A STUDY OF THE INHIBITION OF STREPTOCOCCAL PRO-
TEINASE BY SERA OF NORMAL AND IMMUNE
ANIMALS AND OF PATIENTS INFECTED
WITH GROUP A HEMOLYTIC
STREPTOCOCCI

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Frobisher (1926) demonstrated that certain strains of hemolytic streptococci, when grown in Holman's (1919) meat medium, produce a muscle-digesting enzyme which he named "histase." He showed that sterile filtrates of cultures grown in Holman's medium are capable of digesting muscle, but he was unable to demonstrate a proteolytic action of the enzyme on gelatin or coagulated serum. He also found that histase-producing strains of hemolytic streptococci did not necessarily digest casein; and he therefore concluded that histase was a specific enzyme for muscle tissue.

Seegal and Seegal (1936) described a nephrolytic substance produced by certain strains of group A hemolytic streptococci. They made cultures of streptococci in tubes of meat infusion broth containing thin slices of fresh monkey kidney. After 3 to 5 days' incubation, histological examination showed marked lysis of kidney tissue. Culture filtrates were also slightly nephrolytic. They found that Frobisher's "histase reaction" (digestion of chopped meat) was positive with all the nephrolytic strains tested.

While the present investigation was in progress, Elliott (1945) published his findings that group A streptococci under certain conditions produce an extra-cellular proteolytic enzyme which digests the type-specific M antigens of hemolytic streptococci and also other substrates such as fibrin, casein, milk, gelatin, and streptococcal fibrinolysin. This enzyme was activated under the reducing conditions of active bacterial growth and by cysteine, glutathione, and thioglycolic acid; it was also activated by potassium cyanide but was inactivated by iodoacetic acid. Elliott showed that this enzyme was not streptococcal hemolysin, hyaluronidase, or fibrinolysin.

In the present study Frobisher's experiments were repeated and his results were confirmed. It was also found that the filterable proteinase, which certain

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strains of group A hemolytic streptococci produce in broth cultures, requires for its activation reducing conditions which may be established by the addition of thioglycollic acid or cysteine hydrochloride. In the oxidized state this proteolytic enzyme is inactive. The fact that the enzyme is subject to reversible oxidation and reduction probably furnishes the explanation of Frobisher's failure to demonstrate proteolysis of any substrate except cooked meat, since this was the only substrate used by him which would supply the reducing conditions necessary for activation of streptococcal proteinase. Elliott considered it impossible to determine whether the streptococcal proteinase which he studied was the same enzyme as "histase" described by Frobisher.

It appears evident, however, that the proteolytic enzymes of group A streptococci studied independently and from somewhat different viewpoints by Elliott and by the present author are identical.

The experiments reported in this paper were undertaken to investigate the frequency of occurrence of proteinase-producing strains among hemolytic streptococci belonging to various groups and specific types, and to learn whether or not horses immunized with culture filtrates containing the enzyme would develop antibodies capable of neutralizing the proteolytic activity of streptococcal proteinase. The antiproteinase activity of human serum was also investigated. For this purpose sera were taken from normal persons and from patients with either scarlet fever or rheumatic fever.

Materials and Methods

Strain.—In most of the following experiments a group A type 3 hemolytic streptococcus, designated Cooper 3122,¹ was used for proteinase production. This strain was selected from a total of forty-two strains tested.

Culture Filtrates.—Cultures were grown for 28 hours at 37°C. in Todd-Hewitt (1932) glucose-bicarbonate broth prepared with Difco proteose peptone. They were then filtered through Seitz, Chamberland, or Berkefeld filters. The culture filtrates contained, in addition to streptococcal proteinase, other streptococcal antigens and also antigenic peptones derived from the broth. The pH of the filtrates was usually 6.5. The strain Cooper 3122 did not produce erythrogenic toxin in the broth employed.

Titration of Streptococcal Proteinase.—Two different substrates were employed for titration of enzyme; namely, (a) a crude protein extracted from muscle and (b) diazotized crude proteins derived from skin.

Substrate (a).—600 gm. of finely minced lean beef are placed in 6 litres of distilled water and stirred for 15 minutes to remove excess of phosphates. The meat residue is then recovered by filtration through muslin and resuspended in 2 litres of 20 per cent NaCl solution. The mixture is adjusted to pH 5.0 with N/1 HCl and left at room temperature, with occasional stirring, for 24 hours. It is then filtered through paper pulp and the filtrate is sterilized by

¹ This strain was isolated by Dr. C. A. Green from a patient with acute rheumatic fever. The original culture, which was used for enzyme production, could not be typed because it had no type-specific M substance. The type was finally determined after the culture had been passed through a number of mice when it had regained type-specific M substance and when it no longer produced proteinase (see Elliott, 1945).

Seitz filtration. With aseptic technique the filtrate is adjusted to pH 4.0 with $N/10$ HCl in 20 per cent NaCl solution. The precipitate which appears at this pH is recovered by centrifugation, washed once in sterile 20 per cent NaCl solution, and resuspended in 1 litre of sterile 20 per cent NaCl solution. When this substrate is used, the pH of the system is necessarily on the acid side to prevent solution of the substrate by alkali. For convenience this preparation will be referred to subsequently in this paper as muscle protein substrate.

