5. No increase was found in the antitrypsin level of rabbit sera following feeding of trypsin and a slight increase after repeated daily injections of trypsin. No change was observed in the inhibitory power of guinea pig sera following protein sensitization either before or after the production of anaphylaxis.

6. The sera of certain persons suffering from tuberculosis showed a marked increase in inhibitory power as did the sera of all women examined in the terminal stages of pregnancy.

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Protease Inhibitors

2. BACTERIAL PROTEASES AND THEIR INHIBITORS

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Inhibition of bacterial proteases by normal or immune sera has been studied by various authors, although the results are not always in agreement, probably because of the failure to recognize that the filtrates might contain several proteases, only one of which was inhibited by normal serum. In addition, sensitivity to inhibitor might vary according to the organism and type of culture used. von Dungern (1898) found that the hydrolysis of gelatin by filtrates of *Bacillus anthracis*, *Vibrio cholerae* and *Staphylococcus aureus* was inhibited by immune but not by normal serum. Bertiau (1914) found that normal human serum had no inhibitory action on the proteases of *Bacillus subtilis* and *Pseudomonas pyocyanea*, although specific immune serum inhibited. Blanc & Pozerski (1920) showed that gelatin hydrolysis by filtrates of *Clostridium sporogenes* was inhibited by normal horse serum, which had no action on filtrates of *Cl. histolyticum*. Pozerski & Guelin (1938) showed that raw egg white had similar properties with regard to these filtrates. A more comprehensive study by Maschmann (1937*a*, *b*, 1938*a*, *b*, *c*, *d*, *e*) showed that at least three extracellular proteases could be recognized, and that a bacterial filtrate might contain one or more of these depending on the organism and method of preparation. A summary of Maschmann's findings is given in Table 1.

Smith & Lindsley (1939), in a study of the hydrolysis of gelatin by filtrates of various Clostridia, showed that, whilst normal serum inhibits proteases of non-pathogenic bacteria, it has no action on those of pathogens such as *Cl. histolyticum*, *Cl. welchii* and *Cl. oedematiens*. Filtrates of a rough strain of *Cl. histolyticum* were, however, inhibited by rabbit serum, but experiments showed that,

Table 1. *Classification of bacterial proteases according to Maschmann* (1937*a*, *b*, 1938*a*, *b*, *c*, *d*, *e*)

Protease	Organism	Substrate	Activator	Inhibition by normal serum
(1) 'Pyocyaneus protease'	*Ps. pyocyanea** *Chromobact. prodigiosum** *Ps. fluorescens liquefaciens** *B. mesentericus**	All proteins and peptones	Not required	+
(2) 'Gelatinase'	*Cl. welchii* *Cl. septicum* *Cl. histolyticum* *Cl. chauvei*	Gelatin only	Not required	–
(3) 'Anaerobiase'	*Cl. welchii* *Cl. septicum* *Cl. histolyticum* *Cl. botulinum*	All proteins	Requires low redox potential (SH compounds best)	–

* These organisms are aerobes: all others listed above are anaerobes.

whilst the inhibitory power of serum against the filtrates of a rough strain of *Cl. histolyticum* was lost on storage or on treatment with ammonium sulphate to half saturation, there was no corresponding loss when tested against trypsin. Electrophoretic separation of normal rabbit serum showed that the inhibitory power against trypsin lay entirely in the albumin fraction, whilst that against the protease produced by a rough strain of *Cl. histolyticum* was present also to some extent in the β-globulin fraction.

Some explanation of these findings can be found in the studies of Maschmann (1937*a*, *b*, 1938*a*, *b*, *c*, *d*, *e*), who showed that the pathogenic Clostridia, *Cl. welchii*, *Cl. histolyticum*, produce a specific gelatinase not inhibited by serum. Bidwell & van Heyningen (1948) have since confirmed the substrate specificity of the *Cl. welchii* gelatinase.

EXPERIMENTAL

Culture filtrates. The cultures of *Staph. aureus*, *Chromobacterium prodigiosum* and *Ps. pyocyanea* were grown 18–24 hr. in digest broth continuously shaken. Those of *B. anthracis* and *V. cholerae* were grown in Roux bottles on agar for 18–24 hr. and washed off in saline. *Cl. bifermentans* was grown on the medium of Macfarlane & Knight (1941). *Cl. welchii* Types A and D, and *Cl. histolyticum* were grown 8–18 hr. in a meat infusion broth containing 0·6% (w/v) glucose and 0·001 M-thioglycollic acid. The *Cl. welchii* Type B culture was grown in a glucose peptone broth containing thioglycollic acid and sodium glycerophosphate. All cultures with the exception of those grown on agar were filtered free of organisms; those grown in liquid media were concentrated 5–30 times by pressure dialysis, and all were finally dialyzed against saline and stored under toluene at 4° or frozen.

