

Hematin: Unique Effects on Hemostasis

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Hematin is clinically useful in the treatment of acute intermittent porphyria. Recently, hematin-induced coagulopathy has been reported, and a patient we treated bled during hematin therapy. On 3 separate occasions, infusions of hematin (4 mg/kg) induced thrombocytopenia, prolongation of the prothrombin time, partial thromboplastin time, Reptilase time, and apparent decreases in fibrinogen and increases in fibrin (ogen) degradation products (FDP). However, fibrinogen assayed by heat precipitation was unchanged, the protamine paracoagulation test was negative, there was no red blood cell fragmentation, and plasminogen and antithrombin III remained normal, excluding the presence of disseminated intravascular coagulation. Furthermore, premedication with heparin, 5000 U i.v.,

failed to prevent the lengthening of the Reptilase time and exacerbated the thrombocytopenia. In vitro studies revealed that hematin, 0.1 mg/ml, aggregated platelets and induced the release of ¹⁴C-serotonin and adenosine triphosphate (ATP). Hematin also aggregated washed or gel-filtered platelets but had no effect on formalin-fixed platelets. Aggregation was inhibited by aspirin (0.12 mg/ml), adenosine triphosphate, and apyrase, suggesting that hematin aggregated platelets by inducing adenosine diphosphate (ADP) release. Hematin (0.07 mg/ml) progressively inactivated thrombin and 0.1 mg/ml prolonged the Reptilase time. Thus, hematin is unique in that it both induces platelet aggregation and inhibits coagulation.

HEMATIN, ferric protoporphyrin hydroxide, is effective in the treatment of acute intermittent porphyria.^{1,2} However, disturbing reports have recently appeared that describe a hematin-induced coagulopathy.^{3,4} We observed such a hemorrhagic diathesis in a patient treated with hematin and therefore performed a prospective investigation to elucidate the characteristics of hematin coagulopathy.

MATERIALS AND METHODS

Routine tests of coagulation, including the partial thromboplastin time, fibrinogen, fibrin(ogen) degradation products (FDP), plasminogen, and measurements of factors V, VIII, and antithrombin III were performed as previously described from our laboratories.^{5,6} The thrombin time was performed by adding 0.1 ml of bovine thrombin (Parke-Davis, Detroit, Mich.) to either 0.1 ml of platelet-poor plasma and 0.1 ml of saline, or to 0.2 ml of bovine fibrinogen (Calbiochem, LaJolla, Calif., 98% clottable) dissolved in barbital buffer, pH 7.5. The concentration of the thrombin was adjusted to give clotting times of approximately 15 sec and was usually 1 U/ml. In addition, fibrinogen was also determined by heat precipitation⁷ and the polymerization of fibrin monomer by the method of Cohen et al.⁸ Reptilase-R was obtained from Abbott Laboratories, North Chicago, Ill., and the protamine sulfate precipitation test was performed with the DATA-Fi reagents from Dade Diagnostics, Puerto Rico. Aggregation in platelet-rich plasma (PRP) was examined with a platelet aggregometer⁹ using as inducing agents collagen prepared from human skin¹⁰ and adenosine diphosphate (ADP, Sigma, St. Louis, Mo.). The uptake and release of ¹⁴C-serotonin (Amersham, Arlington Heights, Ill.) was measured according to Jerushalmy and Zucker,¹¹ modified by including 12.5 μM imipramine to prevent reuptake of the serotonin. ATP release was examined with the Lumi-aggregometer (Chrono-log, Havertown, Pa.). Washed platelets were prepared by the method of Rossi,¹² and gel-filtered and formalin-fixed platelets by the techniques of Lages et al.¹³ and Macfarlane et al.,¹⁴ respectively. Inhibition of aggregation was studied using adenosine, apyrase, and adenosine triphosphate (ATP), all purchased from Sigma. Hematin was obtained from Dr. C. A. Pierach, Watson Research Laboratory, Minneapolis, Minn., and the measurements of hematin in serum were performed by his laboratory, as previously described.²

All in vivo experimental procedures were approved by the Institutional Review Board of Northwestern University, and informed

consent was received from the patient prior to performing the procedures to be described.