To test for proteolytic activity, 0.1 cc. volumes of the turbid suspension of muscle protein substrate are mixed with 1.0 cc. volumes of culture filtrate, at pH 6.5, serially diluted with saline. To activate the enzyme, 0.1 cc. of 0.5 M thioglycolic acid, neutralized to pH 7.6 with $N/1$ NaOH, is added to all the tubes. A control series of tubes is set up in which dilutions of uninoculated broth, at the same pH as the filtrate, are used instead of the active culture filtrate; the reagents are otherwise identical with those used in the test. The end-point of the titration is determined by observing the smallest quantity of filtrate which causes diminished turbidity in 24 hours at 37°C. when compared with the corresponding control tube.

Substrate (b).—The second substrate used in these experiments was "azocoll"² prepared as originally described by Oakley, Warrack, and van Heyningen (1946) for titrating collagenase in filtrates from cultures of *Cl. welchii*. Crude commercial "hide powder" is coupled with an azo dye (R salt) to give a reddish purple substrate which can be used as an indicator to detect proteolytic digestion. Culture filtrates containing streptococcal proteinase, when activated by thioglycolic acid and incubated overnight at 37°C. with this substrate, release a deep red color into the fluid adjacent to the sedimented azocoll particles. A similar release of color occurs when the substrate is digested by trypsin, chymotrypsin, pepsin, papain, or human serum proteinase activated by streptococcal fibrinolysin (streptokinase, Christensen and MacLeod, 1945). After overnight incubation, a small amount of color is released from the azocoll in tubes which do not contain any enzyme. This faint color is uniformly distributed throughout the supernatant fluid in contrast to the result of proteolytic digestion, in which case the color is more intense and is localized near the bottom of the tube. In the following experiments the method of Oakley *et al.* was modified by using glycerine instead of manucol to suspend azocoll. Glycerine not only reduces the amount of leakage of color from azocoll but it also supplies an antiseptic agent.

When titrating streptococcal proteinase, serial dilutions of culture filtrate are made in 1.0 cc. volumes in phosphate buffer at pH 7.8. The enzyme is activated by adding 0.5 cc. of 0.1 M thioglycollate solution at pH 7.6 to each tube. Substrate, consisting of 0.5 cc. of a 0.6 per cent suspension of azocoll in 75 per cent glycerine and 25 per cent saline, is then added. The reagents are thoroughly mixed by inverting the tubes several times; the tests are then incubated overnight at 37°C. The end-point of the titration is determined by observing the smallest quantity of filtrate which releases color from azocoll. Table I gives a typical example of the titration of proteinase in a streptococcal filtrate by the use of the two different substrates.

An attempt was also made to estimate quantitatively the activity of streptococcal proteinase by measuring the tyrosine, not precipitable by trichloroacetic acid, liberated from denatured hemoglobin by the enzyme. This method was abandoned because the broth used for culture filtrates itself contained an excess of tyrosine.

Immunisation of Horses.—Cultures of strain Cooper 3122 were grown for 28 hours in 10 to 20 litres of broth; they were then passed through Seitz filters. Thirty-five separate lots of filtrate were prepared in this manner. The proteinase titres of thirty-one of these preparations ranged from 1/200 to 1/1,000; the remaining four filtrates with lower enzyme titres were discarded. Horses were injected intramuscularly with the filtrate commencing with

² The author is greatly indebted to Dr. W. E. van Heyningen and to Dr. Walther F. Goebe for preparations of azocoll.

5.0 cc. and increasing gradually to a maximum of 500 cc. in about 4 months. Injections were given at 2 to 3 day intervals. Regular courses of injections, consisting of eight doses ranging from 100 cc. to 450 cc., with rest periods of 3 weeks between the courses, were instituted after the first 4 months of continuous immunization. Severe local or general reactions were not observed in any of the horses.

Titration of Antiproteinase.—Serum from one of the horses immunized with streptococcal proteinase has been adopted as an arbitrary standard containing 250 units of antiproteinase per cc. of serum. By reference to this standard, the results of all titrations can be recorded in units of antiproteinase per cubic centimeter of serum.

A preliminary standardization of the particular proteinase to be used in any series of titrations is carried out as follows: Serial dilutions of streptococcal filtrate are titrated against

TABLE I
Titration of Proteinase in Streptococcal Filtrates

Enzyme	Substrate	Dilutions of filtrate or broth								
		1/2	1/5	1/10	1/20	1/50	1/100	1/200	1/500	1/1,000
Streptococcal filtrate	Muscle protein	++++	+++	+++	+++	++	++	+	0	0
None (broth control)	" "	0	0	0	0	0	0	0	0	0
Streptococcal filtrate	Azocoll	++++	+++	+++	++	++	++	+	0	0

Incubated overnight at 37°C.

I. Test system for muscle protein substrate:

0.1 cc. acid-precipitated muscle protein.

0.1 cc. 0.5 M thioglycollate.

