Synthesis and Release of Hageman Factor (Factor XII) by the Isolated Perfused Rat Liver

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ABSTRACT The site of synthesis of Hageman factor (HF, Factor XII) has not been previously demonstrated with certainty. We have studied the production and release of HF in the isolated perfused rat liver and have compared rates of synthesis in this system with absolute rates of degradation measured in vivo. Rat livers, perfused for 5 h with a recycling fluid consisting of a perfluorochemical emulsion (Fluosol 43), were used to demonstrate a cumulative increase of HF in the perfusate as measured by a specific and sensitive radioimmunoassay. The rate of increase in the perfusate pool of HF during the final 4 h of perfusion yielded a mean synthetic rate of 3.5 μg/h per 100 g body wt, which was ~0.2% of the synthetic rate of albumin in the same system. The cumulative appearance of albumin and transferrin was linear after 1 h and calculated rates of synthesis were 2,012 μg/h per 100 g and 263 μg/h per 100 g body wt, respectively. *De novo* synthesis of HF was confirmed by demonstrating incorporation of [^{14}C]lysine into specific immunoprecipitates of HF, and by the observations that both specific incorporation of labeled amino acid and net release of immunoassayable HF were inhibited by the administration of cycloheximide. Finally, it was evident that the rates of synthesis observed in the isolated perfused liver agreed closely with absolute rates of degradation of HF measured in vivo with ^{125}I-rat HF (4.0 μg/h per 100 g). From these data we conclude that the liver is the principal site of synthesis of HF.

INTRODUCTION

Hageman factor (HF[1], Factor XII) is a plasma protein with an approximate molecular weight of 80,000 present in the blood of most mammals. Under certain conditions HF triggers several contact-activated plasma reactions such as blood coagulation, kinin generation, and fibrinolysis (2). The normal concentration of HF in human plasma is ~3–4 mg/dl (3) and the plasma half-life of HF in man has been reported to be ~50–60 h following the infusion of normal plasma into individuals with hereditary HF deficiency (4). The site of synthesis of HF is not known with certainty, although earlier observations have indirectly suggested that the liver may be a site of production and secretion.

In this study we have purified HF from rat plasma and raised a monospecific antiserum against rat HF in rabbits, which has enabled us to develop a specific and sensitive radioimmunoassay for rat HF. By means of this assay we have shown that isolated perfused rat liver produces and releases HF into the circulation. *De novo* synthesis of HF was confirmed by demonstrating the incorporation of [^{14}C]lysine into specific immunoprecipitates of HF derived from the circulating perfusate, and by inhibition of synthesis by cycloheximide. Furthermore, we have measured the half-disappearance time of radioiodine-labeled HF in intact rats and have estimated its absolute catabolic rate, which agrees closely with the rates of synthesis determined in vitro in the isolated perfused liver.

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[1] *Abbreviations used in this paper:* HF, Hageman factor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

METHODS

Procoagulant activities measurement. The procoagulant activities of HF (Factor XII), plasma thromboplastin antecedent (Factor XI), prekallikrein, high-molecular-weight kininogen, Christmas factor (Factor IX), and antihemophilic factor (AHF, Factor VIII) were measured by the kaolin-activated partial thromboplastin time using congenital-deficient plasma as substrate (5, 6).

Isolation and characterization of rat HF. Rat HF was isolated from 700 ml of pooled plasma from Sprague-Dawley rats (Pel Freez Biologicals, Rogers, AR) by a method described for human HF (7). The purified preparation had a specific activity of 22 units/mg protein (1 unit being defined as that amount present in 1.0 ml of pooled rat plasma) and it showed a single stained protein band on analytical polyacrylamide disc gel electrophoresis (8). This band contained clot-promoting activity for human HF-deficient plasma, as tested by assaying eluates from 1-mm segments of replicate gels (Fig. 1). Upon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (9) rat HF yielded a single major protein band in the presence and absence of a reducing agent, 2-mercaptoethanol (Fig. 2). The same protein band also yielded a positive periodic acid-Schiff stain (10). The apparent molecular weight of rat HF was ~80,000 and isoelectric focusing (11) revealed a pI of 5.1–5.5. These results suggest that rat HF is a single-chain glycoprotein with a molecular weight of ~80,000, similar to human and bovine HF (12, 13). The amino acid composition of rat HF is also very similar to that of human HF (Table I).

