FIBRINOLYSIS IN GRAM-NEGATIVE BACILLI

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The bacteriology of peritonitis complicating appendicitis has been carefully studied with varying emphasis on the role of each of the more commonly found organisms. Steinberg (1944) and others (Rademaker, 1933; Altemeier, 1938) have emphasized the role of the gram-negative bacilli in peritonitis. Bower and his associates (1928, 1938) have shown the importance of the clostridia, particularly *Clostridium welchii*, both experimentally and clinically, whereas Meleney and his associates (1931, 1932) have re-emphasized the importance of gram-negative bacilli and have shown the importance of synergism of two or more organisms in the mortality associated with peritonitis.

Recently interest has been aroused in the role of fibrinolysis in experimental appendiceal peritonitis by the observations of Kay and Lockwood (1947), who have found little correlation between mortality and the initial flora in the appendix but have emphasized rather the prognostic importance of the fibrinolytic and antifibrinolytic activity of the plasma. Increased fibrinolytic activity before onset and during the disease was of unfavorable prognosis.

Christensen (1945), continuing the original work of Milstone (1941), has demonstrated that fibrinolysis by *beta*-hemolytic streptococci was caused by the activation of plasma fibrinolysin by a specific factor in culture filtrates which he has named *streptokinase*. A similar factor apparently is produced by certain strains of clostridia (Lockwood, 1946; Reed, Orr, and Brown, 1943). Fibrinolysis has also been reported by Neter and Witebsky (1936) to occur with cultures of coliforms, *Pseudomonas pyocyaneus*, *Proteus*, and *Streptococcus enteritidis*. An analysis of the fibrinolytic capacities of various strains of gram-negative bacilli isolated in this laboratory was undertaken to supplement other data on these organisms.

METHODS AND MATERIALS

(1) The main methods used depend upon observing the disappearance of a fibrin clot. The first method employed was described by Tillett and Garner (1933) in their original report on the fibrinolytic activity of streptococci. In some cases the plasma was clotted first, and the culture was layered on top of the clot. The tubes were incubated at 37 C for about 24 hours in most cases. The degree of liquefaction was recorded as 1 to 4 plus, 1 plus being given for each one-fourth of the length of the clot that was lysed in the tube. The decrease in viscosity of gelatin solutions that resulted from the enzyme activity was determined with Ostwald viscosimeters at 37 C in the semiquantitative experiments.

(2) The bacteria employed were organisms obtained from surgical patients

and dogs with peritonitis, and a strain of hemolytic streptococcus, group C, strain P-168, which was used as a source of streptokinase. This is a stock strain from the Department of Bacteriology and was obtained through the courtesy of Dr. H. E. Morton.

(3) The media employed were brain-heart infusion broth (Difco), nutrient broth, and gelatin (Difco). In some mixtures 2 per cent glucose was added to these media, but in others it was omitted.

(4) The substrates used to test lytic activity were 1:4 or 1:5 diluted, citrated dog plasma clotted with human thrombin (Sharp and Dohme). Gelatin (Knox P-20) was used to follow the decrease in viscosity with lysis.

(5) Streptokinase was prepared by a modification of the method of Christensen (1945). Extractions of cultures of *Escherichia coli* were prepared in similar fashion, and by freezing and thawing or prolonged grinding, in order to break up the cells.

(6) The plasminogen was prepared by one-third saturation of human serum with ammonium sulfate. The precipitate was washed in one-third saturated ammonium sulfate, and dissolved in one-tenth the original volume of physiological saline.

It is of interest to note that the plasminogen used in these determinations was prepared from lyophilized serum which had been stored at 5 C since 1939. Active plasminogen preparations could be obtained by ammonium sulfate precipitation, but active plasmin was not found when chloroform extraction was used as it is in fresh serum. Christensen (1946) reported that plasminogen in fresh plasma is destroyed by chloroform. The destruction of the enzyme by chloroform is important in view of the fact that chloroform has been employed to activate plasminogen, and Kay and Lockwood (1947) have recently used chloroform extraction as a test for total proteolytic activity in the blood.

(7) Phosphate buffer (M/10) at pH 7.4 with added 0.9 per cent sodium chloride was used as the solvent for the extraction of the bacterial cultures and for redissolving the plasminogen. Lactate buffers at M/5 concentration and various pH values were also used in some of the experiments.

