PROTEOLYTIC ENZYMES

II. THE PHYSIOLOGICAL SIGNIFICANCE OF THE CONTROL OF THEIR ACTIVITY, ESPECIALLY WITH RESPECT TO BACTERIAL GROWTH

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Müller (1888) first described the presence in human purulent sputum of protease active at neutral pH. Achalme (1899) was the first to find this enzyme in human pus. Jobling and Strouse (1912) showed that extracts of human pus also contain peptidase active at neutral pH. Opie (1905, 1922) showed that polymorphonuclear leucocytes are the chief source of leucoprotease (named by him) and was the first to emphasize the physiological significance of this protease and of its inhibition by serum. He demonstrated that leucoprotease plays a major rôle in the resolution of inflammatory exudates, and that serum (in sufficient concentration) can retard this resolution by virtue of its inhibitory power. More recent workers (Hoimes et al., 1935) have applied these principles to the study of suppurative arthritis and have shown that the presence of antiproteolytic substances in synovial exudates is of great importance in the prevention of damage to joints by protease liberated from the polymorphonuclear leucocytes of these exudates. However, the general problem of the relation of leucoprotease and serum antiprotease to the resolution and absorption of inflammatory exudates and absorbable foreign bodies has not been intensively studied since the work of Opie early in the century. It is hoped that the experiments described in the preceding paper on the control of leucoprotease and serum antiprotease will facilitate such a study by enabling better control of the activity of these important substances than has hitherto been possible.

The experiments of Menkin (1938) and others indicate that the increased capillary permeability and leucocyte infiltration characteristic of inflammation are due to a polypeptide formed by proteolysis at the site of inflammation. Evidence has also been presented (Rocha e Silva, 1941, 1942) that local proteolysis in the tissues liberates histamine, which is thought by some to be important in inflammation and anaphylaxis. The ability to control the activity of leucoprotease and of serum antiprotease may perhaps facilitate further investigation of these phenomena.

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Other physiological and pathological processes in which the ability to control the activity of leucoprotease (or other proteases) and serum antiprotease may prove to be of value include:

(1) The neutralization of the destructive protease liberated in acute pancreatitis and from duodenal fistulae.

(2) The experimental production (Rich and Duff, 1937) of hyaline arteriosclerosis and arteriolonecrosis by subcutaneous injection of proteolytic enzymes. (The local necrosis produced by trypsin has been shown to be inversely proportional to the antiproteolytic activity of the serum (Grob, 1943).)

(3) The coagulation of the blood. Eagle and Harris (1936-37) have presented evidence that, in the course of physiological coagulation, calcium plus thromboplastin constitutes a proteolytic enzyme system (analogous to trypsin) which is believed to be responsible for the transformation of prothrombin to thrombin, itself thought to be a proteolytic enzyme that accelerates the transformation of fibrinogen to fibrin. More recently evidence has been presented (Grob, 1943) that purified serum antitrypsin and pancreatic trypsin inhibitor inhibit the coagulation of plasma in vitro.

(4) The possibility of prolonging insulin action by retarding the breakdown of insulin by serum protease (and other proteases?).

(5) Possibly the release of thyroglobulin by the thyroid gland. Paraaminobenzoic acid, sulfonamides, and thiourea, which have been shown to inhibit leucoprotease, are known (Mackenzie and Mackenzie, 1943, Astwood et al., 1943) to inhibit the activity of the thyroid gland. Dziemian (1943) was able to correlate the activity of the thyroid gland of rats with the proteolytic (catheptic) activity of thyroid extracts. He found that sulfaguanidine decreased both activities, and presented evidence in support of the suggestion of Gersh and Caspersson (1940) that a proteolytic enzyme in the thyroid hydrolyzes thyroglobulin into peptones or polypeptides which are small enough to pass across the cell membranes and are then reconstituted by enzyme action into a larger molecule.

(6) Bacterial growth and sulfonamide action (discussed below).

