SUMMARY

1. The analysis, by silica-gel chromatography, of phospholipids from a human-plasma fraction is described. The fraction was derived from whole blood which had been incubated with sodium [*Me*-^{14}C]acetate for 6·5 hr. The β-lipoproteins had been removed by precipitation with dextran sulphate.

2. All the phospholipids isolated were labelled.

3. The fatty acids of the isolated phospholipids were analysed by vapour-phase chromatography.

4. A comparison of yield, radioactivity and fatty acid distribution with those for the phospholipids of the cells is given.

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Metabolic Heterogeneity of Human γ-Globulin

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Human γ-globulin is known to be heterogeneous according to several immunological and physicochemical criteria. Nevertheless, the labelled protein appears to be eliminated from plasma at a constant rate after the initial phase of equilibration between intra- and extra-vascular pools and this suggests that the molecules have a uniform metabolic fate (Myant, 1952; Dixon, Talmage, Maurer & Deichmiller, 1952; Volwiler *et al.* 1955). Turnover studies based on plasma-elimination data alone cannot, however, detect minor components which are rapidly broken down during the phase of equilibration; nor do such studies constitute a sensitive means of detecting a steady fall in the rate of breakdown such as might occur with a mixed population of molecules.

For assessing the metabolic homogeneity of plasma-protein preparations, radioactive iodine offers advantages over other isotopes. ^{131}I liberated during catabolism is not re-incorporated into protein and, provided that adequate amounts of inactive iodide are administered, the radioactive isotope is quantitatively excreted in the urine (Cohen, Holloway, Matthews & McFarlane, 1956). Daily turnover rates can be calculated by expressing excreted radioactivity as a fraction of the labelled protein remaining in the plasma (McFarlane, 1957). The turnover rate of albumin measured by this method remains constant during 4 weeks of observation in healthy human subjects (S. Cohen, T. Freeman & A. S. McFarlane, unpublished work); turnover rates of γ-globulin, on the other hand, decline progressively both in man and in laboratory animals (McFarlane, 1957).

We have applied this test of metabolic homogeneity to several different preparations of human γ-globulin. The results obtained with fractions prepared by electrophoresis and ion-exchange chromatography, as well as with γ-macroglobulin separated by ultracentrifuging, are described below.

METHODS

Subjects. This study was carried out on fourteen men and one woman (ages 19–76 years). Ten appeared to be in perfect health and were members of the hospital or laboratory staff. The remaining five were patients who had been convalescent for 2–4 weeks before the present investigation was started (Table 1).

Preparation of γ-globulin fractions. γ-Globulin was prepared from the blood of seven different healthy donors by the following methods.

(*a*) Zone electrophoresis. A globulin precipitate was obtained from human serum by adding 26% (w/v) Na_2SO_4 to give a final concentration of 16% of Na_2SO_4; the precipitate was dissolved in 0·9% NaCl solution, dialysed

Table 1. *Experimental data in* 15 *subjects injected with* I-*labelled* γ-*globulin*

Protein fractions were prepared by Na_2SO_4 precipitation and electrophoresis (S.E.), by electrophoresis alone (E), by chromatography (A, B and C) and by ultracentrifuging and electrophoresis (U) (see Methods section). All subjects were males except A16. Normal subjects were members of the hospital or laboratory staff.

Expt. no.	Age (years)	Diagnosis	Body wt. (kg.)	Blood donor	Labelled protein: Fractionation	Labelled protein: Isotope of I	Labelled protein: Radio-activity (μc)	Excretion during initial 24 hr. (% of dose)	Duration of expt. (days)
205A	21	Normal	77	1	S.E.	131	246	6·3	42
205B	19	Normal	68	1	S.E.	131	259	6·4	42
205C	20	Normal	74	1	S.E.	131	257	5·6	30
A10	20	Enuresis	—	2	E	131	82	7·3	18
A11	19	Pneumonia	63	2	E	131	77	7·9	18
A16	39	Cystitis	72	3	A	131	69	5·6	21
A17	43	Normal	109	3	A	131	84	4·1	21
A82	30	Normal	60	4	A	125	25	2·9	21
A23	76	Bronchitis	72	3	B	131	86	7·1	21
A24	42	Coronary thrombosis	71	3	B	131	66	6·8	23
A68	32	Normal	90	5	A	131	83	—	41
				5	B	125	11	—	—
A80	48	Normal	107	4	A	125	38	4·3	24
				4	B	131	22	6·5	—
A85	38	Normal	77	5	A	125	51	2·5	19
				5	C	131	18	3·3	—
A53	28	Normal	77	6	U	131	8	29·0	6
A90	38	Normal	80	7	U	131	24	42·0	8
				4	A	125	24	4·6	10

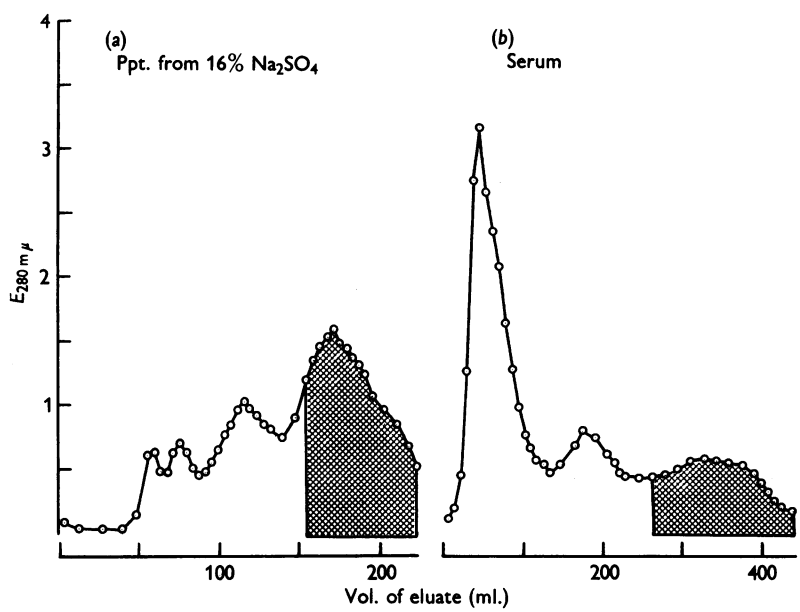

Fig. 1. Zone electrophoresis of a crude globulin precipitate (*a*) and of human serum (*b*). γ-Globulin fractions which were pooled and concentrated by pressure dialysis are shown as shaded areas. See text for experimental details.

against a borate buffer (11·5 mM-$Na_2B_4O_7$–15·5 mM-NaH_2PO_4, pH 8·6) and fractionated by zone electrophoresis on a column of treated cellulose (Porath, 1956). The column measured 40 cm. × 2·5 cm. and was surrounded by a jacket through which tap water was circulated. The eluate was collected in volumes of 4 ml. and protein peaks were located by measurement of the ultraviolet absorption at 280 mμ of volumes (4 ml.) of eluate. The pooled γ-globulin (Fig. 1*a*) was concentrated by pressure dialysis at 4°; this fraction contained 13·6% of the serum protein applied to the column. γ-Globulin was also prepared by zone electrophoresis of 6 ml. of human serum without preliminary salt fractionation and with a cellulose column 83 cm. × 3 cm. This γ-globulin fraction, pooled as shown in Fig. 1*b*, contained 14·9% of the serum protein applied to the column.

