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Cold-Precipitation by Heparin of a Protein in Rabbit and Human Plasma. (21241)

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The earliest pathological event in the generalized Shwartzman reaction, which is produced in rabbits by two intravenous injections of gram negative bacterial endotoxin, is the appearance of intravascular deposits of homogeneous, eosinophilic material with the staining properties of fibrinoid, within the lumen of glomerular capillaries and small vessels of the spleen, liver, and lungs, and in the walls of the coronary arteries(1,2). The morphological situation of the material indicates that it is derived from the circulating blood, and the observation that its deposition in the glomerular capillaries is prevented by heparin(3) suggests that the coagulation mechanism may be involved in its development. In the course of an investigation into possible precursors of fibrinoid, heparinized plasma was obtained from rabbits at various time intervals after an intravenous injection of endotoxin. It was noted that chilling of such plasma specimens at 4°C was followed within less than an hour by the precipitation of gelatinous, flocculant material resembling an incomplete plasma clot. Warming the plasma to 37°C resulted in rapid solution of the material, and chilling again caused precipitation to occur. No cold-precipitation occurred in citrated plasma or in serum. Heparin-precipitable material was not demonstrable in the plasma of a majority of normal rabbits. However, it was encountered in the plasma of normal human beings and, in greater amounts, in the plasma of patients with acute rheumatic fever.

The present paper is a preliminary account of the conditions under which the heparin-precipitable material is demonstrable, with certain observations suggesting that it may be derived from fibrinogen.

Materials and methods. Young hybrid albino rabbits, weighing 1 to 1.5 kilos, were used in all experiments. The animals were maintained on Purina rabbit pellets and water. The following endotoxins were employed: meningococcal "agar washings" toxin prepared as previously described(1) from a strain of meningococcus (44B) supplied by Dr. Gregory Shwartzman, Mount Sinai Hospital, New York, a polysaccharide endotoxin from *S. marcescens,* supplied by Dr. Murray Shear, National Cancer Institute, Washington, and a purified *Sh. paradysenteriae* endotoxin supplied by Dr. Walter Goebel, Rockefeller Insti-

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TABLE I. Cold-Precipitation by Heparin of a Protein in Rabbit Plasma.

Endotoxin*	Time of bleeding	No. of rabbits	Cold-precipitation† —Heparin conc.— 1 mg/cc	.1 mg/cc
	Before endotoxin	20	1	3
Meningococcal	15 min. after endotoxin	10	0	1
	30 " " "	10	1	1
	1 hr " "	20	16	20
	2 " " "	20	20	20
	4 " " "	20	19	20
	6 " " "	10	8	10
	24 " " "	20	5	8
S. marcescens	2 " " "	6	6	6
Sh. paradysenteriae	2 " " "	6	6	6

* The following doses of endotoxin were employed: Meningococcal—2 cc of a 1-200 dilution; *S. marcescens*—0.2 mg; *Sh. paradysenteriae*—0.1 mg.

† Figures refer to No. of animals whose heparinized plasma showed a flocculant precipitate after 2 hr at 4°C.

tute, New York. Dilutions of each endotoxin were made in pyrogen-free saline. All injections were made intravenously, in doses described below. The rabbits were bled by cardiac puncture, using various concentrations of heparin or sodium citrate as anticoagulant. Sterile, pyrogen-free pyrex tubes were used for all manipulations of blood or plasma. Plasma was obtained from whole blood by centrifugation at 2800 rpm (1400 x g) for 15 minutes at room temperature, in a horizontal centrifuge. Heparin solutions containing 10 mg per cc were obtained from 3 commercial sources: Heparin Sodium (Upjohn), Liquaemin (Parke Davis) and Heparin Sodium (Vitarine Company). The preparations showed no differences in the property of causing cold-precipitation in plasma.

