effective whether intracutaneous, subcutaneous or intramuscular. 3. For inducing hemorrhagic skin reactions, 0.03 mg of killed and dried *M. tuberculosis* was necessary in the adjuvant emulsion used for sensitization of male guinea pigs. For male guinea pigs, 0.02 mg of mycobacteria was not effective, but was sufficient for female animals. 4. In guinea pigs sensitized by intracutaneous route using 2.5 mg of egg albumin and 1 mg *M. tuberculosis*, the systemic reaction elicited by subcutaneous injection of 50 mg of egg albumin was not characteristic of protracted anaphylaxis. Body temperatures were normal or slightly above normal, indicating that anaphylactic shock hypothermia is "neutralized" by concurrent systemic "delayed" reactions.

The authors express thanks to Dr. E. M. Lerner for cooperation in preparation of microphotographs.

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Received June 12, 1959. P.S.E.B.M., 1959, v102.

A Fibrinogen Precipitating Factor (FPF) of Group A Streptococci. (25172)

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During experiments designed to test antigenicity of a preparation of Group A, type 1 streptococcal M protein, plasma samples from immunized rabbits were set up in capillary precipitin tubes against homologous M protein. A massive precipitate appeared in each tube 5 minutes after preparation. The magnitude of these reactions far exceeded reasonable expectation of the amount of type-specific antibody present. Furthermore, normal plasma reacted in the same manner, and sera from normal and immunized rabbits proved non-reactive. The nature of this reaction is herein examined.

Materials and methods. Bacterial strains and extracts. Streptococcal, pneumococcal and staphylococcal cultures were obtained from human sources in the course of other studies. All streptococcal cultures were typed by capillary precipitin technic of Swift, Wilson and Lancefield(1), then stored in the lyophilized state. Cultures were initiated in modified Todd-Hewitt broth† and plated for purity. Crude acid extracts were prepared by heating suspensions of bacterial cells in boiling water bath at pH 2(2). M extracts were prepared by repeated alcohol precipitation of crude extracts(3). A partially purified preparation of M protein was prepared by the method of Lancefield and Perlmann(4) from Type 1 Streptococci, strain #2788, kindly supplied, as phenolized cells, by Dr. C. P. Hegarty of Merck, Sharp, and Dohme Labs. Electrophoretic examinations of this material revealed migration as a single peak, but with sufficient boundary spreading to suggest inhomogeneity. Streptococcal T extracts were prepared by slight modification of the method of Pakula(5). Crystalline trypsin in final concentration of 0.01 mg/ml at pH 7.6 was substituted for pancreatic extract. *Enzymes.* Pepsin (Difco 1:10,000) was employed in final concentration of 1% at pH 2. Certain strains and extracts were treated with crystalline trypsin (General Biochemicals, Inc.) to

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† Lean horse meat was substituted for beef hearts.

final concentration of 0.01 mg/ml at pH 7.4. Human fibrinogen (lot #17), obtained from E. R. Squibb and Sons, through the courtesy of American Red Cross, was further purified by the method of Laki(6); the purified material contained 93% clottable protein, and was used in 0.5% concentration in 0.3 M KCl buffer at pH 7.2. *Normal rabbit plasma.* Coagulation of each 5 ml aliquot of blood was prevented with one of the following: 0.05 ml heparin (Abbott 1:1000), 0.5 ml sodium citrate (3.8% solution), 0.5 ml potassium ammonium oxalate solution (2%). Plasma was separated by centrifugation at 1500 g for 15 minutes. *Streptococcal typing sera* were obtained from the Diagnostic Reagents Section, Communicable Disease Center, USPHS, Chamblee, Ga.

Results. Effect of various anticoagulants, and plasmas of different species. In the original observation, partially purified type 1 M protein precipitated with normal rabbit plasma obtained from heparinized blood. Subsequently, oxalated and citrated rabbit plasmas precipitated equally well. Furthermore, all mammalian plasmas tested, *i.e.* man, monkey, guinea pig, rat, and mouse plasma, precipitated strongly with type 1 M protein. Rat plasma behaved differently than the others; an immediate precipitate occurred but disappeared within a few minutes. No activity of M protein against chicken plasma has been found, and addition thereto of varying amounts of normal rabbit serum did not render chicken plasma precipitable. Addition of type 1 M protein to purified human and bovine fibrinogens resulted in immediate, massive precipitates. The material precipitating with plasma or fibrinogen has been designated, for convenience, fibrinogen precipitating factor, or FPF.

