HEMATIN-DERIVED ANTICOAGULANT

Generation In Vitro and In Vivo

By ROBERT L. JONES

From The Rockefeller University Hospital, New York

Metalloporphyrins not only participate in a wide range of essential biochemical reactions, but have also recently received attention as therapeutic agents. Hematoporphyrins, which produce photodynamic destructive effects on cells are under investigation as antineoplastic agents (1, 2). Tin-protoporphyrin IX, a metal substitution of heme, potently inhibits heme-oxygenase and has been suggested in the treatment of hyperbilirubinemia (3–5). And, because heme (ferriprotoporphrin IX) suppresses δ-aminolevulinic acid synthetase, hematin (ferriprotoporphyrin IX hydroxide) is being used to prevent or abate the painful crises of patients wth acute intermittent porphyria (AIP)[1] (6–8).

Although the overall toxicity of hematin infusions appears to be minimal, profound effects on the routine tests of blood coagulation have recently been reported (9–11). Decreased platelet count and fibrinogen concentration, and prolonged thrombin (TT), prothrombin (PT), and activated partial thromboplastin (APTT) times are reported in a picture that mimicks diffuse intravascular coagulation. This study focuses on the mechanism of the fluid phase coagulation defect produced by hematin. The results indicate that, in vitro and in vivo, hematin is oxidatively degraded or metabolized to an anticoagulant, and that hematin itself is not responsible for the prolongation of clotting times. The results also suggest that this anticoagulant is a single derivative of hematin that binds to clotting proteins to reversibly inhibit their function, and which involves iron in its mechanism of action.

Materials and Methods

Hematin. Hematin was prepared by two methods. (*a*) Hemin, assayed 101% purity by pyridine hemochromogen, (Porphyrin Products, Logan, UT) was dissolved in 1 N NaOH (0.2 ml for each milliliter of hematin solution), then 0.8 ml of potassium phosphate buffer (pH 7.5) was added. The final pH, 7.5, was adjusted with 1 N HCl. (*b*) Hemin was dissolved in 1 N sodium carbonate (pH 12). The final pH of 7.5 was obtained by adding 1 N HCl. Tin-protoporphyrin IX, and protoporphyrin IX (Porphyrin Products) were prepared as per preparation (*a*) for hematin. The final concentration for all porphyrin solutions was 10 mg/ml. Hematin concentrations were confirmed by the pyridine hemo-

This work was supported, in part, by a Special Emphasis Research Career Award from the U.S. Public Health Service AM-0059003, by U.S.P.H.S. grants ES-01055, CA29502 (CNRU), RR 00102 (GCRC), and by gifts from the Renfield Foundation and the Dyson Foundations. Address correspondence to R. Jones, The Rockefeller University, 1230 York Ave., New York, NY 10021.

[1] *Abbreviations used in this paper:* AIP, acute intermittent porphyria; APTT, activated partial thromboplastin time; BHT, butylated hydroxytoluene; HDA, hematin-derived anticoagulant; PT, prothrombin time; TBAH, tetrabutylammonium hydroxide; TT, thrombin time.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/86/03/0724/16 $1.00
Volume 163 March 1986 724–739

chromogen assay. (12) Unless otherwise indicated, all other reagents were of the highest grade available from Sigma Chemical Co., St. Louis, MO, or Fisher Scientific Co., Pittsburgh, PA.

Clotting Assays. Clotting times were measured in duplicate with an automated optical coagulation recorder (Biodata Corp., Horsham, PA). Clotting times were also measured in the dark with a mechanical clot detection device (Fibrometer, Becton Dickinson and Co., Sunnyvale, CA). Clotting times were measured using commercial reagents. PT and APTT were measured with Simplastin, lot 4D831; and Automated PTT, lots 4M610 and 4M842 (General Diagnostics, Morris Plains, NJ). TT was measured with Bovine Topical Thrombin (Parke-Davis, Morris Plains, NJ). TT was measured with Bovine Topical Thrombin (Parke-Davis, Morris Plains, NJ). Citrated (3.8%) normal plasma was obtained from normal healthy laboratory personnel and used fresh. TT were also performed with human fibrinogen (Kabi, Stokholm, Sweden) and purified human thrombin (courtesy of Dr. J. Fenton, Albany, NY).

10-μl aliquots of porphyrin solutions were added to 1.0 ml plasma or fibrinogen solution (2.5 mg fibrinogen per milliliter phosphate buffer, pH 7.5) to make final concentrations of 0.01–0.10 mg/ml hematin. This range was chosen to approximate the range of plasma hematin concentrations seen in patients after intravenous hematin infusion. Unless otherwise indicated, anticoagulant activity (hematin-derived anticoagulant HDA) was assessed by the above clotting assays performed immediately after the addition of porphyrin solutions to normal plasma or fibrinogen solutions. Prolongation of clotting times was quantitated by comparing the results with clotting times from plasma that had not been treated with hematin solutions, or to which buffer alone had been added.

In Vitro Generation of HDA (Aging of Hematin). Hematin and other porphyrin solutions were allowed to stand (age) at 4°C exposed to ambient light, exposed to constant incandescent light (40 W lamp positioned 12 inches from the solution), or in total darkness. Hematin solutions were also aged in the presence of 2 mM mannitol, sorbitol, 2-ME, or butylated hydroxytoluene (BHT). Anticoagulant activity of porphyrin solutions was assessed periodically with plasma clotting assays as above for over 6 wk of standing.

Anticoagulant activity (aged hematin) generated in vitro was also tested in the presence of equimolar concentrations of fresh hematin, fresh and aged Sn–protoporphyrin IX, and fresh and aged protoporphyrin IX. Anticoagulant activity was also tested in the presence of varying concentrations of imidazole or desferrioxamine (Ciba Geigy). Anticoagulant activity was also tested in the presence of ferrioxamine, the iron chelate of desferrioxamine. Ferrioxamine was produced by mixing a molar excess of ferric chloride with desferrioxamine, followed by alkalinization with NaOH to pH 8.0 to remove unbound ferric iron. Desferrioxamine or ferrioxamine were added before or after HDA was added to plasma.

TLC of Aged Hematin. Hematin (aged 4 wk) solution containing HDA was spotted on a precoated silica gel 60 plate (E. Merck, Darmstadt, Federal Republic of Germany). A mixture of 2,6-lutidine and water (100 ml/60 ml) was placed in the bottom of chromatographic tank (20 × 20 × 8 cm), and three small beakers containing 7 N NH_4OH were placed in the tank. After saturation of the tank with NH_3 vapor, the TLC plate was placed upright in the tank, and ascending development was carried out for 3 h. Plates were divided into bands, scraped, and the resin was extracted with 1 N NaOH in 95% ethanol. Extracts were dried in a Savant Speed-Vac concentrator, redissolved in equal volumes of phosphosaline buffer (pH 7.4), then tested for HDA by adding aliquots to plasma and measuring APTTs.