CASE REPORT

A previously healthy 39-yr-old black male underwent a technically successful laminectomy at C4-C5 for upper extremity weakness that had been attributed to osteoarthritic spurring. After surgery, abdominal pain, burgundy red urine, and a neuropathy that progressed to quadriplegia suggested the diagnosis of acute intermittent porphyria. The Watson-Schwartz qualitative test for urine porphobilinogen (PBG) was positive and the attack was terminated by high-dose glucose infusion. However, the patient required tracheostomy and temporary mechanical ventilation. Heparin, 5000 U S.C. every 12 hr. was initiated for venous thrombosis prophylaxis.

Three months later, while still on heparin, the patient suffered an acute porphyric attack precipitated by poor caloric intake. At that time he was diaphoretic and hallucinating. The pulse was 112, the blood pressure 150/84, the respiratory rate 38, and the temperature 99°F. The abdomen was diffusely tender with normal bowel sounds and no rebound tenderness. In addition to the abnormal mental status, the neurologic exam was remarkable for quadriplegia, with weakness more marked in the arms than in the legs. Sensation was intact.

The hemoglobin was 12.5 g/dl, hematocrit 36%, and platelet count 176,000/μl. The prothrombin time (PT) was 11.7 sec (control 12.2 sec), and the partial thromboplastin time (PTT) 37.5 sec (control 32.7 sec). Liver function tests were within normal limits. The serum sodium was 126 meq/liter. PBG was 104 μg/dl (normal, 0-4 μg/dl), the delta-aminolevulinic acid (Δ-ALA) 29 μg/dl (normal, 0-10 μg/dl), and the erythrocyte uroporphyrinogen I synthetase 17.3 U (normal, 20.9-42.2 U).

After 72 hr of persistent symptoms despite glucose infusion,

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chlorpromazine, and codeine, hematin, 4 mg/kg (172 mg), was administered intravenously every 12 hr. Seven hours after the fourth dose, the patient was noted to be bleeding from both nares and blood was issuing from his tracheostomy. There was no evidence of trauma or nasopharyngeal pathology and the bleeding stopped without treatment. At that time the blood pressure was 130/70, the PTT was greater than 150 sec, the PT was 18.3 sec (control 11.9 sec), the thrombin time was normal, and the platelet count 154,000. Fibrin(ogen) degradation products (FDP) were less than 10 $\mu\text{g}/\text{ml}$, the hemoglobin was 11.2 g/dl, and the hematocrit 32%. After hematin and heparin were discontinued, laboratory values gradually returned to baseline over 48 hr.

RESULTS

In Vivo Studies

Hematin (4 mg/kg) was infused intravenously over a 15-min period on 3 separate occasions. Blood samples were obtained immediately prior to infusion, and 10 min, 5 hr, 24 hr, and 48 hr postinfusion. The effects of hematin on the prothrombin time, partial thromboplastin time, thrombin time, Reptilase time, levels of factors V, VIII, and fibrinogen, platelet count, and FDP titer are shown in Fig. 1. Ten minutes after the infusion, all clotting times were prolonged, the platelet count and levels of factors V, VIII, and fibrinogen had declined, and the titer of FDP had doubled. By 5 hr, there was partial recovery and complete recovery at 48 hr. Platelet aggregation was impaired with ADP (Fig. 2) and collagen at 10 min, and although improved at 48 hr, a secondary wave of aggregation was still ab-