Estimation of enzyme and inhibitor activity. The majority of estimations were made by the viscosimetric technique already described (Duthie & Lorenz, 1949). A standard preparation of crystalline trypsin which was assigned the arbitrary value of 20,000 units/mg. was the standard of comparison. The casein hydrolysis experiments described in Table 2 had the enzyme concentration given in the second column of the table in a total of 1 ml. of 1% casein, and were incubated 3 hr. at 37°, after which the mixtures were precipitated with 2 vol. of 5% (w/v) trichloroacetic acid. The increase in acid-soluble 'tyrosine' against control mixtures not incubated was estimated by the Folin and Ciocalteu phenol reagent.

All inhibitors were incubated for 10 min. at 37° with the enzyme before the addition of substrate. Milk clotting times were estimated by adding equal volumes of fresh milk to the enzyme concentration given. Collagenase activity was measured as described by Oakley, Warrack & van Heyningen (1946).

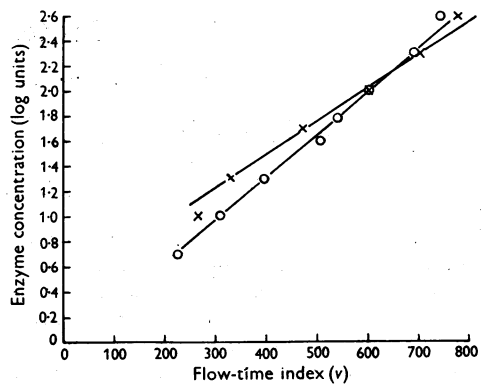

Fig. 1. Relationship between flow-time index (v) and logarithm of enzyme concentration for two bacterial filtrates: ×, *Ps. pyocyanea*; o, *Staph. aureus* (100 units of bacterial filtrate $\equiv 5\,\mu$g. of crystalline trypsin, i.e. a flow-time index of approximately 600).

RESULTS

Measurement of proteolytic activity

Effect of bacterial proteases on gelatin. The examination of a number of bacterial proteases (Fig. 1) showed that the flow-time index (v) plotted against

the logarithm of enzyme concentration gave a straight line over at least part of the range $v = 600$–200. The slope of the line in the case of certain organisms approximates to that for trypsin, lying between 1/380 and 1/450 (Duthie & Lorenz, 1949), but in the case of other organisms, except *Cl. welchii* Type B, it lay between 1/250 and 1/300

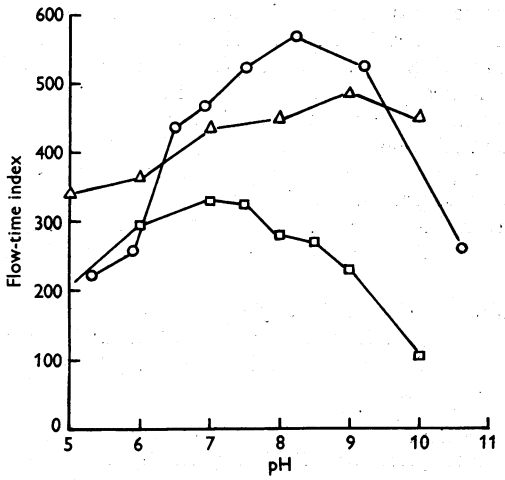

Fig. 2. Effect of pH on enzyme activity of filtrates of aerobic bacteria as measured by the flow-time index. △, *Chromobact. prodigiosum*; □, *Staph. aureus*; ○, *V. cholerae*.

(Fig. 1, Table 2). Thus dilution of these latter decreased the rate of gelatin hydrolysis much less than in the case of trypsin. Quantitative viscosimetric comparisons of protease activity between these two types of filtrate were, therefore, impossible, and all solutions used in Table 2 were standardized so as to have a v value of about 600 after 20 min. hydrolysis of gelatin.