HPLC of Aged Hematin. Tetrabutylammonium hydroxide (TBAH) was purchased from Eastman-Kodak Co., Rochester, NY. Phosphate buffer was prepared using water purified to 18MSL by a Milli-Q filtration system (Millipore Corp., Bedford, MA). All solutions were deaerated by evacuation before use. HPLC conditions included a series 3B solvent distributor (Perkin-Elmer Corp.), a 3u C_{18} column (258-0160, Perkin-Elmer Corp.) a septumless syringe injector (Rheodyne 7105), and LC 75 spectrophotometer (Perkin-Elmer Corp.). The mobile-phase solvent was methanol in 10 mM potassium phosphate buffer (pH 6.0) containing 5 mM TBAH. Methanol concentration was raised from 40 to

99.9% over 10 min with a flow rate of 0.8 ml/min (13). Hematin was detected using absorption at 410 nm. Fractions were collected using a LKB Redi-Rac fraction collector. Data were analyzed by a 3600 Chromatography Data Station (Perkin-Elmer Corp.). Fractions were dried as for TLC, reconstituted in equal volumes of phosphosaline buffer, and then tested for HDA by adding 10-μl aliquots from each fraction to 1.0 ml plasma, and measuring TTs.

Dissociation of HDA from Parent Hematin Compound in Plasma. Aged hematin with HDA was added to fresh normal citrated plasma (0.060 mg/ml) and incubated for 30 min. To assess total available HDA, the incubated plasma was mixed with an equal volume of untreated plasma, and TT was measured. This time was compared with an untreated plasma TT. HDA-treated plasma was clotted with thrombin (5 U/ml) for 60 min, centrifuged at 2,000 × *g* for 30 min, and the serum was separated from the fibrin clot. The serum was then mixed with an equal volume of non–HDA-treated plasma, and TT was measured. This was compared with the TT obtained with serum fron nontreated plasma. Sera (treated and untreated) were also alkalinized with NaOH to pH 12, followed by addition of SDS (0.5% final concentration), and then neutralized with HCl. Total HDA activity was then assessed by measuring TT as for the dissolved clot (see below). This treatment did not significantly increase HDA releasable from serum samples. Plasma and serum hematin concentrations were assessed by measuring absorption at 405 or 610 nm and referring to a standard curve.

The clot from the HDA-treated plasma was solubilized by alkalinization to pH 12 with NaOH and addition of SDS to a final concentration of 0.5%. The solubilized clot was neutralized to pH 7.5 with HCl. The volume of the solubilized clot was adjusted with phosphosaline buffer (pH 7.4) to equal the volume of the plasma from which the clot was obtained. The fibrin clot solution was then tested for HDA by adding to an equal volume of normal plasma and measuring TT as above. This result was compared with TT measured with a clot solution from plasma which had not been treated with HDA. Neither treated nor untreated solubilized clots were clottable with thrombin.

Generation of HDA In Vivo. Male Sprague-Dawlye rats (Taconic Farms, Germantown, NY) (250–300 g) were injected in the jugular vein with freshly prepared hematin (preparation *b*) that had no HDA activity. Each rat was injected with 0.4 ml of a 10 mg/ml solution for a dose of 12 mg/kg. Control rats were given 0.4 ml buffer. At each time point, three rats were anesthetized with ethyl ether and exsanguinated by aortic puncture. Citrated plasma was tested for PT, TT, APTT, and fibrinogen. Plasma fibrinogen was measured by the heat-precipitation method (14). Serum and plasma hematin concentrations were assessed by the pyridine hemochromogen assay (12).

Statistics. Results are expressed as mean and the range of observed values. Where indicated, significance was tested by the student's *t*-test or by analysis of variance.

Results

Generation of HDA In Vitro. HDA was assessed at intervals in hematin solutions that were allowed to stand at 4°C for over 6 wk in ambient light, direct incandescent light, or total darkness. The results of generation of HDA by the aging of hematin solutions in ambient light are seen in Table I. The mean and range of values expressed as percent over control clotting times are shown for aging in ambient light for both preparation *a* (potassium phosphate buffer) and preparation *b* (carbonate buffer). Neither hematin preparation shows any anticoagulant activity (HDA) on day zero. However, by day 50, both preparations have developed sufficient HDA activity to prolong clotting times by up to 188% (APTT) over control when added to a concentration of 0.060 mg hematin per milliliter plasma. Prolongation of clotting times was more pronounced with TT and APTT. Fig. 1 shows the dose-dependent prolongation of clotting times (APTT) when aged or fresh hematin is added to normal plasma. In the aged

TABLE 1

Time Course of HDA Generation from Hematin In Vitro

Age of solution (d)	Control clotting times (s)			HDA activity (percent above control clotting time, and actual clotting time [s])					
				Preparation *a* (phosphate buffer)			Preparation *b* (carbonate buffer)		
	PT	TT	APTT	PT	TT	APTT	PT	TT	APTT
0	13.0	12.1	37.4	4.7 (13.6)	13.3 (13.8)	9.5 (40.9)	0.9 (12.8)	5.5 (12.9)	6.4 (39.8)
4	13.3	13.1	35.4	0.9 (11.7)	7.0 (13.7)	5.5 (37.4)	0.9 (11.7)	5.2 (13.9)	6.4 (37.2)
8	13.5	8.0	40.2	2.8 (13.7)	17.7 (11.1)	21.4 (48.8)	0.9 (12.5)	8.8 (8.7)	14.1 (45.9)
12	13.4	9.5	39.4	2.6 (13.7)	14.3 (10.9)	18.2 (46.6)	0.9 (13.2)	10.6 (10.5)	13.4 (44.7)
16	13.4	15.2	39.0	11.3 (14.9)	18.8 (18.1)	30.0 (50.7)	4.9 (14.1)	12.1 (17.0)	25.5 (48.9)
22	12.0	11.8	33.8	22.0 (14.6)	116.8 (24.5)	71.6 (58.0)	10.5 (13.3)	53.9 (17.4)	71.7 (58.0)
30	11.6	13.2	29.1	27.0 (14.7)	140.6 (31.8)	136.9 (68.9)	18.3 (15.5)	116.5 (28.2)	113.3 (63.0)
50	12.0	10.9	35.8	36.5 (16.4)	154.8 (27.8)	188.0 (103.1)	34.4 (16.1)	137.4 (25.9)	183.0 (101.3)

Generation of HDA in vitro in two types of aging hematin preparations. Hematin was allowed to stand at 4°C in ambient light. Anticoagulant activity was assessed at intervals by measuring PT, TT, or APTT in 1 ml of fresh normal plasma immediately after addition of the equivalent of 0.060 mg hematin. Anticoagulant activity (HDA) is expressed as the percent over a control clotting time, and the actual clotting times (s, in parentheses). Control and HDA clotting times are the mean of three determinations. HDA generation in these samples is representative of 10 such samples of aging hematin solutions. Anticoagulant activity developes slowly in these solutions. The effect on TT and APTT is greater than the effect on PT.