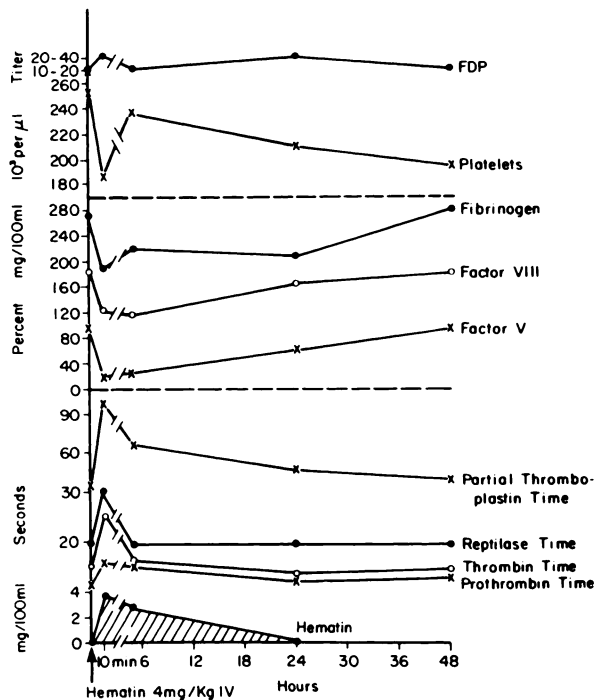


Fig. 1. Serial coagulation analyses after the intravenous infusion of hematin, 4 mg/kg. FDP, fibrin(ogen) degradation products.

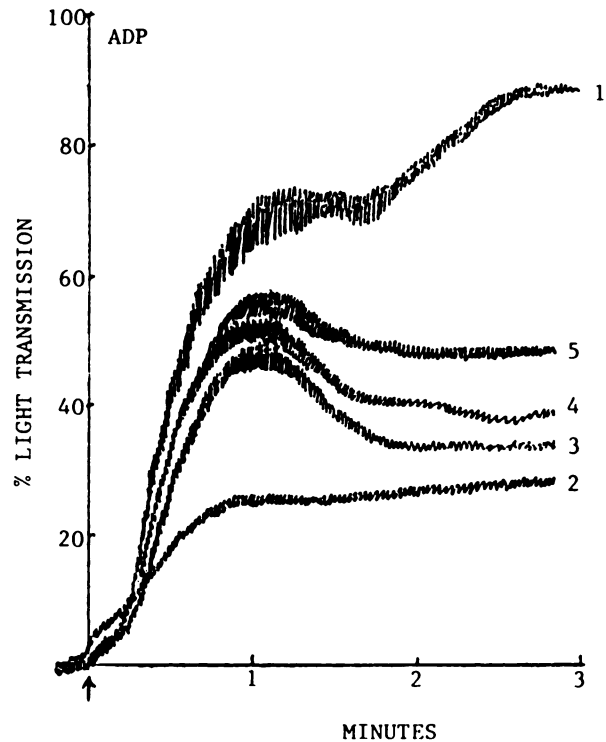


Fig. 2. Aggregation of the patient's PRP with ADP ($2.5 \mu\text{M}$) at various times after hematin (4 mg/kg) infusion. (1) Preinfusion; (2) 10 min; (3) 5 hr; (4) 24 hr; (5) 48 hr.

sent. There were no morphological changes in erythrocytes examined in smears of peripheral blood, and plasma levels of plasminogen and antithrombin III were unaltered.

In the second experiment, the patient was premedicated with aspirin (650 mg) 2 hr prior to the hematin infusion. As can be seen in Table 1, there was once again a marked prolongation of the clotting times, a modest reduction in clotting factor levels, a decrease in the platelet count, and a progressive increase in FDP. Plasma levels of plasminogen and antithrombin III were unaffected. Platelet aggregation studies showed impaired aggregation after aspirin alone, and a further decline in aggregation after the hematin infusion. However, the sample of PRP obtained 2 hr after aspirin ingestion could be aggregated by the *in vitro* addition of hematin in a final concentration of 0.1 mg/ml (see *In Vitro Studies*).

