

## THE FIBRINOLYTIC ACTIVITY OF HEMOLYTIC STREPTOCOCCI

BY WILLIAM S. TILLET, M.D., AND R. L. GARNER, Ph.D.

(From the Biological Division of the Department of Medicine of the Johns Hopkins  
Medical School, Baltimore)

(Received for publication, June 27, 1933)

The findings presented in this communication demonstrate the capacity of broth cultures of hemolytic streptococci to liquefy rapidly the clotted fibrin of normal human plasma. The experiments have been carried out in such a manner as to emphasize the rapidity with which active cultures transform solid clot into a completely liquid state, and to bring out other special characteristics of the fibrinolysis by hemolytic streptococci which differ in some respects from the orderly digestion of solid protein material by proteolytic enzymes. The observations contained in this report are chiefly limited to a consideration of the presence of fibrinolytic substances in cultures and the conditions of fibrin coagulation which influence the occurrence of liquefaction. Additional lines of investigation, suggested by the results, are not yet complete. Consequently the present communication makes only brief mention of some of the results which will be presented in detail in subsequent publications.

The experimental conditions under which the clot-dissolving property of cultures is most strikingly demonstrable consist in mixing the cultures with plasma or fibrinogen before inducing clot formation. By this procedure the organisms and their products are disseminated within the body of the clot as it forms, thus affording maximum surface contact between the active bacterial agent and the fibrin substrate. Under these conditions the quantity of plasma clot employed in the experiments is liquefied in a few minutes, whereas the same amount of plasma, clotted before the addition of cultures, requires several hours incubation to effect the same degree of dissolution.

The strains of *Streptococcus hemolyticus* employed in the tests have

been derived from patients suffering from various manifestations of acute streptococcus infections. The sources of the cultures are given in Table I and the list includes streptococci isolated from cases of septicemia, erysipelas, scarlet fever, acute tonsillitis, cellulitis, and other diseases. In addition to the human strains, hemolytic streptococci of animal origin have also been used in comparable tests. Observations have also been made with strains of other pathogenic bacterial species for the purpose of determining the presence or absence of a similar fibrinolytic property.

Whole plasma from human beings and rabbits, and fibrinogen chemically isolated from the plasma of each species, have served as a source of fibrin. Clot formation was induced by appropriate coagulants which are described in the detailed experiments.

#### *Materials and Methods*

*Cultures.*—NaCl meat infusion broth, adjusted to pH 7.6, and containing 0.05 per cent dextrose, has been the culture medium regularly employed. The broth contains 1 per cent peptone but is not buffered. The final pH, after growth has occurred, rarely goes below 7.2. The tests were performed with fresh cultures which had been incubated 18 to 24 hours. Granular growth was rarely encountered.

*Filtrate.*—Ultrafiltration was carried out in the usual manner, aided by suction. Berkefeld V, Chamberland, and Seitz filters were employed. Sterility of the filtrates was always determined by cultures.

*Anticoagulant.*—Potassium oxalate has been regularly employed in amounts of 0.02 gm. of oxalate to 10 cc. of blood. A 2 per cent solution is made in distilled water. 1 cc. of this solution is placed in small bottles which are heated in a dry air sterilizer until all water has evaporated. 10 cc. of blood, immediately after withdrawal, is mixed with the dried powder. 0.03 to 0.04 gm. of oxalate per 10 cc. of blood is used to obtain rabbit plasma.

*Preparation of Fibrinogen.*—The fraction of plasma proteins precipitated at 25 per cent saturation with ammonium sulfate has proved to be a suitable fibrinogen solution. To oxalated plasma, a saturated solution of ammonium sulfate is added drop by drop until the amount of ammonium sulfate equals one-third the volume of plasma. The mixture is stirred constantly during the addition of ammonium sulfate.

The flocculated protein is collected by centrifugation, and the supernatant liquid is discarded. The precipitate is washed once with 25 per cent saturated ammonium sulfate solution, centrifuged, and drained free of wash liquor. The protein is then dissolved in  $M/100$  phosphate buffered physiological salt solution (pH 7.4). The amount of solvent which is added, makes the final protein solution equal the volume of plasma originally employed.

*Preparation of Thrombin.*<sup>1</sup>—The method consists of two stages.

First, precipitation of prothrombin from plasma. Oxalated plasma is diluted with a tenfold volume of cold, distilled water and maintained at ice bath temperature while carbon dioxide is passed through the mixture. After 10 minutes this operation is interrupted and the flocculent protein collected by centrifugation. The precipitate is dissolved in a volume of physiological salt solution slightly less than the volume of plasma originally employed. The hydrogen ion concentration of this solution is adjusted to pH 7.4 by means of a trace of solid sodium bicarbonate. This method separates prothrombin from the plasma but also precipitates some fibrinogen and other plasma proteins.

