INHIBITION OF PROTEINASES OF CERTAIN CLOSTRIDIA BY SERUM

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Some members of the gas-gangrene group, such as *Clostridium histolyticum*, secrete proteolytic enzymes which cause much tissue damage *in vivo*. However, certain non-pathogenic Clostridia, such as *Clostridium sporogenes*, produce proteolytic enzymes which are as active as those of *Clostridium histolyticum* in the hydrolysis of gelatin or casein, but which are relatively impotent *in vivo*. In regard to the pH optima and activation behavior, no differences have been found between the proteinases of the pathogenic and the non-pathogenic Clostridia (Weil, Kocholaty and Smith, 1939).

Pozerski and Blanc (1920) reported that the proteinase of *C. sporogenes* was inhibited by normal horse serum, while that of *C. histolyticum* was not. This suggested that absence of inhibition by normal serum of the exocellular proteinases of this group of organisms might be correlated with the pathogenicity of the strain producing the proteinase.

Evidence in support of this possibility appeared when, during the prosecution of this study, Grassmann (1938) reported the inhibition of the proteinase of *Clostridium botulinum* by normal horse serum, but could find no inhibition of the enzymes of *Clostridium welchii*. The enzymes of the latter organism, however, were inhibited by immune serum.

MATERIALS AND METHOD

The organisms used in this study, with the exception of the strain of *C. histolyticum*, were obtained from the American Type

Culture Collection. The smooth strains of *C. histolyticum* were obtained from Dr. Weinberg, of the Pasteur Institute, Paris; the rough strains were obtained by colony selection (Hoogerheide, 1937). They were grown in beef-heart infusion broth at 37°C. for 24 to 48 hours. After incubation, the cultures were centrifuged and the supernatant fluids passed through Seitz filters. These filtrates were used as the enzyme solutions.

The method used for the determination of proteolytic activity was a modification of the method of Haines (1932, 1933). The activity of the enzyme solution was calculated from the rate of gelatin liquefaction, measured in Ostwald viscosity tubes. In the comparison of the inhibiting action of certain serum fractions on bacterial proteinases with that on trypsin, Bactotrypsin was diluted until it liquefied gelatin at approximately the same rate as did the enzyme with which it was being compared.

PREPARATION OF GELATIN

Twenty-five grams of Coignet Gold Label gelatin were soaked in 150 ml. of water for three or four hours and then warmed gently until dissolved. A solution of 2.5 grams of phenol in a small quantity of water was added to the gelatin as a preservative. The whole was adjusted to pH 7.0 with 0.1 N NaOH. After dilution to 250 ml. with water, 211 ml. of 0.1 M Na_2HPO_4 and 39 ml. of 0.1 M NaH_2PO_4 were added. The solution was allowed to stand at 37°C. overnight, during which time a precipitate of calcium phosphate formed, which was removed by centrifuging. As was pointed out by Haines (1933), the viscosity properties of gelatin change somewhat with repeated liquefaction and gelation. To avoid this, the stock solution was divided into smaller lots and stored in the refrigerator. For each series of determinations, a fresh tube was liquefied at 37°C.

DETERMINATION OF ENZYME ACTIVITY

Since, when a number of simultaneous determinations were being made, it was necessary to use non-uniform viscosity tubes, these were calibrated by determining the outflow time for distilled

water at 37°C. This factor (t_w = the outflow time in seconds for distilled water, using 5 ml. total volume) was used in calculating the results. Simple Ostwald viscometers were used throughout. Best results were obtained in a given series when the t_w values for the different viscometers were approximately equal, preferably between 40 and 70.

Previous to each experiment, the viscometers and the solutions to be used (gelatin and enzyme) were brought to constant temperature in the thermostat. For each determination, 3 ml. of the 5 per cent gelatin solution, a suitable quantity of the bacterial filtrate (usually 0.1 to 2.0 ml.), and sufficient water to make the total volume exactly 6 ml., were mixed, the time of mixing being noted to the nearest half minute. Five ml. of this mixture were measured into the viscometer. The falling time of the mixture (t) was then measured to the nearest half second, every four or five minutes during the course of the determination. For purposes of calculation, the time (T) at which the viscosity measurement (t) was made, was taken as the interval, in minutes to the nearest half minute, between the mixing time and the mean time of each measurement.