1.0 cc. dilution of culture filtrate or broth.

++++ = complete digestion of substrate with loss of turbidity.

+++ , ++ , + = partial digestion of substrate with incomplete loss of turbidity.

0 = no digestion of substrate; turbidity maintained.

II. Test system for azocoll:

0.5 cc. azocoll suspension.

0.5 cc. 0.1 M thioglycollate.

1.0 cc. dilution of culture filtrate.

++++ = dark red-colored solution extending for a considerable distance above the sedimented azocoll.

+++ , ++ , + = dark colored solution extending for smaller distances above the sedimented azocoll.

0 = no colored solution above the sedimented undigested azocoll.

1 unit of antiproteinase (standard serum diluted 1/250) to determine the dilution of filtrate neutralized by 1 unit of antiproteinase. This dilution of filtrate, in a constant volume of 0.5 cc., is then titrated against serial dilutions of the standard and of the unknown sera in 0.5 cc. volumes.

When using muscle protein substrate, all dilutions are made in saline. A control series of tubes is included containing serum dilutions mixed with uninoculated broth at the same pH and in the same dilution as the streptococcal filtrate. These control tubes are necessary to exclude variations in opacity due to highly colored or lipemic sera. A direct comparison is therefore made between pairs of tubes containing the same quantity of serum mixed with proteinase in the test and with broth in the control. The tubes are incubated in a water bath at 37°C. for 30 minutes to allow time for antigen-antibody combination. Substrate and thioglycollate are then added to all the tubes as described in methods for titrating proteinase in filtrates. The tubes are incubated at 37°C. for 24 hours during which time any residual enzyme digests the substrate. The end-point of the titration is the smallest quantity of serum

which completely prevents digestion of the substrate. This reading would be invalidated if the crude filtrates when mixed with serum formed sufficient precipitate to mask digestion of substrate. The small amounts of precipitate formed are, however, negligible in comparison with the turbidity of the substrate, and the major part of such precipitates is digested by the active enzyme.

When using azocoll as the substrate, all dilutions are made in phosphate buffer at pH 7.8. A negative control containing neither serum or enzyme and a positive control containing enzyme without serum are included. Thioglycollate is added to each tube as described above for proteinase titrations. The mixtures are left at room temperature for 1 hour to allow time for antigen-antibody combination. Azocoll is then added to each tube. After overnight incubation at 37°C., the end-point of the titration is the smallest quantity of serum which completely prevents release of color from the azocoll.

It has been found that the minor differences between the two methods of titrating anti-proteinase, such as the different times and temperatures used for neutralization of the proteinase and the stage at which the enzyme is activated, are immaterial to the tests.

Table II shows the results obtained with the two different substrates when titrating anti-proteinase in sera from immunized horses. In both titrations the results were the same; namely, 250 units per cc. of standard serum and 1,000 units per cc. of serum from horse A.

EXPERIMENTAL RESULTS

Selection of Strains Which Produce High Titled Proteinase.—With muscle protein substrate, a total of forty-two strains of group A hemolytic streptococci, belonging to twenty-five different types, were tested for proteinase production. Of these, thirty strains produced widely varying concentrations of the enzyme with titres ranging from 1/2 to 1/500. The remaining twelve group A strains did not produce any demonstrable proteinase. Eight strains belonging to groups B, C, and G were tested for proteinase production; they were all completely negative. Table III gives the proteinase titres of filtrates from broth cultures of the various strains. The serological group and type, as well as the origin of each strain are included in the table. The serological typing was carried out at the time when the strains were first isolated.

In Table III the strains are divided into three categories: (1) group A strains which have been subcultured for many years, and these include most of Griffith's original type cultures; (2) group A strains, isolated during the last 3 years, which were preserved in the frozen and dried state soon after isolation; and (3) strains belonging to groups B, C, and G. The majority of the filtrates were prepared in a single lot of glucose-bicarbonate broth containing Difco proteose peptone. It will be seen from Table III that in this particular lot of broth the strain Cooper 3122, which was selected for routine preparation of proteinase for immunization of horses, produced less enzyme than the strain E14 (Dochez N. Y. 5). The strain E14, however, was considered unsuitable for these experiments owing to its capacity to produce a large amount of erythrogenic toxin.

Although unconcentrated filtrates may at first appear to contain no proteinase, yet such apparently negative filtrates may yield, on concentration by precipitation with ammonium sulfate, solutions which contain small quantities of

TABLE II
Titration of Antiproteinase in Serum

Enzyme	Serum	Substrate	Antiproteinase units/cc. of serum										Controls	
			200	250	313	400	500	625	833	1,000	1,250	1,590	2,000	Pro- teinase, No serum
Streptococcal filtrate None (broth control)	Standard serum*	Muscle protein	0	0	++	+++	+++	+++	+++	+++	+++	+++	+++	...
	"	"	0	0	0	0	0	...	...	...	...	...	...	...
Streptococcal filtrate None (broth control)	Serum A	"	...	...	...	0	0	0	0	+	+++	+++	...	...
	"	"	...	...	...	0	0	0	0	0	0	0	...	...
Streptococcal filtrate	Standard serum*	Azocoll	0	0	+	++	+++	+++	...	...	...	...	+++	0
Streptococcal filtrate	Serum A	"	...	...	...	0	0	0	0	+	++	+++	+++	0

... = not done.