In the final experiment, heparin (5000 U i.v.) was administered 10 min before the hematin infusion (Fig. 3). While the heparin prolonged the prothrombin time and slightly reduced the apparent factor V activity (measured by a one-stage assay), it did not affect the factor VIII measurement (a two-stage assay), the Reptilase time, levels of fibrinogen, the platelet count, or the FDP titer. However, 10 min after the infusion of hematin, the platelet count plummeted to 30,000/ μl ,

Table 1. Platelet and Clotting Factor Studies After Aspirin (650 mg) and Hematin (4 mg/kg)

Study	Normal Range	Aspirin Baseline	Hematin		
			10 min	5 hr	24 hr
Platelet count	150,000–350,000/ μ l	307,000	107,000	284,000	327,000
Partial thromboplastin time	35–45 sec	37	95	75	41
Prothrombin time	10–12 sec	12	21	17	11
Thrombin time	10–15 sec	12	21	16	11
Factor VIII	50%–150%	120	100	125	125
Fibrinogen	200–400 mg/dl	430	320	370	400
FDP	< 10	< 10	< 10	10–20	20–40
Plasminogen	1.4–3.4 CTA U/ml	1.7	1.5	1.7	1.7
Antithrombin III	> 70%	78	78	81	78

the thrombin-clottable fibrinogen fell to 120 mg/dl, the titer of FDP rose to 1:80–160, the Reptilase time was prolonged, and the factor VIII reduced, and there was a further deterioration in the prothrombin time and factor V activity. Once again, coagulation abnormalities were much improved 5 hr after infusion, although the PTT, which usually returns to normal 6 hr after a single injection of heparin, was still >180 sec at 24 hr. All studies were normal at 48 hr. The changes in the hemostatic parameters closely correlated with the serum levels of hematin (Figs. 1 and 3).

Discrepant results were obtained when these plasma samples were assayed for fibrinogen by the heat precipitation method rather than the routine thrombin-clotting technique. No reduction in precipitable fibrin-

ogen was observed, even in the 10-min specimens. In addition, protamine sulfate precipitation tests of the samples with the highest FDP titers yielded negative results. Taken together, these findings suggested that hematin altered fibrinogen so that it was no longer thrombin-clottable.

In Vitro Studies

The addition of hematin (0.1 mg/ml) to PRP induced platelet aggregation (Fig. 4). This aggregation could not be inhibited by preincubating hematin with either adenosine (1 mM) or apyrase (1 mg/ml), but the presence of either of these agents in PRP in concentrations of 70 μ M and 0.07 mg/ml, respectively, inhibited hematin-induced aggregation as did ATP, 2.5 mM. When aspirin was added to PRP (0.12 mg/ml), or when PRP was prepared from the blood of a donor who had ingested 325 mg of aspirin 2 hr earlier, hematin-induced aggregation was impaired. Similarly, hematin induced the release of 14 C-serotonin from platelets in a dose-dependent fashion; aspirin

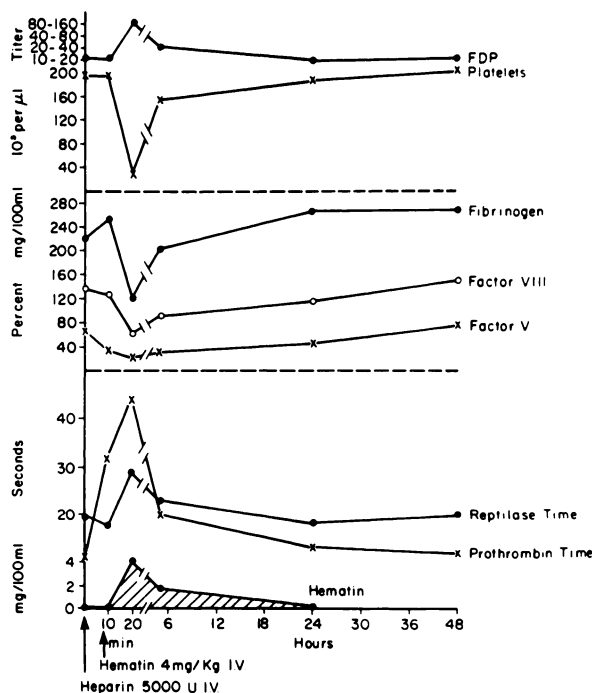


Fig. 3. Serial coagulation analyses before and 10 min after intravenous infusion of 5000 U heparin and then serially after infusion of hematin, 4 mg/kg. FDP, fibrin(ogen) degradation products.