Second, conversion of prothrombin into thrombin. A 2.5 per cent solution of calcium chloride is added in a ratio of 1 cc. for each 10 cc. of prothrombin solution. After 5 to 10 minutes the solution coagulates to a thick gel. By careful manipulation the fibrin is rolled into a compact mass, and the expressed fluid is filtered through paper. This faintly opalescent solution usually possesses a high coagulating activity with either plasma or fibrinogen.

Fibrinogen and thrombin prepared according to the methods just described are sometimes labile and may deteriorate rapidly. Although some lots of each have maintained the original property for several days when kept in the ice box, it is best to use the materials on the day of preparation. In making tests for fibrinolysis, the physiological activity of both fibrinogen and thrombin was first established. Activity was deemed satisfactory when 0.5 cc. of undiluted fibrinogen formed a solid clot with 1 drop of thrombin solution.

*Coagulants.* 1. *Thrombin.*—0.1 cc. of thrombin solution was used to coagulate 0.2 cc. of plasma or fibrinogen. This ratio always insured complete clot formation in 1 to 3 minutes.

2. *Calcium Chloride.*—0.25 cc. of a 0.25 per cent solution of  $\text{CaCl}_2$  in 0.85 per cent salt solution consistently clotted 0.2 cc. of oxalated plasma in 8 to 20 minutes. 0.3 to 0.35 cc. of 0.25 per cent  $\text{CaCl}_2$  solution was added to rabbit plasma since a larger amount of oxalate was mixed with the whole blood. The solution of calcium chloride was sterilized by immersing in boiling water for 30 minutes.

Oxalated plasma which has stood for 3 days or more is rarely coagulable with  $\text{CaCl}_2$ . A distinct delay in clotting is usually noted in 2 day old plasma. The inability of calcium to coagulate old oxalated plasma is usually attributed to the rapid deterioration of prothrombin; the addition of thrombin to old plasma results in the formation of solid clot.

In the experiments reported in this article samples of plasma were always tested within 24 hours of the time of blood withdrawal.

*Description of Test.*—After numerous trials with varying amounts of the different constituents, the following standard procedure was adopted: 0.2 cc. of oxalated

<sup>1</sup> The authors are indebted to Dr. Harry Eagle for the method by which thrombin solutions were prepared. The description, presented here, is given with his kind permission. A more detailed report will be published by Dr. Eagle.

plasma is diluted with 0.8 cc. of physiological salt solution. To this 1 to 5 dilution of plasma, 0.5 cc. of broth culture or sterile filtrate of test organism is added and well mixed. Coagulant is then added and well mixed; if  $\text{CaCl}_2$  is used, 0.25 cc. of 0.25 per cent solution is added, if thrombin is employed, 0.1 cc. is added. The tubes are immediately placed in water bath at  $37.5^\circ\text{C}$ . The time at which solid coagulation occurs is noted. Solid coagulation is considered to be effected when the tube can be inverted without affecting the solid form of clot which adheres to the bottom and sides of the tube; usually no fluid, or only a small drop, escapes from the solid clot on inversion of the tube.

The tubes are allowed to remain in the water bath under continual observation. Complete dissolution of the clot is recorded as the time at which all evidence of fibrin has disappeared, and the contents of the tube are completely fluid. As dissolution begins, the first softening of the clot may be noted by gently shaking the tubes. This movement causes a wave-like motion to pass vertically down through the body of the clot. The coagulum then releases itself from the sides of the tube and usually forms a small mass which, a few minutes later, may be made to disappear by moderate agitation. All tests in which the plasma clot was resistant to dissolution were arbitrarily terminated after 24 hours incubation.

In the tables presented in this communication "complete dissolution" refers to the interval of time between formation of solid clot and complete liquefaction. The time necessary for clot formation is not given special consideration in this record.

By recapitulation, a test is as follows:

0.2 cc. plasma (or fibrinogen) + 0.8 cc. physiological salt solution + 0.5 cc. culture (or filtrate) + 0.25 cc.  $\text{CaCl}_2$  (or 0.1 cc. thrombin).

Unless otherwise specified, all tests were done with standard quantities of the various constituents.

#### *Fibrinolytic Tests with Human Strains of Hemolytic Streptococci*

Twenty-eight strains of hemolytic streptococci of the *beta* type (Brown (1)) have been tested for the capacity to liquefy normal human plasma clot. The results are recorded in Table I.

