Precipitation of Soluble Fibrin Monomer Complexes SFMC by Cellular Basic Proteins, and the Antagonistic Effect of Sulfonated Mucopolysaccharides (35119)

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(Introduced by T. H. SPAET)

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Enzymatic conversion of fibrinogen into fibrin by thrombin is not the only mechanism leading to the formation of fibers showing the ultrastructure characteristic for fibrin. Recently it was demonstrated that fibrinogen, precipitated by dialysis against a neutral buffer of low ionic strength (1) or by addition of protamine sulfate (PS) (2), forms fibers of the same axial periodicity as fibrin. Soluble complexes of fibrin monomer with fibrinogen degradation products FDP, which are unclottable by thrombin, may be transformed into clot-like gels by addition of protamine (3). These "clots" also were shown by electron microscopy to have a highly ordered structure and axial periodicity similar to fibrin (4).

Beside protamines, which are known to occur almost exclusively in fish sperm, some constituents of mammalian cells are able to precipitate soluble fibrin monomer complexes (SFMC). Extracts of platelets, and lysosomes of granulocytes are similarly effective (5, 6). Antiheparin factor 4 of pig platelets was shown to be a potent agent precipitating SFMC (7). This factor is a thermostable protein not yet fully characterized (7, 8). In granulocyte lysosomes, low molecular weight proteins of strongly alkaline character are thought to be responsible for SFMC coagulation and antiheparin activity (6).

In the present investigation we have examined certain rather well-defined, strongly basic cellular proteins, *e.g.*, histones, lysozyme, and cytochrome C with respect to their ability to clot SFMC and to neutralize heparin. Histones were found to be highly active. This study reveals that heparin, chondroitin sulfate (ChS), and crude extracts from aorta wall counteract the precipitation of SFMC by protamine and histones.

Materials and Methods. Fibrinogen degradation products (FDP) obtained by digestion of fibrinogen by plasmin and ^{131}I-labeled bovine fibrinogen (up to one atom of iodine per molecule of fibrinogen; 92–96% clottable) were prepared as described previously (9, 10). Complexes of ^{131}I-fibrin monomer with FDP (^{131}I-SFMC) were obtained as follows: 5 ml of human citrated plasma were mixed with 5 ml of 0.5% ^{131}I-fibrinogen solution, 40 ml of 0.5% FDP, and 5 ml of a 20 NIH units/ml thrombin solution. After 30 min of incubation at 37°, 2 ml of thrombin (200 NIH units/ml) were added, and incubation was continued for 2 hr. Under these conditions thrombin was progressively inactivated by the plasma antithrombins. Small clots formed during this time were removed by centrifugation. Approximately 90% (20,000–30,000 cpm/ml) of the initial radioactivity was recovered in the supernatants, and this radioactivity could not be precipitated by further addition of thrombin.

Histones from calf thymus: Nucleohistones were obtained from thymocytes according to Hnilica (11), and histones from this preparation were extracted by the method of Phillips and Johns (12).

Histones from calf liver were prepared from the nuclear fraction of liver cells, using the procedure of Steele and Busch (13).

Aorta extracts: 2 g of intima and media of fresh bovine aorta caerfully washed with saline were cut into 2 × 2-mm slices and homogenized with 4 ml of 0.15 *N* NaOH using a Potter-type glass homogenizer. The supernatant was obtained by centrifugation

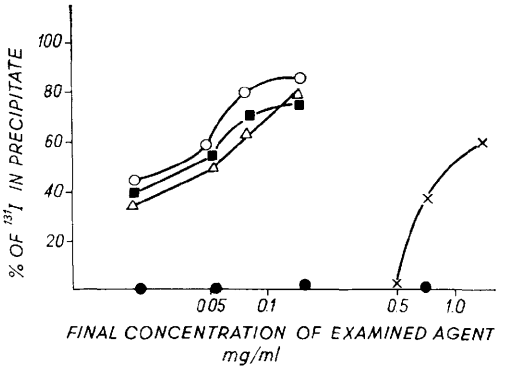

FIG. 1. Precipitation of ^{131}I-SFMC by protamine, histones, lysozyme, and cytochrome C: Protamine (○); thymus histones (■); liver histones (△); lysozyme (×); cytochrome C (●).

for 15 min at 4000 rpm, and was adjusted to pH 7.0 with 0.15 *N* HCl, then diluted with saline to give a final concentration of uronic acids of 200 μg/ml. Cytochrome C from horse heart (Fluka AG), crystalline lysozyme from egg white (British Drug Houses, Ltd), chrondroitin sulfate (Calbiochem), thrombin (Lubelska Wytwórnia Surowic i Szczepionek), heparin (Polfa) were standard commercial preparations.