(8) The indicators used were phenol red, bromphenol blue, nitrazene paper, and "Harleco" liquid indicator.

(9) Bacterial counts were made by the method of Wadsworth (1927), and turbidimetrically.

(10) Inhibitors were added to the cultures to be tested immediately before layering.

(11) Cell-free filtrates were obtained by Seitz filtration.

EXPERIMENTAL RESULTS

Sixty strains of gram-negative bacilli were tested for fibrinolytic capacities, and virulence for mice was determined by intraperitoneal injection for 50 of them. The over-all results are shown in table 1. Fibrinolytic and hemolytic capacities of *Escherichia coli* seem to be associated with virulence. Of these two, fibrinolytic capacity seemed to be the more important because virulence and

fibrinolytic activity disappeared simultaneously in artificial media even though hemolytic capacity remained, and of the ten most virulent strains eight showed no hemolytic capacity whatsoever. Virulence in the non-lactose-fermenting group seemed to bear no relation to hemolytic or fibrinolytic capacities.

It is noteworthy that the virulent strains of *Escherichia coli* compared favorably in virulence with clostridia of various types isolated from similar sources. These strains were resistant to penicillin at levels well above those commonly attained therapeutically in body fluids, and some were resistant to streptomycin.

TABLE 1

Correlation of virulence with hemolytic and fibrinolytic capacities

STRAIN	ORIGIN	VIRULENCE IN MILLIONS	HEMOLYSIS	FIBRINOLYSIS	PENICILLIN SENSITIVITY	STREPTOMYCIN SENSITIVITY	TYPE
18 strains killing mice at 10 or less			38%+	77%+			
Goldstein	Man	10	0	0	50	2	Nonlactose
346	Man	5	0	0	50	100	*Salmonella*
DL_2	Dog	5	0	0	Resistant	—	Nonlactose
DE	Dog	10	+	0	1,000	—	Nonlactose
C_2	Dog	2	0	+	100	4	*A. aerogenes*
Steinberg	300	2	+	+	50	3	*E. coli*
M_4	Dog	2	0	+	50	6	*E. coli*
E_1	Dog	4	0	+	20	4	*E. coli*
K_1	Dog	4	0	+	50	2	*E. coli*
DK_T	Dog	5	0	+	100	—	*E. coli*
Osborne	Man	6.5	0	+	1,000	3	*E. coli*
B_4	Dog	6.5	0	+	500	3	*E. coli*
C_7	Dog	7	0	+	100	—	*E. coli*
P_3	Man	10	+	+	20	2	*E. coli*
288	Dog	10	+	+	50	100	*E. coli*
DL_1	Dog	10	+	0	Resistant	—	*E. coli*
DG_2	Dog	10	0	0	300	—	*E. coli*
N_2	Dog	10	0	0	100	3	*E. coli*
32 strains requiring more than 10			72%+	32%+			

It was observed many times during the course of the study that clotting would not occur if cultures at low pH were added to the plasma. However, when these cultures were neutralized before mixing, clotting occurred normally. This, coupled with the observations of others (Dennis and Adham, 1937; Tillett, 1937), was considered as evidence that this anticoagulant effect was related to low pH and was entirely separate from fibrinolysis.

Lysis was usually associated with a fall in pH early in the period of incubation, which suggested that the whole process might be accounted for by the acidity resulting from bacterial growth (figure 1). This was determined not to be the case by layering sterile lactate buffer at descending pH over plasma clots and determining the pH at which lysis occurred (table 2).

Lysis proceeded at equal rates with the acid neutralized and unneutralized

TABLE 2

Lysis of clots by lactate buffer

	pH								
	4.8	4.5	4.2	4.0	3.9	3.7	3.5	3.3	2.9
8 hours.....	0	0	0	1+	1+	2+	2+	2+	2+
24 hours.....	0	0	±	2+	2+	3+	2+	2+	2+