From the table it can be seen that cultures of all the strains contain substances capable of causing fibrinolysis. Most of the strains effect complete dissolution in less than 20 minutes. With others the speed of clot-liquefaction varies but every strain of hemolytic streptococci derived from patients contains the active principle. Up to the present time no relationship has been noted between the disease source of organisms and fibrinolytic potency.

As noted in the table some of the strains were tested immediately after isolation while others have been in laboratory cultivation for

TABLE I  
*Strains of Hemolytic Streptococcus Isolated from Patients*  
*Fibrinolytic Activity against Human Fibrin Clot*

No.	Patient	Source of culture	Disease	Length of laboratory cultivation	Complete dissolution	
					Clot of	
					Plasma*	Fibrinogen†
1	Co.	Blood	Septicemia	2 yrs.	10 min.‡	2 min.‡
2	Le.	"	"	2 "	10 "	2 "
3	Ma.	"	"	2 "	20 "	
4	Bo.	"	"	1 wk.	10 "	2 "
5	Ot.	Throat	Acute tonsillitis	2 yrs.	15 "	
6	Hi.	"	"	4 days	40 "	5 "
7	Bi.	"	" "	4 "	10 "	2 "
8	Hu.	"	" "	4 "	10 "	
9	Ja.	"	Scarlet fever	1 yr.	30 "	
10	Th.	"	" "	6 mos.	40 "	
11	Sh.	"	" "	6 "	10 "	
12	Mi.	"	" "	1 mo.	10 "	2 "
13	Ur.	"	" "	2 days	50 "	6 "
14	Si.	"	" "	2 "	10 "	2 "
15	Po.	"	" "	2 "	40 "	4 "
16	Pr.	"	" "	2 "	15 "	2 "
17	Zb.	"	" "	2 "	50 "	6 "
18	Shy.	"	" "	2 "	40 "	6 "
19	Ch.	Ear	Otitis media	2 "	20 "	3 "
20	La.	Throat	Erysipelas	2 wks.	10 "	
21	Br.	"	"	1 mo.	10 "	
22	Ro.	Skin	"	6 mos.	25 "	
23	Pr.	Pleural fluid	Empyema	1 wk.	10 "	2 "
24	Sm.	Tonsil	Acute nephritis	7 yrs.	10 "	
25	Ta.	"	" "	7 "	15 "	
26	Mc.	Throat	" "	7 "	10 "	
27	Ba.	Skin pustule	" "	2 mos.	10 "	2 "
28	Lab.	Skin	Cellulitis	3 "	20 "	

All tubes incubated in water bath at 37.5°C.

\* Plasma clotted with CaCl<sub>2</sub>

† Fibrinogen clotted with human thrombin.

‡ Time indicated is interval between formation of solid clot and complete liquefaction. Repeated tests have been made with many of the strains. The average rate of dissolution is noted in the table.

several years. Strain Co., continually highly active for 12 months, has been used in most of the tests. This strain was originally isolated by blood culture from a fatal case of septicemia and has been carried in the laboratory for 2 years.

Although fibrinolytic activity is maximum in freshly transplanted cultures which have been incubated 18 to 24 hours, the property may be retained for several days. Deterioration of the active principle supplied by the organisms, however, is noticeable within 1 to 2 weeks, even at ice box temperature. Consequently, in an attempt to obtain a maximum uniform effect, broth cultures were always used the day following inoculation. In spite of the fact that this procedure necessitated the use of fresh subcultures frequently, the yield of active material maintained a constant high level.

In addition to tests performed with the clot of whole plasma, fibrinogen has been chemically removed from plasma and used as a source of fibrin. Coagulation, in these instances, has been induced by the addition of thrombin solutions, also derived from whole blood. The fibrin formed by the combination of chemically isolated fibrinogen and thrombin solution is called fibrinogen clot in this article, to distinguish it from the clot of whole plasma coagulated by calcium which is referred to as plasma clot. The fibrinogen clot, consisting of fibrinogen plus active culture plus thrombin, has been observed for fibrinolysis in exactly the same manner as that employed with plasma clot.

The results of tests with 15 strains of hemolytic streptococcus against fibrinogen clot are given in Table I and demonstrate that with this material, fibrinolytic activity proceeds with greater rapidity than it does against the fibrin of whole plasma. The culture of Strain Co., for example, which liquefied plasma clot in 10 minutes, dissolved a relatively comparable amount of fibrinogen clot in 2 minutes. The exact amount of fibrin in the plasma clot and the fibrinogen clot was not determined and the comparable experiments recorded in the table indicate only the qualitative character of the phenomenon rather than accurate quantitative titrations, which will be subsequently published. That whole plasma contains a property which may delay or even inhibit the fibrinolytic activity of the organisms will be referred to later in this article.

