

IN VIVO BEHAVIOR OF I¹³¹-FIBRINOGEN

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Conditions in which albumins and γ -globulins can be labeled *in vitro* with I¹³¹ while retaining most of their natural biological properties have been defined, and tests are available for diagnosing the presence of quite small amounts of denatured protein in labeled preparations (1). If a mean of approximately one atom iodine per protein molecule is associated with albumin or γ -globulin in physiological conditions, with steps taken to promote even distribution of the iodine and to minimize subsequent self-radiation damage, less than 3% of the product is denatured, the denatured material representing overiodinated molecules arising from random distribution of the label (2, 3).

By measuring the daily excretion of radioactivity, recipient animals have been shown to catabolize such preparations at steady fractional rates, and when plasmas from these animals, so-called "biologically screened" plasmas (4), were transferred after a few days to secondary recipients, the distribution of labeled proteins between intra- and extravascular spaces in both groups was the same within the limits of animal variation. Finally, satisfactory agreement between distribution ratios and catabolic rates of I¹³¹- and C¹⁴-biosynthetically labeled molecules in the same animal has been demonstrated (2, 5, 6).

Results of similar studies on I¹³¹-fibrinogen are reported here. Madden and Gould (7), after giving S³⁵-amino acids, measured fibrinogen half-lives of 4.2 days in nine dogs and of 5.6 days in three normal humans, but these may be too long, owing to reutilization of the isotope. A half-life of 2.7 days was obtained for this protein by Cohen, Holloway, Matthews, and McFarlane (6) when I¹³¹-labeled whole rabbit plasma was injected into rabbits. Accuracy is likely to be higher in this type of measurement, however, when only the protein under investigation is labeled. Christensen (8), using I¹³¹-human fibrinogen, obtained half-lives of 4.0 to 4.7 days in eight normal humans, and his protein was partitioned at equi-

librium almost equally between the plasma and the extravascular space. This was also broadly the experience of Gitlin and Borges (9) in their nonisotopic study of two afibrinogenemic children, but Hammond and Verel (10), who obtained half-lives of 4.1 to 6.0 days for I¹³¹-fibrinogen in six subjects, found up to 80% of the protein in the extravascular space. In 20 dogs Lewis, Ferguson, and Schoenfeld (11) measured half-lives of 2 to 4 days and found only 34% in the same space. In the studies reported below rabbits were preferred, since their total body radioactivities could be readily measured.

METHODS AND RESULTS

Fibrinogen preparation. Rabbit blood was collected in one-tenth its volume of 3.8% trisodium citrate and centrifuged for 5 minutes to remove red cells. The supernatant plasma was then centrifuged at 4,000 rpm (2,000 g) for 20 to 30 minutes to remove platelets and diluted with two volumes of 0.9% sodium chloride. Fibrinogen was precipitated by dropwise addition of one volume of ammonium sulfate solution saturated at 37° C. After standing one hour, the precipitate was removed and redissolved in one volume of buffer equal to the original plasma volume. This buffer was prepared by mixing 10 ml of 3.8% trisodium citrate and 1 ml of a sodium phosphate buffer, pH 7.6 and ionic strength 0.5, with 90 ml of 0.9% sodium chloride. Reprecipitation of fibrinogen was carried out at 23% saturation ammonium sulfate, and the process was repeated finally at 21% saturation. The product, constituting approximately half the fibrinogen in the original plasma, was dissolved in a citrate-saline buffer at pH 6.0 (5.75 g trisodium citrate·2H₂O plus 3.75 ml 1 N hydrochloric acid plus 27.25 g sodium chloride made up to 1 L) to a concentration of 20 mg per ml and the solution was clarified by high speed centrifugation.

Clotting procedure. The fibrinogen solution was diluted to 1 mg per ml with the citrate-saline buffer, and one drop of a solution of Parke-Davis thrombin (1 mg of thrombin containing 25 U dissolved in 5 ml water) was added to 2 ml. Incubation followed for 2 hours at 37° C and the clot, after contracting onto a glass rod, was washed successively with 0.9% sodium chloride for 24 hours, then with water and ether-ethanol (1:3 by volume) and dried at 80° C before weighing.

Labeling with I^{131} . Fibrinogen, 15 mg, in 0.75 ml of citrate-saline was mixed with 0.2 ml of an alkaline glycine buffer made by mixing 2 ml 1 N sodium hydroxide and 8 ml 1 M glycine in 0.25 M sodium chloride. A stock carrier iodine monochloride solution was prepared by dissolving 150 mg of sodium iodide and 99.0 mg of sodium iodate in 2.5 ml of water and adding 2.40 ml of concentrated hydrochloric acid (specific gravity 1.18) with shaking, the last few crystals of precipitated iodine dissolving on standing overnight. Carbon tetrachloride, 1 to 2 ml, was added as an indicator of free iodine, and 0.1 M sodium iodide was added dropwise until a permanent faint pink color appeared in the organic solvent. The aqueous phase was made up to 10 ml with water and, as required, 1 ml of this stock solution was partially neutralized and diluted by the gradual addition of 2.06 ml 1 N sodium hydroxide with shaking followed by 48.7 ml 1 M sodium chloride. The final reagent contained 0.42 mg of iodine as monochloride per ml and had a pH of approximately 1; the high concentration of chloride in it is essential for stability. Such a diluted stock solution, kept in a glass-stoppered cylinder at 2 to 4° C, has been drawn on intermittently for more than a year with no loss of iodine value. This solution, 0.015 ml, or 7.5 μ g of iodine was used to label 15 mg fibrinogen (12); with a molecular weight of 330,000 and an iodination efficiency of 50% assumed, this implies the substitution of a mean of 0.5 atoms iodine per molecule.

Freedom from reducing agents such as sulfite and thiosulfite which are commonly added to stabilize I^{131} solutions is an advantage, since these take up the isotope for purely oxidative purposes and protein iodination efficiencies are correspondingly reduced. In addition, if reducing agents are present the amounts must be known in advance to be able to effect a predetermined degree of protein substitution. Helmkamp and associates (13) demonstrated that sulfite in I^{131} solutions, supplied by the Oak Ridge National Laboratory, disappeared in a few days as a result of self-irradiation, and thereafter peroxides accumulated. They recommend addition of more sulfite to destroy peroxides and removal of excess sulfite by aeration at 100° C. In the writer's hands this has been an effective procedure preliminary to fibrinogen labeling. However, for present purposes carrier-free I^{131} without reducing agent was obtained from the Radiochemical Centre, Amersham, England, in a concentration of not less than 30 mc per ml of 0.02 M sodium hydroxide.

The requisite volume of I^{131} solution was micro-pipetted into the carrier iodine monochloride and was followed by 0.2 ml of alkaline glycine buffer, pH 9.5, which discharged the faint yellow color of the iodine monochloride. Since the active hypoiodite ion is unstable at alkaline pH's, this adjustment was made immediately before mixing with the protein in the same buffer. The iodine solution, usually 0.3 to 0.4 ml, was injected forcibly into the buffered protein solution, pH 8.5 (1.0 to 1.5 ml), through a finely drawn-out glass pipette.

Routinely, 50 to 60% of the radioactivity became

bound to fibrinogen, but in a few cases, in attempting to incorporate as much as 10 mc into 15 mg fibrinogen with 7.5 μ g of iodine, efficiencies of incorporation were lower, and this was traced to the use of I^{131} which had decayed to specific radioactivities in the region of 5 mc per μ g I^{127} . An excess of I^{127} supplied as ICl over I^{131} present in other forms is necessary to ensure efficient transfer of radioiodine into the cationic form and thus into protein combination. If only low specific activity I^{131} is available, the difficulty can usually be circumvented by adding iodate to the I^{131} followed by HCl to convert all the iodine into ICl. A small excess of iodate at this stage appears not to be harmful to the protein.

Iodide was removed in a few instances by an exchange resin in the chloride form, but since this also removes citrate and may lead to clotting, dialysis was usually employed. Visking tubing, 8 mm diameter, was used with a 6 mm diameter glass rod occupying the center of the sac. With half-hour changes of the outer solution (citrate, phosphate, saline, pH 7.5), all diffusible radioactivity was removed in 3 hours, after which the sac was dismantled, and 2 ml of citrate plasma was added to the labeled fibrinogen for radiation protection. Sterile filtration when carried out was through a 1-mm-thick collodion membrane of suitable porosity and was associated with negligible loss of protein.

Nonprecipitable activity in protein preparations. This was measured by adding 0.2 ml of 100% (w/v) trichloroacetic acid in water to 2.5 ml of the protein solution made up with a commercial albumin to contain at least 25 mg precipitable protein. After centrifugation 2 ml of the clear supernatant fluid was counted and the value multiplied by 1.35 to obtain the total nonprotein count. Less than 0.3% of the activity in the I^{131} fibrinogen preparations failed to precipitate in this way.

