METABOLISM AND DISTRIBUTION OF FIBRINOGEN IN YOUNG AND OLDER MALE RABBITS

by

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Metabolism and Distribution of Fibrinogen in Young and Older Male Rabbits

Thesis directed by Professor E. B. Reeve

This investigation was undertaken to develop methods for studying and characterizing the behavior of fibrinogen in healthy animals. For this purpose, two groups, consisting of young (3 to 5 month) and older (6 to 12 month) rabbits, were selected. Homologous rabbit fibrinogen 93 to 97 per cent clottable was prepared by salt fractionation and labeled with I^{131}-iodide. After intravenous injection of the I^{131}-fibrinogen, the animals were studied for 8 to 10 days. Measurements of the plasma fibrinogen concentration by isotope dilution revealed fluctuations. Plots of the plasma I^{131}-fibrinogen radioactivity against time were fitted by a two or three exponential equation. The excretion of the radioactive breakdown products occurred without delay. A mathematical model based on both these observations and on current knowledge was advanced, and was found to describe the behavior of I.V. I^{131}-fibrinogen. The fractional rate constants were then calculated and used to determine the fluxes of fibrinogen. The transcapillary flux was 171 mg/kg/day in the young and 81 mg/kg/day in the older rabbits. The fibrinogen catabolic flux averaged 56.5 and 39 mg/kg/day in the young and older animals respectively. The interstitial to plasma fibrinogen ratio was 0.20 and 0.36 in the older and young rabbits respectively. The total plasma fibrinogen in 7 older rabbits averaged 106 mg/kg and the total interstitial fibrinogen 22 mg/kg

compared with respective values of 118 and 42 mg/kg in the young rabbit. Both, the transcapillary and catabolic fluxes behave as though governed by either an unsaturated or a volumetric clearance system.

This abstract of about 250 words is approved as to form and content. I recommend its publication.

Signed E B Reeve
Instructor in charge of dissertation

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INTRODUCTION

Properties of the Coagulation Reactants

The formation of fibrin in blood or plasma follows the action of thrombin on fibrinogen. Thrombin, in turn, is the product of a series of reactions not yet well understood. In what follows, the properties of fibrinogen, thrombin, and fibrin will be listed. This will be followed by a brief description of the nature of the reaction between thrombin and fibrinogen, the conditions which influence the reaction, and the physiological significance of their product, fibrin.

Fibrinogen

Fibrinogen has been defined as the plasma protein which forms fibrin when acted upon by thrombin. It has been found under normal conditions in the blood and in lymph, and in the spinal fluids and effusions or exudates in pathological conditions.

Fibrinogen makes up 3 to 6 per cent of the plasma proteins and is readily precipitated with neutral salts. It is heat labile; its isoelectric point occurs at pH 5.5 (1); and its molecular weight has been estimated to be near 350,000 (2). The shape of the molecule has been suggested to be intermediate between an ellipsoid and nodular rod (3). Electron photomicrographs show that the molecule is a rod 475 A° long with two terminal modules of 65 A° in diameter and a smaller centrally located nodule (4).

The aminoacid composition of fibrinogen has been tabulated (5)

and consists of 19 amino acids with terminal tyrosine and glutamic amino acids (6).

Thrombin

Thrombin is the natural coagulant of fibrinogen and manifests its activity only after activation of the clotting mechanism. Thrombin can be obtained experimentally from purified prothrombin in two ways. It can either be obtained by activating prothrombin with tissue (brain or lung) extracts in the presence of calcium ions and factor V to yield "biothrombin" (7), or by activation of prothrombin by high concentrations of citrate to yield "citrate thrombin" (3). The molecular weight of "biothrombin" has been estimated as 62,000 (3) and that of citrate thrombin as 31,000 (3). Thrombin preparations of 15,000 to 20,000 molecular weight without loss of clotting activity have also been described (8).

The functional activity of thrombin is that of a highly specific proteolytic enzyme (7). Its activity is destroyed by strong acids and alkalis and by heating to 60°C. In plasma, the activity of thrombin is neutralized by antithrombins (9). Antithrombins have been shown to neutralize enormous amounts of thrombin (10).

The most familiar substrate of thrombin is fibrinogen, though thrombin has been shown to hydrolyze the synthetic esters of p-toluene sulfonyl-L-arginine (TAME) (11); the lysine ethyl and methyl esters; and lysyl-alanine bond in the oxidized B chain in insulin (8). Disagreement as to significance of the esterase activity of thrombin exists, but some feel clotting and esterase activity are inseparable (12) while others consider that clotting activity functions to release

subunits from prothrombin which posses esterase activity (13).

The Nature of the Thrombin-Fibrinogen Reaction

The way in which the enzymatic action of thrombin leads to the formation of fibrin has now largely been clarified. The first effect of thrombin on fibrinogen is that of partial proteolysis with the rupture of arginyl-glycyl bonds in the fibrinogen molecule with the loss of negative charges due to the removal of peptides (14). The loss of negative charges from the fibrinogen molecules results in the formation of monomers which are led to polymerize by the electrostatic attraction which has replaced the mutual repulsion which existed (15). This polymerization process is thought to take place in stages which depend on the pH and ionic strength of the media. The first stage in polymerization is the end to end alignment of the monomers to form an intermediate polymer (4). At the later stage, the intermediate polymers undergo side to side aggregation to form the coarse fibrin strand seen with the light microscope. If the reaction takes place in the plasma, the fibrin strands undergo further changes. It has been shown that plasma contains a "fibrin stabilizing factor" which renders fibrin insoluble in urea (16). Evidence has been cited that this factor forms S-S bonds (17). The knowledge of the sequence of events leading to the formation of fibrin from fibrinogen has been the result of the work of Mihalyi (19), Lorand (15) (18), Bettelheim (19), Laki (20), Bailey (21), Scheraga (8), Hall and Slayter (4), and their co-workers.

Diagrammatically, the reactions may be listed as follows:

1) proteolysis $F \xrightarrow{T} f$ + Peptides,

2) polymerization $nf \longrightarrow fn$,

3) clotting $mfn \longrightarrow$ fibrin (soluble),

4) stabilization fibrin (soluble) $\xrightarrow{FSF}$ fibrin (insoluble),

where F is fibrinogen, T is thrombin, f is fibrin monomer, m and n are variable integers and FSF is the fibrin stabilizing factor.

Conditions Which Influence the Fibrinogen-Thrombin Reaction

The thrombin-fibrinogen reaction has been studied using both purified fibrinogen solutions and blood plasma.

Using purified fibrinogen solutions, many factors, both real or environmental, have been shown to alter the speed of the reaction. Most purified solutions contain a cold insoluble material (22) which has been termed "profibrin" (23). This profibrin reacts more rapidly with thrombin than with the dissolved fibrinogen (24). Thus, the presence of this material in fibrinogen solutions may influence the overall rate of reaction. Other factors known to influence the speed of the reaction have been discovered. Owren, for instance, found that the concentrations of fibrinogen and thrombin were important and suggested that the optimum fibrinogen concentration was 0.1 per cent when dilute solutions of thrombin were used (24). The concentration of salt in the media may also delay for formation of fibrin (25). Alterations of pH beyond the limit of 6.5 to 7.5 also have marked effects on the formation of fibrinogen and thrombin, and the concentration of calcium are other factors which affect the reaction.

Using plasma, on the other hand, many of the environmental factors are automatically controlled. The salt concentration and pH, for example, are fairly constant for most plasmas. Though these factors are well controlled in plasma, plasma may contain substances which will act on the newly formed or added thrombin and alter the rate of the reaction. Very little is known about the thrombin-fibrinogen reaction in plasma.

Properties of Fibrin and Its Physiological Significance

The physical properties of fibrin are well known. Clots formed by normal blood are able to withstand tensions of about 50 grams per square centimeter (26). Fresh fibrin has strongly adhesive properties and adheres to various surfaces such as glass, metals, and body tissues (27). Electron photomicrographs of fibrin show that fibrin is cross striated with a periodicity of about 230 A° with fine intermediate bands (4). Hall and Slayter (4) suggest that the darker bands correspond to the terminal nodules and the lighter ones to the central nodules of the fibrinogen molecule. If this interpretation is correct, the fibrinogen molecules have been shortened from 475 A° to 230 A° by the action of thrombin or during the polymerization process. As indicated above, fibrin formed from purified solutions is soluble in strong urea solution but that derived from plasma is insoluble in this solvent (16).

The main physiological function assigned to fibrin formation in vivo is that of hemostasis. Blood coagulation in addition to platelet agglutination, contraction of damaged vessels, and adhesion of the walls of minute vessels forms an integral part of

the hemostatic mechanism in mammals.

Another physiological function for fibrin has been postulated, though insufficient evidence in support of this view exists. It has been postulated that fibrin is continually formed within the blood vessels and that a fibrin layer lines the lumen of the vessels (28). Copley considers the fibrin to be essential for the integrity of blood vessels and that it aids in facilitating the flow of blood through the capillaries by reducing the apparent viscosity of blood (29). This idea of simultaneous clotting and fibrinolysis was originated by Nolf in 1908 and he regarded this process as a normal metabolic process (30). In support of this view of continuous formation of fibrin with deposition on the endothelial wall can be cited from the rapid turnover of clotting factors (31) (32) (33). If this view is true, then, the relative rates of fibrin formation and removal play an important part in the maintenance of the vascular patency and endothelial integrity and fibrinolysis then becomes an important antithrombotic activity. The rate of fibrin formation, therefore, must be delicately counterbalanced by the fibrinolytic activity to ensure no net depletion or accumulation of fibrin on the wall of the vessels. An excellent review on fibrinolysis has been published by Von Kaulla (34).

In Vivo Studies of Fibrinogen

Knowledge about fibrinogen has come largely from *in vitro* studies, for instance, of the changes in the rate of fibrin formation in reconstituted synthetic systems and from studies of abnormal

coagulation in disease. Awareness that blood removed from the circulation may not behave the same as circulating blood in the animal, has stimulated investigations of the coagulation system in the intact animal. With the advent of radioactive tracers of isotopes, one approach has been to label fibrinogen with the radioactive isotope and study its rate of disappearance from the animal's plasma. So far, however, only knowledge of the "turnover rate" and the "half-life" of fibrinogen in various animals has been derived from these studies (35) (36) (37) (38) (39).

Among the first to study the fibrinogen system in vivo were Madden and Gould (36). These investigators fed S^{35}-labeled amino acids to dogs and humans and then followed the rate of disappearance of labeled fibrinogen from the circulating plasma by isolating fibrin and determining the number of counts of radioactivity in a given mass of fibrin. They found the half-life in the dog was 4.2 days and in the human 5.6 days. Later, Cohen et al. (39) followed disappearance of C^{14} biosynthetically labeled fibrinogen and compared it with that of in vitro labeled I^{131}-fibrinogen in rabbits. They listed the mean half-lives of I^{131}-fibrinogen as 66 hours and C^{14}-fibrinogen as 68 hours in their rabbits. Other studies using C^{14}, S^{35}, and I^{131} tracer-labeled fibrinogen have been carried out by Dovey et al. (40), Lewis and Ferguson (36), Christensen et al. (37), Gerdes and Maurer (45), and Blomback et al. (41). In most instances, the half-life of the C^{14} and S^{35} labeled fibrinogen is longer than that of the I^{131}-fibrinogen. In all of these studies, the rate of disappearance of the labeled fibrinogen was obtained from specific activity measurements.

Evidence that S^{35} and C^{14}-labeled amino acids liberated by the catabolism of S^{35} and C^{14} tracer-labeled proteins are reutilized by the animal for tissue protein synthesis was presented by Walters et al. (42) and Fleischer et al. (43). These investigators, using doubly-labeled S^{35} and C^{14} homologous albumins and globulins, showed that the ratio of S^{35}/C^{14} in the tissue was 0.9 to 4.6 times greater than the S^{35}/C^{14} ratio of the albumin in the plasma. For doubly-labeled globulins, the tissue S^{35}/C^{14} ratio was from 1.1 to 6.7 times greater than the doubly-labeled plasma globulins. In Keratin, for instance, which contains large amounts of sulfur, the highest S^{35}/C^{14} ratios were found. Theoretically, the tissue to plasma ratio should be equal to 1.0 if S^{35} was not utilized more preferentially than the C^{14}-labeled amino acids which may undergo other metabolic fates than protein synthesis. Using doubly-labeled S^{35} and I^{131}-albumin, these investigators found 9 days after the intravenous injection of the protein that the tissue to plasma S^{35}/I^{131} ratio was 3 to 14 in the rabbit and 50 to 100 times in the rat. They attributed this difference to the more rapid metabolism in the smaller animal and to reutilization of the S^{35}-labeled amino acid for tissue protein synthesis.

These experiments point out, further, that the I^{131}-label is not utilized for tissue protein synthesis, thus, suggest a tracer to circumvent the problem of reutilization. Since the breakdown products of I^{131}-labeled proteins consist largely of inorganic I^{131}-iodide, however, some investigators suspected that this represented a deiodination process. Walters et al. (42) showed, however, that the ratios of I^{131}/S^{35} and I^{131}/C^{14} of doubly-labeled proteins in

the plasma decreased only slightly. This indicated that the disappearance of the I^{131}-labeled protein was not by simple deiodination and that it reflected the true metabolic rate of the protein. This slight decrease in the ratio, I^{131}/C^{14}, in the doubly-labeled plasma proteins can be attributed to slight reutilization of the C^{14}-labeled amino acids liberated after catabolism of the protein. McFarlane, also, has found good correlation in the decline of the specific activity of I^{131}/C^{14} doubly-labeled albumin and fibrinogen (44) (46).

Much of the work done using I^{131}-fibrinogen does not list the iodine atom to fibrinogen molecule ratio of iodine substitution. McFarlane (44) has shown that iodination of albumin with an iodine to protein ratio greater than 3.0 leads to altered biological behavior of the I^{131}-albumin. Over-iodination of fibrinogen may also lead to altered biological behavior of the I^{131}-fibrinogen.

Purpose of the Investigation and Requirements for a Comprehensive Study.

The purpose of this investigation is to establish a method for studying and characterizing fibrinogen in the living animal under normal conditions. This will provide a basis of comparison for later studies of fibrinogen metabolism in disease states and other abnormal conditions, and, it is hoped, will allow intelligent interpretation of such studies. Before a meaningful study of the metabolism and distribution of fibrinogen can be carried out in the living animal methods are required for purifying and labeling fibrinogen, for quantitating plasma fibrinogen, and for

establishing the permissible levels of iodine substitution in fibrinogen. For interpreting the results obtained, on the other hand, a schema of the fibrinogen system which can be described mathematically must be developed and evaluated using tracer labeled fibrinogen. If the tracer model describes accurately the data, the differential equations describing the system will be solved and their solutions used to carry out kinetic analysis of the data. In this manner, it is hoped that the dynamics of the fibrinogen system in the living animal can be characterized more completely.

CHAPTER I

Purification of Fibrinogen

Fibrinogen used for labeling with I^{131}-iodide for *in vivo* metabolic studies must be exceptionally pure and as nearly in the native state as possible.

Fibrinogen was first identified by virtue of its insolubility in concentrated salt solutions (49) and its instability to heat (48). Hammarsten in 1879 separated and purified this protein by simple salting out with half-saturated sodium chloride (22). For many years this was the method used (49) (50) (51) (52); other salts such as ammonium sulfate (53), potassium phosphate (54), and, more recently, the sodium salt of heparin (55) have also been used to purify fibrinogen. Other procedures used employed isoelectric (56), ethanol (57), and ether (58) precipitation and DEAE cellulose column chromotography (59).

