Bacterial Activators (*Lysokinases*) of the Fibrinolytic Enzyme System of Serum.* (17549)

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In recent years, revived interest in a proteolytic enzyme system of the blood has resulted in new data and some difficulties as to terminology.(1,2)

In view of the practical importance of *fibrinolytic* phenomena, we have accepted the "fibrinolysin" rather than the "tryptase" or "plasmin" nomenclatures. A brief summary of these terminologies and our abbreviations follows: 1. *Lysin* or fibrinolysin (tryptase, plasmin): the active proteolytic (fibrinolytic) enzyme; 2. *Antilysin* or antifibrinolysin (antitryptase, antiplasmin): inhibitor of lysin; 3. *Prolysin* or profibrinolysin (tryptogen, plasminogen): the inactive precursor of lysin; 4. *Lysokinase* (tryptokinase): any activator of prolysin, *e.g.* streptokinase,(3) staphylokinase,(4) fibrinokinase;(5) 5. *Antilysokinase* (antitryptokinase): inhibitor(s) of lysokinase(s), *e.g.* antistreptokinase.(6)

The present study concerns methods and results of an extensive survey of available bacterial types for *lysokinase* activity. Earlier studies(3,7) showed that streptokinase specifically activates only human prolysin, while staphylokinase affects prolysin from human, dog, guinea pig, and rabbit sera but not other species tested.(8) This further search for new bacterial lysokinases and particularly for an activator of bovine prolysin has been somewhat discouraging.

Materials. Thrombin. Upjohn's bovine thrombin (courtesy Dr. J. T. Correll), 20 units per cc aqueous (sterile) solution. *Fibrinogen*: 1% aqueous solution of Armour's bovine "Fibrinogen," pH = 7.0 ± 0.2, sterilized by Seitz filtration. *Prolysin preparations*: A. For Survey Tests—(1) Human Plasma Fraction–I† (courtesy Dr. J. T. Edsall), 2% aqueous (sterile) solution, containing fibrinogen as well as prolysin; (2) Dog and Bovine Sera, sterilized by Seitz filtration: B. For Lysokinase Tests—(3) Human, Dog, and Bovine I Serum Fractions, prepared from pooled sera, at 0°C, by precipitation at 25% concentration of ethanol, the deposit after one hour being recovered by centrifugation and redissolved in one-half the original serum volume of borate buffer(7) (pH = 7.75) and stored frozen at −20°C; (4) Bovine II, a fraction prepared by 33% saturation of serum with $(NH_4)_2SO_4$, at 1°C, as previously described.(7)

Bacterial cultures. Stock cultures from the Bacteriology Laboratory of the University of North Carolina were obtained through the courtesy of Drs. D. A. MacPherson and F. L. Rights. The β-hemolytic streptococcus (No. 98) control culture was obtained from Dr. Maxwell Finland of the Thorndike Memorial Laboratory (Boston). Additional cultures isolated from bovine sources were made available through the courtesy of Dr. L. T. Giltner, U. S. Dept. of Agriculture, and Dr. H. E.

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† This product was developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

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Stock medium for survey tests. "Yeast peptone," containing yeast extract (1%), tryptose (2%), dextrose (0.2%), NaCl (1.6%), pH = 7.6 ± 0.2, sterilized by autoclaving. This *double strength* stock medium was diluted by the other materials of the test mixtures (see below). *Un*inoculated mixtures of medium plus fibrin clots, with and without added prolysin (human, dog, bovine), showed no fibrinolysis.

Methods. The first procedure was a general survey of all available bacterial cultures for fibrinolytic activity as manifested during growth in medium + fibrin clots, with and without added prolysins. Secondly, selected cultures showing positive results in the survey test, were followed up with attempted isolation of a partially purified lysokinase by the standard alcoholic precipitation of culture filtrates as used for streptokinase(9) and staphylokinase.(4)

Survey method. For aerobes, sterile fibrin clots (with nutrient medium), in 13 × 100 mm tubes, were inoculated from 24 hour broth subcultures by stabbing and seeding of surface. For anaerobes, the clots were doubled in volume and covered with paraffin. Control (no prolysin) and prolysin-containing clots were incubated at 37°C and observed after 24 and 48 hours. Bacterial tubes in which growth was not satisfactory were repeated or discarded and in those reported growth was successful. Each bacterial strain was tested in the following 4 clot mixtures: I. Control (no prolysin): 1 cc medium + 1 cc fibrinogen + 0.2 cc thrombin; II. Human: 1 cc medium + 1.5 cc Human Plasma Fraction–I (prolysin and fibrinogen) + 0.2 cc thrombin; III. Dog: 0.5 cc medium + 0.5 cc dog serum (prolysin) + 1.0 cc fibrinogen + 0.2 cc thrombin; IV. Bovine: 0.5 cc medium + 0.5 cc bovine serum (prolysin) + 1.0 cc fibrinogen + 0.2 cc thrombin.