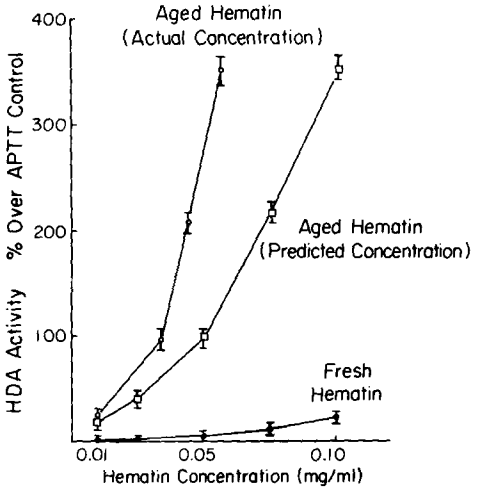

FIGURE 1. Anticoagulant activity (HDA) in aged hematin solutions. Aged (50 d) hematin added to normal citrated plasma shows a dose-dependent prolongation of APTT. Activity is expressed as a percentage over control APTT (untreated plasma APTT, 40.2 sec). Open circles represent actual hematin concentration measured in the aged hematin solution by pyridine hemochromogen. Open boxes represent hematin concentration predicted from the concentration in solution when it was fresh. Closed circles show the activity in a fresh (nonaged) solution. Each point shows the mean and range of determinations on three plasma samples.

preparation, the amount of hematin added to plasma is expressed both as that measured by pyridine hemochromogen in the aged hematin solution, and as the amount predicted from the original hematin concentration when made up fresh. The shift to the left of the actual amount relative to the predicted amount indicates a fall in hematin concentration in the aged solution compared to when it was fresh.

Acceleration and Retardation of HDA Generation. Fig. 2 shows the results of aging hematin solutions in direct incandescent light or in total darkness. Fig. 2*a*

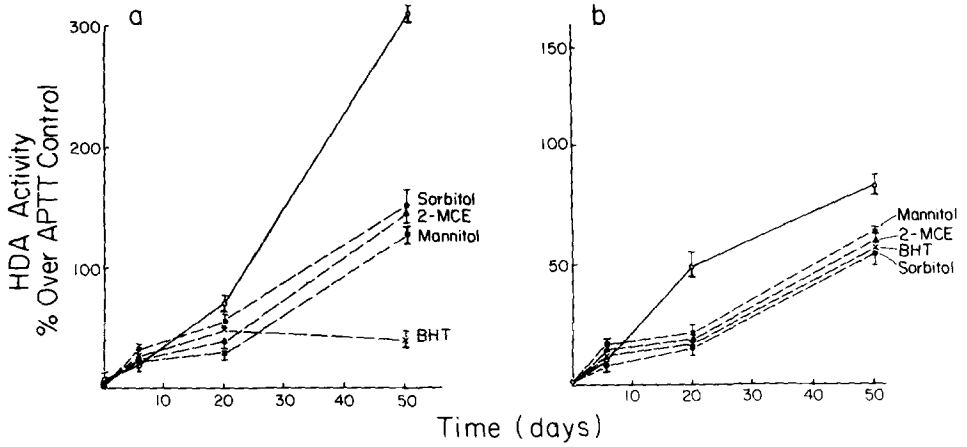

FIGURE 2. Effect of light and antioxidants on the generation of HDA in vitro. Hematin solutions were aged at 4°C in direct incandescent light (40 W lamp at 12 inches) or in complete darkness. Hematin solutions were also aged in the presence of 2 mM concentrations of mannitol (■), sorbitol (●), 2-mercaptoethanol (*2-MCE*) (▲), or BHT (×). The solid line represents hematin allowed to age without antioxidants (○). (*a*) Hematin solutions aged exposed to constant direct light, and (*b*) hematin solutions aged in the dark. The data represent the percent over a control APTT performed at each time point. Control APTT ranged from 34.2–39.8 sec. Each data point shows the mean and range of determinations on three plasma samples.

shows that HDA generation in direct light is greater than that seen with ambient light. Fig. 2*b* shows that aging of hematin in total darkness generates lower levels of HDA activity. While the presence or absence of light had an effect on the generation of anticoagulant activity in aging hematin solutions, clotting times performed with added aged hematin were equally prolonged when performed in the light or in the dark with a mechanical clot detection device. (data not shown) Thus, HDA activity (prolongation of clotting times) found in aged hematin solutions was not affected whether the clotting assays were performed in the absence or presence of light.

Fig. 2 also shows the results of HDA generation in the presence of various antioxidant compounds. At the concentrations used, none of the antioxidants had a significant effect on clotting times when added to normal plasma without hematin solutions present (data not shown). When hematin solutions were aged in the presence of these antioxidants, significant retardation of the development of HDA was observed. Retardation of HDA generation by these compounds was seen in solutions aged both in the light (Fig. 2*a*) or in the dark (Fig. 2*b*). Suppression of the generation of HDA was greatest in the solution aged in the light.

Dissociation and Separation of HDA from Hematin in Aged Hematin Solutions. HDA was dissociated from the parent hematin compound by TLC and HPLC. Fig. 3 shows that the major band of HDA activity (prolongation of APTT) elutes at an appreciably lower R_f than hematin in TLC. It is notable that the band with the highest activity is not associated with pigment. Fig. 4 shows the results of HPLC of fresh and aged hematin solutions. The elution pattern of absorption at 410 nm of fresh hematin solution clearly differs from that of aged hematin.

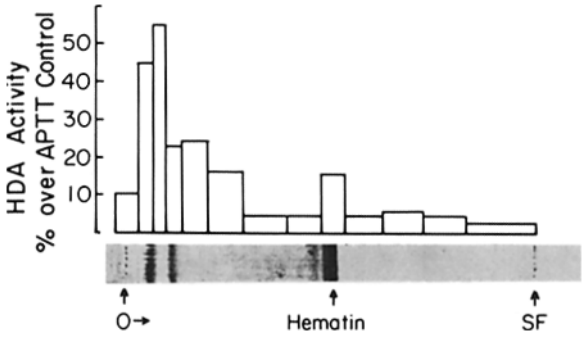

FIGURE 3. TLC of aged hematin solution. Aged hematin (60 d) was eluted on silica gel-60 with 2,6-lutidine and water in a tank saturated with NH_3 vapor. The plates were divided into bands that were scraped, and the resin was extracted for HDA. HDA was tested by adding aliquots from concentrated extracts to plasma and measuring APTTs. The parent compound (hematin) elutes at R_f 0.5. The major band of HDA elutes at a lower R_f, and is not associated with pigment. Similar results were seen with TT determinations.