(*b*) Chromatography. Diethylaminoethylcellulose was prepared according to the method of Peterson & Sober (1956). A volume of normal human serum (42 ml.) containing 3 g. of protein was decreased to 27 ml. by pressure dialysis against 5 mM-sodium phosphate buffer, pH 7·1; 9% of the protein precipitated during dialysis and the remainder was applied to a column (38 cm. × 2·5 cm.) containing 35 g. of cellulose. The buffer used was 5 mM-phosphate, pH 7·1, with a gradient to 0·3 M-NaH_2PO_4; the volume of the mixing chamber was 1100 ml. The ultraviolet absorption of volumes (4 ml.) of the eluate was measured at 280 mμ and γ-globulin fractions were pooled as shown in Fig. 2*a*. Fraction A contained 7·2% of the protein applied to the column and fraction B contained 1·7%; 75% of the total protein applied to the column was eluted.

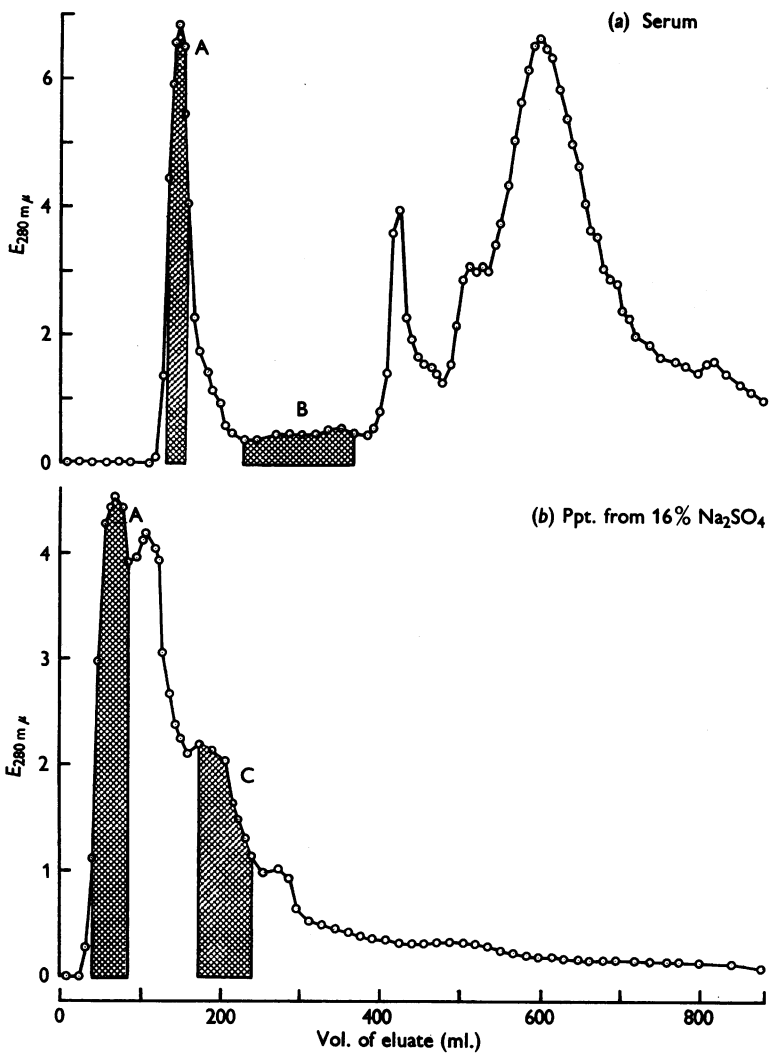

Fig. 2. Chromatography of human serum (*a*) and of globulin precipitated by 16% Na_2SO_4 (*b*). Fractions A, B and C shown as shaded areas were concentrated by pressure dialysis. See text for experimental details.

A globulin precipitate was prepared from normal human serum by precipitating three times in 16% Na_2SO_4. This protein was dissolved in 0·01 M-phosphate buffer, pH 8·3, and dialysed against the same buffer; 2% of the protein precipitated during dialysis and the remainder was applied to a 20 g. diethylaminoethylcellulose column (26 cm. × 2·5 cm.). The buffer was 0·01 M-phosphate, pH 8·3, with a gradient to 0·3 M-NaH_2PO_4; the volume of the mixing chamber was 1100 ml. γ-Globulin fractions were pooled as shown in Fig. 2*b*. Fraction A contained 3·5% of the original serum protein and on rechromatography was identical with fraction A of the previous preparation (Fig. 2*a*); fraction C contained 1·7% of the original serum protein.

(*c*) Ultracentrifuging. Protein solutions, highly enriched in macroglobulin, were prepared from samples (250 ml.) of normal human serum by preparative ultracentrifuging in a Spinco model L ultracentrifuge. The heavy material was spun down eight times at an average centrifugal force of 54 450 *g* for 15 hr. Beginning with the third centrifuging the heavy material was resuspended in 1% of human albumin (Behringwerke, Marburg-Lahn) in an attempt to prevent denaturation (Müller-Eberhard, Kunkel & Franklin, 1956). The final preparation examined in the analytical ultracentrifuge showed one major component having a sedimentation coefficient ($S_{20,w}$) of 17·2 and two minor components having $S_{20,w}$ values of 4·5 (the added albumin) and 24·5; the latter comprised approx. 10% of the total protein. About 10 mg. of the protein prepared by ultracentrifuging was labelled with ^{131}I, mixed with 3 ml. of human serum and separated by zone electrophoresis on a column of treated cellulose (85 cm. × 3 cm.). Labelled albumin, α-macroglobulin and γ-macroglobulin were localized by measurement of radioactivity. The γ-macroglobulin, which contained 14% of the ^{131}I, was pooled as shown in Fig. 3, added to 100 mg. of human albumin (Lister Institute, Elstree, Herts), concentrated by pressure dialysis and passed through a Seitz filter before injection.

Iodination. Protein solutions were labelled with carrier-free ^{131}I or ^{125}I supplied by The Radiochemical Centre, Amersham, Bucks, free from reducing agent and condensed into 0·02 N-NaOH. Iodine monochloride was used as the inactive carrier solution according to the method of McFarlane (1958). Free iodide was removed from labelled protein solutions by passage through anion-exchange columns of De-Acidite (The Permutit Co. Ltd., London, W. 4). After the labelling, 40–60% of the initial radioactivity passed through the column and less than 1·5% of this was present in the supernatant after precipitation with 10% (w/v) trichloroacetic acid. The mean ratio, iodine bound: protein (mol.wt. 150 000) was 1–1·5 g.atom/mole. The labelled proteins were collected in 5% albumin (Lister Institute, Elstree, Herts) and passed through a Seitz filter before injection.