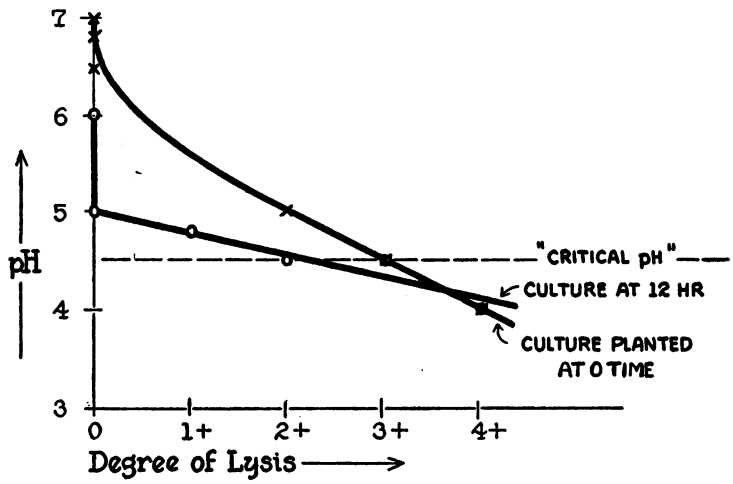

Figure 1. The relation of fibrinolysis to pH in culture of *E. coli* 428. Two and five-tenths ml of a 12-hour culture of *Escherichia coli* 428 in brain heart infusion broth with 2 per cent glucose and 2.5 ml of recently inoculated brain heart infusion broth with 2 per cent glucose were layered over a 2.5-ml clot in separate sterile test tubes. pH determinations, using nitrazene paper and "Harleco" indicator, were made at intervals.

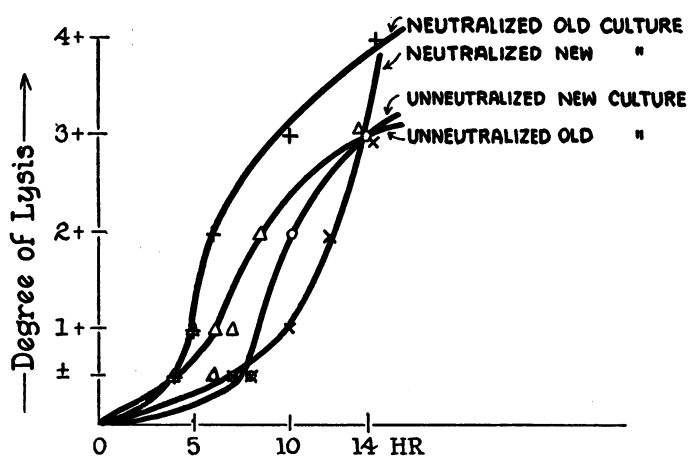

Figure 2. The effect of neutralizing acid in the culture on fibrinolysis by *E. coli* 428. Two tubes were set up with a 2.5-ml clot, over which was layered 2.5 ml of extract broth with 2 per cent glucose. This broth was inoculated with *Escherichia coli* 428 and incubated at 37 C. Two additional tubes were set up as above but with 2.5 ml of a 42-hour culture of *Escherichia coli* 428, which had been stored at 5 C, layered over the clot. The acid was neutralized with 2 N sodium hydroxide as it formed in one tube, but in the other tube it was allowed to increase without neutralization.

(figure 2). Ancillary evidence against acid's being the only cause of lysis is found in the frequent observation of loss of fibrinolytic capacity of *Escherichia coli* maintained on artificial media with retention of acid-forming properties.

Cell-free filtrates of cultures of coliforms were found to have no fibrinolytic activity both with intact bacilli and after repeated rapid freezing and thawing of the cultures in an attempt to break down the cells. The possibility that *Escherichia coli* secreted an activator of the character found by Christensen (1945) to be formed by hemolytic streptococci was considered. To test this hypothesis a vigorously fibrinolytic strain, *Escherichia coli* 428, was grown in 1 liter of brain heart infusion broth with 2 per cent glucose. Acid was neutralized with 5 N sodium hydroxide as it formed. After growth stopped, the supernatant fluid

