples were taken at various intervals up to 5.8h for determination of the plasma-disappearance curve, thus allowing an estimate to be made of the specific protein loss from the intravascular pool during the synthesis interval. Biological screening was carried out by labelling the proteins with ^{125}I (McFarlane, 1964). The labelled protein was injected intraperitoneally into 50g rats and 18h later the rats were exsanguinated under ether anaesthesia, the blood was collected in heparin, the plasma separated and used as a source of labelled screened protein.

Salt precipitation

Fibrinogen and γ -globulins were removed from the plasma by precipitation with 1.2M- $(\text{NH}_4)_2\text{SO}_4$. The $(\text{NH}_4)_2\text{SO}_4$ was added as a solid (Dixon, 1953). The precipitate was removed by centrifugation and discarded (but fibrinogen may be recovered from it as has been done previously; Jeejeebhoy *et al.*, 1970) and the supernatant concentrated to 10–12g of protein/100ml in an Amicon Diaflo cell, by using a U-10 membrane, before treatment by gel filtration as described below.

Preparative methods

Gel filtration. This was carried out with Sephadex G-200 (Pharmacia, Montreal, Canada) in either a K10/100 Pharmacia column at a flow rate of 2ml/min or in a K25/100 Pharmacia column at a flow rate of 0.3ml/min. Buchler Polystaltic pumps (Fort Lee, N.J., U.S.A.) were used to control the flow rate in both cases. The direction of elution was upward and the columns were inverted after each run.

Ion-exchange chromatography. This was carried out with DEAE-Sephadex A-50 (Pharmacia), in K9/15 columns for preparation of the albumin and transferrin used in determining the appearance curves. For preparation of albumin and transferrin for ^{125}I -labelling, K15/30 columns were used.

Polyacrylamide-gel electrophoresis. A 5% gel was prepared in 0.5cm $\times$ 7.5cm tubes and run without using a sample or spacer gel. The ratio of polymer to co-polymer was 40:1. Gels were polymerized with ammonium persulphate. Electrophoresis was carried out at 1mA per tube for 3h at 4°C. Gel buffer was 0.001M-Tris–0.045M-EDTA–0.025M-borate, pH 8.6, and reservoir buffer was 0.05M-sodium borate, pH 9.2. Gels were stained for protein with 1% Amido Schwartz in 7% (v/v) acetic acid (Davies, 1964), for lipid with Oil Red O (Tietz & Fioreck, 1966), and for glycoprotein with periodic acid-Schiff reagent (Zacharius *et al.*, 1969).

For electrophoresis in urea, 8M-urea was incorporated in the gels and the samples were dialysed

against 8M-urea before application. All urea solutions were passed through a column of mixed ion exchanger immediately before use to remove cyanates (Stark *et al.*, 1960). Electrophoresis in urea-gel was performed at room temperature.

Analytical methods

Reduction and alkylation of component M in the macroglobulin peak. To 25 μ l samples containing about 200 μ g of protein were added 100 μ l of, respectively, 0.01 M-, 0.001 M-, 0.0001 M- and 0.00001 M-dithiothreitol in 0.05 M-Tris-HCl, pH 8.2. The samples were then incubated at 37°C for 1 h. A 10% excess of iodoacetamide in 10 μ l of water was added to each tube and the mixtures were kept at 4°C in the dark for 15 min before samples were taken for electrophoretic analysis in polyacrylamide gel. The remainder of the samples to which 0.001 M- and 0.00001 M-dithiothreitol had been added were then pooled, dialysed with 0.9% NaCl and subjected to sedimentation-equilibrium centrifugation (q.v.).

Vertical starch-gel electrophoresis. This was carried out as described by Smithies (1959).

Immunoelectrophoresis. This was carried out in 1.5% agarose in Tris-EDTA-borate buffer, pH 8.6, at 1.5 mA/gel for 1 h, and by using polyvalent rabbit antiserum to rat serum proteins.

Determination of hexose (hexosamine). Total hexose or hexosamine was determined with anthrone reagent (Scott & Melvin, 1953) with galactose as standard.

Determination of protein. Protein was assayed by the method of Lowry *et al.* (1951) with human albumin as a standard.

Determination of fatty acid and phosphorus in α_1 -glycoproteins. Lipid from proteins was removed by chloroform-methanol (2:1, v/v) followed by three extractions with chloroform. The pooled organic phase, after washing with Folch upper phase (chloroform-methanol-0.9% NaCl, 3:48:47, by vol.) three times, was dried down and the lipid taken up in chloroform. Phosphate was determined by the method of Bartlett (1959) and fatty acids were transmethyated (Feldman & Rouser, 1965) and analysed by g.l.c. (Parkes & Thompson, 1970). A sample of pure rat high-density lipoprotein prepared by the method of Koga *et al.* (1969) was included for comparison.