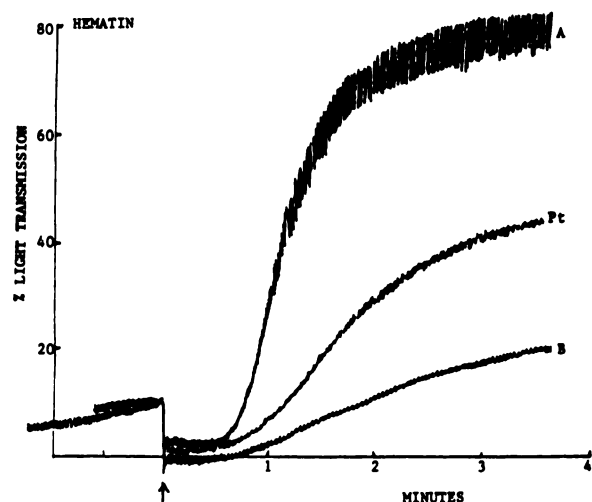


Fig. 4. Hematin-induced platelet aggregation. Hematin (final concentration, 0.1 mg/ml) was added to either normal PRP (subjects A and B) or the patient's PRP (Pt) and the changes in light transmission recorded.

Table 2. Percent ^{14}C -Serotonin Release Induced by Collagen or Hematin: Inhibition by Aspirin

	Collagen	Hematin (mg/ml)			
		0.05	0.10	0.16	0.48
Buffer	51,* 60†	6,-	12,10	77,75	-.82
Aspirin (0.12 mg/ml)	6.6	0,-	7,6	18,14	-.32

*Values for donor A.

†Values for donor B.

inhibited the release (Table 2). Hematin (0.1 mg/ml) also induced the release of ATP (0.7 and 1.3 nmole/ 10^8 platelets; 2 donor plasmas). Washed or gel-filtered platelets were very sensitive to the aggregating effects of hematin (in concentrations of 1 $\mu\text{g}/\text{ml}$), but formalin-fixed platelets were not aggregated by any concentration. Aspirin (0.12 mg/ml) inhibited the aggregation of washed platelets by hematin (2 $\mu\text{g}/\text{ml}$), but this effect could be overcome if the hematin concentration was increased to 5 $\mu\text{g}/\text{ml}$ (Fig. 5). Hematin, in concentrations as low as 0.01 mg/ml, prolonged the thrombin time whether this was done with normal plasma as substrate (Fig. 6), or with purified fibrinogen. Preincubation of thrombin 25 U, with hematin, 0.07 mg/ml, resulted in a time and temperature-dependent lengthening of the thrombin time produced by adding the mixture to substrate plasma (Fig. 7). Since the final concentration of hematin in the substrate plasma was only 0.005 mg/ml, the effects of the hematin are on thrombin itself, rather than on the clotting assay. Similarly, incubation of hematin (0.1 mg/ml) with Reptilase (17 mg/ml) resulted in a