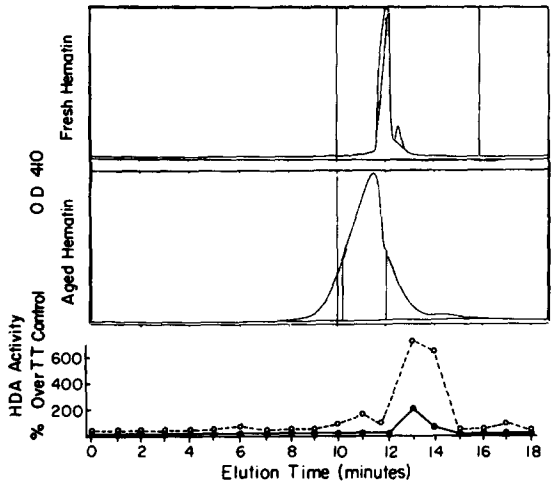

FIGURE 4. HPLC of fresh and aged hematin solutions. Hematin solutions were chromatogrammed on reverse-phase (C_{18}) columns. HDA was tested by adding aliquots from concentrated fractions to plasma and measuring TTs. Closed circles (●) represent fresh hematin and open circles (○) represent aged hematin (50 d). HDA elutes significantly after the major band associated with fresh hematin solution. HDA also elutes in fractions not associated with absorption at 410 nm.

HDA activity (prolongation of TT) elutes after the parent hematin peak in fractions with low absorption at 410 nm.

Dissociation and Separation of HDA from Hematin in Plasma. Normal citrated plasma with added aged hematin solution (0.06 mg/ml) was clotted with thrombin (5 U/ml) for 60 min at 37°C, then centrifuged, and the serum and fibrin clot were separated. HDA was tested in both the serum and dissolved fibrin clot fractions by adding the fractions to an equal volume of normal plasma and measuring TTs. The results are seen in Table II. When compared to the corresponding control serum or dissolved clot fraction (not treated with HDA) the dissolved clot fraction prolonged the thrombin time considerably longer than

TABLE II
Segregation of Hematin and HDA into Serum or Dissolved Clot Fractions of Plasma

Plasma fraction	Control TT (s)	HDA (range)	Hematin concentration (mg/ml)	Hematin added (%)
Whole	10.5	80.5 (64–90)	0.064	100
Dissolved clot	15.3	97.0 (78–109)	0.012	19.4
Serum	12.6	27.6 (18–38)	0.050	78.3

Segregation of HDA into the dissolved clot fraction of whole plasma. Aged hematin solution (0.064 mg/ml) was added to normal plasma and incubated for 30 min at 37°C. The plasma was then clotted with thrombin (5 U/ml) for 60 min, and the clot was separated from the serum. The clot and serum were treated with NaOH and SDS, and HDA activity determined by mixing the fractions with an equal volume of normal plasma. TT of these mixtures were measured and the results were compared with TT measured for the corresponding fraction (whole plasma, dissolved clot fraction, or serum fraction) prepared in the same fashion from untreated plasma (no added hematin solution). HDA activity is expressed as percentage above the corresponding control thrombin time. Treated whole plasma prolonged the thrombin time by 80.5% over the control plasma value. The dissolved plasma clot fraction prolonged the thrombin time by 97%, while the serum fraction prolonged the thrombin time by 27.6% over the corresponding control value. Hematin measured by absorption at 610 nm segregated largely (78.3%) into the serum fraction. The results represent the means of 10 experiments.

the serum fraction (97 vs. 27.6% over control). This suggests that HDA preferentially segregated into the clot fraction. On the other hand, hematin, as measured by absorption at 610 nm, was largely distributed in the serum fraction.

Inhibitors of HDA Prolongation of Clotting Times. Prolongation of clotting times (HDA) found in aged hematin solutions was tested in plasma in the presence of fresh (nonaged) hematin solution, fresh or aged Sn–protoporphyrin IX (Sn-heme) or fresh or aged protoporphyrin IX. The results of the clotting times expressed as percent over control from these experiments are seen in Fig. 5. Neither fresh hematin nor fresh Sn–protoporphyrin IX alone had any significant effect, whereas aged Sn–protoporphyrin IX alone showed a small prolongation of thrombin times. When fresh hematin or fresh Sn–protoporphyrin IX were added in equimolar amounts with aged hematin, there was a significant reduction in HDA activity as measured by plasma thrombin times. Aged Sn–protoporphyrin IX added to plasma with aged hematin showed a small augmentation of HDA. Neither fresh nor aged protoporphyrin IX had an effect on plasma clotting times when added alone or with equimolar concentrations of aged hematin (data not shown).

HDA was also tested in the presence of varying concentrations of imidazole. Dose-dependent reduction of HDA was seen when plasma clotting times were measured with the addition of imidazole (0–50 mM, final concentration) before adding HDA (data not shown). Imidazole was also added to either a purified fibrinogen or a purified thrombin solution 5 min before addition of HDA. Clotting times were measured by adding treated thrombin to untreated fibrinogen or untreated thrombin to treated fibrinogen. In a purified fibrinogen and

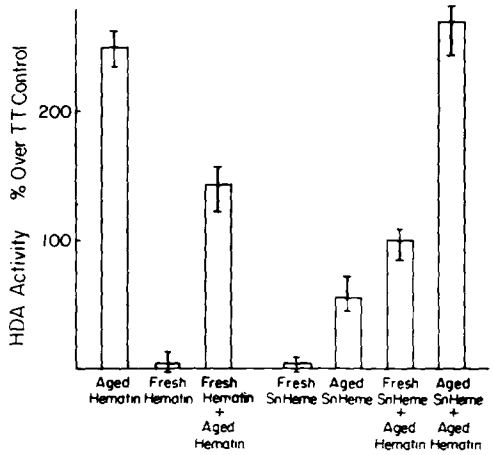

FIGURE 5. Inhibitory effect of fresh heme and tin-heme on HDA. Hematin aged 60 d was added to plasma at 0.06 mg/ml and tested for HDA activity in the presence of equimolar amounts of fresh hematin, fresh tin-heme or aged (60 d) tin-heme solutions. These solutions were also tested for anticoagulant activity without added aged hematin (HDA). The results are expressed as the percent over a control TT (7.6 sec). The data shows the mean and range for TT determinations performed on three samples. Equimolar concentrations of fresh hematin or tin-heme significantly reduce HDA. Aged tin-heme solution alone produces a significant prolongation of thrombin times. Addition of aged tin-heme to aged hematin augments HDA.