Sedimentation equilibrium. Molecular weights were determined by the fringe-displacement method of Yphantis (1964) in a Beckman analytical ultracentrifuge model E at 20°C. Rotor speeds used were 14000, 10000 and 5600 rev./min for α_1 -glycoproteins F, M and S respectively. Sedimentation-equilibrium study was also carried out on a sample of glycoprotein M after reduction by dithiothreitol, at a speed of 14000 rev./min. The partial specific volume (v)

value used in molecular-weight calculations was 0.735.

Sedimentation velocity. By using the instrument just mentioned, sedimentation-velocity runs were carried out for the components of the macroglobulin peak by the boundary-displacement method of Vinograd *et al.* (1963). Rotor speed was 56000 rev./min at 20°C and 1 M-NaCl was used as the displacing solution. The protein concentration in both cases was 0.7 mg/ml of 0.9% NaCl and 30 μ l samples were used.

Determination of plasma [14 C]urea and [6- 14 C]-arginine specific radioactivity of plasma protein. The method used was that described by Jeejeebhoy *et al.* (1970). Using a known amount of nitrogen (0.001 mg in 0.200 ml), which was equivalent to that found when determining the mass of the sixth or guanidine carbon of arginine derived from the α_1 -macroglobulins, we observed a recovery of $80.2 \pm 0.21\%$. Although it leaves room for improvement, we have considered this recovery sufficiently consistent to permit us to measure the specific radioactivity of the small amounts of the different proteins (of the order of 0.1 mg) obtained in our system. This is deemed to be an advance over the gasometric method for determining the sixth or guanidine carbon of arginine as described originally by McFarlane (1963), the procedure then requiring 10–15 mg of protein or else dilution of the $^{14}\text{CO}_2$ with non-radioactive CO_2 , when the amounts of protein being handled were smaller.

Calculations

Calculation of fractional synthesis rates. These rates were calculated from the formula proposed by Koj & McFarlane (1968): fractional synthesis rate of the given protein = (fractional synthesis rate of urea) $\times$ (hypothetical specific radioactivity of the protein's sixth or guanidine carbon) / (hypothetical specific radioactivity of urea carbon).

Hypothetical specific radioactivity of [14 C]urea carbon. This is defined as the specific radioactivity of urea that would be observed if all the labelled urea were distributed in the body water instantaneously. This was calculated as described in Jeejeebhoy *et al.* (1972a).

Hypothetical specific radioactivity of [6- 14 C]-arginine. This is defined as the specific radioactivity of the sixth or guanidine carbon of arginine that would be observed if all the radioactive protein had been distributed instantaneously in the plasma compartment. In essence, the endogenously produced [6- 14 C]arginine specific radioactivity of a given plasma protein is plotted against time, and this appearance curve subjected to exponential analysis. Exponential analysis of the disappearance curve of the same but exogenously ^{125}I -labelled protein is used to correct the appearance curve for ^{14}C -labelled

protein lost from the intravascular system during the interval over which synthesis is measured (about 5h). This blending of the two analyses was done by a deconvolutional technique and computer program developed by a colleague (Norwich, 1972).

Results

Development of fractionation procedures

(1) *Separation of serum proteins by gel filtration.* A flow diagram of the procedures used in the separation of the rat plasma proteins is given in Scheme 1. The very-low-density-lipoprotein- and fibrinogen-free samples described in the Materials and Methods section were passed through a column (10cm $\times$ 100cm) of Sephadex G-200, with 1% NaCl (containing 0.01% NaN_3) as eluent. Four major protein peaks were obtained. Peaks 1 and 2 were designated the macroglobulin fractions (M1 and M2), peak 3 the 7S fraction and peak 4 the 4S fraction. Fractions from the middle of each of the four main peaks were analysed by starch-gel electrophoresis in Tris-EDTA-borate buffer, pH 8.6. The compositions of peaks M1 and M2 were similar, each containing three main components. Since these two peaks were often incompletely resolved they were combined. Peak 3 contained at least seven proteins, including residual γ -globulins. Peak 4 contained only three major components. The fastest was clearly identified as albumin and the two slower components appeared to be

transferrin. The fractions comprising each of these peaks were pooled and concentrated for further analysis.