The initial separation of fibrinogen from plasma using these methods is accomplished readily. Further purification of fibrinogen is made difficult by the ever-present danger of clotting, by the lability of the fibrinogen, and by the presence in the original crude precipitate of small quantities of material which is less soluble than fibrinogen. The danger of clotting, however, may be reduced somewhat by collecting the blood free of tissue fluids (60) and using excess anticoagulant, preferably sodium citrate. Further

procedures for the purification of fibrinogen should involve as few steps and require as little time as possible, and should avoid conditions and repeated use of reagents known to cause denaturation. The problem of material which is less soluble than fibrinogen can be dealt with during the later stages of purification when a reasonably pure fibrinogen preparation has been obtained.

The above considerations ruled out methods of purifying fibrinogen involving the use of acid pH, large surface areas, and organic solvents, since proteins have been known to be denatured by these conditions. Attention was, therefore, focused on methods employing serial precipitation plasma fibrinogen with neutral salts. After several attempts to obtain fibrinogen of high clottability by precipitation with sodium chloride and potassium phosphate had failed, these were abandoned in favor of ammonium sulfate precipitation. Applying serial precipitation to the fibrinogen with this salt, preparations containing 90 per cent clottable protein were obtained. The recovery of the plasma fibrinogen was low, however, and the procedure involves excessive manipulation (7 or 8 reprecipitations) of the protein. Morrison et al. (61) showed that the electrophoretic components present in Cohn Fraction I consisted of 7% albumin, 8% alpha-globulin, 15% beta-globulin, 9% gamma-globulin and 60% fibrinogen. Thus, it seemed reasonable to assume that these proteins would also be present in the crude fibrinogen-salt precipitate. Since all the proteins listed above, with the exception of fibrinogen, are soluble in one-quarter saturated ammonium sulfate, it was decided to extract them from the precipitate with this concentration of ammonium sulfate.

In this manner, fibrinogen preparations of good yield and containing 90 to 93 per cent clottable protein were obtained with less manipulation than serial precipitation. Further reprecipitations and extractions with this concentration of salt failed to improve the clottability of the fibrinogen preparations. Thus, it appeared as if a non-clottable material was present which was less soluble than fibrinogen in this concentration of ammonium sulfate. Morrison (62) in 1947, and Edsall (63) in 1948 noted that a cold insoluble material was present in the Cohn Fraction I and that it precipitated on cooling and dissolved on warming and behaved unlike fibrinogen in many respects. In view of this observation, fibrinogen preparations were placed in the cold at 4°C for 10 hours and the cold insoluble material which formed was centrifuged off at 0°C at 4,500 rpm for 20 minutes. This treatment increased the clottability of the fibrinogen preparations by as much as 6 per cent.

Method for Purifying Fibrinogen

The procedure developed for separating and purifying fibrinogen from platelet-free rabbit plasma was as follows: 30 to 40 ml of rabbit blood were collected by cardiac puncture from a donor rabbit, and immediately mixed with an equal volume of 2.5 per cent sodium citrate in polyethylene centrifuge tubes. The citrated blood was then spun at 2,000 rpm for 30 minutes and the platelet-free plasma was collected and made 1/4 saturated with ammonium sulfate by mixing three parts of plasma with one part of 100 per cent saturated (at 37°C) ammonium sulfate. At this concentration of salt, the fibrinogen precipitate was evident. After allowing the

precipitate to form for 30 minutes at room temperature (25-29°C), it was collected by centrifugation at 2,000 rpm for 10 minutes. The supernatant was discarded by decanting and the test tubes were inverted and allowed to drain over a clean paper tissue for 5 to 10 minutes. The occluded or adsorbed non-clottable proteins were extracted by suspending the precipitate in 10 ml of 1/4 saturated ammonium sulfate for 20 minutes at room temperature, (the precipitate was suspended by dispersing it with a clean, fire-polished, siliconized glass rod). Following two such extractions, the precipitate was dissolved in 15 ml of 0.005 M sodium citrate and reprecipitated by adding 5.0 ml of 100 per cent saturated ammonium sulfate to the solution. This time, the precipitate was allowed to form for 10 minutes instead of 30 minutes. After collection and further extractions of the precipitate with 1/4 saturated ammonium sulfate, the fibrinogen was dissolved in 2 to 3 ml of 0.005 M sodium citrate. The concentration of the fibrinogen solution at this point was near 12 mg/ml and the clottability was usually between 90 and 93 per cent. (If the clottability was below 90 per cent, the solution was diluted to 15 ml with 0.005 M sodium citrate and reprecipitated once more with 4.0 ml of 100 per cent saturated ammonium sulfate*). The preparation was then placed in the cold at 4°C for at least 3 but not more than 12 hours,

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* The clottability of fibrinogen preparations at this stage is not improved by further precipitations with 1/4 saturated ammonium sulfate. Therefore, it sometimes becomes essential to lower the concentration of this salt in order to improve the clottability of fibrinogen preparation.

The cold insoluble material was removed by centrifuging at 0°C and 4,500 rpm for 25 minutes. This yielded a preparation that was 94 to 98 per cent clottable with thrombin.

Criteria for Purity of the Fibrinogen Preparations

As implied above, the ratio of clottable to total protein was taken as a measure of purity. The basis for this assumption is that fibrinogen is the only known plasma protein which forms fibrin when treated with thrombin. Also, it has been shown that 95 to 97 per cent clottable fibrinogen is homogeneous in the ultracentrifuge (64) and it behaves as a homogeneous protein in starch gel and moving boundary electrophoresis. See Figures 1 and 2. The percentage clottability was determined by two independent methods of measuring the clottable and non-clottable protein and excellent agreement was obtained between the two. The methods for measuring the percentage clottability will be described later.

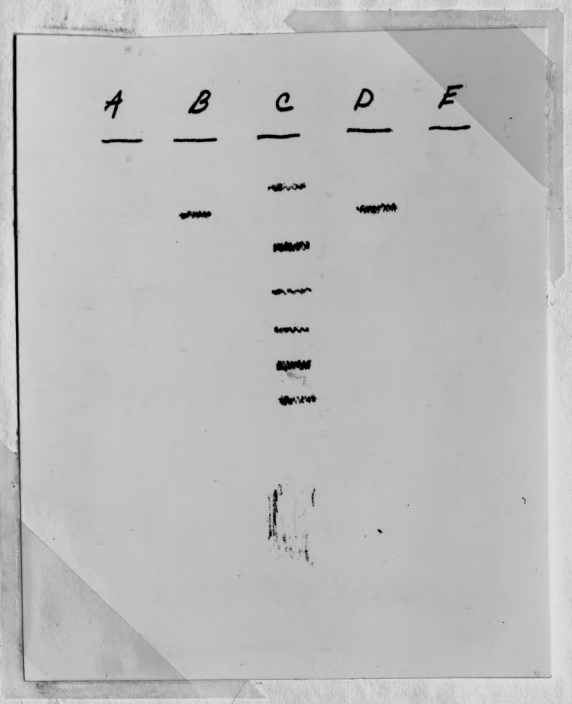

Figure 1. Electrophoretic patterns of fibrinogen which was 95% clottable (B and D) and blood serum (C) using starch gel medium in pH 8.5 borate buffer. (E = 180 volts, i = 5-6 ma, time = 19 hours)

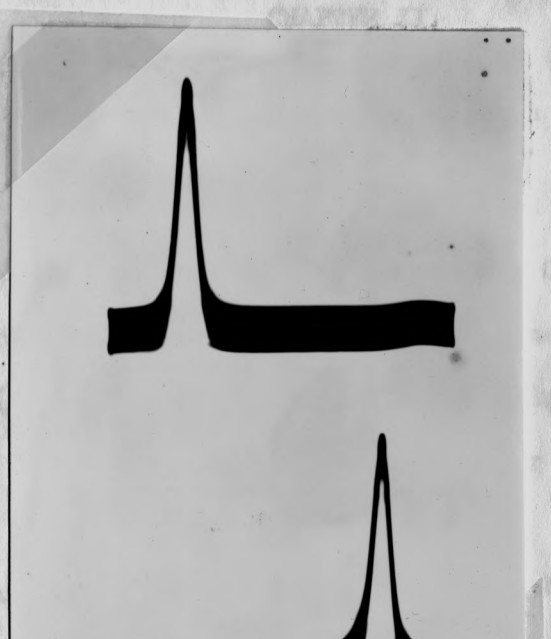

Figure 2. Photograph of an electrophoretic patterns of 1% fibrinogen (94% clottable) in pH 8.5 veronal buffer obtained by tiselius moving boundary method. (Ionic strength = 0.1-0.2, E = 155 volts, i = 8 ma, time = 3 hours)

CHAPTER II

Iodination of Fibrinogen

Various radioactive labels have been used to label proteins. Proteins have been labeled *in vivo* by feeding C^{14} or S^{35}-labeled amino acids or protein hydrolyzates to experimental animals and humans (35) (36). *In vitro* labeling of proteins has been accomplished mainly by using I^{131}-iodine under carefully controlled conditions.

The procedure involved when C^{14} or S^{35}-labeled proteins are used in metabolic studies is to remove the blood from an animal fed radioactive labeled amino acids or protein hydrolyzates after sufficient radioactivity is present in the plasma proteins (65). The desired protein is then fractionated and the purified labeled material is then injected into a recipient experimental animal. Such physiological labeling of proteins is highly desirable; however, it does suffer from the following distinct disadvantages: 1) the possibility of reutilization of the labeled amino acid breakdown products exists; 2) the labeling procedure as well as the counting procedure are time consuming and require expensive radiation detection apparatus; 3) the specific activity of the protein is usually low; consequently, large amounts of the desired protein have to be infused (65); and, 4) some plasma proteins appear to be synthesized from amino acid pools of higher specific activity (66),

and thus contain amino acids of a higher specific activity. Separation of a protein of low specific activity from one of higher specific activity may lead to denaturation and uncertainty about the satisfactoriness of the labeled material.

In vitro labeling of proteins has the following advantages over in vivo labeled proteins: 1) the methods are less time consuming and the protein is labeled with relative ease; 2) the desired specific activity can be introduced so that only traces of the labeled protein need be injected; 3) I^{131}-radioactivity measurement requires less expensive and complicated apparatus and accurate measurement is not difficult; and, 4) the I^{131}-protein breakdown products are quantitatively excreted (67).

Many methods of iodination of proteins have been developed. All of them utilize iodine in the plus (I^+) oxidation state as the active species. The active iodine has either been preformed as I_3^- or prepared by oxidation of the iodide with nitrous acid (68), iodate (69), or hydrogen peroxide (70) just before use. The iodination of proteins is usually performed by mixing the active species of iodine with the protein buffered at a pH of 8 to 10. At this pH, the hydroxyl group of the tyrosyl moiety present in the protein dissociates or ionizes creating an electron density ortho to the hydroxyl group and the electrophilic iodine (I^+) substitutes rapidly (71).

The use of oxidizing agents to oxidize iodide to iodine presents the danger of introducing traces of oxidizing agents which may attack the protein by oxidizing SH groups.

Recently, however, a method which offers the theoretical labeling efficiency of 100 per cent incorporation of the label into proteins was proposed by McFarlane (72). The method employs iodine monochloride, ICl, as carrier. This reagent is prepared in the absence of oxidizing or reducing agents in an excess of HCl. The radioactive iodine monochloride is made by exchange between the iodine monochloride and the radioactive I^{131}-iodide.

Preparation of Iodine Monochloride (72)

The iodine monochloride is prepared according to the following reaction discovered by Andrews in 1903 (73):

$$6HCl + 2\ NaI + NaIO_3 \longrightarrow 3ICl + 3NaCl + 3H_2O$$

In an excess of concentrated HCl (sp. gr. 1.18) the reaction forms mostly ICl instead of I_2 as is the case when dilute acid is used (73).

To prepare iodine monochloride, take 4.97 ml of 0.1 M $NaIO_3$ and mix well with 10 ml of 12 N HCl (sp. gr. 1.18) and add dropwise 1.00 ml of 1.00 M NaI with constant stirring. Transfer the yellow mixture to a glass stoppered graduate cylinder or volumetric flask containing 5.0 ml of chloroform, make to a 50 ml volume with distilled water and shake vigorously. Allow the chloroform layer to settle to the bottom of the cylinder. If the chloroform layer is pink, repeat the treatment with chloroform until it remains colorless. To remove the dispersed droplets of chloroform from the iodine monochloride solution, bubble air through the solution for at least an hour. The solution is 0.033 M in ICl and 3 N in HCl at the final dilution. This solution is exceptionally stable and reacts with proteins in the hypoiodite form only.

The hypoiodite ion is produced by raising the pH of the ICl to pH 8.5 or higher. Above the pH of 10 the ICl solution is unstable.

Removal of Hydrogen Peroxide and Sulfite from NaI^{131}

The removal of peroxide and sulfite is in accordance with the following reaction:

1) $SO_3^{--} + H_2O_2 \longrightarrow SO_4^{--} + H_2O$

2) $SO_3^{--} + 1/2\ O_2 \xrightarrow[pH\ 7.0]{100°C} SO_4^{--}$

The presence of peroxides in aqueous solutions of radioactive material is expected as a result of ionizing radiation on water. However, Helmkamp was first to produce evidence that peroxides were present in sodium I^{131}-iodide obtained from Oakridge (74). He found that an I^{131}-iodide solution of 37.5 millicuries per militer contained 1×10^{-4} m mols of H_2O_2 eight days after it had been produced. In view of this finding, sulfite was added routinely to shipments of NaI^{131} upon arrival. Usually 0.10 ml of 0.05 M Na_2SO_3 was added.

Before using the radioactive material to label proteins the excess sulfite should be removed. The sulfite was removed by reaction 2. It was found that bubbling air through the radioactive iodide at boiling water temperature and neutral pH removed up to 10 mg of sulfite in 20 minutes with little or no loss of radioactivity. The aparatus used to remove the sulfite is shown in Figure 3.

Preparation of $I^{131}Cl$

This solution was prepared by simply mixing the calculated amount of ICl with the desired amount of peroxide and sulfite-free NaI^{131}.

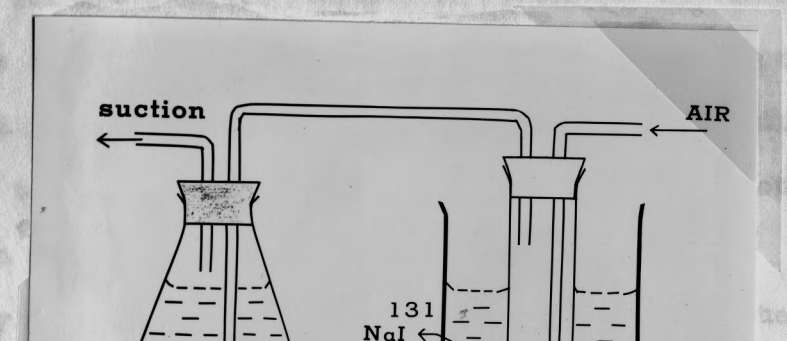

Figure 3. Apparatus for removing sulfite from radioactive I^{131}-iodide solutions.

The solution should be allowed to equilibrate for at least 30 minutes before using it to label proteins.

Iodination of Fibrinogen with ICl

A small volume of sodium iodide-I^{131} of high specific activity and free of peroxides and sulfite was used. The ICl and the radioactive iodide were mixed and cooled to 0°C in an ice bath. The amount of ICl added to the radioactive iodide was calculated to obtain the desired ratio of iodine atoms to protein molecules. Thus, if E is the efficiency of the iodination and m_1 iodine atoms are added to m_2 protein molecules, the iodine labeled protein will contain an average of E m_1/m_2 iodine atoms per molecule. The efficiency of iodination by the method described below when $m_1=m_2$ was 30 to 50 per cent. Hence, when 0.01 ml of 0.0033M ICl is added to 11 mg of fibrinogen (M.W 350,000) the number of iodine atoms is approximately equal to the number of fibrinogen molecules, and the final iodinated protein will contain an average of less than 0.5 atoms of iodine per molecule of fibrinogen.