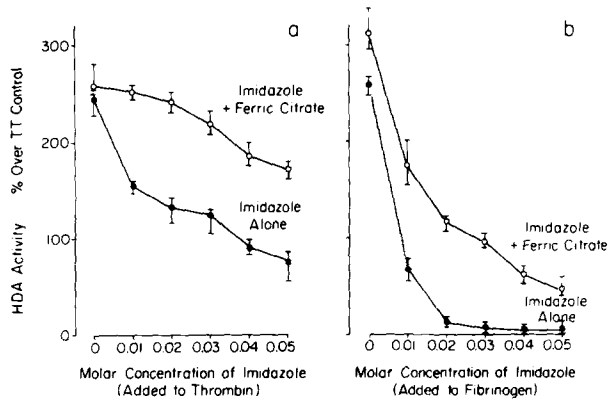

FIGURE 6. Inhibition of HDA by imidazole. Imidazole was added to fibrinogen or thrombin solutions before addition of HDA (hematin aged 50 d, 0.06 mg/ml). TT were measured by adding treated thrombin to untreated fibrinogen (*a*) or by adding untreated thrombin to treated fibrinogen solution (*b*). The results shown are the mean and range of the percent over a control TT (10.5 sec) of three experiments. Imidazole added to achieve concentrations of 10–50 mM produced a dose-dependent inhibition of HDA. Addition of ferric citrate (50 mM) to the treated solutions reverses the inhibition of HDA produced by imidazole.

thrombin system, TT prolongation produced by HDA was inhibited if imidazole was added first either to the fibrinogen or the thrombin (Fig. 6). Control experiments showed no effects on TT when imidazole was added to either the thrombin or fibrinogen solutions in the absence of HDA.

Since iron chelation by imidazole may participate in the inhibition of HDA, these experiments were also performed in the presence of 50 mM ferric citrate.

Ferric citrate alone (no imidazole) produced no additional prolongation of clotting times over that produced by HDA. Fig. 6 shows that ferric iron as ferric citrate reverses the inhibition of HDA produced by imidazole.

To further examine the role of iron in the mechanism, HDA was also tested before (blockade) and after (reversal) the addition of the specific iron chelator, desferrioxamine. Fig. 7 shows the dose-dependent (0–0.3 mg/ml) blockade of anticoagulant activity produced by desferrioxamine when added before HDA in a purified fibrinogen/thrombin time experiment. This figure also shows that ferrioxamine, the iron chelate of desferrioxamine, produced no blockade of HDA activity. Reversal of HDA in plasma was attempted by adding desferrioxamine after the addition of HDA. Desferrioxamine alone added to plasma did not shorten the thrombin time. Table III shows a dose-dependent reversal of

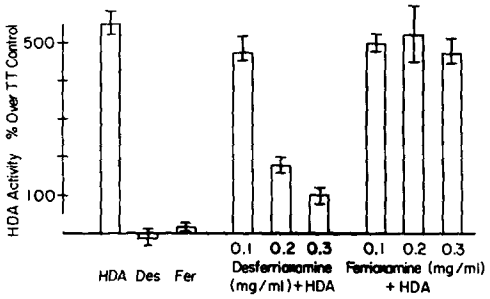

FIGURE 7. Dose-dependent blockade of HDA by desferrioxamine. TT were measured with human fibrinogen (2.5 mg/ml) and thrombin. 5 min before addition of HDA, desferrioxamine or ferrioxamine was added to the fibrinogen solution. HDA (hematin aged 60 d) was added at a concentration of 0.06 mg/ml, and then TT was measured 2 min later. The data represent the mean and range of percent over control TT (6.8 sec) observed for four experimental samples. Neither desferrioxamine (*Des*) nor ferrioxamine (*Fer*) show an effect on the clotting time when added alone (no HDA) to fibrinogen. In combination with HDA, desferrioxamine shows a dose-dependent blockade of anticoagulant activity, whereas the iron chelate, ferrioxamine, shows no inhibition of HDA.

TABLE III

Reversal and Blockade of HDA Activity by Desferrioxamine in Plasma

Desferrioxamine added to plasma (mg/ml)	Remaining HDA activity (prolongation of ATT)			
	Reversal		Blockade	
	Percent	TT (s)	Percent	TT (s)
0.10	80	31.4	75	30.3
0.30	75	30.4	64	27.9
0.50	62	27.5	54	25.8

Reversal and blockade of HDA by desferrioxamine in plasma. Desferrioxamine (0–0.5 mg/ml) was added to citrated plasma 5 min after (reversal) or 5 min before (blockade) the addition of HDA from hematin aged 50 d (0.06 mg/ml). Control TT was 14.5 s. Addition of aged hematin prolonged TT to 35.7 s. Desferrioxamine alone produced a small (4%) prolongation of the thrombin time at 0.5 mg/ml. Data is expressed as percent of the remaining HDA activity (prolongation of 21.2 sec over control, 100%). Actual thrombin times are also shown. The results shown are the means of thrombin times performed on four separate samples. Desferrioxamine reverses as well as blocks HDA activity.

HDA activity in plasma when desferrioxamine (0–0.5 mg/ml) was added 5 min after the addition of HDA. Dose-dependent reversal was also shown by adding desferrioxamine up to 30 min after addition of HDA (data not shown). These results indicate that the mechanism of action of HDA does not involve an irreversible or destructive effect on coagulation proteins.

Generation of HDA In Vivo. Fresh hematin solution was prepared as for infusion into a patient (preparation *b*). This preparation had no HDA activity (prolongation of clotting times) when tested within 1 h after preparation and injection. Rats were given intravenous injections of this hematin preparation (12 mg/kg). Control rats (time zero) were given an equal volume of buffer and showed no anticoagulant effect. There was no effect on plasma PT by hematin infusion. Fig. 8 shows the results of plasma hematin, fibrinogen, TT, and APTT in these experiments. There was a delay between the rise in plasma hematin levels (1 min) and the appearance of anticoagulant activity (3 min). Furthermore, prolongation of the APTT and TT persisted long after the fall in plasma hematin concentration. These results show a temporal dissociation between hematin in the plasma and anticoagulant activity produced by infusion of fresh hematin, and suggests that a modification of hematin leads to HDA in vivo. No change was seen in plasma fibrinogen measured by heat precipitation. This is consistent with previous observations (11) in patients with AIP. Fig. 8 also shows that desferrioxamine (0.5 mg/ml) added to these citrated plasma samples reverses anticoagulant activity produced by HDA in vivo. Reversibility with desferriox-