Anti-rat HF serum. Anti-rat HF serum was raised in New Zealand albino rabbits by injections of purified rat HF. Upon Ouchterlony immunodiffusion, this antiserum showed a single precipitin line of complete identity with purified rat HF and rat plasma (Fig. 3). Furthermore, the antiserum selectively blocked HF clot-promoting activity without significant inhibition of activities of other clotting factors (Table II). These results indicate that this antiserum contains antibodies that are monospecific immunologically and functionally. A crude immunoglobulin fraction of the antiserum was isolated as described earlier (14). A similar fraction of normal rabbit serum was used as control in all experiments involving immunoprecipitation techniques.

Development of a radioimmunoassay (RIA) for rat HF. By the use of purified rat HF and its antiserum, a specific and sensitive RIA was developed as previously described for human HF (3). When pooled rat plasma or purified rat HF was tested in this assay, similar displacement curves were obtained (Fig. 4). The assay is sensitive to 0.05 μg HF/ml or 0.125% of the concentration of HF present in normal pooled rat plasma. The concentration of HF present in rat plasma was estimated to be ~40 μg/ml compared to a stan-

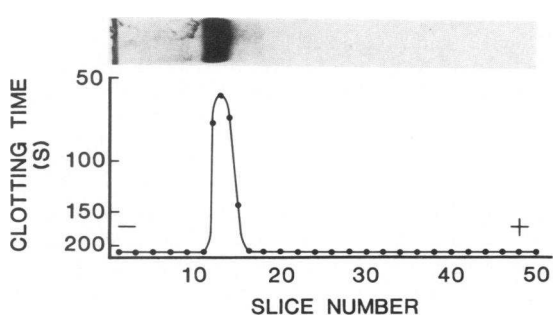

FIGURE 1 Analysis of rat HF on analytical polyacrylamide gel. The gel above was stained with 0.05% Coomassie Blue, while a replicate gel was sliced, eluted, and assayed for HF clot-promoting activity.

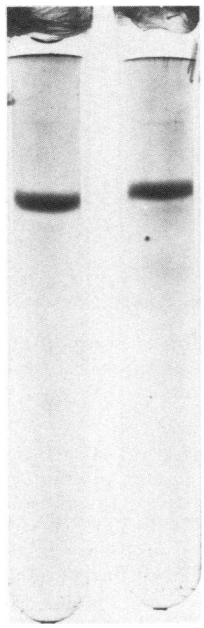

FIGURE 2 SDS-PAGE of rat HF (*A*) before and (*B*) after reduction with 2-mercaptoethanol. Anode is at the bottom.

TABLE I
Amino Acid Analysis of Rat HF

Amino acid	Rat HF*	Human HF‡
	%	
Cysteine	3.9	4.2
Aspartic acid	9.0	6.3
Threonine	5.1	5.8
Serine	5.9	8.5
Glutamic acid	11.0	11.3
Proline	6.9	8.9
Glycine	9.7	10.5
Alanine	7.0	9.2
Methionine	0.8	0.1
Valine	6.4	5.4
Isoleucine	2.4	1.4
Leucine	10.3	7.6
Tyrosine	3.2	2.8
Phenylalanine	3.5	2.9
Lysine	4.2	4.2
Histidine	4.8	4.5
Arginine	6.0	5.9

The techniques used did not permit estimation of tryptophan.
* Data represent mean of two separate batches of rat HF.
‡ Obtained from reference 7.

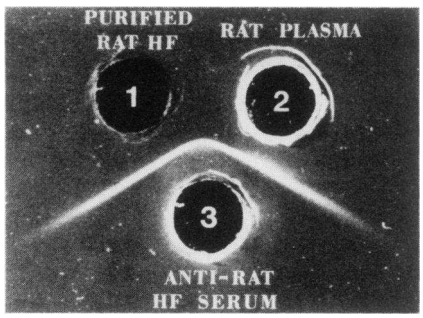

FIGURE 3 Immunodiffusion study of anti-rat HF serum (3) against rat plasma (2) and purified rat HF (1).

dard curve using purified rat HF. This titer is very close to that found in normal human plasma (3).