*Fibrinolytic Tests with Animal Strains of Hemolytic Streptococci*

In addition to the 28 strains of hemolytic streptococci derived from patients, 18 strains from animal sources have been similarly tested for the capacity to liquefy coagulated plasma and fibrinogen. These strains are hemolytic streptococci of the *beta* type and equally as active in producing hemolysis as the human strains. The results of tests with broth culture of the 18 animal strains against human fibrin clot are recorded in Table II.

TABLE II  
*Animal Strains of Hemolytic Streptococcus*  
*Fibrinolytic Activity against Human Fibrin Clot*

No.	Strain	Animal source	Complete dissolution	
			Clot of	
			Plasma*	Fibrinogen†
1	K 226	Rabbit	Neg. 24 hrs.‡	Neg. 24 hrs.‡
2	K 158A	"	" 24 "	" 24 "
3	K 158B	"	" 24 "	" 24 "
4	K 158C	"	30 min.	8 min.
5	K 158D	"	Neg. 24 hrs.	Neg. 24 hrs.
6	K 158E	"	" 24 "	" 24 "
7	K 158F	"	" 24 "	" 24 "
8	V 10	Cow	10 min.	5 min.
9	K 56	Guinea pig	Neg. 24 hrs.	Neg. 24 hrs.
10	K 61	" "	30 min.	8 min.
11	K 64	" "	Neg. 24 hrs.	Neg. 24 hrs.
12	K 104	" "	" 24 "	" 24 "
13	P 454	" "	" 24 "	" 24 "
14	J 20	" "	" 24 "	" 24 "
15	Br 1	Rabbit	" 24 "	" 24 "
16	Br 2	Guinea pig	" 24 "	" 24 "
17	H 1	Horse	" 24 "	" 24 "
18	R 1	Rabbit	" 24 "	" 24 "

All tubes incubated in water bath at 37.5°C.

\* Plasma clotted with CaCl<sub>2</sub>.

† Fibrinogen clotted with human thrombin.

‡ Time indicated is interval between formation of solid clot and complete liquefaction. All experiments terminated after 24 hours incubation.

Strains 1 to 14 were kindly supplied by Dr. R. C. Lancefield, Hospital of The Rockefeller Institute, New York.

Strains 15 and 16 were kindly supplied by Dr. J. Howard Brown, Department of Pathology and Bacteriology, Johns Hopkins Medical School, Baltimore.

From the table it can be seen that 15 of these strains are totally incapable of inducing clot liquefaction even though the method of experimental procedure was identical with that used in testing the human strains.

Concerning the 3 strains of animal source which liquefied human clot, it is interesting to record that their cultivation on blood agar yielded, with each of the strains, two distinct types of colony; one type was small with a wide zone of hemolysis; the other type was larger, tended to be mucoid, and had a relatively smaller zone of hemolysis. When these two types of colonies were isolated and grown in broth, the small colony yielded a culture which was inert against clot, whereas broth transplant of the larger type of colony was highly active in dissolving coagulated plasma and fibrinogen. The significance of this behavior of the culture is not yet understood. The 15 strains of animal origin which were inactive produced colonies similar to the smaller, strongly hemolytic type.

The result of the observations with animal strains is interesting in that it demonstrates that the capacity to liquefy human clot is absent or suppressed in some strains of *Streptococcus hemolyticus* even though the organisms conform in other cultural respects to active strains; fibrinolysis does not necessarily parallel hemolysis.

#### *Fibrinolytic Tests with Other Human Bacterial Species*

In order to determine whether or not the fibrinolytic substances are limited to hemolytic streptococci, comparable tests have been performed with other pathogenic bacteria derived from patients.

All of the tests have been completely negative. The list of other organisms consists of:

- 6 strains of *Streptococcus viridans*.
- 2 strains of non-hemolytic streptococci.
- 6 strains of streptococci with weak hemolyzing property, some of which have been identified as *alpha* prime (Brown (1)) variety.
- 12 strains of pneumococci of different types (two of Type I strain, one of Type II, two of Type III, one each of Types VI, VIII, and XIV, three untyped Group IV strains, and one R strain of pneumococcus originally derived from Type II).
- 2 strains of typhoid bacillus.



- 2 strains of colon bacillus.
- 1 strain each of paratyphoid bacillus A and B.
- 1 strain each of Shiga and Flexner dysentery bacillus.
- 2 strains of Freidländer bacillus, Types A and B (Julianelle (2)).
- 2 strains of hemolytic influenza bacillus.

Altogether 38 strains of organisms other than hemolytic streptococci have been examined and in no instance have any of these cultures affected either clotted plasma or the more susceptible fibrinogen clot during the 24 hour test period.

