THE ANTIPROTEOLYTIC ACTIVITY OF SERUM

I. The Nature and Experimental Variation of the Antiproteolytic Activity of Serum

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I. REVIEW OF THE LITERATURE

1. Nature and Experimental Variation

Hildebrandt (1893), Camus and Gley (1897), and Landsteiner (1900) were the first investigators to observe that normal serum markedly inhibits the action of trypsin and of yeast proteases. Opie (1905) and Jochmann (1908) demonstrated its ability to inhibit leucoprotease. Study of numerous species of animals (Launoy, 1919) showed antiproteolytic activity to be a constant property of mammalian and bird serum, but innumerable investigations have as yet led to little success in the identification of the substance or substances responsible for this property.

The earliest, and still prevalent, theory of the origin of serum antiprotease is that it is a protective antibody formed by the body against proteolytic ferments emptied into the blood stream. This idea had its inception in the work of Achalme (1901) and Meyer (1909), who reported an increase in the antiproteolytic[1] activity of serum in response to intraperitoneal injection of trypsin into guinea pigs. This finding was refuted by some investigators (*e.g.*, Bergell and Schutze, 1905; Young, 1918), but confirmed and extended by Jochmann and Kantorowicz (1908), who demonstrated a rise in activity following subcutaneous injection of leucoprotease into rabbits, and who concluded from this that leucoprotease serves as the normal antigen in response to which antiprotease is produced.

Others (*e.g.*, von Bergmann and Gulecke, 1910) postulated that the pancreas rather than the leucocytes supplied the hypothetical protease antigen, and offered evidence that the antiproteolytic activity on the serum increases following implantation of the pancreas into the peritoneal cavity.

This seemed to be supported by the work of Cobliner (1910), who showed that serum antiproteolytic activity decreases considerably following extirpation of the pancreas.

Indirect evidence against the antibody theory has come from Landsteiner's re-

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* Henry Strong Denison Scholar for 1941–42.

[1] The term "antiproteolytic" is here used to represent the ability to inhibit many animal and plant proteases which are active at about neutral pH. Trypsin is the protease used in this, and most other experimental work, but inhibition of leucoprotease and of other proteolytic enzymes has repeatedly been demonstrated.

peatedly confirmed observation (1900) that antiprotease activity is a property of the albumin fraction of serum, whereas antibody activity is a property of the globulin fraction. Furthermore, there is general agreement that increased enzyme-inhibiting action of serum, when it occurs, rarely exceeds three or four times the original degree of inhibition, in contrast to true antibodies, which increase to the extent of thousands of times the original antibody content of the blood in response to injections of antigen.

Impressed by these considerations, and by his discovery that charcoal inhibited trypsin by adsorbing it, Hedin (1906–07) attempted to prove that serum exerted its antiprotease action by adsorption of the enzyme, supposedly by serum albumin. Many of the more recent workers, notably Banting and Gairns (1930), Fine (1931), and Chrometzka ("neurohormonal regulation" theory, 1932) have accepted Hedin's idea, largely on theoretical grounds. But Hanson (1918) was unable to find any variation in the concentration of serum protein when the antiproteolytic activity of the serum was doubled, or even tripled.

A third group of workers followed the lead of Schwartz (1909) and Bauer (1910), who observed that after serum antiproteolytic activity had been "extracted" with ether, it could be restored by the addition of lipoids to the serum. This group attempted to prove (Jobling and Petersen, 1914) that the antiproteolytic activity of serum is due to its content of unsaturated fatty acids. They demonstrated that unsaturated fatty acids have an inhibitory action on trypsin or leucoprotease which is proportional to their iodine values and which can be destroyed by saturating their double bonds. But that lipoids are not concerned in the antiproteolytic power of serum was shown by Meyer (1909), Cobliner (1910), and Teale and Bach (1919–20). They demonstrated that lipoid solvents destroyed rather than extracted serum antiprotease; that lipoid solvents had no such effect on dried serum; and that the kinetics of the action of unsaturated fatty acids on trypsin is very different from that of serum.

A fourth line of investigation was opened by Bayliss' discovery (1904), (confirmed by Abderhalden and Gigon (1907)) that trypsin is inhibited by the products of protein digestion. Bayliss assumed that amino acids were responsible. This led Rosenthal (1910) to suggest that serum owes its antiproteolytic action to its amino acid content ("amino acid theory"). But Walters (1912) and Northrop (1921–22) showed that the amino acids themselves have little, if any, effect on tryptic action. Northrop demonstrated that some other constituent of the digest mixture, (unidentified, but apparently a polypeptide), is responsible, combining reversibly with trypsin in accordance with the law of mass action. Shortly afterwards (1922–23) Hussey and Northrop observed that the antitrypsin of normal serum behaves quantitatively like the trypsin inhibitor produced during the tryptic digestion of proteins. More recently Northrop and Kunitz (1932–33) have isolated a polypeptide trypsin inhibitor from the pancreas, and Schmitz (1938), employing their method, has extracted from beef blood small amounts of a substance resembling pancreatic trypsin inhibitor.