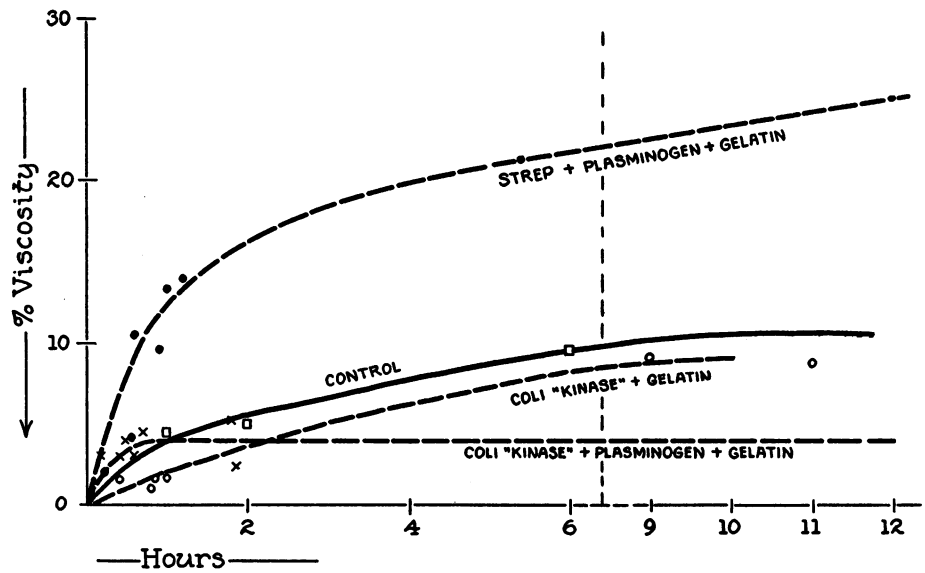

Figure 3. The fibrinolytic activity of extracts of culture of *E. Coli* 428. The fibrinolytic activity of cell-free extracts of *Escherichia coli* 428 with and without added plasminogen are compared with the effects of streptokinase in a similar system.

was decanted from the bacterial sediment and saturated with ammonium sulfate. The precipitate was collected and dissolved in phosphate buffer at pH 7.4. One-half ml of this preparation was mixed with 1.0 ml of plasma, and, after clotting, observed for fibrinolysis. None was found to occur.

The production of proteolytic enzyme by *E. coli* 428 was considered as a possible explanation of this phenomenon. Therefore gelatin media (Difco) with a solution of 2 per cent glucose was inoculated with this organism. No lysis of the gelatin occurred over a period of 3 weeks, although good growth was obtained.

Semiquantitive tests were undertaken to determine if any significant proteolysis occurred with the protein extract of the cultures. Decrease in viscosity of gelatin solutions was measured. To test for fibrinolytic activity of the type found by Christensen (1945) these experiments were repeated with the addition

TABLE 3

Fibrinolysis with various inhibitors

SUBSTANCE	COAGULATION			FIBRINOLYSIS		
	In vitro	*In vivo*	Alone	$ChCl_3$ act.	Strep.	Coli
Polysulfonic acids						
Heparin	−	−	0	−	−	0
Chlorazole	−	−	0	−	−	−
Trypan blue	−	−	0	−	−	+
Polyamino heterocyclic bases						
Methylene blue	+	0	0	−	−	−
Toluidine blue	+	0	0	−	−	−
9-Aminoacridine	+	0	0	−	−	−
Lipids						
Unsat. fatty acids	0	0	0	−	Not run	Not run
Cholesterol	0	0	0	−	−	−
Cholesterol esters	0	0	0	0	0	−
Mixed phospholipids	0	0	0	0	0	Not run
Cephalin	0	+	0	0	0	Not run
Thromboplastin	+	+	0	−	−	−
Lyso phosphatides	0	0	0	0	−	+
Compounds active in clot formation						
Vitamin K (2-methyl-1,4-naphthoquinone)	0	+	0	Not run	0	Not run
Thrombin	+	+	0	−	−	−
Dicoumarin	0	−	0	Not run	0	Not run
SH active						
Adenylic acid	−	Not run	0	+	+	0
KCN	−	Not run	0	+	+	+
Cystine	0	0	0	−	−	−
Cysteine	−	Not run	0	0	+	0
Antibiotics						
Sulfonamides						
Sulfanilamide	0	0	0	0	+	+
Sulfasuccidine	0	0	0	0	+	+
Sulfapyridine	0	0	0	Not run	0	−
Sulfathiazole	0	0	0	Not run	0	−
Sulfadiazine	0	0	0	Not run	+	+
Others						
Penicillin G	Not run	+	0	−	−	−
Streptomycin	+	Not run	0	−	−	−
9-Aminoacridine	+	0	0	−	−	−

of plasminogen derived from human plasma. The data obtained are shown in figure 3. There was no lysis of the gelatin comparable to that found with streptokinase under the conditions of the experiment.

