varied independently and was never higher than 1:20. We have reasons to believe that the activity against gram-positive and gram-negative organisms is due to two different substances which might be separated by their selective solubility in organic solvents. The substance active against staphylococci is also active against *M. tuberculosis* BCG. The anti-coli substance is less stable and has not yet been investigated for activity against BCG.

Both substances can be obtained in crude form from the medium, (1) by extraction with chloroform, either directly or after preliminary concentration of the medium; (2) by adsorption onto Norit and subsequent elution with chloroform and (3) by saturation with ammonium sulfate and extraction of the precipitate with chloroform. Chloroform extracts (1) and (3) can be partially decolorized by treatment with Norit with hardly any loss of activity. The active substance seems to be dialysable through Cellophane membranes.

Probably identical substances can also be extracted from the mold itself by alcohol, acetone, chloroform or ether, supporting Vaudremer's[2] observation made with press juice of the same mold.

At present our efforts are directed mainly towards the final purification of the active substance, as the work with crude products has only presumptive value.

The activity of the partially purified preparations was established by the following methods: (1) for staphylococci and *B. coli:* (a) by serial dilutions in papain broth[3]; (b) by a new method, using the permeability of soft agar for testing growth inhibitory substances, the particulars of which will be published separately. (2) For acid-fast organisms by two methods: (a) bacteriostatic action was investigated by making serial dilutions of the substance in Kirchner's[4] medium and inoculating the tubes with a suspension of BCG. Preliminary readings were taken after 5 to 10 days and final examinations for growth were made after 6 weeks; (b) bactericidal action was estimated by incubating a heavy suspension of BCG with different dilutions of the substance for 24 hours and subsequently inoculating Petragnani slants with 0.1 ml of the mixture. Results were read after 6 weeks.

Our experiments with the crude preparations seem to indicate that: (1) they possess a high degree of activity against staphylococci, preventing their growth in 1:700,000 of the dry crude substance; (2) their bacteriostatic activity against BCG appears to be higher, preventing growth in at least 1:1,400,000 dilution; (3) their bactericidal action against BCG is equal to or slightly lower than their anti-staphylococci activity.

[3] I. N. Asheshov, *Can. Publ. Health Jour.*, 32: 468, 1941.

[4] O. Kirchner, *Zbl. f. Bakt.*, I Orig., 124: 403, 1932.

Often a BCG emulsion treated with a 1:500,000 dilution of the active substance produced no growth on Petragnani slants, but occasionally isolated colonies appeared on slants inoculated with lower dilutions. We attribute this phenomenon to imperfect emulsion of the BCG culture, resulting in lumps which protect the bacterial cells from the action of the antibiotic.

No bactericidal action on avian type of *M. tuberculosis* was observed even in dilutions as low as 1:100.

The active substance is poorly soluble in water: the activity of aqueous extracts of the dry substance against staphylococci and BCG never exceeded 1:40,000.

We consider animal experiments on toxicity and activity of the crude substance valueless and are postponing them until preparations of a greater purity are obtained.

The active substance investigated may be similar to fumigacin or helvolic acid (Waksman;[5] Chain *et al.*[6]), though some of its properties seem to indicate that the two antibiotics are not identical.

Some other, as yet unidentified, molds were found to produce active substances against tubercle bacilli and are now under investigation.

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A QUANTITATIVE STUDY OF THE FIBRINOLYSIN-ANTIFIBRINOLYSIN REACTION[1]

THE antifibrinolysin test devised by Tillett and Garner[2] is based upon the observation that Group A hemolytic streptococci produce a substance, fibrinolysin, which dissolves the plasma clot of normal individuals, whereas the plasma clot of individuals convalescent from hemolytic streptococcal infections is generally resistant to lysis. This resistance is attributed to the presence in the blood of specific antibody, antifibrinolysin.

In 1938, Milstone[3] reported that the process of streptococcal fibrinolysis required the presence of an accessory lytic factor normally present in human

[5] S. A. Waksman, SCIENCE, 99: 220, 1944.

[6] E. Chain *et al.*, *Brit. Jour. Exp. Path.*, 24: 108, 1943.

[1] This investigation was supported through the Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of The Surgeon General, United States Army, and by grants from the Commonwealth Fund, the W. K. Kellogg Foundation, the John and Mary R. Markle Foundation and the International Health Division of the Rockefeller Foundation to the Board for the Investigation and Control of Influenza and Other Epidemic Diseases for the Commission on Acute Respiratory Diseases.

[2] W. S. Tillett and R. L. Garner, *Jour. Exp. Med.*, 58: 485, 1933.