To label fibrinogen of 93 to 98 per cent clottability, take 11 to 15 mg of fibrinogen in a volume of from 0.5 to 1.0 ml, cool the solution to 0°C in an ice bath, and make to pH 9.0 with 0.2 ml of 1.0 M glycine buffer (v/v 8 parts M glycine in 1.5 per cent NaCl to 2 parts N NaOH). At the same time, the iodine monochloride-I^{131} mixture is brought to pH 8.5 with 0.2 ml of 1.0 M glycine buffer (v/v 9 parts M glycine to 1 part N NaOH). The protein and $I^{131}Cl$ are then jet mixed by forcible injection of the $I^{131}Cl$ through a narrow glass nozzle or Pasteur pipette into the protein solution.

The substitution of iodine into the protein is instantaneous. The volume of the solution is now immediately brought to 6.0 ml with 0.005 M sodium citrate and the fibrinogen is precipitated by adding 2.0 ml of saturated ammonium sulfate. The unbound I^{131} which remains in the supernatant after centrifugation is removed as completely as possible. The precipitate is then suspended in 1/4 saturated ammonium sulfate and centrifuged down. This step is repeated till less than 1 per cent of the total activity in the solution is not protein bound. The unbound activity is that activity which is not precipitable with 10 per cent trichloroacetic acid.

Determination of Clottability

Percentage clottability was used as a measure of the purity of the fibrinogen preparation. It was measured either from the change in soluble I^{131}-fibrinogen content or the change in soluble protein content after the addition of thrombin solution.

Two equal volumes of a fibrinogen or I^{131}-fibrinogen preparation were diluted to 4.0 ml with 0.005 M citrate. In one, the radioactivity (cpm/ml) of the solution was measured and to the other 0.2 ml of thrombin (20 NIH units/ml in 50 per cent glycerol) was added. After some five minutes, the opaque fibrin clot was collected on a glass rod as described below. The solution was then allowed to sit for one hour longer, in order to allow the thrombin-fibrinogen reaction to proceed to completion, and any additional fibrin which formed during this time was also collected. The radioactivity (cpm/ml) of the clot liquor was then measured. The per cent

clottability (%C) was then given by:

$$\%C = \frac{100\ (R_1 - R_2)}{R_1}$$

where R_1 = radioactivity (cpm/ml) before the addition of thrombin

and R_2 = radioactivity (cpm/ml) after the addition of thrombin and removal of the fibrin clot.

When the protein method was used, equal volumes of the fibrinogen preparation or labeled I^{131}-fibrinogen were diluted as described above with 0.005M citrate. Then the optical density, e_1, of the fibrinogen solution was measured at 280 mu wave length in one cm optical path length, quartz, cuvets. To the other test tube and a blank (4.0 ml of citrate) was added 0.2 ml of bovine thrombin. The fibrin was collected as described above and the optical density, e_2, of the clot liquor was measured against the citrate blank. The %C was then calculated as follows:

$$\%C = 100\ \frac{(e_1 - e_2)}{e_1}$$

Excellent agreement between the clottability measurements by both methods was found. See Table I.

TABLE I

CLOTTABILITY RATIOS OF FIBRINOGEN AND I^{131}-FIBRINOGEN PREPARATION: BEFORE AND AFTER IODINATION

	Optical Density e_1	Optical Density e_2	Change in optical Density $e_1 - e_2$	Ratio $\frac{e_1 - e_2}{e_1}$	C.P.M. Fibrinogen R_1	C.P.M. in Clot liquor R_2	$R_1 - R_2$	$\frac{R_1 - R_2}{R_1}$
1	.855	.023	.832	.978	1,045,000	43,000	1,002,000	.959
2	.545	.019	.526	.965	927,000	39,000	888,000	.958
3	.578	.033	.545	.943	614,000	33,170	586,630	.946
4	.880	.041	.839	.953	564,600	25,020	539,600	.956
5	.738	.025	.713	.965	86,800	7,650	79,150	.920
6	.591	.031	.560	.948	75,276	3,350	71,726	.958
7	.610	.050	.560	.918	55,276	2,962	52,113	.946
8	.815	.041	.774	.950	695,500	38,860	656,640	.944
9	.650	.021	.629	.968	271,000	15,360	255,640	.943
10	.798	.024	.774	.969	952,000	42,350	909,650	.955
11	.859	.036	.823	.958	349,500	35,780	313,720	.940
Mean				.956				.948

CHAPTER III

Isotope Dilution Method for Measuring Plasma Fibrinogen

For studies of the metabolism and distribution of fibrinogen in animals, an accurate method of quantitating the plasma fibrinogen concentration was required. The current methods, the better of which are listed in Table II do not give accurate results. These methods depend on separating the fibrinogen from the plasma by heat coagulation, salt precipitation or fibrin formation and then determining the quantity of the separated protein by weight, or chemical or physical measurements. To determine the accuracy of these methods, measurements of the completeness of fibrinogen separation from plasma and of the extent of occlusion of other plasma proteins in the separated fibrinogen were made, using the three most often cited principles for separating fibrinogen.

Completeness of Fibrinogen Separation from Plasma by Heat Coagulation, Thrombin, and Salt Precipitation

This was measured by adding a small amount of I^{131}-fibrinogen which was 96 per cent clottable, before and after labeling with I^{131}, to a number of citrated rabbit plasma samples. The fibrinogen was then separated from the plasma samples by standard methods and the amount of radioactivity remaining in the plasma after removal of the fibrinogen precipitate or fibrin was measured. This radioactivity measurement was then corrected for the percentage, %C, of

TABLE II

METHODS FOR MEASURING FIBRINOGEN

Method Isolating Fibrinogen	Method of Quantitation	Investigator
1) Heat Coagulation	dry weight	Fredericq (78)
	Nitrogen content	Gram (79), Whipple (75), Lewis and Ferguson (35)
	Turbidimetry	Stirland (76)
2) Salt Precipitation	dry weight	Rehye (77) and Lewinski (80)
	Nitrogen content	Howe (81), Florkin (82), Campbell and Hanna (83)
	Turbidimetry	Podmore (84)
3) Thrombin - induced Fibrin Formation	dry weight	Szecsenyi-Nagy (85) Foster and Whipple (86)
	Nitrogen content	Cullen and Van Slyke (87) Howe (88) and Jones and Smith (89)
	Tyrosine content	Wu (90), Wu and Ling (91), and Ratnoff (92)
	Turbidimetry	Losner et al. (92) and Klinke and Elias (94)
	Clotting time	Adamis (95)

the fibrinogen preparation that was not clottable.

Then, $$\frac{\%C \times \text{cpm separated from the plasma}}{\text{cpm of } I^{131}\text{-fibrinogen in the plasma}}$$

gives the percentage removal of fibrinogen from the plasma. After adding the I^{131}-fibrinogen to each plasma sample, the sample was then divided into three portions. The fibrinogen in the first portion was heat coagulated as described by Lewis and Ferguson (35), that in the second was treated with bovine thrombin as described by Ratnoff (92), and the fibrinogen in the third portion was precipitated with one-quarter saturated ammonium sulfate, after the plasma had been diluted ten-fold with 0.005 M sodium citrate. Measurements were made in triplicate. Table III records the results and shows that an average of 19.5, 11.0, and 54 per cent of the total fibrinogen was not separated by heat, thrombin and ammonium sulfate treatment respectively; or with an average fibrinogen level of 300 mg per 100 ml of plasma, 59, 33, and 150 mg per 100 ml were not separated.

Occlusion of Proteins Other than Fibrinogen in Separated Fibrinogen or Fibrin

The proteins in rabbit blood serum were labeled with I^{131}-iodide and small amounts of the labeled serum were added to a series of citrated rabbit plasmas. The samples were again divided into three portions and the fibrinogen was separated by heat coagulation and fibrin formation as described above. (Studies on ammonium sulfate precipitation of fibrinogen were not done because of the large errors noted above.) The coagulum and fibrin were then separated by centrifugation, washed three times with 0.9 per cent NaCl solution

TABLE III

PERCENT SEPARATION OF FIBRINOGEN FROM PLASMA BY HEAT, BY THROMBIN AND BY ONE-QUARTER SATURATED AMMONIUM SULFATE

Sample No.	Heat Coagulation	Thrombin	Ammonium Sulfate
1	79.4%	88.7%	75%
	78.8	89.9	40
	78.8	90.2	52
	80.3	87.3	42
	80.2	88.3	51
2	79.0	89.4	35
	82.4	89.1	39
3	82.8	89.3	33
	83.0	88.6	50
Mean	80.5	89.0	46.3

and counted in well scintillation counter. The radioactivity found in the separated fibrin or precipitate was taken as an approximate measure of occluded protein. Table IV shows the results of these experiments when the occluded protein was calculated from:

$$\frac{\text{cpm of } I^{131} \text{ found in precipitate or fibrin} \times S}{\text{cpm of } I^{131} \text{ per ml of plasma}}$$

where S is the average serum protein concentration per ml and was taken as 70 mg/ml. It is seen that an average of 1.36 mg of protein was occluded in the heat coagulum from 1.0 ml of plasma and 3.66 mg in the fibrin clot formed from 1.0 ml of plasma, or roughly speaking, the fibrinogen concentration would be 1.45 and 2.22 times its true value by the two methods respectively.

It is thus clear that incomplete separation of fibrinogen, on the one hand, and occlusion of other plasma proteins on the other, cause serious errors in measurements of plasma fibrinogen concentration. The errors are in opposite directions and will only by chance cancel each other out.

Isotope Dilution Method

A possible way of circumventing the difficulties of incomplete separation and protein occlusion in the determination of fibrinogen is to use the isotope dilution principle. In accordance with this principle, if a small quantity, r_I, of pure radioisotope in a volume V_I is mixed thoroughly with an unknown quantity, Q, of non-radioisotope contained in a given volume, V_s, one can readily determine the quantity of Q provided the substance can be isolated in pure form. For if the radioisotope is evenly distributed and behaves

TABLE IV

PERCENT OF OTHER SERUM PROTEINS* OCCLUDED IN FIBRIN CLOTS AND HEAT PRECIPITATES

Sample No.	Heat Coagulation	Thrombin Treatment
1	3.5%	6.1%
	1.6	3.8
	1.7	3.6
2	1.5	5.0
	1.6	11.3
	2.4	4.4
3	1.8	3.3
	1.8	4.9
	1.6	4.7
Mean	1.9	5.2
Calculated weight of occluded non-fibrinogen protein per 100 ml of plasma:	136 mg	366 mg

*Expressed as per cent of total non-fibrinogen proteins present. The total serum protein was taken as 70 mg/ml.

identically as the rest of the non-radioactive atoms, the material need not be isolated completely, because any fraction of the material isolated will contain the same ratio of radioactive to non-radioactive atoms. Thus, if one measures the radioactivity per unit volume, N_I (cpm/ml), of the mixture and the radioactivity per unit mass, N_2 of the isolated substance (cpm/mg), the quantity, Q (mg/ml), can be calculated from the following equation:

$$Q = (N_I/N_2 - r_I/V_I + V_s)\,(V_I + V_s)/V_s$$

where Q = mg/ml of substance being measured
N_I = cpm/ml of solution
N_2 = cpm/mg of substance isolated (specific activity)
r_I = mg of radioisotope added
V_I = volume of the radioactive solution added (ml)
V_s = volume of the solution containing the non-radioactive substance (ml)

The above equation contains corrections for dilution from the addition of V_I, and for the addition of the radioactive material r_I. This principle cannot be applied to the quantitation of proteins readily, since only few proteins can be isolated in pure form. However, fibrinogen can be converted to fibrin and fibrin can be isolated reasonably free from other plasma proteins. Therefore, if a small quantity, f_I of I^{131}-fibrinogen containing the optimum amount of iodine atoms per molecule of fibrinogen, is thoroughly mixed with the unlabeled fibrinogen in a volume V_p of plasma containing F mg/ml of fibrinogen, the quantity F can be calculated as described above. On the one hand, the quantity N_I (cpm/ml) in the plasma is measured directly, and on the other, the quantity N_2 (cpm/mg) of fibrinogen can be determined from fibrin. That is:

$$F = C\,(N_I/N_2 - f_I/V_I + V_p)\,(V_I + V_p)/V_p = \text{mg/ml of plasma fibrinogen}$$

where C = the fractional clottability of the I^{131}-fibrinogen

N_I = cpm/ml of plasma

N_2 = cpm/mg of fibrin

f_I = mg of I^{131}-fibrinogen added to the plasma

V_I = the volume of I^{131}-fibrinogen solution added

V_p = volume of plasma

This formula can be used to calculate the quantity of fibrinogen but for <u>in vitro</u> measurements the parameters f_I, V_I, and V_p have to be measured accurately. For <u>in vivo</u> studies, however, since the quantities f_I and V_I are very small compared to the total quantity of fibrinogen and the plasma volume of the experimental animal, the equation above can be simplified to:

$$F = C\,(N_I/N_2) \quad \text{because} \quad f_I/V_I + V_p \cong 0,$$

$$\text{and} \quad \frac{V_I + V_p}{V_p} \cong 1.0$$

for all practical purposes.

<u>Method</u>

The experimental rabbits were injected with 25 to 30 uc of I^{131}-fibrinogen containing 4 to 8 mg of fibrinogen. The volume of radioactive fibrinogen injected was usually 2 to 3 ml.

The radioactivity, N_I, in one militer plasma was measured directly by simply removing a small volume (.100 to .200 ml) of plasma, placing it in a counting vial, making it up to 1.0 ml volume with 0.1 N NaOH, and counting the radioactivity in a gamma scintillation counter.

The specific activity, N_2 (cpm/mg), measurements, on the other hand, were more involved. Usually 0.5 ml of radioactive plasma was taken and diluted to 6.0 ml with 5.5 ml of 0.005 M citrate. The fibrinogen in the solution was then precipitated by adding 2.0 ml of 100 per cent saturated ammonium sulfate. After agitation, the precipitate was allowed to stand at room temperature for at least 30 minutes or in the cold for as long as 12 hours. The precipitate which formed during this time was separated by centrifugation at 2,000 rpm for 10 minutes. After discarding the supernatant, the precipitate was washed twice with 8 ml volumes of 1/4 saturated ammonium sulfate. The supernatant from the last washing was removed as completely as possible in order to reduce the quantity of salt present, which may interfere with the action of thrombin on fibrinogen (25). After this treatment, the precipitate was dissolved in 4 to 5 ml of 0.005 M citrate and clotted with 0.2 ml of bovine thrombin solution (20 NIH units/ml), containing 6 to 10 ug of protein. The fibrin was collected as it formed on a glass rod and the fluid in the clot was expressed from it by rotating the glass rod and at the same time squeezing the clot between the rod and the test tube wall. The fibrin was then washed by first dipping the glass rod in 5 to 10 ml of 0.9 per cent NaCl and allowing it to stand for 20 to 30 minutes. The glass rod and fibrin were then placed in distilled water for another 20 to 30 minutes. When necessary, the fibrin was stored at temperatures below zero till the time for analysis.

The specific activity, N_2, of the fibrin was determined after the fibrin had been dissolved in 40 per cent alkaline urea

solution (40 per cent urea in 0.2 N NaOH). First the radioactivity of the fibrin-urea solution was measured directly by transferring to a counting vial 1.00 ml, accurately measured, of urea-fibrin solution. The concentration of fibrin in the urea was obtained from measurements of the density of the alkaline urea-fibrin using 1 cm optical path length quartz cuvets.