In a previous paper (Grob, 1943) evidence was presented that:

(1) Heating diluted serum at 80°C. for 10 minutes makes it a better medium for bacterial growth. This is believed to have been at least partly due to destruction of the serum antiprotease by heat. (2) Growth is accelerated and proceeds further in the presence of trypsin. (3) Growth is somewhat retarded in the presence of pancreatic trypsin inhibitor. (4) The bacteriostatic action of sulfathiazole in serum is reduced by heating the serum at 80°C. for 10 minutes and much more markedly by adding trypsin. It is greater in serum and albumin than in peptone and meat infusion.

These findings were taken to indicate that the products of tryptic digestion promote bacterial growth, and directly and indirectly inhibit the bacteriostatic action of sulfathiazole. On the basis of these findings it was concluded that the growth of bacteria...
in the body will be more rapid, and the inhibition of sulfathiazole will be greater: (a) when considerable leucoprotease (and peptidase?) is present; (b) when the organism itself produces active protease (and peptidase?); (c) when the inflammatory exudate is small and poor in antiprotease; (d) when the source of antiprotease (the blood) is poor in this constituent; and (e) when the medium is rich in non-protein nitrogen (especially para-aminobenzoic acid?).

These conclusions would not be valid if leucoprotease could not be shown to have the same effect on bacterial growth and sulfonamide action as was shown for crude trypsin. To investigate this the growth of *Escherichia coli, Proteus vulgaris, Staphylococcus aureus, Streptococcus pyogenes*, and *Clostridium welchii* was followed in the following media:

1. 10 per cent human serum (serum diluted with isotonic phosphate buffer and heated at 56°C. for 30 minutes to destroy complement, so as to eliminate the action of normal bactericidal antibodies).
2. 1 heated at 80°C. for 10 minutes to destroy antiproteolytic activity.
3. 1 + sulfathiazole (0.1 mg./cc.).
4. 2 + sulfathiazole (0.1 mg./cc.).

Growth was followed in these media in the absence of added enzyme, and in the presence of crude trypsin (1.0 mg./cc.), crystalline trypsin (0.25 mg./cc.), and leucoprotease (cat, lyo-, equivalent to 0.2 mg./cc. crude trypsin). Bacterial growth was determined by measuring the turbidity of bacterial suspensions after 24 hours' incubation at 37°C. by means of a photoelectric nephelometer. The opacity of the suspension is represented as the number of 24 hour old organisms (determined by direct count) which, when suspended in the same medium, allowed the same amount of light to be transmitted. A small correction was made for changes in the opacity of the medium resulting from incubation alone (especially for heated serum plus enzyme), by incubating under toluene control tubes of similar composition. The error produced by the digestion of dead bacteria by protease (resulting in some decrease in turbidity) was not corrected for, but several pour plate counts of viable bacteria indicated that this error did not affect the conclusions drawn from the experiment.

The initial concentration of bacteria in each case was approximately 5 million/cc. Except for *Clostridium welchii* (which was obtained from thioglycollate medium) the organisms were obtained from 48 hour old agar slant cultures, and all were washed repeatedly in saline before addition to the test media. *Clostridium welchii* was grown under anaerobic conditions.

Examination of the data (Table I) reveals that:

(a) Growth proceeded further in serum that had been heated at 80°C. for 10 minutes to destroy serum antiprotease than in serum that had merely been heated sufficiently to destroy complement. (However, the destruction of serum antiprotease cannot be said to be alone responsible for the increased growth, since denaturation of proteins by heat alters the rate at which they are hydrolyzed, as well as many other of their properties.)
(b) Growth proceeded further in media to which crude trypsin, crystalline trypsin, or leucoprotease had been added.

(c) The bacteriostatic action of sulfathiazole was: (1) somewhat reduced by destruction of the antiproteolytic activity of the serum (by heat, which altered the serum in other ways too); (2) reduced by the presence of crude trypsin, leucoprotease, or (to a lesser extent) crystalline trypsin; (3) not influenced by boiled leucoprotease, which also had no appreciable effect on bacterial growth.