Treatment of plasma samples. Four ml of blood was taken into 0.025 ml of heparin 10 minutes after injecting I^{131} -fibrinogen into the opposite ear vein, similar samples being taken at 6 and 18 hours and thereafter every second day at the same time for 15 to 18 days. Two-ml plasma samples were counted in a well-type scintillation counter and were then precipitated by diluting with 4 ml 0.9% sodium chloride and adding 2 ml saturated ammonium sulfate. The proportion of nonprecipitable activity in the plasma was estimated by adding 0.3 ml 100% trichloroacetic acid to 3 ml of the ammonium sulfate supernatant. The crude fibrinogen precipitate was reprecipitated with ammonium sulfate at 23% saturation as in the original fibrinogen preparation. A third precipitation was avoided, however, since it effects only a small additional purification and in small samples is associated with serious fibrinogen losses. The fibrinogen was dissolved in 5 ml citrate saline, pH 6.0, clarified, and clotted as described. The dry salt-free fibrins were transferred to pointed agglutination tubes and weighed on a microbalance, the yields corresponding to approximately 2 mg of fibrin per ml of plasma. The tubes were supported centrally in the counter well with the fibrin pellet in the tip and counted for I^{131} activity, the result

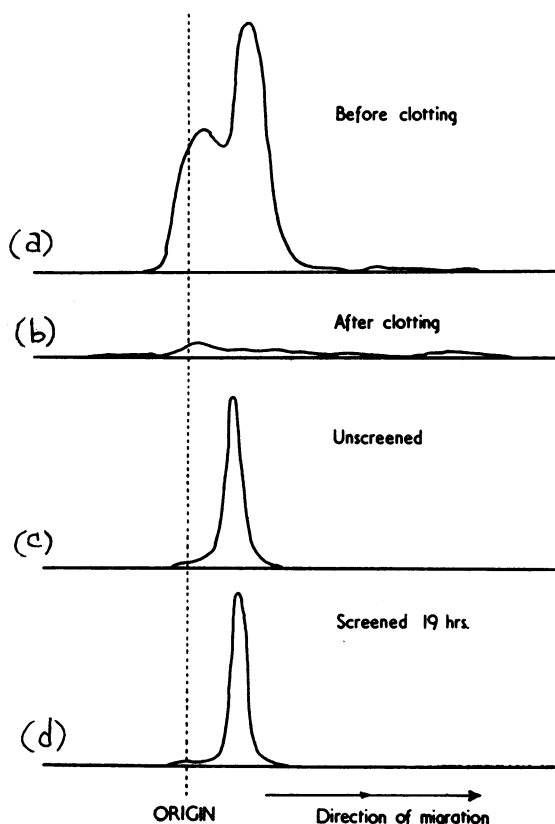


FIG. 1. CURVES OF I^{131} RADIOACTIVITY DISTRIBUTED ALONG PAPER ELECTROPHORETIC STRIPS. (a) 0.6 atom I^{131} -fibrinogen added to inactive plasma; (b) supernatant fluid from the same mixture after clotting; (c) a different I^{131} -fibrinogen showing a minimal proportion of "origin component"; and (d) plasma from a rabbit at 19 hours after receiving the preparation shown in (c).

being expressed as counts per minute per milligram of fibrin. The pellets were then tipped into platinum boats and combusted to carbon dioxide in a dry-combustion microanalysis tube. The gas, which was dried by passing through a glass spiral immersed in dry ice-ethanol mixture and further purified by contact with a red hot copper wire, was transferred first to a constant volume manometer and then into a proportional gas counter tube as described elsewhere (14). Results in this case were expressed as counts per minute per milligram of fibrin carbon (see Figure 10).

Animals. Male Sandylop rabbits of the Mill Hill strain (weight, 2.7 to 3.5 kg) were used. These were maintained on drinking water containing 0.005% sodium iodide and 0.45% sodium chloride starting 3 days before the fibrinogen injection.

Total body radioactivities were measured daily at the same time by putting the rabbit in the center of a shielded ring of six vertical Geiger tubes.

General plan. A series of experiments was carried out with I^{131} -fibrinogen alone, varying the conditions of

iodination, and deducing from excretion data and by means of screening tests the proportions of denatured material present. When it became evident that what appeared to be an essentially undenatured product could be obtained, this material was then injected along with C^{14} -fibrinogen.

Preparation of C^{14} -fibrinogen. Because of the high cost of C^{14} -aminoacids, only one preparation was undertaken. One mc C^{14} -algal protein obtained from the Radiochemical Centre, Amersham, England, was digested for 20 hours at 37° C in 0.1 M ammonium carbonate with 1% by weight of an exoendopeptidase obtained from *Streptococcus griseus*.¹ The hydrolyzate was then given by stomach tube to a rabbit weighing 2.9 kg, which nineteen hours earlier had received 1.9 mc of I^{131} -fibrinogen labeled at 0.5 atoms iodine per molecule. Immediately before passing the stomach tube, 40 ml of blood was removed from the ear vein to stimulate fibrinogen synthesis. Five and one-half hours later, another 40 ml of blood was taken from the ear and 1 ml of heparin containing 5,000 international units plus 100 mg of pentobarbital sodium in 1.5 ml was injected. The chest was then opened, and a further 45 ml of blood recovered from the heart by syringe. The ante-mortem blood sample yielded 22 ml of plasma, of which 5 was injected at once into each of two rabbits (M and N) and the remainder was pooled with 29 ml of plasma from the heart sample. This pool was used to prepare fibrinogen by triple ammonium sulfate precipitation as described and yielded after dialysis 82 mg of fibrinogen (180 μ c) in 4.6 ml, which was divided equally between two rabbits, one (P) receiving it intravenously and the other (O) in five widely distributed subcutaneous sites.

In vitro properties of I^{131} -fibrinogens. All preparations after addition of unlabeled plasma were subjected to paper electrophoresis with a Veronal buffer (1.84 g diethylbarbituric acid and 10.30 g barbitol sodium in 1 L), pH 8.6, to which was added 100 ml of 3.8% trisodium citrate per 2 L of buffer. The paper strips were scanned for radioactivity, variable amounts being observed at the origin in addition to the dominant fibrinogen peak. Figure 1 shows two extremes in the proportion of mobile to immobile activity. The preparation with the highest proportion of origin component was clotted, and the supernatant fluid, when examined in identical electrophoretic conditions in the presence of carrier plasma, was found to have little or none of it. Biological screening for 19 hours of a fibrinogen labeled at 0.6 atoms per molecule also effected no change in the proportion of origin components, and we conclude that fibrinogen is partly adsorbed on the paper at the point of application. Traces of a labeled γ -globulin contaminant could have been masked to some extent by this phenomenon, but these could not have been associated with more than 5% of the total activity.

¹ Kindly supplied by Dr. Nomoto of the Institute of Physical and Chemical Research, Komagome, Bunkyo-Ku, Tokyo, Japan.

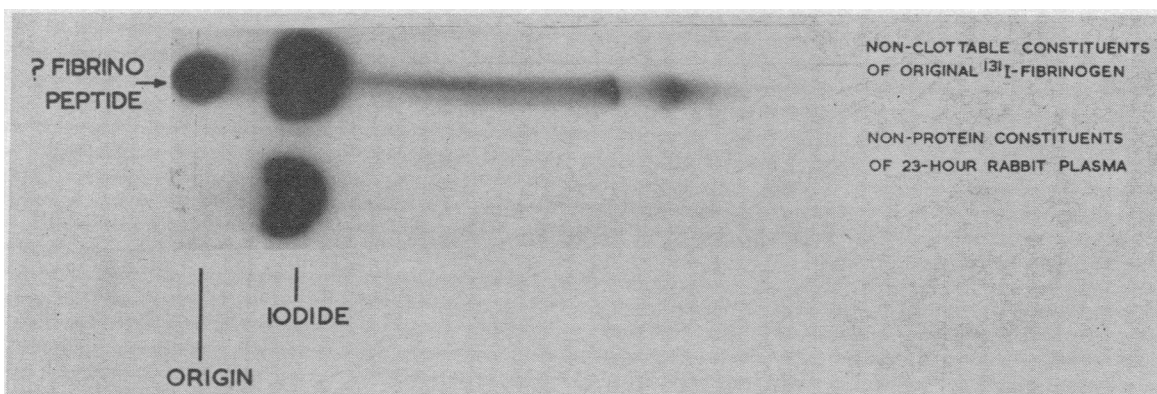


FIG. 2. RADIOAUTOGRAPH OF BUTANOL-ACETIC CHROMATOGRAMS OF THE SUPERNATANT FLUID FROM A I^{131} -FIBRINOGEN CLOT AND FROM A TRICHLOROACETIC ACID PRECIPITATE OF A 23-HOUR RABBIT PLASMA SAMPLE.

