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Hydrogen ion Concentration and Anticoagulating and Fibrinolytic Action of Cultures of Streptococci and Pneumococci.

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Recently Neter and Witebsky[1, 2] have reported that many strains of *Streptococcus viridans* and pneumococcus were found to be fibrinolytic for human fibrin provided the organisms with which the tests were performed were grown in broth containing 2% dextrose. Tunnicliff[3] reported that strains of *Streptococcus viridans* were incapable of dissolving normal fibrin clot but that cultures of smooth forms inhibited coagulation. The latter author used for liquid culture medium meat extract broth containing one percent dextrose. Dennis and Berberian[4] reported that some strains of *Streptococcus hemolyticus* and *viridans* inhibited the formation of fibrin in plasma. They stated that 2% dextrose broth gave more active cultures than did 0.5% dextrose broth.

Because of the experience in this laboratory, using culture media containing 0.05% dextrose, that neither strains of *Streptococcus viridans* nor Pneumococcus have been encountered which were capable of dissolving human fibrin under the experimental conditions previously described,[5] additional observations have been made with special consideration being given to the dextrose content of the culture media in which the organisms were cultivated in relation to anticoagulating and fibrinolytic activity of strains of *Streptococcus hemolyticus, Streptococcus viridans,* and Pneumococcus.

Culture Media. Plain meat infusion broth containing one percent neo-peptone but no buffer, has been employed. Before use, the pH was adjusted to 7.4-7.6. Dextrose has been added to make concentration of 0.05%, 1.0%, and 2%.

Strains: Streptococcus hemolyticus. Ten strains derived from patients suffering from acute infections were employed. These strains were all known to be actively fibrinolytic.

Streptococcus viridans. Thirty-three strains, all of which were derived from patients with endocarditis, were tested.

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1 Neter, E., and Witebsky, E., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 549.
2 Witebsky, E., and Neter, E., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 858.
3 Tunnicliff, R., *J. Inf. Dis.*, 1936, **58**, 92.
4 Dennis, E. W., and Berberian, D., *J. Exp. Med.*, 1934, **60**, 581.
5 Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, 1933, **58**, 485.

Pneumococcus. Ten strains, all derived from patients with lobar pneumonia, were used.

All the cultures were approximately 18 to 24 hours old when used.

Test: The method was the same as previously described[5] and as used by the authors previously mentioned.[1-4] Immediately after $CaCl_2$ was added to the mixture of plasma and culture the tubes were placed in a waterbath at 37.5°C. and kept under continuous observation. The tubes were inspected at frequent intervals in order to note whether clot formation developed or was inhibited. Since the occurrence of fibrin dissolution necessitates the formation of fibrin, it is obvious that when coagulation failed to take place, the presence or absence of fibrinolysin could not be determined.

pH Determination: The broth cultures were centrifuged at high speed until the supernatant fluid was clear. 5-10 cc. were pipetted off and used in the tests for pH. LaMotte colorimetric block was used for most of the determinations. The range was from 4.4 to 8.4. Methyl red, chlorphenol red, and phenol red were used as indicators. In the experiment in which determination of pH in plasma-culture mixtures was made Beckman's glass electrode pH meter was employed.

The pH was determined on all cultures which were cultivated in broth containing 0.05%, 1.0%, and 2.0% dextrose. The tests with plasma concerning anticoagulating or fibrinolytic effect were carried out regularly at the same time with the whole broth culture; additional tests were also frequently made with the supernatant fluid of cultures which had been centrifuged.

The correlation between the pH of the full grown cultures and the result of tests for the prevention of coagulation or fibrinolytic action are presented in Table I.

An analysis of the table reveals the following: *Streptococcus viridans:* when grown in 0.05% dextrose broth the final pH of the cultures ranged from 6.6 to 7.2; coagulation occurred in every instance; dissolution of fibrin occurred in no instance.

When the same strains were grown in 1.0% dextrose broth the pH with 6 of the strains ranged between 5.0 and 5.6. With these 6 strains coagulation occurred, but no dissolution of fibrin. With the remaining 27 strains the acidity of the culture was found to range from pH 4.4 to 4.9. When tests were made with these cultures, coagulation did not occur. Since fibrin was not formed it is obvious that determination of fibrinolytic activity could not be made.