Liver perfusion studies. Livers isolated from fed male Sprague-Dawley rats (400–500 g body wt) were perfused through the portal vein with recycling perfusate (~80 ml) of a heparinized (1,000 U) fluorocarbon emulsion (Fluosol 43; Green Cross Corp., Osaka, Japan), which is free of plasma proteins and blood cells. The perfluorocarbon components (perfluorotributylamine and pluronic F-68) were dialyzed overnight against deionized water, centrifuged at 2,700 *g* to remove nonemulsifed material, filtered through Whatman No. 1 filter paper (Whatman Chemical Separation, Inc., Clifton, NJ) and mixed with the aqueous annex solution to give a final concentration of the following constituents (in grams per deciliter); perfluorotributylamine (20.0); pluronic F-68 (2.56); NaCl (0.6); KCl (0.034); $CaCl_2$ (0.028); $MgCl_2$ (0.02); $NaHCO_3$ (0.21); glucose (0.18); hydroxyethyl starch (3.0). The perfusate was maintained in gaseous equilibrium at 37°C with 95% O_2/5% CO_2 by means of a thin-walled silastic tubing membrane oxygenator (25 ft of coiled tubing, caliber 0.058-in. i.d., 0.077-in. o.d., Dow Corning Corp., Midland, MI). Portal vein inflow Po_2 of >350 mmHg was maintained by this method at hepatic perfusate flow rates of 15–20 ml/min and portal vein pressures of 15 cm of H_2O. Before the liver was placed in the perfusion circuit it was flushed with 20 ml of oxygenated fluorocarbon to wash out residual blood. Measured additions to the perfusate consisted of sodium taurocholate (18.6 mM, 0.28 μmol/min) to stimulate bile flow, and $NaHCO_3$ (1.2 M) to adjust the monitored pH to 7.4 at hourly intervals. In some experiments, all glass surfaces in the perfusion system were washed with 100 μg/ml of hexadimethrine bromide (Polybrene, Aldrich Chemical Co. Inc., Milwaukee, WI) and rinsed with deionized water before use.

Samples of perfusate (3–5 ml) were withdrawn at hourly intervals and centrifuged at 12,000 *g* for 30 min to sediment the suspended perfluorochemicals. The clear supernatant, to which aprotinin (Sigma Chemical Co., St. Louis, MO) was added (10 units/ml), was assayed for HF by RIA and for albumin and transferrin by radial immunodiffusion (15). Preliminary experiments showed that rat HF added to the fluorocarbon suspension was completely recovered in the supernatant and that the presence of the supernatant or cycloheximide in the assay mixture did not interfere with the RIA. Assay of HF standards gave identical results in the presence or absence of the fluorocarbon supernatant. The rates of HF, albumin, and transferrin production and release, expressed as micrograms per hour per 100 g body wt, were calculated from the rate of increase in the size of the respective cumulative pools, which was obtained by multiplying the perfusate specific protein concentration by the supernatant volume, with allowance made for previous sampling.

Effect of cycloheximide upon production and release of HF. In four experiments, cycloheximide was added to the perfusate after 1 h to give a final concentration of 18 μM. This concentration of cycloheximide has previously been shown to inhibit albumin and plasminogen synthesis and release in the isolated perfused rat liver (16).