Fibrin prepared from purified fibrinogen solutions dissolved in alkaline-urea and measured within one to four hours of solution has an extinction coefficient, $E^{1\%}_{1cm}$, at 282 mu wave length of 16.17 (ie. the alkaline urea containing 1 mg of fibrin per ml of solution has an optical density of 1.617). Thus, the concentration, f, of fibrin in alkaline urea solution was determined from the optical density, e, of the solution and the extinction coefficient according to the equation $f = e/1.617$ (mg/ml). The radioactivity r_a of the dissolved I^{131}-fibrin was measured by counting 1.00 ml of the urea-fibrin solution in a gamma scintillation counter.

Having measured the concentration, f, and the radioactivity, r_a, of the dissolved fibrin, the specific activity N_2 is found from:

$$N_2 = f/r_a \text{ (cpm/mg)}$$

Knowing the radioactivity, N_I(cpm/ml), in the plasma and the specific activity N_2 of fibrin, F, the concentration of plasma fibrinogen, is found from:

$$F = C\ (N_I/N_2)$$

Test of the Isotope Dilution Method

a) The Extinction Coefficient of Rabbit Fibrin To determine the extinction coefficient of rabbit fibrin, fibrin was prepared from a purified fibrinogen solution of high clottability. The rabbit fibrinogen was clotted with thrombin (6 to 10 ug) and dialyzed free of salts, dehydrated by treatment with a (v/v 3 parts to 2 parts) mixture of ethanol and ether and dried in an oven at 110°C for 24 hours. The bovine fibrin was prepared from a 1 per cent solution of Armour bovine fibrinogen dissolved in 0.005 M sodium citrate, and then treated exactly as the rabbit plasma. Weighed portions of dry fibrin were dissolved in alkaline urea solutions and the extinction coefficients were measured in the Beckman DU spectrophotometer at 282 mu wave length. Reasonable agreement was obtained between the values of $E^{1\%}_{282\ mu}$, found by Blomback and Blomback (96) and those listed in Table V.

b) Errors Arising from Occlusion of Other Plasma Proteins Blood serum and purified gamma globulin were labeled with I^{131}-iodine and mixed with heparinized rabbit plasma. The fibrinogen was then separated, purified and clotted as described above and the radioactivity in the fibrin was estimated. Table VI shows that small amounts of other serum proteins are occluded in the fibrin clot, and that errors from this cause are, therefore, negligible.

c) Recovery of Fibrinogen from Plasma, 0.005 M Citrate, and Reconstituted Serum Measured amounts of purified fibrinogen were added and mixed with 0.005 M citrate, commercial reconstituted serum (Labtrol-Dade reagent) and to citrated and heparinized plasma.

TABLE V

EXTINCTION COEFFICIENT OF FIBRIN IN ALKALINE UREA SOLUTION AT 280 mu

Species	Number of Measurements	Extinction Coefficient Mean and Range	Reference
Rabbit	5	16.09	-
		15.99 to 16.32	
Bovine	3	16.59	-
		16.36 to 16.88	
Bovine	-	16.17	Blomback and Blomback (96)

The extinction coefficient $E^{1\%}_{282} = \frac{\text{Log}_{10} I_o/I_t}{1 \text{ cm } (f_n)}$; where,

1 cm = light path length,

f_n = concentration of fibrin (10 mg/ml) in alkaline urea,

$\text{Log}_{10} I_o/I_t$ = optical density at 282 mu wave length,

I_o = incident light,

I_t = transmitted light.

TABLE VI

PER CENT OF CONTAMINATING TOTAL SERUM PROTEINS AND GAMMA-GLOBULIN FOUND IN FIBRIN CLOTS AFTER SEPARATION OF THE FIBRINOGEN FROM PLASMA AS IN THE ISOTOPE DILUTION METHOD

Sample No.	Occluded total Serum Protein	Occluded Gamma-globulin**	Occluded Protein* in mg/100 ml
1	0.17%	-	12 mg
2	0.35	-	24
3	0.30	-	21
Mean	0.27	-	19
4	-	0.5%	5 mg
5	-	1.1	11
6	-	0.9	9
Mean	-	0.8	8

*Calculated from an initial plasma total protein concentration of 7 gram/100 ml.

**Calculated from an initial plasma gamma-globulin concentration of 1 gram/100 ml.

The quantity of fibrinogen present initially in the plasma was measured by the isotope dilution method, and that present in the purified fibrinogen solution was determined from the clottability and the optical density at 282 mu in alkaline urea. A mean of 101 per cent of the added fibrinogen was recovered as determined in 33 analyses, and the precision of a single measurement was plus or minus 3.75 per cent. See Table VII.

The isotope dilution method was, therefore, considered satisfactory for quantitating the concentration of fibrinogen in plasma. It has a reasonably high precision, performs well as tested by recovery experiments, is sensitive, and does not suffer from the two major errors that beset the previous methods of measurements of fibrinogen, namely, losses of fibrinogen during its isolation and occlusion of contaminating proteins in the separated fibrinogen or fibrin. Further, it is not affected by anticoagulants such as heparin since these are removed during the fractionating process.

TABLE VII

RECOVERIES OF PURIFIED FIBRINOGEN ADDED TO CITRATE SOLUTION, RECONSTITUTED SERUM AND PLASMA

Experiment No.*	Initial Solution	Fibrinogen Concentration mg/ml	Fibrinogen Added mgs	Fibrinogen Found mg	Percent Recovery
1	.005M Citrate	0	3.53	3.77	107
2	.005M Citrate	0	3.45	3.55	103
3	.005M Citrate	0	2.04	2.04	100
4	.005M Citrate	0	3.89	3.91	100
5	.005M Citrate	0	1.74	1.81	104
6	.005M Citrate	0	3.82	3.76	99
7	Reconstituted Serum	0	2.00	2.22	111
8	Reconstituted Serum	0	3.95	4.08	103
9	Blood Plasma	.89	1.69	2.55	99
10	Blood Plasma	.89	1.69	2.47	96
11	Blood Plasma	.89	3.38	4.08	98
Mean					101

*Mean of triplicate analyses standard deviation ± 3.75%.

CHAPTER IV

Tracer Studies With I^{131}-Fibrinogen in Rabbits: Standard Procedure

The experimental animals were white New Zealand male rabbits weighing from 1.6 to 3.7 kilograms. The young animals were 3 to 5 months old, and the older animals were 6 to 12 months old. They were fed Purina Chow and fresh greens, and food and water were given ab libitum. Two weeks before, and during the course of the experiment the animals were housed in individual metabolic cages. For three days before and during the experiment their drinking water contained 200 mg of potassium iodide and 1.8 grams of sodium chloride per liter to prevent I^{131}-iodide recycling and to encourage urinary flow.

The metabolic cages were of stainless steel with screened floors of mesh small enough to retain feces and yet permit the passage of urine into collecting receptacles. Daily collections of the urine and feces were made. The urine was collected in a polyethylene jar containing 20 to 30 ml of 1 per cent potassium iodide solution and 2 to 5 ml of toluene preservative. At the time of the urine collection, the cages were rinsed out with 200 ml of 1 per cent potassium iodide solution and the rinsings were added to the urine. The feces were homogenized in a Waring blendor in 100 to 150 ml of distilled water containing a few crystals of potassium iodide. The homogenized feces were then transferred to a large counting bottle

and made to 300 ml with distilled water for counting.

Two to four ml containing 1.5 to 4 mg of I^{131}-fibrinogen were injected, usually within one hour after iodination, into the marginal ear vein of the rabbits. The quantity of radioactivity injected was determined from the weight of the syringes before and after filling, less the residual volume determined from the activity washed out of the syringes by 0.1N NaOH. Duplicate standards for counting were prepared by diluting portions of the I^{131}-fibrinogen solution with carrier serum and dilute NaOH solution. Following the injection, heparinized blood samples of 2 to 3 ml were withdrawn at accurately noted times over a period of 8 to 10 days. Some or all of the following measurements were made: hematocrit values, plasma total protein concentration, plasma fibrinogen concentration, and the radioactivities of whole plasma, separated plasma fibrinogen and of the protein-free plasma. The plasma total protein concentration was determined by the $CuSO_4$ method of Van Slyke (97), and the plasma fibrinogen concentration was measured by the isotope dilution method described above. The radioactivities of the plasma, urine, feces, and fibrin were all measured with a Nuclear Chicago well scintillation system consisting of a Model 1810 radiation analyzer, a 2-inch sodium iodide crystal, and a Model 183 binary scaler. The number of counts of radioactivity in the samples was corrected for I^{131} decay by comparing them to radioactive standards prepared from the I^{131}-fibrinogen injection material.

CHAPTER V

Tests of Iodine-Labeled Fibrinogen

A sensitive method for testing the quality of iodinated proteins is to measure the I^{131}/C^{14} count ratio of injected doubly-labeled plasma proteins over a period of time in experimental animals. For this test, the I^{131}-protein is injected intravenously into a donor animal that is at the same time started on a diet containing C^{14}-labeled amino acids. After the incorporation of sufficient C^{14}-amino acids into the protein, the doubly-labeled protein is isolated, purified, and reinjected I.V. into a recipient animal. The decline in the specific activity of the doubly-labeled material is then studied and the changes in the I^{131}/C^{14} count ratio are calculated.

A second method is to use "biologically screened" I^{131}-labeled proteins. This method is based on McFarlane's (44) observations that over-iodinated or altered proteins are removed rapidly from the circulation. The method consists of injecting I.V. I^{131}-labeled proteins into a donor animal, removing some of the donors plasma after 1 to 2 days, then partially purifying the protein under study and transferring this or some of the donors plasma to a second animal. If the plasma radioactivity curves differ from those of the second animal given the unscreened I^{131}-preparation, it can safely be assumed that the donor animal has recognized and removed a portion of the injected material that was different from

the rest of the labeled molecules. The transferred protein or plasma is referred to as "screened" labeled protein by McFarlane (44). If the labeled protein removed by screening is also rapidly catabolized, the body fluids and urine of the animal given the unscreened protein will early show higher levels of radioactivity unbound to protein than the body fluids and urine of the animal given the screened I^{131}-protein.

McFarlane (44) has shown, using both of these methods, that for plasma albumin only certain levels of iodine substitution are permissible if the I^{131}-labeled albumin is to mimic the behavior of C^{14}-biosynthetically labeled albumin or screened I^{131}-albumin. In view of this finding and because fibrinogen is less stable than albumin, various levels of iodine substitution were tested to determine the optimum level of iodination of fibrinogen. The criterion used was to compare the behavior of the in vivo screened I^{131}-fibrinogen with that of unscreened I^{131}-fibrinogen. For this test, young rabbits were chosen since they eliminated the labeled material more rapidly than older rabbits.

Effect of Overlabeling Fibrinogen with Iodine

To study the effect of over-iodinating fibrinogen, fibrinogen was labeled at various levels of iodine substitution, which are defined by the I/P ratios. An I/P ratio of 1.0 indicates an average substitution of one iodine atom per fibrinogen molecule. The I/P ratio of I^{131}-fibrinogen was varied from 0.3 to 8.0. This material was then injected intravenously into animals of comparable size, age and sex and its behavior was compared with that of screened

I^{131}-fibrinogen preparations with an I/P ratio of 0.5.

Figure 4 shows the results. The values of the screened fibrinogen are represented by the small open circles and the top shaded area represents the range of variation of the values from three different animals given the same screened preparation. The stars, *, on the graph represent measurements made on rabbits given unscreened I^{131}-fibrinogen with an I/P ratio of ≤ 0.5. The lower points indicated by the dark dots and the more darkly shaded area represent measurements made on animals given unscreened I^{131}-fibrinogen with an I/P ratio ≥ 1.0.

Inspection of the figure clearly shows that fibrinogen labeled with a mean of 1/2 atom or less of iodine per molecule of fibrinogen behaves as the screened preparations.

Figure 5 shows a comparison of optimally labeled I^{131}-fibrinogen ($I/P \leq 0.5$) and overlabeled I^{131}-fibrinogen in older animals. Again, the top parts are the values of the optimum labeled fibrinogen, and the shaded area represents the range of values obtained from these animals. The lower parts represented by the +'s are the values of the overiodinated preparation, and the shaded area which includes them represents the variations in values obtained from three animals given I^{131}-fibrinogen with I/P ratios of 1.0, 2.0, and 8.0.

On the basis of these experiments, the optimum level of iodination was chosen as less than 1/2 atom of iodine per molecule of fibrinogen.

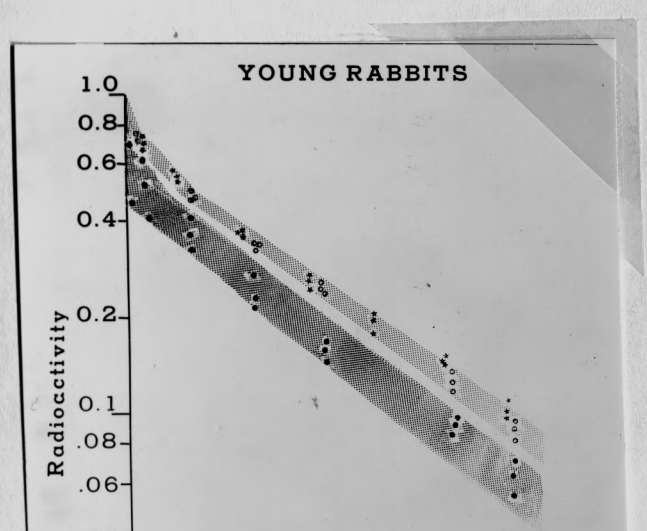

Figure 4. Comparison of values found when young rabbits were given I.V. "biologically screened" (o) and unscreened (*,●) I^{131}-fibrinogen. The stars, *, represent measurements made when optimally labeled ($I/P \leq 0.5$) I^{131}-fibrinogen was used and the dots, ●, the values obtained when overiodinated ($I/P \geq 1.0$) fibrinogen was injected. The shaded areas represent the range of variation of the values found.

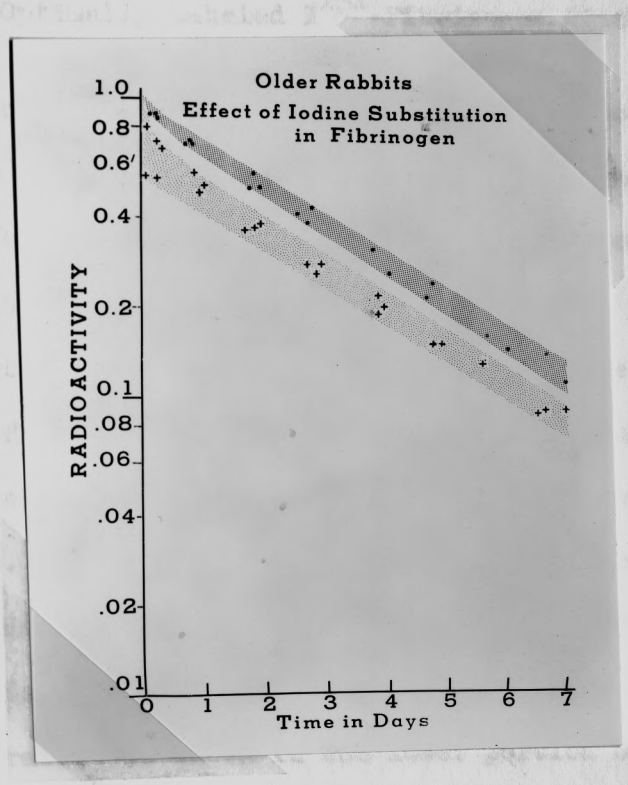

Figure 5. Comparison of values obtained when optimally labeled (•) and overlabeled (+), I^{131}-fibrinogen was given I.V. to older rabbits. The shaded areas represent the range of variation of these values.