Effect of pH. The filtrates chosen for comparison were selected from those bacteria which showed well-marked proteolytic activity in crude unconcentrated filtrates. The majority of such filtrates showed optimal activity between pH 7 and 9 (Figs. 2, 3), although a filtrate of *Cl. welchii* Type A

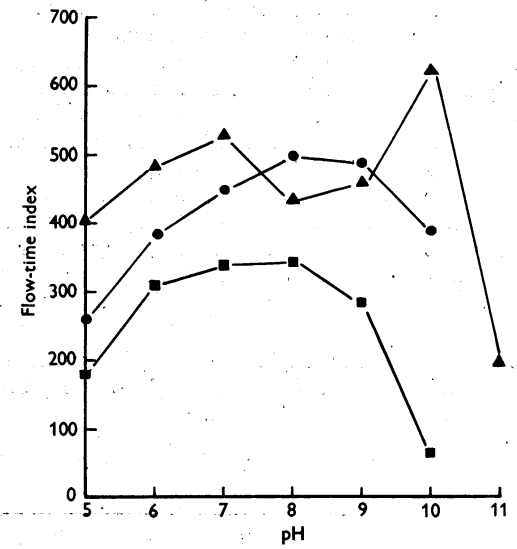

Fig. 3. Effect of pH on enzyme activity of filtrates of anaerobic bacteria as measured by the flow-time index. ▲, *Cl. welchii* Type A filtrate; ■, *Cl. welchii* Type B filtrate; ●, *Cl. histolyticum*.

showed two optima, one at pH 7 and the other at pH 10, indicating the presence of two enzymes. Walbum & Reymann (1934) obtained similar curves for this organism, although their optima were slightly lower. All filtrates showed good activity around pH 7·6, which was that used in the majority of experiments.

Table 2. *Effect of the same concentration of different bacterial filtrates on different substrates and a comparison of the inhibitory activity of normal sheep serum*

Organism	Gelatinase activity (flow-time index)		Slope of gelatin activity curve	Activity against casein measured as μg. 'tyrosine' liberated/ml. digest		Milk clotting time (min.)
	Enzyme alone	Enzyme and 10% (v/v) sheep serum		Enzyme alone	Enzyme and 10% (v/v) sheep serum	
Staph. aureus (113344)	612	406	1/270	102	48	—
B. anthracis	603	0	1/250	110	0	18
Chromobact. prodigiosum	646	0	1/440	118	18	15
Ps. pyocyanea	620	0	1/380	111	0	30
B. megatherium	598	0	1/260	100	0	3
Cl. welchii Type A	600	550	1/450	0	0	—
Cl. welchii Type B	570	0	1/110	80	39	3
Cl. bifermentans	613	0	1/280	84	0	4
Cl. histolyticum	645	325	1/410	66	0	—
Crystalline trypsin	606	0	1/400	81	0	—

Serum inhibition

Action of serum. The addition of serum to a final concentration of 10 % (v/v) affected the hydrolysis of both gelatin and casein in the case of all the bacterial filtrates studied (Table 2). Complete inhibition of gelatin hydrolysis was found in the case of four of the five filtrates of aerobic bacteria and in two of the anaerobic bacteria. Wide variation in sensitivity to serum was encountered among those completely inhibited by 10 % sheep serum; a 50 % inhibition was produced in 100 units of a filtrate of *Chromobact. prodigiosum* by a final dilution of 1·25 % serum, whereas the same effect was produced in 100 units of a filtrate of *Ps. pyocyanea* by as little as 0·05 % serum. The inhibitory power of different animal sera varied according to the species (Table 3), sheep being very much more inhibitory as

Table 3. *Comparison of inhibitory effect of normal animal sera on a culture filtrate of* Ps. pyocyanea *as measured by flow-time index*

(Filtrate 100 units/ml.: sera 0·16 % (v/v).)

	Sera			
	Rabbit	Human	Horse	Sheep
Flow-time index	385	390	330	190

in the case of crystalline trypsin (Duthie & Lorenz, 1949). The interaction between serum and a filtrate of *Ps. pyocyanea* was immediate, there being no increase in inhibition if the mixture was incubated for varying periods prior to the addition of gelatin.

Effect of age and heating on serum. Specimens of horse and human sera kept at 4° rapidly lost part of their inhibitory power against bacterial filtrates, though this was not so noticeable in the case of sheep serum. Table 4 shows that, in a comparison

Table 4. *Comparison of loss in inhibitory effect of stored normal sera on a bacterial filtrate* (Ps. pyocyanea) *and on crystalline trypsin*

(Sera stored 1 year at 4°. Enzyme 100 units/ml.)

	Loss in inhibitory power (%)	
	Human serum	Sheep serum
Filtrate of *Ps. pyocyanea*	66	80
Crystalline trypsin	30	Nil

of fresh and stored human and sheep sera, the loss in inhibitory activity is very much greater when measured against a bacterial filtrate than against crystalline trypsin. The comparison is based on the assumption that the stored sera had originally the same inhibitory power as the fresh sera used. Even if this were not the case, the figures show clearly that inhibitory activity against trypsin and a bacterial filtrate are unrelated, since the fresh sheep serum was equal in inhibitory power to the old serum when measured against crystalline trypsin, though five times as active when measured against the bacterial filtrate. The inhibitor against bacterial filtrates was very sensitive to age and to acidity, more than 90 % of the activity of sheep serum being lost after 2 hr. at 37° and pH 4·6 or 30 min. at 70° in neutral solution. The loss in activity against trypsin under these conditions was less than one third (Duthie & Lorenz, 1949).