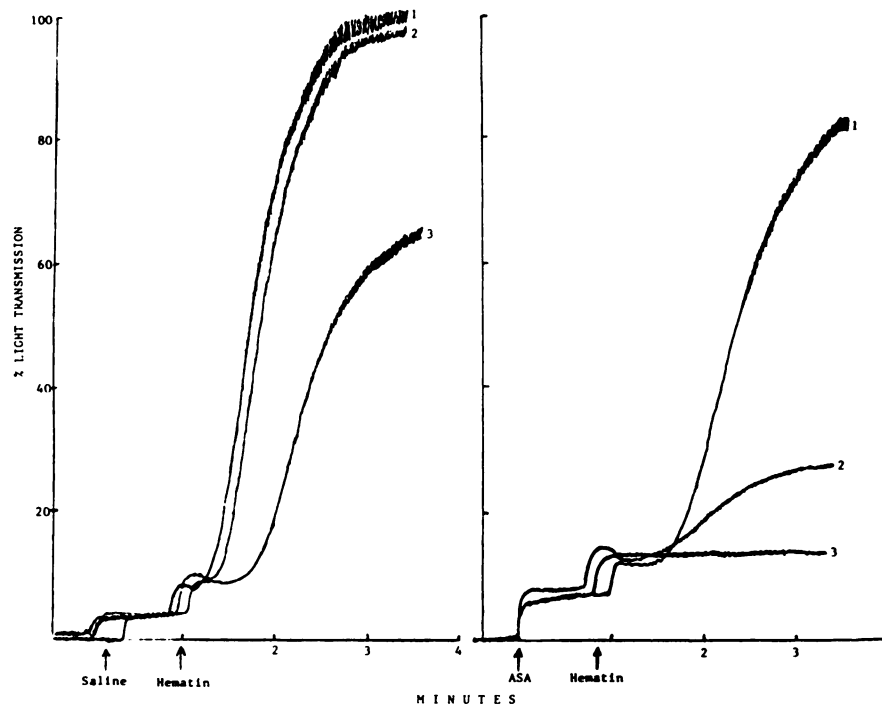


Fig. 5. Effect of aspirin on hematin-induced aggregation of washed platelets. Saline or aspirin (0.12 mg/ml) was added to washed platelet suspensions, and aggregation was induced with various concentrations of hematin: (1) 5 $\mu\text{g}/\text{ml}$; (2) 2 $\mu\text{g}/\text{ml}$; (3) 1 $\mu\text{g}/\text{ml}$.

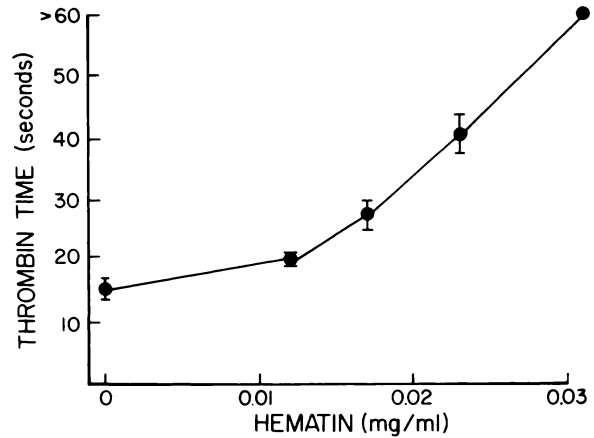


Fig. 6. Prolongation of the thrombin clotting time in the presence of various concentrations of hematin. The mean \pm SD of determinations with 5 normal plasmas is shown.

marked prolongation of the Reptilase time (from 25 sec to 50 sec after 15 min at 37°C).

No inhibition of fibrin polymerization was found when fibrin monomer, solubilized in 5 *M* urea and adjusted to pH 5.3 with monochloroacetic acid, was incubated with hematin, 0.02–0.09 mg/ml, and the mixture polymerized by the addition of buffered saline.

DISCUSSION

Acute intermittent porphyria is an autosomal dominant disorder with a gene frequency of 1:10,000 to 1:50,000.¹⁵ The biochemical lesion consists of a partial

