PROTEOLYTIC ENZYMES

I. The Control of Their Activity

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Because enzymes are among the most important regulators of animal and plant life processes, the results of the ability to control and utilize them may well prove to be far reaching. Such control and utilization can only follow an understanding of their mode of action, and of the conditions affecting their activity.

One of the most important and best studied groups of organic catalysts is the proteolytic enzymes.

A. CLASSIFICATION

Proteolytic enzymes, or proteases, are those enzymes which break down protein by hydrolyzing peptide linkages. The work of Bergmann and his coworkers (1936, 1937) has demonstrated that two general classes of proteases can be differentiated, the proteinases and the peptidases.

Proteinases hydrolyze peptide linkages anywhere in the protein molecule. Usually the linkages are within the molecule, *i.e.* adjacent to another peptide bond, so that these enzymes have been called "endopeptidases." They may be further classified on the basis of animal or plant origin, extracellular or intracellular action, optimal action at neutral pH or in varying degrees of acidity, and activation or inactivation by various compounds. The extracellular proteinases include pepsin (active in acid medium), trypsin and chymotrypsin (most active in neutral or slightly alkaline medium), and some yeast, mold, and bacterial proteases. The intracellular proteinases include those of animal origin, called cathepsins (found in all tissues and organs, and most active in acid medium at pH 5 to 6), and those of plant origin, including papain (from the papaya plant, optimum pH 5 to 6), ficin (from figs), bromelin (from pineapple), most yeast and mold proteases, and most bacterial proteases. Leucoprotease, the proteolytic enzyme of white blood cells (most active at neutral pH), probably functions within as well as outside of the cells, but because its properties are similar to those of trypsin it has usually been classified with this extracellular enzyme.

Peptidases hydrolyze only peptide linkages that are adjacent to the end of

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the protein molecule, and have therefore been called "exopeptidases." They may be classified on the same bases as the proteinases, and are as widely distributed. Both proteinases and peptidases, especially the latter, have been further classified in terms of the specificity of the substrate that is hydrolyzed. Thus, carboxypeptidases hydrolyze substrates with a free carboxyl group adjacent to the linkage to be split, aminopeptidases hydrolyze substrates with a free amino group, and dipeptidases, substrates with a free carboxyl and a free amino group.

B. CONDITIONS AFFECTING THE ACTIVITY OF PROTEOLYTIC ENZYMES (REVIEW OF LITERATURE)

The influence of temperature and of hydrogen ion concentration on the activity of proteolytic enzymes has been carefully studied. Most of these enzymes are very active at 37°C., slightly increased in activity by short exposure to a temperature between 37 and 60°C., slightly impaired by a temperature of 65°C., and destroyed (in solution, though not in dry state) above 70°C., the necessary temperature varying inversely with the length of time of exposure. The non-specific influence of adjacent ions and molecules on the thermodynamic environment, and the specific influence of many ions, especially metallic ones, has also frequently been evaluated. Realization of the significance of oxidation-reduction systems is more recent, and while the intracellular proteinases have been intensively investigated in this regard, the important extracellular proteinases and leucoprotease have been relatively neglected.

Consideration of the importance of oxidation-reduction systems began with the discoveries that HCN (Vines, 1903, 1905), H_2S (Mendel and Blood, 1910), cysteine, and HS glutathione (Grassman, von Schoenebeck, and Eibeler, 1931) increased the activity of papain and of cathepsin (Grassman *et al.*, 1930; Waldschmidt-Leitz, 1929). The physiological significance of these findings was emphasized by the demonstration (Grassman, Dyckerhoff, and von Schoenebeck, 1930) that many intracellular proteases, both plant and animal, are accompanied by sulfhydryl compounds which serve as natural activators, presumably by virtue of their reducing action. A theory for the mechanism of the activation of papain by reducing agents, and of the reversible inactivation of papain by careful use of oxidizing agents such as iodine or hydrogen peroxide (Bersin, 1933, 1934) was developed by Bersin (1935) and Hellerman (1937), who suggested that the disulfide form of the enzyme (En—S—S—En) is inactive, and that reduction of disulfide groups to active sulfhydryl groups activates the enzyme:

$$\text{En—S—S—En} + 2\text{H}^+ + 2(\text{e}) \underset{\text{oxidation}}{\overset{\text{reduction}}{\rightleftharpoons}} 2\text{En} - \text{SH}$$

This sulfhydryl mechanism has been extended by Hellerman (1937) and has been applied to many other enzymes. Further evidence for this mechanism is provided by the reversible inactivation of these enzymes (papain, cathepsin, urease) by mercaptide-forming reagents (such as benzyl mercuric chloride, *p*-tolyl mercuric chloride, cuprous oxide, etc., represented below as Me) and their reactivation by substances which decompose the mercaptides (Hellerman, 1939):

$$\underset{\text{active}}{\text{En} - \text{SH}} + \text{Me}^+ \rightleftharpoons \underset{\text{inactive}}{\text{En} - \text{S} - \text{Me}} + \text{H}^+$$

Objections to the sulfhydryl theory (Bergmann and Fruton, 1941), based on varying specificities of the activators unrelated to reducing potential, and on the mechanism of activation by HCN, will not be considered at present. It is desired mainly to emphasize the significant rôle which biologically important thiol-sulfhydryl compounds (glutathione, ergothionine, cysteine, cysteine peptides, sulfhydryl-containing proteins, etc.) and other oxidation-reduction compounds (*e.g.* ascorbic acid) play in the control of enzyme action. For the sake of simplicity such control will be assumed to be exerted by virtue of control of the oxidation-reduction system, though other mechanisms of action are not excluded.

Table I attempts to summarize the literature on the importance of the oxidation-reduction system in the control of the activity of many enzymes. First are listed the enzymes whose reduced form is more active than the oxidized form; then the enzymes whose oxidized form presumably is more active. Activation and inhibition have in most cases been shown to be reversible and reciprocal, particularly in that enzymes which are activated by reducing agents (especially sulfhydryl compounds) are inhibited by oxidizing agents (especially inhibitors of sulfhydryl groups).

As previously mentioned, the study of the influence of the oxidation-reduction system on extracellular proteinases and on leucoprotease has been relatively neglected. Grassman, Dyckerhoff, and von Schoenebeck (1930) demonstrated that cysteine, H_2S, HCN, and pyrophosphate reduced the activity of trypsin, while cystine increased its activity, but they attributed these findings to the inactivation of trypsin kinase by the reducing agents and activation by cystine, rather than to any direct effect on the enzyme. Search of the literature revealed no report on the influence of the oxidation-reduction system on leucoprotease, or on the inhibitors of trypsin and leucoprotease, including serum antiprotease.

Table II attempts to summarize the literature on substances which, in suitable concentration, have been found to influence the activity of trypsin and leucoprotease.

Because of similarity of many properties, leucoprotease and trypsin have been thought by some (Willstätter and Rohdewald, 1932) to be identical enzymes. Others (Stern, 1931) have pointed to the difference in the effect of Mn^{++} and Fe^{++} (in suitable concentrations) on these enzymes and have concluded that they are different. Comparison is difficult because crude trypsin, and leucoprotease as extracted from white blood cells, are mixtures of enzymes, and furthermore the leucoproteases of different animals may differ considerably in the kinds and amounts of enzymes present. Thus the proteinase activity of the polymorphonuclear leucocytes of carnivorous animals at pH 7 is said to be over twice as high as that of herbivorous animals (Willstätter and Rohdewald, 1932). Barnes (1940) has compared the proteinases of rabbit and cat white blood cells as follows:

	Rabbit		Cat	
	Lympho-cytes	Poly-morphs	Lympho-cytes	Poly-morphs
Proteinase active at pH 4 ("cathepsin")	+	+	+	+
Proteinase active at pH 8 ("tryptase")	−	−	−	+++

Parker and Franke (1917) also found no "tryptase" in rabbit polymorphs, but Weiss (1939) has reported the presence here of a "tryptase" which digests casein or gelatin