The various I^{131} -fibrinogens were 85 to 95% clottable by weight and 90 to 95% clottable by radioactivity. Figure 2 presents a butanol-acetic acid chromatogram of the fluid from the clot, and shows that the nonclottable radioactivity consisted mainly of iodide and an origin component which is presumed to be the tyrosine-containing fibrinopeptide B of Lorand and Middlebrook (15).

As will be discussed below, it is important to know the form in which I^{131} liberated by protein catabolism circulates in the blood. The companion chromatogram in Figure 2 of nonprecipitable material from a plasma sample at 23 hours shows the radioactivity to be ex-

clusively in the form of iodide, bearing out Stanbury, Kassenaar, and Meijer (16) that mono- and diiodotyrosine, whether of endogenous origin or injected parenterally, have only a fleeting existence before being deiodinated.

In vivo behavior of I^{131} -fibrinogens. Preparation F₁, labeled at 2.5 atoms per molecule, was injected into two rabbits, 68 mc into rabbit C and five mc into a second rabbit for screening purposes. The screening animal was bled at 17 hours, and 16 ml of its plasma containing 134 μ c was transferred to rabbit D. A second bleeding of the screening rabbit at 120 hours provided 35 ml of plasma

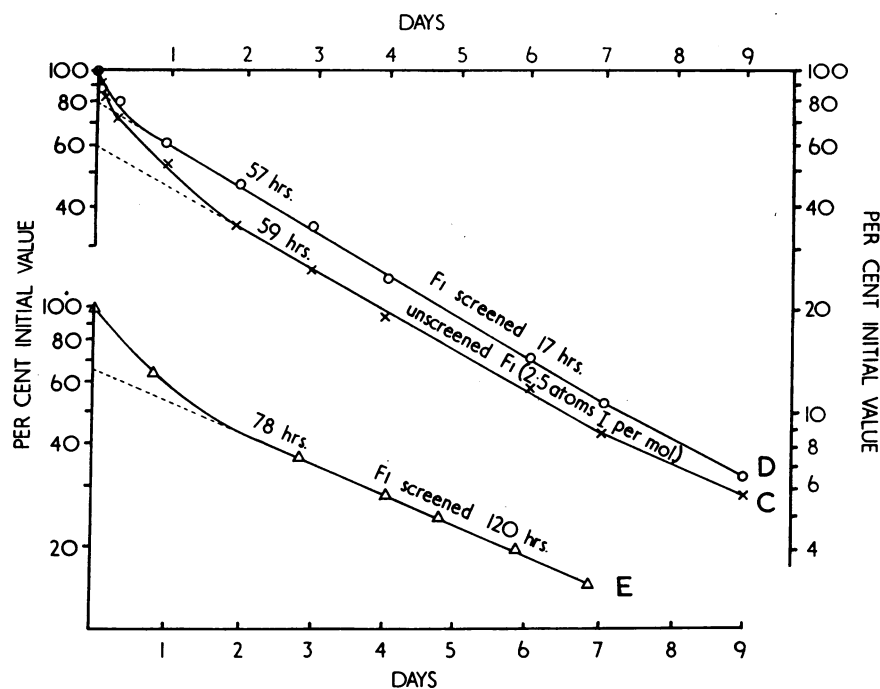


FIG. 3. PLASMA RADIOACTIVITIES OF RABBITS AFTER RECEIVING 2.5-ATOM I^{131} -FIBRINOGEN UNSCREENED AND SCREENED FOR 17 AND 120 HOURS.

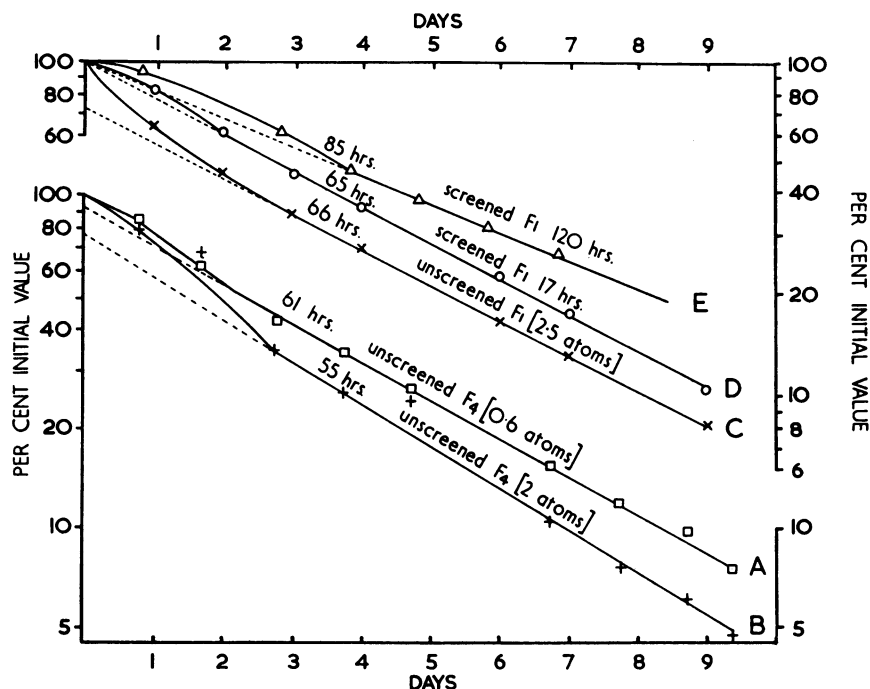


FIG. 4. TOTAL BODY RADIOACTIVITIES OF RABBITS INJECTED WITH SCREENED AND UNSCREENED 2.5 ATOM I^{131} -FIBRINOGEN (F_1) PLASMAS AND WITH ANOTHER FIBRINOGEN PREPARATION (F_4) CONTAINING 0.6 AND 2.0 ATOMS IODINE.

containing $67 \mu\text{c}$ which was transferred to rabbit E, which had been partially prepared by removing 35 ml of its blood. Rabbits C, D, and E were then sampled and ring-counted for 2 to 3 weeks.

Plasma radioactivity curves of C and D (Figures 3) become exponential after the second day with closely similar slopes, curve D extrapolating to 80% while C extrapolates to 60%. The initial fall in both curves is attributable to reversible equipartition of molecules between the plasma and extravascular compartments, and also to catabolism of any denatured material which may have been injected. That more than half of it in the case of rabbit C was due to the latter cause is evident in Figure 4 from the fact that this animal lost 27% more of its body radioactivity in the first 2 days than did D. Since the subsequent behavior of plasma and total body radioactivities in C and D were sensibly the same, it is evident that the screening period of 17 hours was adequate for removal of all denatured material. After seven days the half-life in both animals showed a tendency to lengthen, suggesting the presence of small amounts of longer-lived material, and this was confirmed by the findings in rabbit E in which the half-life after 3 to 4 days was 78 hours. At the fourth day this rabbit had retained 45% of the injected activity, which was itself a selected material representing only 20% of the original labeled fibrinogen. Thus the longer half-life was only clearly apparent when the original population of labeled molecules had been decimated. Most probably it

reflects the presence of traces of labeled γ -globulin in the original fibrinogen preparation, these being associated with up to 5% of the total radioactivity during labeling. Rabbit γ -globulin has a half-life of 160 to 200 hours and presumably the half-life in E would ultimately have lengthened to this value had later measurements been possible. The plasma exponential of rabbit γ -globulin extrapolates to between 40 and 50%, so that 65% is reasonable for the mixture of fibrinogen and γ -globulin surviving in E. It will be noted that plasma half-lives in C and D were significantly shorter than total body half-lives, suggesting a possible plasma protein or water imbalance.

