A New Technic for the Production of an in Vivo Labeled Fibrinogen

By Henry Gans, James Mc Leod and James T. Lowman

ACCURATE IN VIVO KINETIC STUDIES of plasma proteins have been greatly facilitated by the use of radioactive labeled proteins. This labeling has been accomplished in vitro using iodine-131 (I^{131}) or chromium-51 (Cr^{51}) and in vivo with phosphorus-32 (P^{32}), tritium (H^{3}) or carbon-14 (C^{14}). Proteins labeled in vivo when compared with those labeled in vitro frequently demonstrate differences in physiologic clearance rates.[1-3] In vitro labeled proteins, as a rule, exhibit faster turnover rates than in vivo labeled material. It is assumed that denaturation of the protein, elution of the label, or inadequate removal of the unbound isotope from the protein solution produces this inconsistency of the in vitro labeled material. This problem does not exist when in vivo biosynthetic labeled plasma constituents are used. The in vivo labeling, however, has inherent disadvantages: (1) It requires large amounts of labeled amino acids to produce high specific activity material. The injected labeled precursors may be lost in the urine or removed by organs which do not contribute to plasma protein production, and the tagged proteins may be diluted by unlabeled proteins from the existing plasma and extravascular pools. (2) The labeling isotope has most frequently been a soft beta emitter which requires special, time-consuming preparation for counting and accurate quantitation.

The search for a labeled fibrinogen preparation of high specific activity led to the present study. Four major modes of attack were undertaken.

1. *Choice of isotope:* An energetic gamma emitter, selenium-75 (Se^{75}), as selenomethinonine was selected as the labeling precursor. Se^{75} has the added advantage of a 120 day half-life.

2. *Enhancement of fibrinogen formation:* Acceleration of fibrinogen formation was artificially induced. This condition provided the labeled amino acid with the opportunity to be incorporated at a faster rate and in greater concentration.

3. *Prevention of elimination:* Urinary loss of the radioactive amino acid was prevented by nephrectomizing the injected animals.

4. *Providing constant high concentrations of labeled amino acid:* In order to

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provide a labeled amino acid pool during the entire period of new fibrinogen formation, a constant infusion of selenomethionine was undertaken.

An in vivo labeled, gamm-emitting fibrinogen of relatively high specific activity and with a long half-life was harvested.

Materials and Methods

A 17 Kg. mongrel dog and retired Holtzman breeder rats were used for all experiments. The rats were fed Purina rat chow and the dog a standard laboratory formulation.

Surgical Procedures: Bilateral nephrectomies were carried out under nembutal anesthesia through small midline incisions. The animals were fully awake 2 hours after the operation and were active prior to thrombin-induced defibrination. Femoral vein cutdowns were performed on the restrained dog and rats. In the former a #15 polyethylene catheter and in the latter a #27 (P.E.10) polyethylene catheter was inserted. The catheters were held in place with silk ligatures.

Defibrination: Defibrination of the rats was obtained by a single injection of 2500 units of bovine thrombin* into the peritoneal cavity. The intravenous infusion of bovine thrombin over a 2-hour period into the dog has been previously described.[4]

Labeling Material: L-seleno-75 methionine† was used as the labeled amino acid in all experiments. The material had a specific activity of 1.0 to 1.9 mc./mg. methionine. Prior to its use, radioactivity of this material was determined in a well counter. Amounts used in rats ranged from 50–200 microcuries per animal, and in the dog 900 microcuries were used. Slow, continuous infusions were carried out with the use of a Harvard infusion pump.

Blood Sampling: Blood samples for fibrinogen determinations were obtained from the tail vein of the rat. At the end of the labeling experiment, the rats were anesthetized with ether and the peritoneal cavity was opened and inspected for evidence of peritonitis. In the absence of peritonitis, the animals were exsanguinated from the abdominal aorta using a 20-gauge needle and an EDTA-containing syringe (0.5 milliliters of 10 per cent EDTA per 11.5 milliliters of blood). In the dog a femoral artery catheter was used to collect blood samples.