2. Physiological and Pathological Variation

Widespread attention was first drawn to variations in the antiproteolytic activity of the serum by the observation of Brieger and Trebing (1908) that this activity increases markedly in the presence of malignant tumors. This was repeatedly con-

firmed, and was, for a time, unfortunately used as a diagnostic test for malignancy. However, it was not long before a similar increase was observed in many cases of acute infection, especially if accompanied by high fever (Hort, 1909); anemias (Brenner, 1909); late syphilis (Fuerstenberg and Trebing, 1909); tuberculosis and hyperthyroidism (Waelli, 1912); organic diseases of the central nervous system (Bolten, 1918); anaphylaxis (Pfeiffer and Jarisch, 1913); protein shock therapy (Jobling and Petersen, 1914); x-ray irradition (Herzger, 1924); pregnancy (Gräfenberg, 1909), especially when complicated by eclampsia; after very severe muscular exercise (Preti, 1912); and just before menstruation (Daniel and Florian, 1935–36). An increase after meals and a decrease during starvation have been reported by Glaessner (1903) Rosenthal (1910) and Remedi and Bolognesi (1911). The significance of these variations in the antiproteolytic activity of the blood has been little understood, although speculation has been rife. It has been regarded by many as an indicator of cachexia, especially in chronic diseases.

II. DATA ON EXPERIMENTAL VARIATION

1. Determination of Antiproteolytic Activity

It has been shown (Robertson, 1912) that the refractive index of a solution of sodium caseinate is unaltered by tryptic digestion. It is possible to estimate the extent of hydrolysis in such a solution by precipitation of the undigested casein and determination of the refractive index of the filtrate. The amount of casein digested is proportional to the difference between the index of refraction of this filtrate, and that of a control filtrate, from which the casein is precipitated at once.

The antitryptic action of a given serum may be measured by its suppression of casein digestion as compared with a similar solution in which the serum is replaced by saline (Robertson and Hanson, 1918).

The contribution of the serum protein to the substrate (a factor neglected by Robertson and Hanson) may be corrected for by a third digest solution which contains an equal amount of serum whose antitryptic activity has been entirely destroyed by heat (serum diluted and heated at 80°C for 10 minutes). The contribution of the trypsin to the index of refraction (aside from its enzymatic activity) may be corrected for by adding trypsin to the control whose casein is precipitated at once.

The refractometric measurements indicate the extent of protease, but not of peptidase activity, and hence will allow investigation of the antiprotease action of serum only. However, Jobling, Petersen, and Eggstein (1915) have demonstrated the absence of antipeptidase activity by the serum. In fact, the serum contains a small amount of active peptidase (Jobling and Strouse, 1912).

The formula derived by Robertson and Hanson (1918) for the determination of the antitryptic activity of serum has been found by Fine (1931) and others to provide only an approximation of the true value, and to be quite unreliable when the concentration of serum is below 10 per cent.

The following derivation of a formula for antiproteolytic activity, based on Hussey and Northrop's convincing demonstration (1922–23) that tryptic protease and its inhibitor in serum combine reversibly in accordance with the law of mass action (the inhibitor serving as a "buffer" of the enzyme), may perhaps more accurately serve the desired purpose.

If we assume that one molecule of tryptic protease (T) combines reversibly with one molecule of serum antiprotease (A') to form one molecule of protease-antiprotease compound (TA'):

$$T + A' \rightleftharpoons TA'.$$

By the law of mass action

$$\frac{C_T \times C_{A'}}{C_{TA'}} = K.$$

If: E = concentration of total trypsin (free and combined)
T = concentration of free (and hence active) trypsin
A = concentration of total antiprotease $(C_{A'} + C_{TA'})$

We may rewrite the equilibrium equation as:

$$\frac{(T)(A - (E - T))}{E - T} = K$$

or

$$A = (E - T)\left(1 + \frac{K}{T}\right)$$

The value of E is known. T is calculated from the experimental results with the aid of Schutz's "law":

$X = k\sqrt{T}$ (at constant incubation time)
where X = amount of protein digested.
and T = concentration of free enzyme.

Table I illustrates the calculation. The total volume of each solution was 3 cc. In each serum-added series the serum was at 1.33 per cent concentration. All mixtures were at pH 8 (phosphate buffer), and were incubated for 4 hours at 37°C. The undigested casein of each was precipitated with acetic acid and filtered off. The index of refraction of each filtrate was then determined. These readings were corrected as previously described.