0 = no digestion of substrate.

+, ++, +++ = degrees of partial digestion of substrate.

++++ = complete digestion of substrate.

* The standard horse serum contains 250 units of antiproteinase per cc. For details of test, see Table I and Methods.

proteinase. It is therefore probable that the majority of strains produce proteinase, and that differences in production are usually quantitative rather than qualitative.

Antiproteinase in Normal Horse Sera.—In order to determine the average antiproteinase titres of normal horse sera, samples from ninety-nine uninocu-

TABLE III
Production of Proteinase by Various Strains of Hemolytic Streptococci

Group A strains after subcultivation for many years	Serological type	Proteinase titres*	Origin of culture	Group A strains frozen and dried soon after isolation	Serological type	Proteinase titres*	Origin of culture
S.F.130	1	0	Scarlet fever	Richards	3	20	Puerperal fever
S.F. 22	2	50	" "	Cooper 3122	3	200	Rheumatic fever
Lewis	3	0	" "	Tanck	6	0	" "
N.W.28	4	100	" "	1RSC86	6	0	" "
Franklin	5	0	Puerperal "	1RSC176	17	2	" "
283T	6	20	Scarlet "	1RSC174	19	2	" "
S43	6	0	Pneumonia	1RSC178	19	10	" "
S.F.4	8	50	Scarlet fever	1RSC165	24	5	" "
Symons	9	100	Sore throat	1RSC169	30	50	" "
E14	10	500	Scarlet fever	1RSC173	36	100	" "
Blackmore	11	50	" "	Kil.3779	...	100	" "
Kemball	11	100	" "	Adams	...	20	" "
Dobson	11	0	" "	Dvorak	...	100	" "
S.F.42	12	100	" "	Obee	...	50	" "
Anderson	13	100	Tonsillitis	Noris	...	100	" "
Williams	14	0	Puerperal fever	Groups B, C, and G strains			Origin of culture
Gillard	17	100	" "				
Hutchinson	18	0	Tonsillitis	O90	B	0	Scarlet fever
S.F.73	19	0	Scarlet fever	Brownnett	B	0	Endocarditis
63T	22	100	" "	E3	C	0	Erysipelas
King	23	50	" "	Azgazdah	C	0	Puerperal fever
G.54	24	0	Tonsillitis	Harrison	G	0	" "
Matthews	25	100	Normal throat	Havjll	G	0	Normal throat
S.F.40	27	100	Scarlet fever	Sears	G	0	" "
Small	28	50	Tonsillitis	Valente	G	0	Vagina
Gay	30	0	Bronchopneumonia				
S.F.13	47	50	Scarlet fever				

... = not typed.

0 = no digestion of substrate by undiluted filtrate.

27 group A strains after prolonged subcultivation, 66 per cent proteinase positive.

15 " " " frozen and dried, 86 per cent " " .

* Proteinase titre = reciprocal of highest dilution of filtrate which digested substrate.

lated horses were titrated for their ability to prevent the digestion of muscle protein substrate by streptococcal proteinase. The antistreptolysin O titres were also determined (Todd, 1938).

It will be seen from Table IV that the majority of the normal sera (53) contained 1 unit or less of antiproteinase per cc. of serum, while a minority (46) contained more than 1 unit of antiproteinase. The highest titre was 30 units per cc. of serum. There was no correlation between antistreptolysin O, which

is usually a good index of previous infection by streptococci of groups A, C, and G, and the antiproteinase titres of the sera. It therefore seems probable that the relatively high antiproteinase titres which occurred among the normal horse sera were not due to previous recent infections with group A hemolytic streptococci.

Antiproteinase in Sera from Horses Immunized with Streptococcal Filtrates.—In accordance with the usual practice of selecting for immunization horses with the highest titres of "natural antibody," the seven normal horses with the highest antiproteinase levels were used in the following experiments.

Table V gives the volumes of filtrates, prepared from strain Cooper 3122, which were injected intramuscularly into horse A, and also the titres of antiproteinase and antistreptolysin O of samples of serum taken during the course of

TABLE IV
Antiproteinase and Antistreptolysin O Titres of Normal Horse Sera

Antiproteinase titres	No. of horses in each group	Antistreptolysin O titres		
		Lowest	Highest	Mean
<i>units/cc.</i>		<i>units/cc.</i>	<i>units/cc.</i>	<i>units/cc.</i>
0	37	0	100	26
1	16	4	160	40
2	31	3	200	52
5	10	3	60	30
10	2	6	50	...
20	1	...	100	...
25	1	...	12	...
30	1	...	8	...

0 = less than 1 unit per cc. of serum.

immunization. The antiproteinase titre rose slowly to a maximum of 1,000 units per cc. which was not attained until the horse had been immunized for 8 months. On the other hand, the antistreptolysin O titre rose rapidly to a high level, and after fluctuating for several months, it gradually fell to a level which may be found in apparently normal uninoculated horses. This shows that there is no correlation between antiproteinase and antistreptolysin O.