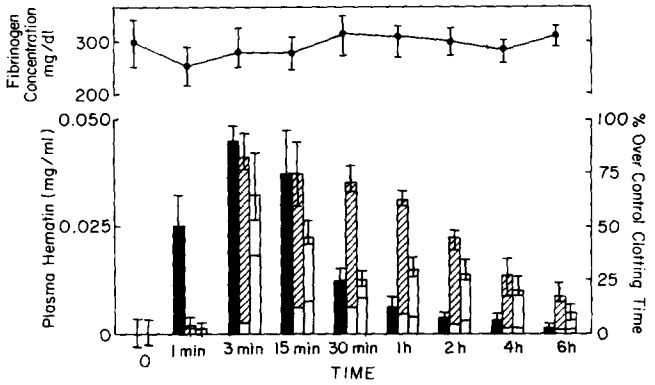

FIGURE 8. Generation of HDA in vivo. Fresh hematin (no HDA) was injected intravenously by jugular vein into rats and samples were drawn for plasma hematin (pyridine hemochromogen), clotting times and plasma fibrinogen. Plasma fibrinogen was measured by heat precipitation. Each histogram shows the mean and range of determinations from three rats sacrificed at each time point. The clotting time data are presented as the percent over control clotting times (APTT, 17.3 sec, TT, 14.0 sec). Anticoagulant activity (prolonged APTT [striped bars] and TT [open bars below APTT]) appears 3 min after hematin injection, whereas plasma hematin (solid bars) levels are elevated after 1 min. Plasma fibrinogen levels (●——●) show no change measured by heat precipitation (analysis of variance). Clotting times (open bars) are prolonged considerably after the fall in plasma hematin concentration. There is a greater effect on APTT compared to TT. There is statistically significant prolongation of clotting times ($p < 0.05$, student's *t*-test) for all points after hematin infusion except for 1 min and 6 h (TT). Shaded areas within the clotting time histograms represent the mean clotting time after desferrioxamine (0.5 mg/ml) was added to the plasma. Desferrioxamine reverses the prolongation of clotting times produced by in vivo generated HDA. This effect is more dramatic with APTT.

amine suggests that HDA activity seen in vivo and HDA produced in vitro in aging hematin solutions, have similar mechanisms.

Discussion

Recent clinical trials have shown that infusions of hematin (ferriprotoporphyrin IX hydroxide) are effective in preventing or abating the painful crises associated with AIP. (6–8) Preparations of hematin have now become commercially available, and it is expected that the use of this compound for therapy in porphyria patients will become more widespread. With the limited use thus far, few adverse reactions have been reported after infusions of hematin. Chemical phlebitis and reversible acute renal insufficiency have been rarely reported (15, 16). More recently however, hematin infusion has been reported to be associated with profound effects on coagulation tests. (9, 10) Glueck et al. (11) reported elevation of the standard clinical clotting assays, elevation of fibrin degradation products, decreases in the levels of clotting factors, including fibrinogen, and thrombocytopenia. These observations suggested a picture of intravascular coagulation with consumption of clotting factors. On the other hand, when these investigators measured fibrinogen concentration by heat precipitation, no decrease was found. This suggests that the prolongation of clotting times produced by hematin is due to interference with coagulation factor mechanisms rather than consumption of clotting factors. The present study indicates that prolongation of clotting times produced by hematin is due to a degradation product or derivative of hematin (HDA) that reversibly inhibits the action of one or more coagulation factors.

HDA was generated in vitro by allowing solutions to stand (age) at 4°C. The generation was time dependent on and accelerated by light. The acceleration by light suggests that an oxidation or free radical–mediated alteration of hematin is responsible for the generation of HDA. Retardation of HDA generation by mannitol, sorbitol, 2-ME, or BHT supports this idea, since these compounds are antioxidants and free radical scavengers. Acceleration by light also suggests that generation of HDA in vitro is promoted by a photodynamic effect. However, a photodynamic effect is not responsible for the anticoagulant action of HDA, because clotting times measured in the dark are equally prolonged compared to those measured in the light. This distinguishes HDA from the photodynamic destructive effects on clotting proteins and the prolongation of clotting times described for hematoporphyrin (17, 18).

Dissociation of HDA from the parent compound hematin was also demonstrated when plasma containing HDA was clotted with thrombin. Although the interpretation of such experiments may be complicated by the presence of procoagulants and/or inhibitors generated by the clotting process, these factors were controlled for by comparing the experimental clotting times with the results produced by the appropriate untreated clot or serum fractions. Using these controls, the results show that HDA activity largely segregated into the fibrin clot fraction, whereas hematin remains largely in the serum. This suggests that HDA binds to clottable protein or fibrinogen.

TLC and HPLC experiments also showed clear separation of HDA from the parent hematin compound when aged hematin solutions were chromatographed. Besides showing a distinction between hematin and HDA, the results of the TLC

and HPLC experiments also suggest that HDA may be due to a single derivative or degradation product of the in vitro hematin aging process. Unfortunately, little is known about nonenzymatic degradation of heme or other porphyrins in vitro. In a study of methemalbumin, Rosenfeld and Surgenor found that hematin was transformed to a product that did not have the albumin-binding characteristics of heme (19). This unidentified product was formed when hematin was allowed to stand for several hours under conditions similar to the aging process described above. Under these conditions, it is possible that hematin undergoes attack by oxygen or hydroxyl radicals. Some of these reactions may take place at the α-methene carbon, leading to products similar to those generated by enzymatic degradation (20). Hematin in aqueous solution is also known (21) to form soluble and insoluble polymers or aggregates. However, these polymers seem unlikely candidates for HDA, since the TLC and HPLC experiments showed HDA eluting in fractions not associated with pigment or absorption at 410 nm.

Further evidence that HDA is a derivative of hematin rather than the parent compound is provided by the experiments showing inhibition by freshly prepared solutions of hematin and Sn-protoporphyrin. It is apparent that a breakdown product will bear some structural similarity to the parent compound. Therefore, the observed inhibition of anticoagulant activity suggests that nondegraded metalloporphyrins interact with or block specific sites on coagulation proteins that must be available for HDA activity. Also, neither fresh nor aged protoporphyrin IX showed inhibition of HDA when added in equimolar amounts. The lack of anticoagulant activity or inhibition of anticoagulant activity produced by aged or fresh protoporphyrin IX indicates that a metal must be present in the porphyrin ring to cause breakdown to HDA or inhibition of HDA by the fresh porphyrin.

Imidazole showed a dose-dependent inhibition of HDA. More specifically, imidazole prevents or retards the inactivation of thrombin or fibrinogen, and it appears that this effect is more pronounced with fibrinogen (Fig. 6). In the hemoglobin molecule, heme binds to globin partially through an interaction of the iron of heme and a histidine residue (imidazole) of globin (22, 23). Inhibition of HDA by imidazole, and the observed reversal of this inhibition by iron suggests that iron or a metal atom may play an important role in the action of HDA. This result also suggests the possibility that HDA may interact with histidine residues of clotting proteins to produce prolongation of clotting times.