This experiment showed, therefore, that fibrinogen labeled at 2.5 atoms per molecule contained at least 20% of a denatured material and a few per cent of a more slowly catabolized component, probably γ -globulin. An unusual appearance which will be further discussed was noted in the total body activity curve of the screened material, a concave section, in the first few days. One-half of another fibrinogen preparation was labeled at 0.6 atoms and the other at 2.0 atoms iodine per molecule, and these were injected into rabbits A and B (Figures 4 and 5). 3.7 mc of the 2.0-atom material was also injected into a screening rabbit from which 51 ml of blood was taken into citrate at 41 hours. Since the screened fibrinogen in this blood was required partly for other experiments in the form of a concentrate, the total plasma was diluted and precipitated once with ammonium sul-

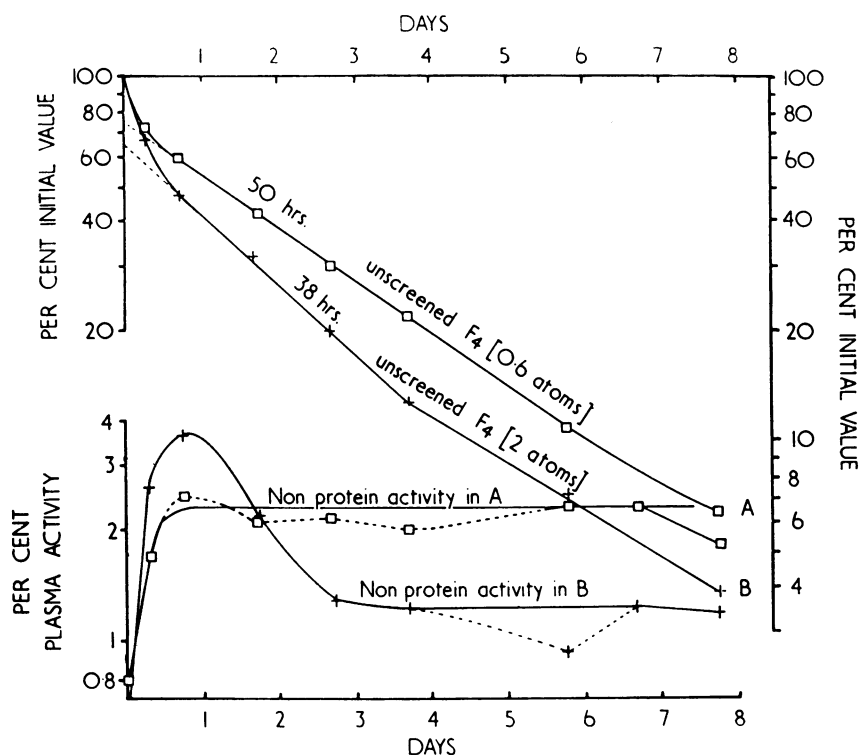


FIG. 5. PLASMA TOTAL AND NONPROTEIN RADIOACTIVITIES OF RABBITS INJECTED WITH 0.6 AND 2.0 ATOM I^{131} -FIBRINOGEN (F_4).

fate at 25% saturation, and the crude precipitated protein was then dialyzed free of sulfate. Seven mg of this crude fibrinogen associated with $27.3 \mu\text{c}$ in 0.9 ml and containing an additional $1.15 \mu\text{c}$ of nonprecipitable activity was injected into rabbit C. In all three recipients in this experiment, measurements were made for the first time of levels of nonprecipitable activity in the plasma samples. It is evident in Figure 5 that the plasma radioactivity curve of unscreened 0.6-atom material in A, in extrapolating to 75%, is similar to those of screened 2.5- and 2.0-atom materials in Figures 3 and 6, indicating that 0.6-atom fibrinogen contains little denatured material. This was supported by the behavior of the total body radioactivity curve of A (Figure 4), which extrapolated to 93%.

The plasma curve of the 2-atom preparation in Figure 5 shows two exponential segments with an abrupt change of slope occurring between them at 3.8 days. The explanation is best obtained by reference to the nonprotein activity curves in the same figure. The proportion of this activity in the plasma of A rose to a maximum of 2.3% and then remained constant, presumably owing to establishment of an equilibrium between iodide arriving by catabolism of labeled protein and departing by renal excretion. In B, however, an initially higher value of 3.6% at 2 hours was not maintained, and only after 3 to 5 days was a plateau established at 1.2%. Evidently, labeled protein was being catabolized at a

much higher rate in the first few days in B than subsequently.

Total body radioactivity measurements (Figure 4) indicate that 20% more was catabolized in the first 3 days in B than in A, while extrapolation of both exponential segments of the plasma curve of B and of the single exponential of A suggests 25% plus 10%, i.e., the differences in the three intercept values. The total of 35% may well be an overestimate for reasons which will be discussed, but it is clear that at least 20% of the protein injected into B was more rapidly catabolized than the remainder, confirming the result obtained in the first experiment using 2.5 atom fibrinogen.

Nonprotein plasma activities. The abrupt change of slope at 3.8 days in the plasma curve of B coincides with the establishment of an equilibrium in the nonprotein activity curve and with the end of an initial convexity in the total body radiation curve (Figure 4). These coincidences suggest that the convexity is due to retention of radioiodide in the body water and as will be shown, should be more noticeable with increasing values for the nonprotein activity in the plasma. On the other hand, the presence of a convexity could be concealed by a superimposed concavity such as might arise from unusually rapid initial catabolism of denatured protein. This presumably has occurred in curve C (Figure 4), but not as might have been expected in curve B. Although the critical second and third points on the B curve may have

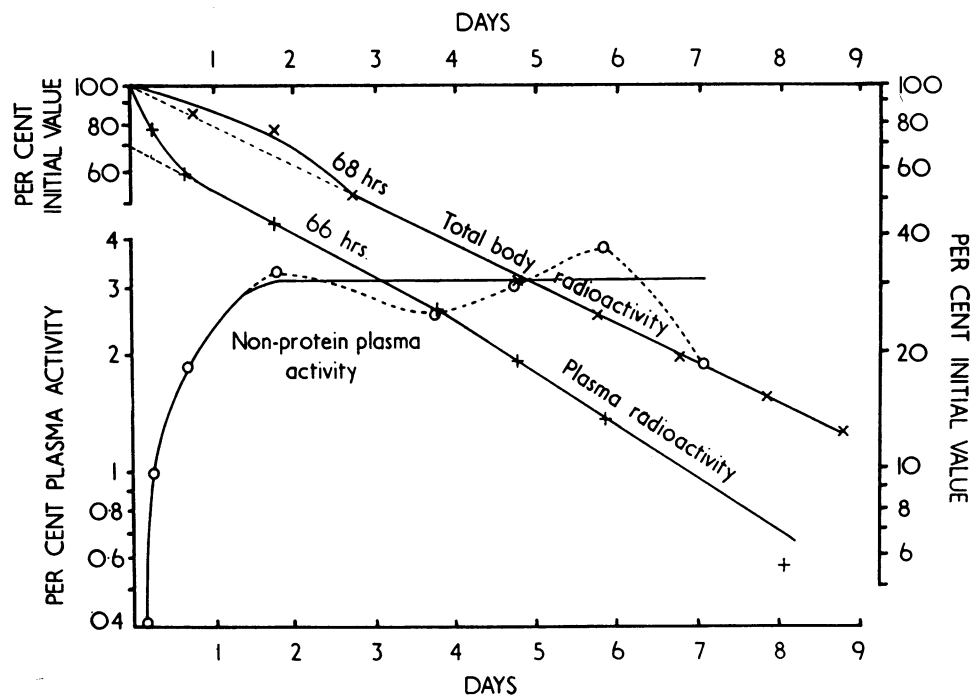


FIG. 6. TOTAL BODY AND PLASMA PROTEIN AND NONPROTEIN RADIOACTIVITIES OF A RABBIT RECEIVING 41-HOUR SCREENED I^{125} -FIBRINOGEN (F_4 -2.0 ATOMS) INJECTED IN THE FORM OF A CONCENTRATE.

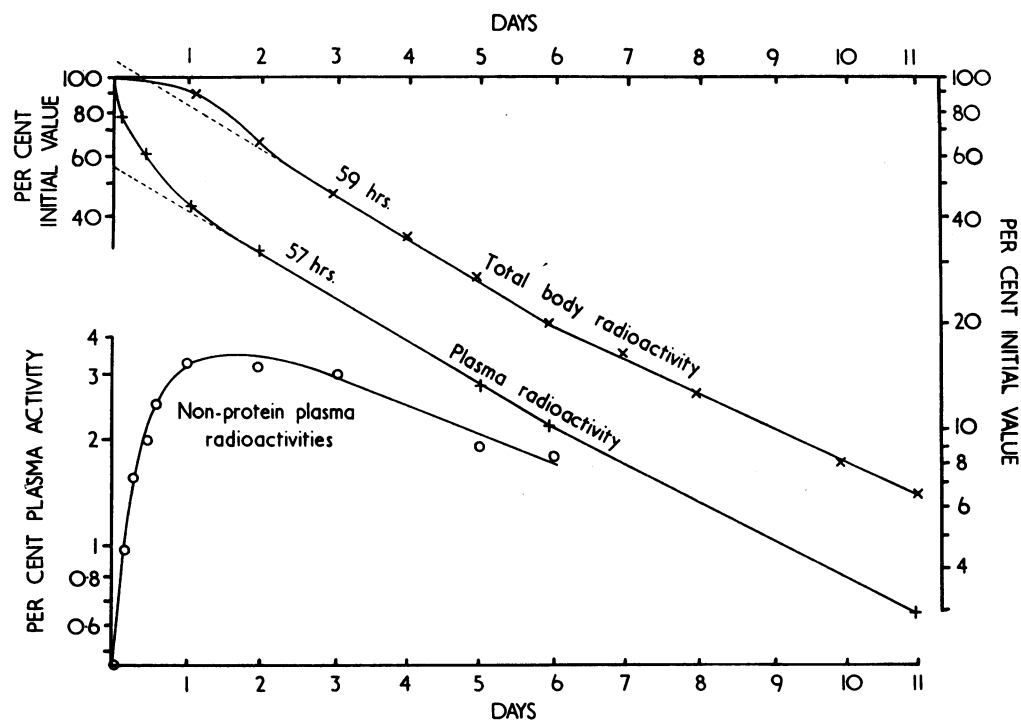


FIG. 7. TOTAL BODY AND PLASMA PROTEIN AND NONPROTEIN RADIOACTIVITIES OF A RABBIT RECEIVING A 90-HOUR SCREENED I^{125} -FIBRINOGEN (F_4 -3.3 ATOMS) PLASMA.

