

Isotopic Studies of Therapeutic Anticoagulation with a Coagulating Enzyme

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ABSTRACT The kinetics of the depletion of plasma fibrinogen were studied in seven patients who received fibrinogen-¹³¹I 1 hr before an intravenous injection of the coagulating enzyme (CE) derived from the venom of the pit viper, *Agkistrodon rhodostoma*. Disappearance of the clottable radioactively labeled fibrinogen from the circulation conformed to an exponential decay with an average half-life of 0.85 hr. The mean clearance rate for protein-bound radioactivity, composed of fibrinogen and its split products, was 12% of the intravascular pool per hour. The breakdown products of fibrin produced by CE inhibited polymerization of fibrin in vitro.

Studies in five patients performed between the 3rd and 10th day following the administration of CE revealed that the absolute catabolic rates of fibrinogen were subnormal initially, but gradually increased as the fibrinogen concentration returned to normal.

In rabbits, after the administration of CE, regeneration of the fibrinogen pool was markedly prolonged. This delayed regeneration time was not influenced by an excess of antivenene, but rapid regeneration to pretreatment values of plasma fibrinogen was immediately initiated by stimulating fibrinogen synthesis with subcutaneous turpentine.

INTRODUCTION

The coagulation impairment produced by the coagulating enzyme (CE) (Arvin), fractionated from the crude venom of *Agkistrodon rhodostoma*, resides in its ability to affect circulating fibrinogen directly. The CE produces progressive depletion of the fibrinogen pool, probably through the formation of an atypical fibrin monomer

differing from that produced by thrombin (1-5). Kinetic data obtained from animal experiments employing labeled fibrinogen indicate that fibrinogen depletion can, under optimal circumstances, proceed at exceptionally rapid rates, corresponding to half-times of 5-15 min (3, 6, 7). However, these values were obtained using large amounts of CE in radiated animals and are not representative of the therapeutic doses employed in man by Bell, Pitney, and Goodwin (8) and Sharp, Warren, Paxton, and Allington (9).

Bell and his associates (5, 8) found that the time required for the fibrinogen pool to regenerate was unexpectedly long after discontinuation of therapy with CE. The cause of the delay was investigated in the kinetic studies described in this report, which include observations in patients as well as in experimental animals.

METHODS

The CE used in all studies was prepared from a single batch of crude venom from *Agkistrodon rhodostoma*. It was supplied as Arvin¹ in sterile ampoules containing 75 U (1 U containing 2 µg protein) dissolved in 1.0 ml of 0.03 M sodium phosphate/0.13 M sodium chloride at pH 7.0. Clotting time measurements,² using purified human fibrinogen as a substrate, indicate that 2 µg of CE correspond to 1 NIH U of thrombin. CE was administered intravenously to all patients and animals at a dose of 1 U/kg body weight. In patients the initial dose was given in 100 ml of sterile 0.9% sodium chloride and infused slowly over a period of 4-6 hr by means of a constant infusion pump (B. Braun type 1830, Apparaturbau Melsungen, West Germany). Upon completion of the infusion, a second dose was injected in 20 ml of 0.9% sodium chloride over 10-15 min and repeated every 12 hr for the duration of therapy. In animals, the initial dose was given in 10 ml of 0.9% sodium chloride over 30 min and subsequent doses, when given, were administered in 1-2 ml of 0.9% sodium chloride over 5 min.

¹ Supplied by Prof. F. A. Robinson, Twyford Laboratories, London, N.W. 10, England.

² Performed by Mr. G. W. Tunnah, Twyford Laboratories, London, N.W. 10, England.

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Antivenene was unfractionated horse serum produced by immunization against crude *Agkistrodon rhodostoma* venom,³ and diluted 1 in 3 with 0.9% sodium chloride. 1 ml of the antivenene neutralized 260 μg (130 U) of the CE in vitro as estimated by fibrinogen clotting times. After routine skin testing in patients, antivenene was administered intravenously in 50 ml of 0.9% sodium chloride over 30 min. No special precaution was observed in animals, and a volume of 1–2 ml was infused into a peripheral ear vein.