Blockade and reversal of HDA by desferrioxamine is further evidence that iron is involved in the mechanism of HDA activity. Failure of ferrioxamine (the iron chelate of desferrioxamine) to inhibit HDA shows that the iron-binding capacity of desferrioxamine is necessary for the inhibition. Reversal of the activity indicates that the interaction of HDA with coagulation proteins is not destructive, and that the structural integrity of the coagulation proteins affected is maintained. The apparent role of iron in the mechanism of HDA and the observed reversibility of HDA activity are notable in light of the coagulation defect seen in acute iron toxicity. A recent report by Rosenmund et al. documents that ferric iron autoxidized from ferrous sulfate causes a functional impairment of thrombin and other coagulation proteases which prolongs coagulation times (24). Similarly to HDA, this functional impairment was also reversible with chelation of iron.

The lowest level of free iron used in their study was 8–80-fold higher than those that are possible from the hematin used herein. It is therefore unlikely that free iron from degraded hematin is responsible for HDA activity.

In the experiments in rats, when fresh hematin solution containing no HDA was injected as an intravenous bolus, anticoagulant activity appeared in the plasma after the rise in plasma hematin concentration. Furthermore, anticoagulant activity persisted after the fall in plasma hematin. This indicates a temporal dissociation of prolongation of clotting time activity (HDA) from the parent hematin compound in vivo. Although the time sequence of prolonged clotting times after hematin infusion has not been precisely defined, the results in rats reported here are consistent with published information from human studies. The very rapid time course of generation of HDA in vivo, suggests a metabolic conversion of hematin that leads to HDA activity rather than the slow degradation or derivatization seen in vitro. Bilirubin and other bile pigments are the major metabolic products recovered when heme is administered intravenously to rats (25). These pigments are produced by the microsomal heme-oxygenase system, which is thought to play the dominant role in physiologic heme catabolism (26). Recently, (27, 28), however, NADPH-cytochrome c reductase has also been found to degrade heme in a less specific manner to a mixture of dipyrrolic propentdyopents. This system is distinct from the heme-oxygenase catabolism of heme, and bile pigments are not intermediates in this pathway. Propentdyopents have been found in gallstones and urine, and this mode of heme catabolism has been suggested (29) to be operative at low levels in normal metabolic states. Therefore, these products as well as the bile pigments must be considered as candidates for HDA.

Although the structural identity of HDA was not identified by this study, in vitro (TLC and HPLC) experiments indicate that HDA is a single derivative of hematin. Furthermore, it is also probable that this compound contains iron, or that iron is necessary for the anticoagulant action. Reversal of HDA activity in plasma from the hematin-treated rats by desferrioxamine suggests that a common mechanism and derivative is responsible for HDA activity produced in vitro and in vivo. The mechanism for the anticoagulant action is also likely related to a reversible binding of HDA to clotting proteins. In a recent study of hematin effects on hemostasis, Green and associates (30) showed that the parent compound binds to fibrinogen and albumin in vitro. Müller-Eberhard and others (31, 32) have shown that, in vivo, hematin (heme) is bound most specifically to hemopexin and albumin. This binding and subsequent uptake by the liver is the major pathway for clearance of hematin from the plasma. Since HDA segregates largely into the clot fraction of whole plasma, it appears that HDA binds preferentially to fibrinogen and/or other clotting proteins, with a resultant anticoagulant effect.

The results outlined above have obvious clinical implications. To avoid infusion of hematin that has anticoagulant activity, one should use material as freshly prepared from hemin (ferriprotoporphyrin IX chloride) as possible. We have recently examined the commercially available product Panhematin (Abbott Laboratories), which is a lyophilized preparation of hematin. This product, when freshly dissolved with sterile H_2O as for infusion, showed prolongation of plasma

clotting times in vitro (40% increase in APTT at a plasma concentration of 0.078 mg/ml). Furthermore, the anticoagulant activity detected in this preparation eluted in an identical fraction to HDA in the HPLC system described above. This indicates that stored hematin may degrade to HDA before or after lyophilization, and that only products freshly prepared from hemin will be free of HDA. On the other hand, the experiment in rats suggests that even freshly prepared hematin may be metabolically converted to produce an anticoagulant (HDA?) in vivo. Thus, this potential toxicity of hematin infusion may not be avoidable. Finally, if it is confirmed that the appearance of HDA in vivo is due to metabolic conversion of hematin, it is possible that endogenous heme or other porphyrins are converted to HDA. Confirmation that these molecules and their effects on hemostasis exist in normal or disease states awaits purification and structural identification of HDA from in vitro and in vivo sources. The fact that the anticoagulant effect of hematin (heme) derivatives may be inhibited by pharmacologic agents, such as desferrioxamine or the synthetic analogue of heme, Sn-protoporphyrin, raises the possibility that such agents may also be useful to suppress HDA in clinical circumstances where HDA may lead to increased risk of hemorrhage.

Summary

Prolongation of clotting times produced by hematin was investigated both in vitro and in vivo. Hematin-derived anticoagulant (HDA) was found to be due to a degradative product or derivative of hematin, and was generated in vitro in standing (aging) aqueous solutions of the parent compound. Generation of HDA in vitro was inhibited by antioxidants. The anticoagulant effect of HDA was inhibited by freshly prepared hematin, fresh Sn-protoporphyrin, imidazole, or the iron chelator desferrioxamine. Ferrioxamine did not inhibit HDA, and inhibition by imidazole was reversed with ferric citrate, suggesting a role for iron in the mechanism of HDA activity. HDA activity was dissociated from hematin in plasma by clotting with thrombin. HDA segregated into the clot fraction, whereas hematin remained largely in the serum fraction, suggesting that HDA may preferentially bind to fibrinogen. TLC and HPLC showed a single peak of HDA activity that was not associated with the parent compound. Evidence for HDA generation in vivo was found when rats were injected with fresh (no HDA) hematin. Prolongation of clotting times appeared after hematin appeared in the plasma, and anticoagulant activity persisted after a fall in plasma hematin concentration. Thus, there was a temporal dissociation between hematin and HDA, suggesting that a modification of hematin must occur in vivo before an anticoagulant effect is produced. Generation of HDA in vitro has implications for hematin preparation and administration. Generation of HDA in vivo suggests that similar modifications of endogenous heme or other porphyrins may occur to produce HDA under physiologic or pathophysiologic conditions.

I wish to acknowledge the advice and support of Drs. Peter Harpel of the Cornell University Medical College, and Drs. Attallah Kappas and Shigeru Sassa of the Rockefeller University Hospital in carrying out this investigation. Dr. Sassa kindly carried out the

TLC and HPLC analysis. In addition, I thank Martin Sorette for his invaluable technical contributions.