TABLE I

Proteinases activated by reducing agents
(*a*) Papain proteinase (79, 59, 35)
(*b*) Cathepsin (35, 82)
(*c*) Ficin and bromelin proteinase (41)
(*d*) Yeast proteinase (33)
(*e*) Proteinases of anaerobic bacteria, and of aerobic bacteria in presence of Fe^{++} (85)
Peptidases activated by reducing agents
(*a*) Some yeast and mold dipeptidases (also activated by Zn^{++} or Mn^{++} (33)
(*b*) Some intestinal dipeptidases (also activated by Mn^{++} (31)
(*c*) Animal intracellular ("catheptic") peptidases (29)
(*d*) Bacterial peptidases, especially of anaerobic bacteria (also activated by Zn^{++}, Fe^{++}, Pb^{++}, Cu^{++}, Mn^{++}, Sn^{++}, Cd^{++}, Hg^{++}) (9)
(*e*) Most *d*-aminopeptidases (11)
Other enzyme systems activated by reducing agents
(*a*) Pneumococcal hemolysin (73)
(*b*) Lysozyme (60)
(*c*) Cerebrosidase (78)
(*d*) Muscle glycolysis (32)
(*e*) Urease (40)
(*f*) Serum complement (24)
(*g*) Stability of virus proteins (increased by reducing agents) (71, 3, 18, 61, 91)
(*h*) Liver pyruvate, malate, and ketoglutarate oxidase; adenosinetriphosphatase (5)
(*i*) *d*-amino acid, *l*-glutamic acid, and monoamine oxidase; transaminase (5)
(*j*) Yeast alcohol, liver choline, and bacterial glycerol oxidase (5)
(*k*) Liver and bacterial (*E. coli*) stearate, bacterial oleate, heart β-hydroxybutyrate oxidase; ? pancreatic lipase (5)
(*l*) Acetylcholine esterase; ? serum mono-*n*-butyrin and liver esterase (5)
Proteinases inhibited by reducing agents
(*a*) Trypsin (kinase said to be inhibited) (34)
(*b*) Proteinases of aerobic bacteria in absence of Fe^{++} (85)
Peptidases inhibited by reducing agents
(*a*) Some yeast and mold dipeptidases (also inhibited by Mn^{++} and polypeptidases (33, 10)
(*b*) Intestinal leucine aminopeptidase (33), effect counteracted by Mn^{++} and Mg^{++} (9)
(*c*) Papain peptidase (12)
Other enzyme systems inhibited by reducing agents
(*a*) Protein synthesis by papain (80, not confirmed in 77)

at pH 8. Human polymorphs (obtained from the blood of myeloid leukemia patients) have been found to contain proteinase most active at pH 7 to 8, other proteinase most active at pH 4, and peptidase most active at pH 7 to 8 (Husfeldt, 1931).

No discussion of the inhibition of proteolytic enzymes would be inclusive without

TABLE II

Substances which have been found to inhibit the activity of trypsin
(*a*) Serum and plasma (44, repeatedly confirmed)
(*b*) Charcoal (39)
(*c*) Unsaturated fatty acids (49)
(*d*) Tryptic digests of proteins (7, 47)
(*e*) Pancreatic trypsin inhibitor (crystallized—63)
(*f*) Serum trypsin inhibitor (crystallized—72)
(*g*) Egg white antitrypsin (said to act by inhibiting trypsin kinase—2)
(*h*) Cysteine, H_2S, HCN, and pyrophosphate (said to act by inhibiting trypsin kinase—34)
(*i*) Hydrogen ion concentration other than the optimum of pH = 8 (37)
(*j*) Alcohol, formaldehyde, thymol, chloroform, toluol, some alkaloids, *e.g.* hordenine sulfate (26)
(*k*) Glycerol (81)
(*l*) Triglycerides, sugar (concentrated), asparagine, glutamic acid (27)
(*m*) Glycylglycine, leucylglycine, and alanylglycine (84)
(*n*) F^-, $Cl^- > I^- > Br^-$; $NH_4^+ > K^+ > Na^+$ (21)
(*o*) Cu^{++}, Zn^{++} (76)
(*p*) Mn^{++} (89)
(*q*) Hg^{++} (25)
(*r*) Lecithin (75)
(*s*) Heparin (45)
(*t*) Quinine salts (55) (act by changing pH, according to 74)
(*u*) Urea salts (74)
(*v*) Germanin (Bayer 205) (8)
(*w*) Azodyes (56, 68)
(*x*) Acridine dyes (trypaflavin, rivanol) and Congo red (6)
(*y*) X-rays and ultraviolet light (19)
(*z*) Bacteria (53)
Substances which have been found to increase the activity of trypsin
(*a*) Cystine (said to act by activating trypsin kinase—34)
(*b*) Asparagine, aspartic acid, glutamic acid, cysteine, and CN^- (accelerate the first stage of hydrolysis, according to 27)
(*c*) Fe^{++}, Se^{++} (slightly) (89)
(*d*) Ca^{++}, Mg^{++} (22)
(*e*) Ag^+ (76)
(*f*) Primary phosphate (28)
Substances which have been found to inhibit the activity of leucoprotease
(*a*) Serum and plasma (65, repeatedly confirmed)
(*b*) Tryptic digests of proteins (according to 69)
(*c*) Unsaturated fatty acids (50)
(*d*) Hydrogen ion concentration other than the optimum of pH = 7 (76)
(*e*) Fe^{++} (89)
(*f*) Quinine salts (55) (act by changing pH according to 74)
(*g*) Phosphatide of tubercle bacillus (70)
Substances which have been found to increase the aetivity of leucoprotease
(*a*) Mn^{++}, Se^{++} (89)

reference to serum antiprotease. Mammalian and bird serum and plasma have long been known to strongly inhibit many animal and plant proteases, including those of pancrease (trypsin), yeast, leucocytes (leucoprotease), and tissue cells (cathepsin) (Jochman, 1908). Also present in the serum are small amounts of proteases active at neutral pH and in acid medium (Opie and Barker, 1908), but these are neutralized by a considerable excess of serum antiprotease. The nature, and the reasons for the physiological and pathological variation of the antiproteolytic activity of the serum have been the subject of much dispute. Evidence has been presented (Grob, 1943) that products of protein hydrolysis (probably one or more polypeptides) formed in the intestine and parenterally are an important factor contributing to this activity of the serum. Elucidation of the mode of action of the antiproteolytic activity of the serum would throw considerable light on conditions affecting the activity of such important proteolytic enzymes as leucoprotease and trypsin.

C. THE CONTROL OF THE ACTIVITY OF TRYPSIN, LEUCOPROTEASE, AND SERUM ANTIPROTEASE (EXPERIMENTAL DATA)

1. Determination of Proteolytic Activity

Proteolytic and antiproteolytic activity were determined by following the digestion of casein nephelometrically by modification of the method of Wunderly (1936). Digest mixtures were prepared by adding the following reagents in the order given:

(*a*) 0 to 2 cc. of the solution to be tested for influence on protease action,

(*b*) 3.5 cc. of M/15 KH_2PO_4 – K_2HPO_4 buffer (pH 7.5),

(*c*) 0 to 2 cc. of 0.85 per cent NaCl,

(*d*) 1 cc. of enzyme solution,

(*e*) 3.5 cc. of 0.5 per cent casein solution in 0.006 N NaOH (pH 10.4),

(*f*) 5 drops of toluol (did not influence digestion, and prevented bacterial growth if tubes were stoppered).

The total volume of each digest mixture was 10 cc. Ten minutes were allowed to elapse between the addition of the enzyme and the casein solution, thus allowing exposure of enzyme to inhibitor for an appreciable time interval before addition of substrate. Immediately after the addition of the substrate and mixing, 2 cc. of the mixture were removed for determination of pH (by Beckman glass electrode), and another 2 cc. were removed, added to 2 cc. of 25 per cent HCl and 1 cc. of 20 per cent sulfosalicylic acid, and the turbidity of the resulting suspension of undigested casein determined with the aid of a photoelectric nephelometer. Similar determinations of the concentration of undigested casein were again made after an interval of digestion at 37°C. Calibration of the nephelometer, *i.e.* translation of turbidity measurements into corresponding concentrations of casein, was possible, since known concentrations of casein were found to transmit light in accordance with Lambert-Beer's law, the concentration of turbid particles being proportional to the logarithm of the per cent of incident monochromatic light transmitted. A green filter which transmitted light of 540 millimicrons wave length gave the greatest range of scale readings. The logarithm of the per cent of incident monochromatic light absorbed (R) was translated into corresponding concentration of casein (C) by means of the calibration factor

($F = C/R$). Concentrations of solution (a) in the digest mixture which caused the pH to vary outside of the range 7.4 to 7.9 are not included in the tables. The pH of the digest mixture in the absence of solution (a) was 7.8.