Measurement of radioactivity. ^{131}I and ^{125}I were measured by scintillation counting with a well-type NaI crystal and a pulse-height analyser having a 9 v channel width. Measurements of ^{131}I were made with the channel in the region of the 0·36 Mev γ-ray peak and ^{125}I was measured in the region of the 0·035 Mev peak. In the samples which contained both isotopes ^{125}I measurements were corrected for ^{131}I counts (equivalent to 18·5–20% of the count rate observed in the ^{131}I channel). ^{131}I measurements, on the other hand, did not require correction for ^{125}I, as all

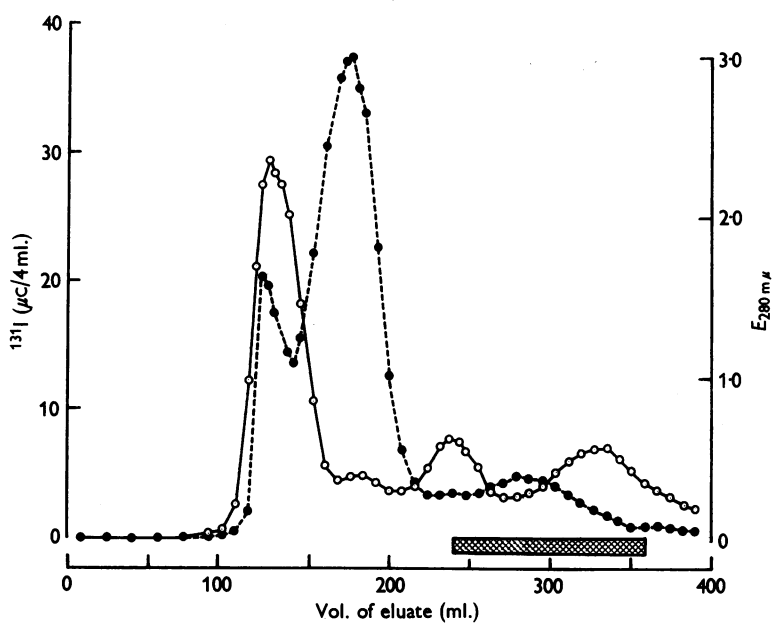

Fig. 3. Zone electrophoresis of a ^{131}I-labelled macroglobulin preparation which contained some added human albumin. About 10 mg. of labelled macroglobulin was mixed with 3 ml. of human serum. The labelled γ-macroglobulin which was concentrated by pressure dialysis is indicated by the shaded area. ●, Radioactivity; ○, extinction.

batches of the latter isotope, when used, gave a negligible counting rate in this channel (less than 0·2 % of the rate observed in the ^{125}I channel). The standard deviation of counts recorded in this study did not exceed ±3 %.

Hexosamine estimations. These were performed by the method of Rondle & Morgan (1955) after hydrolysis of γ-globulin in 4 N-HCl for 4 hr.

Paper electrophoresis. Serum samples were analysed by paper electrophoresis according to the method of Jencks, Jetton & Durrum (1955) and the proportion of γ-globulin was estimated by the elution of strips stained with bromophenol blue. The distribution of radioactivity along strips was determined by scanning before a Geiger counter shielded by lead with a 1·5 mm. window and connected to a ratemeter with pen recorder.

Ultracentrifugal analysis. Solutions containing approx. 10 mg. of protein/ml. were dialysed against 0·15 M-NaCl and examined at room temperature in a Spinco model E ultracentrifuge. Sedimentation coefficients expressed as Svedberg units and corrected to 20° ($S_{20,w}$) were calculated according to the method described by Charlwood (1955).

Experimental procedure. Subjects received NaI (45 mg./day) for 2 days before the injection of labelled γ-globulin and subsequently throughout the period of blood sampling. A volume of 2–5 ml. of labelled γ-globulin (2–8 mg. of protein) was injected into the antecubital vein; residual radioactivity retained in the syringe, needle and ampoule was measured and the dose injected (Table 1) calculated by difference. Blood samples were collected into tubes containing 0·05 ml. of heparin (5000 i.u./ml.). The first was taken 5–10 min. after injection and subsequent samples were collected at intervals of 1–2 days for up to 6 weeks (Table 1). Plasma-protein concentration was determined by a biuret method (Gornall, Bardawill & David, 1949). Radioactivity measurements were made on samples (2 ml.) of plasma and expressed as μc/ml.; during the initial 5 days in which measurements could be made less than 1·5 % of the plasma radioactivity was present in the supernatant after precipitation with trichloroacetic acid at a final concentration of 10 %. Collection of pooled 24 hr. samples of urine was continued for 14–25 days; the radioactivity of samples (2 ml.) was measured. In two experiments (A 10 and A 11) the faeces contained 0·7–0·9 % of the total radioactivity excreted during the first 5 days; faecal analyses were not performed in subsequent experiments.

Analysis of data. The following calculations were made:

Plasma volume =

$$\frac{\text{Injected dose } (\mu c)}{\text{Plasma radioactivity } (\mu c/\text{ml.}) \text{ after 5–10 min.}}$$

Plasma-protein mass = plasma vol. × protein concn.

Plasma-γ-globulin mass = plasma-protein mass × % of γ-globulin (by paper electrophoresis).

Total body radioactivity = injected radioactivity − cumulative urinary excretion of labelled I.

Extravascular radioactivity = total body radioactivity − intravascular radioactivity.

The fractional turnover rate (fraction of the total or intravascular γ-globulin broken down per day) and the mass ratio of extra- to intra-vascular γ-globulin were calculated by the following methods.

(i) From the slope of the plasma specific activity curve (Sterling, 1950).

(ii) By analysis at the 'equilibrium time', i.e. the time at which total activity in the extravascular pool reaches a maximum. As shown by Campbell, Cuthbertson, Matthews & McFarlane (1956) there is no net transfer of radioactivity between intra- and extra-vascular compartments at this time. The rate of change of plasma specific activity at the equilibrium time is therefore equal to the fractional turnover rate and the ratio of intra- to extra-vascular total activities provides a measure of the mass ratio of protein in the two compartments (Fig. 6).

(iii) By resolution of the plasma specific activity curve into two or more exponential functions of time; the rate constants of the system are calculated from the slopes and coefficients of these exponentials (Matthews, 1957). In the present study this analysis was performed by means of an analogue computer in which pool masses and exchange rates are simulated by means of a capacity resistance network (Perkins & Piper, 1959). The plasma specific activity curve was drawn on a graticule in front of the cathode-ray tube and the electrical parameters were adjusted to match the experimental curve. Pool masses were then derived from the relative values of the capacities; the resistance values gave the fractional turnover rate as well as exchange rates between pools.

(iv) Fractional turnover rates were also calculated from the daily urinary excretion of radioactive iodine expressed as a fraction of the mean total intravascular radioactivity during the corresponding period.