CHAPTER VI

Behavior of Optimally Labeled I^{131}-Fibrinogen After I.V. Injection

Figure 6 shows the typical behavior of intravenously injected I^{131}-fibrinogen in young and older rabbits. The data on each rabbit is represented by two plots: one is the plasma radioactivity curve, x(t), and the other is the plot of the radioactivity remaining in the animal, the 1.0 - u(t) curve. On the ordinate is plotted the logarithm of the fraction of the radioactivity injected which remains in the animals, and on the abscissa is plotted the time of the measurement. The graph shows the results obtained from rabbits 779_s and 786. Rabbit 779_s was injected with screened I^{131}-fibrinogen and this is indicated by the subscript s. The plot of the data from the older rabbit is shown in the lower portion of the graph.

Analysis of the plasma activity curve by standard methods shows that the x(t) curve is usually fitted by an exponential function of time $x(t) = C_1e^{-a_1t}+C_2e^{-a_2t}$. The smooth curve, x(t), drawn by means of this equation, with the constants determined by graphical analysis, closely describes the data during the experimental period, as shown in the figure, both for the older animal 786 and the young rabbit. To allow comparison of the experimental data in different animals, the initial samples were assigned unit radioactivity at zero time (10 minutes after injection of the labeled fibrinogen), and the radioactivities of subsequent samples were plotted as the

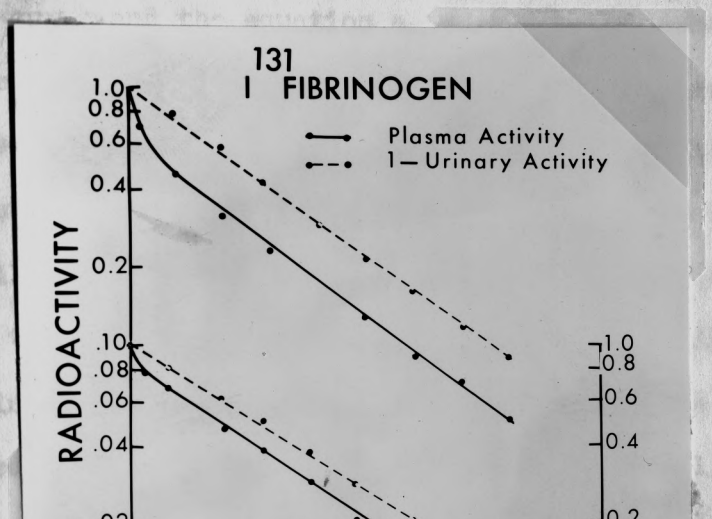

Figure 6. Typical behavior of optimally labeled I^{131}-fibrinogen in young and older rabbits after I.V. injection. The top two curves are those of the young rabbit and the lower two of the older rabbit.

logarithms of the fractions of the amount in the initial sample. The constant C_1 and the slow exponent, a_1, of the linear portion of the curve were obtained by extrapolating the tail of the curve to time zero to obtain the zero intercept, C_1, and from the half-life of the curve and the equation $a_1 = .693/t\text{-}1/2$. The half-life is read directly from the graph, and is the time corresponding to $C_1/2$. The second constant, C_2, and the faster exponent, a_2, were found by subtracting the fitted values of $C_1e^{-a_1t}$ from the curve fitted to all the experimental data, and plotting this difference between the two curves against time. In most cases this difference curve was linear and could be fitted by a function, $C_2e^{-a_2t}$, where C_2 is defined by $1 - C_1$, and a_2 is defined by $0.693/t\text{-}1/2$, where $t\text{-}1/2$ is the half-life of this faster slope. Occasionally extrapolation through this difference curve to zero time led to an intercept C_3 that was less than $1\text{-}C_1$. Then subtraction of the curve defined by $C_2e^{-a_2t}$ from the difference curve left a small remaining curve that could be fitted in the same manner by a 3rd exponential $C_3e^{-a_3t}$.

Table VIII summarizes the values for the constants, C_1, C_2, C_3, a_1, a_2, and a_3, and it can be seen that in two and possibly a third young rabbit the x(t) curve is a three exponential curve. The values for C_1 range from 0.68 to 0.90 in the older rabbits and from 0.57 to 0.82 in the young animals. The values for the linear slope a_1 range from 0.273 to 0.396 in all the experimental animals. The values for the biological "half-life" are also given and range from 1.96 to 2.54 days in the older and 1.75 to 2.54 days in the

TABLE VIII. In this table s = rabbits given screened labeled fibrinogen; a, b, and d = rabbits given the same labeled fibrinogen preparation; and * = average does not include rabbits number 726 and 780s.

TABLE VIII

I^{131}-FIBRINOGEN AND PLASMA RADIOACTIVITY, x(t), DATA IN YOUNG AND OLDER RABBITS

Rabbit No.	20 min Clottability C	I/P	days t-1/2	$x(t) = C_1e^{-a_1t} + C_2e^{-a_2t} + (C_3e^{-a_3t})$ C_1	a_1	C_2	a_2	C_3	a_3
Older Rabbits									
179b	0.93	0.54	2.20	.68	.314	.32	2.93	-	-
181b	0.93	0.54	1.96	.70	.354	.30	4.16	-	-
151	0.93	0.45	2.19	.86	.317	.14	8.00	-	-
795a	0.94	0.42	2.48	.79	.280	.21	2.08	-	-
787	0.95	0.32	2.31	.90	.303	.10	10.00	-	-
797	0.96	0.50	2.34	.82	.297	.18	4.15	-	-
786d	0.96	0.40	2.54	.90	.273	.10	8.30	-	-
Mean	0.94	0.45	2.28	.81	.305	.19	5.66	-	-
Young Rabbits									
725	0.96	0.50	1.76	.83	.395	.17	5.55	-	-
724a	0.94	0.42	1.75	.71	.396	.29	8.00	-	-
726	0.95	0.46	2.42	.58	.287	.25	1.75	.17	8.0
780s	0.94	0.50	2.29	.57	.302	.20	1.38	.20	5.6
784d	0.96	0.40	2.12	.68	.326	.32	8.30	-	-
781s	0.94	0.50	2.06	.66	.336	.34	4.15	-	-
779s	0.94	0.50	1.90	.67	.365	.33	4.75	-	-
788s	0.94	0.30	2.54	.66	.273	.34	3.34	-	-
787	0.97	0.42	2.27	.71	.305	.29	8.30	-	-
Mean	0.95	0.44	2.12	.70*	.331	.30*	6.05*		

young rabbits.

The Radioactive Breakdown Products in the Plasma and Tissue Fluids

It has been shown that the radioactive breakdown products of I^{131}-albumin given intravenously to rabbits consist of more than 80 per cent inorganic iodide and the remainder of small organic metabolites, (67), (98). This is probably also true for I^{131}-fibrinogen (46). These breakdown products are distributed in a volume of 8 to 10 times the plasma volume and are rapidly excreted (67). Figure 15 (see page 80) shows the behavior of the total quantity of the radioactive breakdown products represented by the curve z(t). The points on the z(t) curve are expressed as decimal fractions of the total radioactivity injected. These values were calculated from the measurements made of the radioactivity in the plasma which was not precipitable with 10 per cent trichloroacetic acid multiplied by the distribution volume of the breakdown products. From the figure it can be seen that maximum retention of the breakdown products in these animals occurred about 12 hours after the injection. When the unbound radioactivity was measured in the plasma at this time it accounted for only 1 to 2 per cent of the total radioactivity in the older animals, but in some of the younger it was found to make up 4 to 6 per cent of the total activity.

Excretion of the Radioactive Breakdown Products

The upper curves in Figure 6 show the disappearance rate of the injected radioactivity from the animals as measured from the activity in the excreta.

The curve is a plot of the amount injected, normalized to 1.0, minus the cumulative excretion of the radioactivity excreted, u(t). The 1 - u(t) curve has a slope a_u and is seen to parallel the slope, a_1, of the plasma activity curve. The radioactivity was excreted rapidly with little or no delay as shown by the plots of the cumulative rates of excretion, u(t), given in Figure 7. Table IX summarizes the values for the daily excretion of breakdown products, and it can be seen that on the average 15 to 30 per cent of the radioactivity injected appears in the urine during the first day in animals exhibiting no urine retention. The majority of the radioactivity excreted was found in the urine and only very small amounts in the feces. After the first day or two, the curve describing the excretion of radioactive breakdown products is given by $u(t) = 1.0 - Ke^{-a_u t}$ where K is some constant.

Extravascular I^{131}-Fibrinogen

The extravascular I^{131}-fibrinogen cannot be measured directly, but may be calculated from measurements of x(t), the plasma activity z(t), the radioactive breakdown products activity, and u(t), the fraction of the injected radioactivity excreted up to a given time, t, since by definition y(t), the interstitial I^{131}-fibrinogen is equal to 1.0 - x(t) - z(t) - u(t). Good measurements of the total plasma and urine activities are readily made, but the amount of radioactivity in the plasma due to breakdown products is low and accurate measurements of z(t) are difficult to make. Hence, the values found for the interstitial fibrinogen determined in this manner are only approximate. Figure 8 shows the results of such calculations of the total interstitial activity in one rabbit.

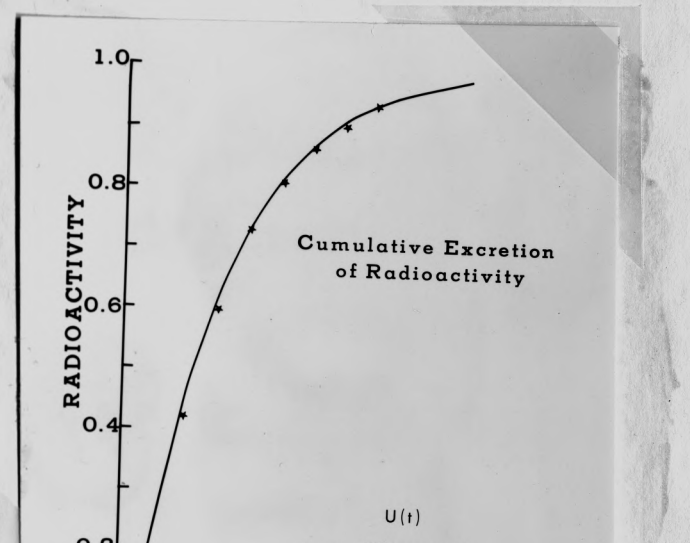

Figure 7. Plot of the cumulative rate of excretion of radioactive breakdown products. The stars, *, represent the observed values and the smooth curve the values calculated from equation 18.

TABLE IX. In this table, * = the time of urine collection (the morning following the I.V. injection of I^{131}-fibrinogen) is expressed as a fraction A.

TABLE IX

CUMULATIVE EXCRETION OF RADIOACTIVITY, AFTER I.V. I^{131}-FIBRINOGEN, EXPRESSED AS DECIMAL FRACTION, u(t)

Rabbit No.	Fraction Day 1* A	Fraction of Radioactivity excreted in A	1 + A	2 + A	3 + A	4 + A	5 + A	6 + A	7 + A	8 + A
Older Rabbits										
179b	0	0	.196	.425	.569	.672	.753	.817	.859	.881
181b	0	0	.293	.492	.643	.732	.795	.857	.895	.921
151	.79	.200	.388	.501	.619	.699	.773	.868	.938	-
795a	.66	.093	.162	.479	.595	.703	.764	-	.889	-
787	.87	.236	.324	.551	.657	.732	.828	.887	.912	.937
797	.79	.152	.384	.502	.618	.696	-	.794	-	853
786d	.75	.159	.331	.454	.587	.696	-	.818	-	.928
Young Rabbits										
724a	.66	.057	.322	.532	.637	.724	-	.808	-	.870
726	.71	.217	.401	.582	.689	.754	-	.852	-	.911
780s	.94	.216	.425	.540	.629	.734	.793	.835	.865	-
784d	.75	.183	.401	.568	.673	.750	-	.887	-	-
781s	.94	.156	.328	.502	.638	.792	.862	.910	.942	-
779s	.94	.177	.410	.581	.717	.798	.853	.894	.926	-
788s	.83	.260	.420	.590	.700	-	-	-	-	-
787	.66	.230	.450	.600	.720	.800	.855	-	.930	-

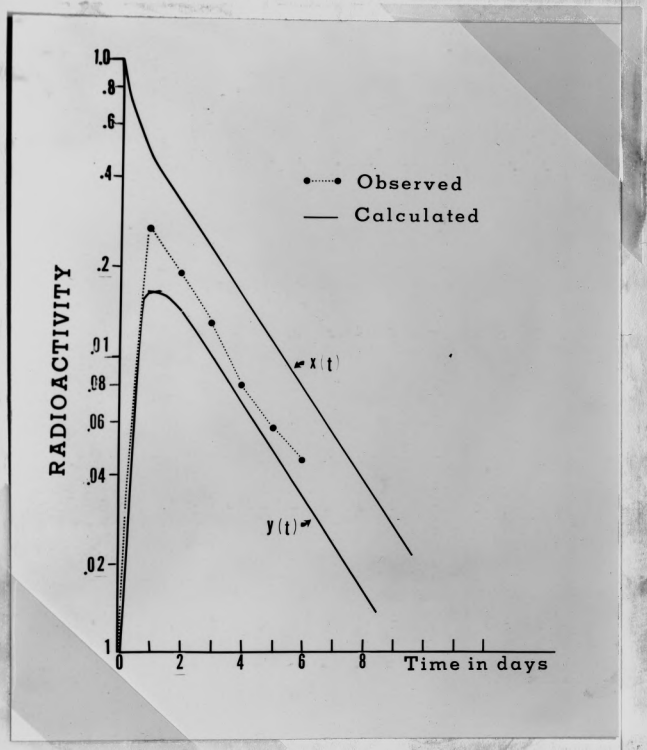

Figure 8. Extravascular I^{131}-fibrinogen: The dots, •, represent the values found from $y(t) = 1.0 - x(t) - z(t) - u(t)$ and the smooth curve those calculated from equation 17.

CHAPTER VII

Model Describing the Distribution and Catabolism of Fibrinogen

It has been shown that fibrinogen is made in the liver (99) (100) by the liver parenchymal cell (101). The fibrinogen, newly synthesized, is then transferred to the systemic circulation either by the hepatic lymphatics (102) (103) (104), or by direct transfer into the blood stream (105) (106). Whatever the mode of transfer, the fibrinogen must reach the circulation rapidly and once it has entered the circulation, it is further distributed throughout the blood stream. From there, the fibrinogen passes to the interstitial fluids by transcapillary transfer, and then returns to the venous circulation mostly via the lymphatics. Evidence for this was obtained by Lewis and Furguson (35) who recovered I^{131}-fibrinogen given intravenously from the thoracic duct lymph. Additional evidence is found when lymph is treated with thrombin and fibrin clots form. Fibrinogen is also continuously catabolized. The catabolism of fibrinogen may occur either directly by fibrinogenolysis, or, after fibrin formation, by fibrinolysis. Some of the views on the modes and sites of fibrinogen catabolism have already been summarized. In healthy animals, the rate of fibrinogen synthesis must equal the rate of fibrinogen catabolism and the rate of fibrin deposition must equal the rate of removal. Otherwise a net depletion or accumulation of fibrinogen and fibrin would occur.

Simple Models Describing the Behavior of Fibrinogen and I^{131}-Fibrinogen

The behavior of fibrinogen as described above may be presented diagrammatically using a compartmental model as in Figure 9 and assigning varying rates of transfer and catabolism to fibrinogen. The rates can be considered as velocities requiring a definite time for a molecule of fibrinogen to reach its destination.