When serum, or serum fractions, were used as inhibiting agents, they were added to the enzyme solution two minutes before the addition of the gelatin. The length of this time interval was purely arbitrary. One-tenth ml. of serum, or a corresponding amount of serum fractions, was used in each experiment.

CALCULATION OF RESULTS

The experimentally observed outflow time (t, in seconds) is plotted against the square root of the elapsed time (T, in minutes). A straight line results, the slope of which is calculated from the relation

$$t_1 - t_2 = k(\sqrt{T_2} - \sqrt{T_1}) \tag{A}$$

The magnitude of the constant, k, depends, of course, on the constant of the particular viscometer used. As is shown below, it is also directly proportional to the square root of the enzyme

quantity used in the determination. To correct for these factors, use is made of the equation,

$$\text{(B)} \qquad \frac{100\,k}{t_w} = K\sqrt{V}$$

where t_w is the viscometer constant, and V is the volume in milliliters of the enzyme solutions used. The factor 100 is introduced merely so that the values for K, the enzyme activity, will in general be above unity.

The equations presented above are forms of Schütz's law (1885). Haines (1932, 1933) has shown that equation (A) holds for the liquefaction of gelatin by filtrates of a variety of microorganisms. It was found to hold also for the bacterial enzymes

TABLE 1

Influence of amount of enzyme on gelatin liquefaction

VOLUME OF FILTRATE	k	t_w	$\frac{k}{t_w}$	K
ml.				
2.0	4.89	55.6	0.088	6.20
1.5	4.35	57.3	0.076	6.23
1.0	2.41	40.8	0.059	5.90
0.75	2.41	45.5	0.053	6.16
0.50	1.92	49.4	0.043	6.08
0.25	1.24	42.9	0.029	5.94

used in this study. Haines carried out his work using a constant volume (2.5 ml.) of enzyme solution throughout. This procedure is justified when filtrates of moderate enzymatic activity are used. It is often necessary, however, to measure very high or very low enzyme activities. To keep the viscosity change within convenient limits, the amount of enzyme must be increased or decreased. In order to obtain comparable results in such cases, it was necessary to establish the relation between the rate of gelatin liquefaction and the volume of enzyme solution used. The results are given in table 1.

EXPERIMENTAL

The inhibition of the proteolytic enzymes of a number of anaerobic spore-formers by fresh, normal rabbit serum was investigated. The results are given in table 2.

No non-pathogenic organism in the above list possesses proteolytic enzymes which are able to resist inhibition by normal serum. Of the pathogens tested, *Clostridium histolyticum*, *C. welchii*, and *C. oedematis-maligni* possess proteolytic enzymes which are capable of hydrolyzing proteins in the presence of normal serum. It is probable that this characteristic is an important one in pathogenesis, enabling the members of this group to establish a foothold in the body of the host and to obtain those breakdown products of protein which are their main source of energy. *C. botulinum*, although usually considered as pathogenic, nevertheless is so only by virtue of its toxin, which must be produced *ex vivo*.

TABLE 2

Inhibition of the proteinases of Clostridia by normal rabbit serum

ORGANISM	ENZYME ACTIVITY	ACTIVITY WITH 0.1 ML. SERUM
C. aerofetidum	23.5	<1
C. botulinum	7.5	<1
C. fallax	1.9	<1
C. histolyticum R-	35.0	3.0
C. histolyticum S-	33.0	33.0
C. oedematis-maligni	2.0	2.0
C. putrificum	22.0	3.0
C. sporogenes	45.0	<1
C. welchii	15.0	12.0

Pozerski and Guelin (1938) reported that the escharotic properties of culture filtrates of the spore-forming anaerobic bacteria bore little or no relation to their proteolytic activity. They did not, however, determine whether there was any inhibition of the proteolytic enzymes by normal serum, although they did report, in a separate paper, that the proteinases of *Clostridium sporogenes*, *C. bifermentans*, and *C. sordelli* were inhibited by egg albumin, while those of *C. histolyticum* were not.