Fibrinogen from human and other species was prepared in the Biophysics Division at the National Institute for Medical Research at Mill Hill from fresh oxalated plasma by fractionation with ammonium sulphate, as described (10). Each preparation contained 10 mg of protein in 0.25–0.3 ml vol and was iodinated with ¹²⁵I- or ¹²⁵I-labeled monochloride (11) so that substitution rates did not exceed one atom of iodine per molecule of fibrinogen (12). Unbound radioactivity was removed by passing the preparations through columns (4 × 0.7 cm) of a strong anion-exchange resin,⁴ which were thoroughly washed before loading with 0.9% solution of sodium chloride containing 0.005 M trisodium citrate. Labeled fibrinogen preparations for injection were sterilized by filtration⁵ and refrigeration at –25°C and used within 48 hr. Of the protein-bound radioactivity 92–94% was clottable with thrombin in the case of the human and 94–97% in the case of the rabbit fibrinogen preparations. Carrier-free ¹²⁵I and ¹³¹I were obtained commercially.⁶

10-mg portions of a commercially available human albumin preparation⁷ were labeled with ¹³¹I at mean degrees of substitution ranging between 1 and 1.5 atoms of iodine per molecule of albumin using the same methods described above for labeling fibrinogen.

CE releases more peptides from fibrinogen than does thrombin (4), and to qualify the use of labeled fibrinogen in connection with CE it was necessary to determine whether these peptides contained any of the isotopic label. Labeled fibrinogens from different species (human, monkey, rabbit, sheep, and dog) were clotted with 5 NIH U thrombin and 10 μg CE, respectively, and the nonprotein residue of the clotting mixtures was determined using previously published methods (10). These experiments showed that the release of radioactivity from fibrinogen on clotting followed the general pattern found earlier with thrombin (10): no losses were encountered with fibrinogens in which fibrinopeptide B either lacks a tyrosine residue or this residue is esterified with sulfuric acid (primates, rabbit). Sheep and dog fibrinogens containing free tyrosine residue (13) in the B peptide lost some label on clotting with CE but less than when thrombin was used to initiate clotting.

Assay of plasma radioactivity. 5 ml of venous blood samples were drawn from the subjects who had received labeled fibrinogen and placed into test tubes containing 4 mg of dry dipotassium EDTA as an anticoagulant for each milliliter of blood. After separation by centrifugation, 0.5 ml of the plasma was immediately clotted in a counting tube containing 1.5 ml of phosphate buffer (0.054 M sodium phosphate/0.073 sodium iodide at pH 6.1) in which 50

NIH U thrombin⁸ and 1 mg of crystalline soybean trypsin inhibitor⁹ were dissolved. In view of the low fibrinogen concentrations often encountered, the samples were allowed to remain initially at room temperature for 6 hr and then stored at 4°C for several days before processing them. The latter consisted of separating radioactivity associated with clottable, nonclottable but protein-bound, and nonprotein fractions, using methods described elsewhere (14).

Turnover rates were calculated by graphically analyzing the data of clottable protein-bound radioactivity, as described by Matthews (15).

Plasma fibrinogen concentration was measured using a modification of Jacobsson's technique (16) as described elsewhere (14).

Animals. Male Sandylop rabbits of the Mill Hill strain, weighing between 2.9 and 3.7 kg were used. The animals were kept individually in metabolic cages and received a standard pellet diet and drinking water containing 0.005% sodium iodide and 0.45% sodium chloride.

Rate of regeneration of plasma fibrinogen. In six rabbits, the plasma fibrinogen concentration was measured before and at frequent intervals for 9 days following two intravenous doses (2 $\mu\text{g}/\text{kg}$) of CE separated by an interval of 30 min. In six other rabbits, plasma fibrinogen concentrations were measured in an identical manner before and following a single injection of 1550 NIH U thrombin. In four other rabbits, when the plasma fibrinogen concentration reached its nadir following the second dose of CE, a subcutaneous injection of 2 ml of turpentine was administered and the plasma fibrinogen concentration was monitored at frequent intervals. Fibrinogen turnover rates were calculated in all animals and as well as in six control animals receiving subcutaneous turpentine alone.