The *FPF positive reaction* in capillary tubes appears within 5 minutes at room temperature when purified M protein and fibrinogen are used. This reaction is not inhibited by diisopropylfluorophosphate (DFP), nor will a known positive M protein hydrolyze synthetic substrate, *i.e.,* tosyl-arginyl-methyl ester (TAME).‡ Strongly positive FPF reactions appear as gross floccules throughout the tube; in weaker reactions a fine haze appears. Fibrin-like retraction may be seen with a hand lens after a short time. The precipitated material, examined under phase microscope, consists of considerable amorphous material together with some characteristic fibrin needles.

Occurrence of FPF in various organisms. Subsequent to finding FPF in a purified M protein preparation, it was demonstrated in crude acid extracts of some but not all beta hemolytic streptococci prepared for routine grouping and typing. To determine distribution of FPF among streptococci, pneumococci, and staphylococci, crude acid extracts of 18 hour Todd-Hewitt broth cultures of these organisms were prepared, and tested in capillary tubes against purified human fibrinogen. The tubes were incubated 2 hours at 37°C, then overnight at room temperature. Estimates of amount of precipitates varied from 1+ to 4+. Of 146 strains of beta hemolytic streptococci tested, 131 were of Group A; the remainder were distributed among Groups B, C, D, F, G, and K (Table I). It should be noted that typing of Group A organisms was performed prior to lyophilization and storage; not all strains were typable when the lyophile tubes were opened and cultured. Reactivity of a particular extract with fibrinogen did not correlate, however, with its ability to react with type-specific antiserum. FPF was found in at least one strain of each type of Group A streptococci when 2 or more strains were available for testing, except for 5 typable strains of Type 18; crude acid extracts of these 5 strains proved nonreactive. However, M extracts prepared from 2 of these crude extracts by repeated alcohol precipitation both precipitated (weakly) with fibrinogen.

Of 15 non-group A streptococcal extracts, only one from a Group G organism proved reactive. Acid extracts of 5 coagulase-positive strains of staphylococci, 4 typed strains of pneumococci, and 5 strains of alpha-hemolytic streptococci did not react with fibrinogen or plasma. Eleven T extracts prepared by Pakula's method from FPF positive strains of

‡ These determinations were kindly performed by Dr. Jules P. Gladner, Laboratory of Physical Biology, Nat. Inst. of Arthritis and Metab. Dis., N.I.H., Bethesda, Md.

TABLE I. Distribution of Fibrinogen Precipitating Factor in Extracts of Hemolytic Streptococci, Pneumococci, and Staphylococci.

Organism	Putative type*	No. strains tested	No. of extracts reactive with: Homol. antiserum	Fibrinogen
Hemolytic streptococci				
Group A	1	4	4	3
	2	4	0	3
	3	3	3	2
	4	29	29	28
	5	4	4	2
	6	5	5	3
	8	3	No serum	3
	11	5	5	4
	12	28	28	6
	13	4	0	3
	14	1	1	0
	15	1	1	0
	17	2	0	1
	18	5	5	0
	19	4	3	2
	22	3	1	2
	24	1	1	0
	23	4	3	2
	25	3	0	2
	26	1	1	0
	28	2	2	2
	29	2	2	1
	30	1	1	0
	31	2	2	1
	32	1	1	0
	36	1	1	0
	37	1	1	0
	39	1	1	0
	40	1	1	0
	43	1	1	0
	44	2	No serum	0
	46	1	1	0
	47	1	1	0
Group B		2	2	0
C		4	4	0
D		2	2	0
F		2	2	0
G		4	4	1
K		1	1	0
Viridans streptococci		5		0
Staphylococci		5	All coag. pos.	0
Pneumococci		4	4	0

* All strains typed prior to storage.