RESULTS

γ-Globulin prepared by electrophoresis. γ-Globulin prepared by electrophoresis showed one predominant component in the ultracentrifuge ($S_{20,w}$ 6·75) and about 5 % of heavier material ($S_{20,w}$ 19). The hexosamine content was 1·48 % (Table 2). The ^{131}I-labelled protein was mixed with serum and analysed by paper electrophoresis; radioactivity was confined to the γ-globulin region (Fig. 4). On chromatographic analysis the labelled γ-globulin was widely distributed (Fig. 5); 64 % of the eluted radioactivity emerged with the initial γ-globulin peaks, 25 % was associated with β-globulin and 9 % was eluted beyond the albumin peak in the region of the eluate which contained γ-macroglobulin (see below). The β-globulin area of the chromatogram (Fig. 5, 240–280 ml. of the eluate) was concentrated by pressure dialysis, mixed with unlabelled serum and analysed by paper

Table 2. *Hexosamine content and sedimentation coefficients of γ-globulin preparations*

A, B and C refer to chromatographic fractions (Fig. 2*b*).

γ-Globulin fraction	Hexosamine (%)	Sedimentation coefficient ($S_{20,w}$)
Electrophoresis	1·48	6·75 and 19
A	1·18	6·10–6·40*
B	1·23	6·30–6·62*
C	1·62	6·30

* Range in three separate samples.

electrophoresis; radioactivity was again found to be confined to γ-globulin.

Three subjects received ^{131}I-labelled γ-globulin prepared by sodium sulphate precipitation and electrophoresis; two were injected with material prepared by electrophoresis alone (Table 1). The results were similar in all subjects. Plasma specific activities plotted on a semi-logarithmic scale showed the usual rapid initial decrease associated with mixing of labelled molecules with extravascular protein (Fig. 6*a*). This phase was followed after 7–10 days by an apparently linear rate of decrease; half-lives calculated from this portion of the plasma curve varied from 15 to 21 days (mean 18 days). The apparent mass ratio of extra- to

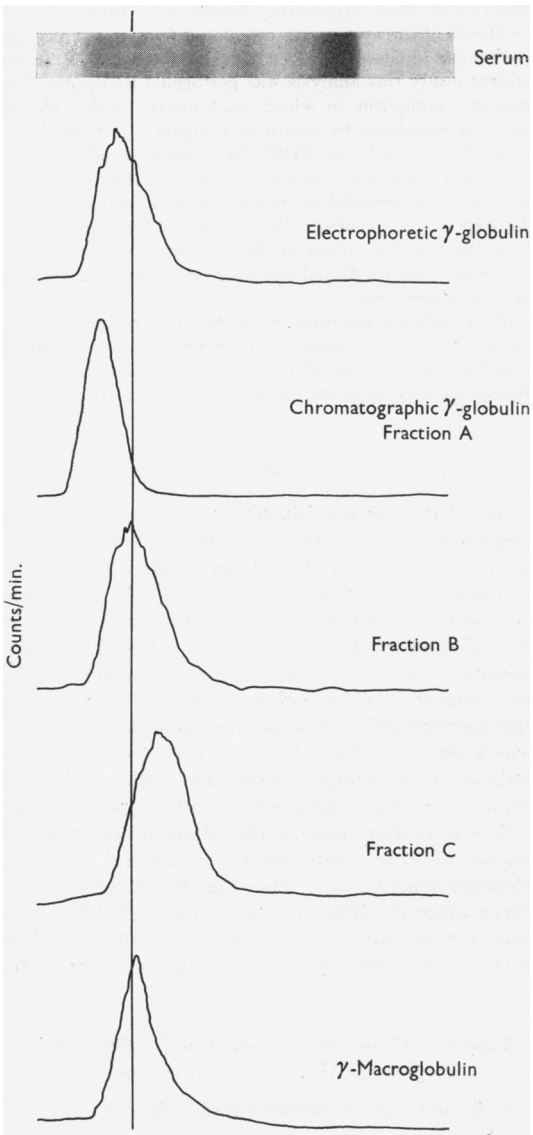

Fig. 4. Electrophoretic analysis and radioactive assay of labelled γ-globulin preparations. A tracer amount of each labelled γ-globulin preparation (see Methods section) was added to normal human serum and analysed by paper electrophoresis. The distribution of radioactivity on the paper strips was determined by scanning before a Geiger counter shielded by lead with a 1·5 mm. window.

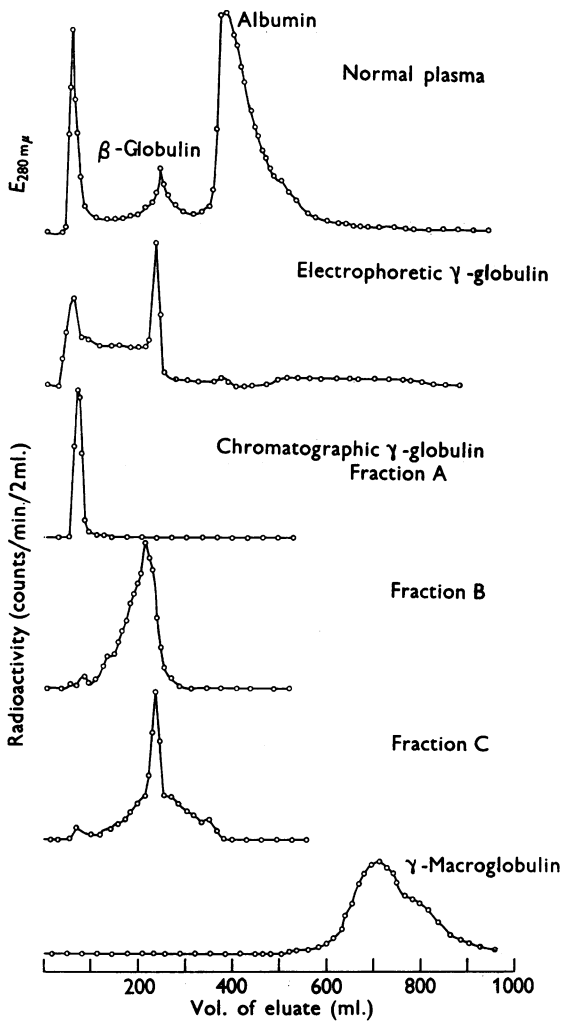

Fig. 5. Chromatographic analysis and radioactive assay of labelled γ-globulin preparations added to normal human serum. Protein distribution in normal serum is shown above. Less than 1 mg. of labelled γ-globulin was mixed with 10 ml. of human serum and analysed on 20 g. cellulose columns. The buffer was 5 mM-phosphate, pH 7·1, except with the electrophoretic fraction, in which 0·01 M-phosphate, pH 8·3, was used; the gradient was to 0·3 M-NaH_2PO_4. Radioactivity was measured by scintillation counting on volumes (2 ml.) of the eluate.