(2) *Separation of components in the 4S fraction.* These were resolved by ion-exchange chromatography. After addition of a drop of $^{59}\text{FeCl}_3$ in 0.1M-HCl to serve as a marker for transferrin the sample was equilibrated by dialysis with 0.03M- K_2HPO_4 , pH 7.5, before being applied to a column (1.5cm $\times$ 30cm) of DEAE-Sephadex A-50, previously equilibrated with the same buffer. Elution was carried out by using a linear gradient of chloride-free Na_2HPO_4 , increasing in concentration from 0.03 to 0.08M. Only two peaks were eluted during this gradient. The first comprised the two transferrins, identified on the basis of their electrophoretic mobility in starch gel, both bearing the ^{59}Fe label. The single component in the second peak was identified as albumin. The albumin and transferrin prepared by this chloride-free Na_2HPO_4 buffer each gave a single precipitin line with polyvalent anti-serum on immunoelectrophoresis (Plate 1a).

(3) *Fractionation of the 7S fraction.* An attempt to separate the components of this fraction by DEAE-Sephadex A-50 was only partially successful.

Polyacrylamide-gel electrophoresis proved to be the most effective method for the resolution of the macroglobulin fraction. Electrophoresis in 5% polyacrylamide gel gave good separation (Plate 1b). The three major components observed were designated F (fast), M (medium) and S (slow) according

Scheme 1. Flow diagram showing the sequence of procedures used in the separation of rat plasma proteins
VLDL, very-low-density lipoprotein.

EXPLANATION OF PLATE 1(a)

Immunoelectrophoresis of purified proteins from the 4S fraction prepared by DEAE-cellulose chromatography as described
Antiserum in both troughs was rabbit anti-(whole rat serum). In the wells of slide (I), the top one held rat serum, the bottom one transferrin, and in those of slide (II), the top one held albumin, the bottom one rat serum.

EXPLANATION OF PLATE 1(b)

Polyacrylamide-gel-electrophoretic analysis of the macroglobulin fraction from Sephadex G-200

(I) Electrophoresis of the whole macroglobulin fraction; (II) electrophoresis of the main components S, M and F recovered from sections of gels; (III) electrophoresis of components F and M recovered from sections in which M was run as the starting material.

EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of (a) samples of component M after reduction with various concentrations of dithiothreitol and alkylation with iodoacetamide and (b) samples of components S, M and F in 8M-urea and in 8M-urea containing 0.013M-mercaptoethanol

(a) Concentrations of dithiothreitol used: (A) 0; (B) 0.00001M; (C) 0.0001M; (D) 0.001M; (E) 0.01M.

to their mobility towards the anode. Attempts to scale up this separation by using a preparative gel were unsuccessful. To obtain the individual components, 96 analytical gels were run simultaneously. One gel, stained with Coomassie Blue, was used as a template and sections were cut from the other gels corresponding to the protein bands on the stained gel. The sections corresponding to each component were combined and homogenized in 0.9% NaCl in the ratio of 1.5 ml of gel to 30 ml of 0.9% NaCl. The gels were homogenized in 10 ml of saline at a time and stirred overnight at 4°C to allow the protein to diffuse out of the gel. The gel was spun off and re-extracted twice more in the same way. The combined supernatants were pooled and concentrated to ½ ml in an Amicon Diaflo (R) cell. The amount of protein recovered amounted to 4 mg in 0.5 ml of final concentrate. The three proteins obtained this way were re-examined on analytical polyacrylamide gel (Plate 1b). Component F was found to be entirely free from other components. Component S was free from the other major components but retained a small amount of other proteins. Component M invariably gave rise to two major bands, one corresponding to component M and the other to F. To elucidate the relationship between components M and F and the reason for the presence of component F in all preparations of component M, a number of gels containing purified component M were run and the M and F zones were sectioned and eluted again. When component M was eluted and run again, more component F was generated (Plate 1b). Visually the amount of component F generated varied from preparation to preparation. However, when component F was prepared again in this fashion and rerun, it continued to give rise only to component F.

Investigation of the relationship between components M and F

After reduction and alkylation component M was converted almost wholly into F. The result of reduction with increasing amounts of dithiothreitol is shown in Plate 2(a). As the concentration of reducing agent increased, the intensity of the M band decreased, with corresponding increase in intensity of the F band. At 0.001 M- and 0.01 M-dithiothreitol, the M band was barely visible (Plate 2a). After complete reduction and alkylation in the presence of 8 M-urea both components M and F gave the same electrophoretic pattern consisting of two very closely spaced bands appearing to be equal in amount (Plate 2b). The electrophoretic behaviour of components F and M was not altered when run in gels prepared in 8 M-urea in the absence of reducing agent (Plate 2b) and there appeared to be no change in the relative amounts of components F and M present when

component M was rerun in gels containing only 8 M-urea.

To further establish the relationship between components F and M, samples of components F, M and S were subjected to immunological analysis by double diffusion in 1.2% agarose against anti-rat serum. Component S gave a precipitin line, which crossed that of the other two components. Components F and M each gave a single precipitin line which joined at the corner with no spurs.