Lysokinase study. Bacteria selected as a result of the survey test (see above) were cultured for 24 hours in 500 cc Bacto heart infusion broth and centrifuged subsequently to remove suspended bacteria. The selected anaerobe (*Cl. botulinus*) was grown in thioglycollate broth. The "lysokinase," in each instance, was prepared by the alcoholic precipitation method of Garner and Tillett,(9) yielding a dry powder, subsequently prepared for use as a 1% solution in borate buffer and tested for activation of human, dog, and bovine prolysins (see *Materials*), utilizing methods previously described.(7)

Results. Survey study. Seventy-seven cultures of 40 types of bacteria were satisfactorily submitted to the lysis tests of the 4 types noted under *Methods.* Some common organisms were omitted because cultures were unavailable at the time or, in some cases, because of failure to grow under the test conditions. Completely negative results (no significant lysis of any of the clots) were obtained with: *Streptococcus pyogenes* (5 strains, incl. 1 erysipelatis—but *cf.* Table I), *Strep. faecalis, Strep. acidominium* (uberis), *Strep. mitis* (viridans—3 strains—but *cf.* Table I); *Micrococcus* (Staphylococcus) *citreus, Micr. pyogenes, var. albus* (*Staph. albus*—2 strains); *Gaffleya tetragena; Neisseria catarrhalis; Corynebacterium pseudodiphtheriticum; Vibrio metschnikovii; Alcaligenes faecalis; Escherichia coli* (3 strains); *Aerobacter aerogenes* (3 strains); *Proteus vulgaris* (1 strain); *Salmonella (Eberthella) typhosa* (2 strains); *Shigella paradysenteriae* (7 strains), *Shig. sonnei* (2 strains); *Pasteurella multocida (suilla), Past. pseudotuberculosis; Brucella melitensis,*‡ *Bruc. abortus,*‡ *Bacillus megatherium; Mycobacterium phlei.*

Table I summarizes the survey tests on all bacteria which produced significant fibrinolysis in any of the clots, whether in the control (I), without prolysin, or in the presence of human (II), dog (III), or bovine (IV) prolysin. The degree of lysis is roughly quantitated 0——+-+-+-+. Under *Comment* is noted the ability to liquefy gelatine, and the lack of correlation between this property and the fibrinolytic phenomena is noteworthy. Individual cultures of the same type often varied in the results of these tests. The strains asterisked in Table I were selected for the

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‡ Grown both aerobically and in CO_2.

TABLE I.
Survey of Fibrinolytic (I) and Lysokinase (II-IV) Effects of Certain Bacteria (See text).

Bacterial type	I Control	II Human	III Dog	IV Bovine	Comment
**Streptococcus pyogenes* (β-hemolytic)	0	++++	0	0	No. 98 (Finland)
**Staphylococcus aureus* (hemolytic) (*Micrococcus pyogenes, var. aureus*)	0	++++	++++	0	Gelatine liq.
**Staphylococcus aureus*† (hemolytic)	0	++++	++++	0	,, ,,
,, ,,	0	++	++++	0	,, ,,
,, ,,	0	++	++++	0	,, ,,
Streptococcus pyogenes	0	++++	0	0	
,, *mitis* (*viridans*)	0	+++	0	0	
**Sarcina lutea*	0	++++	+	0	,, ,,
**Pseudomonas aeruginosa*	+	+	++++	+	,, ,,
**Klebsiella pneumoniae* (*friedlanderi*)	++	++	+++	++	
,, ,,	0	0	+++	0	
**Serratia marcescens*	++	++++	0	0	,, ,,
**Salmonella paratyphi*	0	+++	++	0	
,, ,,	0	0	+	0	
,, ,,	++	++	++	0	
,, *schottmuelleri*	++	0	+	0	
* ,, *typhimurium* (*aertrycke*)	+++	+	+	+	
,, ,,	++	0	++	0	
,, *choleraesuis*	+++	+	+++	0	
,, ,,	++	++	0	0	
,, *enteritidis* (*gaertneri*)	+	+++	0	0	
,, *anatis*†	+++	0	++	0	
**Shigella ambigua*	0	+++	0	0	
,, ,,	+	+	+	0	
Brucella (*abortus*) *suis* (in O_2)	++++	0	0	0	
,, ,, ,, (in CO_2)	++++	0	0	0	
**Bacillus subtilis*	+	0	++++	+	,, ,,
,, ,,	+	0	+++	0	,, ,,
,, ,,	++++	++	++++	+	,, ,,
Clostridium septicum	++++	++++	++++	+++	,, ,,
* ,, *botulinum*	++++	++++	++++	+++	,, ,,
,, ,,	++++	++++	++++	+	,, ,,
,, *perfringens* (*welchii*)	++++	++++	+++	+++	,, ,,