The concentration of crude trypsin in the digest mixture was 0.01 mg./cc., of crude papain (partly activated by dissolving in water containing a few milligrams of cysteine which was then removed by dialysis) 0.1 mg./cc., and of crystalline trypsin 0.005 mg./cc. The concentration of leucoprotease (cat) that was used produced approximately the same degree of casein proteolysis in 17 hours as was produced in 7 hours by 0.01 mg./cc. of crude trypsin, in 17 hours by 0.1 mg./cc. of crude papain when fully activated, and in 1 hour by 0.005 mg./cc. of crystalline trypsin. (These were the digestion times, at 37°C., used throughout the experiment.)

The initial concentration of casein in each digest mixture was 1.75 mg./cc. The concentration of casein after partial digestion was determined nephelometrically, as described above. Subtraction disclosed the concentration of casein that was digested by the enzyme. This enabled calculation of the concentration of free, *i.e.* active, enzyme with the aid of Schutz's "law":

$$X \; K\sqrt{T} \text{ (at constant incubation time)}$$

where X = concentration of protein digested,

T = concentration of free (*i.e.* active) enzyme,

K = a constant which is determined by digesting casein with known concentrations of each enzyme for the time intervals used experimentally, (*e.g.* 0.01 mg./cc. of crude trypsin digested 0.306 mg./cc. of casein in the standard time of 7 hours so that K = 3.06 for these conditions).

Since the initial concentration of protease (E) in each case was 0.01 mg./cc. of crude trypsin, 0.005 mg./cc. of crystalline trypsin, 0.1 mg./cc. of papain, and an equivalent in leucoprotease activity, the per cent of E that was free (*i.e.* active) in the presence of solution (a) is equal (for crude trypsin) to $\frac{T}{0.01} \times 100$ per cent $= T \times 10^4$ per cent, for crystalline trypsin $\frac{T}{0.005} \times 100$ per cent $= 2 \times T \times 10^4$ per cent, and for papain $\frac{T}{0.1} \times 100$ per cent $= T \times 10^3$ per cent. T was calculated for each digest mixture (and expressed in equivalent weight of trypsin in the case of leucoprotease), and the results are recorded in the tables as "the per cent of enzyme activity."

Wherever serum or plasma was used in these experiments the source was human.

2. Preparation of Leucoprotease

The proteolytic enzymes that were studied by this method were crude trypsin (Fairchild, beef), crystalline trypsin, lyo-leucoprotease, desmo-leucoprotease, and crude papain. Sufficient crystalline trypsin was available for only part of the experiments. Results obtained with desmo-leucoprotease were essentialy the same as those obtained with lyo-leucoprotease, so that only the latter are recorded.

Polymorphonuclear leucocytes were obtained from rabbits and from cats by injecting 10 cc. of 5 per cent aleuronat and 5 per cent tragacanth into each pleural and peritoneal cavity of 8 animals (4 of each species), killing the animals after 24 hours by bleeding from the carotid artery, collecting the exudates in isotonic saline plus citrate, centrifuging, and washing the sedimented leucocytes several times with isotonic sodium chloride to thoroughly remove the supernatant fluid, which has been found (Opie, 1905, 1906) to be high in antiproteolytic activity. Smears of the sediment showed over 90 per cent polymorphonuclear leucocytes, the remainder being mononuclears and red blood cells. Leucoprotease may be obtained from the sediment by allowing the cells to autolyze, or by extraction with glycerol. The method of extraction was used, the sediment being shaken for several hours in 100 per cent glycerol and then centrifuged. The supernatant contains the lyo-leucoprotease ("free" leucoprotease) of Willstätter and Rohdewald (1932). The resulting sediment was then extracted with 60 per cent glycerol and centrifuged, the supernatant now containing the desmo-leucoprotease ("bound" leucoprotease).

Both the lyo and desmo preparations from rabbit leucocytes failed to show appreciable protease activity (on casein substrate at pH 7.8). This is confirmatory of the findings of Parker and Franke (1917) and Barnes (1940), who used other methods of preparation. Preparations from cat leucocytes were high in protease action at neutral pH, and are discussed below. It is interesting in this respect to note that skin and subcutaneous abscesses (as produced by injection of staphylococci) were found to be grossly different in rabbits and in cats. In cats (as in man) resolution, or liquefaction, of such abscesses commonly took place, presumably because of proteolysis by leucoprotease. In rabbits such liquefaction was found to be much slower and less marked, with the result that the abscess contents had a caseous, or "cheesy," appearance. It is suggested that this difference is due to the absence from rabbit polymorphonuclear leucocytes, and the presence in cat (and human) polymorphs, of protease active at neutral pH.

3. Substances Which Inhibited Leucoprotease and Trypsin

(*a*) *Reducing Agents.*—Cysteine, sodium thioglycollate, H—S glutathione, ascorbic acid, sodium cyanide, and hydrogen sulfide, (in concentrations indicated in Table III), were found to be strongly inhibitory of leucoprotease and trypsin. These reducing compounds (with the exception of ascorbic acid) considerably increased the activity of partly activated papain, showing the contrast in the influence of the oxidation-reduction system on these proteases.

The reducing capacity of each digest mixture was determined by its ability to decolorize a 0.0005 M solution of sodium-2,6-dichlorophenol indophenol. Charts 1 and 2 show that for each reducing agent the degree of inhibition of leucoprotease and trypsin, and the degree of activation of papain, are propor-

tional to the reducing capacity of the medium. The results also indicate that there is no relation between the reducing capacity of different reductants and

TABLE III

The Influence of Reducing Agents on the Activity of Leucoprotease, Trypsin, and Papain

(The per cent of enzyme activity is recorded. The reducing capacity is recorded as the cubic centimeters of 0.0005 M Na-2,6-dichlorophenol indophenol decolorized by 1 cc. of digest mixture.)

Compound	Concentration in digest mixture	Reducing capacity of digest mixture	Leucoprotease	Crude trypsin	Crystalline trypsin	Papain
	gm./100 cc.					
None (control)	—	0	100	100	100	3
Cysteine	0.10	5.5	0	44	41	100
"	0.05	2.9	32	70		
"	0.04	2.5	38	79		77
"	0.02	1.3	60	90		
"	0.01	0.5	72	100		47
"	0.005	0.1	79			
Sodium thioglycollate	0.60	1.2	76			
" "	0.40	0.75	90	66	74	100
" "	0.10	0.25	95			
" "	0.04	0.10	106	100		82
Glutathione	0.14	0.5	53	79	83	98
"	0.035	0.3	72	88		75
"	0.014	0.1	105	96		2
Ascorbic acid	0.10	9.5	31	89	70	0
" "	0.05	4.8	44	92		0
Ascorbic acid + Fe^{++}	0.10 + 0.01 Fe^{++}	9.0	1	52	42	13
" " "	0.05 + 0.005 Fe^{++}	4.5	17	70		0
" " "	0.02 + 0.002 Fe^{++}	0.5	62	90		0
NaCN	0.25	0	8	28	50	106
"	0.125	0	14	55		98
H_2S	1/5 saturated		18	60	93	28
"	1/50 saturated		87	106		13

their effect on the enzymes. This is no doubt due in part to differences in the reducing potential of each reductant. For instance, ascorbic acid, half reduced, has an E_0 of +0.06 volt at pH 7, while cysteine has an E_0 of −0.3 volt, so that it is not surprising that ascorbic acid has less effect on the enzymes