Heparin-Precipitable Protein in Rabbit Plasma. Blood was obtained from 20 normal rabbits, and from these and other groups of animals at various times after an intravenous injection of 2 cc of a 1-200 dilution of meningococcal endotoxin. At each bleeding, two 5 cc samples of blood were placed in tubes containing, respectively, 5.0 and 0.5 mg of heparin dissolved in 0.5 cc physiological saline. The blood was centrifuged immediately, at room temperature, and each plasma specimen was divided into 2 equal portions. One part was kept at 37°C, and the other placed in a 4°C refrigerator. The results are summarized in Table I. In all of the plasma samples obtained 2 or 3 hours after the injection of endotoxin, cold-precipitation occurred within less than an hour after chilling the tubes. No precipitate appeared in plasma kept at 37°C. In most instances, the plasma became turbid within 15-20 minutes after chilling, and during the next 15 minutes numerous small, glistening floccules appeared throughout the sample. These floccules then coalesced to form an opaque, gelatinous mass which settled to the bottom of the tube. The appearance of precipitates one hour after chilling is illustrated in Fig. 1. When tubes containing freshly precipitated material were warmed to 37°C, the precipitate went back into solution within a few minutes, and when chilled again, precipitation again occurred. In plasma chilled for 24 hours or longer, the precipitates were more solid in appearance and contained strands of fibrous material which usually failed to redissolve completely when warmed.

It will be noted in Table I that the heparin-precipitable material was not demonstrable until one hour after the injection of endotoxin; plasma samples obtained at 15 and 30 minutes were negative. The quantity of precipitate was much diminished in plasma obtained at 6 hours or later, and the majority were negative 24 hours after endotoxin. Precipitation was observed in a few plasmas from normal rabbits, as shown in Table I. In contrast to the rapidly appearing voluminous flocculation which occurred in plasma after an injection of endotoxin, the precipitates in normal plasma were usually small and did not appear

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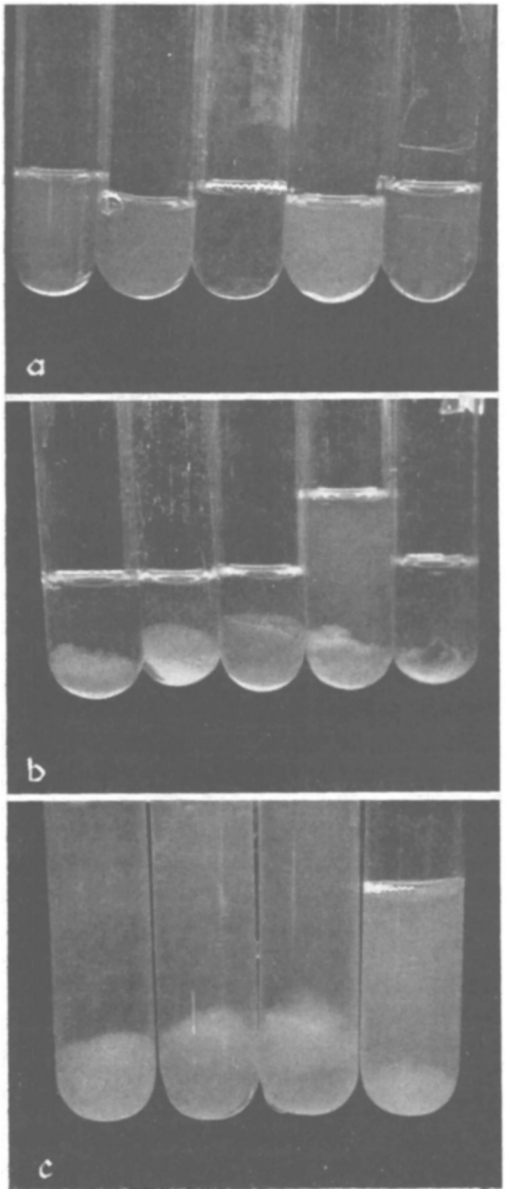

FIG. 1. Cold-precipitation by heparin in rabbit plasma. a. Plasma from 5 normal rabbits, containing 1 mg heparin/cc, photographed after chilling at 4°C for 2 hr. No visible precipitate. b. Heparinized plasma from the same rabbits shown above, obtained 2 hr after intrav. inj. of 2 cc 1-200 meningococcal endotoxin, photographed after chilling for 2 hr. Note flocculant precipitate in each tube. c. Heparinized plasma from 4 rabbits, obtained 2 hr after an intrav. inj. of 100 μg of *S. marcescens* polysaccharide endotoxin, photographed after chilling for 2 hr.

until 4 hours or more after chilling. The effect of the concentration of heparin on the degree of cold precipitation is indicated in Table I. It will be noted that the highest incidence of precipitation occurred in plasma containing 0.1 mg heparin per cc, while with 1 mg/cc precipitation failed to occur in several instances. Concentrations lower than 0.1 mg could not be tested for cold precipitation because of the frequent occurrence of clotting in plasma from animals injected with endotoxin.