These results indicate that the products of protein hydrolysis by leucoprotease accelerate bacterial growth and inhibit sulfonamide action, in a manner similar to that previously described for the products of protein hydrolysis by crude trypsin. They also suggest that while the products of hydrolysis by crystalline trypsin accelerate bacterial growth to about the same extent as those of crude trypsin, the former (higher) degradation products are not as effective in the inhibition of sulfonamide action.

Evidence was previously presented (Grob, 1943) that trypsin inhibitor possesses no bactericidal activity, and trypsin no antibactericidal activity. Although corresponding experiments were not performed for leucoprotease, it is probable that this protease would also be found to have no bactericidal or

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**TABLE I**

**The Influence of Proteolytic Enzymes and Serum Antiprotease on Bacterial Growth and Sulfonamide Action**

<table>
<thead>
<tr>
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</thead>
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<td>Intact</td>
<td>0</td>
<td>Intact</td>
<td>0</td>
<td>Intact</td>
</tr>
<tr>
<td>Enzyme .............</td>
<td>Sulfathiazole</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None .............</td>
<td>115</td>
<td>380</td>
<td>125</td>
<td>155</td>
<td>105</td>
</tr>
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<td>55</td>
<td>45</td>
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<tr>
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<td>580</td>
<td>135</td>
<td>185</td>
<td>125</td>
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<tr>
<td>0.1 mg./cc. .............</td>
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<td>264</td>
<td>25</td>
<td>165</td>
<td>85</td>
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<tr>
<td>Crystalline trypsin .............</td>
<td>125</td>
<td>520</td>
<td>145</td>
<td>285</td>
<td>105</td>
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<tr>
<td>0.1 mg./cc. .............</td>
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<td>185</td>
<td>45</td>
<td>40</td>
<td>65</td>
</tr>
<tr>
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<td>580</td>
<td>145</td>
<td>300</td>
<td>105</td>
</tr>
<tr>
<td>0.1 mg./cc. .............</td>
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<td>185</td>
<td>75</td>
<td>200</td>
<td>65</td>
</tr>
<tr>
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<td>125</td>
<td>55</td>
<td>150</td>
<td>90</td>
<td>100</td>
</tr>
</tbody>
</table>
antibactericidal activity. However, the polymorphonuclear leucocytes of
many animals do contain a bactericidal substance ("leukin"), which may be
identical with lysozyme (Barnes, 1940) and which will be present at the site of
inflammation and in extracts of the polymorphs.

The inhibitory effect of peptone and of various bacterial extracts on sulfonamides
has been the subject of intensive study (Lockwood, 1938; McIntosh and Whitby,
1939; Stamp, 1939). Woods (1940) has advanced evidence that this inhibitory effect
is due to the presence of para-aminobenzoic acid, and has attributed to the presence
of para-aminobenzoic acid preformed in the medium the observed (Lockwood et al.,
1937) difficulty of inhibiting bacteria by the use of sulfonamide in peptone and in
lesions characterized by considerable tissue destruction. MacLeod (1940) has cor-
related sulfonamide inhibition with the degree of tissue autolysis (which, he believes,
liberates para-aminobenzoic acid from a preexisting conjugated form). Others
(Lockwood and Lynch, 1940) have pointed out that organisms known to be susceptible
to sulfonamides are in general not actively proteolytic, and it is believed (MacLeod,
1940) that they are probably not capable of producing much para-aminobenzoic acid.

Evidence has been presented that para-aminobenzoic acid also inhibits the "bac-
teriostatic" action of sulfonamides (Fitzgerald and Feinstone, 1943) and promin
(Steenken and Heise, 1943) on tubercle bacilli, and of sulfonamides on the Plasmodium
of bird malaria and on the virus of lymphogranuloma venereum (Maier and Riley,
1942).

Green and Bielschowsky (1942) have emphasized that the antisulfonamide activity
of bacterial and yeast extracts is exerted directly by para-aminobenzoic acid and simi-
lar sulfonamide inhibitors, and indirectly by substances which stimulate bacterial
growth, even in an optimal nutrient medium. (Para-aminobenzoic acid itself
stimulates the growth of very few organisms (Rubbo and Gillespie, 1940; Sevag and
Shelburne, 1942; Rantz, 1942.) These latter substances which stimulate growth will
also exert their indirect inhibiting effect against other bacteriostatic agents than
the sulfonamides; e.g., against penicillin (Green and Bielschowsky, 1942).