It is seen that complete digestion of 50 mg./cc. of sodium caseinate resulted in an increase in the index of refraction of the solvent of 0.0064 units, so that an increase of 0.0001 unit corresponds to the digestion of 0.78 mg./cc. of casein. This enables easy calculation of X, the concentration of digested casein, and

of $\frac{X}{\sqrt{E}}$, which is seen to be constant, (until excess enzyme was added). Since $\frac{X \text{ control}}{\sqrt{E}} = \frac{X \text{ serum}}{\sqrt{T}}$, T is readily calculated for each concentration of enzyme. This leaves two unknowns in the equilibrium equation, A and K. A is constant for any one serum so that values for E and T can be entered for two different enzyme concentrations (calculated for $T > 0.0002$ mg./cc., since concentrations of active enzyme smaller than this were considered negligible), and K determined by eliminating A by subtraction. This was done for several sera, and K was relatively constant at 0.0018. Using this value for K, A was calculated for these and other sera, and was constant for each serum (within

TABLE I

The Effect of Serum on Varying Concentrations of Trypsin

	(E) Concentration of crude trypsin	Concentration of casein	Control (no serum)			Serum 1 (1.33 per cent)				Serum 2 (1.33 per cent)			
			I.R.*	(X) Digested casein	$\frac{X}{\sqrt{E}}$	I.R.	X	T	A	I.R.	X	T	A
	mg./cc.	mg./cc.		mg./cc.									
1	0	50	1.3338	0	—	1.3338	0	—	—	1.3338	0	—	—
2	0.017	50	1.3360	17.6	135	1.3339	0.78	0.000036	—	1.3338	0		
3	0.033	50	1.3370	25.0	139	1.3340	1.6	0.00014	—	1.3340	0.00014	—	—
4	0.067	50	1.3383	35.1	135	1.3340	1.6	0.00014	—	1.3340	0.00014	—	—
5	0.133	50	1.3400	48.4	134	1.3350	9.4	0.005	0.179	1.3358	15.6	0.013	0.137
6	0.167	50	1.3402	50	122	1.3360	14.8	0.014	0.172	1.3368	23.4	0.031	0.144
7	0.200	50	1.3402	50	113	1.3370	25	0.034	0.176	1.3380	32.7	0.060	0.144

* Index of refraction.

the error of the index of refraction—about 5 per cent) regardless of the concentration of enzyme.

A represents the concentration of serum antiprotease in the digest mixture. It cannot be expressed in grams or other definable unit. The law of mass action is defined in molarity, but since it is not possible to accurately express concentration of trypsin or casein in moles, milligrams per cubic centimeter were used throughout, resulting in a change in the constants, but not in the validity of the equation. The concentration of antiprotease in the serum can be represented as

$$S = \frac{A}{C}$$

where S = concentration of antiprotease in serum (units/cc.)
A = concentration of antiprotease in digest mixture (units/cc.)
C = cc. of serum in 1 cc. of digest mixture.

TABLE II

The Antitryptic Activity of Varying Concentrations of Serum (Control As in Table I)

E	Casein	Serum 3	I.R.	*T*	*A*	*S*
mg./cc.	*mg./cc.*	*per cent*				
0.067	50	13.33	1.3340	0.00014	—	—
0.067	50	6.67	1.3340	0.00014	—	—
0.067	50	3.33	1.3340	0.00014	—	—
0.067	50	1.67	1.3340	0.00014	—	—
0.067	50	0.83	1.3358	0.014	0.054	6.8
0.067	50	0.42	1.3371	0.039	0.028	7.0
0.067	50	0.21	1.3376	0.052	0.015	7.5
0.133	50	1.33	1.3375	0.049	0.085	6.6
0.200	50	1.33	1.3394	0.113	0.087	6.7

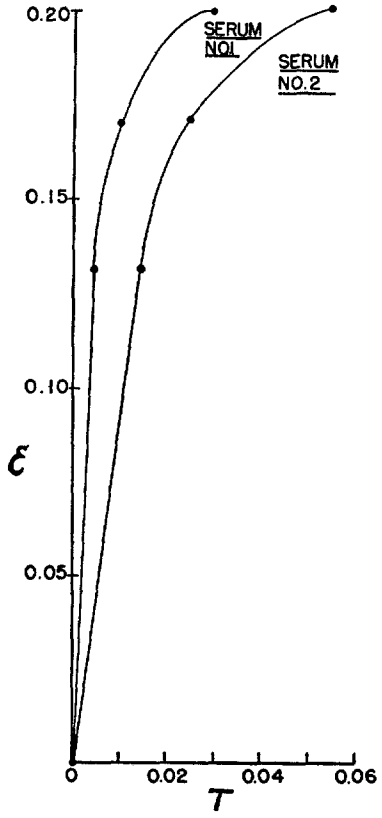

CHART 1. The effect of serum on trypsin

Table II illustrates the approximate constancy of *S*, regardless of the concentration of serum (provided that the change in the index of refraction is greater

than the limit of accuracy of the measurement). Measurements over a wider range, however, show relatively more activity at low concentration than at high, in accordance with the law of mass action.