A number of serum samples taken from horse A during the course of immunization were also tested by Proom's flocculation method (1941) for antibody to erythrotoxic toxin. In these titrations, graded dilutions of serum were mixed with a constant volume of erythrotoxic toxin which had previously been absorbed with antiserum horse serum. None of the samples of antiproteinase serum tested contained any antibody to erythrotoxic toxin; this was in accordance with the observation previously made that the strain Cooper 3122 did not produce erythrotoxic toxin in Todd-Hewitt broth.

TABLE V
Immunization of Horse A with Filtrates Containing Streptococcal Proteinase

Dates of injection or bleeding	Volumes injected	Antiproteinase titres of serum	Antistreptolysin O titres of serum
1943	cc.	units/cc.	units/cc.
Nov. 8	...	5	30
" 22, 24, 26, 29	5, 5, 5, 10	8	100
Dec. 1, 3, 6	15, 20, 25	6	250
" 8, 10, 13	25, 35, 35	10	500
" 15, 17, 20	50, 50, 75	16	4,000
" 22, 27	75, 75	20	3,000
" 29, 31	75, 100	...	...
1944			
Jan. 3	100	40	2,500
" 5, 7, 10	100, 100, 150	50	2,500
" 12, 14, 17	*150, 150, 200	100	2,500
" 19, 21, 24	200, 200, 250	125	2,500
" 26, 28, 31	250, 250, 300	200	2,500
Feb. 2, 4, 7	300, 300, 350	250	3,000
" 9, 11, 14	350, 400, 400	300	2,000
" 16, 18, 21	450, 450, 450	400	2,000
" 23, 25	450, 500	...	...
" 28	...	600	1,500
Mar. 2	...	600	1,250
" 6	...	500	1,000
" 16, 18, 20, 22	100, 150, 200, 250,	...	...
" 24, 27, 29, 31	300, 350, 400, 450	...	...
Apr. 3, 6, 10	...	625, 800, 625	6,250, 4,000, 3,000
" 20 to May 5	Full course†	...	...
May 8, 11, 15	...	625, 800, 625	4,000, 3,000, 2,500
" 25 to June 9	Full course†	...	...
June 12, 15, 19	...	625, 500, 625	3,000, 3,000, 2,000
July 6 to July 21	Full course†	...	...
" 24, 27, 31	...	800, 1,000, 800	800, 625, 625
Aug. 10 to Aug. 25	Full course†	...	...
" 28, 31, Sept. 4	...	1,000, 800, 800	400, 300, 300
Sept. 14 to Sept. 29	Full course†	...	...
Oct. 2, 5, 9	...	1,000, 1,000, 800	250, 250, 200
" 19 to Nov. 3	Full course†	...	...
Nov. 6, 9, 13	...	800, 800, 625	...
" 23 to Dec. 11	Full course†	...	...
Dec. 12, 15, 19	...	1,000, 1,000, 1,000	...
" 28 to Jan. 12	Full course†	...	...
1945			
Jan. 15, 18, 21	...	600, 600, 600	...
Feb. 2 to Feb. 17	Full course†	...	...
" 20, 23, 27	...	400, 500, 400	...

... = no injection or titre not determined.

During the earlier stages of immunization the injections and bleedings were done on the same day. Under these circumstances the last date on each line in the table is the day of bleeding.

* This and all subsequent injections contained alum.

† Full course = eight doses ranging from 100 cc. to 450 cc. at intervals of 2 to 3 days as shown for Mar. 16 to Mar. 31, 1944.

Six other horses were immunized by intramuscular injections of streptococcal filtrates containing proteinase. The volumes injected, the addition of alum to

the filtrate, and the intervals between injections were similar to those described in Table V. The resulting antiproteinase titres are recorded in Table VI.

It will be seen from Table VI that the horses varied in their ability to produce antiproteinase. The highest titres of the individual horses in units per cc. of serum were 1,000, 500, 300, 125, 600, 500, and 125 units. If satisfactory antiproteinase titres were not obtained after immunizing a horse for several months, the injections were stopped. This occurred in the cases of horses D and G, with maximal titres of 125 units per cc., and of horses C and F, with falling titres following earlier high antibody levels. Sera from all the horses acquired increased antiproteinase activity during the course of immunization.

Immunization of Rabbits.—Three rabbits were immunized by subcutaneous

TABLE VI
Antiproteinase Titres of Seven Horses Undergoing Immunization with Streptococcal Filtrates

Time in relation to immunization			Units of antiproteinase/cc. of serum						
			Horse A	Horse B	Horse C	Horse D	Horse E	Horse F	Horse G
Before immunization			5	20	10	10	25	30	5
After	1 month's	"	6	20	20	8	60	50	20
"	2	"	50	50	20	...	160	160	30
"	3	"	250	200	50	10	300	400	100
"	4	"	500	500	200	60	400	500	125
"	6	"	625	300	300	125	300	300	...
"	8	"	1,000	400	300	...	400	250	...
"	10	"	1,000	500	160	...	600	...	...
"	12	"	800	400	...	...	500	...	...
"	14	"	600	300	...	...	300	...	...