Model I in Figure 9 describes the fibrinogen system. Fibrinogen made in the liver is shown to enter the blood compartment in which there is $\bar{x}$ grams of fibrinogen. Here it is rapidly mixed. Fibrinogen passes into the interstitial fluids at the rate $r_1\bar{x}$ and is returned to the plasma at the rate $r_2\bar{y}$ where $\bar{y}$ if the total interstitial fibrinogen. Fibrinogen also passes to catabolic sites, $\bar{v}$, at the rate $r_3\bar{x}$ and to tissue fibrin, $\bar{w}$, at the rate $r_4\bar{x}$. The fibrin in compartment w is then shown to be depolymerized at a rate $r_7\bar{w}$ and transferred to the same breakdown site, $\bar{v}$, as that for fibrinogen where both fibrinogen and fibrin monomers are broken down further. All the r's have dimensions of day^{-1}. The model thus suggests two possible catabolic pathways for fibrinogen and the rates imply a dynamic system which can be subjected to kinetic analysis by using I^{131}-labeled fibrinogen.

The system describing the behavior of injected I^{131}-fibrinogen is presented as Model II in the Figure 10. In this, the liver compartment has been deleted and two others, the radioactive breakdown products and the I^{131} excretion compartments, have been added. The total radioactivity in each compartment is designated as a function of time. Thus, the total I^{131}-fibrinogen radioactivity at time, t, in the plasma is x(t) and that in the interstitial

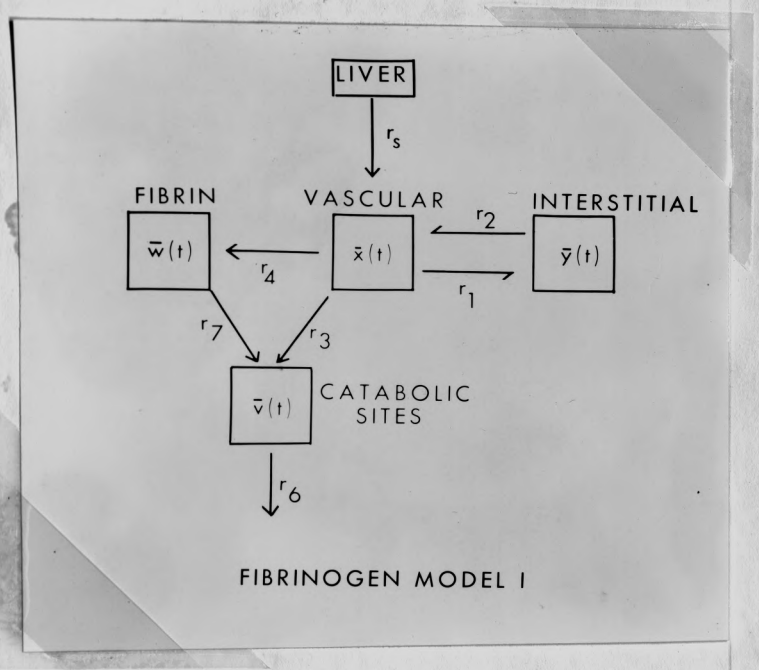

Figure 9. Model I: Schema describing the distribution and transfer of fibrinogen in the living animal in the steady state.

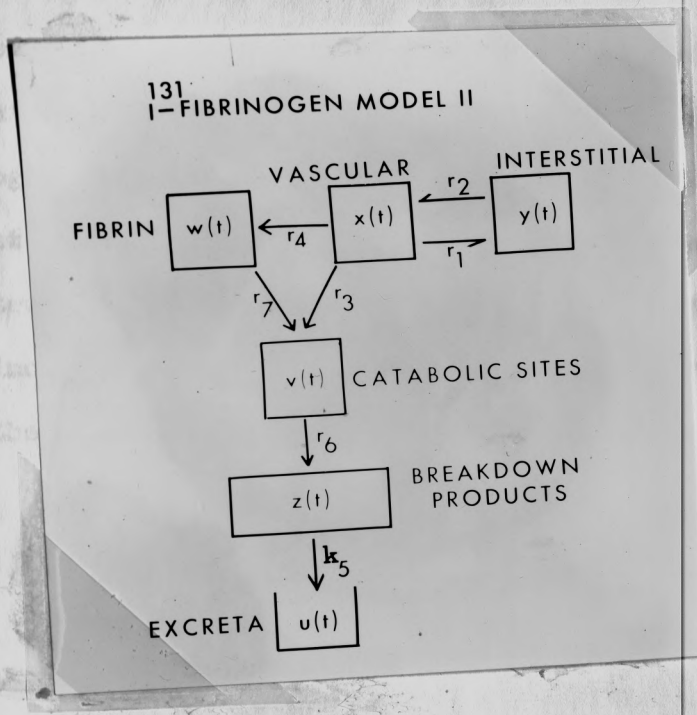

Figure 10. Model II: Schema of the distribution, transfer and excretion of radioactivity following I.V. injection of I^{131}-fibrinogen.

fluids y(t). The I^{131}-fibrin radioactivity is w(t) and that in the catabolic compartment v(t). The radioactivity of the breakdown products in the tissue fluids is z(t), and the cumulative radioactivity passing into the excreta is u(t).

Since the rates represent the fractional rate of removal of the labeled material the product $r_1x(t)$, for instance, defines the flux of fibrinogen radioactivity into the interstitial fluids per day. Such a system can be described by a series of differential equations. Before attempting to find the solutions for these, analysis of the I^{131}-fibrinogen data may suggest a simpler model which will adequately describe the observed data.

CHAPTER VIII

Analysis of the Radioactivity in the Various Compartments

Examination of the Plasma Radioactivity and Fibrinogen Concentration

Figure 11 shows a plot of the plasma activity, cpm/ml, (the top curve) and the I^{131}-fibrinogen specific activity, cpm/mg, (the lower curve), in rabbit 781_s. Below the two curves are plotted the plasma fibrinogen concentrations at the time of blood sampling. These findings are typical in rabbits showing fluctuations in the concentration of plasma fibrinogen.

The most striking feature of Figure 11 is the effect that changes in the plasma fibrinogen concentration produce on the specific activity curve. Notice that as the fibrinogen concentration rises, the specific activity curve falls more rapidly than the plasma activity curve, and conversely, as the fibrinogen concentration falls the rate of decline of the specific activity curve is decreased. However, the fluctuations do not affect the plasma activity curve, which as noted above is described by a 2 or 3 exponential equation. A possible cause for these observations is that the I^{131}-fibrinogen is selectively removed from the circulation. But since it behaves as biosynthetically labeled C^{14}-fibrinogen and screened I^{131}-fibrinogen, such a process is very unlikely. Other causes of the findings are that the I^{131}-fibrinogen system behaves as either an unsaturated first order process or a volumetric clearance system.

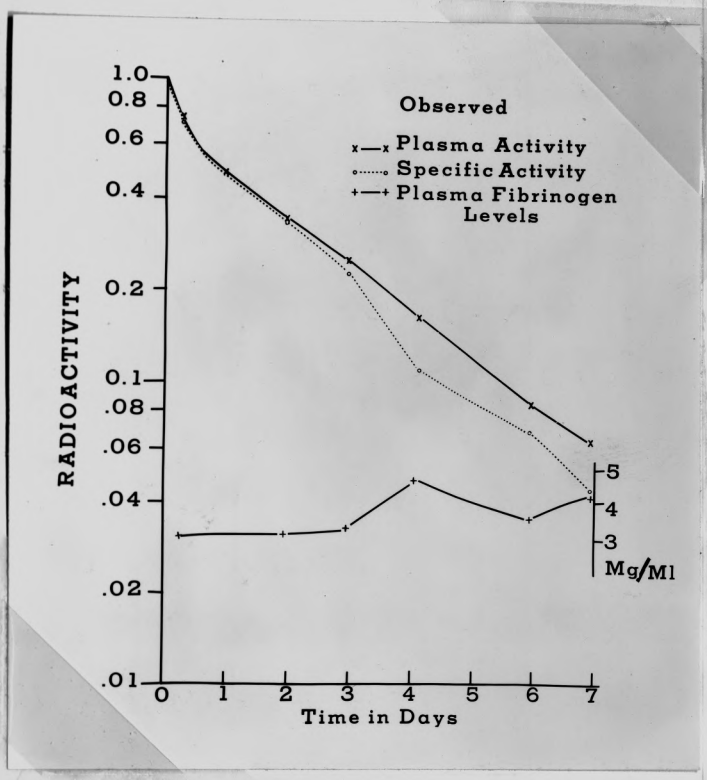

Figure 11. Fluctuations in fibrinogen concentration and their effect on the plasma activity and I^{131}-fibrinogen specific activity curves.

Both types of processes will now be briefly discussed.

a) The Unsaturated I^{131}-Fibrinogen Capillary Transfer System The transcapillary transfer system is not difficult to conceive if one assumes that "pores" are available for the passage of fibrinogen across the capillary wall. If many pores exist in the endothelial wall, these would be difficult to saturate with fibrinogen molecules since capillaries permeate almost every tissue. In fact, it has been estimated that the total endothelial area of the capillaries of human muscle is about 6,000 square meters (107), while the fibrinogen concentration in the plasma ranges from 300 to 500 mg per 100 ml. If the ratio of I^{131}-fibrinogen to unlabeled fibrinogen molecules was halved by doubling the concentration of fibrinogen by new synthesis, by the law of mass action, the total number of molecules colliding with the endothelial wall would double, but the fraction of I^{131}-fibrinogen molecules colliding in unit time would be unchanged. Such an effect would maintain the rate of I^{131}-fibrinogen transfer the same as it was before the increase in fibrinogen concentration. Thus, the plasma radioactivity curve would be unaffected by the fluctuation in fibrinogen concentration.

b) The Unsaturated Catabolic System An unsaturated catabolic system might consist of a large number of catabolic sites. These could be distributed either intravascularly or extravascularly. When plasma containing I^{131}-fibrinogen is removed from healthy experimental animals and incubated at 37°C for 24 hours, no increase in the non-precipitable radioactivity is observed, which excludes the

the presence of an enzyme in the plasma (in health) that breaks down I^{131}-fibrinogen. Evidence in favor of vascular endothelial catabolic sites has been obtained. Thus Copley and others (29) (103) have shown that fibrinolytic activity is closely associated with the capillary wall. Copley maintains further that this fibrinolytic activity is essential to maintain the patency of the capillary wall and to remove any fibrin deposited on the endothelial wall. At the present there is insufficient evidence about the nature of the extravascular catabolic sites if they occur.

c) A Volumetric I^{131}-Fibrinogen Clearance System A volumetric capillary transfer system might be due to vesicle transport system occurring in the capillary endothelial cells. Each pinocytotic vesicle would "drink" a certain volume of plasma and the vesicle would be passed across the capillary endothelium. Thus a given fraction of I^{131}-fibrinogen would be passed across the capillary membrane, which would be independent of the fibrinogen concentration. Electron microscope studies of the endothelial cells have shown the presence of intracellular vesicles of about 650 Å in diameter. These vesicles, in tightly packed layers, are seen to face both the perivascular space and the capillary lumen (109) (110) (111). Close examination suggests that many of them open at the perivascular surface of the endothelial cell membrane. The transport of fibrinogen thus may depend on innumerable small volumes of plasma being taken up in vesicles by pinocytosis and extruded on the perivascular side of the cell.

Against the pinocytotic theory two points can be advanced.

First it is not clear whether the pinocytotic process occurs sufficiently rapidly to explain the rate of transcapillary passage. Second, it implies that any proteins in the pinocytotic vesicles would be "cleared" at the same rate from the plasma. On present evidence this does not appear to be true.

d) The Volumetric Catabolic System This would occur by vesicular transport to a catabolic site, but at the present evidence in favor of this is lacking.

Examination of the Radioactive Breakdown Products Released by the Catabolism of I^{131}-Fibrinogen

a) Catabolism Via Fibrin Formation If formation of fibrin is prerequisite to fibrinogen breakdown and occurs outside the vascular compartment as shown in Model I, this can be closely approximated by forming an I^{131}-fibrin blood clot subcutaneously. In this manner some insight into the mechanism of fibrinogen catabolism may be obtained.

I^{131}-fibrinogen mixed with rabbit citrated blood was recalcified and the mixture immediately injected subcutaneously. This produced a firm blood clot containing radioactivity which could be measured at the site of the clot with a scintillation probe. The measurements made with the probe could then be compared with the excretion of the breakdown products.

Figure 12 shows the results of such an experiment. The top curve represents the radioactivity remaining in the animal and was obtained by subtracting the fraction of the total activity excreted from 1.0, the initial radioactivity in the clot. The lower curve

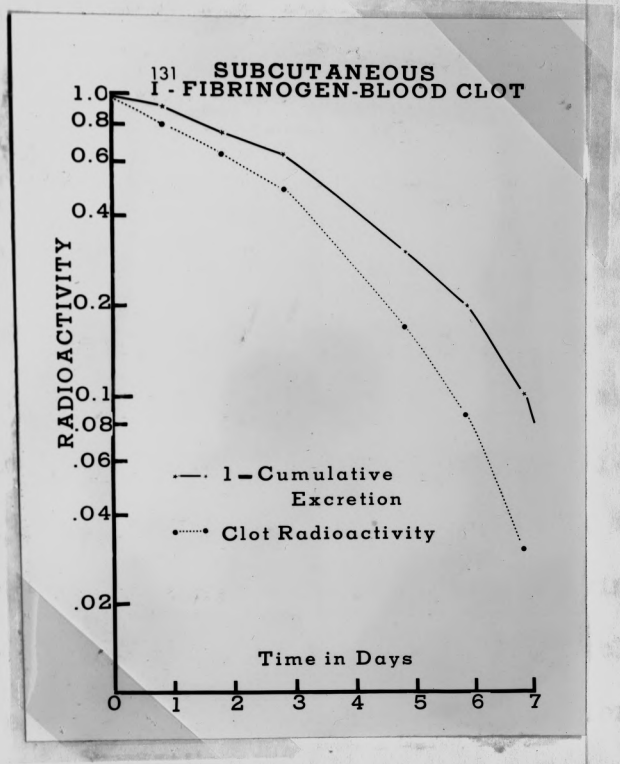

Figure 12. Disappearance of radioacitivity from site of clot compared with that remaining in the animal.

is the radioactivity measured at the site of the clot with the probe, which disappeared over 7 to 8 days. Measurements of the plasma activity during this time showed that it was not precipitable by trichloroacetic acid, and, therefore, the clot was broken down in situ with the release of radioactive breakdown products unbound to soluble protein. Though the radioactivity began to disappear from the clot almost immediately, some time was required for the radioactivity to appear in the urine. This indicates that if I^{131}-fibrinogen is broken down via extravascular fibrin formation, a lag in the excretion of the breakdown products might be detectable in the animals given I^{131}-fibrinogen intravenously.