It will be noted that strains of *Staphylococcus aureus* and *Staphylococcus albus* are not included in the list. Interesting properties by which cultures of staphylococcus affect plasma have been reported by Much (3), Gratia (4), Gross (5), and others. They have found that these organisms are capable of coagulating plasma spontaneously and some of the articles have reported that liquefaction may also occur over a period of several hours. These properties of staphylococci are being investigated separately in this laboratory. Up to the present time, fibrinolytic activity has been found in only occasional strains and, when present, causes dissolution irregularly and requires 18 to 24 hours or longer to act.

With the possible exception, then, of staphylococci, hemolytic streptococci appear to be unique among the species of bacteria usually pathogenic for human beings in the capacity to transform rapidly, solid clotted fibrin into a thin fluid state.

#### *Fibrinolytic Tests with Sterile Filtrates of Hemolytic Streptococci*

The observations so far described have been made by adding to plasma, whole broth cultures containing both living organisms and the bacterial products present in the medium. Additional investigations have been carried out with sterile, cell-free filtrates, with washed organisms, and with cultures grown on solid media.

The sterile filtrates of 6 strains of hemolytic streptococcus, grown in broth, have been tested for the presence of fibrinolytic substances by the use of human plasma clot and also human fibrinogen clot. The results are presented in Table III. Filtration has been made through Berkefeld V, Chamberland, and Seitz filters. The records

contained in the table were obtained with material passed through Berkefeld V candles.

From the table it may be seen that, in most instances, 0.5 cc. of cell-free filtrate liquefies normal human fibrin clot as rapidly as does

TABLE III  
*Fibrinolytic Activity of Sterile Filtrates of Active Strains of Hemolytic Streptococcus against Human Fibrin Clot*

Strains	Amount of filtrate	Amount of whole broth culture	Complete dissolution	
			Clot of	
			Plasma*	Fibrinogen†
Co.	0.5	—	10 min.‡	2 min.‡
	—	0.5	10 "	2 "
Le.	0.5	—	10 "	2 "
	—	0.5	10 "	2 "
Br.	0.5	—	12 "	2 "
	—	0.5	10 "	2 "
Ba.	0.5	—	15 "	4 "
	—	0.5	10 "	2 "
Ma.	0.5	—	30 "	6 "
	—	0.5	20 "	4 "
Hi.	0.5	—	1 hr. 10 min.	10 "
	—	0.5	40 min.	8 "

All tubes incubated in water bath at 37.5°C.

\* Plasma clotted with CaCl<sub>2</sub>.

† Fibrinogen clotted with human thrombin.

‡ Time indicated is interval between formation of solid clot and complete liquefaction. Repeated tests have been made with many of the strains. The average rate of dissolution is noted in the table.

0.5 cc. of whole broth culture; with less active strains, the filtrate acts at a slightly slower rate than does whole culture. The presence of the fibrinolytic substances in the filtrate evidences the fact that the active principle is elaborated by growing organisms and is liberated freely into the surrounding medium.

*Titration of Fibrinolytic Activity of Sterile Filtrates*

When whole broth cultures were employed in attempts to titrate quantitatively the amount of fibrinolytic substance present, the presence of living organisms detracted from the accuracy of the results. In such attempts the additional multiplication of bacterial cells which might take place at 37.5°C. was uncontrolled. By the use of sterile filtrates, however, the degree of activity has been more satisfactorily estimated.

Titration of filtrates have been done both by testing varying dilutions of filtrate against a constant quantity of plasma and fibrinogen, and by testing a constant quantity of filtrate against varying amounts of plasma and fibrinogen. The results are presented in Table IV.

The observations given in the table demonstrate that, against the clot contained in 0.2 cc. plasma, 0.01 cc. of filtrate effects fibrinolysis almost as rapidly as does 0.5 cc. of filtrate. 0.005 cc. is slowly active, whereas 0.001 cc. is ineffectual. When progressively decreasing quantities of filtrate were tested against fibrinogen clot, even so small an amount as 0.0005 cc. of filtrate induced lysis in an hour and 5 minutes. Smaller quantities of filtrate have not been tested. The greater susceptibility of fibrinogen-clot is also brought out in the lower half of Table V, where the increasing amounts of fibrin substrate are exposed to a constant quantity of active filtrate. In these experiments, 0.5 cc. of filtrate required 3 hours and a half to liquefy 2 cc. of plasma clot; whereas 0.5 cc. of filtrate dissolved 10 cc. of fibrinogen clot in 30 minutes.

The titration of many lots of active filtrate has resulted in some variation in quantitative activity. The results given in Table IV were obtained with a highly active product; in a few instances even more potent material has been obtained.