* Cultures subsequently selected for *lysokinase* tests.
† Bacteria isolated from *bovine* sources.

subsequent lysokinase studies (see below). The streptococcus and staphylococcus strains listed separately at the top of Table I are typical good lysokinase producers and have been used repeatedly in this laboratory for preparation of streptokinase and staphylokinase, respectively. Strains isolated from bovine sources are indicated (†).

Lysokinase study. As indicated in Table I, lysokinase studies (see *Methods*) were attempted on alcohol-precipitated material isolated from 12 bacterial strains, namely, 1. β-hemolytic *Strep. pyogenes* (human source), 2. hemolytic *Staph. aureus* (human source), 3. hemolytic *Staph. aureus* (bovine source), 4. *Sarcina lutea,* 5. *Pseudomonas aeruginosa,* 6. *Klebsiella pneumoniae* (Friedlander), 7. *Serratia marcescens,* 8. *Salmonella paratyphi,* 9. *Salm. typhimurium* (Aertrycke), 10. *Shig. ambigua,* 11. *Bacillus subtilis,* 12. *Clostridium botulinum.*

Fibrinolytic tests(7) were performed after preliminary incubation of "lysokinase" preparations with the 4 prolysins (see *Materials*: B. (3), (4)) as follows: (1) no incubation (tested immediately), (2) 20 min. at 37°C, (3) 1 hour at 29°C, (4) 24 hours at 6°C. The staphylokinase of bovine origin (hemolytic *Staph. aureus*) activated both human and dog prolysins to approximately the same lytic strength as our standard (human) staphylokinase. It did not affect bovine prolysin, however. The material from *Clostridium botulinum* (anaerobic) cultures was

actively fibrinolytic but lysed clots containing prolysins in longer times than the prolysin-free control (lysis time: 15 min.) so that its action is presumably due solely to a proteolytic enzyme from the bacteria themselves. All other materials gave negative results in concentrations of 0.06-1.0%. There were insufficient materials for testing at higher concentrations.

Discussion. Omitted from these studies were a number of bacterial types, some of which were known to have been tested by earlier workers with the older "fibrinolytic" technics. Tillett(10) reviews a number of these and particularly states that the pneumococcus gives negative results. Our data clearly show that fibrinolytic activity is encountered in cultures of a wide variety of bacteria, but only in the case of certain hemolytic streptococci and staphylococci can a definite lysokinase be demonstrated. The results with *Clostridium botulinum* show the need for the prolysin-free control test. Fibrinolytic activity appears to be unrelated to the gelatine liquefaction test.

Summary. In a survey test of 77 satisfactory cultures of 40 bacterial types, fibrinolytic phenomena were encountered in 19 types (33 cultures) of bacteria tested. Only certain hemolytic streptococci and staphylococci gave positive results with a specific test for *lysokinase,* however, and these organisms were not directly fibrinolytic nor able to activate bovine profibrinolysin. *Direct* fibrinolytic activity may occur in cultures when (except in the case of *Cl. botulinum*) it is not found in the alcoholic precipitate. The failure to obtain a lysokinase in at least 6 types of bacterial cultures which gave good results in the survey test is disappointing, as is the failure to find a kinase for bovine profibrinolysin.

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A Substance in Egg Yolk Which Inhibits Deterioration of Aureomycin Activity.* (17550)

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In the treatment of chick embryos infected with psittacosis virus(1) the single dose of aureomycin required to protect 50% of infected embryos for 7-9 days was found to be one-third to one-fourth as great as the dose of chloromycetin required to produce the same effect. This finding was unexpected in view of the rapidity with which aureomycin deteriorates *in vitro* at 37°C under neutral or slightly alkaline conditions(2-6) as well as the relative stability of chloramphenicol under similar circumstances.(7-9) It appeared possible, therefore, that some factor, present in the embryonated egg, protected aureomycin from deterioration. In preliminary experiments, de-

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