Figure 13 compares plots of the excretion of the radioactive breakdown products in experimental animals receiving I^{131}-fibrinogen intravenously and subcutaneous I^{131}-fibrin blood clot. No lag in excretion is apparent in the animal given I^{131}-fibrinogen I.V. On the basis of these findings, it is concluded that I^{131}-fibrinogen before being catabolized does not pass through an extravascular fibrin compartment, w, and this is excluded from Model II.

b) Existence of the v Compartment, The Breakdown Compartment

If a breakdown compartment, v, exists, according to Model II, the I^{131}-fibrinogen would be transported to this at a rate $r_3x(t)$, catabolized, and the breakdown products released at the rate $r_6v(t)$. This process is time consuming and a delay in the excretion of radioactive breakdown products should be observed if it is in operation. To test the importance of the v compartment, Figure 7 compares a plot of the observed rates of excretion with values

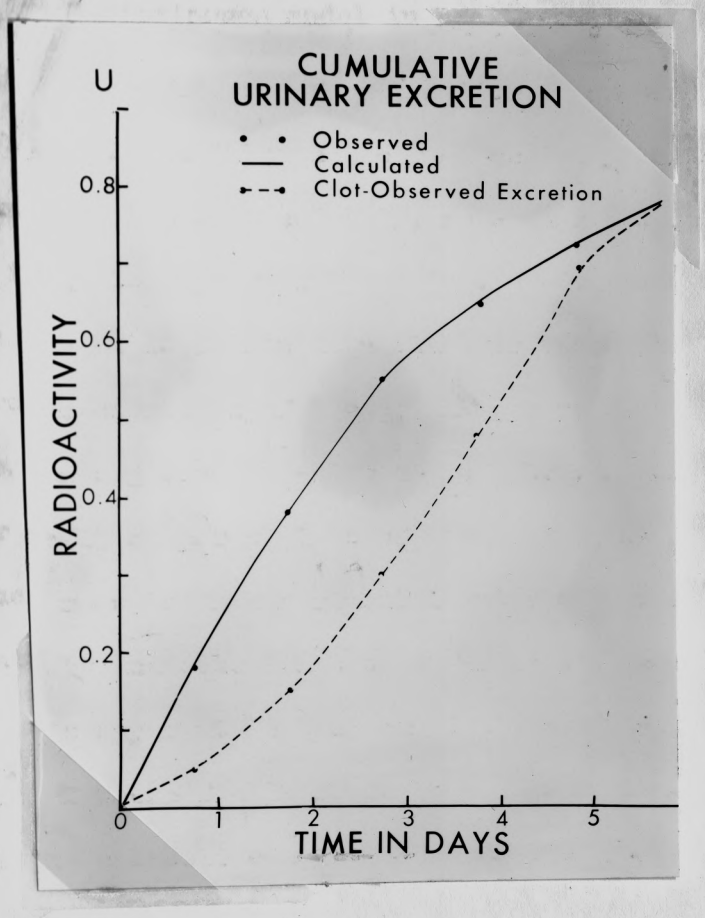

Figure 13. Cumulative excretion of radioactive breakdown products in the urine of rabbits given I^{131}-fibrinogen I.V. and those with a subcutaneous I^{131}-fibrin blood clot.

calculated on the assumption that breakdown occurs in the blood stream. Agreement is good and thus evidence that the v compartment in Model II, if it exists, is small and can be neglected.

The I^{131}-fibrinogen model in health thus may be simplified to a four compartment model by omitting the fibrin compartment, w, and the breakdown compartment, v, from Model II, though this may not be true in disease. The observations first described in this chapter indicate that the rate constants, r_1, r_2, and r_3 are first order whatever their underlying physiological basis; k_5 is also first order and is the fractional rate of excretion of the radioactive breakdown products. It ranges between 2.0 to 3.0 day^{-1} (67). The four compartment model will now be presented diagrammatically, the mathematical equations will be written, and the predictions of the equations will be tested by experiment.

CHAPTER IX

Model Describing the Behavior of I^{131}-Fibrinogen

Model III in Figure 14, consists of four compartments only, the x(t), y(t), z(t), and u(t) compartments which have already been defined. The arrows indicate the direction of flow of I^{131}-fibrinogen from the vascular compartment.

Mathematically, Model III is described by the following differential equations:

1) $dx(t)/dt = r_2 y(t) - (r_1 + r_3)\, x(t)$

2) $dy(t)/dt = r_1 x(t) - r_2 y(t)$

3) $dz(t)/dt = r_3 x(t) - k_5 z(t)$

4) $du(t)/dt = k_5 z(t)$

Solutions of the Differential Equations Describing the Model

Equations 1 and 2 in operator form become equations 5 and 6:

5) $\left[D + (r_1 + r_3) \right] x(t) = r_2 y(t)$,

6) $(D + r_2) y(t) = r_1 x(t)$.

Solving equation 6 for y(t), $y(t) = r_1 x(t)/ (D + r_2)$, and substituting y(t) into equation 5 one obtains equation 7.

7) $(D + r_2) \left[D + (r_1 + r_3) \right] x(t) = r_1 r_2 x(t)$ which simplifies to

8) $\left[D^2 + (r_1 + r_2 + r_3)\, D + r_2 r_3 \right] x(t) = 0.$

Equation 8 is a linear homogeneous differential equation with the roots of the auxiliary equation, m_1 and m_2 real and distinct. It

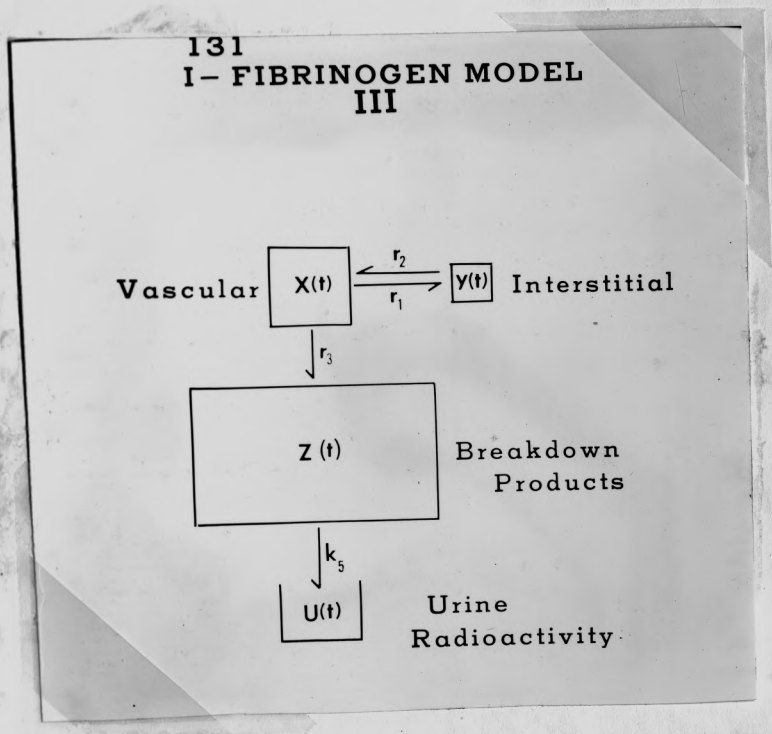

Figure 14. Model III: Simplified model describing the behavior of I^{131}-fibrinogen in the living animal.

has the general solution, $x(t) = C_1 e^{m_1 t} + C_2 e^{m_2 t}$. Letting $m_1 = -a_1$ and $m_2 = -a_2$ where both a's are greater than zero to ensure that the rates, r, are also greater than zero, then x(t) becomes

9) $x(t) = C_1 e^{-a_1 t} + C_2 e^{-a_2 t}$. This agrees with the form of the plasma activity curve.

The solutions for the rate constants, r_1, r_2, and r_3 will now be calculated from equation 1 and the relations between the roots of the x(t) curve, and the initial and final conditions.

From equation 1 and by differentiating equation 9, equation 10 is obtained.

10) $dx(t)/dt = -C_1 a_1 e^{-a_1 t} - C_2 a_2 e^{-a_2 t} = r_2 y(t) - (r_1 + r_3)\, x(t)$.

From initial conditions that at t = 0, y(0)=0, x(0)=1, and $e^{-0}=1$, equation 10 becomes equation 11.

11) $r_1 + r_3 = C_1 a_1 + C_2 a_2$ and $r_1 = C_1 a_1 + C_2 a_2 - r_3$.

Since the amount of radioactivity injected has been normalized to 1.0, as time, t, increases x(t) goes to zero and u(t) to 1.0. Therefore, the total quantity of I^{131}-fibrinogen broken down at the rate r_3 cannot exceed unity; ie, $r_3 \int_0^\infty x(t)dt = 1.0$. For a two exponential x(t), $\int_0^\infty x(t)dt = C_1/a_1 + C_2/a_2$, and thus the fractional rate r_3 is found by the reciprocal of this value.

12) $$r_3 = \frac{1}{C_1/a_1 + C_2/a_2}$$

The solution for r_2 is found from equation 12 and the relation between the roots of the auxiliary equation and the x(t) equation. If $m_1 m_2 = a_1 a_2 = r_2 r_3$, then $r_2 = a_1 a_2 / r_3$. Substituting this into equation 12, the solution for r_2 is found to be,

13) $r_2 = a_2 C_1 + a_1 C_2$.

The values for the rate constants r_1, r_2, and r_3 can be found from the C's and a's which are obtained from the experimental x(t), the plasma activity, by graphical analysis and have been presented in Table X (see page 82).

Solutions for y(t), z(t) and u(t)

Taking x(t) as a two exponential function the solution for equations 2, 3, and 4 can be found by standard methods. Only one solution will be carried out in detail and the other solutions will be given.

Setting equation 3 in operator form, it becomes,

14) $(D + k_5)z(t) = r_3x(t) = r_3(C_1e^{-a_1t} + C_2e^{-a_2t})$. Equation 14 is a non-homogeneous linear differential equation and can be solved by the method of undetermined coefficients. The general solution of equation 14 is $z = z_c + z_p$ where z_c can be obtained at once from the values of the root of the characteristic equation, which for equation 14 is $-k_5$. Hence, the complementary function for 14, z_c, has the following general solution,

15) $z_c = C_ze^{-k_5t}$.

To find the particular solution z_p, let $z_p = Ae^{-a_1t} + Be^{-a_2t}$ which has a first derivative, $z_p' = -(Aa_1e^{-a_1t} + Ba_2e^{-a_2t})$. By substituting z_p for z(t) and z_p' for D in equation 14 and solving for A and B, it is found that,

$A = r_3C_1(k_5-a_1)$ and, $B = r_3C_2(k_5-a_2)$. Since the solution for $z(t) = z_c + z_p$ the solution for z(t) can be obtained by substitution and solving for C_z and is,

16) $z(t) = A(e^{-a_1t} - e^{-k_5t}) + B(e^{-a_2t} - e^{-k_5t})$.

Solutions for equations 2 and 4 can be obtained in a similar manner. The solution for y(t) is given by equation 17,

17) $y(t) = G_1(e^{-a_1 t} - e^{-r_2 t}) + G_2(e^{-a_2 t} - e^{-r_2 t})$ where

$G_1 = r_1 C_1/(r_2 - a_1)$ and $G_2 = r_1 C_2/(r_2 - a_2)$.

The solution for equation 4 is given in equation 18,

18) $u(t) = 1.0 - k_5 \left[A/a_1 e^{-a_1 t} + B/a_2 e^{-a_2 t} - (B+A)/k_5 e^{-k_5 t} \right]$

where A and B are the constants described for z(t) in equation 16.

CHAPTER X

Test of the Model: Comparison of the Observed and Calculated z(t), u(t), and y(t) the Tracer Rate Constants in Young and Older Rabbits

Agreement between x(t), the plasma activity curve, found experimentally and that calculated has already been mentioned above. Figure 6 shows the comparison. The experimental values are represented by the dots, and the fitted curve by the continuous line.

Comparison of Observed and Calculated z(t)

Figure 15 compares the observed z(t) and z(t) calculated from equation 16. The measured z(t) values are plotted as fractions of the total activity injected. The plots in Figure 15 are for rabbits number 779_s, the young rabbit, and number 797, an older rabbit. Since the measured count rate for the plasma unbound I^{131}-racioactivity ranged from 10 to 50 counts per minute against a background of 20 cpm, the accuracy of the measurements is not great. From the figure it can be seen that the calculated and observed z(t) agree reasonably well.

Comparison of Observed and Calculated u(t)

In Figure 7, the measured and the calculated rates of urinary excretion of the breakdown products are plotted. The calculated u(t) was found from equation 18 for rabbit 784, is based on the assumption that the fibrinogen is broken down in or very near the vascular compartment with the immediate release of the radioactive

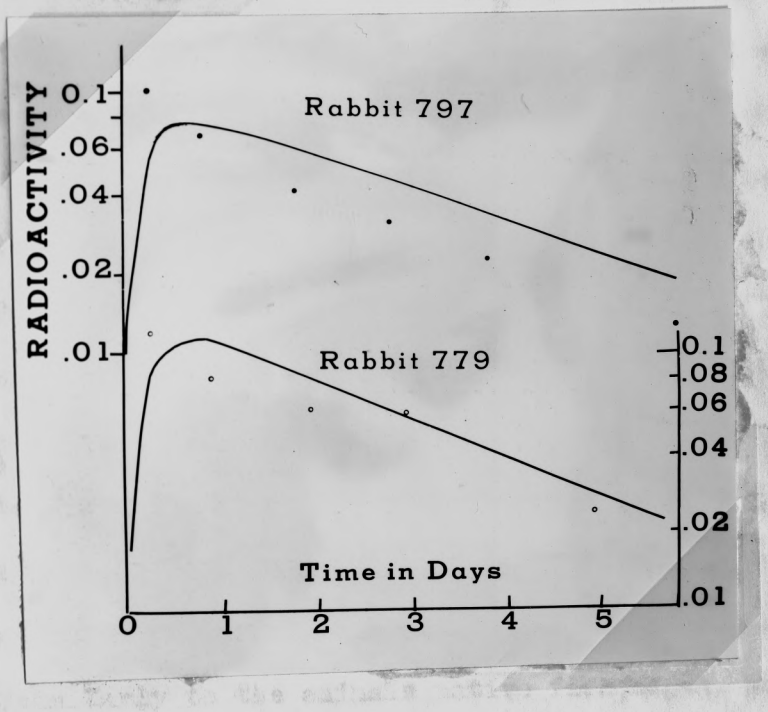

Figure 15. Comparison of Values for z(t) calculated from equation 16 with those obtained experimentally. The calculated values are shown by the continuous black line and observed values by the broken line.

breakdown products, which then pass directly into the breakdown products compartment. The agreement between the calculated and the observed values is excellent.

Comparison of Observed and Calculated y(t)

Figure 8 compares for rabbit 779_s the calculated y(t) curve obtained from equation 17 and that obtained from the experimental values were $y(t) = 1.0 - x(t) - z(t) - u(t)$. The observed values of y(t) are represented by the dots and the broken line, and the calculated values are represented by the smooth curve. The observed and calculated values agree reasonably well though as noted below the measurements of z(t) are only approximate.

Since the agreement between the observed values and those calculated from the equations derived from the model is good, Model III is accepted as describing the behavior of the I^{131}-fibrinogen in vivo. Further since by all tests, the I^{131}-fibrinogen behaves similarly to the animals native fibrinogen, the rate constants, the r's, can be used to determine the fluxes of the native fibrinogen. Before this can be done, the r's must be determined by equations 11, 12 and 13 from the tracer data.