[3] H. Milstone, *Jour. Immun.*, 42: 109, 1941.

serum. Studies from this laboratory[4] and those by Christiansen[5] have indicated that, in the fibrinolytic process, the lytic factor is converted by fibrinolysin into an active tryptase. It was shown that the mechanism of streptococcal fibrinolysis consists of two stages:

(1) Activation of the lytic factor by fibrinolysin with the production of an active tryptase.
(2) Dissolution by the tryptase of the fibrin clot.

The present report is concerned with a study of (1) the factors participating in the inhibition of the fibrinolytic reaction, as well as the application of these observations to the formulation of an accurate method for the estimation of serum antifibrinolysin; and (2) observations concerning the relation of specific strains of β-hemolytic streptococci to the antifibrinolysin response in man.

(1) Fibrinolysin-Antifibrinolysin Reaction

Lyophilized preparations of human fibrinogen[6] were kindly supplied by Drs. E. J. Cohn and S. Howard Armstrong, Jr. The fibrinogen contained the lytic factor in adequate and constant amount. The thrombin employed was a highly active commercial preparation (Lederle) from rabbit blood. Fibrinolysin was obtained by alcoholic precipitation according to the procedure of Garner and Tillett.[7]

Observations made on the problem of the resistance of plasma clots to lysis indicated that the inhibition of either one of the two stages of the fibrinolytic mechanism manifested itself as an apparent resistance to the action of fibrinolysin. Thus, the resistance conferred on a fibrin clot by a given serum was found to result from: (a) the presence in the serum of a specific antifibrinolysin; or (b) the presence of antiproteases. Sera having high antiprotease titers measured against preparations of chloroform-activated serum tryptase[8] also yielded high "antifibrinolysin" titers, as might be expected. However, sera possessing elevated antifibrinolysin titers showed no correlation with their antiprotease titers. It was further observed that the resistance to fibrinolysin of sera from cases of pneumococcal pneumonia could be attributed to an elevated antiprotease concentration. In general, the normal sera and the acute and convalescent sera obtained from cases of hemolytic streptococcal infections yielded negligibly low antiprotease titers which did not interfere with the determination of antifibrinolysin.

Antifibrinolysin was found to combine rapidly and specifically with fibrinolysin in multiple proportions depending upon the ratio in which these two substances were mixed. The reaction was thus similar to toxin-antitoxin reactions *in vitro*. The combinations of fibrinolysin and antifibrinolysin was approximately 85 per cent. complete after incubation for thirty minutes at 37° C.

A method was devised for the quantitative determination of antifibrinolysin in serum by the proper control of the concentrations and activities of the reagents participating in the fibrinolytic process. The procedure required the standardization of the three factors: fibrinogen, lytic factor and fibrinolysin. Since the fibrinogen product contained the lytic factor in constant and adequate amount, the use of a single preparation throughout the study simultaneously controlled the fibrinogen and lytic factor concentrations. A solution containing 0.18 per cent. fibrinogen (0.60 g of Fraction I per 100 ml) was employed since it gave a clot which was both firm and readily susceptible to lysis. For standardization, the solution of fibrinolysin was diluted progressively and added in 0.5 ml amounts to a series of tubes containing 0.5 ml of fibrinogen solution and 1.0 ml of buffered saline. The clot was produced by further addition of 0.2 ml of thrombin. Incubation was carried out for 30 minutes at 37° C. The highest dilution permitting complete dissolution of the fibrin clot contained the required concentration of fibrinolysin and this amount was termed one unit.

The procedure for determination of the antifibrinolysin titer of serum was as follows: 0.5 ml amounts (1 unit) of the proper fibrinolysin dilution were added to a series of tubes containing 1.0 ml of sucessive dilutions of serum. After incubation for 30 minutes at 37° C., 0.5 ml of fibrinogen and 0.2 ml of thrombin were added to each tube to form the standard clot. The tubes were then incubated at 37° C. for 60 minutes. The highest dilution of serum which completely prevented lysis of the standard clot after 60 minutes incubation was taken as the titer of the serum. A clot was regarded as lysed if it "ran" or "slid" in the slightest degree when the tube was inverted for ten seconds. The range of serum dilutions employed extended from 1/50 to 1/2,000 (initial dilutions).

This method of determining antifibrinolysin has given reliable and reproducible results. A detailed analysis of the antifibrinolysin titers obtained in a group of 3,504 sera collected from approximately 900

[4] M. H. Kaplan, *Proc. Soc. Exp. Biol. and Med.*, 57: 40, 1944.
[5] L. R. Christiansen, *Jour. Bact.*, 47: 65, 1944.
[6] The product employed was Fraction I of the plasma proteins prepared by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, from blood collected by the American Red Cross, under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.
[7] R. L. Garner and W. S. Tillett, *Jour. Exp. Med.*, 60: 239, 1934.
[8] H. J. Tagnon, C. S. Davidson and F. H. L. Taylor, *Jour. Clin. Invest.*, 21: 525, 1942.

individuals, including normal men as well as those with respiratory diseases, is being presented elsewhere.