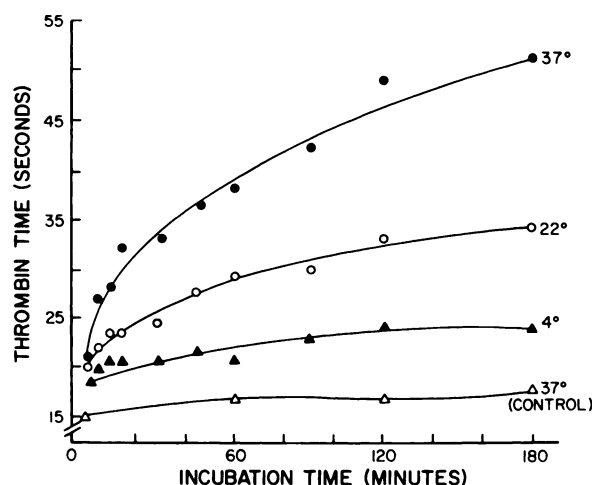


Fig. 7. Hematin was diluted in Tris buffer, pH 7.4, and incubated with an equal volume of thrombin so that the final concentration of hematin was 0.07 mg/ml and of thrombin, 25 U/ml. The control was thrombin incubated with Tris buffer alone. The results of repeated thrombin times performed by adding these mixtures, after incubation at the indicated temperatures, to substrate plasma are shown (mean values using 2 different substrate plasmas).

deficiency of uroporphyrinogen I synthetase, an enzyme responsible for tetrapyrrole formation in the heme biosynthetic pathway. Depletion of a proposed "regulatory heme pool" by stimuli that induce heme-containing enzymes results in derepression of Δ -aminolevulinic acid-synthetase (Δ -ALA-S) and massive production of porphyrin precursors proximal to the defective enzyme.¹⁶ The biochemical abnormalities (increased serum and urine ALA and porphobilinogen) and the clinical manifestations of an acute porphyric attack are often dramatically improved by the administration of hematin.^{2,17-20} It is undetermined whether this beneficial effect is due to repression of Δ -ALA-S and reduction of possibly toxic porphyrin products or to repletion of a systemic heme deficiency.^{17,21,22}

Adverse effects due to hematin have been uncommon: chemical phlebitis¹⁹ and one case of transitory renal insufficiency²³ have been reported. Recently, however, hematin has been associated with hemostatic defects characterized by elevated PT and PTT and minimally abnormal FDP levels.⁴ Pierach³ has reported that the anticoagulant effect of hematin is not associated with thrombocytopenia, evidence of disseminated intravascular coagulation (DIC), or clinically evident bleeding. Our data indicate that hematin is a potent anticoagulant with unique effects on the hemostatic system.

When hematin, either alone or preceded by aspirin, was administered intravenously in the usual therapeutic dose of 4 mg/kg, the platelet count fell and the PT, PTT, Reptilase, and thrombin times were pro-

longed (Fig. 1, Table 1). The degree and rate of resolution of these abnormalities closely paralleled the serum hematin levels: they were most marked 10 min after infusion, substantially resolved at 5 hr, and back to baseline at 48 hr. The anticoagulant effects were amplified by premedication with heparin and included a dramatic thrombocytopenia and extreme prolongation of all clotting times. Resolution was less rapid than after hematin alone, but still complete by 48 hr.

The potent anticoagulant effect of hematin and heparin together is emphasized by the hemorrhage our patient sustained while receiving this combination of drugs. Spontaneous bleeding has not previously been reported in association with hematin therapy. This bleeding diathesis is explained by the dual lesion hematin inflicts on the hemostatic system: direct platelet aggregation and interference with the clotting cascade.

Hematin aggregated platelets in normal platelet-rich plasma (PRP) at concentrations approaching therapeutic serum levels (Fig. 4). The eradication of this effect by preincubation of PRP with adenosine, apyrase, or ATP indicates that it is mediated by activation of the platelet ADP release mechanism. The release of ¹⁴C-serotonin and ATP by hematin and the absence of agglutination of formalin-fixed platelets are consistent with this interpretation. Aspirin, which decreases production of the prostaglandin endoperoxides responsible for degranulation and ADP release, inhibited platelet aggregation by low concentrations of hematin (1–2 μ g/ml), but not by higher concentrations comparable to the patient's serum levels (Fig. 5). Thus, aspirin pretreatment failed to prevent the thrombocytopenia induced by hematin.