When organisms are removed from broth by centrifugation, washed, and resuspended in physiological salt solution, their capacity to dissolve fibrin clot is considerably delayed. In such experiments liquefaction required 6 to 10 hours. This was interpreted as being chiefly due to the fact that most of the preformed fibrinolytic property was discarded, and that the slow rate of liquefaction depended upon

the speed with which additional active material could be formed by the organisms and excreted into the clot. A similar delayed rate of dissolution occurred when streptococci grown on blood agar were washed off and tested for fibrinolytic activity.

TABLE IV  
*Titration of Fibrinolytic Activity of Sterile Filtrate against Human Fibrin Clot*

Amount of filtrate	Amount of plasma*	Time of complete dissolution	Amount of filtrate	Amount of fibrinogen†	Time of complete dissolution
Decreasing amounts of filtrate against constant quantity of plasma clot and fibrinogen clot					
cc.	cc.		cc.	cc.	
0.5	0.2	8 min.‡	0.5	0.2	2 min.‡
0.1	0.2	10 "	0.1	0.2	2 "
0.05	0.2	10 "	0.05	0.2	4 "
0.01	0.2	30 "	0.01	0.2	7 "
0.005	0.2	6 hrs.	0.005	0.2	10 "
0.001	0.2	Neg. 24 hrs.	0.001	0.2	40 "
0.0005	0.2	—	0.0005	0.2	11 hr. 5 min.
Constant amount of filtrate against increasing quantities of plasma clot and fibrinogen clot					
0.5	0.2	7 min.	0.5	0.2	2 min.
0.5	0.5	15 "	0.5	0.5	2 "
0.5	1.0	30 "	0.5	1.0	3 "
0.5	2.0	3 hrs. 30 min.	0.5	2.0	4 "
0.5	5.0	20 hrs.	0.5	5.0	5 "
0.5	10.0	—	0.5	10.0	30 "

All tubes incubated in water bath at 37.5°C.

\* Plasma clotted with CaCl<sub>2</sub>.

† Fibrinogen clotted with human thrombin.

‡ Time indicated is interval between formation of solid clot and complete liquefaction.

In each instance the plasma and fibrinogen was diluted 5 times. The amount of coagulant necessary was calculated on the basis of a standard test (see pages 487 and 488).

That the active substances contained in broth cultures may be separated from the living bacterial cells has facilitated chemical studies on the nature of the fibrinolytic property elaborated by hemolytic streptococci. Even though the interaction between

cultural material and fibrin is comparable to enzyme activity, the fibrinolytic principle has special characteristics different from the metabolic proteolytic enzymes of hemolytic streptococci as described by Tongs (6), West and Stevens (7), and Frobisher (8). The investigation has, therefore, been extended to include a study of the nature and properties of the active substance. A detailed report will follow this preliminary record.

*Resistance to Fibrinolysis by Plasma of Patients Recovered from Hemolytic Streptococcus Infections*

In addition to observations on the occurrence and properties of the fibrinolytic substance, the blood of patients and of normal individuals has been investigated for the purpose of determining whether or not infection with hemolytic streptococci is followed by changes in plasma which are demonstrable by tests for susceptibility to fibrinolysis. Tests have, therefore, been made on samples of plasma taken at repeated intervals before and after recovery from infection. The results demonstrate that the plasma clot from the blood of patients recovered from hemolytic streptococcus diseases is often completely resistant to dissolution by active culture. An analysis of many tests on samples of blood taken during the course of disease is now in preparation and will form a separate communication. At the present time it is interesting to record an instance of resistance and also to note the fact that the serum of an individual, whose plasma is resistant, may render normal plasma clot unsusceptible.

An example of the resistant effect is given in the following protocol, which consists of tests done with the plasma and serum of a patient convalescent from erysipelas.

1. Patient's plasma + culture +  $\text{CaCl}_2$ : Negative 24 hours.
2. Normal plasma + culture +  $\text{CaCl}_2$ : Complete dissolution in 10 minutes.
3. Normal plasma + culture + 0.1 cc. patient's serum +  $\text{CaCl}_2$ : No dissolution in 24 hours.
4. Normal plasma + culture + 0.1 cc. normal serum +  $\text{CaCl}_2$ : Complete dissolution in 10 minutes.

Standard quantities of constituents were employed in the experiment.

Resistance to the fibrinolytic activity of hemolytic streptococci exemplified in the protocol just given has been observed with the

plasma of other convalescent patients. The absence of fibrinolysis may also be strikingly demonstrated by the use of normal animal plasma.