Salt fractionation. Precipitation with ammonium sulphate provided further proof (Table 5) that the serum inhibitor against a bacterial filtrate was different from that against crystalline trypsin since

Table 5. *Comparison of inhibitory power of sheep serum fractions measured against* 100 *units of crystalline trypsin and a culture filtrate of* Ps. pyocyanea

(Fractions separated by $(NH_4)_2SO_4$ and activity expressed as percentage of inhibitory activity of whole serum.)

	Globulin	Albumin precipitated by complete saturation with $(NH_4)_2SO_4$ at pH 4·2 (Fraction C_1)
Crystalline trypsin	20	45
Ps. pyocyanea	38	2·8

the more soluble albumin fraction C_1, precipitated by full saturation with ammonium sulphate at the isoelectric point, contained nearly half the original activity against trypsin but only 3 % of the original activity when measured against the bacterial filtrate. Similar results were obtained with fractions separated electrophoretically. Thus while both globulin and albumin fractions are inhibitory, though in different degree, to crystalline trypsin, the bulk of the inhibitory action against bacterial proteases is associated with the globulin.

Effect of other inhibitors

Egg white and soya bean. Neither soya-bean trypsin inhibitor nor pancreatic trypsin inhibitor had any action on the proteases of either *Staph. aureus*, *Ps. pyocyanea* or *Chromobact. prodigiosum*. Raw egg white resembled serum with regard to the varying sensitivity of different bacterial filtrates (Table 6). Ovomucoid purified according to the method of Lineweaver & Murray (1947) was active against 100 units of crystalline trypsin at 1:2,000,000, but was without activity against bacterial filtrates at 1:1000.

Table 6. *Comparison of sensitivity of different bacterial filtrates to serum and to egg white*

	Concentration of inhibitor (%)	Inhibition of proteolysis (%)		
		Ps. pyocyanea	*Chromobact. prodigiosum*	*Cl. welchii* Type A
Human serum	1·25	100	50	Nil
Egg white	2·5	62	33	Nil
Soya bean	0·1	Nil	Nil	Nil

Specific antisera. When specific antiserum is added to a constant amount of a culture filtrate of *Staph. aureus* or of *Cl. welchii* Type A, and the flow-time index is plotted against the units of antiserum added, the curves obtained are not dissimilar from those obtained by the addition of normal serum to either crystalline trypsin (Duthie & Lorenz, 1949) or to filtrates of bacteria (Fig. 4). The viscosimetric

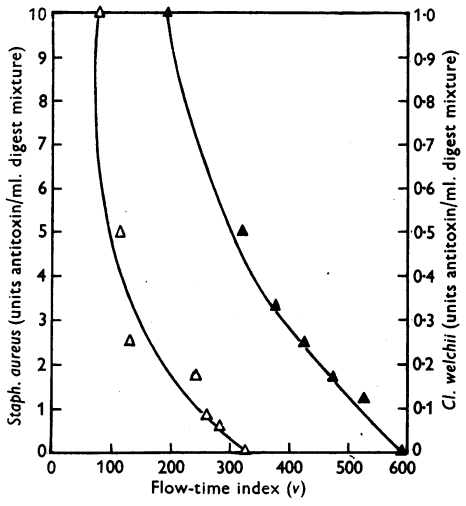

Fig. 4. Effect of increasing amounts of specific antiserum on filtrates of *Staph. aureus* and *Cl. welchii* Type A. (Staphylococcal antiserum was a globulin fraction, *Cl. welchii* antiserum was a pepsin-digested globulin.) △, *Staph. aureus*; ▲, *Cl. welchii*.

method can thus be used in the estimation of the antiprotease component of any antiserum. Both staphylococcal and *Cl. welchii* Type A antisera were specific in their antiprotease activity and the *Cl. welchii* Type A antiserum did not neutralize the proteases in *Cl. welchii* Type B filtrates.