Heiden (1905, 1906) reported that trypsin was inhibited by serum albumin. This inhibition was studied in detail by Hussey and Northrup (1923), who concluded that the inhibition was due to the relative indigestibility of the serum albumin, to which the trypsin became attached. It was thought that the "anti-trypsin"

of the serum might be the substance responsible for the inhibition of certain of the bacterial proteinases. In making the comparison of the inhibition of trypsin and a bacterial proteinase which was inhibited by normal serum, the filtrate of a rough strain of *C. histolyticum* was used. The proteinase of this organism was active in liquefying gelatin, was almost totally inhibited by fresh serum, and was readily obtainable.

Serum which had stood for about a week at room temperature, or for two or three months in the refrigerator, inhibited the bacterial proteinase hardly at all, while it did inhibit trypsin almost as well as fresh serum. Half saturation with ammonium sulfate also served to destroy the ability to inhibit the bacterial proteinase, but did not affect the "anti-tryptic" activity.

These results made it seem possible that there might be two substances in serum, one inhibiting trypsin and the other inhibiting the bacterial proteinase. However, all attempts to separate the two were unsuccessful. Both were destroyed by heat at the same rate, being half destroyed by an exposure of 10 minutes at 64°C., and being almost completely destroyed by an exposure of 30 minutes at this temperature.

Serum was separated into its constituent fractions by electrophoresis in the Tiselius apparatus.[1] These fractions were tested for the inhibition of trypsin, and for the inhibition of the proteinase of the rough strain of *C. histolyticum*. The results are given in table 3.

The inhibition of the bacterial proteinase by the β globulin could not be represented quantitatively. There was an almost complete inhibition of gelatin liquefaction for thirty minutes. At the end of this time, liquefaction began and proceeded almost as rapidly as if no inhibiting substance were present. The activation shown by the Δ globulin, although slight, was consistent. It was also found with globulin prepared from normal horse serum.

The parallelism of the inhibition of trypsin and of the bacterial proteinase by different fractions of normal rabbit serum, as shown

[1] The authors are indebted to Dr. Laura Krejci and Mr. Robert Jennings for preparing the serum fractions.

in table 3, indicates that the inhibition of these two enzymes is primarily due to a single substance, the albumin of the serum, although the β globulin inhibited the bacterial proteinase more than it did the trypsin. Serum albumin prepared in the Tiselius apparatus is submitted to a minimum of manipulation and exposure to adverse conditions, and contains no more than traces of the other protein constituents. However, if the albumin of the serum is responsible for the inhibition of both of these enzymes, then certainly the mechanism of inhibition cannot be the same. It is possible that certain labile groups which can be altered by treatment with ammonium sulfate or by standing at room temperature, are responsible for the inhibition of the bacterial proteinase, but have no effect on the inhibition of trypsin.

Immune serum prepared by the injection of filtrates of *C. histolyticum*, smooth, into rabbits, inhibited the enzymes of this

TABLE 3

Inhibition of bacterial proteinase and trypsin by serum fractions

ENZYME	ORIGINAL ACTIVITY	WHOLE SERUM	ALBUMIN	β	$\beta+\gamma$	$\gamma+\Delta$	Δ
Trypsin	17	<1	<1	13	10	15	18
C. histolyticum R-	13	<1	<1	1–14	7	13	15

organism. Inhibition of the proteolytic enzymes of bacteria by immune sera has been reported by Bertiau (1914) for *Pseudomonas aeruginosa*, Blanc and Pozerski (1920) for *C. histolyticum*, Dukes (1922) for *P. aeruginosa*, and Grassmann (1938) for *C. welchii*.

The inhibition of the proteinases of *C. histolyticum* was specific, the serum of the rabbit immunized to *C. histolyticum* inhibiting the proteinase of this organism, but not that of *C. sporogenes*, *C. welchii*, or even that of rough strains of *C. histolyticum*. This experiment was performed with antiserum which had stood sufficiently long in the cold to have lost its ability to inhibit the proteinases of the non-pathogenic organisms.