Chemical analysis of F, M and S components of the macroglobulin fraction

The results of analyses of components F, S and a preparation of M containing F are shown in Table 1. The lipid analysis by using g.l.c. of a chloroform-methanol extract showed that despite the fact that the protein bands in polyacrylamide gel could be stained with Oil Red O, the lipid content of eluted protein was extremely low. A sample of rat high-density lipoprotein prepared by the method of Koga *et al.* (1969) was included in these analyses for comparison. Components F, M and S contain less than 2% of the lipid present in the high-density lipoprotein. Hexose analysis by the H₂SO₄-anthrone method (Scott & Melvin, 1953) showed the following compositions: 5.4, 6.3 and 4.6% hexose for components F, M and S respectively. All three components gave positive staining with periodic acid-Schiff reagent (Zacharius *et al.*, 1969).

Sedimentation studies

(a) *Molecular-weight determination.* The molecular weights of the three components F, M, S and reduced M were determined by the high-speed equilibrium method. The plots of ln (fringe displacement) against the square of the radial distance were linear throughout for components F and S, shown in Figs. 1(a) and 1(b) respectively. A similar plot for component M was curved, indicating inhomogeneity in the preparation. An equilibrium run on a preparation of component M that had been reduced and alkylated

Table 1. *Chemical composition of components F, M, S and rat high-density lipoprotein*

Amounts in 1 mg of protein. HDL, high-density lipoprotein.

Component	Fatty acid (µg)	Inorganic phosphate (µg)	Hexose (µg)
F	11.4	<0.02	54.4
M	15.7	<0.02	62.5
S	14.4	<0.02	45.7
Rat HDL	999.6	30.6

Fig. 1. Ultracentrifuge studies on components F and S from the macroglobulin fraction: sedimentation-equilibrium analysis

Plots of $\ln$ (fringe displacement) $[\ln(Y - Y_0)]$ versus the square of the radial distance ($R^2/2$) for (a) component F, (b) component S.

gave a more linear plot, having a slope almost identical with that obtained at the same speed for component F. Because of the carbohydrate content, which was similar to that of human α_2 -macroglobulin, a value of 0.735 for the partial specific volume, $\bar{v}$, was assumed (Schoenenberger *et al.*, 1958). With this value of $\bar{v}$ an average of the molecular weights for component M (with its F component) was calculated to be 330700. The molecular weights of F, M and S components of the α_1 -macroglobulin fraction were found to be 180600, 300000 and 739000 respectively. When the M component was reduced its molecular weight was 188700 which was similar to that of the F component.

(b) *Sedimentation - coefficient determination.* Samples of components S and M were studied by the boundary-displacement method of Vinograd *et al.* (1963). The sedimentation coefficient, $s_{20,w}$, for component S was 18.1S. Two sedimentation coefficients were obtained for component M owing to the presence of two peaks migrating from the centre of rotation, one corresponding to component M and the other to component F generated by M, and these coefficients were 10.7 and 8.0S respectively.

Fig. 2. Disappearance curves of ^{125}I -labelled albumin (○) and ^{125}I -labelled transferrin (●)

Fig. 3. Appearance curves of ^{14}C -labelled albumin (○) and ^{14}C -labelled transferrin (●)

Fractional synthesis rate studies

(1) *Components of the 4S fraction.* The disappearance curves of ^{125}I -labelled albumin and ^{125}I -labelled transferrin, and the appearance curves of albumin [$6\text{-}^{14}\text{C}$]arginine specific radioactivity and transferrin [$6\text{-}^{14}\text{C}$]arginine specific radioactivity are shown in Figs. 2 and 3 respectively. The peak of the albumin specific radioactivity was observed at 2.2h after the injection of $\text{Na}_2^{14}\text{CO}_3$. In contrast, that of transferrin was at 3.5h. The fractional synthesis rates for albumin and transferrin were calculated as noted in the Materials and Methods section and are given in Tables 2 and 3 respectively.

(2) *Components of the 7S fraction.* Since the 7S fraction has not yet been resolved satisfactorily,

Table 2. Fractional synthesis rate of albumin

The hypothetical specific radioactivity of the plasma protein [6-¹⁴C]arginine (column 3) is the measured [6-¹⁴C]specific radioactivity (column 2) corrected for losses of label into the extravascular pool and by catabolism during the period over which synthesis is measured. The method of correction used was that of Jeejeebhoy *et al.* (1972a). Synthesis (column 6) (as % of intravascular pool/day) = column 3/column 4 × column 5 × 24h × 100, [calculated by the method of Koj & McFarlane (1968)].