Studies in patients Measurements with labeled fibrinogen were performed during the institution (seven patients) and after completion (five patients) of therapy with CE. In each instance, thyroid uptake of ¹²⁵I or ¹³¹I was prevented by oral administration of potassium iodide 180 mg every 8 hr. Permission for the administration of the various radioactivities to patients was granted by the Committee on Isotopes for the Medical Research Council. Informed consent was obtained from each patient before the institution of the various therapeutic procedures. To examine the effect of the initial dose of CE on circulating fibrinogen, 50–60 μCi of labeled fibrinogen was injected 1 hr before the infusion of CE. Plasma samples were taken at frequent intervals over a period of 7 hr, and in some patients, an additional sample was obtained at 13 hr.

Turnover studies during the regeneration phase (time of return of plasma fibrinogen to normal pretreatment concentrations following discontinuation of CE) were commenced after an interval of 3 days in four patients and 2 days in one patient between the last dose of CE and the labeled fibrinogen (70–90 μCi). This interval was probably adequate to exclude the possibility that trace amounts of CE were still circulating. As an additional precaution to insure the exclusion of trace amounts of CE 1 ml of antivenene was administered 1 hr before the labeled fibrinogen.

Disappearance of labeled albumin from the plasma during the infusion of CE was studied in four patients by injecting 30–40 μCi of labeled albumin 1 hr before commencing treatment. Plasma samples were measured for albumin at fre-

³ Obtained commercially from the Pasteur Institute, Paris, 15, France.

⁴ De-Acidite FF, Permutit, London, England, 100–200 mesh, chloride form.

⁵ Millipore Corp., Bedford, Mass. Pore size 0.22 μ .

⁶ The radiochemical Centre, Amersham, England.

⁷ Behringwerke, Marburg, Germany.

⁸ Leo Pharmaceutical Ltd., Copenhagen, Denmark. Specific activity: 120 NIH U/mg.

⁹ Worthington Biochemical Corp., Freehold, N. J.

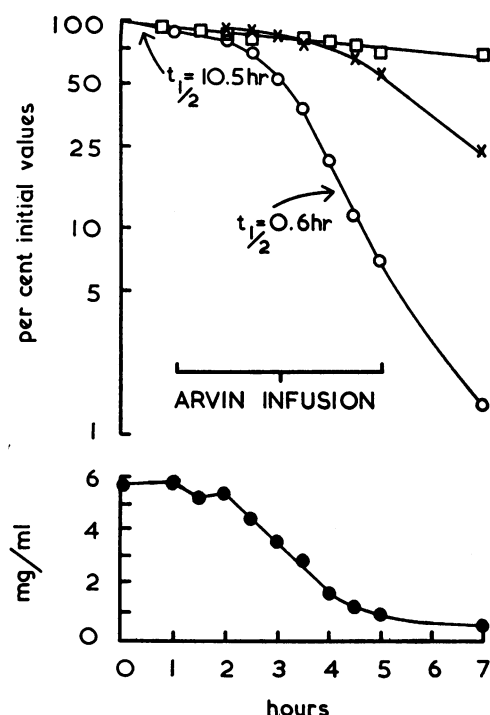


FIGURE 1 Effects of infusing CE on circulating fibrinogen-¹²⁵I in a patient. The semilog curves are: clottable radioactivity per milliliter of plasma (○), sum of clottable and nonclottable protein-bound radioactivities (□), fibrinogen specific activity (×), and plasma fibrinogen concentration (●).

quent intervals together with hematocrit values. Normal values for the rate-labeled albumin disappearance were obtained from two control patients free from organic disease.