Incorporation of [^{14}C]lysine into HF. In five experiments, 50 μCi of L-[^{14}C(U)]lysine (>300 mCi/mmol, New England Nuclear, Boston, MA) was added to the reservoir at the start of the perfusion. After 5 h of perfusion, ~40 ml of perfusate supernatant containing free and incorporated [^{14}C]lysine was exhaustively dialyzed against 1 mM nonradioactive lysine in barbital-saline buffer (0.025 M sodium barbital, 0.125 M sodium chloride, pH 7.4). 1 ml of human HF-deficient plasma was added, as a source of carrier proteins, to 10 ml of dialyzed perfusate and the 0–60% ammonium sulfate fraction was precipitated. The precipitate was dissolved in barbital-saline buffer, dialyzed against the same buffer, and divided in two aliquots. One aliquot was incubated with 0.025 ml of rabbit anti-rat HF IgG (17.5 mg/ml) for 1 h at 37°C and then overnight at 4°C. The other aliquot was incubated with normal rabbit IgG (17.0 mg/ml) in a similar manner. Goat anti-rabbit IgG serum, 0.1 ml (Antibodies Inc., Paris, CA) was then added to each tube and the mixtures were incubated overnight at 4°C. The resulting immunoprecipitates were centrifuged and washed three times with ice-cold saline. The washed immunoprecipitates were dissolved in 0.3 ml of hyamine hydroxide (New England Nuclear) and counted in a liquid scintillation spectrometer after the addition of Aquasol scintillator (New England Nuclear). The results were expressed as a percentage of the radioactivity in immunoprecipitates relative to that contained in the initial ammonium sulfate-precipitated protein fraction.

TABLE II
Functional Specificity of Anti-Rat HF Serum

Clotting factor	Activities: Normal plasma + anti-HF serum*	Activities: Normal plasma + normal rabbit serum*
	%	
HF	<1	100
Factor XI	100	100
Prekallikrein	95	100
High-molecular-weight kininogen	95	100
Christmas factor	94	100
Factor VIII	90	100

* Adsorbed with $Ca_3(PO_4)_2$ and kaolin, and heated at 60°C for 1 h.

A 1:1 mixture (vol/vol) of normal pooled rat plasma and either rabbit anti-HF serum or normal rabbit serum was incubated at 37°C for 60 min, and was then tested for various clotting factor activities after appropriate dilutions.

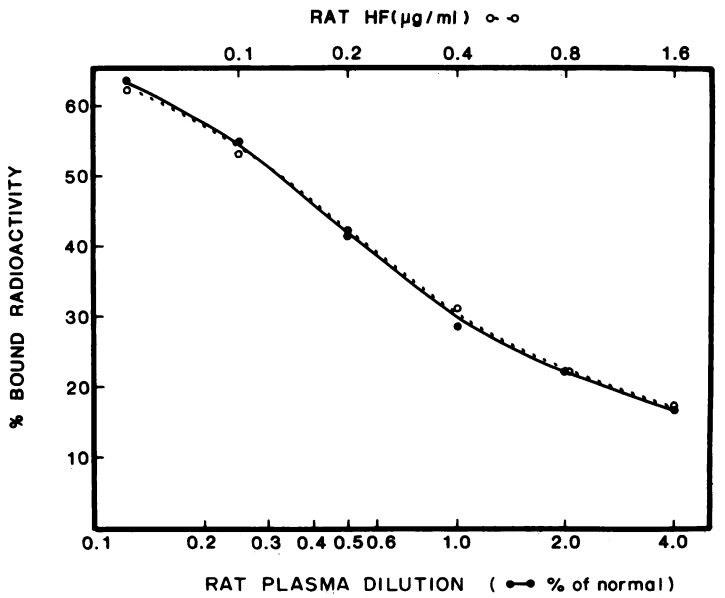

FIGURE 4 RIA of rat HF. Ordinate, percentage of bound radioactivity relative to the total radioactivity added; abscissa, percentage of pooled normal rat plasma (*bottom*) or micrograms rat HF per milliter (*top*).

Measurement of in vivo catabolic rate of HF. Rat HF (63 μg) purified as described above was labeled with ^{125}I by the chloramine-T method (17), at a molar ratio of iodine to protein of no >0.33. The final dialyzed ^{125}I-rat HF was >99% precipitable with 5% (wt/vol) trichloroacetic acid (TCA) and had a specific radioactivity of 1.46×10^6 cpm/μg protein. Four rats were injected with 3.75 μg of ^{125}I-HF via the tail vein. Blood samples (~150 μl) were taken into heparinized capillary tubes at intervals for ~120 h. Plasma samples were obtained after centrifugation, and 50-μl aliquots were counted in a gamma spectrometer. Samples of plasma at 10 min after injection were subjected to SDS-PAGE. This confirmed that the radioactivity was confined to the 80,000-mol wt band. Sequential plasma counts were expressed as a percentage of the value obtained 10 min after injection. Fractional rates of degradation were calculated by linear regression of the terminal exponential of the plasma decay curves. Absolute rates of degradation were calculated from the product of this value and the mean plasma HF concentration of 40.0 μg/ml and a plasma volume of 3.8 ml/100 g body wt (18).