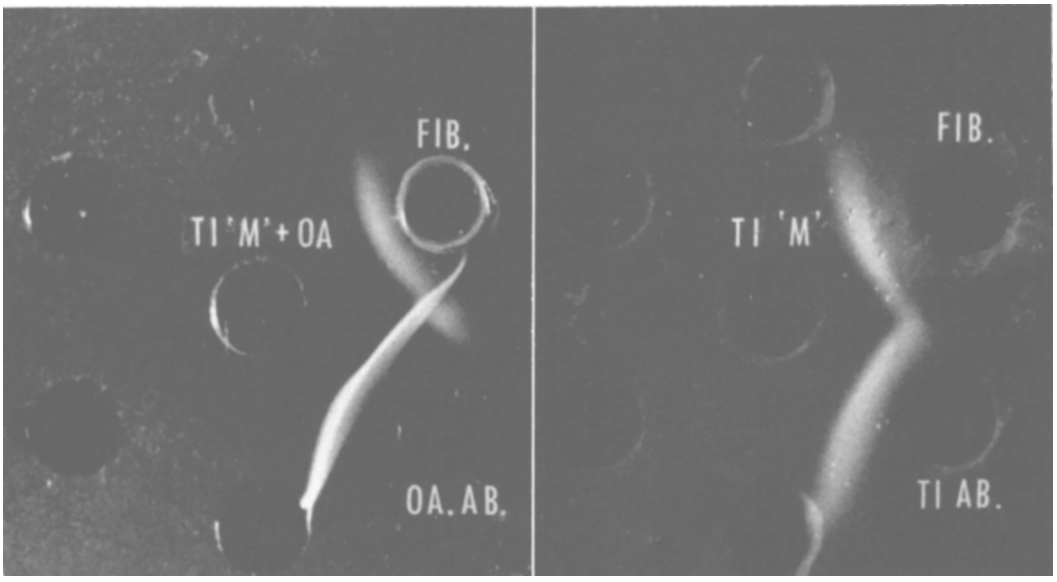
Group A streptococci did not precipitate fibrinogen.

Effect of heat, pH, and proteolytic enzymes. Treatment of partially purified type 1 M protein with pepsin at pH 2 for 2 hours at 37°C with subsequent neutralization, removed both FPF and the type-specific material. Trypsin was similarly effective. FPF could not be acid-extracted from trypsinized streptococcal cells known to contain this factor prior to trypsin treatment. Heating for 35 minutes at 95°C at pH 2 did not affect either FPF or M protein.

Identity. Fibrinogen precipitating factor could not be separated from M protein. Absorption of a 0.1% solution of partially purified Type 1 M protein with an equal volume of 0.5% solution of human fibrinogen completely removed both M protein and FPF; 2 absorptions of M protein solution with equal volumes of type-specfiic antiserum also removed both M protein and FPF. Finally, agar diffusion experiments illustrated in Figs. 1a and 1b demonstrate that FPF and M protein represent 2 activities resident in the same moiety. In Fig. 1a, a mixture of 0.1% M protein in saline, and 0.5% saline solution of ovalbumin, in ratio of 4:1, was pipetted into the center well. A solution of 0.5% fibrinogen was placed in the well marked "FIB.," and undiluted rabbit antiovalbumin serum was placed in the well marked "OA. AB." The reaction between FPF and fibrinogen in no way interfered with the immune precipitate formed between ovalbumin and homologous antiserum. However, when M protein alone is pipetted into the center well, (Fig. 1b), type 1 antiserum placed in the well marked "Tl. AB.", and fibrinogen placed in the well marked "FIB.," the reaction between FPF and fibrinogen interferes with the immune precipitate line, which ends abruptly at the intersection of the 2 precipitates. It is clear that fibrinogen is capable of absorbing M protein so that none is available for reaction with type-specific antiserum in the region peripheral to the FPF-fibrinogen precipitate line.

Inhibition of FPF reaction. The weak reactivity of M extracts of two Type 18 streptococcal strains, and the lack of reactivity of crude acid extracts of these same strains suggested the presence of inhibitors. Since both these strains were highly mucoid, possible inhibition by hyaluronic acid was investigated as follows: a 3 ml suspension of known FPF positive Type 1 bacterial cells was divided into 3 equal samples. To Sample I, 1 ml of 0.1% hyaluronic acid§ was added; to Sample

§ Highly purified potassium hyaluronate from human umbilical cord (Schering Corp., West Berlin) was kindly supplied by Dr. Emily Emmart, Lab. of Pharmacol. and Toxicol., Nat. Inst. of Arthritis and Metab. Dis., N.I.H.



a. b.

FIG. 1. Agar-gel precipitin reactions of type 1 M protein with fibrinogen and type-specific antiserum. T1 M, .1% Type 1 M protein; FIB., .5% Fibrinogen; OA., .5% Ovalbumin; T1 AB., Type 1 Antiserum; OA. AB., Ovalbumin Antiserum.