Although cold-precipitation was not demonstrable in chilled citrated plasma obtained after endotoxin, it occurred promptly after the addition of heparin, in concentrations ranging from 1.0 to 0.01 mg/cc. Precipitation did not occur in citrated plasma from normal rabbits when the concentration of citrate was sufficiently high to maintain complete incoagulability. Thus, the addition of heparin to normal plasma containing 0.4% sodium citrate did not produce cold-precipitation. However, in normal plasma containing 0.2% citrate, which usually formed partial clots after several hours, cold-precipitation by heparin was frequently encountered. The phenomenon of cold-precipitation could not be brought about *in vitro* by the addition of endotoxin to heparinized or citrated plasma.

Relation of heparin-precipitable material to fibrinogen. The precipitated material was washed by repeated centrifugation at 4°C, and preliminary biochemical studies of its nature were made. The quantity of precipitate formed in plasma during 4 hours at 4°C, estimated by dry weight, ranged between 0.75 and 1.50 mg/cc; when estimated as protein by the biuret reaction the range was between 0.6 and 1.0 mg/cc. The following observations indicate that the material may be a protein related to or closely associated with fibrinogen. As mentioned above, it is present only in plasma and cannot be demonstrated in serum. Washed samples of the material, dissolved in warm saline, when subjected to paper electrophoresis were found to migrate in a manner similar to fibrinogen. Furthermore, the addition of thrombin to solutions of the material resulted in partial clotting. The occurrence of cold precipitation by heparin in normal plasma containing inadequate amounts of citrate is consistent with the view that the

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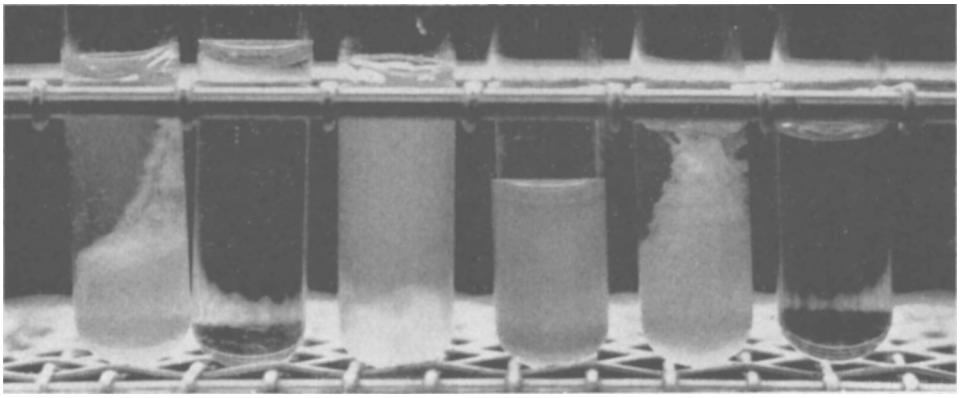

FIG. 2. Disappearance of heparin-precipitable protein after intrav. inj. of Liquoid. Three pairs of heparinized rabbit plasma are shown; 1a, 2a and 3a are plasmas obtained 3 hr after intrav. inj. of meningococcal endotoxin. Each rabbit then received 8 mg Liquoid intrav., and 1b, 2b and 3b are the heparinized plasmas obtained 10 min. after Liquoid. All photographed after chilling at 4°C for 18 hr.

precipitable material may represent a stage in the transition of fibrinogen to fibrin.