RESULTS

Initial changes in the fibrinogen pool. The effects of CE on circulating fibrinogen were studied in seven patients and representative results in one patient are illustrated in Fig. 1. In all seven patients, the effect of CE in reducing the clottable radioactively labeled fibrinogen increased gradually. The degree of alteration in the circulating level of fibrinogen observed during the first 2 hr of the enzyme infusion (half the dose) was small. Then the slope increased progressively until only a small fraction of the original clottable radioactively labeled fibrinogen remained. This residual fraction disappeared more slowly. In all patients, the clottable radioactively labeled fibrinogen was decreased to 1–5% of the initial value by the 7th hour of the experiment.

In view of the manner in which CE altered the level of circulating radioactively labeled fibrinogen (Fig. 1), the disappearance of the clottable protein could not be described by a single rate constant. However, the nearly

linear slope between the initial and terminal inflexions was a useful relative measure for expressing the rate at which the preponderance of the coagulable protein disappeared. The half-lives indicated by these slopes detailed in Table I, suggest a maximal mean disappearance rate of 82% (53–115%) of the clottable intravascular fibrinogen pool per hour.

Normally, the plot of the sums of the clottable and nonclottable protein-bound radioactively labeled components yield a slope which runs parallel and very close to the slope obtained by plotting the clottable radioactively labeled protein alone. The profound difference between these curves in the patients (Fig. 1) reflects the presence of incoagulable degradation products which also affected the clottability of the intact circulating fibrinogen. The half-lives of this plot representing the clearance of both fibrinogen and fibrinogen breakdown products are also listed in Table I, and the $t_{1/2}$ value corresponds to 12% (7–19) of the intravascular pool per hour.

Alterations in the plasma concentrations of labeled proteins during the infusion of CE. Soon after initiating the infusion of CE in four of seven patients, a small and transitory rise in the concentration of the labeled fibrinogen was observed. The duration of this rise was approximately 1 hr (Fig. 2). This phenomenon was not specific for fibrinogen, since similar observations were made in three of four patients receiving labeled albumin under the same conditions (Fig. 3). In these patients, the increase in labeled albumin concentration occurred during the latter half of the infusion of CE. However, the increments of per cent change in the concentration of both fibrinogen and albumin were minimal and, by calculation, were only a few per cent of the circulating radioactivities. Hematocrit values measured at 5-min intervals beginning at 1 hr after starting the

TABLE I
Approximate Half-Lives of the Clottable (C) and of the Sum of the Clottable and Nonclottable Protein-Bound (C + PB) Radioactivities in Seven Patients during Infusion with Arvin

Patient no.	Sex	Age yr	Body wt kg	Plasma fibrinogen concn* mg/ml	Half-life of (C) hr	Half-life of (C + PB) hr
1	M	57	62	2.94	0.67	3.6
2	M	68	81	5.15	0.63	3.7
3	F	24	52	3.81	1.30	4.2
4	F	63	64	4.16	0.75	6.1
5	F	25	61	5.77	0.60	10.5
6	M	45	103	3.15	0.80	5.2
7	F	66	91	3.90	1.21	6.4
Mean					0.85	5.7

* Measured a few minutes before starting the infusion.

infusion of CE and continued for 3 hr revealed no statistically significant change.

Fibrinogen turnover following discontinuation of CE. The return of plasma fibrinogen concentration to normal following the discontinuation of CE is exceedingly slow (8). The data on the metabolic turnover of fibrinogen during this regeneration phase are summarized in Table II. These data demonstrate that the fractional catabolic rates of fibrinogen were significantly increased in each of the five patients studied. The absolute catabolic rates were only moderately elevated. To explain this discrepancy, it must be pointed out that the absolute catabolic rates in Table II are over-all balances for the observation periods during which quantities of fibrinogen available for catabolism progressively increased from subnormal to normal levels while the simultaneous fractional catabolic rates remained essentially the same (Fig. 4). Absolute catabolic rates thus were minimal at the initial portion of the regeneration phase, but progressively increased to a substantial magnitude toward the end of the experiments. The distribution of the labeled protein between the intra- and extravascular pools was slightly shifted in favor of the extravascular compartment. The capillary transfer rate of fibrinogen averaged at 59% of the intravascular pool per day, this being similar to the corresponding value found in normal subjects (17).