In Chart 1, the values for T and E given in Table I are plotted against each other. Similar curves, of varying initial slopes, were obtained for the many other sera studied. It is evident that the extent to which $\frac{\Delta E}{\Delta T}$ (*i.e.*, the slope of the curve) exceeds 1 is dependent on the concentration of antiprotease present. When T is small compared to E, *i.e.* when small amounts of trypsin are acting in the presence of serum, $(E\text{-}T)$ approaches E, and A becomes proportional to $\frac{E}{T}$, producing a straight line curve whose slope $\left(\frac{E}{T}\right)$ is proportional to the concentration of antiprotease. As E increases, T increases, and the reaction

$$T + A' \rightleftharpoons TA'$$

is driven toward the right. The concentration of free antiprotease approaches zero, and the curve approaches a straight line with a slope of 1, increments of E producing equal increments of T. The shape of the curve reflects the fact that the greater the concentration of trypsin, the less is the per cent inhibition, despite the higher total concentration of trypsin inhibited.

The calculation of S with the aid of the above formula is not limited to the refractometric method of following digestion, but should be equally applicable to any method that measures protease activity.

2. Procedure and Results

Reports on the variation of antiproteolytic activity in response to injection of trypsin have been far from unanimous. This may in large part be due to failure to appreciate that normal variations in antiproteolytic activity are constantly occurring, that different samples of equally active crude trypsin are frequently inhibited to different extents by the same serum (Young, 1918), and that the choice of method has a very important effect on the results obtained (Fine, 1930).

Hanson (1918) reported that the normal variation in antitryptic activity of the serum of several rabbits over a period of weeks was approximately 50 per cent. He also reported that subcutaneous injection of large amounts of crude trypsin resulted in a marked fall in the antitryptic activity, followed, if injection was continued, by a rise within a few days. This rarely exceeded three times the average normal variation during a similar period. Then, despite continued injection, a rapid return to the normal range occurred. These results have been confirmed by some workers, and refuted by others.

The effect on the antitryptic activity of the following procedures was studied, and it was hoped that this might not only indicate the existence and magnitude

of a response to trypsin, but might also furnish a clue to the mechanism of the action of trypsin and to the nature of the antitryptic substance: (1) Intramuscular injection of trypsin. (2) Intravenous injection of trypsin. (3)

TABLE III

Daily Normal Variation in Antiproteolytic Activity

Day	1	2	3	4	5	6	7	Average daily variation	
								Units	Per cent
Rabbit 1	6.0	7.1	7.6	8.1	6.3	5.7	5.2	0.8	12
" 2	3.5	4.8	4.5	3.8	2.8	3.0	4.2	0.8	21
" 3	5.3	5.1	4.5	4.9	3.5	4.7	4.8	0.7	15
" 4	6.8	6.6	8.2	6.8	5.6	5.0	5.7	0.95	15

TABLE IV

Response to Intramuscular Injection of Trypsin (25 Mg./Day; 2.5 Cc. of 1 Per Cent Solution in 0.85 Per Cent NaCl; Seitz Filtered; Sterile)

Rabbits 1–6. Daily injection of active trypsin for 52 days.
7 and 8. Daily injection of active trypsin for 30 days.
9 and 10. Daily injection of denatured trypsin for 52 days.