Determination of Radioactivity: Radioactivity of 1 ml. aliquots of each blood sample was determined in a well counter with a single-channel-analyzer unit. The samples were prepared in identical counting vials and samples of different geometry were corrected to equal 1 ml. Each sample was counted to a total of 5000 counts or greater and background was subtracted. This resulted in a counting error of $\pm$ 1.4 per cent as one standard deviation. In order to determine the amount of activity excreted by the rats, with and without nephrectomy, the animals immediately following injection were counted on a 5 $\times$ 5 in. sodium iodide crystal and counted again immediately before sacrifice. The counts were standardized by counting an equivalent sample of standard material and appropriate corrections were calculated where necessary. Specific activities reported are net counts per mg. of material. The full energy peak efficiency for this isotope in the equipment described is 28 per cent.

Total Protein Determinations: Total protein concentration of plasma samples was determined by the micro Kjeldahl technic.

Fibrinogen Determinations: Fibrinogen was determined by the procedure described by Jacobsson,[5] using resin thrombin[6] to clot the fibrinogen. The clot was washed for several hours against frequent changes of saline.

Results

Rate of Fibrinogen Regeneration Following Thrombin-Induced Defibrination of Blood

As previously reported,[4] a 2-hour intravenous infusion of 2500 units of thrombin in dogs produced a profound defibrination. Infusion of 3500 units re-

*Parke-Davis and Co.

†E. R. Squibb and Sons, New Brunswick, N. J.

Table 1.—*Role of Incorporation of Radioactivity into Plasma Proteins and Fibrinogen during Fibrinogen Regeneration in Nephrectomized, Defibrinated* Dog†*

Blood Sample	Fibrinogen Concentration in mg. Per Cent	Counts/ Min./Ml. Plasma	Counts/ Min./Mg. Fibrinogen
1: Before thrombin infusion	345		
2: Immediately after thrombin infusion	63		
3: Two hours after thrombin infusion	69	960	105
4: Four hours after thrombin infusion	94	7083	606
5: Six hours after thrombin infusion	115	16033	1272
6: Eighteen hours after thrombin infusion	269	57937	2796
7: Twenty-two hours after thrombin infusion	299	64519	3348

*Defibrination by means of intravenous infusion of 3500 units of bovine thrombin over a 2-hour period.

†Weight of the dog: 37 pounds.

900 μc. of Se^{75} methionine was infused intravenously iver a 17-hour period.

sulted in nearly complete defibrination as measured by this method. Following the defibrination, a gradual rise in fibrinogen concentration was observed. Fibrinogen levels returned to or above preinfusion values within 24 hours. During this recovery phase circulating fibrinogen concentration increased at an average rate of 10–15 mg. per cent per hour (Table 1).

Injection of 2500 units of thrombin into the peritoneal cavity of rats resulted in nearly complete defibrination of the blood in 2 hours. Twenty-four hours later, fibrinogen concentration in the intact rats reached an average of 145 per cent of the preinfusion value (Table 2). Average fibrinogen values following nephrectomy was higher than in nonoperated animals. Fibrinogen regeneration in the nephrectomized group was found to be less than in the nonoperated controls. In the nephrectomized animals an average fibrinogen concentration of 75 per cent of the prethrombin value was observed at 24 hours following thrombin-induced defibrination (Table 3).

Labeling Experiments

Because of the limited blood volume of the rat, it was deemed inadvisable to introduce additional changes by obtaining frequent blood samples. Instead, the changes in fibrinogen levels and fibrinogen radioactivity were studied in a defibrinated, nephrectomized dog. The dog received a slow infusion of 900 μc. Se^{75} methionine intravenously beginning 2 hours following defibrination and continuing over a 17-hour period. In this way, return of circulating fibrinogen could be studied with more precise definition of the temporal changes. The rise in fibrinogen concentration was noted to parallel closely the increase in the specific activity (Table 1).