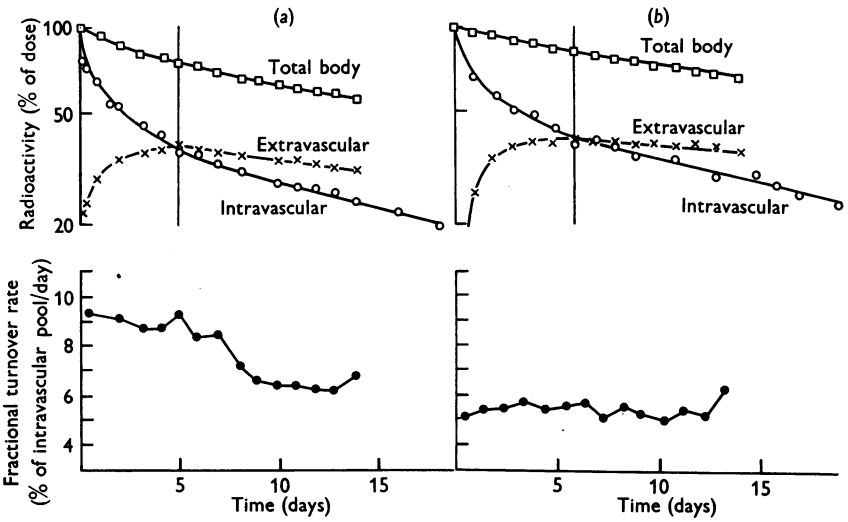

Fig. 6. Turnover data obtained with ^{131}I-labelled γ-globulin prepared by zone electrophoresis (*a*) and by chromatography (*b*). The equilibrium time is shown by a vertical line (see text).

intra-vascular γ-globulin, estimated by extrapolation of the linear portion of the plasma specific activity curve, was 1·2–1·6 (mean 1·4). Extravascular activity increased progressively to reach a maximum value after 3–5 days and thereafter declined more slowly than the intravascular activity.

Between 5·6 and 7·9% of the injected ^{131}I was excreted in the urine within 24 hr. (Table 1). In the three subjects injected with γ-globulin prepared by sodium sulphate precipitation and electrophoresis, the fractional turnover rate calculated from the urinary excretion of ^{131}I declined progressively for 11–13 days (Fig. 7); strict control of urine collection was not possible in these subjects. The experiment was therefore repeated in two hospitalized subjects in whom urine collection was supervised (Expts. A10, A11, in Table 1). γ-Globulin prepared by zone electrophoresis was used; in both experiments the fractional turnover rate fell progressively for 7 days and then remained constant during a further week of observation (Fig. 7).

γ-Globulin prepared by chromatography. The three subfractions of γ-globulin prepared by chromatography and referred to as fractions A, B and C (Fig. 2) contained no rapidly sedimenting material when examined in the ultracentrifuge and each appeared as a single symmetrical peak; $S_{20,w}$ values were between 6·10 and 6·62 (Table 2). The hexosamine content of these fractions increased progressively from 1·18 to 1·62% (Table 2); this finding could be correlated with increasing electrophoretic mobility (Fig. 5) and progressively later elution from the chromatographic column. Fraction A, when labelled and re-examined by chromatography, was confined to the first peak, fraction B emerged partly with β-globulin and fraction C extended to the albumin peak (Fig. 5).

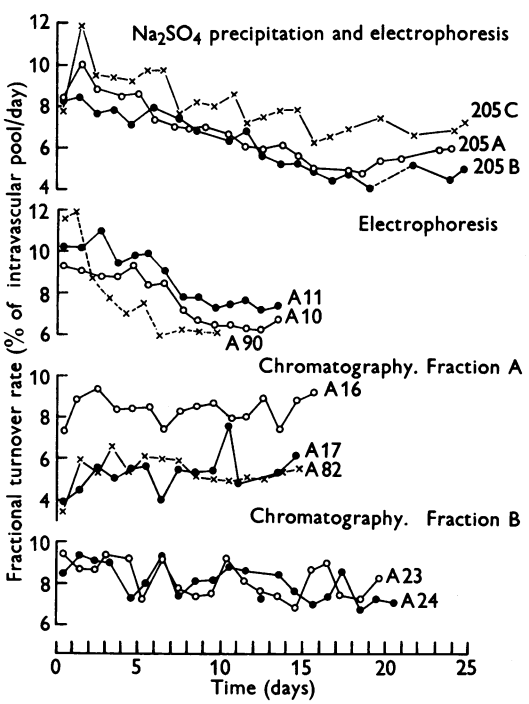

Fig. 7. Fractional turnover rate of γ-globulin preparations calculated from urinary excretion of ^{131}I. The broken line shows the fractional turnover rate obtained with a mixture of chromatographic fraction A and γ-macroglobulin (see text).

Five subjects were injected with either fraction A or B labelled with ^{131}I (Table 1); results obtained in an experiment with fraction A are shown in Fig. 6*b*. The turnover rate in all experiments was relatively constant throughout and did not show the initial progressive fall observed with electrophoretic γ-globulin (Fig. 7). The mean turnover rate varied from 5·2 to 8·4% in three subjects who received fraction A, and from 8·1 to 8·3% in two subjects injected with fraction B. Individual variation made it impossible to decide whether the two fractions were broken down at significantly different rates in healthy subjects. Fractions A and B were therefore separately labelled with either ^{131}I or ^{125}I, injected together and turnover rates compared directly in two subjects (Table 1 and Fig. 8, Expts. A68 and A80). In both experiments the two fractions were eliminated from the plasma at identical rates. In Expt. A80 (Fig. 8) in which urine analyses were performed, the excretion of ^{131}I (fraction B) exceeded that of ^{125}I (fraction A) by 3·8% of the dose during the first 3 days of the experiment; thereafter, the excretion rates of the two isotopes were closely similar. The initial difference was probably due to the presence of denatured material in the ^{131}I-labelled protein as a similar result was observed in another subject given the same preparation. In Expt. A85 (Fig. 8) fractions A and C prepared by salt precipitation and chromatography (Fig. 2*b*) gave identical plasma elimination curves and fractional turnover rates throughout.

Different methods of analysis gave similar values for the fractional turnover rate and distribution ratio of chromatographic γ-globulin (Table 3). The turnover rate was relatively high in the three convalescent patients (A16, A23 and A24). In the remaining eight experiments performed on five normal subjects, the half-life of γ-globulin was 21–26 days (mean 23 days). The mass ratio of extra- to intra-vascular γ-globulin was 0·6–1·1 (mean 1·0) and the exchange rate between intra- and extra-vascular pools was equivalent to 25% (range 19–33%) of the circulating γ-globulin per day. Estimates of the absolute breakdown rate calculated from the slope of the plasma specific activity curve were always about 10% higher than values obtained by three other methods; the latter gave an absolute rate of 1·5–2·5 g./day (mean 2·1 g./day).

γ-Macroglobulin. The γ-macroglobulin prepared by ultracentrifuging and zone electrophoresis had an electrophoretic mobility similar to that of fraction B described above (Fig. 4). On chromatographic analysis, however, the macroglobulin was widely separated from γ-fractions of lower molecular weight and was eluted in the region beyond the albumin peak (Fig. 5).