	Before injection			Daily injection							After injection					
Day	7	4	1	1	6	11	16	21	26	31	6/36	9/39	16/46	22/52	26/4	29/7
Rabbit 1	6.0	7.6	5.2	5.8	6.7	6.5	7.1	8.6	12.2	13.0	10.2	8.8	8.6	9.0	7.1	5.2
" 2	3.5	3.8	4.2	4.0	3.5	5.2	7.3	9.0	13.1	13.5	9.8	8.5	8.2	8.8	6.4	5.1
" 3	5.3	4.9	4.8	5.5	6.2	6.0	6.9	9.3	11.6	13.3	10.8	9.0	9.1	9.5	7.2	4.7
" 4	6.8	6.8	5.7	5.0	5.3	6.3	6.1	9.8	13.6	13.3	12.1	10.0	11.0	10.8	8.2	7.0
" 5	5.2	—	6.2	6.6	—	6.8	—	7.6	—	11.0	9.1	8.0	Died		—	—
" 6	5.8	—	6.0	6.7	—	7.0	—	7.3	—	10.8	8.8	7.9	8.4	8.5	5.6	7.0
" 7	7.2	—	8.1	9.1	—	9.6	—	10.3	—	14.0	8.1	7.0	7.4	—	—	—
" 8	6.5	—	5.7	6.0	—	6.4	—	8.2	—	12.9	6.6	6.2	5.8	—	—	—
" 9	5.7	—	5.3	5.0	—	4.2	—	6.0	—	4.9	—	6.7	—	5.8	—	—
" 10	6.0	—	6.6	6.2	—	7.5	—	7.2	Died	—	—	—	—	—	—	—

Oral administration of trypsin. (4) Denatured trypsin, (*a*) administered intramuscularly, (*b*) administered intravenously, (*c*) administered orally.

In addition, the local reaction to trypsin injection was studied at different blood levels of antiproteolytic activity.

The results obtained are recorded in Tables III–VII. Antiproteolytic activity is expressed in *S* units/cc. of serum. Crude trypsin (Fairchild) was used throughout.

TABLE V

Response to Oral Administration of Trypsin (200 Mg./Day via Stomach Tube)

Rabbit 1–4. Daily administration of active trypsin for 4 days.
5 and 6. Daily administration of active trypsin for 8 days.
7 and 8. Daily administration of denatured trypsin for 4 days.

	Before	Daily administration						After		
Day	1	1	2	3	4	2/6	4/8	6/2	9/5	12/8
Rabbit 1	8.7	8.3	10.3	10.5	12.0	12.5	10.9	10.3	7.9	—
" 2	6.0	6.5	6.8	10.3	11.6	12.6	10.6	7.5	7.2	—
" 3	7.5	8.3	9.8	10.8	14.8	10.2	9.3	8.0	6.4	—
" 4	7.0	6.8	10.3	11.5	10.6	8.1	7.0	7.2	6.1	—
" 5	7.7	7.9	10.4	10.1	14.8	14.6	14.4	14.0	8.3	8.5
" 6	5.0	5.8	6.3	8.9	12.7	13.1	12.5	12.0	8.7	4.2
" 7	6.6	7.5	7.3	8.5	6.8	7.6	—	—	—	—
" 8	6.2	5.5	6.3	4.9	5.3	7.1	—	—	—	—

TABLE VI

Response to Intravenous Injection of Trypsin (50 Mg./Day)

Rabbits 1–3. Daily injection of active trypsin for 15 days.
4 and 5. Daily injection of denatured trypsin for 15 days.

	Before	Daily administration					After
Day	1	1	3	7	10	15	2
Rabbit 1	7.6	8.0	8.9	8.6	8.0	9.1	8.2
" 2	7.0	6.1	5.5	Died	—	—	—
" 3	5.8	6.4	4.7	7.3	8.1	8.3	7.7
" 4	4.5	5.1	5.8	4.9	5.6	4.0	3.4
" 5	4.3	6.2	Died	—	—	—	—

TABLE VII

Local Reaction 24 Hours after the Subcutaneous Injection of Trypsin (25 Mg.) (2.5 Cc. of 1 Per Cent Solution in 0.85 Per Cent NaCl, Seitz Filtered) at Different Blood Levels of Antiproteolytic Activity

Rabbit 1			Rabbit 2		
Day	S	Size of resulting ulcer (mm.²)	Day	S	Size of resulting ulcer (mm.²)
1	4.3	250	1	9.6	70
4	7.0	30	3	6.9	320
5	9.3	18	13	11.2	6
Rabbit 3			**Rabbit 4**		
Day	S	Size of resulting ulcer (mm.²)	Day	S	Size of resulting ulcer (mm.²)
1	3.8	1000	1	8.8	240
13	9.3	250	11	10.8	130
19	8.0	320	17	12.0	90

III. DISCUSSION

The results recorded in the tables are reproduced in part in Charts 2 to 4, and they show the following:

1. Daily variations in antiproteolytic activity of approximately 20 per cent may be considered to be normal, so that experimental variations must exceed this in order to be significant. There is not sufficient data to evaluate the