Groups of two rats Column no. ... (1)		Specific radioactivity of plasma albumin [6- ¹⁴ C]arginine (d.p.m./mg of C)		Plasma [¹⁴ C]urea specific radioactivity		Fractional synthesis (% of intravascular pool/day) (6)
		Measured (2)	Hypothetical (3)	At time <i>t</i> ₀ (d.p.m./mg of C) (4)	Slope ' <i>k</i> ' (0.693/ <i>t</i> _½ in h) (5)	
A	7960	14300	122000	0.235	66.0	
B	7380	13200	106000	0.235	70.4	
C	8360	15000	110000	0.235	77.2	
D	8230	14800	159000	0.235	52.2	
E	11190	20100	133000	0.235	85.1	
Mean±s.d.					70.2±12.4	

Table 3. Fractional synthesis rate of transferrin

Details were as for Table 2.

Groups of two rats Column no. ... (1)		Specific radioactivity of plasma transferrin [6- ¹⁴ C]arginine (d.p.m./mg of C)		Plasma [¹⁴ C]urea specific radioactivity		Fractional synthesis (% of intravascular pool/day) (6)
		Measured (2)	Hypothetical (3)	At time <i>t</i> ₀ (d.p.m./mg of C) (4)	Slope ' <i>k</i> ' (0.693/ <i>t</i> _½ in h) (5)	
A	12000	18900	122000	0.235	87.2	
B	10000	15800	106000	0.235	83.9	
C	11000	17300	110000	0.235	89.0	
D	10600	16400	159000	0.235	57.9	
E	9390	14600	133000	0.235	61.8	
Mean ± s.d.					76.0 ± 14.9

Fig. 4. Disappearance curves of ¹²⁵I-labelled macroglobulin components M (●) and S (○)

Fig. 5. Appearance curves of ¹⁴C-labelled macroglobulin components M (●) and S (○)

Table 4. *Fractional synthesis rate of glycoprotein S*

Details were as for Table 2.

Groups of two rats Column no. ... (1)	Specific radioactivity of plasma glycoprotein S [$6\text{-}^{14}\text{C}$] arginine (d.p.m./mg of C)		Plasma [^{14}C] urea specific radioactivity		Fractional synthesis (% of intravascular pool/day) (6)
	Measured (2)	Hypothetical (3)	At time t_0 (d.p.m./mg of C) (4)	Slope ' k ' ($0.693/t_{1/2}$ in h) (5)	
A	10400	16100	122000	0.235	73.9
B	11300	17400	106000	0.235	92.5
C	10700	16500	110000	0.235	85.0
D	9970	18400	159000	0.235	66.1
E	6720	12400	133000	0.235	52.6
Mean $\pm$ S.D. 73.8 $\pm$ 15.8					

Table 5. *Fractional synthesis rate of glycoprotein M*

Details were as for Table 2.

Groups of two rats Column no. ... (1)	Specific radioactivity of plasma glycoprotein M [$6\text{-}^{14}\text{C}$] arginine (d.p.m./mg of C)		Plasma [^{14}C] urea specific radioactivity		Fractional synthesis (% of intravascular pool/day) (6)
	Measured (2)	Hypothetical (3)	At time t_0 (d.p.m./mg of C) (4)	Slope ' k ' ($0.693/t_{1/2}$ in h) (5)	
A	7320	12500	122000	0.235	58.0
B	10600	18200	106000	0.235	96.9
C	7780	13300	110000	0.235	68.6
D	10500	17900	160000	0.235	63.4
E	10400	17400	133000	0.235	75.5
Mean $\pm$ S.D. 72.5 $\pm$ 15.1					

no metabolic studies were carried out with proteins of this fraction.

(3) *Components of the macroglobulin fraction.* The disappearance and appearance curves for components M and S are shown in Figs. 4 and 5 respectively. The peak ^{14}C radioactivity occurred after 3 h for both components M and S. The fractional synthesis rates for these two proteins are shown in Tables 4 and 5. Component F was not so examined, since it was considered to be an artifact of preparation and probably not truly native (see the Discussion section).

Discussion

The present paper describes a fractionation scheme for the separation of four plasma proteins, namely albumin, transferrin and α_1 -glycoproteins M and S, from a single sample, for the purpose of measuring their synthesis rates. The method is based on a two-step process, i.e. gel filtration and anion-exchange chromatography or polyacrylamide-gel electrophoresis, and allows the separation of a number of proteins without loss of the remaining ones. Thus although the 7S peak has not at this time been

resolved, further development of the conditions for DEAE-Sephadex chromatography should allow its analysis in the future. Meanwhile studies can continue on the named fractions and these will be unaffected by any subsequent development in the purification of other components.

Identification of separated proteins

The 4S fraction had the simplest composition, containing two major proteins, albumin and transferrin. The latter occurred as two distinct electrophoretic components, a characteristic of rat transferrin previously noted by Gordon & Louis (1963). Both proteins were immunoelectrophoretically pure and immunologically identical.