Two subjects were injected with ^{131}I-labelled γ-macroglobulin (Table 1, A53 and A90). The distribution and turnover of this protein was strikingly different from other γ-globulin fractions studied (Fig. 9). The total radioactivity and intravascular radioactivity declined at similar rates and the slightly higher level of the former during the first 3 days can probably be attributed to the diffusion into the extravascular fluid of free iodine liberated during protein breakdown. This data

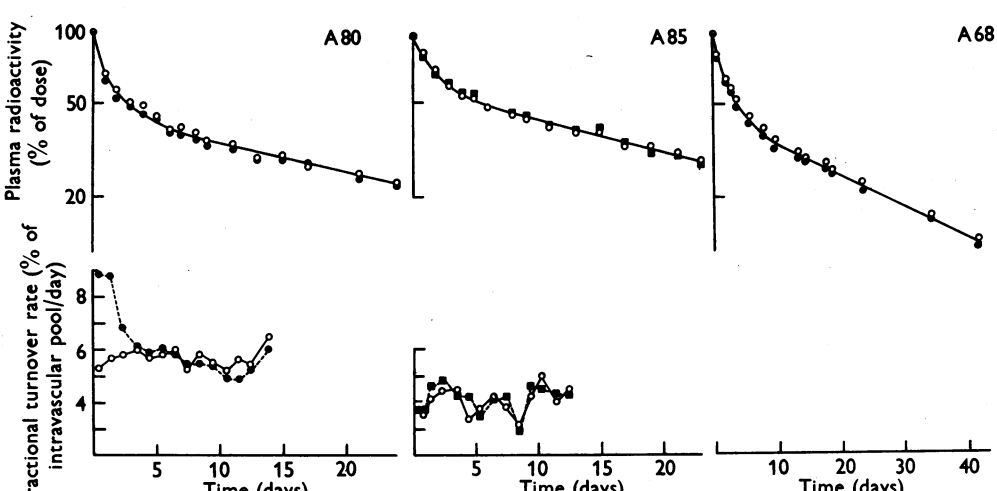

Fig. 8. Results in three subjects each injected with two different γ-globulin fractions prepared by chromatography. A68, ^{131}I-labelled fraction A and ^{125}I-labelled fraction B; A80, ^{125}I-labelled fraction A and ^{131}I-labelled fraction B; A85, ^{125}I-labelled fraction A and ^{131}I-labelled fraction C. ○, Fraction A; ●, fraction B; ■, fraction C.

Table 3. *Turnover data in eight subjects injected with chromatographic subfractions of γ-globulin*

Intravascular γ-globulin mass = total intravascular protein × % of γ-globulin determined by paper electrophoresis. Distribution ratio (extra- to intravascular protein mass) was determined by (1) extrapolation of the linear portion of the plasma activity curve, (2) equilibrium-time method, (3) mathematical analysis (see text). Fractional turnover rate (FTR) was calculated from (1) half-life of plasma activity curve (% of total pool), (2) equilibrium-time method, (3) mathematical analysis, (4) urinary excretion of labelled iodine (mean value). Absolute turnover rate was determined from: (1) total exchangeable γ-globulin × FTR (method 1); (2) intravascular γ-globulin × FTR (mean of methods 2, 3 and 4). Exchange rate = % of intravascular γ-globulin passing into extravascular pool/day.

Expt. no.	Intravascular protein: Total (g.)	Intravascular protein: γ-Globulin (g.)	Fraction	Plasma vol. (l.)	Half-life (days)	Distribution ratio: 1	Distribution ratio: 2	Distribution ratio: 3	Fractional turnover rate/day: 1	Fractional turnover rate/day: 2	Fractional turnover rate/day: 3	Fractional turnover rate/day: 4	Absolute rate: 1	Absolute rate: 2	Exchange rate (%/day)
A16	195	27	A	2·58	14	1·2	0·9	0·7	5·0	9·1	9·8	8·4	3·0	2·5	28
A17	242	33	A	2·96	21	1·2	1·1	0·7	3·3	6·0	6·8	5·2	2·4	2·0	24
A82	186	28	A	2·48	26	1·1	0·9	0·8	2·7	5·3	5·5	5·4	1·6	1·5	33
A23*	156	24	B	2·34	—	—	1·1	1·1	—	7·5	9·5	8·3	—	2·0	30
A24*	197	29	B	2·78	—	—	1·3	1·4	—	8·4	8·6	8·1	—	2·4	28
A68	293	44	A	3·44	21	1·0	—	0·8	3·3	5·3	5·7	—	2·9	2·4	22
			B	3·40	21	1·2	—	0·8	3·3	5·6	5·7	—	3·2	2·5	22
A80	290	41	A	3·81	25	1·2	1·0	0·9	2·8	5·2	5·8	5·7	2·5	2·3	31
			B	3·80	25	1·2	1·0	0·9	2·8	5·5	5·8	6·1	2·5	2·4	31
A85	262	38	A	3·62	24	0·9	0·8	0·6	2·9	4·5	5·4	4·0	2·1	1·8	19
			C	3·65	24	0·8	0·9	0·6	2·9	4·0	5·4	4·1	2·0	1·7	19
Mean (excluding convalescent patients A16, A23, A24)				—	23	1·1	1·0	0·8	3·0	5·2	5·8	5·1	2·4	2·1	25

* In these subjects plasma specific activity did not decline at an exponential rate and the half-life could not therefore be determined.

Table 4. *Theoretical elimination rate of a minor component in electrophoretic γ-globulin*

For experimental details see text.

Expt. no.	Day	Observed excretion/24 hr. (% of dose)	Major component excretion/24 hr. (% of dose)	Minor component excretion/24 hr. (% of dose)	Minor component excretion/24 hr. (% of 9 % of dose)	Minor component retained (%)
A10	1	7·3	5·2	2·1	23	77
	2	5·5	5·0	1·5	17	60
	3	4·7	3·6	1·1	12	48
	4	3·9	2·9	1·0	11	37
	5	3·6	2·7	0·9	10	27
	6	3·2	2·5	0·7	8	19
A11	1	7·9	5·8	2·1	23	77
	2	5·9	4·3	1·6	18	59
	3	5·2	3·4	1·8	20	39
	4	4·0	3·1	0·9	10	29
	5	3·8	2·8	1·0	11	18
	6	3·3	2·7	0·6	7	11

indicates that intact γ-macroglobulin does not penetrate into extravascular tissues, but remains confined to the circulation. The fractional turnover rate calculated from urinary ^{131}I fell progressively from an initial level of 35–38 % of the intravascular pool per day to 15–18 % after 5–7 days.

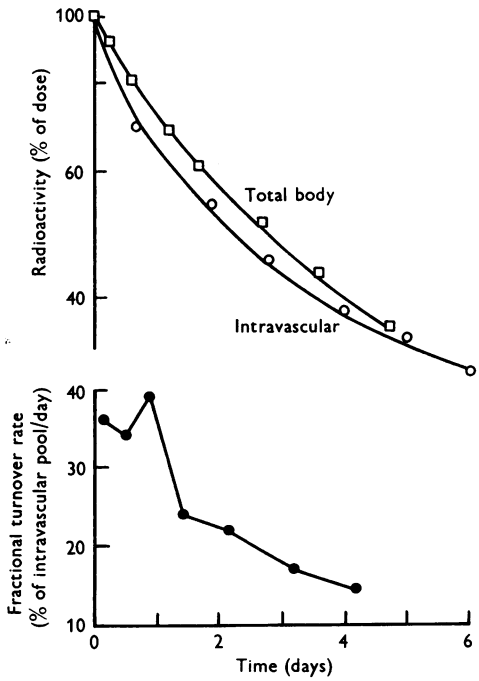

Fig. 9. Total body and intravascular radioactivity and fractional turnover rate in a subject (A 53) injected with ^{131}I-labelled γ-macroglobulin.