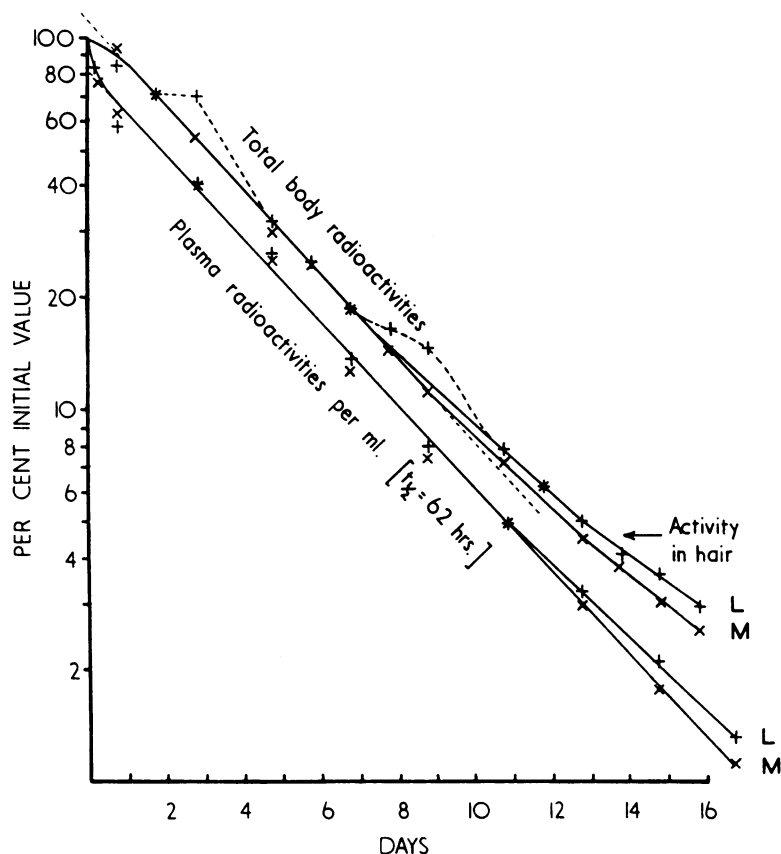


FIG. 8. TOTAL BODY AND PLASMA RADIOACTIVITIES OF TWO RABBITS RECEIVING 24-HOUR SCREENED I^{131} -FIBRINOGEN (F_5 -0.5 ATOMS) PLASMA. Values for L are marked by + and for M, by \times .

been affected by experimental errors, the fact that rabbit B is catabolizing undenatured fibrinogen faster than C must be taken into account, and also the possibility that B may have a lower renal clearance rate for iodide than C—both tending to increase the nonprotein activities.

Additional experiments. In another experiment 41 hours screening removed the denatured component in the 2-atom fibrinogen (F_4) and abolished the initial peak in the nonprotein activity curve (Figure 6).

A third preparation of fibrinogen (F_3) was labeled at 3.3 atoms per molecule, screened for 90 hours, and injected into rabbit T. The results, which are shown in Figure 7, confirm those obtained with F_1 and F_4 . In this case plasma and total body radioactivity half-lives were sensibly the same and changed to slightly longer values after 6 days, suggesting that this preparation contained more γ -globulin than did F_1 and F_4 . Once again the maximal concavity seen in the first few days of the total body curve coincided with the maximum in the nonprotein activity curve, supporting the view that the concavity is due to iodide retention.

Comparison of I^{131} - and C^{14} -fibrinogens. In the final experiment a comparison of I^{131} - and C^{14} -fibrinogens was

made in the same animals. I^{131} -labeling was at a level of 0.5 atoms, and in case traces of denatured protein were present, the preparation was conveniently screened for 24 hours in the animal which was synthesizing C^{14} proteins. In this way, as explained before, a plasma was prepared which contained 24-hour screened I^{131} -fibrinogen and C^{14} -fibrinogen maximally labeled. This was injected without additional treatment or storage into rabbits L and M with I^{131} -results which are shown in Figure 8. As anticipated both total body curves extrapolated to more than 100% and both plasma curves to slightly over 80%. Rabbit L retained its urine erratically giving rise to a few aberrant points on the total body curve which have no counterparts on the plasma curve. Terminal curvature appearing after 10 days in the total body curve coincided with the appearance of organically bound iodine in the hair, a phenomenon which we have seen also in rats, and the nature of which was confirmed by shaving the hair and counting it. Half-lives of total body and plasma exponentials in both animals were the same.

The remaining active plasma was fractionated to prepare a fibrinogen concentrate suitable for subcutaneous injection. This was injected into multiple sites on the

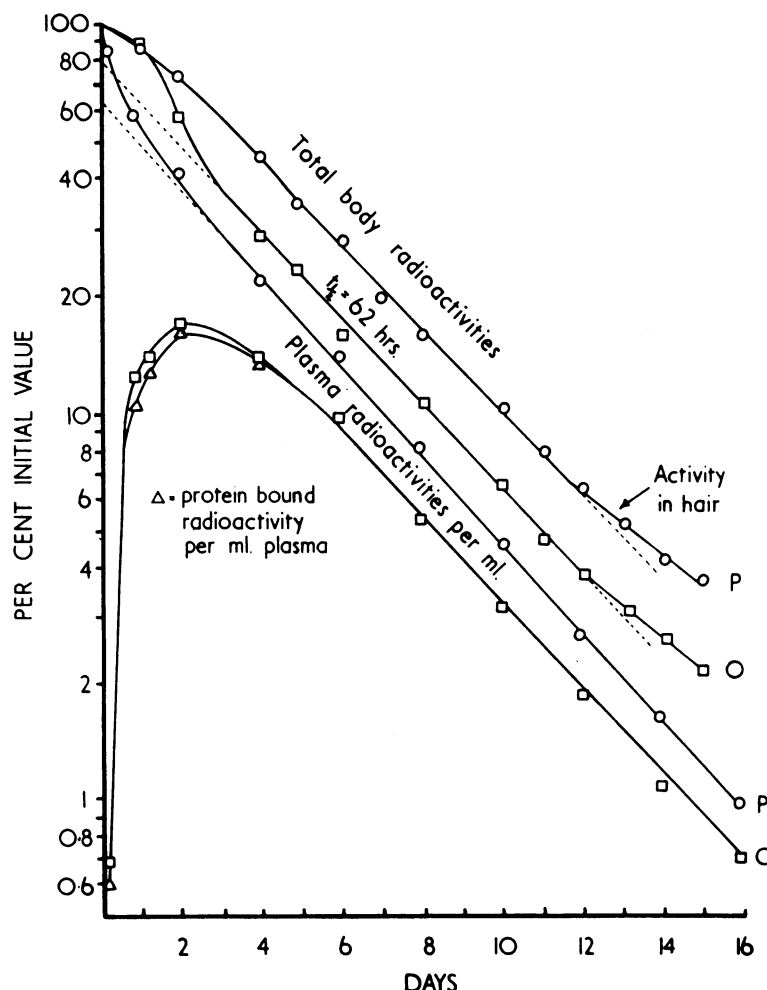


FIG. 9. TOTAL BODY AND PLASMA RADIOACTIVITIES OF TWO RABBITS RECEIVING 24-HOUR SCREENED I^{131} -FIBRINOGEN (F_2 -0.5 ATOMS) IN THE FORM OF A CONCENTRATE. One (O) received it intravenously and the other (□) subcutaneously in multiple sites.

belly and flanks of rabbit O, and an equal volume was given intravenously to rabbit P (Figure 9). The total body curve of P was very similar to that of L and M (Figure 8), which received the unfractionated plasma ($t_{1/2}$ in both = 62 hrs), but its plasma curve extrapolated to 62%. However, when fibrin specific activities were measured instead of activities per ml of plasma, the curve had two components, the first extrapolating to 80% and the second to 70% (Figure 10). Possibly these differences were due to an unbalance associated with the injection of a concentrate containing 41 mg fibrinogen into each rabbit in the second part of the experiment and only 15 mg fibrinogen in 5 ml of plasma in the first. Injection of the concentrate must have increased the blood fibrinogen pool by 14%, with effects on plasma volume and thus on radioactivity per ml of plasma which could have persisted for several days.