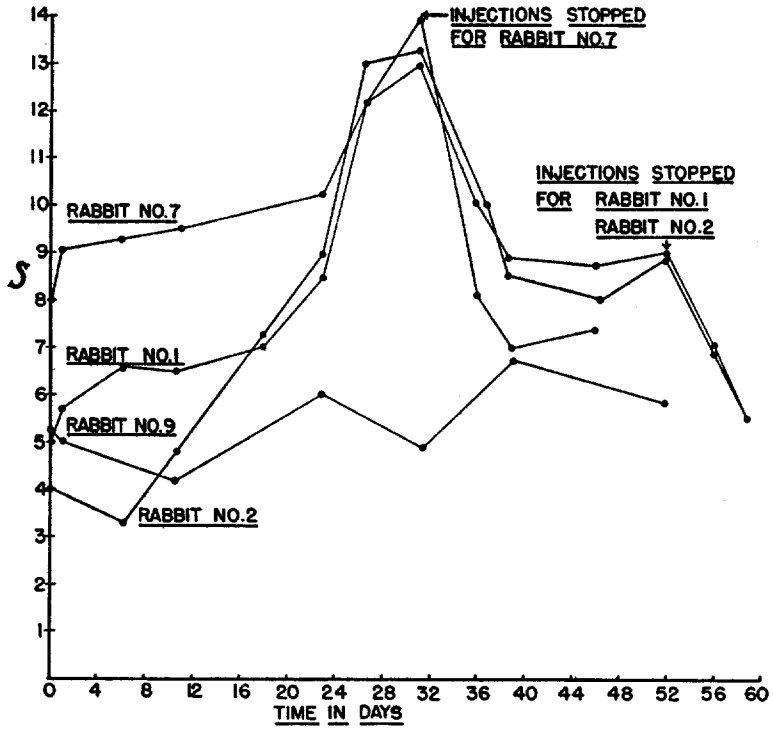

CHART 2. Response to intramuscular injection of trypsin

average variation over days and weeks, but there is strong indication that this rarely exceeded 50 per cent of the average value.

2. Daily intramuscular injection of a solution of 25 mg. of crude trypsin produced little significant change in the antiproteolytic activity for about the first 11 days. Following this period the activity gradually increased, reached a maximum (representing an increase of from 70 per cent to 250 per cent over the average normal value) at about the 4th week, and then declined during the next 10 days to a value which was only about 40 per cent to 120 per cent above the average normal, and remained relatively fixed at this level during the next 2 weeks. If the injections were stopped when the antiproteolytic

activity was at its height, the activity fell off much more sharply than if injection were maintained, and returned to the normal range within a week. Cessation of injections at the 52nd day also resulted in return to the norm in less than a week. The intramuscular injection of denatured trypsin did not have any significant effect on the antiproteolytic activity of the serum.

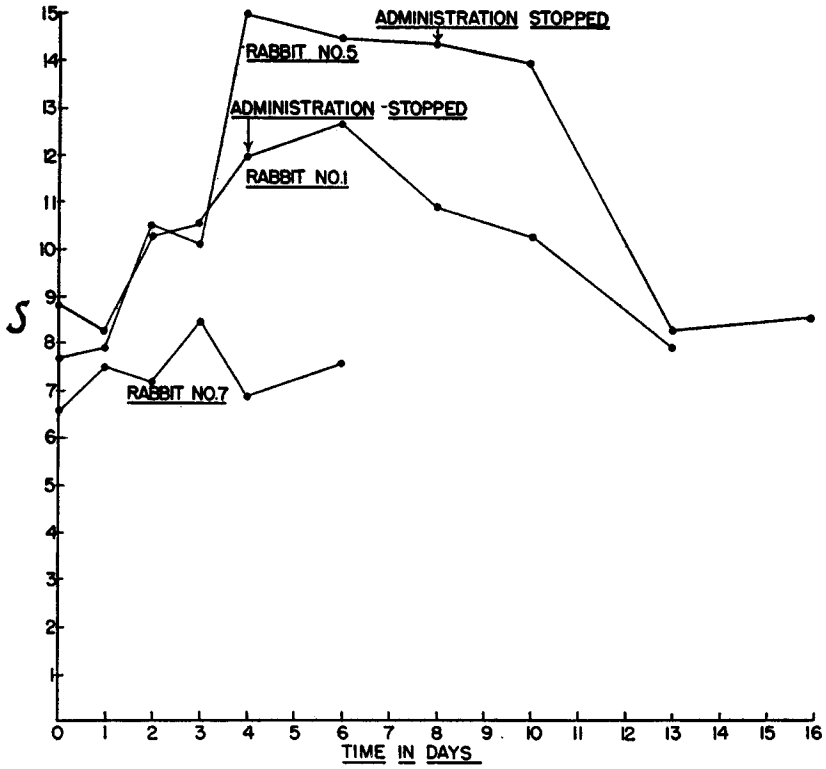

CHART 3. Response to oral administration of trypsin

3. Daily oral administration of 200 mg. of trypsin resulted in a rapid rise in antiproteolytic activity within the first day or two, which was maintained during the 4 or 8 days of administration, and which returned to the normal range within a week after this was stopped. Denatured trypsin had no such effect.