*Resistance to Fibrinolysis by Plasma of Rabbits*

Table V contains the results of fibrinolytic tests done on rabbit plasma clot and rabbit fibrinogen clot with 6 human strains and 3

TABLE V  
*Fibrinolytic Activity of Human and Animal Strains of Hemolytic Streptococcus against Rabbit Fibrin Clot*

No.	Strain	Source	Activity against human fibrin clot	Rabbit fibrin clot Complete dissolution	
				Clot of	
				Plasma*	Fibrinogen†
1	Co.	Human	++++	Neg. 24 hrs.‡	Neg. 24 hrs.‡ or 10-20 hrs. Complete
2	Le.	"	++++	" 24 "	" "
3	Br.	"	++++	" 24 "	" "
4	Ba.	"	++++	" 24 "	Neg. 24 hrs.
5	Hu.	"	++++	" 24 "	" 24 "
6	Si.	"	++++	" 24 "	" 24 "
7	K 158A	Rabbit	Neg.	" 24 "	" 24 "
8	K 158B	"	"	" 24 "	" 24 "
9	K 158C	"	+++	" 24 "	" 24 "
10	K 226	"	Neg.	" 24 "	" 24 "
11	K 61	"	++++	" 24 "	" 24 "
12	V 10	Cow	++++	" 24 "	" 24 "

All tubes incubated in water bath at 37.5°C.

\* Clotted with CaCl<sub>2</sub>.

† Clotted with rabbit thrombin.

‡ Experiments arbitrarily terminated at end of 24 hour incubation.

++++ indicates maximum activity (see Table I).

animal strains of hemolytic streptococci highly active against human clot, and with 3 rabbit strains inactive against human fibrin.

The complete insusceptibility of rabbit plasma clot to the fibrinolytic property of the organisms contrasts strikingly to the rapid liquefaction of human clot. Plasma from fifteen adult rabbits and

three young rabbits, 1 month old, has been tested in order to determine individual variation. The results have been uniformly negative.

The coagulation of separated rabbit fibrinogen, in the presence of active culture, by rabbit thrombin results in the formation of a fibrinogen clot which, in most instances, has been as resistant to dissolution as the plasma clot. Exceptions to this experience have been encountered, however, which are indicated in Table V by the record of dissolution of fibrinogen clot in 10 to 20 hours. That rabbit fibrinogen clot may occasionally be slowly liquefied, whereas whole rabbit plasma clot is not, indicates that the factor responsible for resistance is greater in whole plasma than in the constituents which have been chemically isolated. The behavior of the rabbit fibrinogen-thrombin mixtures, although not identical with the results obtained with human material, tends to conform with the results presented in Table I. An understanding of the phenomenon must await an analysis of the factors which influence the fibrinolytic process.

#### *Relation of Thrombin to Resistance*

Additional evidence as to the complexity of the mechanism which determines the susceptibility or resistance of clot to dissolution is brought out in experiments in which coagulation is induced by different agents. In the observations so far presented human plasma has been clotted by the addition of  $\text{CaCl}_2$ . By this method, calcium promotes the transformation of the prothrombin of the plasma into thrombin, which in turn changes the fibrinogen into fibrin. The whole process of coagulation is effected by homologous native agents. Similarly rabbit plasma clotted with calcium behaves in an identical manner. Where fibrinogen has been used for fibrin clot, coagulation has been effected by homologous thrombin.

Interesting differences in the susceptibility of clot to the fibrinolytic substance of streptococci are brought out by interchanging human and animal materials which go to form the fibrin clot. The results are contained in the protocol given below. In all of the tests listed, the coagulation was complete and, from the gross appearance of the clot, the occurrence of fibrinolysis could not be anticipated. The experiments were controlled with tests, in which physiological salt

solution or sterile uninoculated broth was substituted for active filtrate. The clot, formed under these conditions, remained intact.

				Time of	
				Clot formation	Clot dissolution
1. Human plasma	+	filtrate	+ CaCl <sub>2</sub>	10 min.	10 min.
2. " "	+	"	+ human thrombin	1 "	6 "
3. " "	+	"	+ rabbit "	1 "	10 "
4. Rabbit plasma	+	filtrate	+ CaCl <sub>2</sub>	10 "	Neg. 24 hrs.
5. " "	+	"	+ human thrombin	1 "	45 min.
6. " "	+	"	+ rabbit "	1 "	Neg. 24 hrs.
7. Human fibrinogen	+	filtrate	+ human thrombin	1 "	2 min.
8. " "	+	"	+ rabbit "	1 "	4 "
9. Rabbit fibrinogen	+	filtrate	+ human thrombin	1 "	4 "
10. " "	+	"	+ rabbit "	1 "	Neg. 24 hrs. or complete dissolution in 10-20 hrs.