... = not titrated or immunization not continued.

injections of filtrates from cultures of the strain designated Cooper 3122 grown in chopped rabbit meat medium for 7 days. The meat was digested as described by Frobisher (1926) and the filtered digest was used for immunization. The commencing inoculum of 0.5 cc. was gradually increased over a period of 42 days up to 5.0 cc. The total volume of filtrate injected into each rabbit was 40 cc. Bleedings were taken 7 days after the last injection. Sera taken from the rabbits before immunization contained no demonstrable antiproteinase; after immunization the antiproteinase titres were 5, 5, and 2 units per cc. of serum.

Relation of Streptococcal Antiproteinase to Serum Trypsin Inhibitor.—Interpretation of the inhibitory effect of specific immune sera on a proteolytic enzyme necessarily involves a consideration of the possible rôle of the trypsin inhibitor which occurs in normal serum. Since it is known that the trypsin inhibitor in

human serum may be increased in acute illness and in cachexia, it seemed possible that immunization of horses might cause a similar increase of inhibitor for streptococcal proteinase. For this reason, experiments were carried out to determine whether the antiproteinase in immune horse sera has the properties of an antibody or of a naturally occurring inhibitor.

The serological specificity of the antiproteinase was investigated by examining the sera of horses immunized with antigens other than the streptococcal proteinase, and further by testing the effect of antiproteinase sera on other proteolytic enzymes. Samples of serum from horses immunized with (a) tetanus toxoid, (b) diphtheria toxoid, (c) erythrogenic streptococcal toxin, contained respectively 10, 10, and 0 units of antiproteinase per cc., while a sample of serum from a normal horse in the same stables contained 10 units of antiproteinase per cc.³ It has been found that strains of group A streptococci which produce proteinase in Todd-Hewitt proteose peptone broth do not produce any demonstrable proteinase in O'Meara's (1934) broth. A horse was immunized for 15 months with streptococcal erythrogenic toxin prepared in O'Meara broth. Both before and after immunization the serum of this horse contained no demonstrable antiproteinase. A sample of concentrated serum from the pooled bleedings of horses immunized with erythrogenic toxin prepared in O'Meara broth contained 3,300 units of antibody to erythrogenic toxin and only 20 units of antiproteinase.

It appears from these experiments that antiproteinase activity does not develop in the sera of horses immunized with antigens which do not contain streptococcal proteinase.

Experiments were also carried out to determine the neutralizing action of antiproteinase sera on crystalline trypsin and chymotrypsin and on crude preparations of papain and *Cl. welchii* collagenase. A preliminary experiment was performed to determine the highest dilution of each enzyme which digested azocoll. Neutralization experiments were then carried out using ten minimal digestive doses of each enzyme in a constant volume of diluent mixed with serial dilutions of three different sera which contained various quantities of antiproteinase. The usual technique for neutralization experiments with azocoll substrate was employed. The results of this experiment are shown in Table VII.

The three sera contained respectively 10, 1,000 and 1,500 units of antiproteinase per cc. With chymotrypsin this order of neutralizing activity was reversed. For trypsin⁴ and for papain the highest titred antiproteinase serum

³ These sera were obtained through the kindness of Dr. Jules Freund who informs me that the sera contained (a) 700 to 800 units of tetanus antitoxin per cc. and (b) 620 units of diphtheria antitoxin per cc.

⁴ It will be seen from Table VII that both normal and immune sera, when tested by the azocoll method, showed unexpectedly high antitryptic activity. The results of antitrypsin titrations in these experiments were therefore difficult to interpret as they do not conform with the antitryptic determinations obtained by the usual methods.

had the lowest neutralizing capacity. Collagenase was neutralized to an equal extent by sera containing 10 and 1,000 units of antiproteinase per cc. of serum. This experiment again demonstrates the specificity of antiproteinase sera.

Landsteiner (1900) and many subsequent workers (see Grob, 1943) have shown that serum trypsin inhibitor is associated with the albumin fraction of serum. It has also been established that the inhibitory effect of serum is readily destroyed by heating, by acidification below pH 3, and by shaking with certain

TABLE VII
Specificity of Streptococcal Antiproteinase. Neutralization Tests with Various Enzymes
Azocoll Method

Enzymes	Horse sera		
	Normal	Unconcentrated antiproteinase*	Concentrated antiproteinase†
Streptococcal proteinase.....	10	1,000	1,500
Crystalline chymotrypsin (0.05 mg.).....	20	10	2
“ trypsin (0.005 mg.).....	1,000	1,000	200
Crude papain (extract from 0.045 mg.).....	50	50	0
<i>Cl. welchii</i> collagenase§.....	10	10	200

Figures are reciprocals of serum dilutions which neutralized ten minimal digestive doses of each enzyme.

0 = no neutralization by undiluted serum.

* Unconcentrated antiproteinase serum from immunized horse A.