Total body radioactivities of O were remarkable in dropping after 72 hours to values 30% below the corresponding ones of P. Evidently, 30% more of the fibrinogen injected into O was catabolized in the first 24 hours and the associated iodide largely excreted in the next 48. The plasma curve shows a maximum of 17% of the injected radioactivity in the plasma at 44 hours, at which time the nonprecipitable proportion was 7.3%. In the sample at 20 hours when 12.5% of the dose was in the plasma, the nonprecipitable fraction was as high as 17%, confirming that an unusually large amount of labeled iodide had been liberated into the body water in the first day. When I^{131} -albumin or γ -globulin was injected subcutaneously, the labeled protein was ultimately distributed between extra- and intravascular spaces, as if it had been injected intravenously and no abnormal catabolism was observed. This suggests the possibility that clotting

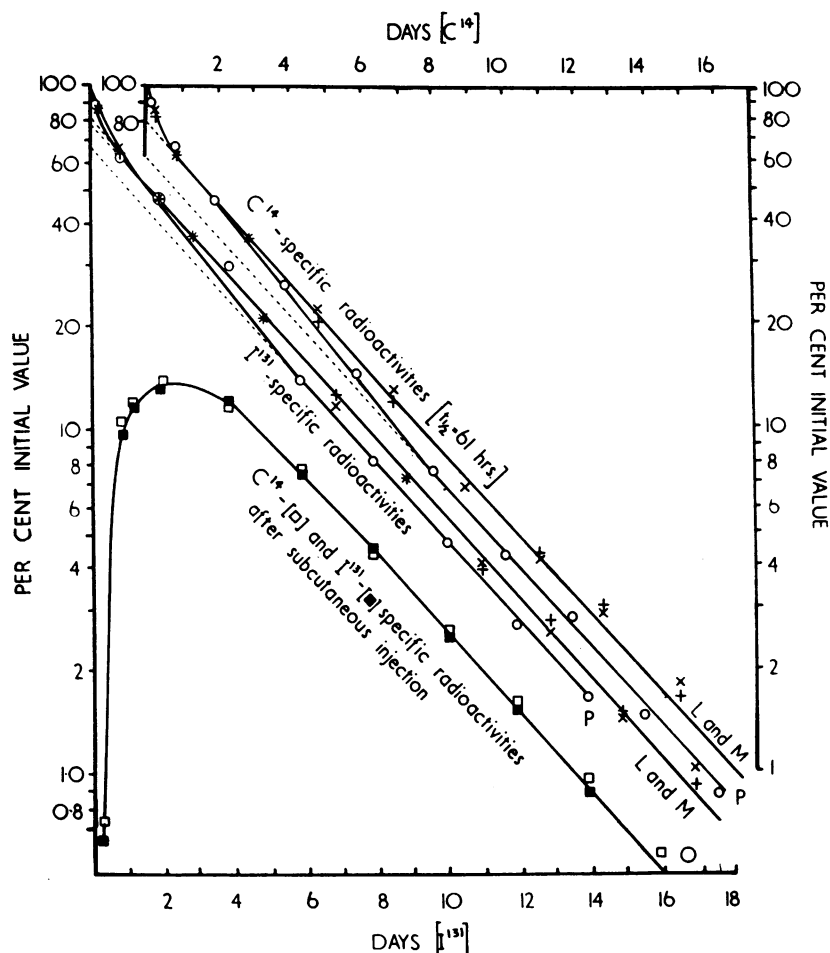


FIG. 10. C^{14} - AND I^{131} -SPECIFIC RADIOACTIVITIES (COUNTS PER MINUTE PER MILLIGRAM) OF FIBRINS PREPARED FROM PLASMA SAMPLES FROM FOUR RABBITS RECEIVING A MIXTURE OF 24-HOUR SCREENED I^{131} -FIBRINOGEN (F_0 -0.5 ATOMS) AND C^{14} -BIOSYNTHETIC FIBRINOGEN INJECTED INTRAVENOUSLY (+, \times , AND \circ) IN THE FORM OF WHOLE PLASMA (+ AND \times) AND OF A CONCENTRATE (\circ), AND INJECTED INTO MULTIPLE SUBCUTANEOUS SITES (\square FOR C^{14} AND \blacksquare FOR I^{131}).

took place at the site of subcutaneous fibrinogen injection and that active iodide was liberated during resolution of the clot.

Finally, all plasma samples were fractionated for fibrinogens which were clotted, and the fibrins were counted as described, with results which are shown in Figure 10. To avoid superimposing C^{14} and I^{131} values, in this figure a time scale for C^{14} specific radioactivities has been used which is displaced by two days from that for I^{131} values. C^{14} and I^{131} values were very similar throughout, leaving no doubt that all four animals were unable to discriminate between fibrinogens labeled in these two ways.

Distribution and renal excretion of iodide. Since importance seemed to attach to iodide parameters in the rabbit, excretion rates and space ratios were measured by injecting I^{131} -iodide containing insignificant amounts

of carrier and following total body and plasma radioactivities for 5 to 7 days. Considerable variability in excretion rates was observed in rabbits of similar weight, and this was found to depend to a large extent on random variations in dietary chloride intake and hardly at all on deliberate changes in the level of iodide in the drinking water. To study this chloride effect further, rabbits of similar weights were placed on a constant level of iodide and increasing levels of chloride in the drinking water for 3 days before the injection and subsequently throughout the period of observation. Figure 11 shows results obtained with iodide alone and with the maximal additional concentration of sodium chloride used, 0.9%. With high chloride levels, equilibration of radioiodide between plasma and body water occurred more rapidly and half-lives were generally shorter than with low levels. Half-lives were approximately the same

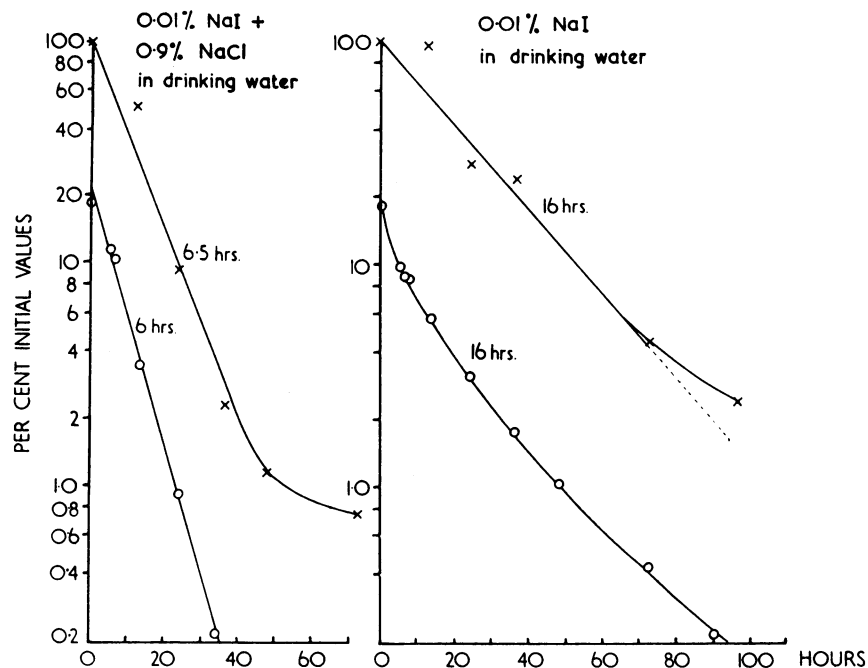


FIG. 11. TOTAL BODY (X) AND PLASMA (O) RADIOACTIVITIES AFTER INJECTING I^{131} -SODIUM IODIDE INTO TWO RABBITS BOTH ON DRINKING WATER CONTAINING 0.01% SODIUM IODIDE AND, IN ONE CASE, 0.90% SODIUM CHLORIDE.

for plasma and total body radioactivities, and varied between 6 and 16 hours corresponding to fractional elimination rates of 277 and 104% per day. The Y-axis intercept of the plasma curve represents the proportion which the activity of iodide in the plasma bears to the total iodide activity. Values obtained for the inverse ratio, or so-called iodide space factor, varied between 5 and 9. In these experiments, also, terminal changes of slope in the total body curves were observed and shown to coincide with the appearance of activity in the hair.