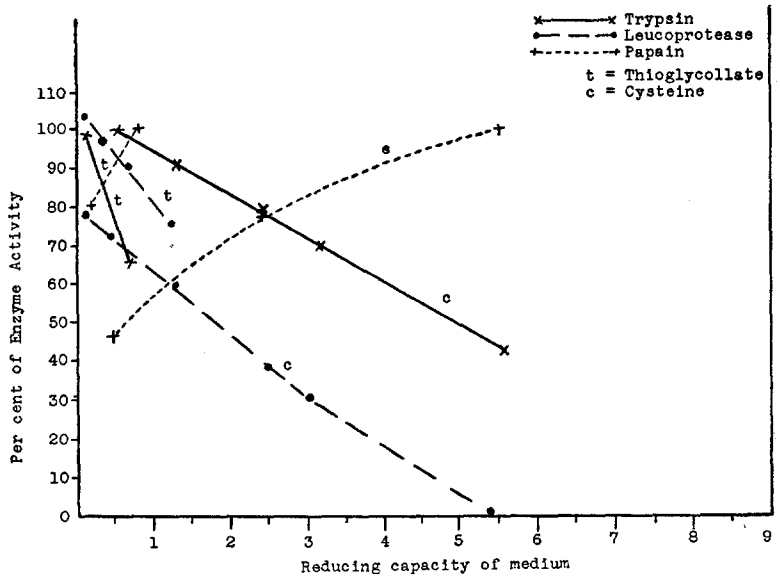

CHART 1. The influence of reducing agents (cysteine and sodium thioglycollate) on the activity of leucoprotease, crude trypsin, and papain. (The reducing capacity of the medium is recorded as the cubic centimeters of 0.0005 M Na-2,6-dichlorophenol indophenol decolorized by 1 cc. of digest mixture.)

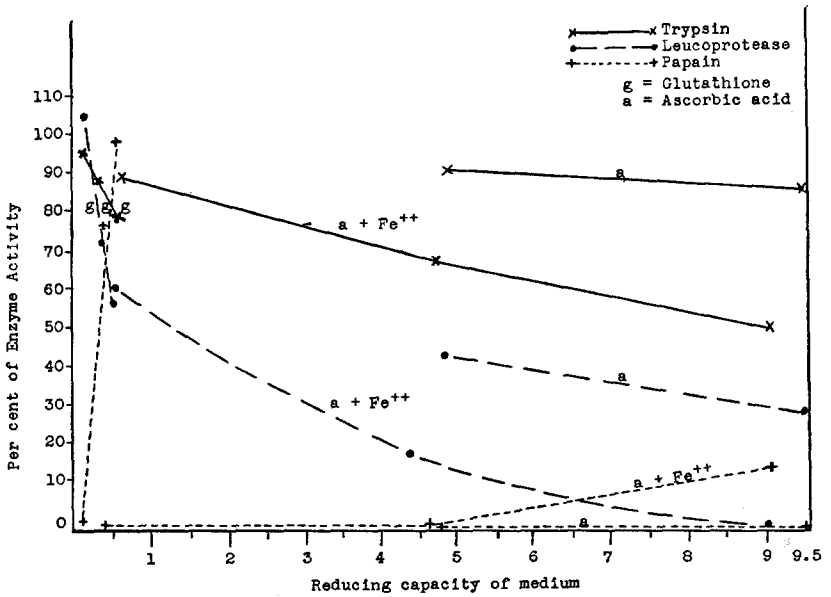

CHART 2. The influence of reducing agents (glutathione, ascorbic acid, and ascorbic acid + Fe^{++}) on the activity of leucoprotease, crude trypsin, and papain. (The reducing capacity of the medium is recorded as the cubic centimeters of 0.0005 M Na-2,6-dichlorophenol indophenol decolorized by 1 cc. of digest mixture.)

than cysteine even when the reducing capacity of the former is higher. However, that other mechanisms may also be involved is suggested by the finding that the addition of a few milligrams of ferrous salt increased the inhibition of leucoprotease and trypsin by ascorbic acid (in the case of trypsin considerably in excess of the additive effect (see Table V)), and caused papain to be slightly activated by ascorbic acid, without changing the reducing capacity of the medium. A possible mechanism for the greater inhibition of trypsin and the beginning activation of papain is suggested by the observation (Parr, 1935) that the ferrous-ascorbic acid complex is a much more potent reducer of disulfide to thiol groups than ascorbic acid alone. Any inactive disulfide compounds in the mixture would thereby be converted into potent thiol compounds.

The contention of Grassman, Dyckerhoff, and von Schoenebeck (1930), that cysteine, H_2S, HCN, and pyrophosphate retard tryptic activity because they inhibit trypsin kinase is disproved by demonstration of the inhibition of crystalline trypsin and of leucoprotease, both of which exist in the activated state and require no kinase for their action. (Willstätter and Rohdewald, 1932, demonstrated that leucoprotease does not require kinase.)

The physiological significance of this inhibition of trypsin and leucoprotease is made evident by recalling that these enzymes probably never act in the body without the presence of appreciable concentrations of biologically important thiol-sulfhydryl reductants (glutathione, ergothionine, cysteine, cysteine peptides, sulfhydryl-containing proteins), as well as ascorbic acid. The thiol-sulfhydryl compounds were known to activate the intracellular cathepsins (just as they activate papain) but their significance as inhibitors of trypsin and leucoprotease has not been appreciated. Similarly ascorbic acid has been known to activate cathepsin and to inhibit papain (except in the presence of sufficient ferrous ions (Maschmann and Helmert, 1934) and disulfide-thiol compounds (Parr, 1935)), but its inhibition of trypsin and leucoprotease has not previously been evaluated.

(*b*) *p-Aminobenzoic Acid, Sulfonamides, and Diphenyl Sulfones.*—Evidence has been presented (Grob, 1943) that the growth of bacteria in the body will be more rapid, and the inhibition of sulfathiazole will be greater, when considerable protease is present and when the fluid inflammatory exudate is small and poor in antiprotease. Protease is believed to exert this effect because the products of proteolysis both accelerate bacterial growth (especially when the medium is poor in non-protein nitrogen) and inhibit sulfonamide action. Because of this physiological significance of protease, the influence on leucoprotease of the sulfonamides and related compounds was studied. Results are recorded in Table IV. They show that *p*-aminobenzoic acid and the sulfonamides tested (especially sulfathiazole) inhibited leucoprotease in physiological concentrations. Promin and a number of other diphenyl sulfones (kindly provided by Dr. Eleanor Bliss) were similarly tested, in an effort to find a

compound of greater inhibitory power that might be used to inhibit leucoprotease when desired, for instance in assisting the action of sulfonamides in purulent lesions. The sulfones that were tested inhibited leucoprotease no more than the sulfonamides, and except for promin, the sulfoxalate condensation

TABLE IV

The Influence of p-Aminobenzoic Acid, Sulfonamides, and Diphenyl Sulfones on Leucoprotease and Trypsin

(The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture	Leucoprotease	Crude trypsin
	gm./100 cc.		
Control	—	100	100
p-Aminobenzoic acid	0.040	20	97
" "	0.010	90	
" "	0.004	107	
Sulfathiazole	0.040	10	104
"	0.020	17	
"	0.010	25	
"	0.004	33	
Sulfanilamide	0.040	18	110
"	0.020	21	
"	0.010	37	
"	0.004	59	
Sulfadiazine	0.040	25	90
"	0.020	40	
"	0.010	60	
"	0.004	87	
Sulfaguanidine	0.040	4	100
"	0.020	9	
"	0.010	22	
"	0.004	54	

product of diphenyl sulfone, *p,p'*-diglucosaminodiphenyl sulfone, and *p,p'*-diaminodiphenyl sulfone salicylate, they have the disadvantage of being much less soluble.

The compounds that were tested inhibited (crude) trypsin slightly or not at all, so that values obtained for trypsin are for the most part omitted. It may be that smaller concentrations of trypsin would have been inhibited. (The initial concentration of trypsin in the digest mixtures was greater than that of

leucoprotease, since leucoprotease digests were incubated longer than the tryptic digests in order to reach the same extent of casein digestion.)