† Concentrated antiproteinase globulin was prepared from a pool of selected bleedings taken from various horses which had attained high antiproteinase levels after prolonged immunization with streptococcal filtrates. It was “refined” by the pepsin digest process, and subsequently concentrated by ultrafiltration through collodion-coated Berkefeld candles. It contained 15.8 per cent of protein and 22.8 percent of total solids.

§ This preparation, which was obtained through the kindness of Dr. van Heyningen, contained ten minimal digestive doses in 0.5 cc. of 1/250 dilution. It contained in addition to collagenase the α and θ toxins of *Cl. welchii*.

organic reagents such as chloroform. These properties provide convenient criteria for differentiating between inhibitor and antibody.

Normal and immune horse sera containing 10 and 1,000 units per cc. of antiproteinase respectively were subjected to fractionation with ammonium sulfate. In each case no demonstrable antiproteinase was found in the albumin fraction, while the globulin fractions contained antiproteinase in the same proportions as the original sera. Furthermore, heating at 56°C. for 30 minutes or acidification to pH 2.75 resulted in no material loss of the antiproteinase activity of the serum. It would appear that the antiproteinase does not have the properties usually ascribed to trypsin inhibitor. However, these results are rendered less conclusive by the fact that in the azocoll system used in these ex-

periments, the behavior of serum trypsin inhibitor diverges somewhat from that previously described. Thus, while heat and acidification cause some decrease in the titre of inhibitor, as measured by its effect on the activity of crystalline trypsin, the residual titre after treatment remains relatively high.

Antiproteinase Activity of Human Sera

In addition to the study of the antigenicity of streptococcal proteinase for horses and of the properties of the immune horse serum, determinations were made of the level of antiproteinase in normal human sera, in sera from patients with scarlet fever, with streptococcal pharyngitis, and with acute rheumatic fever. Muscle protein substrate was used in all these antiproteinase titrations. No attempt was made to distinguish between a specific antibody and a possible serum inhibitor for streptococcal proteinase.

Normal Human Serum.—The sera of twelve normal persons, who were donors to the blood transfusion service, were tested for antiproteinase and antistreptolysin O. Eleven of these sera had low antiproteinase titres of 1 unit or less per cc. of serum and antistreptolysin O titres within the normal range. The remaining serum contained 2 units of antiproteinase per cc.; and in this case the antistreptolysin O titre of 310 units per cc. indicated a probable recent infection with hemolytic streptococci. In addition, twenty-seven sera which had been taken for Wassermann reactions were titrated for antiproteinase. Twenty-four of these sera contained 1 unit or less and the remaining three contained 2 units of antiproteinase per cc. Of the thirty-eight normal sera tested, twenty-nine contained no detectable antiproteinase.

Sera from Patients with Scarlet Fever and Streptococcal Pharyngitis.—The sera of nine patients with scarlet fever were obtained both in the acute stage of the disease on admission to the hospital and during convalescence immediately before discharge. Only one serum had an antiproteinase titre of over 2 units per cc. There was little evidence of increasing antiproteinase during convalescence; on the contrary, the titres of sera from five of the patients were higher during the acute stage of the infection than they were during convalescence. This suggests the presence of proteinase inhibitor in the sera, since antibody would be more likely to appear during convalescence.

Tests of single serum specimens from five additional patients with scarlet fever or streptococcal pharyngitis showed titres ranging from 2 to 10 units per cc. There was no correlation between the antistreptolysin O and antiproteinase levels of the patients' sera.

Sera from Patients with Acute Rheumatic Fever.—Sera were obtained at weekly intervals from eighteen patients with acute rheumatic fever and titrated both for antiproteinase and antistreptolysin O. The highest antiproteinase titre recorded was 20 units per cc., while two patients had no detectable antiproteinase in their sera during the whole course of the disease. Again, as in the

case of the scarlet fever patients, the antiproteinase titres were low in general and there was no correlation with the antistreptolysin O.

The results of the antiproteinase titrations in the various human sera are summarized in Table VIII. The majority of normal individuals had no demonstrable antiproteinase in their sera; on the other hand only six of thirty-two patients infected with hemolytic streptococci had antiproteinase negative sera. There is no significant difference between the titres of sera from rheumatic fever patients and sera from other streptococcal infections. Thus, it is unlikely that the development of antiproteinase is related to the etiology of rheumatic fever.

Treatment of Rheumatic Fever Patients with Antiproteinase Serum.—Further studies were undertaken to investigate the influence of the antibody to streptococcal proteinase in rheumatic fever by injecting high titred horse serum into patients. A clinical trial with pooled horse serum "refined" by the pepsin

TABLE VIII
Antiproteinase in Human Serum

Source of sera	Total No.	Antiproteinase positive	Antiproteinase negative
Normal individuals.....	38	9	29
Patients with scarlet fever or pharyngitis.....	14	13	1
Patients with rheumatic fever.....	18	13	5

The patients' sera were all taken during the 3rd week of illness with the exception of four sera from patients with pharyngitis which were taken on the 4th day of disease.

digest process and containing 1,500 units of antiproteinase per cc. was carried out by Dr. A. L. Jacobs in ten cases of rheumatic fever. The administration of serum in amounts sufficient to cause readily measurable increases in the antiproteinase content of the patients' sera did not appear to have any effect on the clinical course of rheumatic fever. The antiproteinase titres after treatment ranged from 2 to 12 units per cc. of serum; the averages of titres before and after treatment were 0.9 and 7.1 units respectively. Combined treatment with serum and penicillin in three cases again failed to produce beneficial results which could be attributed to the serum.