The Fractional Rates, r_1 and r_3, of I^{131}-Fibrinogen

The values for the fractional transcapillary transfer rate, r_1, were calculated from equation 11 for each experimental animal and are listed in Table X. In the older animals these values range from 0.32 to 1.03 day^{-1} with a mean of 0.77 day^{-1}. In the young rabbits, however, the values calculated were greater, ranging from 0.81 to 2.20 day^{-1} with a mean of 1.44 day^{-1}. Thus, in the young

TABLE X

FRACTIONAL RATES OF I^{131}-FIBRINOGEN
CALCULATED FROM EQUATIONS 11, 12 AND 13

Rabbit No.	Transcapillary r_1 = day^{-1}	r_2 = day^{-1}	Catabolic r_3 = day^{-1}
Older Rabbits			
179b	.71	2.09	.44
181b	1.01	3.02	.49
151	1.03	6.90	.37
795a	.32	1.70	.34
787	.94	9.00	.33
797	.63	3.45	.36
786d	.77	7.50	.30
Mean	.77	4.80	.375
Young Rabbits			
725	.81	5.27	.47
724a	2.05	5.79	.35
726	1.51	-*	.48
780s	1.08	-*	.48
784d	1.58	5.72	.47
781s	1.58	2.85	.49
779s	1.28	3.30	.52
788s	.92	2.29	.40
787	2.20	6.00	.42
Mean	1.44	4.46	.45

* Rabbits had 3 exponential x(t) curves thus r_2 is not defined by equation 13.

s = rabbits given screened labeled fibrinogen; a, b, and d = rabbits given the same labeled fibrinogen preparation.

rabbits the fractional transcapillary rate of I^{131}-fibrinogen is almost twice as fast as that calculated for the older rabbits.

The values for r_3, the fractional catabolic rate, calculated from equation 12 are also listed in Table X. Again note that the young rabbits have a faster r_3. In the young animals r_3 ranges from 0.35 to 0.52 day^{-1} with a mean of 0.45 day^{-1} compared to the range of r_3 found in the older rabbits of 0.30 to 0.49 day^{-1} with a mean of 0.375 day^{-1}. Though the fractional catabolic rate is faster in the young rabbits, the difference is not as large as that seen in the transcapillary transfer fractional rate.

Since the I^{131}-fibrinogen injected into these animals has been shown to be as near to the native fibrinogen, according to all the tests performed, these values will now be used to calculate the fluxes of fibrinogen in these animals.

CHAPTER XI

Results

Plasma Fibrinogen Concentration

The plasma fibrinogen concentration fluctuated in some animals and remained reasonably constant in others. In a few animals the fibrinogen concentration doubled in 24 hours but then returned to normal within 24 to 48 hours. In most of the rabbits, however, only small fluctuations were observed. The mean plasma fibrinogen concentrations for each experimental animal over the course of the experiment are listed in Table XI. The values found range from 2.40 to 4.35 mg/ml with a mean of 3.44 mg/ml in the entire group of animals.

Total Fibrinogen in the Plasma

The total plasma fibrinogen was calculated from the measured plasma volume and the mean plasma fibrinogen concentrations. The values are listed in Table XI and are higher in the older rabbits. For instance, in the older rabbits the total plasma fibrinogen ranged from 260 to 441 mg with a mean of 376 mg, compared to a range of 210 to 318 mg with a mean of 245 mg in the young rabbits. This difference is primarily the results of the smaller plasma volume of the young rabbits, from their smaller size. When the plasma fibrinogen per kg body weight is determined, the difference was not as large. In the older animals, the range was from 76 to 122 mg/kg with a mean

TABLE XI. In this table s = rabbits given screened labeled fibrinogen; a, b, and d = rabbits given the same labeled fibrinogen preparation.

TABLE XI

FIBRINOGEN DATA IN YOUNG AND OLDER RABBITS

Rabbit No.	Bodyweight kg	Plasma Volume V_p	Plasma Fibrinogen: Total Fibrinogen $\bar{x}$	Plasma Fibrinogen: mg/ml $\bar{x}/V_p$	Plasma Fibrinogen: mg/kg $\bar{x}$/kg	Fibrinogen Fluxes: mg/kg/day $r_1\bar{x}$/kg	Fibrinogen Fluxes: mg/kg/day $r_3\bar{x}$/kg
Older Rabbits							
179b	3.33	131	379	2.88	114	81	50
181b	3.43	86	260	3.00	76	77	37
151	3.52	99	359	3.63	102	105	38
795a	3.58	97	362	3.73	101	32	34
787	3.60	106	434	4.06	120	113	40
797	3.60	110	441	4.01	122	77	44
786d	3.79	127	400	3.14	105	81	31
Mean	3.55	108	376	3.49	106	81	39
Young Rabbits							
725	1.60	51	222	4.35	139	113	65
724a	1.67	53	223	4.21	134	275	74
726	1.97	68	210	3.08	106	160	51
780s	2.07	70	213	3.04	103	111	49
784d	2.20	91	259	2.84	128	202	60
781s	2.23	73	239	3.27	107	169	53
779s	2.30	83	318	3.82	139	177	72
788s	2.48	96	232	2.41	94	86	38
787	2.58	82	290	3.53	112	246	47
Mean	2.12	72	245	3.39	118	171	56.5

of 106 mg/kg, compared to a range of 94 to 139 mg/kg with a mean of 118 mg/kg in the young rabbits.

The Total Interstitial Fibrinogen $\bar{y}(t)$

The total interstitial fibrinogen, $\bar{y}(t)$, cannot be measured directly but was calculated from the following equations,

19) $r_1\bar{x}(t) = r_2\bar{y}(t)$, from which

$\bar{y}(t) = r_1\bar{x}(t)/r_2$,

20) $\bar{y}(t) = r_1\bar{x}(t)\ (D_1/d_1 + D_2/d_2)$ (112).

Equation 19 is applied when x(t) is a 2 exponential equation, and equation 20 when x(t) is a 3 exponential equation. The values for D_1, D_2, d_1 and d_2 are calculated from the 3 exponential x(t) as described elsewhere (112).

The values for $\bar{y}(t)$ calculated from these equations are listed in Table XII which shows that the total interstitial fibrinogen in the older animals ranges from 11 to 39 mg/kg with a mean of 22 mg/kg, but in the young rabbits was somewhat larger, with a mean of 42 mg/kg and a range of 21 to 59 mg/kg.

The total interstitial fibrinogen can also be expressed in terms of the extravascular to vascular fibrinogen ratio. Table XII shows that this ratio in the older rabbits ranges from 0.10 to 0.34 with a mean of 0.20, and in the young rabbits ranges from 0.15 to 0.55 with a mean of 0.36. In comparable older rabbits the ratio of extravascular to intravascular albumin is about 1.5 (113). Thus, fibrinogen must be distributed in much less of the interstitial fluids than albumin.

TABLE XII. In this table s = rabbits given screened labeled fibrinogen; a, b, and d = rabbits given the same labeled fibrinogen preparation; and * = average does not include rabbits number 726 and 780s. For calculations of D_1, d_1, D_2, and d_2 see (112).

TABLE XII

EXTRAVASCULAR FIBRINOGEN CALCULATED FROM EQUATIONS 19 AND 20

Rabbit No.	r_1	r_2	mg/kg $\bar{y}$	$\bar{y}/\bar{x}$	D_1	d_1	D_2	d_2
Older Rabbits								
179b	.71	2.09	39	.34	-	-	-	-
181b	1.01	3.02	25	.33	-	-	-	-
151	1.03	6.90	17	.15	-	-	-	-
795a	.32	1.70	20	.19	-	-	-	-
787	.94	9.00	12	.10	-	-	-	-
797	.63	3.45	22	.18	-	-	-	-
786d	.77	7.50	11	.10	-	-	-	-
Mean	.77	4.80	22	.20	-	-	-	-
Young Rabbits								
725	.81	5.27	21	.15	-	-	-	-
724a	2.05	5.79	47	.35	-	-	-	-
726	1.51	-	35	.33	.71	6.74	.29	1.30
780s	1.08	-	45	.44	.71	4.62	.29	1.03
784d	1.58	5.72	36	.28	-	-	-	-
781s	1.58	2.85	59	.55	-	-	-	-
779s	1.28	3.30	54	.39	-	-	-	-
788s	.92	2.29	38	.40	-	-	-	-
787	2.20	6.00	41	.37	-	-	-	-
Mean	1.44	4.46*	42	.36	.71	5.68	.29	1.51

The Distribution and Fluxes of Fibrinogen

Analyses of the I^{131}-fibrinogen data have shown that the transcapillary transfer and the catabolic systems were not affected by fluctuations in fibrinogen concentration, which are defined in the symbols previously used as $\bar{x}(t)/V_p$ where $\bar{x}(t)$ is the total plasma fibrinogen at time t, and V_p is the plasma volume. Flux to the interstitial fluids over the interval t_1 to t_2 is given by

$$r_1 \int_{t_1}^{t_2} \bar{x}(t)dt/(t_2-t_1)$$

and the flux to the catabolic sites as

$$r_3 \int_{t_1}^{t_2} \bar{x}(t)dt/(t_2-t_1),$$

where the time interval is measured in days. These fluxes may be pictured as the fractions of the volumes of plasma r_1V_p and r_3V_p, "cleared" per day to enter the interstitial fluids and catabolic sites respectively.

The Transcapillary and Catabolic Fluxes of Fibrinogen

Average values for the transcapillary flux, $r_1\bar{x}$, are listed in Table XI, which shows that a mean of 81 mg/kg/day of fibrinogen in the older animals plasma passed to the interstitial fluids compared to a mean of 171 mg/kg/day in the younger animals.

Table XI also shows that a mean of 39 mg/kg/day was catabolized by older rabbits, compared with a mean of 56.5 mg/kg/day by the young rabbits.

CHAPTER XII

Discussion

Preparation and Test of I^{131}-Fibrinogen

a) Preparation of Fibrinogen The purity of the fibrinogen used for labeling is important since iodination will label all proteins present. If the half-lives of the other proteins are longer than that of fibrinogen their presence will extend the apparent half-life of fibrinogen. Thus, suppose that the iodinated fibrinogen contained 10 per cent albumin (half-life 8 days) and 90 per cent fibrinogen (half-life 2 days). Then, after eight days, the albumin radioactivity in the circulating plasma would make up 50 per cent of the total plasma radioactivity, and the values for the slow part of the I^{131}-fibrinogen curve described by $C_1 e^{-a_1 t}$ would be obtained that were much too long.

b) Tests of the I^{131}-Fibrinogen These tests, with the results of their application, have already been described. These studies confirm those of McFarlane (46) in showing that to obtain I^{131}-fibrinogen suitable for tracer studies in vivo, the iodine atom to protein molecule ratio must be 1/2 or less. This has only been appreciated within the last 2 years, and makes the results of the most earlier studies with I^{131}-fibrinogen of uncertain significance. The effect of overiodination of fibrinogen is shown in Figures 4 and 5. If such preparations were used to calculate the fibrinogen

fluxes too high values would be obtained.

In previous studies with tracer-labeled fibrinogen information about fibrinogen concentrations, plasma volume, the constants of the x(t) curve, and the I/P ratio is all or in part lacking. In studies using biosynthetically labeled fibrinogen the disappearance of the radioactivity was obtained from specific activity measurements, which are affected by fluctuations in fibrinogen and by reutilization of the C^{14} or S^{35}-labeled amino acids. In these studies half-lives for labeled fibrinogen of 53 to 86 hours were reported (36) (39) (44) (45) but fibrinogen fluxes and distribution cannot be calculated.

In studies with I^{131}-fibrinogen the disappearance rates of injected radioactivity, for the most part, were obtained from specific activity measurements. The I^{131}-fibrinogen was 85 to 90 per cent clottable, and when noted (35), the I/P ratio was above the optimum level. Half-lives of 48 to 66 hours were given but again distribution and fluxes cannot be calculated.

These criticisms do not apply to the recent study of A.S. McFarlane (46), but his work was concerned with the level of iodination of fibrinogen, and not, except indirectly, with defining the behavior of fibrinogen in healthy animals.

Models Describing the In Vivo Behavior of Fibrinogen

Studies with tracer-labeled fibrinogen can only be interpreted with the aid of a satisfactory model of the biological behavior of fibrinogen. Up to now, though models for albumin metabolism and distribution have been described (113) (114), no studies have been made of the fibrinogen-fibrin system. The studies described here

have permitted the development of such a model. The increase in knowledge of albumin metabolism and distribution has depended largely on the development of models which describe its behavior and the same should be true for fibrinogen.

The Fluxes and Distribution of Fibrinogen in Healthy Young and Older Rabbits

The data of Tables XI and XII provide normal values for young and older rabbits. Of interest are the more rapid transcapillary and catabolic fluxes found in the young rabbits. Thus, the mean transcapillary flux in the young rabbits was 171 mg/kg/day compared to 81 mg/kg/day in the older rabbits. The mean catabolic flux in the young rabbits was 56.5 mg/kg/day compared to 39 mg/kg/day in the older rabbits. A possible explanation is that greater activity in the young animals, or perhaps greater porosity or pinocytosis in the capillary endothelium, is responsible for the increased transcapillary flux. A possible explanation for the increased catabolic flux is greater utilization of fibrinogen during growth. Both these explanations require experimental demonstration.

Significance of the Findings for Fibrinogen Catabolism

The possible mechanisms underlying the first order tracer rate constants have already been discussed. Here it is noted that the findings on catabolism of I^{131}-fibrinogen, namely that this is a first order process, and that catabolism of I^{131}-fibrinogen leaving the plasma occurs without observable delay, would fit a current theory of fibrinogen breakdown. This theory, championed by Copley (29), holds that fibrinogen is continually deposited as fibrin

on the vascular endothelium and continually broken down by fibrinolysis. If the fibrinolytic enzyme is unsaturated by its fibrin substrate this picture would explain the findings. Other mechanisms of fibrinogen catabolism must occur in disease, for instance during inflammation, which may resemble more closely the events that occur after subcutaneous formation of I^{131}-fibrin blood clots.

These mechanisms require further study.

SUMMARY

The object of this study was to define the behavior of I^{131}-fibrinogen and fibrinogen in healthy rabbits. Rabbit fibrinogen of high clottability was first prepared by ammonium sulfate fractionaction and its purity was examined with starch gel zone and moving boundary electrophoresis. The purified fibrinogen was then labeled with I^{131}-iodine and the permissible level of iodination was established at 0.5 atom of iodine per molecule of fibrinogen by "biological screening". Above this level of iodination the *in vivo* behavior of the I^{131}-fibrinogen did not reflect the behavior of native fibrinogen. Standard methods of measuring fibrinogen concentration were tested and found to be inaccurate. An isotope dilution method for measuring the plasma fibrinogen concentration was therefore developed. On test this method gave accurate measurements of plasma fibrinogen concentration. Studies of the behavior of the I^{131}-fibrinogen after intravenous injection were made in 7 older and 9 young rabbits. The animals were studied for 8 to 10 days and the following measurements were made: the plasma fibrinogen concentration, the plasma I^{131}-fibrinogen and I^{131} unbound to protein and the urinary and fecal excretion of radioactivity. The disappearance of I^{131}-fibrinogen from the plasma was described by a 2 or 3 exponential equation. On the basis of present knowledge models of fibrinogen and I^{131}-fibrinogen behavior were advanced. These models were modified by reference to the observed behavior of I^{131}-fibrinogen

and equations were developed to describe them. Using these equations, the fractional rates of I^{131}-fibrinogen transcapillary passage, and catabolism were calculated, and from these the fluxes of fibrinogen into the interstitial fluids and to catabolic sites were calculated. The transcapillary flux averaged 171 mg/kg/day in the young rabbits and 81 mg/kg/day in the older. The fibrinogen catabolic flux averaged 56.5 and 39 mg/kg/day in the young and older rabbits respectively. The equations of the model allow calculation of the total interstitial to plasma fibrinogen ratio. This averaged 0.20 and 0.36 in the older and young animals respectively. In the older animals the total plasma fibrinogen per kilogram bodyweight averaged 106 mg and the total interstitial fibrinogen per kilogram bodyweight 22 mg. In the young animals the values were 118 mg and 42 mg respectively.

The studies show that the I^{131}-fibrinogen system behaves either as an unsaturated or a volumetric clearance system with respect to transcapillary passage and fibrinogen catabolism. The physiological significance of this is examined.

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