The inhibiting factor in this serum was probably antibody, since it was associated with the globulins of the serum, being precipitated by half saturation with ammonium sulfate. It was

fairly stable, being present in practically full amount in serum which had been kept sterilely in the cold for over a year.

Dukes considered that the inhibition of the enzymatic activity was not due to an antibody which inactivated the enzyme, but rather to a precipitin which removed the enzyme from solution, and consequently, from contact with the substrate. In our experiments with immune sera, no visible precipitate was ever formed. While this does not invalidate Dukes' contention, nevertheless, the sharp specificity shown by the anti-enzyme sera indicates strongly that these enzymes are capable of acting as specific antigens, and of inducing the production of specific antibodies.

SUMMARY

1. The proteolytic activity of culture filtrates of various spore-forming anaerobes was determined by the gelatinase method of Haines, so modified as to permit the determination of gelatinase in solutions of widely differing enzymatic activity.

2. Fresh, normal rabbit serum inhibited the proteinases of *Clostridium aerofetidum, Clostridium botulinum, Clostridium fallax, Clostridium putrificum, Clostridium sporogenes,* and a rough strain of *Clostridium histolyticum.* The β globulin and albumin of the serum were responsible for this inhibition, the latter accounting for the most of it. Trypsin was inhibited only by the albumin.

3. Normal serum did not inhibit the proteinases of *Clostridium histolyticum, Clostridium oedematis-maligni,* or *Clostridium welchii.*

4. Immune serum specifically inhibited the proteinase of *C. histolyticum.*

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REFERENCES

BERTIAU, P. 1914 Les ferments bactériens qui liquéfient la gélatine et leurs antiferments. Zentr. Bakt. Parasitenk., Orig., **74**, 374–82.

BLANC, J., AND POZERSKI, E. 1920 Sur les ferments protéolytiques du *B. sporo-*

genes et du *B. histolyticus*, action empêchante des sérums normaux et specifiques. Compt. rend. soc. biol., **83**, 1369–70.

DUKES, C. E. 1922 The proteolytic enzyme of *Bacillus pyocyaneus*: the inhibition produced by normal and immune serum. J. Path. Bact., **25**, 258–65.

GRASSMANN, W. 1938 Über Bakterienproteasen. III. Die proteasen des *B. perfringens*. Biochem. Z., **295**, 351–68.

HAINES, R. B. 1932 The influence of the medium on the production of bacterial gelatinase. Biochem. J., **27**, 466–74.

HAINES, R. B. 1933 Further studies of the effect of the medium on the production of bacterial gelatinase. *Ibid.*, **27**, 466–74.

HEDIN, S. G. 1905 On the antitryptic action of serum-albumin. J. Physiol., **32**, 390–4.

HEDIN, S. G. 1906 Trypsin and antitrypsin. Biochem. J., **1**, 474–84.

HOOGERHEIDE, J. C. 1937 Variability in morphological and biochemical properties of *Clostridium histolyticum*. J. Bact., **34**, 387–407.

HUSSEY, R. G., AND NORTHRUP, J. H. 1923 A study of the equilibrium between the so-called "anti-trypsin" of the blood and trypsin. J. Gen. Physiol., **5**, 335–51.

POZERSKI, E., AND GUELIN, A. 1938 Action empêchante de l'ovalbumine crue sur les ferments gélatinolytiques de quelques microbes anaérobies. Compt. rend. soc. biol., **128**, 504–6.

POZERSKI, E., AND GUELIN, A. 1938 Contribution a l'étude des filtrats de quelques microbes anaérobies. Gélatinase et pouvoir escarrifiant. *Ibid.*, **128**, 842–44.

SCHÜTZ, E. 1885 Eine Methode zur Bestimmung der relativen Pepsinmenge. Z. physiol. Chem., **9**, 577–590.

WEIL, L., KOCHOLATY, W., AND SMITH, L. DS. 1939 Studies on proteinases of some anaerobic and aerobic micro-organisms. Biochem. J., in press.