Effect of Increasing Doses of Se^{75} Methionine on Specific Activity

Four nephrectomized rats were subjected to thrombin-induced defibrination followed by the slow intravenous infusion of 100 μc. Se^{75} methionine over a 17-hour period with the aid of a Harvard infusion pump. The animals were exsanguinated 24 hours after thrombin injection by means of aortic puncture.

Table 2.—*Effect of Intraperitoneal Administration of Thrombine* on the Circulating Fibrinogen Concentration of Rats*

Rat No.	Fibrinogen Concentration of Rat Tail Vein Blood in Mg. Per Cent			
	Before Thrombin*	2 Hours after Thrombin*	6 Hours after Thrombin	24 Hours after Thrombin*
3	310	64		420
4	256	0		446
5	357	0		409
6	221	83		
7	220	0		
8	260	0		
9	299	0		329
10	202	0	30	343
11	248	0	9	357
12	232	0	7	345
Average value	260	16	15	378

*Bovine thrombin (Parke Davis Co.) 2500 units in 1 ml. N saline.

Table 3.—*Effect of Intraperitoneal Administration of Thrombin on the Circulating Fibrinogen Concentration of Nephrectomized Rats*

Rat No.	Fibrinogen Concentration of Rat Tail Vein Blood in Mg. Per Cent		
	Before Thrombin	2 Hours after Thrombin	24 Hours after Thrombin
1R	274	0	218
2R	281	0	271
5R	372	0	143
7R	409	50	242
8R	359	0	253
9R	304	0	221
10R	350	0	391
11R	437	0	271
Average value	336	6	257

The mean specific activity of the fibrinogen prepared from the plasma of these animals was 7715 counts per minute per milligram of fibrinogen. The range of counts was 7000 to 8120.

Two animals were prepared in similar fashion and received 200 μc. Se^{75} methionine each. The specific activity of the fibrinogen prepared from the plasma of these animals was 13,220 and 20,762 counts per minute per milligram fibrinogen. These represent values of twice that observed following the injection of 100 μc. (Fig. 1).

Effect of Flash Labeling Technic

Results obtained with flash labeling were compared with those obtained with the continuous infusion of the labeled amino acid. Two nephrectomized, defibrinated rats received 100 μc. Se^{75} methionine as a single intraperitoneal injection. Activity of the fibrinogen prepared from the plasma of these animals was 4929 and 6103 counts per minute per mg. fibrinogen. This yielded an aver-

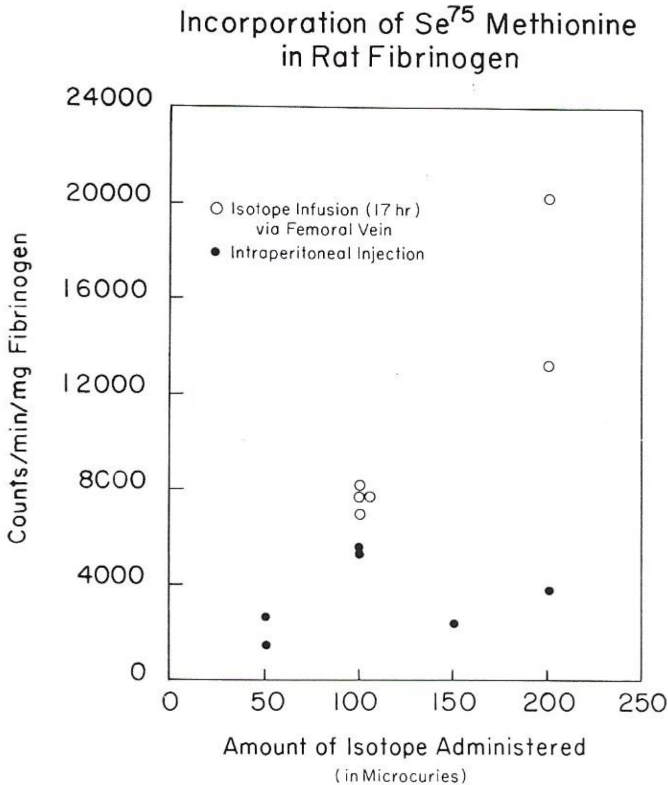

Fig. 1.—The specific activity of isolated rat fibrinogen obtained following either a single injection (flash labeling) or slow infusion at various isotope doses are compared. The slow infusion of a large amount of Se^{75} methionine yields the highest specific activity as counts/min./mg. fibrinogen.

age specific activity of 5516 counts/min./mg., which is less than the 7715 counts/min./mg. obtained when the isotopically labeled amino acid was infused slowly during the regeneration phase (Fig. 1).