RESULTS

Appearance of HF in the perfusate. The amount of HF present in the perfusate increased progressively during 5 h of perfusion (Fig. 5). The HF titers measured in the perfusate in four separate experiments were 10.0±1.0 μg/100 g body wt at 60 min, 13.7±1.4 μg at 120 min, 17.3±1.7 μg at 180 min, 21.3±3.0 μg at 240 min, and 23.9±4.2 μg at 300 min (mean±SEM). The mean synthetic rate of HF, calculated from the total increase in the perfusate pool between 60 and 300 min was 3.5 μg/h per 100 g body wt. From these data it was apparent that after an initial rapid appearance of protein during the first 60 min there was a continued linear appearance of HF at a somewhat lower rate for the remaining 240 min of perfusion.

Effect of cycloheximide upon the accumulation of HF in the perfusate. In four perfusion experiments, 18 μM cycloheximide was added to the perfusate after the 60-min samples were taken (as indicated by the arrow in Fig. 5). In these experiments the HF titer at 60 min was 7.8±1.1 μg/100 g body wt (mean±SEM) and was not significantly different from that in the control experiments ($P > 0.05$). The amount of HF in

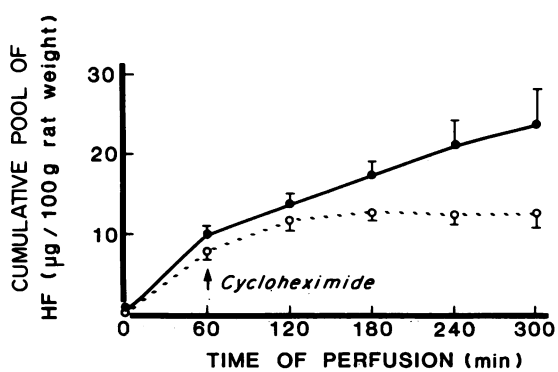

FIGURE 5 Time course of the appearance of rat HF in the perfusate of rat liver in the presence and absence of cycloheximide (added 60 min after the start of the perfusion). Bars represent mean±SEM ($n = 5$). Control perfusion (●——●); cycloheximide perfusion (O - - - O).

the perfusate, however, did not increase after 120 min in the cycloheximide-treated group and there was a statistically significant difference between the control and the cyclohexmide-treated groups at 180, 240, and 300 min ($P < 0.025$). These data indicate that cycloheximide effectively blocked the appearance of HF in the perfusate, and confirmed that the increase in titer of HF under normal conditions represents *de novo* synthesis and not predominant release of preformed protein.

Similar cumulative plots of appearance of protein were demonstrated for both albumin and transferrin (Fig. 6). The synthetic rates derived by linear regression of the cumulative appearance curves for all three proteins were 2,012±280 μg/h per 100 g body wt (albumin), 263±23 μg/h per 100 g (transferrin), and 3.5±1.3 μg/h per 100 g (HF). Therefore, compared in this way, the synthetic rate of HF was ~0.2% of that of albumin and 1.4% of that of transferrin.

Properties of HF released by isolated perfused livers. When an aliquot of 300-min perfusate was filtered through a column of Sephadex G 150, HF clot-promoting activity and immunoreactive HF eluted at the same place and had an apparent molecular weight of ~100,000. This experiment suggests that HF released by liver is functional and that it is approximately the same size as plasma HF (3). When serial dilutions of a similar aliquot of perfusate were tested by RIA, the slope of the displacement curve was similar to that of plasma HF, suggesting that they have similar immunological properties (data not shown).