It is known that cold-insoluble globulins exist in crude fibrinogen preparations, sometimes referred to as "contractinogen"(4) or as the "non-clottable fraction"(5). Electrophoretically this component is closely associated with fibrinogen but is clottable by thrombin to a lesser extent or not at all. Although in the present study no cold-precipitation in plasma occurred in the absence of heparin, a cold-insoluble fraction was found in a commercial preparation of bovine fibrinogen (Armour). The degree of cold-precipitation from this sample was enhanced by the addition of heparin. A heparin precipitable fraction was noted in a sample of commercial human fibrinogen (Cutter). On the other hand, heparin did not cause precipitation of bovine fibrinogen from which the cold insoluble fraction had been removed by the procedure of Laki(6).

Disappearance of heparin-precipitable material after injecting "Liquoid". Liquoid (Hoffman-LaRoche) is a synthetic polymer, sodium polyanethol sulfonate, which possesses anticoagulant properties similar to those of heparin and, in addition, has the property of causing precipitation of fibrinogen from plasma *in vitro*(7,8). Repeated injections of large quantities of Liquoid, in rabbits, were shown by Tausman and Dreyfuss(9) to cause occlusion of glomerular capillaries by homogeneous eosinophilic material. In this laboratory, the combined injection of Liquoid and small quantities of endotoxin has been found to produce the typical lesions of the generalized Shwartzman reaction(8). The addition of Liquoid to rabbit plasma containing the heparin-precipitable material resulted in prompt precipitation of this protein as well as fibrinogen. It was therefore of interest to determine whether the precipitable material was removed *in vivo* following an intravenous injection of Liquoid. Eight rabbits were injected with 2 cc of a 1-200 dilution of meningococcal endotoxin, and 2 hours later blood was obtained for heparinized plasma. They then received 8 mg Liquoid contained in 2 cc saline by vein, and were bled again 10 minutes later. In each instance, the chilled plasma taken before the injection of Liquoid contained large amounts of precipitate, while none was demonstrable in the plasma 10 minutes after Liquoid. Photographs illustrating the observation are shown in Fig. 2.

Heparin precipitable protein in human plasma. Heparinized plasma, containing 0.1 mg heparin per cc, was obtained from a group of 12 normal adult human beings, 6 human infants of 1 year or less, 6 children with acute rheumatic fever, and 1 child with acute rheumatoid arthritis. Aliquots of each were placed at 4°C and observed for precipitation. In

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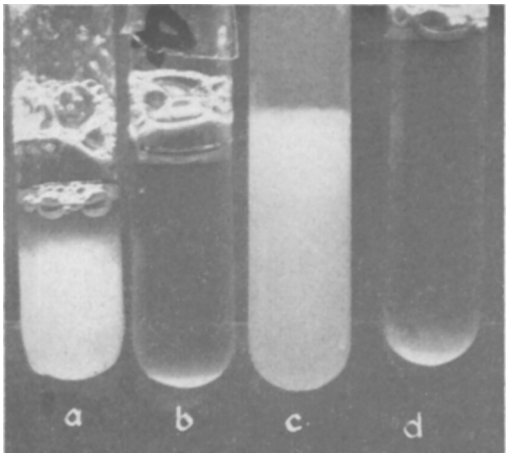

FIG. 3. Cold-precipitation by heparin in plasma of a patient with acute rheumatic fever. a. Chilled heparinized plasma (0.1 mg heparin/cc) obtained during first week of illness, prior to therapy. b. Plasma obtained 3 days after instituting salicylate treatment, at a time when patient was afebrile. c. Plasma 3 wk later, during exacerbation of the disease, while still receiving salicylate. Cortisone therapy was started after this specimen was obtained. d. Plasma after 3 days of cortisone treatment; patient afebrile at this time.

every instance, a flocculant, opaque precipitate formed within less than 4 hours after chilling, which was completely redissolved within a few minutes after rewarming the tubes. The amount of cold-precipitation appeared to be greater in the plasma of adults than in that of the normal infants. The heaviest precipitates occurred in the plasma of the patients with acute rheumatic disease, and precipitation became visible within a shorter time after chilling the plasma. The washed heparin-precipitable protein was examined by paper electrophoresis, and the migration of the material resembled that observed with a partially purified solution of human fibrinogen. In one patient with rheumatic fever, specimens of heparinized plasma were obtained at 4 different periods during his illness: at the time of admission to the hospital, several days after the administration of full therapeutic doses of salicylate, at the time of an exacerbation of the disease during salicylate therapy, and two days after subsequent treatment with cortisone. The cold-precipitation in these samples of plasma is shown in Fig. 3. It will be seen that the abundant precipitation present on admission decreased after salicylates, reappeared during the exacerbation, and diminished again after cortisone. Further studies along similar lines are currently in progress and will be described in a later communication.