Precipitation of ^{131}I-SFMC was accomplished in a mixture containing 0.1 ml of precipitating agent, 0.2 ml of saline, and 1.0 ml of ^{131}I-SFMC. The samples were incubated for 2 hr at room temperature and centrifuged for 10 min at 4000 rpm. Radioactivity was measured in a well-type scintillation counter (ECKO), and percentage of initial radioactivity in the precipitate was calculated by standard methods. Three-tenths ml of heparin, ChS, or aorta extract, instead of saline were mixed with 0.1 ml of precipitating agent and preincubated for 3 min before addition of ^{131}I-SFMC, when the inhibitory effect of these substances on SFMC precipitation was to be examined. Neutralization of anticlotting activity of heparin was estimated according to Saba *et al.* (14). Protein content of histone preparations was determined by the method of Lowry *et al.* (15). Calf thymus histones were used for making a standard calibration curve. Uronic acids were quantitated according to Dische (16). All test reagents were dissolved just before use in isotonic saline and adjusted to pH 7.0 with 0.15 *N* HCl or 0.15 *N* NAOH. Saline was used for further dilutions.

Results. Precipitation of ^{131}I-SFMC. Figure 1 shows that PS and histones isolated from thymus and liver precipitate large amounts of ^{131}I-SFMC. Approximately 75% of radioactively labeled fibrin monomer is removed from the fluid phase when the concentration of histones attains 0.16 mg/ml. Protamine at the same concentration precipitates 84% of ^{131}I-SFMC. Lysozyme is much less effective, and a tenfold higher concentration induces the precipitation of only 60% of ^{131}I-SFMC. Cytochrome C is without any effect on ^{131}I-SFMC partition between the fluid and solid phase.

It is worth noting that formation of clot-like precipitates was observed when more than 15% of the initial radioactivity was removed from the solution by the precipitating agent.

Neutralization of heparin anticlotting activity. Figure 2 shows that all the agents tested neutralize the anticoagulant action of heparin. Protamine, both types of histones, and cytochrome C exert similar effects at the same order of concentration. Lysozyme is less active and must be used at a tenfold higher concentration to produce similar shortening of the clotting time of heparinized plasma.

Significantly, cytochrome C, which does

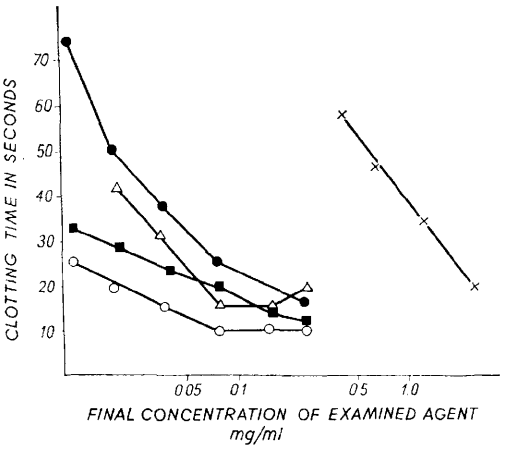

FIG. 2. Neutralization of anticlotting activity of heparin by protamine, histones, lysozyme, and cytochrome C: Protamine (○); thymus histones (■); liver histones (△); lysozyme (×); cytochrome C (●).

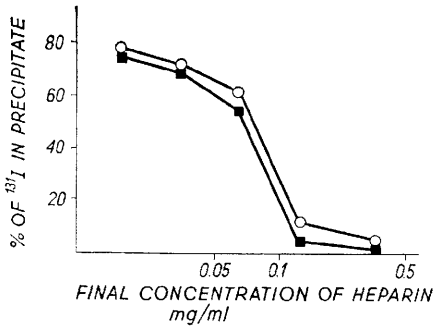

FIG. 3. Influence of heparin on ^{131}I-SFMC precipitation induced by protamine and thymus histones: (○) protamine final concentration, 0.05 mg/ml; (■) thymus histones final concentration, 0.35 mg/ml.

not precipitate ^{131}I-SFMC at all, neutralizes anticoagulant action of heparin nearly as efficiently as histones and PS.

Influence of heparin, chondroitin sulfate (ChS), and of aorta extract on the ability of protamine and histones to precipitate ^{131}I-SFMC.

It is well known that neutralization of heparin by protamine leads to the formation of an insoluble complex of these substances. Similar complexes of histones with heparin and other sulfonated polysaccharides, namely with sulfonated dextran, have been described (17, 18). Accordingly, the next step in our investigation was to check whether heparin, ChS, and the extract from the aorta wall counteract the precipitation of ^{131}I-SFMC induced by protamine and histones. The data presented in Figs. 3 and 4 show that heparin and ChS at appropriate concentrations abolish the ability of protamine and histones to precipitate ^{131}I-SFMC. A similar, although weaker, effect is exerted by aorta extract (Fig. 5).