II, 1 ml of saline was added; the third sample was untreated. Reaction of each sample was lowered to pH 2 with N HCl and they were extracted in a boiling water bath for 10 minutes. Subsequent to cooling, Sample III received 1 ml of hyaluronic acid. All samples were neutralized with N/5 NaOH to pH 7.4 and volumes equalized with saline. These samples were set up in capillary tubes with fibrinogen and with type-specific antiserum. All extracts reacted strongly with type-specific antiserum. Sample I reacted minimally with fibrinogen when compared with the other 2 samples. The presence of hyaluronic acid prior to acid extraction at 95°C impaired reactivity of the resulting extract with fibrinogen. Addition of hyaluronic acid to Sample III after acid extraction had no apparent effect.

Discussion. An active principle has been found in various streptococci which precipitates the plasmas of several different animals. This material is closely related, if not identical to M protein. Reactivity of crude streptococcal extracts with type-specific antiserum did not correlate, however, with ability to precipitate fibrinogen. An explanation for this lack of correlation may be the interference, by inhibitors, with the demonstration of FPF in typable extracts. The experimental data favor this explanation: 1) Purification of two type 18, FPF negative, crude extracts by repeated alcohol precipitation resulted in fibrinogen reactive substances. 2) Addition of hyaluronic acid to a known FPF positive suspension of bacteria prior to acid extraction resulted in virtual absence of FPF activity in the crude extract. Since strains of streptococci vary considerably in production of hyaluronic acid, this material may be one explanation for the presence of type-specific substance in an extract devoid of fibrinogen activity.

Reaction of streptococcal M protein with fibrinogen suggests an analogy with staphylococcal coagulase. Although both materials are present chiefly in virulent strains, inhibit phagocytosis(7,8), are trypsin sensitive, and poorly antigenic(9,5), they are not analogous. M protein is located in the cell wall, while staphylocoagulase is an extracellular product. Coagulase activity results in a typical fibrin gel, whereas FPF precipitates with fibrinogen. Furthermore, coagulase requires a serum co-factor, coagulase reacting factor (CRF), for fibrinogen conversion; this

factor is not present (in adequate amounts) in rat, mouse, and chicken plasma(10). Our experience, cited above, shows that FPF precipitates rat and mouse plasma. Chicken plasma reacts with neither coagulase nor FPF; however, addition of normal rabbit serum renders chicken plasma clottable with coagulase, but does not impart FPF reactivity.

The significance of the fibrinogen precipitating factor in streptococcal infections and the non-suppurative sequalae of such infections is the subject of current investigations.

Summary. 1) An active factor was found in streptococci which precipitates various mammalian plasmas, and human and bovine fibrinogens. Fibrinogen precipitating factor (FPF) was found in crude acid extracts of several strains of group A, and one strain of group G streptococci; extracts of staphylococci, pneumococci, and viridans streptococci have proven non-reactive. 2) Streptococcal M protein and FPF appear inseparable, although presence of hyaluronic acid in a suspension of streptococcal cells prior to acid extraction may inhibit the fibrinogen precipitating reactivity of the extract, without significantly affecting the typing reaction. FPF and staphylocoagulase share certain properties, but they are not analogous.

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Received July 9, 1959. P.S.E.B.M., 1959, v102.

Properdin Levels in Splenectomized Persons. (25173)

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Our previous studies(1) showed that antibody response to tularemia vaccine in 92 of 105 splenectomized persons was similar to that observed in 47 normal controls. Failure of 13 persons to exhibit antibodies was attributed to the underlying disease for which splenectomy was performed rather than to absence of the spleen. This present study is concerned with determination of properdin levels in the same 105 splenectomized subjects.

Materials and methods. The 105 splenectomized persons included 35 males and 70 females, 2 to 77 years old and 2 months to 18 years post-splenectomy. Blood samples for properdin determinations were obtained prior to tularemia vaccination(1). Healthy non-splenectomized controls consisted of 100 males and 13 females, 18 to 40 years of age. All blood samples were centrifuged 5-7 minutes at 2000-2200 rpm in an International, Size 2 centrifuge and the serum frozen and stored at −22°C until tested. Properdin levels were determined by the Pillemer zymosan assay technic(2). Serum reagents RP and R3 were prepared from pools of normal human serum(3) and standardized by the Pillemer method(2). Serial 2-fold dilutions of test sera(4) were used. The amounts of zymosan* found optimal were 1 mg and 2.5 mg/ml of normal human serum in preparation of R3 and RP respectively, and 6 mg/ml of RP in the assay proper. Sensitivity and validity of the test were controlled by daily assays of 1 serum sample of known properdin content and 2 synthetic samples prepared by adding known

* Nutritional Biochemicals Corp., Cleveland, O.