Rate of recovery of the plasma fibrinogen concentration in rabbits. The results of experiments examining the regeneration of the intravascular fibrinogen pool following discontinuation of treatment with CE are sum-

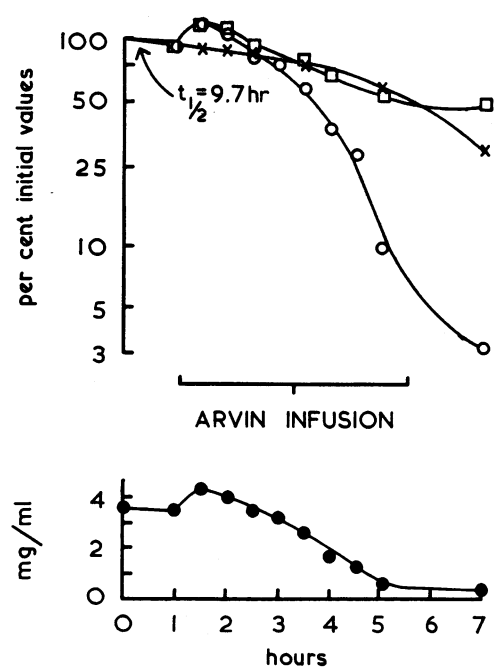


FIGURE 2 Redistribution of intravascular fibrinogen- ^{125}I activity in a patient in response to infusion of CE. For symbols, see Fig. 1.

marized in Fig. 5. It is evident that the plasma fibrinogen concentration increased slowly, and even 9 days later, only 70–80% of the initial value was reached. The rate of regeneration was not accelerated by antivenene injected intravenously 30 min after CE. In striking contrast,

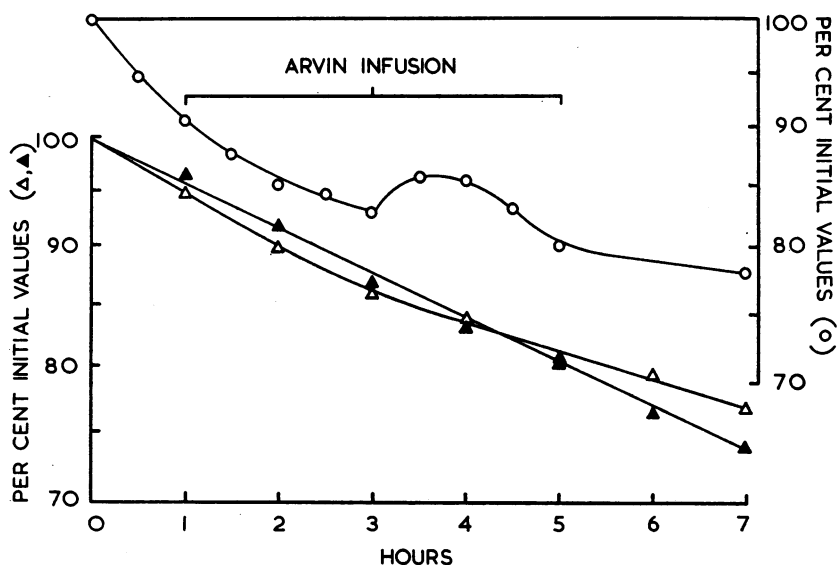


FIGURE 3 Disappearance of albumin- ^{125}I from the plasma during CE infusion, the upper diagram showing the mean curve from three patients (○) compared with two disappearance curves (▲, △) from normal subjects in the lower diagram.