Incorporation of [^{14}C]lysine into HF. Perfusate at 300 min from five labeling experiments were immunoprecipitated by the sequential addition of either rabbit anti-rat HF IgG or normal rabbit IgG and goat anti-rabbit IgG serum. There was significantly higher

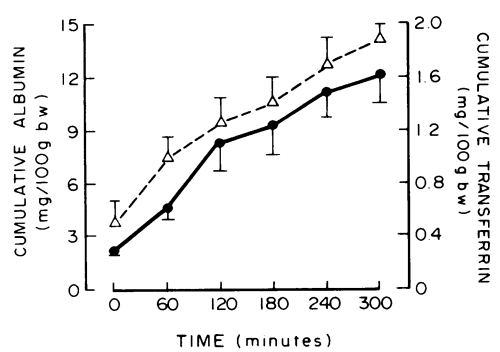

FIGURE 6 Time course of appearance of rat albumin (●——●) and transferrin (▲- - -▲). Rates of synthesis calculated by linear regression of the cumulative perfusate pools between 60 and 300 min were 2.01±0.28 mg/h per 100 g and 0.26±0.02 mg/h per 100 g (mean±SEM, $n = 5$), respectively.

TABLE III
Immunoprecipitation of [^{14}C]Lysine-labeled Proteins

Percent relative radioactivity* in immunoprecipitates	
Rabbit anti-HF IgG + Goat anti-rabbit IgG	Rabbit normal IgG + Goat anti-rabbit IgG
4.1±1.0	1.8±0.4
$P < 0.025$	

Data represent mean±SEM; $n = 5$.
* Relative radioactivity is expressed as a percentage of the initial counts per minute in equal aliquots of 0–60% ammonium sulfate fraction prepared from 10 ml of the 300-min perfusate supernatant.

^{14}C-radioactivity in the specific immunoprecipitates (4.1±1.0%, mean±SEM) than in the control immunoprecipitates (1.8±0.4%, $P < 0.025$) (Table III). Furthermore, the addition of cycloheximide virtually abolished the incorporation of ^{14}C-radioactivity into both specific and control immunoprecipitates.

Absolute catabolic rate of HF. The plasma decay curve for the mean catabolic data is shown in Fig. 7. The two exponentials yielded plasma half-lives of 1.4 and 27.0 h, respectively. Since it was apparent that plasma HF radioactivity had fallen to ~14.5% of the 10-min value after ~4 h, it was difficult to explain the initial rapid decay on the basis solely of distribution kinetics. It seems likely that other nonidentified routes of loss, including rapid degradation of some HF denatured in the purification process, were responsible for early disappearance. Therefore, the terminal slower exponential was used to calculate and approximate fractional catabolic rate, on the assumption that this represented the degradation of residual native HF. The mean rate constant of this exponential was 0.026/h. The mean absolute catabolic rate calculated from this on the basis of a plasma volume of 3.8 ml/100 g body wt (18) was 4.0 μg/h per 100 g.

DISCUSSION

The present study was designed to determine the role of the liver in the synthesis of HF by a method that avoids the nonspecificity of functional assay techniques. As such, it confirms and extends the observations of Owen and Bowie (19, 20) that the prolonged clotting time of human HF-deficient plasma can be corrected by the addition of perfusate from isolated rat liver. However, since the liver produces many clotting factors (19), such a functional assay may be affected by the presence of other activated procoagulants released by the liver. In contrast, the present studies evaluated the presence of newly synthesized HF, quantified its rate of synthesis by a RIA, and correlated