Discussion. A gelatinous, opaque protein precipitate occurs in chilled heparinized plasma obtained from rabbits between 1 and 6 hours after an intravenous injection of endotoxin derived from gram negative bacteria. The material is promptly redissolved on warming the plasma to 37°. It is not demonstrable in chilled citrated plasma until heparin is added.

The existence of the heparin-precipitable protein in plasma, but not in serum, suggests a possible relationship to fibrinogen. This receives further support from the observation that the washed, redissolved material migrates on paper electrophoresis in a manner similar to fibrinogen. Moreover, the washed protein is partially clottable by thrombin.

Other investigators have suggested that varying degrees of polymerization of fibrinogen may occur under certain conditions, resembling interrupted stages in the conversion of fibrinogen to fibrin(10). It is conceivable that the material under study may represent such an alteration in fibrinogen, with the formation of molecular aggregates capable of combining in an unstable complex with heparin.

The heparin-precipitable protein is to be differentiated from the cold-insoluble "nonclottable" fraction known to be present in crude fibrinogen preparations, since no precipitation occurs in chilled plasma in the absence of heparin. However, it should be noted that cold-precipitation of this fraction in bovine and human fibrinogen has been found in this laboratory to be augmented by heparin.

The mechanism by which heparin causes cold-precipitation is not clear. Walton(11) has shown that dextran sulfate preparations of large molecular size, with anticoagulant properties similar to those of heparin, cause reversible precipitation of fibrinogen at neutral pH by the formation of a complex, perhaps by coacervation. This investigator also found that heparin prevented precipitation by dextran sulfate, indicating a competitive affinity

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of the two acidic polymers for fibrinogen. It is possible that a similar affinity may be responsible for cold-precipitation by heparin under conditions of increased molecular aggregation or polymerization of fibrinogen.

The possibility that the heparin-precipitable protein may be a precursor for the fibrinoid-like material which occludes the glomerular capillaries in the generalized Shwartzman reaction is under investigation. It is of interest that Liquoid, which precipitates the protein from plasma *in vitro,* produces the lesions of the generalized Shwartzman reaction when injected by vein in combination with endotoxin (9). Similar results, to be described elsewhere, have been obtained with combination of intravenous endotoxin and dextran sulfate of large molecular size. The observation that the heparin-precipitable protein disappears from the blood within 10 minutes after an injection of Liquoid is consistent with the view that it may be precipitated by Liquoid *in vivo.*

It is not known whether the heparin-precipitable protein in rabbit plasma is the same component as that which exists in normal human plasma. Preliminary paper electrophoretic studies of the latter material suggests a similar close relationship to fibrinogen. The marked increase in precipitable material observed in the plasma of children with acute rheumatic disease, and the diminution which was observed in one patient during salicylate and cortisone therapy suggest that a qualitative alteration in fibrinogen may have occurred in association with the disease. Further investigations of the protein in human plasma are in progress.

Summary. 1. Cold-precipitation of a mass of gelatinous protein material is produced by heparin in plasma obtained from rabbits between one and 6 hours after an injection of endotoxin derived from gram negative bacteria. The precipitate is redissolved on warming the plasma. It is demonstrable within 15-30 minutes after chilling heparinized plasma, or citrated plasma to which heparin has been added. 2. The heparin-precipitable protein is not demonstrable in serum. Its possible relationship to fibrinogen is indicated by similar migration in paper electrophoresis, and by the fact that it is partially clottable by thrombin. 3. The material disappears from the blood within 10 minutes after an intravenous injection of Liquoid (Na polyanethol sulfonate), an acidic polymer known to be capable of precipitating fibrinogen. 4. A heparin-precipitable protein of similar appearance is present in normal human plasma, and is much increased in the plasma of patients with acute rheumatic disease. In one patient with rheumatic fever, the amount of precipitate diminished during salicylate and cortisone therapy.

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