TABLE II
*Turnover of Iodine-Labeled Fibrinogen during Regeneration of the Fibrinogen Pool
in Patients after Treatment with Arvin*

Patient no.	Sex	Age	Body wt	Duration of treatment	Half-life of final slope	Fractional capillary transfer rate	Pool ratio EV/IV	Fgn* concn	IV† pool	Catabolic rate	
										Fractional	Absolute
		yr	kg	days	hr			mg/ml	g		mg kg ⁻¹ day ⁻¹
1	M	58	61	6	47	0.48	0.37	2.64	5.79	0.52	49.3
2	F	47	61	8	41	0.82	0.32	1.50	3.29	0.52	28.0
3	M	68	81	6	65	0.45	0.21	2.45	7.14	0.33	29.1
4	M	45	103	12	60	0.51	0.32	2.08	7.71	0.41	30.6
5	M	38	56	14	37	0.69	0.28	1.89	3.81	0.60	40.8
Mean values					50	0.59	0.30	2.11		0.47	35.5

EV = extravascular; IV = intravascular. Fractional rates are expressed as those of the intravascular pool per day.

* Calculated by measuring the area under the curves of plasma fibrinogen concentration over the observation period.

† Calculated from the prevailing mean fibrinogen concentration during the experiment.

following the infusion of thrombin, there was a prompt return of plasma fibrinogen to the preinfusion value. When, following the infusion of CE, fibrinogen synthesis was stimulated by subcutaneous turpentine, the response was noted within a few hours and within 2 days had exceeded the preinfusion value. During the response of fibrinogen synthesis, the fractional catabolic rate of fibrinogen was 110% per day; this explains that the maximum fibrinogen concentration reached was only

4.7 mg/ml, contrasted with 9.1 mg/ml in the control group receiving turpentine but no CE. It is apparent that the action of the CE on fibrinogen, unlike that of the naturally occurring enzyme thrombin, is followed by a retarded return of the plasma fibrinogen concentration to normal. However, CE per se does not paralyze the fibrinogen synthesizing mechanism, since significant production was observed when adequate stimulation was provided by subcutaneous turpentine.

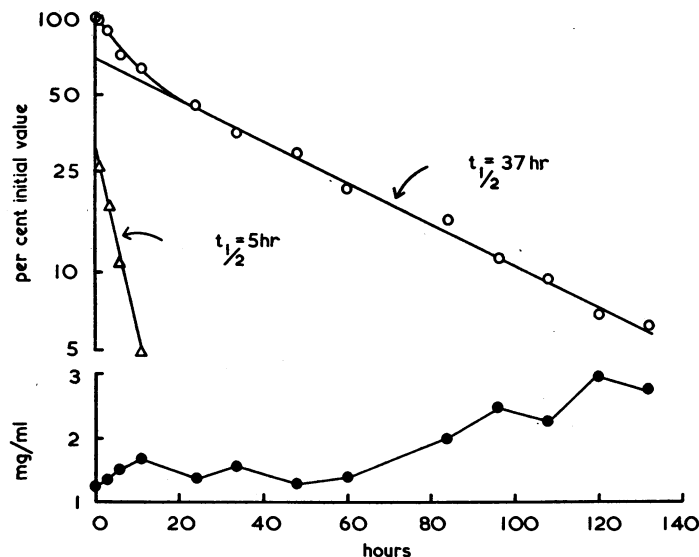


FIGURE 4 Turnover of fibrinogen-¹²⁵I in a patient after treatment with CE for 14 days, the labeled protein being injected 3 days after completion of the therapy. Upper diagram: graphical analysis of the intravascular radioactivities (for details see Matthews [15]). Lower diagram: plasma fibrinogen concentration.