When the 33 strains of *Streptococcus viridans* were grown in 2% dextrose broth, the 8 cultures, which did not reduce the pH lower

TABLE I.
pH of Cultures in Relation to Coagulation and Fibrinolysis of Plasma.

% dextrose broth	*Strept. viridans*				Pneumococcus				*Strept. hemolyticus*			
	No. of strains	Range of pH	Coagulation*	Fibrinolysis†	No. of strains	Range of pH	Coagulation	Fibrinolysis	No. of strains	Range of pH	Coagulation	Fibrinolysis
0.05	33	6.6-7.2	33+	0	10	6.8-7.2	10+	0	10	6.6-7.2	10+	10
1.0	6	5.0-5.6	6+	0	6	5.1-5.6	6+	0	2	5.0-5.1	2+	2
									4	4.9	4+ Partial	4
	27	4.4-4.9	27—	X	4	4.8-5.0	4—	X	4	4.7-4.8	4—	X
2.0	8	5.1-6.0	8+	0	6	5.0-5.4	6+	0	4	5.1-5.2	4+	4
									4	4.9	4+ Partial	4
	25	4.4-4.8	25—	X	4	4.8	4—	X	2	4.8	2—	X

*Numerals refer to number of strains. + indicates clot formation. — indicates no clot formation.
†0 indicates no fibrinolysis occurred. X indicates no test since no clot formed. Numerals indicate number of strains causing fibrinolysis.

than 5, did not interfere with coagulation nor did fibrinolysis occur. The 25 strains which did form acid to the extent of lowering the pH below 5 inhibited coagulation.

The same intimate correlation between the pH of the test culture and the occurrence or inhibition of clot formation in the culture-plasma mixtures which occurred with the strains of *Streptococcus viridans* also held for the observations made with the 10 strains of Pneumococcus and 10 strains of *Streptococcus hemolyticus*. When the final pH was below 5.0, coagulation was prevented; when above 5.0, clotting occurred. *When coagulation occurred,* dissolution was brought about by the 10 strains of *Streptococcus hemolyticus* but the fibrin was unaffected by the 10 strains of Pneumococcus.

Additional evidence of the significance of the degree of acidity is brought out by tests for anticoagulating and fibrinolytic effect made with the following materials: (1) Sterile, uninoculated broth of varying pH; (2) Dextrose broth cultures with a final pH below 5, which were adjusted to above 5; (3) Plain broth cultures with a final pH above 5 which were adjusted to below 5. N/20 NaOH or N/1 acetic acid was used to adjust arbitrarily pH when necessary.

	pH 7.0		6.0		5.5		5.0		4.8-4.5	
	Coag.	Dis.	Coag.	Dis.	Coag.	Dis.	Coag.	Dis.	Coag.	Dis.
Sterile broth	Yes	No	Yes	No	Yes	No	Partial or No	No*	No	X
Strept. vir.	,,	,,	,,	,,	,,	,,	,,	,,	,,	X
,, *hem.*	,,	Yes	,,	Yes	,,	Yes	,,	Yes*	,,	X
Pneumococcus	,,	No	,,	No	,,	No	,,	No*	,,	X

*Result dependent upon whether or not partial coagulation occurred.

In the above experiment the formation of fibrin or inhibition of coagulation was directly related to the pH regardless of whether the degree of acidity was due to bacterial growth or was arbitrarily adjusted. Fibrinolysis occurred only with strains of *Streptococcus hemolyticus* at pH above 5. Below this degree of acidity the failure of coagulation to occur rendered impossible the determination of fibrin dissolution. When the dextrose broth culture of the strains of *Streptococcus viridans* and Pneumococcus, having pH below 5 and inhibiting coagulation were adjusted to pH above 5, coagulation occurred but no dissolution. When plain broth cultures of the same organisms, having pH usually 6.5 to 7.0 and not interfering with clot formation nor causing dissolution, were made acid to a degree of pH below 5, no clotting occurred and consequently lysis was not determinable. It may be further noted that sterile uninoculated broth, when adjusted to varying degrees of acidity, gave results indistinguishable from those obtained with cultures of *Streptococcus viridans* or Pneumococcus.