DISCUSSION

Total body radioactivity curves. From these results it appears that total body radioactivity measurements are in general more reliable indicators of protein catabolism than measurements made on plasma samples. In principle this is to be expected, since they are unequivocal measurements of activity remaining in the animal, and the plasma values are affected by variations in plasma pool parameters. Nevertheless, it is clear that confusing factors also affect total body measurements, the most important being retention of diffusible activity in the body water. Fortunately, as the chromatogram in Figure 2 shows, all the nonprotein activity in the body is sensibly present

as I^{131} -iodide; addition of carrier iodide to the drinking water before and during the experiment is effective in depressing thyroid and circulating hormone activities.

Since iodide retention represents a net balance of iodide arriving by catabolism and departing by renal excretion, it will be directly proportional to the protein catabolic rate and inversely to the iodide excretion rate. With a relatively rapidly catabolized protein like fibrinogen, retention in rabbits is sufficient to alter the slope of the total body exponential in the first few days so that a convexity appears. This phenomenon has not been observed with albumin or γ -globulin preparations in these animals even after screening, presumably because of the much lower rates at which these proteins are catabolized.

Substantial retention can also occur when partly denatured proteins are injected, since the denatured material is largely catabolized in the first day, simulating a high catabolic rate. In human investigations commercially labeled albumins and globulins are often used which are partly denatured, up to 15% of the injected dose being excreted in the first day, compared with 3 to 4%

when an undenatured protein is used. Experience with overiodinated fibrinogen suggests that even such high urinary values probably provide underestimates of the true proportion of denatured protein present, since much of the active iodide arising from catabolism of the denatured material takes several days to be excreted. Even with undenatured proteins, when these are injected into patients with renal impairment, iodide excretion rates may be so low as to markedly affect the slope of total body radioactivity curves. Other confusing factors affecting total body measurements are mostly peculiar to animals, since these concern the appearance of radioactivity in the hair, retention of urine, and soiling of the skin, all of which have been sporadically seen in these studies. In general they are recognizable and assessable so that they do not seriously interfere with the general usefulness of this type of measurement in experimental studies of plasma protein catabolism.

Retained iodide. Total body radioactivities can only be correlated quantitatively with protein catabolism by estimating the proportion of activity present as iodide in the body water. Estimates of total iodide activity are made by multiplying nonprecipitable activities for the whole plasma by plasma:total body iodide ratios, the latter being obtained by injecting labeled iodide. As shown in Figure 11, a stable equilibrium is established within 20 minutes when chloride is added to the drinking water, whereas without chloride, the iodide space appears to increase for many hours. Ideally, the ratio should be measured at the time of the metabolic experiment by injecting independently labeled iodide, e.g., with I¹²⁵ or I¹³². However, on a regime of chloride added to the drinking water, the value is constant over long periods in the same animal, and indeed does not vary greatly between individuals. From 27 measurements on 10 rabbits placed on .005% sodium iodide and 0.45% sodium chloride drinking water, Regoeczi (personal communication) found a mean ratio of 6.65 ± 0.71 . A similar degree of variation has been observed in 12 normal humans (17).

When undenatured fibrinogen was injected into rabbits on similar drinking water, a steady value of 2.5% for nonprecipitable activity in the plasma was reached at 24 hours. With 6.65 for the iodide space ratio of the recipient animals, 16.5%

of the plasma activity at 24 hours or 13% of the injected activity was present at this time as iodide in the body water. Catabolism was at a steady rate of 33% of the plasma protein per day (see Figure 10) and should have liberated iodide equivalent to approximately 26% of the injected activity in the first day so that 13%, or half the total iodide liberated, should have appeared in the urine. This value is also found by reference to the total body curve. Clearly, the convexity which is apparent in the total body curve when undenatured I¹³¹-fibrinogen is injected is explained by iodide retention. Terres, Hughes, and Wolins (18) also observed that the total body radioactivity exponential of mice injected with I¹³¹ bovine serum albumin extrapolated to more than 100% and concluded that this effect was due to retention of iodide in the body water.

Plasma radioactivities. These were essentially specific activities, whether measured as counts per milliliter of plasma or per milligram of fibrin, and as such can only be expected to reflect protein catabolism unequivocally if the pool of fibrinogen or plasma water remains constant. Since blood samples were small, there was no reason to anticipate significant changes of plasma volume during the experiment, but changes could not be excluded at and immediately following injections of materials screened for long periods. These contained fibrinogens of such low specific activity that only by injecting relatively large amounts of fibrinogen in the form of concentrate or large volumes of plasma could the requisite activities be transferred. These transfers could have given rise to intravascular and extravascular movements of fibrinogen or water, or both, or they could have depressed the normal synthesis rate of the protein so that specific activities would be altered. Fibrinogen concentrations are also liable to change abruptly after minor trauma such as might be associated with the injection procedure. One or another of these factors probably explains inconsistencies which are visible in Figures 8, 9, and 10. Thus, whereas the counts per minute per milliliter curve of P in Figure 9 extrapolates to 62%, the counts per minute per milligram fibrin curve from the same plasma samples is biphasic, with the first component extrapolating to 80% (Figure 10). Also, whereas the counts per minute per milliliter plasma curve for the two rab-

bits which received whole plasma (Figure 8) extrapolated to 80%, the similar curve for rabbit P (Figure 9), which received the same fibrinogen but in the smaller volume of a concentrate, extrapolated to 62%. The suspected, almost unavoidable, displacements of water or protein equilibria seem to offer a serious obstacle to quantitative deductions based on whole plasma measurements, and with one exception, only qualitative conclusions will be attempted.

Preparation of undenatured I^{131} -fibrinogen. Increasing the proportion of substituted iodine above 0.5 atoms per molecule consistently reduced the extrapolation values of both total body and plasma exponentials, as would be expected to occur if denatured material were present and were being rapidly catabolized. The presence of this material is further indicated by maxima appearing at the end of the first day in nonprecipitable plasma activities and rapid falls which were evident thereafter. In one instance (see curve B in Figure 5) the proportion of denatured material was high enough and its catabolism sufficiently prolonged to produce two exponentials in the plasma curve. In other examples the effect of overiodination was to prolong the time required for truly exponential elimination to be established beyond the 24 to 36 hours seen with 0.5-atom material.

All appearances suggestive of overiodination were in general abolished or reversed by screening which uniformly raised the extrapolation values to over 100% in the case of the total body curves and up to a maximal value of 80% in the case of the plasma activity curves. The plasma and total body curves of 0.6-atom unscreened fibrinogen and of 0.5-atom 24-hour screened fibrinogen were very similar to each other. This and all other aspects of the behavior of these two proteins suggest that they contained practically no denatured protein. Evidently fibrinogen cannot be associated with much more than a mean 0.5 atoms iodine per molecule and certainly not with 2 atoms per molecule if denaturation is to be avoided. Fibrinogen thus appears to be more sensitive than albumin to substitution with iodine, but possibly not more so than transferrin is. Johnson, Day, and Pressman (19) have shown that above 2 atoms of iodine per molecule, antibody globulins begin to show a fall in precipitin titer.

Validity of the I^{131} label. Charlwood (20) has demonstrated that fibrinogen is not polymerized

by labeling with iodine. The possibility that some other change had been brought about, causing the protein to be unnaturally confined to the plasma, could only be excluded by comparing its behavior with that of the C^{14} biosynthetically prepared protein. The result was unequivocal, and it is clear that if precautions that have been outlined are taken in the iodine-labeling procedure, then carbon- and iodine-labeled fibrinogens behave identically so far as their distribution in the body is concerned and are catabolized at the same rate. Since the behavior of the biosynthetic protein is probably also that of the native protein, it follows that at least 80% of the total fibrinogen in the rabbit is normally present in the circulating plasma. Similar studies with human I^{131} -fibrinogen, which will be reported elsewhere, indicate that this is also true in man.

Rate of catabolism. A half-life of 61 hours (Figure 10) corresponds to an elimination rate of 27.3% of the activity in the animal per day. Since 80% of the fibrinogen was present in the blood and with the assumption that catabolism of body fibrinogen takes place exclusively from this pool (5), then 34% of the plasma fibrinogen must be catabolized per day. A qualification to this calculation is that the use of the extrapolation device by which the proportion of circulating fibrinogen is determined requires that fibrinogen specific activities inside and outside the circulation after the preliminary equilibration period should be the same. This assumption, which may not be justified, can be avoided by a more rigorous approach (21) based on graphical resolution of the plasma curve into two exponential components having intercepts at 20% and 80% and half-lives of 6 and 61 hours, respectively. The fractional catabolic rate is now given by the inverse fraction of $0.8/0.273 + 0.2/2.77$, or 0.333 per day. The difference in the two values is small due to the fact that extravascular fibrinogen represents only one-fifth of the total and that the fractional rate of its transcapillary exchange is ten times greater than of its catabolism. Thus the extrapolation method first introduced by Sterling (22) and much criticized for estimating the size of the extravascular albumin pool gives, in the case of fibrinogen, a close approximation to the true value.