Effect of Nephrectomy

Labeling was carried out in thrombin-induced defibrinated non-nephrectomized animals. Two rats received 100 μc. $seleno^{75}$ methionine intraperitoneally. Results obtained with this procedure were compared with those obtained in nephrectomized animals. Average activity of the fibrinogen prepared from the plasma of these animals was 4691; the counts were 4995 and 4387 per min./per mg.

Total body counting of defibrinated, intact and nephrectomized rats following an intraperitoneal injection of Se^{75} methionine was performed. Total body retention of the labeled material at the time of sacrifice of the animal was determined. Average loss of radioactivity in 4 nephrectomized rats receiving selenomethionine in doses ranging from 10–200 μc. amounted to only 8 per cent of the injected dose. In contrast, two non-nephrectomized animals receiving

100 μc. each had losses averaging 35 per cent. The difference in the results obtained in these two groups of animals thus represents loss of radioactivity via the kidneys. This loss may consist of the labeled amino acid or its breakdown products.

Specific Activity of Fibrinogen vs. Other Plasma Proteins

The radioactivity of 1 ml. of plasma and the TCA-soluble fraction was determined. The activity of the plasma minus that of the TCA-soluble fraction yielded the activity of the protein in each sample. This value, when divided by the total protein concentration of the aliquot, gives the specific activity of the protein as counts/min./mg. of plasma protein. The fibrinogen specific activity over the plasma protein specific activity yields a comparative ratio for specific labeling of the fibrinogen. Two determination using 100 μc. of Se^{75} methionine yielded ratios of 2.3 and 2.1; one determination with 200 μc. yielded a ratio of 1.7.

Discussion

The impressive mass of clinical and experimental evidence summarized by Madden and Whipple[7] and Miller and Bale[8] strongly supports the hypothesis that all plasma proteins, except the globulins, are formed by the liver. More direct evidence that the liver produces fibrinogen was recently demonstrated by Forman and Barnhart.[9]

The exclusive role of the liver in the biosynthesis of fibrinogen has been affirmed by Miller and Bale[8] in isolated liver perfusion experiments. These investigators noted that the normal rate of fibrinogen formation could be enhanced 3 to 4 times in livers perfused with defibrinated blood. It was felt that the hypofibrinogenemia provided the stimulus for the organ's accelerated rate of fibrinogen formation. If a similar response could be elicited in the living animals, it would serve an important means for obtaining fibrinogen production at a significantly accelerated rate.

Previously, McFarlane[10] observed in rabbits that 80 per cent of all fibrinogen is present in the blood. If this same situation pertains to the rat, thrombin-induced defibrination of blood would rid the animal forthwith of at lesat four-fifths of its fibrinogen stores. Thus, near complete elimination or removal of the entire pool of unlabeled fibrinogen can be obtained. This would provide the necessary stimulus for the liver to produce fibrinogen at a much higher rate. In addition, this procedure eliminates the major share of unlabeled fibrinogen from decreasing the specific activity of newly-formed labeled fibrinogen.

Under normal conditions, regeneration of rat fibrinogen takes place at a rapid rate. Campbell and associates[11] found that the turnover of rat fibrinogen approximates 50 per cent in 24 hours. Following complete defibrination of rats, we found that average plasma fibrinogen values increased to 145 per cent of predefibrination values within 24 hours (Table 2).