Not only the properties of the medium, but also the properties of the bacteria in-
fluence sulfonamide action. It is believed (Woods, 1940; Green and Bielschowsky,
1942) that the degree of sensitivity of bacteria to sulfonamides is determined by the
extent to which para-aminobenzoic acid and related "essential metabolites" are
necessary to the life cycle of the bacteria, and by the rate at which para-aminobenzoic
acid is synthesized and released into the surrounding medium. Many investigators
have attempted to discover a chemotherapeutic agent which would react with para-
aminobenzoic acid, and/or reduce the rate of synthesis of para-aminobenzoic acid by
bacteria. It is thought that azochloramide and related halogen compounds (Schmelkes
and Wyss, 1942), and perhaps urea (Tenenberg et al., 1942) are capable of neutralizing
the action of para-aminobenzoic acid and may thereby prove to be useful as poten-
tiators of the sulfonamides.

The experiments on leucoprotease described above indicate that leucopro-
tease activity, such as occurs at any site of bacterial growth with its concomitant
inflammation, results in the production of protein degradation products which
stimulate bacterial growth, and directly and indirectly inhibit sulfonamide action. (A third way in which leucoprotease activity increases bacterial growth in the presence of sulfonamides is by causing an accumulation of acid products of hydrolysis, which decrease sulfonamide action since sulfonamides have been shown (Schmelkes et al., 1942) to be less active at decreased pH. Of course, when the pH falls too low, as in a poorly buffered medium, bacterial growth will be retarded.)

It is to be expected that substances which inhibit the activity of leucoprotease e.g., serum, reducing agents, heavy metals, etc.) will inhibit the production of protein degradation products at the site of bacterial growth and inflammation, and will thereby act to prevent the acceleration of bacterial growth, and the inhibition of sulfonamide action, that these products cause. It is suggested that the search for sulfonamide potentiators be directed not only toward the discovery of agents which will destroy sulfonamide inhibitors once formed, but also toward the utilization of substances which will prevent the formation of these inhibitors (and of the degradation products which stimulate bacterial growth) by retarding the action of leucoprotease (and perhaps of bacterial proteases as well). This would seem to be especially desirable in purulent lesions, where the sulfonamides have proved least efficacious. With respect to the use of bacteriostatic agents which are not inhibited in purulent lesions (e.g. penicillin) it would still seem desirable to control the production by proteolysis of degradation products which have been shown to directly stimulate bacterial growth. It is hoped that the data presented in the preceding paper on the control of the activity of leucoprotease and serum antiprotease may prove applicable in these respects.

SUMMARY

It has been suggested that the ability to control the activity of leucoprotease and serum antiprotease may prove useful in the further study and understanding of such phenomena as: (1) inflammation; (2) the resolution of inflammatory exudates and the absorption of absorbable foreign bodies; (3) the protection of joint and other structures from the proteolytic action of leucoprotease; (4) the neutralization of the destructive protease liberated in acute pancreatitis and from duodenal fistulae; (5) experimental arteriosclerosis and arteriolonecrosis; (6) the coagulation of the blood; (7) the possibility of prolonging the action of insulin; (8) the release of thyroglobulin by the thyroid gland; (9) bacterial growth and sulfonamide action.

In this last respect evidence has been presented that the products of the hydrolysis of protein by leucoprotease stimulate bacterial growth and directly and indirectly inhibit sulfonamide action, and the hope has been expressed that the ability to control leucoprotease action may contribute to the more successful use of chemotherapeutic agents in purulent lesions.
It is a pleasure to acknowledge again my gratitude to Dr. J. Howard Brown for his encouragement and assistance in the performance of these and previous experiments. I am also indebted to Dr. Leslie Hellerman for the interest he has taken in this work, and for the help he has given.

REFERENCES