(*c*) *Heavy Metals.*—Aluminum, manganous, ferrous, mercuric, zinc, and cupric salts, in the concentrations shown, inhibited leucoprotease and trypsin

TABLE IV—*Concluded*

Substance	Concentration in digest mixture	Leucoprotease
	gm./100 cc.	
Promin	0.025	74
"	0.012	84
"	0.002	101
Diphenyl sulfones		
p,p'-Diaminodiphenyl	0.020	87
p,p'-Diacetylamino	"	76
p,p'-Diformylamino	"	72
p,p'-Dipropionylamino	"	65
p,p'-Dibutyrylamino	"	72
p-Acetoamino *p'*-amino	"	56
p-Sulfoxalate	"	68
p-Benzylideneamino *p'*-amino	"	65
2,4-Dinitro 4'-amino	"	76
N_1 Benzol *p,p'*-diamino	"	84
p-Propionylamino *p'*-amino	"	78
p-Butyryl *p'*-amino	"	92
p-Acetylamino *p'*-hydroxy	"	68
Disulfonamido	"	76
Diphenyl sulfone	"	84
p-Cinnamalamino *p'*-amino	"	74
p-(*N*-*p*-tolylglycyl)amino *p'*-amino	"	81
p,p'-Diglucosamino	"	74
p,p'-Diaminodiphenyl sulfone salicylate	"	80
p,p'-Diethanolamino	"	84
2,4-Dinitro 4'-acetoamino	"	74
p-Monoacetylamino	"	56

(Table V). Manganous and ferrous salts had a much greater inhibitory effect on leucoprotease than on trypsin.

(*d*) *Other Inhibitors.*—Other substances which were found to inhibit leucoprotease (and usually trypsin) are listed in Table VI. These include serum and plasma (which are equally active in the inhibition of leucoprotease and trypsin and are among the most potent inhibitors of these enzymes), thiourea, heparin, glutamic acid, tyrothricin, ammonium salts, calcium chloride, asparagine, bile salts and acids, and (to a lesser degree) organic mercury compounds.

(*e*) *Polypeptide Trypsin Inhibitors.*—Polypeptide inhibitors of trypsin can be

prepared in concentrated form from a tryptic digest of casein (Hussey and Northrop, 1923). Such a concentrate was prepared, and similar concentrates were made from tryptic digests of egg albumen and serum albumin. A trypsin inhibitor preparation was also extracted (by the method of Balls and Swenson, 1934) from the thin fraction of egg white, which is believed to be a proteolytic product of the more viscid fraction of egg white. These preparations were found to inhibit trypsin to a much greater degree than leucoprotease (Table

TABLE V

The Influence of Heavy Metals on the Activity of Leucoprotease and Trypsin

(The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture	Leucoprotease	Crude trypsin
	gm./100 cc.		
None (control)	—	100	100
$AlCl_3$	2.0	9	20
"	0.05	12	
"	0.02	40	77
"	0.01	103	
$Mn(Ac)_2 \cdot 4H_2O$	0.20	0	28
"	0.04	0	104
$FeSO_4$	0.10	0	66
"	0.02	0	97
$HgCl_2$	0.30	0	0
"	0.04	6	0
$ZnCl_2$	Suspension		
	0.05	66	50
$CuSO_4$	0.05	16	48

VII), a finding which lends support to the belief that although leucoprotease and trypsin are very similar, they are probably not identical enzymes.

An interesting observation was that the boiling of solutions of tryptic digests of proteins increased their power of retarding the activity of trypsin. This is in contrast to serum, which loses its antitryptic and antileucoprotease activity when heated at 80°C. for 10 minutes. These findings will be discussed later, under consideration of the nature of serum antiprotease.

Egg white antitrypsin inhibited crystalline as well as crude trypsin. This disproves the theory of Balls and Swenson (1934) that egg white antitrypsin

acts by inhibiting trypsin kinase, since crystalline trypsin requires no kinase for its action.

TABLE VI

The Influence of Some Other Substances on the Activity of Leucoprotease and Trypsin

(The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture	Leucoprotease	Crude trypsin	Crystalline trypsin
	gm./100 cc.			
None (control)	—	100	100	100
Serum	0.5 per cent	3	3	6
Serum heated at 80° for 10 min.	0.5 per cent	105	112	108
Plasma	0.5 per cent	3	3	7
Plasma heated at 80° for 10 min.	0.5 per cent	103	110	106
Urea	2.0	81	105	
"	0.5	89	98	
"	0.2	87	108	
Thiourea	1.0	45	112	
"	0.25	58	106	
"	0.10	72	94	
Heparin	0.06	72	90	
"	0.015	80	95	
"	0.006	100	98	
Glutamic acid	0.20	20	30	
" "	0.02	54	100	
Quinine mono-HCl	0.20	46	19	
" "	0.02	63	91	
Penicillin	500 units/100 cc.	78	104	
"	50 units/100 cc.	73	91	
Tyrothricin	0.008	14	70	
"	0.0008	18	94	
$NH_4H_2PO_4$	0.20	4	70	
"	0.05	68	84	
"	0.02	74	104	
"	0.005	82	95	
$(NH_4)_2SO_4$	0.05	82	95	
"	0.01	94	94	
NH_4NO_3	0.05	72	74	
"	0.01	89	82	
NH_4 citrate	0.10	39	75	
" "	0.02	74	99	
NH_4 oxalate	0.10	72	100	
" "	0.02	100	101	
NH_4 tartrate	0.10	68	92	
" "	0.02	93	94	

TABLE VI—*Concluded*

Substance	Concentration in digest mixture	Leucoprotease	Crude trypsin	Crystalline trypsin
	gm./100 cc.			
$CaCl_2$	0.05	53	64	
"	0.02	88	89	
Na_2SO_4	0.20	68	104	
Asparagine	0.40	6	98	
"	0.04	15	104	
Na glycocholate	0.10	8	49	
" "	0.02	21	104	
Na taurocholate	0.10	52	98	
" "	0.02	82	102	
Cholic acid	0.10	0	85	
" "	0.02	11	105	
Desoxycholic acid	0.50	6	104	
" "	0.05	11	108	
Boiled crude trypsin	0.004	75	90	
Benzyl HgCl	1/50 saturation	66	80	100
p-tolyl HgCl	" "	82	75	90
Hg di-*p*-Tolyl	" "	84	92	102

TABLE VII

The Influence of Prepared Trypsin Inhibitors on the Activity of Leucoprotease and Trypsin

(The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture	Leucoprotease	Crude trypsin	Crystalline trypsin
	gm./100 cc.			
None (control)	—	100	100	100
Egg white antitrypsin	0.2	100	17	35
" " "	0.05	101	35	
Boiled egg white antitrypsin	0.2	101	17	
Tryptic digest of egg albumen	0.2	100	67	84
Boiled tryptic digest of egg albumen	0.2	98	37	
Tryptic digest of serum albumin	0.2	91	84	86
Boiled tryptic digest of serum albumin	0.2	95	73	
Tryptic digest of casein	0.2	98	86	35
Boiled tryptic digest of casein	0.2	93	47	

4. Substances Which Accelerated Leucoprotease and Trypsin

A number of oxidizing agents were found, in suitable concentration, to increase the activity of leucoprotease and trypsin (Table VIII). This was in direct contrast to their inhibiting effect on papain. Whether the oxidants

exerted their effect directly on the leucoprotease and trypsin, or by oxidizing unknown reductant inhibitors present in the digest mixtures, was not ascertained. Higher concentrations, especially of stronger oxidizing agents, inhibited and could even completely prevent the action of these two proteases.