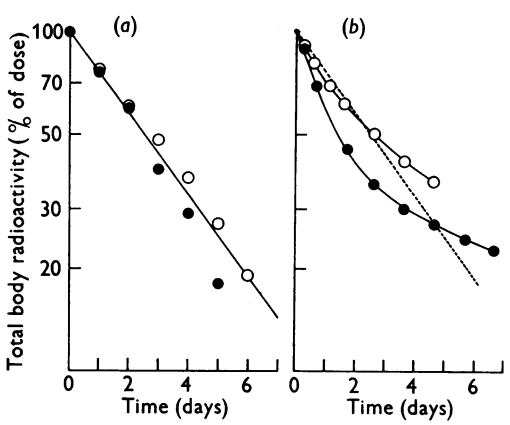

Fig. 10. (*a*) Theoretical elimination rate of a minor component responsible for the fall in the fractional turnover rate observed with electrophoretic γ-globulin. ○, Subject A 10; ●, subject A 11. (*b*) Total body radioactivity in two subjects injected with ^{131}I-labelled γ-macroglobulin. The theoretical elimination rate of the minor component of electrophoretic γ-globulin is shown as a broken line. ○, Subject A 53; ●, subject A 90.

An attempt has been made to calculate whether the initial fall in the fractional turnover rate observed with electrophoretic γ-globulin can be ascribed to the small amounts of γ-macroglobulin known to be present in these preparations. Chromatographic analysis of ^{131}I-labelled γ-globulin, prepared by electrophoresis, has shown that 9 % of the isotope is eluted as a broad peak in the same region as γ-macroglobulin (Fig. 5). From the data of experiments A 10 and A 11 it has been assumed that 91 % of the labelled protein has a constant turnover rate corresponding to the mean value observed during the second week of the experiment (6·6 %/day in A 10 and 7·5 %/day in A 11, Fig. 7). The daily excretion of ^{131}I resulting from the breakdown of this major component was calculated from $E' = P$/fractional turnover rate where E' is the daily excretion of ^{131}I from the major component (% of dose) and P is the mean level of intravascular radioactivity (% of dose) and the fractional turnover rate is that of the major component (% intravascular pool/day). The difference between E' and the observed daily excretion represents the radioactivity derived from breakdown of the minor component. This was expressed as a fraction of 9 % of the administered dose to obtain the theoretical elimination rate of the minor component (Table 4). This rate was similar for both experiments (Fig. 10*a*) and approximated to the observed elimination rate of γ-macroglobulin (Fig. 10*b*).

In one subject (A 90) ^{131}I-labelled γ-macroglobulin was injected together with chromatographic ^{125}I-labelled fraction A. The latter was eliminated at a constant rate equivalent to 6 % of the intravascular pool per day. The fractional turnover rate was calculated for a mixture of the two proteins assuming that 9 % of the total radioactivity was associated with macroglobulin. The result is plotted as a broken line in Fig. 7. The progressive fall in the fractional turnover rate during the first 6 days, characteristic of electrophoretic γ-globulin, was simulated by the mixture, but the rate of decline of the latter was more rapid.

DISCUSSION

A basic assumption in this investigation is that labelled γ-globulin preparations were not adversely affected by preliminary fractionation and labelling and were therefore metabolized in the same way as the corresponding native proteins. Electrophoresis and ion-exchange chromatography can be regarded as comparatively mild forms of protein fractionation. The turnover of rat albumin prepared by chromatography is unaffected by preliminary biological screening which indicates that the

original labelled material is virtually free of denatured protein (Cohen, 1958). Liver-perfusion experiments have shown that albumin, prepared either by chromatography or electrophoresis, contains less than 3% of molecules which have a relatively rapid rate of breakdown (Cohen & Gordon, 1958).

The validity of metabolic data obtained with ^{131}I-labelled proteins has been questioned in recent years (Goldsworthy & Volwiler, 1957). Studies in this Laboratory, however, have shown in animals that the behaviour of ^{131}I-labelled albumin and globulin is almost identical with that of the same proteins biosynthetically labelled with ^{14}C (Cohen *et al.* 1956; Campbell *et al.* 1956). Moreover, the elimination of ^{14}C- and ^{131}I-labelled antibody globulins is identical either in the presence or absence of an immune response (McFarlane, 1957; Humphrey, 1957). More recently, it has been shown that a subject with congenital analbuminaemia eliminated ^{131}I-labelled and unlabelled albumin at the same rate (Freeman, Matthews, McFarlane, Bennhold & Kallee, 1959; Bennhold & Kallee, 1959).

These experiments show that the radioactive iodine, attached under defined conditions, can be used as a reliable indicator of the metabolic behaviour of unlabelled protein molecules. In this connexion it may be noted that half-lives in this study fall within the range observed with biosynthetically labelled γ-globulin (Volwiler *et al.* 1955). The initial fall in the fractional turnover rate of electrophoretic γ-globulin therefore indicates that this preparation contains molecules which, in the native state, would have different rates of breakdown. This labelled γ-globulin had a wide distribution on chromatographic analysis corresponding with that described for unlabelled human γ-globulin (Fahey, McCoy & Goulian, 1958); the chromatographic similarity of ^{131}-labelled and unlabelled human albumin has been demonstrated (Cohen, 1959).

Turnover rates were constant with three chromatographic subfractions of γ-globulin and each must be regarded as metabolically homogeneous. The fact that a constant fraction of the plasma radioactivity was excreted each day, even during the process of equilibration between intra- and extra-vascular pools, indicates that γ-globulin breakdown occurs at a site in close functional proximity to the bloodstream; the same conclusion has been reached about the site of albumin catabolism (Berson & Yalow, 1954; Campbell *et al.* 1956; Lewallen, Berman & Rall, 1959). With a double-labelling technique the three chromatographic fractions were shown to have identical plasma-elimination and turnover rates. These fractions contained 10% of the serum protein applied to chromatographic columns from which the total recovery of protein was about 75%; taken together the fractions cover 90% of the electrophoretic γ-globulin chromatogram (Fig. 5). The results therefore indicate that the bulk of human γ-globulin has a uniform distribution and breakdown rate despite the fact that it can be resolved by chromatography into subfractions which have different electrophoretic mobility, total hexose (Fahey & Horbett, 1959) and hexosamine content, and distinct immunological activity (Humphrey & Porter, 1957; Sober & Peterson, 1958; Fahey & Horbett, 1959). This conclusion concerning the metabolic homogeneity of the bulk of human γ-globulin is not in accordance with the observation that individual antibodies are degraded at significantly different rates in hypo-γ-globulinaemic patients injected with pooled human γ-globulin (Martin, Gordon, Felts & McCullough, 1957).