Since whole cultures were fibrinolytic and since the ammonium sulfate precipitate was not, it seemed that there might be an active, nondialyzable substance in the supernatant. Accordingly the supernatant fluid was dialyzed against water until it was free of ammonium sulfate. The final solution showed no fibrinolytic activity when tested by the usual methods.

DISCUSSION

Too little attention has been paid in the past to the fibrinolytic activity of gram-negative organisms. Anticoagulant and fibrinolytic activities have been confused by many previous investigators. Neter and Witebsky (1936) reported fibrinolytic activity for cultures of many organisms, both gram-positive and gram-negative, including *Escherichia coli*, if 2 per cent glucose were included in the medium. These investigators apparently considered the test for fibrinolysis as positive if failure of coagulation occurred on the addition of calcium to mixtures of plasma and cultures. In a later study Witebsky and Neter (1936) reported two fibrinolysins for streptococci, one active in clot dissolution, the other in preventing clot formation. This clot inhibition had been previously reported for streptococci by other investigators (Dennis and Berberian, 1934; Tunnicliff, 1936). Dennis and Adham (1937) further studied the anticoagulant effect of streptococci grown in glucose broth and concluded that the inhibition of clotting was primarily due to lactic acid, and the determining factor was total acid rather than low pH. Tillett (1937) studied the anticoagulant and fibrinolytic activity of *Streptococcus hemolyticus*, *Streptococcus viridans*, and pneumococci. He concluded that the anticoagulant effect was due to pH below 5.0. Our results confirm Tillett's point of view.

Fibrinolytic activity was found in 31 of 60 strains of *Escherichia coli* tested by our methods. The experiments herein reported show that fibrinolysis does occur with *Escherichia coli*. There are several differences between *Escherichia coli* fibrinolysis and hemolytic streptococcus fibrinolysis, however. The lysis by *Escherichia coli* proceeds at a slower rate, and instead of starting as a generalized softening of the clot, followed by the disappearance of formed fibrin, it begins on the surface of the clot and proceeds from there. Lysis with streptococci occurs when they are grown on 0.05 per cent glucose media, but *Escherichia coli* will not consistently produce fibrinolytic activity at this glucose level. The extracts of cultures of *Escherichia coli* were inactive, but the cultures of the hemolytic streptococcus always yielded extracts that were active. No lysis occurred with *Escherichia coli* cultures in the absence of living, metabolizing bacteria, which may indicate that the process is related to the intrinsic enzyme systems of the bacteria. There was no effect on gelatin by any of the gram-negative bacilli studied. In table 3 it may be seen that various substances inhibited lysis by streptococci but increased lysis by *Escherichia coli*. Other substances, for example, cysteine and adenylic acid, showed the opposite effect. These differences support the contention of Tillett (1938) that no fibrinolytic substance equivalent to that of the hemolytic streptococci is found in *Escherichia coli*.

There is some positive correlation between fibrinolysis and virulence. However, the problem of *in vivo* activity is not solved in these experiments. In order to

produce maximum and consistent lysis with *Escherichia coli*, glucose is required in the medium in concentrations far exceeding the 100 mg per cent found in the body; and if the organisms metabolize *in vivo* by the same mechanisms used *in vitro*, it is extremely unlikely that much lysis of fibrin can occur. It may be that the *in vitro* fibrinolysis simply reflects some difference in the organism giving it greater pathogenic powers.

SUMMARY

Dissolution of fibrin by strains of *Escherichia coli* occurs.

This fibrinolytic capacity is separate and distinct from the acid-forming properties of the strains.

The fibrinolytic property is more often seen in strains most virulent for mice.

The lysis proceeds without significant trypsinlike activity.

The process differs from that seen with streptococci in several other important respects: (1) The lysis with coliforms is slower. (2) No cell-free extract was found to have activity. (3) To produce lysis the coliforms are best grown on a high glucose medium. (4) Significant differences in inhibition by trypan blue, lysophosphatides, adenylic acid, cysteine, and two sulfonamides exist.

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