TABLE VIII

The Influence of Oxidizing Agents on the Activity of Leucoprotease and Trypsin

(The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture	Leucoprotease	Crude trypsin	Crystalline trypsin
	gm./100 cc.			
None (control)	—	100	100	100
l-Cystine	0.02	107	120	110
"	0.01	106	112	
"	0.004	103	105	
I_2 in KI	0.004 (each)	145	77	83
ICl, tri-,	0.01	40	20	41
"	0.001	115	106	
IBr	0.02	0	0	0
"	0.001	110	105	100
H_2O_2	3 parts in 100	106	108	103
Dakin's solution	1 part in 1,000	107	107	104
Dichloramine T	0.015	105	110	105
Azochloramide	0.06	121	114	110
$NaClO_3$	0.40	108	102	
"	0.04	123	115	106
$NaBrO_3$	0.40	30	85	
"	0.04	105	110	105
Na per ClO_3	0.40	20	105	
" " "	0.04	110	115	107
Cu_2O (suspension)	0.40	92	104	
" "	0.04	104	110	98
Ferricyanide	0.20	70	85	
"	0.02	106	104	100
o-Iodosobenzoic acid	0.20	82	116	
" "	0.02	109	118	

The increase of leucoprotease and tryptic activity in the presence of mild oxidizing agents corresponds with the finding of their inhibition by reducing agents, and supports the contention that the oxidation-reduction system is important in the control of the activity of these proteolytic enzymes.

That the oxidation-reduction system may be important in protein synthesis too is suggested by a report (Voegtlin *et al.*, 1932) that oxygenation favors the synthesis of protein by papain from protein digests and tissue extracts. Others

(Strain and Linderstrøm-Lang, 1938) have failed to reproduce this finding, but in view of the fact that proteolytic enzymes have been found by Wasteneys and Borsook (1930) to synthesize proteins from split products (by means of equilibrium systems which could continuously remove the synthetic product), it is very possible that the oxidation-reduction system will be found to be significant in protein synthesis, as it is in proteolysis.

5. Substances Which Inhibited the Antiproteolytic Activity of the Serum

The cause of the antiproteolytic activity of the serum has been the subject of much dispute. This activity has been attributed to antibodies to trypsin and/or leucoprotease (Jochman, 1908), to adsorption of the enzyme by serum albumin (Hedin, 1907), to unsaturated fatty acids (Jobling and Petersen, 1914), and to products of protein digestion, probably polypeptide in nature, and probably produced both in the intestine and parenterally (Grob, 1943).

(*a*) *Inhibitors of the Antiproteolytic Activity of the Serum.*—In Table IX are listed the substances which, in suitable concentration, were found to inhibit the antiproteolytic activity of the serum. These are seen to be, for the most part, oxidizing agents. (Dakin's solution was especially effective, which is interesting in view of its ability to accelerate the solution of pus.) An effort was made to use concentrations of oxidant which decreased rather than increased the activity of the proteases, so as to differentiate between the direct effect on the enzymes and the effect on the antiproteolytic activity of the serum. In addition to the oxidants, three other substances of special interest: zinc chloride, copper sulfate, and *o*-iodosobenzoic acid, inhibited the antiproteolytic activity of the serum. These substances are special inhibitors of sulfhydryl groups (Hellerman *et al.*, 1941).

(*b*) *The Nature of the Antiproteolytic Substance in the Serum.*—Evidence has been presented that: (1) Leucoprotease and trypsin are readily inhibited by reducing agents, including thiol-sulfhydryl compounds and ascorbic acid; (2) the antiproteolytic activity of the serum is inhibited by oxidizing agents and by some special inhibitors of sulfhydryl groups; and (3) the antiproteolytic activity of the serum is due in part to protein degradation products (Grob, 1943) which are most likely polypeptides.

On the basis of this evidence it is suggested that the antiproteolytic activity of the serum is at least partly due to reducing agents in the serum, including polypeptides (particularly those containing thiol-sulfhydryl compounds such as cysteine) and to a lesser degree ascorbic acid. That the part played by ascorbic acid in this activity is a minor one is indicated by the evidence that the antiproteolytic activity of the serum (as tested against trypsin) differs from ascorbic acid in several respects. The antiproteolytic activity is largely confined to the albumin fraction of the serum (Landsteiner, 1900); it is not dialyzable (Fujimoto, 1918); it is not freely diffusible in the body except when

inflammation or other pathologic process increases capillary permeability (Weinberg and Laroche, 1909); and it could be accounted for to only a small extent by the concentration of ascorbic acid in the serum, which is 1 to 2 mg. per cent when the body is saturated with it. Serum polypeptides, on the other hand, would behave like serum antiprotease if the evidence advanced by Loiseleur and Colliard (1937) that these polypeptides are normally adsorbed to the serum proteins is confirmed, for they would then be fractionated with the proteins; they would not be freely dialyzable; and they would not be freely diffusible in the body except when capillary permeability is increased.

TABLE IX

The Influence of Oxidizing Agents and Some Special Sulfhydryl Inhibitors on the Antiproteolytic Activity of the Serum

(The per cent of enzyme activity is recorded.)

Substance	Concentration in digest mixture	Leucoprotease	Leucoprotease +0.5 per cent serum	Crude trypsin	Cryde trypsin +0.5 per cent serum
	gm./100 cc.				
None (control)	—	100	3	100	3
Serum heated at 80° for 10 min.	0.5 per cent	105		112	
Carboxypeptidase (incubated with serum for 24 hrs.)	Few milligrams	105	23	96	25
ICl, tri	0.001	115	20	106	18
"	0.0005	98	148	99	30
IBr	0.001	110	26	105	8
ICl, mono	0.001	101	18	70	6
I_2 in KI	0.004 each	145	65	77	9
H_2O_2	3 parts in 100	106	40	108	14
Dakin's solution	3 parts in 1,000	76	67	75	42
Azochloramide	0.006	99	16	97	8
Chloramine T	0.02	92	2	94	14
Dichloramine T	0.015	105	23	110	15
$NaClO_3$	0.40	108	20	102	6
$NaBrO_3$	0.40	70	30	85	9
Na per ClO_3	0.40	115	20	105	8
Cu_2O	0.40	92	15	104	6
o-Iodosobenzoic acid	0.20	82	4	116	10
" "	0.02	109	4	118	9
$ZnCl_2$ (suspension)	0.05	66	18	50	8
$CuSO_4$	0.05	16	9	48	7

(*c*) *The Nature of Other Trypsin Inhibitors.*—Reference was previously made to a number of polypeptide trypsin inhibitors which have been prepared from tryptic digests of proteins, and from the thin fraction of egg white. The latter,

egg white antitrypsin, has been studied analytically (Balls and Swenson, 1934) and has been found to be a polypeptide rich in cysteine and in thiol groups. It may well owe its antitryptic activity to the presence of active sulfhydryl groups. The crystalline trypsin inhibitors of pancreas (Northrop and Kunitz, 1932) and serum (Schmitz, 1938) have probably also been studied analytically, but any such data were not readily available.

The increase in the activity of trypsin inhibitor solutions (prepared from protein digests) as a result of boiling is demonstrated in Table VII. It has been shown (Dubos, 1929) that boiling solutions of peptone increases their reducing capacity. This would be expected to increase the antiproteolytic activity, if such activity were due to reducing substances. The destruction of

TABLE X

Substances Which Were Found to Have No Effect on the Antiproteolytic Activity of the Serum (Other Than an Additive Effect on the Enzyme Used in Testing)

Substance	Concentration
Na iodate	0.25
Ferricyanide	0.20
"	0.05
l-Cystine	0.01
p-Tolyl HgCl	1/50 saturation
Benzyl HgCl	1/50 saturation
Hg di-*p*-tolyl	1/50 saturation
Sodium selenite	0.05
Cysteine	0.05
Ascorbic acid	0.05
Na oxalate	0.05
Na citrate	0.05
Sulfathiazole	0.012

the antiproteolytic activity of the serum by heat (80°C. for 10 minutes) is not at variance with these findings, since evidence has been presented (Grob, 1943) that this destruction is due to the denaturation by heat of serum proteins (especially albumin), to which serum antiprotease is probably bound.

(*d*) *Objections to the Reducing Agent-Sulfhydryl Theory of the Antiproteolytic Activity of the Serum, and Further Work That Is Indicated.*—More convincing evidence for attributing the antiproteolytic activity of the serum to the action of sulfhydryl compounds would be the demonstration of inactivation of these compounds by mercaptide-forming reagents (*e.g.* benzylmercuric chloride, *p*-tolyl mercuric chloride, cuprous oxide), but not by symmetrically substituted mercury compounds (*e.g.* mercury di-*p*-tolyl), which cannot convert mercaptans to substituted mercaptides. These four reagents were tested, the mercury compounds in concentration of 1/50th saturation, and only the cuprous oxide was found to inhibit serum antiprotease. Table X lists some of the

compounds which, in the concentrations studied, had no observed effect on serum antiprotease. These included three inhibitors of sulfhydryl groups; namely the two mercaptide-forming mercury reagents and sodium selenite. However, a wider range of concentration of these compounds must be tested before it can be said that they are not effective against serum antiprotease.