Received for publication 4 November 1985.

References

1. Kessel, D., and T. Chou. 1983. Tumor-localizing components of the porphyrin preparation hematoporphyrin derivative. *Cancer Res.* 43:1994.
2. Zanelli, G. D., and A. C. Kaelin. 1981. Synthetic porphyrins as tumour-localizing agents. *Brit. J. Radiol.* 54:403.
3. Drummond, G. S., and A. Kappas. 1981. Prevention of neonatal hyperbilirubinemia by tin protoporphyrin IX, a potent competitive inhibitor of heme oxidation. *Proc. Natl. Acad. Sci. USA.* 78:6466.
4. Drummond, G. S., and A. Kappas. 1982. Chemoprevention of neonatal jaundice: Potency of tin-protoporphyrin in an animal model. *Science (Wash. DC).* 217:1250.
5. Sassa, S., G. S. Drummond, S. E. Bernstein, and A. Kappas. 1983. Tin-protoporphyrin suppression of hyperbilirubinemia in mutant mice with severe hemolytic anemia. *Blood.* 61:1011.
6. Watson, C. J., G. J. Dhar, I. Bossenmaier, R. Cardinal, and Z. J. Pertyka. 1973. Effect of hematin in acute porphyria relapse. *Ann. Internal Med.* 79:80.
7. Watson, C. J., C. A. Pierach, I. Bossenmaier, and R. Cardinal. 1978. Use of hematin in the acute attack of the "inducible" hepatic porphyrias. *Adv. Internal Med.* 23:265.
8. McColl, K. E. L., M. R. Moore, G. G. Thompson, and A. Goldberg. 1981. Treatment with haematin in acute hepatic porphyria. *Quart. J. Med.* 198:161.
9. Pierach, C. A., I. Bossenmaier, R. Cardinal, M. Weiner, N. W. Shilling, and J. R. Edson. 1981. Hematin, an anticoagulant. *Hepatology.* 1:536(Abstr.).
10. Morris, D. L., M. D. Dudley, and R. D. Pearson. 1981. Coagulopathy associated with hematin treatment for acute intermittent porphyria. *Ann. Internal Med.* 95:700.
11. Glueck, R., D. Green, I. Cohen, and C. Ts'ao. 1983. Hematin: Unique effects on hemostasis. *Blood.* 61:243.
12. Fuhrhop, J. H., and K. M. Smith. 1975. *In* Laboratory Methods in Porphyrin and Metalloporphrin Research. Elsevier Biomedical Press, New York, pp. 48.
13. Chiba, M., and S. Sassa. 1982. Analysis of porphyrin carboxylic acids in biologic fluids by high-performance liquid chromatography. *Anal. Biochem.* 124:279.
14. Searcy, R. L., N. M. Simons, and E. M. Y. Low. 1967. A simple method for rapid detection of hypofibrinogenemia. *Am. J. Med. Technol.* 33:326.
15. Dhar, G. J., I. Bossenmaier, Z. J. Petryka, R. Cardinal, and C. J. Watson. 1975. Effects of hematin in hepatic porphyria: further studies. *Ann. Internal Med.* 83:20.
16. Dhar, G. J., I. Bossenmaier, R. Cardinal, Z. J. Petryka, and C. J. Watson. 1978. Transitory renal failure following rapid administration of a relatively large amount of hematin in a patient with acute intermittent porphyria in clinical remission. *Acta Med. Scand.* 203:437.
17. Zieve, P. D., and H. M. Solomon. 1965. Effect of hematoporphyrin and light on human fibrinogen. *J. Clin. Invest.* 44:1391.
18. Davis, R. B., R. Biggs, and K. W. E. Denson. 1967. "Inhibitor-like" effects of hematoporphyrin on factor VIII (Antihemophilic globulin). 1967. *Thromb. Diath. Haemorrh.* 17:299.
19. Rosenfeld, M., and D. M. Surgenor. 1950. Methemalbumin: interaction between human serum albumin and ferriprotoporphyrin IX. *J. Biol. Chem.* 183:663.
20. Yoshida, T., and G. Kikuchi. 1978. Purification and properties of heme oxygenase from pig spleen microsomes. *J. Biol. Chem.* 253:4224.

21. Shack, J., and W. M. Clark. 1947. Metalloporphyrin. Cycles of changes in systems containing heme. *J. Biol. Chem.* 171:143.
22. Brittain, T. 1981. A comparison of the binding of imidazole to valence hybrid and methemoglobin: An assignment of individual heme reactivity. *J. Inorg. Chem.* 15:243.
23. Friedman, J. T., T. W. Scott, R. A. Stepnoski, M. Ikeda-Saito, and T. Yonetani. 1983. The iron-proximal histidine linkage and protein control of oxygen binding in hemoglobin. *J. Biol. Chem.* 258:10564.
24. Rosenmund, A., A. Haeberli, and P. W. Straub. 1984. Blood coagulation and acute iron toxicity. Reversible iron-induced inactivation of serine proteases in vitro. *J. Lab. Clin. Med.* 103:524.
25. Guengerich, F. P. 1978. Destruction of heme and hemoproteins mediated liver microsomal reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase. *Biochemistry.* 17:3633.
26. Masters, B. S., and B. A. Schacter. 1976. The catalysis of heme degradation by purified NADPH-cytochrome c reductase in the absence of other microsomal proteins. *Ann. Clin. Res.* 8(Suppl. 17):18.
27. Guengerich, F. P., and T. W. Strickland. 1977. Metabolism of vinyl chloride: destruction of the heme of highly purified liver microsomal cytochrome P-450 by a metabolite. *Mol. Pharmacol.* 13:993.
28. Yoshinaga, T., S. Sassa, and A. Kappas. 1982. A comparative study of heme degradation by NADPH-cytochrome c reductase alone and by the complete heme oxygenase system. *J. Biol. Chem.* 257:7794.
29. Lightner, D. 1978. Derivatives of bile pigments. *In* The Porphyrins. D. Dolphin, editor. Academic Press, New York. pp. 521.
30. Green, D., N. Reynolds, J. Klein, H. Kohl, and C. Ts'ao. 1983. The inactivation of hemostatic factors by hematin. *J. Lab. Clin. Med.* 102:361.
31. Müller-Eberhard, U., and H. H. Liem. 1974. Hemopexin, the heme binding serum B-glycoprotein. *In* Structure and Function of Plasma Proteins. A. C. Allison, editor. Plenum Press, Lond. pp. 35.
32. Müller-Eberhard, U., H. H. Liem, A. Hanstein, and P. A. Saarien. 1969. Studies on the disposal of intravascular heme in the rabbit. *J. Lab. Clin. Med.* 73:210.