The rate of catabolism can be calculated in another way. After 24 hours a constant proportion of nonprecipitable activity was observed in the

plasma, suggesting that active iodide arriving in the body water as a result of protein catabolism at a steady fractional rate is exactly balanced by the iodide activity cleared by the kidneys, also at a steady fractional rate. This is not necessarily true, however, since in equilibrium conditions the animal is only known to excrete a constant mass of iodide per day. A more detailed mathematical treatment is required, and this is appended by Dr. P. Charlwood. It will be seen that his equation confirms the experimental result that the ratio of nonprecipitable to protein-bound activity in the plasma becomes constant with increasing values of time, but is not exactly equal to the quotient of catabolic rate and iodide excretion rate.

A term Y is involved which depends on the catabolic rate of the protein as well as on iodide parameters of the recipient animal, and some idea of its magnitude can be obtained by substituting mean experimental values. In the experiments described where undenatured fibrinogens were used, a mean plateau value of .022 was obtained for the ratio of nonprecipitable to protein-bound activity (X_2/X_1) in the plasma, and a mean value of 16.6 was obtained by Regoeczi in experiments already mentioned for the fraction of plasma iodide cleared per day (K_{25}). The product is 0.36 or 36% per day and represents a hypothetical catabolic rate. When Dr. Charlwood's equation is used along with a b_1 value for fibrinogen of 0.27 and representative values of 200 for K_{24} and 40 for K_{42} , the effect of the term Y is to reduce the hypothetical value by 10%. If a lower value is used for b_1 , for example, the values for albumin or γ -globulin in rabbits, the correspondence between hypothetical and true catabolic rates becomes closer. This suggests that for these proteins, both in rabbits and in man, the simple expedient of multiplying the renal clearance rate by the plateau value for the nonprecipitability ratio in the plasma gives a sufficiently close approximation to the true catabolic rate for most purposes. The generalization is also broadly justified that in animals with similar iodide parameters, the proportion of nonprecipitable activity in the plasma after the first day reflects directly the catabolic rate of the labeled protein.

SUMMARY

1. Rabbit fibrinogens labeled with 2 to 3 atoms of iodine per molecule were found to contain 20%

or more protein which was catabolized unusually rapidly.

2. I¹³¹-fibrinogens surviving in plasmas from rabbits which had digested this protein were catabolized at constant fractional rates of 30 to 40% of the plasma fibrinogen per day. They also partitioned between the plasma and extravascular space in a ratio of 4 to 1.

3. Fibrinogens labeled at mean substitutions of 0.5 atoms iodine per molecule behaved like the *in vivo* screened proteins.

4. Screened 0.5-atom fibrinogen behaved identically with C¹⁴-biosynthetically labeled fibrinogen in the same animal whether the proteins were injected intravenously or subcutaneously.

5. Only small amounts of subcutaneously injected fibrinogen passed back into the blood stream and much more was catabolized in the first few days when given in this way than when given by the intravenous route.

6. I¹³¹ released from protein combination by catabolism was found exclusively in the form of iodide, half or more of the activity so released in the first 24 hours being retained in the body water. After an initial equilibration period, the ratio of iodide- to protein-bound activity in the plasma was constant and approximately equal to the quotient of protein catabolic rate and renal clearance rate for iodide.

7. An equation is appended by which approximate protein catabolic rates can be calculated from plasma precipitability ratios if iodide excretion and distribution parameters of the recipient animal are known. Values obtained are within 10% of the true catabolic rates in the case of fibrinogens, and should be closer in the case of more slowly catabolized proteins such as albumins and γ -globulins.

8. Labeling with iodine, if proper precautions are observed, does not alter the clottability of fibrinogen nor detectably affect its biological behavior.

9. Eighty % of the total fibrinogen in the rabbit is confined to the plasma.

ACKNOWLEDGMENT

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MATHEMATICAL APPENDIX

By P. A. CHARLWOOD

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Theoretical treatments of the time dependence of the distribution of activity among a set of communicating compartments after initial injection of labeled material into one of them have been given by several authors. Rescigno (23) has a general solution for the following system of equations which applies under the conditions given below:

$$\frac{dX_i}{dt} = \sum_{j=1}^n K_{ji}X_j - \sum_{j=1}^n K_{ij}X_i \quad (i = 1, 2, \dots, n).$$

Here the subscripts denote compartments, X_i = fraction of injected activity (corrected for radioactive decay) in compartment i , and K_{ij} = fraction of the contents of compartment i passing into compartment j per unit time. K 's with subscripts i and j identical are zero. The case considered here is one in which an over-all steady state exists, i.e., total mass of protein (or breakdown products) in any given compartment is invariant, except in the final compartment, which corresponds with breakdown products excreted. These assumptions imply that the rate constants K are themselves independent of time.

In the model system treated here (Figure 12), intravascular protein and iodide compartments are shown separated, the unidirectional arrow connecting them implying that iodide is not reincorporated into protein. For the system

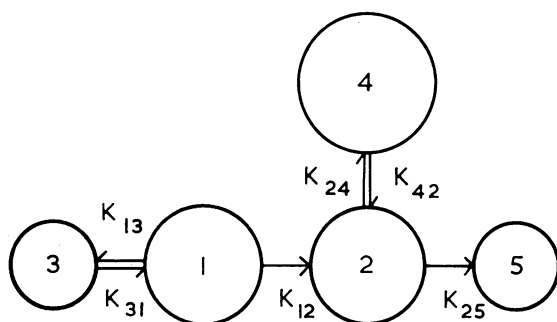


FIG. 12. INTERCONNECTIONS OF COMPARTMENTS WHICH REFER TO [1] INTRAVASCULAR PROTEIN [2] INTRAVASCULAR IODIDE [3] EXTRAVASCULAR PROTEIN [4] EXTRAVASCULAR IODIDE AND [5] EXCRETED IODIDE.

shown, Rescigno's solutions are expressed most succinctly in terms of the determinant:

$$\Delta = \begin{vmatrix} s + K_{12} + K_{13} & 0 & -K_{31} & 0 & 0 \\ -K_{12} & s + K_{24} + K_{25} & 0 & -K_{42} & 0 \\ -K_{13} & 0 & s + K_{31} & 0 & 0 \\ 0 & -K_{24} & 0 & s + K_{42} & 0 \\ 0 & -K_{25} & 0 & 0 & s \end{vmatrix}$$

Then

$$X_i = \frac{S_{n-1:i}}{S_{n-1}} + (-1)^{i+1} \sum_{p=1}^4 \frac{\Delta_{1:i}}{\Delta'} \bigg|_{s=-b_p} e^{-b_p t}.$$

The first term is unity for $i = 5$ and zero for the other values of i , $\Delta_{1:i}$ is the determinant obtained by omission of the first row and the i th column from Δ , and $\Delta' = d\Delta/ds$. The roots of $\Delta = 0$ are $-b_1, -b_2, -b_3$, and $-b_4$ in ascending order of (absolute) magnitude.

When t is sufficiently large, X_1 and X_2 can be represented with sufficient accuracy by the exponential term involving b_1 . The ratio X_2/X_1 is then the ratio of the appropriate coefficients, which is

$$\frac{K_{12}(K_{42} - b_1)}{K_{25}K_{42} - b_1(K_{24} + K_{42} + K_{25}) + b_1^2}.$$

As the rate constants relating to iodide are large in comparison with b_1 , negligible error is introduced if we write

$$\frac{X_2}{X_1} = \frac{K_{12}K_{42}}{K_{25}K_{42} - b_1(K_{24} + K_{42} + K_{25})}$$

or

$$X_{12} = \frac{X_2}{X_1} K_{25} \left\{ 1 - b_1 \frac{(K_{24} + K_{42} + K_{25})}{K_{25}K_{42}} \right\} = \frac{X_2}{X_1} K_{25} (1 - Y).$$

A more usual way of determining protein breakdown rate is to express the activity excreted per unit time as a fraction of the plasma protein activity. This, in the limit, measures $(1/X_1)(dX_5/dt)$. Since, however, $dX_5/dt = K_{25}X_2$, it follows that $(1/X_1)(dX_5/dt) = X_2K_{25}/X_1$, showing that the factor $(1 - Y)$ must be introduced in calculating the true breakdown rate. This factor seems inescapable in any method of assessing protein catabolism in which iodide activity in one part of the system only, for example in the plasma or excreted in the urine, is measured.

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