Serum inhibits papain, though to a much lesser degree than it inhibits trypsin or leucoprotease. The inhibition of papain must be explained by some other mechanism than sulfhydryl groups, since papain is activated rather than inactivated by sulhydryl compounds. It is probable that the antiproteolytic activity of the serum is the sum of two or more factors, and at least one of these factors (ascorbic acid) inhibits papain in spite of the fact that it is a reducing agent that also inhibits trypsin and leucoprotease.

The sulfhydryl theory would be supported by demonstration of the reversibility of the inhibition of serum antiprotease. Experiments were planned: (*a*) to test the reversibility of the inhibition of serum antiprotease; (*b*) to test the reversibility of the inactivation of leucoprotease and trypsin by reducing agents and their activation by oxidizing agents; (*c*) to study the possible inhibition of the trypsin inhibitors prepared from tryptic digests of proteins and from egg white, in a manner similar to the study of the inhibition of serum antiprotease; and (*d*) to study the antiproteases that might be prepared from leucoprotease digests of proteins. However, time was not available for their execution.

6. The Influence of pH on Leucoprotease, Trypsin, Papain, and Serum Antiprotease

Leucoprotease has long been known to have its optimum pH at 7, trypsin at 8, and papain at 5 to 6. Northrop (1922) has shown that the inhibition of trypsin by the products of tryptic hydrolysis of proteins is not affected by pH between 6 and 10. Weiss (1927) has demonstrated that adjusting the reaction to a pH of 5 or less causes serum to lose its antitryptic power.

The influence of pH on leucoprotease, trypsin, papain, and serum antiprotease was studied by following the digestion of casein (nephelometrically) in media buffered at various pH values The results are recorded in Tables XI and XII (and reproduced in Charts 3A, 3B, and 3C). Chart 3A shows the activity of the three proteases at varying pH, and the optima are seen to be pH 8 for trypsin, pH 7 for leucoprotease, and pH 6 for papain, with rather restricted range of activity for leucoprotease and trypsin. Chart 3B shows the activity of the enzymes at various pH values in the presence of serum (0.2 per cent). It is seen that the serum has markedly inhibited trypsin and leucoprotease, and has only slightly inhibited papain. Furthermore the presence of serum has caused the pH optima of leucoprotease and trypsin to shift to the acid side, to a pH of 6 to 6.5, and it has caused the range of activity to widen slightly in both acid and alkaline reactions.

The reason for these latter changes is made clear by study of the effect of exposing serum to different pH values for 1 hour, then adjusting the reaction

TABLE XI

The Influence of pH on the Activity of Leucoprotease, Trypsin, and Papain, in the Absence of Serum and in the Presence of Serum

(The per cent of enzyme activity is recorded.)

pH	Leucoprotease	pH	Leucoprotease + 0.2 per cent serum	pH	Crude trypsin	pH	Crude trypsin +0.02 per cent serum	pH	Papain	pH	Papain + 0.2 per cent serum
1.8	0	2.0	0	2.5	0	2.1	0	2.0	0	3.3	8
3.1	0	3.5	0	4.1	0	4.9	0	4.5	68	4.5	40
4.7	0	5.3	15	5.8	4	5.4	3	5.6	92	5.6	51
6.0	60	5.8	35	6.5	70	5.8	25	6.0	100	6.0	71
6.3	80	6.3	55	6.6	74	6.3	36	6.3	95		
6.5	88			6.8	88	6.8	36	6.8	90	6.7	83
7.0	100	7.4	37	7.4	98	7.4	29	7.4	74	7.4	67
8.0	80	8.0	27	7.9	100	8.0	20	7.9	50	8.0	56
9.2	28	9.2	14	8.6	80			9.2	0	9.2	42
9.5	0	9.8	0	9.1	4	9.1	12	9.5	0		
10.8	0	10.9	0	9.3	0	9.5	0			10.0	0

TABLE XII

The Influence of pH on the Antiproteolytic Activity of the Serum

(Serum exposed to indicated pH for 1 hour, neutralized, added to digest mixture, and protease action determined at pH 7.8. The per cent of enzyme activity is recorded.)

pH to which serum was exposed	Leucoprotease +0.1 per cent serum	Crude trypsin +0.1 per cent serum	Papain +0.1 per cent serum
2.6	96	100	100
3.7	96	97	98
4.5	91	90	96
5.9	90	85	94
6.6	85	77	94
7.4	80	75	90
8.0	80	75	90
9.7	85	80	92
10.7	95	88	94
11.2	100	100	95

back to neutral and adding the serum to digest mixtures (to reach a concentration of 0.1 per cent), and determining the protease action (at pH 7.8). The results of this procedure are reproduced in Chart 3C, which shows that the antiproteolytic activity of the serum (against leucoprotease, trypsin, and

papain) is progressively weakened by exposure to hydrogen ion concentrations below pH 6.5 or above pH 9.7.

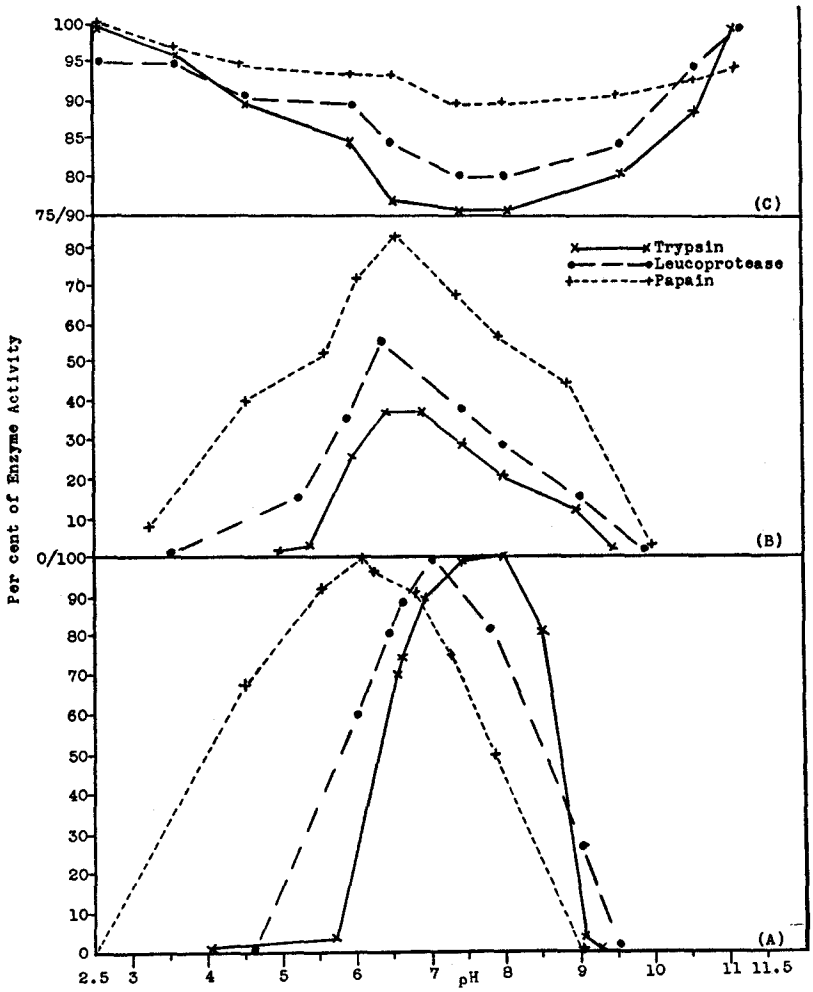

CHART 3. A, the influence of pH on the activity of leucoprotease, crude trypsin, and papain. B, the influence of pH on the activity of leucoprotease, crude trypsin, and papain in the presence of serum (0.2 per cent). C, the influence of pH on the antiproteolytic activity of serum (0.1 per cent). (Serum exposed to indicated pH for 1 hour, neutralized, added to digest mixture, and protease action determined at pH 7.8.)