The failure of the patient's post-hematin PRP to demonstrate a secondary wave of aggregation (Fig. 2) is consistent with the thesis that hematin causes platelet aggregation by release of storage pool ADP. In vivo formation of platelet aggregates is the most likely explanation for hematin-induced thrombocytopenia. Rapid restoration of the platelet count may be due to dissociation of these aggregates as plasma hematin levels fall. The dramatic thrombocytopenia observed when hematin and heparin were administered together is consistent with the known potentiating effects of heparin on ADP-induced platelet aggregation.^{24,25}

The synergistic thrombocytopenic effect of hematin and heparin was accompanied by a marked prolongation of all clotting times (Fig. 3). This potent anticoagulant effect is explained by the fact that both agents inhibit thrombin. While heparin inhibits fibrinogen proteolysis by potentiating antithrombin III activity, hematin exerts its anticoagulant effect by inactivating thrombin. At concentrations as low as 0.01 mg/ml,

hematin significantly prolonged the thrombin time of normal plasma (Fig. 6) or purified fibrinogen (lacking in antithrombin III). Furthermore, hematin appeared to inhibit other components of the clotting factor cascade. Factors V and VIII consistently declined after hematin administration. This effect may have been due to the direct complexing of hematin with the respective clotting factors, since the effects were apparent even when the samples were highly diluted. By contrast, heparin inhibition of clotting factor assays is reduced by dilution. This difference between the actions of hematin and heparin is best illustrated in Fig. 3, which shows that the two-stage factor VIII assay (an assay using highly diluted samples) gave normal values for factor VIII 10 min after heparin injection, but a decreased factor VIII 10 min after hematin infusion.

While on first inspection hematin produces a pattern of abnormalities suggestive of disseminated intravascular coagulation (DIC), its mechanisms of action, as described above, are entirely distinct. The hallmark of DIC is a pathologic activation of coagulation that results in fibrinolysis, depletion of coagulation factors, and secondary thrombocytopenia. Hematin, on the other hand, affects platelets directly, and its interference with the clotting cascade is mediated by the drug itself.

In each of the hematin experiments, antithrombin II and plasminogen levels remained normal, findings inconsistent with the increased thrombin and plasmin activity expected in DIC. Furthermore, the discrepancy between fibrinogen measured by thrombin-clotting (low) and fibrinogen measured by heat precipitation (normal) indicates that fibrinogen had not been physically consumed, but had become nonclottable by thrombin. This would also account for the positive Fi-test for FDP, and explain why the protamine sulfate precipitation test, which detects soluble complexes of

fibrin monomer, was negative. The absence of fragmented erythrocytes and the worsening rather than improvement of hemostatic parameters with heparin pretreatment support the conclusion that DIC is not the operative mechanism of hematin coagulopathy. The way in which hematin alters fibrinogen has not been established; in preliminary studies we could not detect inhibition of fibrin monomer polymerization.

Thus, thrombocytopenia and interference with the clotting cascade are characteristic of the unique and potent anticoagulant activity of hematin. These effects have significant implications for the treatment of the acute hepatic porphyrias. At the very least, frequent monitoring of platelet count and hemostatic indices should be routine whenever hematin is used. Variations in response to hematin's anticoagulant effect may render some patients perilously susceptible to hemorrhagic complications. Therefore, the dosing regimen should be tailored to each patient's hemostatic response. Bleeding diatheses and surgery should be relative contraindications to the use of hematin and, of course, concomitant heparin use must be strictly avoided.

Aspirin delayed the increase in FDP following the *in vivo* infusion of hematin, blunted the release of ¹⁴C-serotonin from hematin-stimulated platelets, and inhibited platelet aggregation by low concentrations of hematin. These results suggest that other antiplatelet agents or higher doses of aspirin may diminish the anticoagulant effect of hematin. Further studies to address this issue, and caution in the use of hematin, are warranted by the findings of the present report.

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