4. Daily intravenous injection of 50 mg. of trypsin produced no variation in activity comparable to intramuscular or oral administration. Denatured trypsin was likewise without effect.

5. The subcutaneous injection of 25 mg. of crude trypsin into animals at different blood levels of antiproteolytic activity resulted in the production of

ulcers varying from 6 mm.^{2} in area to large sloughs up to 1000 mm.^{2} in area. Microscopic examination of the underlying subcutaneous tissues showed in-

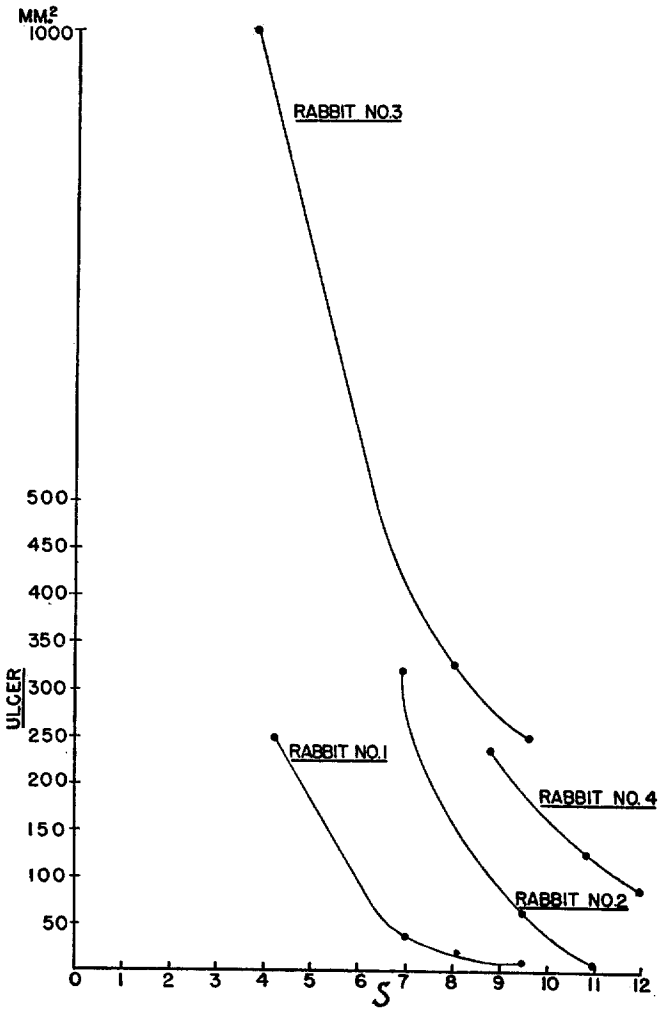

CHART 4. Local reaction to trypsin

flammation and necrosis, corresponding in severity to the size of the ulcer. Comparison between the size of the ulcer and the antiproteolytic activity of the serum shows a rough inverse correlation (in the four animals studied) between the activity of the serum of any one rabbit and the size and severity of the ulcer in that animal, low antiproteolytic activity being associated with a severe local reaction, and *vice versa*.

The experimental results outlined above strongly indicate that an antiproteolytic response to trypsin occurs only when the enzyme is active, and when it is administered to the animal in such a way as to allow enzymatic action to occur. The ineffectiveness of intravenous injection of trypsin could well be explained by the prompt neutralization of the enzyme by the antiproteolytic action of the circulating blood and its maintenance in inactive combination due to the excess of antiprotease. The decline in antiproteolytic activity after a maximum has been reached as a result of daily intramuscular injection could also be explained by decreased proteolysis by the enzyme, here resulting from the increased neutralizing action of the blood, and hence of the exudate that responds to the local necrotizing action of trypsin.

The work of Bayliss (1904) demonstrating that trypsin is inhibited by the products of protein digestion, and of Northrop (1921–22) identifying the trypsin inhibitor as a polypeptide, has already been referred to. The results of experimental variation of antiproteolytic activity that have been described are compatible with this mechanism of antiprotease production. They suggest that the products of protein hydrolysis in the intestine and parenterally are an important factor in the antiproteolytic activity of the serum. These products are most likely polypeptides similar to the trypsin inhibitor produced *in vitro* by Northrop. The rapid increase in serum antiprotease following oral administration of trypsin does not contraindicate a polypeptide inhibitor, since the intestinal absorption of peptides, and even of small amounts of larger molecules, is now recognized (Sussman *et al.*, 1928).