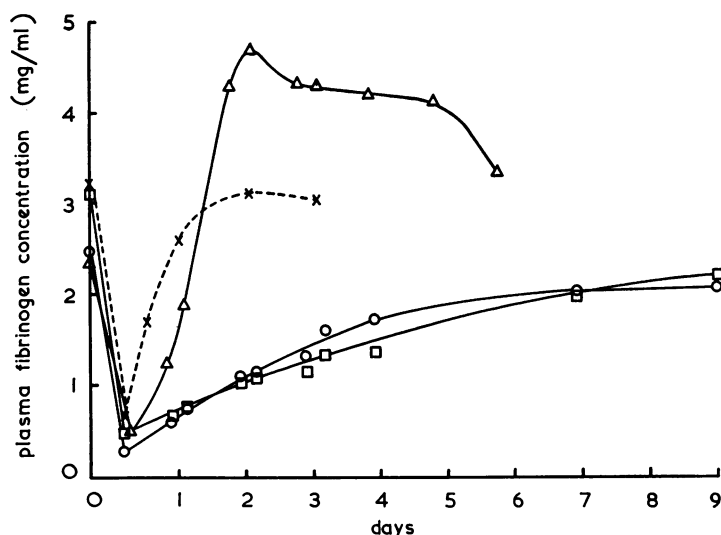


FIGURE 5 Regeneration of plasma fibrinogen in rabbits after two injections of $1 \mu\text{g}$ CE/kg spaced 30 min apart. The curves, each representing mean values from two animals, show: CE alone (○); CE followed 30 min later by 0.2 ml antivenene (□); CE and antivenene as in the previous group, followed 10 hr later by 0.5 ml of turpentine/kg subcutaneously (△); 1550 NIH U of thrombin infused through the jugular vein (×).

DISCUSSION

The fate of fibrin formed in the blood depends in part on the dynamic balance between the rate of formation and the capacity of the body to clear fibrin from the circulation. When coagulation takes place at a slow rate, the small polymers that are formed do not obstruct the microcirculation and are rapidly cleared by the reticuloendothelial system (18, 19). As a result of the intravascular coagulation, the disappearance of labeled fibrinogen from the plasma is accelerated (20): the radioactivity of clottable and nonclottable protein decreases in the plasma in a continuous manner. This is in contrast to the phenomena accompanying rapid rates of defibrinogenation, with formation and temporary deposition of micro-clots, which on dissolution give rise to a paradoxical increase (rebound peak) in radioactivity of nonclottable protein in the plasma. Such rebound peaks have been described after infusion of thromboplastin (21), crude *Aegistrodon rhodostoma* venom (3), and thrombin (22).

In the present studies no patient demonstrated a rebound peak¹⁰ (Figs. 1 and 2). Thus, the present data provide no evidence for gross deposition of fibrin during the initial stages of administration of CE. However,

¹⁰ The early abrupt increase in fibrinogen activity seen in four patients, and illustrated in Fig. 2, preceded reduction in labeled fibrinogen and is not related to release of radioactivity from the dissolution of microclots. Obviously, such a rebound peak cannot appear in advance of a substantial reduction in labeled fibrinogen from the circulation.

transitory deposition of small quantities of fibrin can escape detection by this technique, unless the solubilization of the fibrin deposits is prevented by blockade of the fibrinolytic system. This was not attempted in these studies.

The reason for the transient elevations of radioactivity of fibrinogen (Fig. 2) and albumin (Fig. 3), respectively, during the infusion of CE is not clear. Even less obvious is why these two proteins were affected in similar fashion, but at different times with respect to the initiation of the infusion of CE. It is conceivable that CE, like many enzymes with arginine esterase activity, directly or indirectly liberates some biologically active peptide(s) that have the capacity to alter capillary permeability or the composition of the blood. Further studies are required to delineate whether CE is capable of activating kininogens as do other coagulant and fibrinolytic substances (23–26). As an alternative source of vasoactive peptides, the release of small polypeptide fragments from fibrinogen by CE may be considered (27, 28). In view of the absence of sufficient knowledge concerning the possible effects of such peptide hormones on the intravascular distribution and transcapillary diffusion of labeled plasma proteins, interpretation of the present observations must be speculative.