Discussion. The presence of soluble complexes of fibrin monomer with fibrinogen or its degradation products in the circulating blood has been demonstrated in pathological conditions connected with intravascular activation of coagulation (19, 20). Binding of fibrin monomer into a soluble complex by fibrinogen or its degradation products may be considered as one of the protective mechanisms preventing fibrin deposition and maintaining blood fluidity (3). This mechanism may fail when factors able to precipitate SFMC are leased from the tissues into the blood stream. Observations indicating that experimental leukopenias (21) as well as thrombopenias (22) may prevent the occurrence of the Shwartzman phenomenon have drawn more attention to the role of cellular components in thrombotic phenomena and consumption coagulopathy.

The demonstration that factor 4 of platelets (7) and granulocyte lysosomal proteins (6) precipitate SFMC was in agreement with the assumption that deposition of fibrin due to this mechanism may be of importance for thrombotic phenomena in Swartzman reaction. It was found, however, that isolated granules of polymorphonuclear leukocytes do not restore the ability of leukopenic rabbits to respond with renal necrosis, to injections of endotoxin while intact leukocytes do (21). The significance of platelet activation, or even destruction, for disseminated intravascular clotting is not well documented. Indeed, Hjort was unable to induce consumption coagulopathy by infusion of significant amounts of disrupted platelets into the circulation of rabbits (23). Our results indicate that beside platelets and granulocytes, other tissues, such as lymphatic and liver cells, may be the source of substances precipitating SFMC.

We have examined the ability of histones, lysozyme and of cytochrome C to precipitate

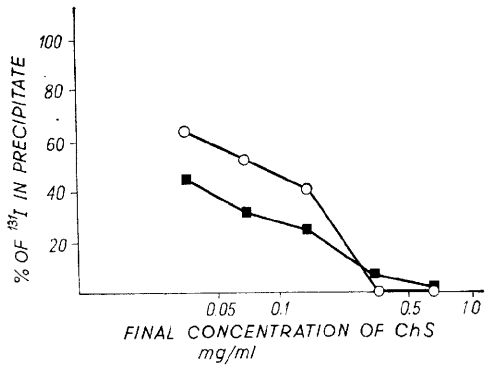

FIG. 4. Influence of chondroitin sulfate on ^{131}I-SFMC precipitation induced by protamine and thymus histones: (○) protamine final concentration, 0.05 mg/ml; (■) thymus histones final concentration, 0.35 mg/ml.

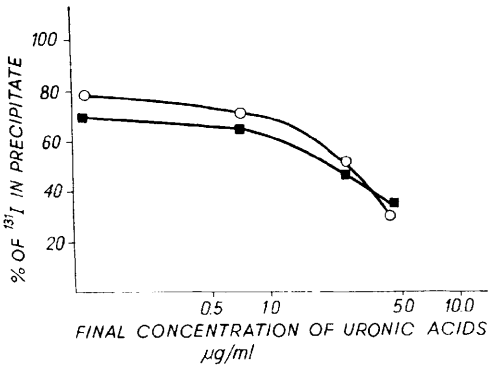

FIG. 5. Influence of aorta extract on ^{131}I-SFMC precipitation induced by protamine and thymus histones: (○) protamine final concentration, 0.05 mg/ml; (■) thymus histones final concentration, 0.35 mg/ml.

SFMC and to neutralize heparin. These three proteins are of similar low molecular weight and are strongly basic. They are variously localized in cells. Histones are found exclusively in the nuclei, lysozyme mainly in lysosomes, and cytochrome C in mitochondria. It was found that histones from thymus and liver precipitate SFMC very efficiently, lysozyme is less active and cytochrome C is without influence on SFMC solubility. All three show pronounced antiheparin activity which does not correlate with the ability to precipitate SFMC. Cytochrome C, devoid of the latter, was found to be only slightly weaker as an antiheparin agent than histones, but stronger than lysozyme.

It may be of interest that granulocyte lysosomes and platelets contain not only the proteins precipitating SFMC, but also acid mucopolysaccharides similar to chondroitin sulfate (24, 25). In the present investigation it is shown that heparin, chondroitin sulfate, and crude extracts from aorta wall inhibit the precipitation of SFMC induced by protamine sulfate and histones. This indicates that heparin treatment not only inhibits thrombin formation and its activity, but may also prevent the deposition of already formed fibrin monomer by maintaining it in solution as soluble complex with fibrinogen or FDP.

It is well known that acid mucopolysaccharides present in the vessel wall inhibit blood coagulation (26–29). The presented results demonstrate that chondroitin sulfate and aorta extract may also counteract the precipitation of SFMC. The role of substances originating from cell destruction precipitation SFMC or counteracting this phenomenon remains to be demonstrated in experiments *in vivo*.

Summary. Histones from calf thymus and liver were found to precipitate ^{131}I-SFMC and to neutralize anticlotting activity of heparin nearly as efficiently as protamine. Lysozyme showed both activities but had to be used in tenfold higher concentrations to induce similar effects. Cytochrome C neutralized heparin but did not precipitate SFMC. Heparin, chrondroitin sulfate, and bovine aorta extracts inhibited precipitation of SFMC induced by protamine and histones.

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