The behaviour of the three chromatographic fractions does not explain the metabolic heterogeneity of electrophoretic γ-globulin. None of the chromatographic fractions contained macroglobulin, which was found to be eluted late in the chromatogram; other macroglobulin preparations have shown similar chromatographic behaviour on diethylaminoethylcellulose (Lospalluto & Ziff, 1959; Fahey & Horbett, 1959). Interpretation of the γ-macroglobulin turnover data must be qualified by the fact that the protein may have been altered during the repeated process of preparative ultracentrifuging despite the precautions taken to prevent denaturation. Thus the declining fractional turnover rate does not necessarily indicate that native macroglobulin would show similar metabolic heterogeneity. The fact that the elimination was initially somewhat faster than the theoretical rate (Fig. 10*b*) may be due to partial denaturation of the macroglobulin; similarly, the fractional turnover rate during the first 6 days fell more rapidly with a mixture of chromatographic and macroglobulin than with electrophoretic γ-globulin (Fig. 7). In most forms of denaturation altered protein molecules are very rapidly removed from the circulation (Freeman, 1959). The fact that the breakdown rate in two subjects was equivalent to 15–18% of the intravascular pool per day after 5–7 days indicates that the macroglobulin in its native form has a turnover rate at least three times as great as γ-globulin of smaller molecular size. A similar difference in the half-lives of small- and large-molecular-weight antibodies has been reported in rabbits (Taliaferro & Talmage, 1956).

The macroglobulin also differed from smaller-molecular-weight γ-globulin in its failure to penetrate into extravascular tissues. This apparent inability to pass across capillaries can be explained

in terms of the pore theory of permeability (Pappenheimer, 1953), but it is not easily accounted for by a more recent theory in which the transfer of droplets of plasma across the endothelium is postulated (Palade, 1956).

Methods of analysing turnover data rest upon the assumption that rates of synthesis, breakdown and exchange remain constant during the experiment. Analysis of the data obtained with a heterogeneous protein in terms of constant rates gives rise to considerable error; thus, for example, the conventional method of obtaining the pool ratio by extrapolation (Sterling, 1950) gives a value for electrophoretic γ-globulin of 1·22–1·66, whereas the same ratio for homogeneous protein fractions is 0·8–1·2. Calculations based upon the slope of the plasma specific activity curve are also dependent upon the assumption that specific activities are equal throughout the exchangeable protein pool after the period of equilibration. However, if protein breakdown is largely confined to the intravascular pool this assumption is not permissible (McFarlane, 1957); the extent of the resultant error is a function of the rates of turnover and exchange between compartments (Matthews, 1958). With human γ-globulin these rates are such that the error, as predicted by Matthews (1958), is small and the conventional analysis (Sterling, 1950) gives results comparable with those obtained by three other methods.

SUMMARY

1. ^{131}I-Labelled γ-globulin prepared by zone electrophoresis is metabolically heterogeneous; the fractional turnover rate falls progressively in healthy human subjects during the first 7–13 days after injection. Such γ-globulin preparations contain macroglobulin ($S_{20,w}$ 19) and after the labelling about 9% of the ^{131}I is attached to this fraction.

2. Three subfractions of γ-globulin of increasing electrophoretic mobility and hexosamine content, but having similar sedimentation coefficients ($S_{20,w}$ 6·10–6·62), were prepared by anion-exchange chromatography. These fractions, which comprised about 90% of the total γ-globulin, were metabolically homogeneous and all were identical in distribution and turnover in healthy subjects.

3. Four different analytical methods gave similar turnover data for the chromatographic γ-globulin fractions. The half-life was 21–26 days (mean 23 days), the extra- to intra-vascular mass ratio of γ-globulin was 0·6–1·1 (mean 1·0) and the exchange rate between intra- and extra-vascular pools was equivalent to 19–33% (mean 25%) of the circulating γ-globulin/day. The fractional turnover rate (% of intravascular pool/day) in normal subjects was 4·0–6·8% (mean 5·4%) and the absolute turnover rate was 1·5–2·5 g./day (mean 2·1 g./day).

4. γ-Macroglobulin was chromatographically distinct from the bulk of γ-globulin; its turnover rate was more rapid and it did not equilibrate with an extravascular pool.

5. These findings indicate that γ-globulin prepared by electrophoresis consists of two metabolically distinct groups of molecules which differ in regard to turnover rate and distribution between extra- and intra-vascular pools.

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The Lamellibranch Crystalline Style

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The crystalline style in many lamellibranchs exists as an elongated rod in a diverticulum of the stomach, the anterior end jutting into the stomach. It is rotated by cilia of the style sac, so that at the higher pH of the stomach, and by friction against the gastric shield, the one end frays and disperses, entangling food particles. The structure is constantly renewed by the epithelial cells lining the sac (see Nelson, 1918), and in anaerobiosis may disappear and then reappear under favourable circumstances. The style occurs in a few, but not many, gastropods, e.g. in the slipper limpet, *Crepidula fornicata*, L., *Patella* (Mackintosh, 1925), *Melanoides tuberculata* Müller (Fish, 1955).

The function of the style has been considered by many workers, and it is now generally agreed that its purpose is digestive. Coupin (1900) found in the style of *Cardium* amylase activity, as did Mitra (1901) in *Anodonta*. The properties of this enzyme in the oyster were studied by Yonge (1926), who also reported the absence of catalase, protease and lipase from the style. Though it is quite certain that proteolytic activity is absent, a lipolytic enzyme was found by George (1952) in the style of *Crassostrea virginica* Gmelin, the ribbed mussel *Modiolus demissus* Dillwyn and of other species; also by Hozumi (1959) in that of the clam *Venerupis philippinarum* Adams and Reeve.

Lavine (1946), noticing that Visking casing became weakened by style extracts from certain clams including *Mya arenaria* L., suggested the presence of a cellulase, though pure cellulose fibre was not digested. Since then, cellulase has been reported in the styles of *Ostrea edulis* L. and *Mytilus* (Newell, 1953); in the lamellibranch *Caelatura*, the gastropod *Melanoides* (Fish, 1955) and in the wood-boring lamellibranch *Bankia indica nair* (Nair, 1955). In *Crassostrea virginica*, Dean (1958) noted that various coloured algae suspended in a drop of sea water containing solid crystalline style became non-motile and the limiting membrane was indistinguishable after 10–15 min. This effect, which he calls 'a new property,' might appear too rapid to signify a cellulase, and it may well be due to an action on cell-wall lipid. It is not certain that the cellulase is derived from the secretory apparatus of the style sac, for it could be a component of the symbiotic spirochaetes usually found in the style. Walker & Warren (1938) studied a rather similar spirochaete (*Cytophaga*) isolated from soil, which decomposed cellulose partly to carbon dioxide and partly to a non-reducing mucilage.

A reinvestigation of phenol-oxidase activity in the style, first demonstrated by Berkeley (1923), has shown that for *Mytilus* and *Modiolus* at least, the enzyme has the character of an *ortho*-phenolase (Johansson, 1945). Its role is obscure.

Coupin believed that the style consisted mainly of a mucous substance 'saturated' with digestive fluid, and Barrois (1889) considered the style matrix to consist of globulin with a trace of 'mucin' or 'chondrin'. Mackintosh (1925) came to a similar conclusion, but in reality the tests employed were not strictly specific for true globulin. There seems no doubt, however, that a mucoprotein is present. During an investigation of the tropomyosin A (paramyosin) component of the