Although it was possible to identify the components of the 4S peak by analogy with human proteins, the identification of components of the macroglobulin fraction has not been so easy. Horne & Toner (1972) have referred to this fraction as slow α_1 -globulin and have measured the change by this fraction as a whole in nephrotic rats but they have not characterized it further nor measured its synthesis

relative to other proteins. This fraction contained three major proteins, designated components, F, M and S on the basis of their electrophoretic mobility in polyacrylamide gel. All three had large molecular weights and contained significant amounts of carbohydrate. The finding that they were glycoproteins was confirmed by positive staining of the protein bands in polyacrylamide gel with periodic acid-Schiff reagent. Despite positive staining with Oil Red O, chloroform-methanol extracts of these proteins contained virtually no fatty acid, as determined by g.l.c., and no phospholipid, on the basis of phosphate measurements. A sample of rat serum high-density lipoprotein prepared by the method of Koga *et al.* (1969), containing significant amounts of both these components, served as a positive control for this test. The electrophoretic mobility of rat high-density lipoprotein was distinct from that of components F, M and S and also it was dispersed compared with the well-defined bands of our glycoproteins in the macroglobulin peak.

Two of these components, i.e. F and M, appeared to be related. On immunodiffusion both components F and M gave a single precipitin line which fused at the junction, indicating immunological identity. The relationship between components F and M was further revealed on repeated electrophoresis in polyacrylamide gel. When component M was rerun, it consistently gave rise to quantities of component F. Component F, on the other hand, invariably ran true on rerunning.

The conversion of component M into F was neither enhanced nor reversed by 8M-urea, hence the interaction is not a simple one of non-covalent forces. Reduction with dithiothreitol showed that at a 0.001M concentration of the reducing agent, component M completely disappeared, with concomitant increase in the intensity of the F band in polyacrylamide gel, indicating that component M was made up of subunits of component F joined through disulphide linkages. However, it is not clear why component M would split under non-reducing conditions to give quantities of component F unless limited disulphide interchange was occurring. When components F and M were reduced in 0.013M-mercaptoethanol in 8M-urea both gave rise in polyacrylamide gels to two very closely spaced components which appeared to be identical whether derived from components F or M, suggesting that components F and M have the same basic structure, being made up of two different polypeptide chains linked.

Since electrophoresis on cellulose acetate showed that both components F and M had α_1 mobility, the separation seen in polyacrylamide gel was based almost entirely on differences in size. Such a difference was demonstrated too by sedimentation-equilibrium studies of these two components. The molecular weight calculated for component F was 180 600, by

using a $\bar{v}$ value of 0.735, which was that calculated for human α_2 -macroglobulin by Schoenenberger *et al.* (1958). This $\bar{v}$ value was chosen because component F had a similar hexose content to that of the human α_2 -macroglobulin, i.e. 5.4% as compared with 3.6%. The plot of $\ln$ (fringe displacement) versus r^2 for component F was linear throughout, whereas that for component M was curved. The molecular weight of component M calculated from the top part of the slope was 330 700. Because of the presence of a smaller component, that part of the slope used for calculating the molecular weight of component M might have been somewhat smaller. Hence the true molecular weight of component M might be a little higher than 330 700 and it is likely that component M has twice the molecular weight of component F, i.e. 360 000. After reduction, the molecular weight of component M was calculated to be 188 700. This further suggests that component M might have a molecular weight double that of component F. Sedimentation-velocity studies carried out on a sample of component M also revealed the presence of two components, one representing the larger molecule M, and the other F. $s_{20,w}$ values so obtained were 10.7 and 8.0S respectively. No literature value for these proteins was available for comparison.

The S component was demonstrated to be completely distinct from components M and F. It gave a single precipitin arc which crossed that of components M and F, showing no relationship to them. When a sample of S was re-examined in polyacrylamide gel, it showed no evidence of generating other components. It too had an α_1 mobility on cellulose acetate. Sedimentation showed it to have a molecular weight of 739 000 and an $s_{20,w}$ value of 18.1S, which was consistent with the 18.3 reported by Boffa *et al.* (1964) for rat α_1 -macroglobulin. It is not known whether this rat α_1 -macroglobulin is analogous to human α_2 -macroglobulin, since in the rat an α_2 -macroglobulin is also present but only in pregnant (Beaton *et al.*, 1961) or tumour-bearing rats (Menninger *et al.*, 1970). A value of 0.735 for $\bar{v}$ was again used in the calculation of the molecular weight of component S, since the latter contained 4.6% hexose.