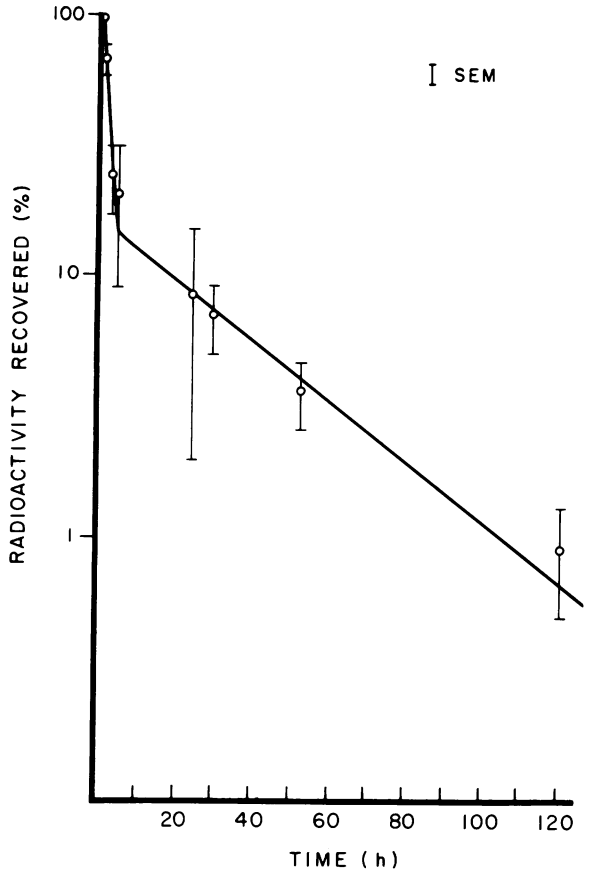

FIGURE 7 Plasma decay curve of ^{125}I-HF in the rat. Semilogarithmic plot of percentage of the 10-min plasma radioactivity (ordinate) is shown at timed intervals (abscissa) in plasma samples of rats injected intravenously with ^{125}I-HF. Bars represent the mean±SEM, n = 4. The fractional rate of degradation was calculated by linear regression of the terminal exponential of the plasma decay curve (mean rate constant 0.026/h, n = 5).

such assayable protein with functional activity based upon HF clot-promoting activity.

The validity of our data is intrinsically dependent upon the specificity of the rabbit anti-rat HF serum and RIA technique used. The rat HF used for immunization and for the RIA was judged to be a single homogeneous protein on disc gel electrophoresis, SDS-PAGE, and immunodiffusion studies. Amino acid analysis of this preparation was very similar to that of human HF. Furthermore, the anti-rat HF serum raised in rabbits showed a single precipitin line of complete identity with both purified rat HF and whole rat plasma. Similarly, the monospecificity of this source of antibodies was confirmed by demonstrating that the slopes of the displacement curves for both rat plasma and purified HF were identical.

The liver perfusion system used a chemically defined artificial Krebs-Ringer bicarbonate perfusate in which O_2 transport and delivery was the property of a perfluorocarbon, and oncotic pressure was exerted by hydroxyethyl starch. Thus, the possibility that HF is generated by contaminating blood cells (21) is unlikely. A similar preparation has been shown to maintain viability of the perfused liver for at least 5 h (22), and previous studies in this laboratory have demonstrated that the liver in this system synthesizes plasminogen at physiologic rates (16). Furthermore, similar rates of production of albumin were observed in the present study, as were found in an earlier study that used a more physiological perfusate of plasma and erythrocytes (23). In addition, it is apparent that although the initial rate of appearance of HF in the perfusate is rapid and may be partially accounted for by release of preformed protein, the linear appearance of HF over the subsequent 240 min represents *de novo* synthesis of protein. This was confirmed by the efficacy of cycloheximide, an inhibitor of protein synthesis, in almost totally abolishing the appearance of HF within 60 min of administration.

Finally, an attempt was made to use the incorporation of [^{14}C]lysine into HF as qualitative evidence for *de novo* synthesis. In these experiments the entire 300-min perfusate was harvested for double-antibody immunoprecipitation of labeled HF. To allow for nonspecific coprecipitation of other labeled proteins of liver origin present in the ammonium sulfate fraction, a control precipitation was carried out by adding normal rabbit IgG to an equal aliquot followed by goat anti-rabbit IgG. The percent radioactivity precipitated by the specific anti-HF IgG was significantly higher in all five experiments. In other words, the difference in radioactivity incorporated in these two groups of immunoprecipitates represents [^{14}C]lysine specifically incorporated into HF. Furthermore, this specific incorporation was also inhibited by cycloheximide.

The hepatic synthetic rate of HF of 3.5 μg/h per 100 g body wt calculated from the rate of increase in the cumulative pool of HF in the isolated perfused rat liver is in close agreement with the estimated absolute rate of catabolism measured in vivo of 4.0 μg/h per 100 g. Although the latter calculation is based upon the slowest component of the plasma half-life; it has been argued that such a method can be expected to yield very similar results to that obtained from measurements of whole body radioactivity (24). The close agreement between this calculated rate of degradation and the rate of synthesis observed in the perfusion system leads us to conclude that the liver is the principal site of HF synthesis and secretion in the rat.

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