The use of labeled proteins in non-steady states needs to be justified, since the application of kinetic models under these circumstances can give misleading results. Fibrinogen, however, may be regarded as an exception

for several reasons. (a) Fibrinogen catabolism is a well established first-order kinetic process (14, 29, 30), implying that the mass of fibrinogen catabolized is directly proportional to the concentration of this protein in the plasma; (b) the amount of fibrinogen passing the capillary barrier is directly proportional to the plasma level of fibrinogen (31); and (c) the interstitial passage time of fibrinogen is remarkably short, 90% of the filtered fibrinogen returning to the intravascular pool within 24 hr (17). Moreover, the rate of synthesis of fibrinogen is subject to considerable, apparently physiological, variation, so that a steady-state condition in fibrinogen metabolism is much less apparent than it is, for example, in albumin metabolism (29, 30). Mathematical analysis has shown that the reability of isotopic data is not seriously affected so long as the non-steady state is due to continuous, rather than to abrupt, changes (29, 32).

For the above reasons, measurements of catabolic rates during the regeneration of the body fibrinogen pool should be possible. The average fractional catabolic rate of 47% per day, during the regeneration period between the 3rd and 10th day following discontinuation of CE, the fractional catabolic rates were normal.

To explain the accelerated disappearance of labeled fibrinogen in man, it is tempting to assume that CE continued to act at a reduced rate for some time after administration was discontinued. This is favored by the observation that CE-¹²⁵I was not completely eliminated from the circulation after 1 wk (33). CE returning slowly from intravascular sites into the plasma over several days may have affected circulating fibrinogen together with the labeled dose by rendering it susceptible to accelerated metabolism despite our effort to neutralize CE with an excess of antivenene at the beginning of these studies. This is a likely explanation for the unexpected finding that the plasma slope of the injected labeled fibrinogen failed to slow down with increasing time (Fig. 4).

Hypercatabolism is probably in part the cause of delayed regeneration of the fibrinogen pool following treatment with CE. This may be concluded from the extent of the rise in plasma fibrinogen concentration following stimulation with turpentine: fibrinogen rose to a maximum of 4.7 mg/ml in rabbits pretreated with CE (fractional catabolic rate of fibrinogen: 110%), in contrast to 9.1 mg/ml in the control animals (fractional catabolic rate: 46%). It is realized, however, that the negative effect on the speed of regeneration of a 2.4-fold increase in the catabolic rate could easily be overcome by the large reserve capacity of the liver, which is capable of synthesizing fibrinogen 7–8 times above the normal rate (34). While a potent stimulation of this reserve capacity was observable after infusing thrombin, the hypofibrinogenemia induced by CE has hardly chal-

lenged the synthesis rate of fibrinogen. From the data shown in Fig. 5, it was calculated that the net increase in the body fibrinogen pool of the CE-treated rabbits on the 2nd day of regeneration averaged 49 mg; since during the same time they catabolized 116 mg fibrinogen, the synthesis rate must have been about 165 mg/day. This value is only slightly above the normal rate (145 mg/day) reported by others (34).

Thus, it appears that the abolition of comparable fractions of the body fibrinogen pool using thrombin and CE, respectively, affects the fibrinogen-synthesizing mechanism quite differently. At present, the interpretation of this finding is difficult because the regulation of fibrinogen synthesis is largely unknown. Nevertheless, the widely recognized stimulatory effect of cell damage prompted Gordon and Koj (35) to postulate the release of a hypothetical factor from injured cells initiating increased synthesis of the acute phase proteins, including fibrinogen, by a mechanism yet to be identified. This theory could provide a satisfactory explanation for changes in the synthetic rate of fibrinogen following the infusion of thrombin, this enzyme causing aggregation and the release of the contents of the platelets (36). The fact that CE has no such effects (5) gives rise to the speculation that hypofibrinogenemia per se is perhaps not a stimulus of fibrinogen synthesis at all, and that the commonly observed speedy recovery of the fibrinogen pool following trauma is brought about more by components released by injured tissue rather than by the net losses in coagulable protein. The implications of this preliminary conclusion must be elaborated in further experiments.

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