Metabolic studies

The slope of the endogenous plasma [^{14}C]urea curve was essentially a single exponential over the 5h period, indicating the rapidity of distribution and very low degree of reutilization of the label. This was in agreement with findings by Jeejeebhoy *et al.* (1970). Hence the ' k ' value ($0.693/t_{1/2}$) used for the calculation of synthesis rate was obtained from this curve of the [^{14}C]urea, generated endogenously after the injection of $\text{Na}_2^{14}\text{CO}_3$.

The validity of the fractionation procedure was tested by comparing the fractional synthesis rates of albumin and transferrin prepared by this new method with those obtained previously (Jeejeebhoy *et al.*, 1972b) in this laboratory for albumin and transferrin separated earlier (Jeejeebhoy *et al.*, 1972a) by other procedures. The radioactivity of albumin and transferrin, prepared by the new method and labelled with ^{125}I , declined in a manner indistinguishable from that of the earlier preparations. ^{125}I excretion in urine was not measured. This shows that even though the two proteins had not been previously screened biologically, they contained no grossly denatured material, in which case a steep multi-exponential curve would be apparent in the early stages of the experiment. ^{125}I -labelled albumin and ^{125}I -labelled transferrin were found to have a half-life of 4.8 and 4.7 h respectively, and their fractional synthesis rates were 70.2 ± 12.4 and $75.9 \pm 14.0\%$ respectively (mean $\pm$ s.d.) of the intravascular pool per day. Both sets of values were not significantly different from previous findings (Jeejeebhoy *et al.*, 1970, 1972a). This demonstrates that enlargement of the scheme to include separation of proteins in addition to albumin and transferrin has not detectably altered or denatured these latter two.

Since component M invariably gave rise to component F and not vice versa, and almost complete conversion of component M into F was demonstrated by 0.001 M-dithiothreitol in the absence of urea, component M was presumed to be the naturally occurring species. Therefore metabolic studies were carried out with only components M and S of the macroglobulin fraction. The disappearance curves of these two α_1 -glycoproteins were obtained by using material that had been biologically screened for 18 h. A curve for unscreened component M was included for comparison, and it indicated the presence of some denatured protein or free ^{125}I by having a steeper initial slope by comparison with curves obtained using the screened product. The half-lives of components M and S were 13.8 and 17.2 h respectively and their plots suggested that their extravascular spaces were probably divided into multi-compartments with variable permeability. The fractional synthesis rates of components M and S were calculated to be 72.5 ± 15.1 and $73.8 \pm 15.8\%$ of the intravascular pool per day respectively. No published comparable rates were found for comparison.

All four proteins studied, i.e. albumin, transferrin and α_1 -glycoproteins M and S, appear to have similar fractional synthesis rates, and these will serve as control values for comparison with rates obtained under the influence of various factors. It has been shown in this laboratory that ethanol (Jeejeebhoy *et al.*, 1972a), cortisol (Jeejeebhoy *et al.*, 1972b), growth hormone (Jeejeebhoy *et al.*, 1970) and protein deprivation (Jeejeebhoy *et al.*, 1971, 1973) affect the

fractional synthesis rates of albumin, transferrin and fibrinogen independently and their effect on the synthesis rate of the two α_1 -glycoproteins is being awaited with interest.

It is possible to extend this fractionation scheme to encompass, in addition to the above-mentioned proteins plus fibrinogen, those proteins occurring in the 7S fraction including the lipoproteins, high-density lipoprotein, low-density lipoprotein and very-low-density lipoprotein which may be fractionated by flotation ultracentrifugation.

We are grateful for generous financial support from our own Department of Medicine, from the Medical Research Council of Canada (Grants MA 785, MA 3204 and MT 1361) and the Bickell Foundation. We thank Dr. R. Baker for lipid analyses and Mrs. J. Chrupala for secretarial aid.