This impressive gain was applied in the present study to obtain enhanced incorporation of the labeled, essential amino acid L-Se^{75} methionine into fibrinogen. In rat fibrinogin, methionine and the products of its metabolism,

cysteine and cystine, account for approximately 5 per cent of the amino acid composition.[12]

Labeled amino acids injected into animals are rapidly cleared from the blood by various organs. Those organs which exhibit active protein metabolism show a preferential uptake. The liver, as a source of plasma protein production, takes up a large share of this material. However, Tarver and Reinhardt[13] found that intravenous methionine was utilized by hepatectomized dogs at about the same rate as in normal, nonoperated dogs. In the hepatectomized dog, the material was predominantly present in pancreas, intestinal mucosa and kidneys.

From these and our own observations, it would appear that the effect of nephrectomy is twofold: First, it reduces the loss of total body radioactivity. At least part of this reduction resulted from the prevention of elimination of labeled amino acid by the kidney. Thus, fuller utilization, including a possible reutilization of the amino acid by the liver, can now take place.[14] Second, the removal of the kidneys eliminates an important extrahepatic source of methionine uptake.

Constant infusion of the labeled amino acid provided a preparation with a specific activity greater than that obtained when "flash" labeling technics were used. This finding suggests that the constant replenishing of the labeled amino acids makes this material more readily available for incorporation. Previously, Kinsell and co-workers[15] noted that following one injection of S^{35} methionine maximum incorporation into plasma protein was obtained in 8 hours with little change thereafter. Miller and Bale observed that the production of labeled plasma proteins by an isolated perfused rat liver gradually declined and practically ceased after 4 hours. However, it would resume immediately its former rate when fresh, labeled amino acid with a greater opportunity to become incorporated.

The results of these studies indicate that each individual step of the procedure—defibrination, nephrectomy, and prolonged infusion of a labeled amino acid—enhances the incorporation of the label into rat fibrinogen. The combination of these steps has provided us with a technic for obtaining biosynthetically labeled fibrinogen of high specific activity.

Summary

The fact that in vitro labeled proteins, as a rule, exhibit faster turnover rates than in vivo labeled materials led us to explore means of obtaining in vivo labeled fibrinogen of high specific activity. It was found that defibrination of the rat provides a stimulus for the liver to regenerate fibrinogen at an accelerated rate. Administration of seleno^{75} methionine shortly after thrombin-induced defibrination of the animal resulted in the incorporation of large quantities of the label. The rate of incorporation was further increased if the amino acid was administered as a slow infusion during the entire period of fibrinogen regeneration. In addition, prior nephrectomy of the animal would appear to result in a slight increase in specific activity of the fibrinogen preparation obtained.

The results of these studies indicate that defibrination, nephrectomy, and

the prolonged infusion of the labeled amino acid selenomethionine provided us with a technic for obtaining a biosynthetically labeled, γ-emitting, fibrinogen preparation of high specific activity.

Summario in Interlingua

Le facto que, a generalmente parlar, proteinas marcate in vitro manifesta plus accelerate transitos metabolic que materiales marcate in vivo ha inducite nos a explorar methodos pro le obtention de fibrinogeno a marcage in vivo e de alte activitate specific. Esseva trovate que defibrination del ratto provide un stimulo pro le hepate a regenerar fibrinogeno acceleratemente. Le administration de seleno^{75}-methionina brevemente post le defibrination del animal inducite per thrombina resultava in le incorporation de grande quantitates del marca. Iste incorporation esseva accelerate additionalmente quando le amino-acido esseva administrate per un methodo de lente infusion durante le integre periodo del regeneration de fibrinogeno. In plus, le subjection preparatori del animal a nephrectomia pare resultar in un leve augmento in le activitate specific del finalmente obtenite preparato de fibrinogeno.

Le resultatos del presente studios indica que defibrination, nephrectomia, e le lente infusion del marcate aminoacido seleno^{75}-methionina ha providite nos con un technica pro le obtention de un biosyntheticamente marcate preparato de fibrinogeno que emitte radios γ e possede un alte activitate specific.

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