Comparison of filtrate activities

Table 2 shows that for all five filtrates of aerobic bacteria examined there was a very good correlation between casein and gelatin hydrolysis, comparing the v value and acid-soluble 'tyrosine' liberated, indicating that the same enzyme is probably involved. The filtrates from Clostridia showed a much greater degree of variation and, in addition, a lower ratio of casein to gelatin hydrolysis. Of these four filtrates, only *Cl. welchii* Type A failed to hydrolyze casein, and to hydrolyze gelatin in the presence of serum. The hydrolysis of gelatin by a filtrate of *Cl. histolyticum* was only partly inhibited by serum, and both filtrates presumably contain a specific gelatinase. These two filtrates attacked native collagen in the form of muscle and tendon, but collagenase and gelatinase properties are not due to the same enzyme, since two purified preparations of collagenase received from Miss E. Bidwell had lost completely their power of attacking native collagen following 4 months' storage at 4°, while retaining their gelatinase properties unimpaired.

None of the filtrates in Table 2 showed increased proteolysis in the presence of thioglycollic acid. Only certain filtrates clotted milk, and then the rate of reaction was not related to protease activity as measured either on gelatin or casein. The clotting of milk was inhibited by normal sera and especially by the globulin fraction. The albumin fraction used in the experiment of Table 5 had little or no inhibitory effect.

DISCUSSION

In contrast to serum antitrypsin, which is associated with the more soluble albumin fraction, the serum inhibitor of bacterial proteases is associated mainly with the globulins. This property is also much more labile than in the case of serum antitrypsin, but it does not disappear completely on storage as stated by Smith & Lindsley (1939). Likewise, whilst considerable differences exist between the inhibitory power of the sera of different species, it is never absent as claimed by Maschmann (1937*b*), and such discrepancies as have been reported are due either to varying sensitivity of the proteases tested or to failure to use fresh serum, since the inhibitory titre of horse serum can fall very rapidly even at 4°. The rapid combination of the serum inhibitor of bacterial proteases with enzyme resembles that of an antigen-antibody reaction, and further distinguishes this inhibitor from serum antitrypsin. Todd (1947) has found that the serum of about 5% of normal horses and 25% of normal humans investigated contained

an inhibitor for a streptococcal protease which he studied. The inhibitor also lay in the globulin fraction and suggested some form of 'natural antibody'. Wide variations in antiprotease titre, such as were found by Todd, were not noticed in the present studies, but sufficient work has not been done on this point. All human and animal sera investigated inhibited susceptible bacterial proteases, and the inhibition would, therefore, appear to be part of a non-specific immunity reaction.

Recently Haas (1946) has studied the inhibitory action of serum on hyaluronidase both of testicular and of bacterial origin. Haas finds that, like the reaction between serum antitrypsin and trypsin, the amount of inhibition increases with time of contact of enzyme and serum prior to incubation with substrate. Similarly, the inhibitor is destroyed by short treatment with acid and by heating to 53° for 15 min. It is apparently assumed that the same substance is responsible for the inhibition of both testicular and bacterial hyaluronidase, since the source of the enzyme used in these experiments is not specified. On the basis of these findings, Haas concludes that the serum inhibitor of hyaluronidase is an enzyme which he terms antivasin 1. Since the inhibitory properties of serum for both trypsin and bacterial proteases are analogous to and closely resemble those antihyaluronidase properties described by Haas, one might equally regard them as being due to an enzyme. While such a possibility might be considered, it is felt that the evidence produced by Haas is insufficient.

The filtrates of *Cl. welchii* Type A and *Cl. histolyticum* clearly contain the substrate-specific gelatinase described by Maschmann (1937*a*, *b*, 1938*b*), which is only partly inhibited by very large amounts of normal serum such as 10% (v/v) used in Table 2. Although gelatinase is closely associated with collagenase in the purification process used by Bidwell & van Heyningen (1948), the two are not identical, since collagenase activity alone is lost on storage or on dialysis at pH 10·0 (Bidwell, personal communication).

SUMMARY

1. The flow-time index relationship described by Swyer & Emmens (1947) can be used for the viscosimetric assay of bacterial proteases and their inhibitors.

2. The serum inhibitor active against bacterial proteases is a labile component of the globulin fraction, in contrast to the relatively more stable serum antitrypsin, which is found mainly in the albumin fraction. The serum of different animal species varies in its inhibitory power.

3. Bacterial proteases vary in their sensitivity to the serum inhibitor, the specific gelatinase found in certain Clostridia being relatively insensitive; filtrates sensitive to serum are also inhibited by egg white, but all are unaffected by ovomucoid, soya bean or by pancreatic trypsin inhibitors.

4. *Cl. welchii* Type A gelatinase is inhibited by specific antiserum, and the potency of different antisera can be compared viscosimetrically. The gelatinase activity is independent of collagenase activity.

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