References

- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Beaton, G. H., Selby, A. E., Veen, M. J. & Wright, A. M. (1961) *J. Biol. Chem.* **236**, 2005–2008
- Boffa, G. A., Jacquot-Armand, Y. & Fine, J. M. (1964) *Biochim. Biophys. Acta* **86**, 511–518
- Davies, B. K. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
- Dixon, M. (1953) *Biochem. J.* **54**, 457–458
- Feldman, G. L. & Rouser, G. (1965) *J. Amer. Oil Chem. Soc.* **42**, 290–293
- Gordon, A. H. & Louis, L. N. (1963) *Biochem. J.* **88**, 409–414
- Horne, C. H. W. & Toner, P. G. (1972) *Clin. Sci.* **42**, 743–749
- Jeejeebhoy, K. N. (1964) *Brit. Med. J.* **1**, 30–35
- Jeejeebhoy, K. N., Boucher, B. J. & Hartog, M. (1965) *Metab. Clin. Exp.* **14**, 67–74
- Jeejeebhoy, K. N., Samuel, A. M., Singh, B., Nadkarni, G. D., Desai, H. G., Bokhar, A. V. & Mani, L. S. (1969) *Gastroenterology* **56**, 252–267
- Jeejeebhoy, K. N., Bruce-Robertson, A., Sotke, U. & Foley, M. (1970) *Biochem. J.* **119**, 243–249
- Jeejeebhoy, K. N., Bruce-Robertson, A., Ho, J. & Sotke, U. (1971) *Clin. Res.* **19**, 792 (Abstr.)
- Jeejeebhoy, K. N., Phillips, M. J., Bruce-Robertson, A., Ho, J. & Sotke, U. (1972a) *Biochem. J.* **126**, 1111–1124
- Jeejeebhoy, K. N., Bruce-Robertson, A., Ho, J. & Sotke, U. (1972b) *Biochem. J.* **130**, 533–538
- Jeejeebhoy, K. N., Bruce-Robertson, A., Ho, J. & Sotke, U. (1973) in *Protein Turnover; Ciba Symp.* **9**, pp. 217–247, Elsevier, Amsterdam
- Jones, E. A., Craigie, A., Tavill, A. S., Franglen, G. & Rosenoer, V. M. (1968) *Gut* **9**, 466–469
- Kirsch, R., Frith, L., Black, E. & Hoffenberg, R. (1968) *Nature (London)* **217**, 578–579
- Koga, S., Horwitz, D. & Scanu, A. (1969) *J. Lipid Res.* **10**, 577–588
- Koj, A. & McFarlane, A. S. (1968) *Biochem. J.* **108**, 137–146
- Laster, L., Waldmann, T. A., Fenster, L. F. & Singleton, J. W. (1966) *J. Clin. Invest.* **45**, 637–644
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

- McFarlane, A. S. (1963) *Biochem. J.* **89**, 277-289
- McFarlane, A. S. (1964) in *Mammalian Protein Metabolism* (Munro, H. N. & Allison, J. B., eds.), vol. 1, pp. 297-341, Academic Press, New York
- McFarlane, A. S., Irons, L., Koj, A. & Regoeczi, E. (1965) *Biochem. J.* **95**, 536-540
- Menninger, F. F., Jr., Esber, H. J. & Bogden, A. E. (1970) *Clin. Chim. Acta* **27**, 385-394
- Norwich, K. H. (1972) *Biochem. J.* **126**, 1124-1126
- Parkes, J. G. & Thompson, W. (1970) *Biochim. Biophys. Acta* **196**, 162-169
- Picou, D. & Waterlow, J. C. (1962) *Clin. Sci.* **22**, 459-468
- Reeve, E. B., Pearson, J. R. & Martz, D. C. (1963) *Science* **139**, 914-916
- Rothschild, M. A., Schreiber, S. S., Oratz, M. & McGee, H. L. (1958) *J. Clin. Invest.* **37**, 1229-1235
- Rothschild, M. A., Oratz, M., Mongelli, J. & Schreiber, S. S. (1968) *J. Clin. Invest.* **47**, 2591-2599
- Schoenenberger, M., Schmidtberger, R. & Schultze, H. E. (1958) *Z. Naturforsch., B* **13**, 761-772
- Scott, T. A. & Melvin, E. H. (1953) *Anal. Chem.* **25**, 1656-1661
- Sellers, A. L., Bonoris, G. & Katz, J. (1969) in *Physiology and Pathophysiology of Plasma Protein Metabolism* (Birke, G., Norbert, R. & Plantin, L.-O., eds.), pp. 95-99, Pergamon Press, Braunschweig
- Smithies, O. (1959) *Biochem. J.* **71**, 585-587
- Stark, G. R., Stein, W. H. & Moore, S. (1960) *J. Biol. Chem.* **235**, 3177-3181
- Tavill, A. S., Craigie, A. & Rosenoer, V. M. (1968) *Clin. Sci.* **34**, 1-28
- Tietz, N. W. & Fiereck, E. A. (1966) *Clin. Chim. Acta* **13**, 352-358
- Vinograd, J. R., Bruner, R., Kent, K. & Weigle, H. (1963) *Proc. Nat. Acad. Sci. U.S.* **49**, 902-910
- Wochner, R. D., Weissman, S. M., Waldmann, T. A., Houston, D. & Berlin, N. I. (1968) *J. Clin. Invest.* **47**, 971-982
- Yphantis, D. A. (1964) *Biochemistry* **3**, 297-317
- Zacharius, R. M., Zell, T. E., Morrison, J. H. & Woodlock, J. J. (1969) *Anal. Biochem.* **30**, 148-152