(2) Production of Antifibrinolysin

In previous studies,[9] it was demonstrated that bacteriological and clinical evidence of β-hemolytic streptococcal infection is not necessarily sufficient to establish an etiological diagnosis; rather, the development of specific antibody during convalescence is required. In known streptococcal infections, such as scarlet fever and epidemic sore throat, there is a significant increase in antistreptolysin antibodies in about 85 per cent. of the convalescent sera. For the purpose of the present study, patients with exudative tonsillitis or pharyngitis from whom types 3, 5, 19 or 12[10] streptococci were isolated in one or more of three cultures of the throat, and who developed antistreptolysin antibodies during convalescence, are included as *proved* instances of streptococcal infections. In all, 110 hospitalized soldiers are included in this analysis.

The distribution of the cases according to type of streptococcus and the results of the antifibrinolysin determinations are recorded in Table 1. The type 3

TABLE 1

Antifibrinolysin Response in Streptococcal Infections

Type of Group A β-Hemolytic Streptococcus	Occurrence of cases	Antifibrinolysin test		
		No. of cases positive	No. of cases negative	Per cent. positive
3	Endemic	1	9	10
5	Epidemic	15	61	20
19	Endemic	8	5	62
12	Epidemic	10	1	91

and type 19 infections occurred endemically during 1943 and 1944. In contrast, the type 5 infections were the result of a food-borne epidemic, and at least nine of the type 12 infections occurred in a single small outbreak. Presumably, the sporadic cases were produced by several strains of the given types, while the epidemic cases resulted from a single strain.

The variation in the antifibrinolysin response in these subjects was marked. Only one of the type 3 infections exhibited a significant response, whereas a rise in antibodies was demonstrated in 62 per cent. of the type 19 infections. Similarly, there was a difference between type 5 epidemic cases with only 20 per cent. positive antifibrinolysin tests and type 12 infections with 91 per cent. These marked differences in antibody formation suggested that the development of antifibrinolysin in man might be related to the fibrinolytic capacity of the infecting organism.

[9] Commission on Acute Respiratory Diseases, *Jour. Am. Med. Assn.*, 125: 1163, 1944.

[10] Type specific rabbit serums were made available through the generosity of Drs. Homer T. Swift and Rebecca C. Lancefield.

A test was devised therefore to measure the amount of fibrinolysin produced by these streptococci *in vitro*. The type 12 strains were not available for study. The average production of fibrinolysin of nine of the type 3 strains was found to be 40 units per ml of culture medium, that of the type 5 strains 90 units, and of the type 19 strains 180 units. These results suggest that the antifibrinolysin response in the subjects reported here is related to the ability of the homologous organism to produce fibrinolysin *in vitro*. It should be emphasized, however, that the amount of fibrinolysin produced is not necessarily a property of a given Lancefield type, but may be a strain characteristic. For example, some carrier strains of type 3 streptococci, isolated from the same population groups supplying the above cases, have the ability to produce large amounts of fibrinolysin.

Summary

The results of a study of the streptococcal fibrinolysin reaction and its inhibition by sera containing specific antibody are presented. It was possible to devise a quantitative antifibrinolysin test by controlling the various factors influencing the reaction. In man, the antifibrinolysin response was found to vary according to the strain of streptococcus responsible for the infection.

Commission on Acute Respiratory Diseases[11] in Collaboration with
Melvin H. Kaplan

THE IMMUNIZING EFFECT OF CALCIUM PHOSPHATE ADSORBED INFLUENZA VIRUS[1, 2]

For the purpose of enhancing the antigenic activity of certain proteins, toxins and infectious agents a variety of adjuvants have been employed. As applied to influenza virus vaccines, Friedewald[3] has described the adjuvant effect of oily substances and acid-fast bacilli when combined with formalin-inactivated virus. In view of the local reactions resulting from subcutaneous injections of such mixtures in animals, Friede-

[11] Members and professional associates of the Commission on Acute Respiratory Diseases are: John H. Dingle, Major, M.C., A.U.S., Director; Theodore J. Abernethy, Major, M.C., A.U.S.; George F. Badger, Captain, M.C., A.U.S.; Joseph W. Beard, M.D.; Norman L. Cressy, Major, M.C., A.U.S.; A. E. Feller, M.D.; Irving Gordon, M.D.; Alexander D. Langmuir, Captain, M.C., A.U.S.; Charles H. Rammelkamp, Jr., M.D.; Elias Strauss, Captain, M.C., A.U.S.; and Hugh Tatlock, 1st Lieutenant, M.C., A.U.S.

[1] From the Department of Epidemiology and the Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor.

[2] These investigations were aided through the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

[3] Wm. F. Friedewald, SCIENCE, 99: 453, 1944.