From this list it can be seen that when the constituents of the clot are composed of human material, liquefaction occurs promptly. Fibrinolysis is rapidly induced by filtrate whether the fibrinogen and thrombin are resident in whole plasma or are first chemically separated and then recombined. On the contrary, when the coagulum is made up entirely of rabbit substances, the clot remains intact. An exception to this is found in the few instances, already referred to, where rabbit fibrinogen plus rabbit thrombin has produced a clot which is finally dissolved after 10 to 20 hours. The use of human thrombin in the coagulation of rabbit plasma or fibrinogen, however, results in the formation of a clot which active filtrate is able to liquefy with great rapidity. Repeated observations have uniformly demonstrated that the addition of a clot constituent from a susceptible blood to a resistant blood results in a coagulum which active cultural material can successfully liquefy.

At the present time the investigation has not proceeded far enough to permit discussion of the mechanism of susceptibility and resistance which is manifest under the experimental conditions just described. The fibrinogen and thrombin preparations are not purified and undoubtedly contain other blood constituents, the importance of which is as yet undetermined. The observations have, however, been re-



peatedly made and the facts, which have been established, serve as a basis for additional investigations.

#### SUMMARY

Broth cultures of hemolytic streptococci derived from patients are capable of rapidly liquefying normal human fibrin clot.

The active fibrinolytic principle is also contained in sterile, cell-free filtrates of broth cultures. The degree of activity of filtrates parallels the activity of whole broth cultures sufficiently closely to indicate that large amounts of the fibrinolytic substance are freely excreted into the surrounding medium and pass readily through Berkefeld V, Seitz, and Chamberland filters.

The occurrence of fibrinolysis is most strikingly observed when plasma or fibrinogen is mixed with active cultural material before clot formation is effected. Under the standard experimental conditions described, complete dissolution of human plasma clot (whole oxalated plasma +  $\text{CaCl}_2$ ) occurs in about 10 minutes; complete dissolution of human fibrinogen clot (chemically isolated fibrinogen + thrombin) takes place in about 2 minutes. Titration of filtrate activity is recorded in Table IV.

Twenty-eight strains of *Streptococcus hemolyticus*, isolated from patients suffering from various manifestations of streptococcus infection, have been tested for the capacity to liquefy fibrin clot. Broth cultures of all of the strains induced fibrinolysis.

Of 18 strains of *Streptococcus hemolyticus* of animal origin, only three were capable of causing dissolution of clot.

Completely negative results were obtained with 38 strains of other bacterial species. The list is presented on pages 492 and 493.

The plasma of many patients recovered from acute hemolytic streptococcus infections, when clotted in the presence of active cultures, is highly resistant to fibrinolysis. Furthermore, serum, derived from patients whose plasma clot is resistant, often confers on normal plasma clot an antifibrinolytic property. One example of the resistance possessed by the blood of convalescent patients is presented in this report. A second paper, now in preparation, will give in detail a large number of observations on the relation of in-

fection to the development of resistance to the fibrinolytic activity of hemolytic streptococci.

In contrast to the susceptibility of normal human fibrin clot to liquefaction by active culture, normal rabbit fibrin clot is totally resistant to dissolution when tested under comparable conditions. The insusceptibility of rabbit fibrin clot is manifest provided the coagulum is composed of rabbit constituents. When human thrombin is used to clot rabbit plasma or fibrinogen in the presence of active cultures, fibrinolysis is not prohibited. The rôle of thrombin in determining the resistance or susceptibility of rabbit fibrin to dissolution offers a suggestive approach to problems relating to the underlying mechanism.

#### REFERENCES

1. Brown, J. H., The use of blood agar for the study of streptococci, Monograph of The Rockefeller Institute for Medical Research, No. 9, New York, 1919.
2. Julianelle, L. A., *J. Exp. Med.*, 1926, **44**, 113.
3. Much, H., *Biochem. Z.*, 1908, **14**, 143.
4. Gratia, A., *Compt. rend. Soc. biol.*, 1919, **82**, 1245, 1247, 1393; 1920, **83**, 585; 1921, **85**, 25.
5. Gross, H., *Z. Immunitätsforsch.*, 1928, **59**, 510; *Klin. Woch.*, 1933, **12**, 304.
6. Tongs, M. S., *J. Am. Med. Assn.*, 1919, **73**, 1277.
7. West, R., and Stevens, F. A., *Proc. Soc. Exp. Biol. and Med.*, 1920-21, **18**, 234.  
Stevens, F. A., and West, R., *J. Exp. Med.*, 1922, **35**, 823.
8. Frobisher, M., *J. Exp. Med.*, 1926, **44**, 777.