A fuller account of the cases treated by the injection of high titred antibody to streptococcal proteinase will be published later by Dr. A. L. Jacobs.

DISCUSSION

Many strains of group A hemolytic streptococci produce a proteolytic enzyme when grown in Todd-Hewitt broth. This streptococcal proteinase is inactive in the oxidized state and is activated by reducing agents such as thioglycollic acid and cysteine hydrochloride. When horses are immunized with crude culture filtrates containing streptococcal proteinase, they gradually develop an

increased antiproteinase activity in their sera. Methods are described in this paper for titrating the amount of streptococcal proteinase in culture filtrates and for measuring the antiproteinase activity of serum. The majority of normal horse sera have very little antiproteinase activity, yet about 5 per cent of such sera have titres of antiproteinase ranging from 10 to 30 units per cc. Parallel titrations of these sera for antistreptolysin O suggest that the relatively high antiproteinase titres are not caused by recent infections with group A streptococci. The question therefore arises as to whether this comparatively uncommon antiproteinase action of normal serum is due to "natural antibody" or whether it is a proteinase inhibitor similar to the trypsin inhibitor of serum. The same question arises when considering the antiproteinase titres of sera from horses undergoing immunization with streptococcal proteinase, as it is known that considerable variation in the antitryptic activity of serum occurs. Certain distinguishing characteristics between antibodies and inhibitors of enzymes have therefore been studied.

Antiproteinase is in the globulin fraction of both normal and immune sera; it therefore resembles antibody in this respect. Conversely the serum albumin of normal and immune sera has no demonstrable neutralizing activity for streptococcal proteinase, although it has been shown by other investigators to contain all the trypsin inhibitor found in untreated serum. Treatment of high titred antiproteinase serum by heat and by acid under conditions which have been shown by other workers to destroy trypsin inhibitor does not destroy the antiproteinase activity of the serum. A further distinction between antibody and trypsin inhibitor is the degree of increase in neutralizing power which occurs in the sera of animals immunized with enzymes (Grob, 1943). In the present study the antiproteinase activity of serum from horses immunized with streptococcal proteinase was increased as much as 200 times above the original level.

These large increases in antiproteinase activity, in contrast to the results obtained by Grob with trypsin, suggest that horses immunized with streptococcal proteinase gradually form considerable amounts of antibody to the enzyme. It is possible, however, that serum may contain an inhibitor to streptococcal proteinase with properties entirely different from those of trypsin inhibitor.

The existence of antibodies capable of neutralizing the biological activity of enzymes was denied by a number of earlier workers in this field. It is now known, however, that certain bacterial toxins are enzymes, and that they are nevertheless capable of exciting the production of potent and effective antitoxins when injected into animals. A notable example is the α toxin of *Cl. welchii*, which has been shown by Macfarlane and Knight (1941) to be a lecithinase. Other examples are the lecithinase of snake venom and the collagenase of *Cl. welchii* (Oakley *et al.*, 1946). The neutralization of an enzyme by a specific antibody is therefore not without precedent. The evidence presented in this paper suggests that the neutralization of streptococcal proteinase by high titred immune sera is due to the action of specific antibody.

Although the antiproteinase titres of sera from patients infected with group A hemolytic streptococci were found to be raised slightly above the normal level, yet the highest of such titres observed were low in comparison with those of immunized horses. No significant difference was found between the antiproteinase titres of sera from rheumatic fever patients and sera from other streptococcal infections. Because rheumatic fever patients do not develop any more or any less antiproteinase in their sera than is produced by scarlet fever patients, it seems evident that failure to produce this antibody cannot account for the peculiar symptom complex of rheumatic fever. This view is supported by the observation that high titred antiproteinase serum, prepared in horses, has no therapeutic value in the treatment of rheumatic fever.

SUMMARY

Antiproteinase sera were prepared by immunizing horses with filtrates from a selected strain of group A streptococcus. This strain, which produced high titred proteinase but no erythrogenic toxin, was selected from forty-two strains of group A streptococci which produced varying amounts of proteinase. A few strains belonging to groups B, C, and G were also tested; they were all proteinase-negative. Methods are described for titrating streptococcal proteinase in crude culture filtrates and for measuring the antiproteinase activity of serum.

The antiproteinase titres of sera from immunized horses ranged from 125 units to 1,000 units per cc. in contrast to the low titres of normal horse sera, only 5 per cent of which had titres as high as 10 to 30 units per cc.

The available evidence suggests that the antiproteinase activity of immune sera is dependent on the action of specific antibody for streptococcal proteinase.

Patients infected with group A streptococci do not develop high antiproteinase titres. There appears to be no correlation between the occurrence of rheumatic fever and the antiproteinase titre of the patient's serum.

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