The activity of protease in the presence of serum and at various pH values will be directly proportional to the activity of the enzyme at any given pH, and inversely proportional to the activity of serum antiprotease at that pH. The resultant activity will be the summation of these two factors. At neutral

pH leucoprotease and trypsin are most active, but serum antiprotease is also at its optimum activity. At a pH of 6 to 6.5 the enzymes are somewhat less active, but the serum antiprotease is weakened to a greater degree, so that the resultant activity in the presence of serum exceeds that at neutral pH. At pH values below 6.5 and above 9.7 serum antiprotease is increasingly inactive, and the presence of serum may then actually help enzyme action by virtue of its buffering power.

The influence of pH on the antiproteolytic activity of the serum, and the influence of the presence of serum on the pH optimum of leucoprotease, are significant because all three factors—leucoprotease, antiprotease, and hydrogen ion concentration—are variables of great importance wherever polymorphonuclear leucocytes accumulate in the body. The interplay of these factors may well explain many of the obscure phenomena of inflammation: for instance, to name only one, why proteolysis frequently proceeds more rapidly in exudates (especially purulent ones) after the accumulation of some protein digest products has rendered the medium *more* acid than the optimal pH for leucoprotease activity.

The hydrogen ion concentration not only exerts a direct influence on the activity of leucoprotease and serum antiprotease, but by shifting oxidation-reduction equilibria exerts an additional and important indirect effect. The sensitivity of oxidation-reduction potentials to hydrogen ion concentration has been fully demonstrated by Clark and his coworkers (1928).

D. THE CONTROL OF THE ACTIVITY OF LEUCOPEPTIDASE (EXPERIMENTAL DATA)

Peptidase activity has been found in extracts of human polymorphonuclear leucocytes (Husfeldt, 1931). Human serum has also been found to contain a small amount of active peptidase (Jobling and Strouse, 1912). It is claimed (Jobling, Petersen, and Eggstein, 1915) that serum possesses no antipeptidase activity.

In view of the physiological significance of the enzymes of the polymorphonuclear leucocytes, the control of the activity of the peptidase of these cells ("leucopeptidase") was studied. It was originally intended to repeat with leucopeptidase the experiments described above (performed with leucoprotease). However time permitted only a few preliminary experiments on the control of the activity of leucopeptidase, and of pancreatic peptidase (in crude trypsin).

Leucopeptidase and pancreatic peptidase activity were determined by adding glycerol extracts of cat polymorphonuclear leucocytes (the "leucoprotease" described above), and crude trypsin (Fairchild, beef) to a substrate of peptic digest of edestin (prepared after Anson, 1937), stopping the digestion after a given time by the addition of formaldehyde, and finally adding phenolphthalein and determining how much sodium hydroxide was needed to make the solution as pink as a standard solution. The titration was begun with

strong alkali and completed with 0.02 N NaOH, in terms of which the results are recorded. This was done in order to avoid increasing the volume, which would produce the so called "water error" (Harris, 1923).

TABLE XIII

Preliminary Experiment on the Influence of Reducing and Other Substances on Leucopeptidase and Pancreatic Peptidase

(Enzyme activity is recorded as the cubic centimeters of 0.02N NaOH (or its equivalent) required to titrate digest mixture of 8 cc. peptic digest of edestin + 1 cc. enzyme + 1 cc. substance to be tested, after 20 hours incubation at 37°C. under toluene.)

Substance	Concentration in digest mixture	Leucopeptidase	Pancreatic peptidase
	gm./100 cc.		
None (control)	—	3.0	4.0
Cysteine	0.03	5.2	5.6
Ascorbic acid	0.05	6.3	7.4
Serum	1.0 (per cent)	4.0	4.8
Sulfathiazole	0.010	3.1	3.9
Thiourea	0.50	2.5	2.8

TABLE XIV

Summary of the Influence of Reducing and Oxidizing Agents on the Systems Studied

Source	Enzyme	Influence of reducing agents	Influence of oxidizing agents
Polymorph. leucocytes	Leucoprotease	−	+
" "	Leucopeptidase	+	(Not studied)
Pancreas	Trypsin (proteinase)	−	+
"	Erepsin (peptidase)	+	(Not studied)
Papaya plant	Papain proteinase	+	−
" "	" peptidase	− (Bergmann and collaborators, 1935–36)	+ (Bergmann and collaborators, 1935–36)
	Enzyme inhibitor		
Serum	Antiprotease	+ (Additive)	−

+ = increased activity.
− = decreased activity.

The influence of cysteine, ascorbic acid, serum (human), sulfathiazole, and thiourea on leucopeptidase and pancreatic peptidase was studied. Results are recorded in Table XIII. The few experiments that were performed indicate that extracts of cat polymorphonuclear leucocytes contain active peptidase, and that the activity of this leucopeptidase, as well as of pancreatic

peptidase, is increased by reducing agents such as cysteine and ascorbic acid, and is not influenced appreciably by serum, sulfathiazole, or thiourea. (The slight increase in activity caused by serum may be due to serum peptidase.)

The presence in extracts of polymorphonuclear leucocytes and of pancreas of proteinase (leucoprotease and trypsin) which is inhibited by reducing agents, and of peptidase (leucopeptidase and erepsin) which is activated by reducing agents, recalls another reciprocal relationship in the control of the activity of proteinase and peptidase: namely, the presence in crude papain preparations of proteinase which is activated by reducing agents and peptidase (Bergmann and collaborators, 1935–36) which is inactivated by reducing agents. Table XIV lists these enzymes (and serum antiprotease) and summarizes the influence of reducing and oxidizing agents. The reciprocal relationships that exist in the control of these enzyme systems are no doubt significant physiologically, and perhaps teleologically as well.

SUMMARY

1. The literature on conditions affecting the activity of proteolytic enzymes has been reviewed.

2. Experimental data on the control of the activity of trypsin, leucoprotease, papain, serum antiprotease, leucopeptidase, and pancreatic peptidase have been presented. These data indicate that:

(*a*) The polymorphonuclear leucocytes of the cat contain abundant proteinase and peptidase active at neutral pH; those of the rabbit lack proteinase active at neutral pH.

(*b*) Reducing agents, including several biologically important thiol-sulfhydryl compounds and ascorbic acid, inhibit the activity of leucoprotease and trypsin. For each reductant the degree of inhibition is proportional to the reducing capacity of the medium.

(*c*) *p*-Aminobenzoic acid, sulfonamides (especially sulfathiazole), and many diphenyl sulfones inhibit the activity of leucoprotease.

(*d*) Serum, plasma, several heavy metals, ammonium salts, asparagine, thiourea, heparin, glutamic acid, tyrothricin, calcium chloride, and bile salts and bile acids also inhibit the activity of leucoprotease, and in most cases of trypsin too.

(*e*) Preparations of tryptic digests of proteins, and egg white trypsin inhibitor, inhibit trypsin to a much greater degree than leucoprotease.

(*f*) Mild oxidizing agents increase the activity of leucoprotease and trypsin.

(*g*) Oxidizing agents and some inhibitors of sulfhydryl groups inhibit the antiproteolytic activity of the serum. It is suggested that serum antiprotease may consist (chiefly) of reducing agents, including thiol-sulfhydryl peptides which exert their antiproteolytic activity by virtue of the presence of sulfhydryl groups.

(*h*) The antiproteolytic activity of the serum is progressively weakened by exposure to a hydrogen ion concentration below pH 6.5 or above pH 9.7. Because of this the pH optima of leucoprotease and trypsin are shifted in the presence of serum from pH of 7 and 8 to pH of 6 to 6.5, and the range of activity of these enzymes is slightly widened, in both acid and alkaline reactions.

(*i*) Reducing agents increase the activity of leucopeptidase and pancreatic peptidase. Serum, sulfathiazole, and thiourea have little or no effect.

3. The significance of the oxidation-reduction system in the control of the activity of leucoprotease, trypsin, serum antiprotease, leucopeptidase, and pancreatic peptidase has been emphasized.

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