The final experiment consisted of a determination of the pH of plasma and cultures of *Streptococcus viridans,* the measurement being made after the materials were mixed. The results, correlated with the occurrence or absence of clot formation using the same materials, are contained in the following protocol:

Green	strep.	+	plasma	—	pH	of	mixture	7.20	—	clot	15	min.
,,	,,	+	,,	—	,,	,,	,,	7.16	—	,,	,,	,,
,,	,,	+	,,	—	,,	,,	,,	6.18	—	,,	30	,,
,,	,,	+	,,	—	,,	,,	,,	6.14	—	,,	60	,,
,,	,,	+	,,	—	,,	,,	,,	6.11	—	,,	45	,,
,,	,,	+	,,	—	,,	,,	,,	5.65	—	no	clot	
,,	,,	+	,,	—	,,	,,	,,	5.57	—	,,	,,	

The results indicate the delayed effect on coagulation when the pH is 6.1 + or —, and the absence of coagulation at pH 5.5 to 5.6.

In a review of the physiology and pathology of blood coagulation, Wöhlisch[6] refers to the several investigators who have reported that pH 6.4-6.6 is optimal for fibrin formation. Eagle,[7] using fibrinogen and thrombin preparations, has found that fibrin formation is delayed at pH less than 6.2 and does not usually occur below 5.6. It is well known that streptococci and pneumococci produce a relatively high degree of acidity when grown in culture media containing appropriate amounts of sugar. It seems reasonable, therefore, to interpret the results which have been presented as indicating that the inhibition of coagulation by cultures grown in 1% or 2% dextrose broth is due not directly to a special property of the organism (antithrombin or antiprothrombin) which interferes with the phenomenon, but to the degree of acidity of the culture, which on addition to plasma, denatures the fibrinogen or other components involved in the process of blood coagulation. Recently Dennis and Adham[8] have reported results which emphasize the importance of acid contents of cultures in relation to anticlotting activity. The observations recorded in this article lend support to their findings.

It should also be emphasized that when the pH of any of the cultures was below 5.0, the failure of solid fibrin to form rendered impossible a determination of fibrinolytic activity. When the sugar content of the culture was so slight that growth of the organism did not lower the pH below 5.0 or when the acidity of the 1 or 2% dextrose broth cultures was arbitrarily altered to a pH sufficiently high to exclude the denaturing effect of acid, none of the strains of *Strep-*

6 Wöhlisch, E., *Ergebn. der Physiol.,* 1929, **28**, 443.

7 Eagle, H., *J. Exp. Med.,* 1937, **65**, 613.

8 Dennis, E. W., and Adham, L. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 84.

tococcus hemolyticus, viridans, or Pneumococcus inhibited coagulation; only the strains of *Streptococcus hemolyticus* induced fibrin dissolution.

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Bactericidal Action of Human Serum on Hemolytic Streptococci. Active Principle Obtained by Fractionation of Sera.

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The present report is part of an investigation concerning the capacity of serum from patients who are acutely ill to destroy hemolytic streptococci of the *beta* type. Previous reports[1] have demonstrated that the bactericidal property under consideration was demonstrable in the serum derived from patients during the period of active disease due to a variety of infections, but that following recovery from illness the streptococcidal activity was greatly diminished, as measured by the methods which were employed. Furthermore, normal sera have been found to be essentially devoid of streptococcidal activity and have served as controls throughout the observations.

The previous articles have described the technical procedures. In the present study a strain of *Streptococcus hemolyticus,* which has been found to be uniformly highly sensitive to the killing effect of patients' sera, has been used in all of the experiments. The samples of sera were derived from patients who were acutely, and usually severely ill from diseases such as pneumonia, or pyogenic infections due to different kinds of organisms.

Studies have been carried out in an attempt to isolate the active principle in serum which is responsible for the streptococcidal activity. Observations have been made with a protein-fraction and a non-protein constituent. The materials have been used separately and in combination.

The protein-fraction most regularly employed in the tests has been obtained by precipitation of serum at low temperatures with alcohol

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1 Tillett, W. S., *J. Exp. Med.*, 1937, **65**, 147, 163.