If the above mechanism of antiprotease production is true, it would be expected that oral administration of trypsin to animals deprived of food would produce no rise in antiproteolytic activity. Two rabbits that were so studied after 4 days of starvation showed no increase in serum activity after 3 days of trypsin *per os* (200 mg./day). Unfortunately, they died on the 8th day of starvation, so that the experimental results are only suggestive.

The physiological and pathological variations in the antiproteolytic activity of the serum that have been reported are not at variance with a "proteolytic" theory of the origin of this activity. The postprandial increase in activity and the decrease that occurs during starvation (Glaessner, 1903; Rosenthal, 1910; and Remedi and Bolognesi, 1911) would be attributed to the rôle that intestinal digestion of proteins plays in the production of the antiprotease. The proteolytic enzymes that are abundant in placental tissue (Maeda, 1923) would be responsible for the increase during pregnancy. The increase in hyperthyroidism (Waelli, 1912), in many cases of acute infection with high fever (Hort, 1909), and after very severe muscular exercise (Preti, 1912) would be due to increased protein catabolism, which might also result in the formation of antiprotease. That the marked rise accompanying malignancy may well be due to resultant tissue necrosis and autolysis is supported by the observation

of Raab (1935) that the increase of serum antiproteolytic activity in malignant disease is rougly proportional to the degree of disintegration and proteolysis in the tumor. The importance of tissue autolysis in pathological increases of antiproteolytic activity has been emphasized by Meyer (1909), by Braunstein and Kepinow (1910), who increased serum activity by injecting intravenously the products of autolysis of tumors, liver tissue, and other protein-rich substrates, and by Opie, Barker, and Dochez (1911), who showed that substances such as chloroform, which cause necrosis of liver cells, cause a parallel increase in serum antiproteolytic activity. Finally the increased serum antiprotease observed in advanced tuberculosis (Waelli, 1912), and in other conditions associated with "cachexia," may perhaps be due to the increased protein catabolism associated with the wasting that is a prominent feature of these conditions.

That the increase in antiproteolytic activity in many of the above conditions might, at least in part, be due to decreased serum protease, rather than increased antiprotease, is contraindicated by the work of Falls (1915) and of Jobling, Petersen, and Eggstein (1915), who have reported that serum protease increases in many of these conditions.

The possibility that antiprotease is an antibody produced in response to trypsin is rendered highly unlikely by the nature, magnitude, and variation of the antiprotease response. Nevertheless, precipitin tests between the serum of rabbits, before and after trypsin injections, and various dilutions of trypsin were performed. Outside of a weak precipitin reaction with serum of animals that had received trypsin intravenously they were all negative, and the positive precipitin reactions showed no parallelism to antiprotease activity. It is furthermore probable that the antibodies that were observed were produced not in response to trypsin, but to other proteins in the crude trypsin, particularly since TenBroeck (1934) was unable to obtain positive precipitin reactions following injection of crystalline trypsin, although he obtained positive Dale anaphylactic tests. And that such antibodies are responsible for the antitryptic activity of the serum and for the ability of injected rabbits to neutralize the local action of trypsin, as was believed by Achalme (1901) and by Young (1918), seems very unlikely in view of the great dissimilarity between the antiprotease response to trypsin, described above, and the antibody response to the enzyme, described by TenBroeck.

Northrop (1929–30) has discovered that when pepsin is injected into an animal it is almost immediately denatured, so that the antibodies which result are largely antibodies to the denatured pepsin. That this is probably not true in the case of trypsin is indicated by the failure of the precipitin reaction to become any more positive when denatured instead of active trypsin is employed.

Animals to whom trypsin had been previously administered did not show

increased response when they received the enzyme again at a later date (by any route).

A few findings present objections to the "polypeptide" theory of serum antiprotease. Benetato, *et al.* (1937) have reported an inverse relationship between serum antiproteolytic activity and serum non-protein nitrogen. However, this work is at least partly refuted by the observations of Shono (1933), and, in any event, awaits confirmation. More serious objection is raised by the fact that serum antiprotease is not dialyzable, and is destroyed by heating serum (after dilution to prevent coagulation) at 80°C. for 10 minutes (Fujimoto, 1918), neither of which would be expected if it were a simple polypeptide. This would be explained, however, if the evidence advanced by Loiseleur and Colliard (1937) that polypeptides in the blood are normally adsorbed to the plasma proteins is confirmed, and is shown to apply to serum antiprotease. Attempts which were made to dissociate such a combination by lowering the pH of the serum (or of the plasma, which has the same antiproteolytic activity) below 3, at which pH the compound of trypsin and pancreatic trypsin inhibitor dissociates, resulted in an irreversible loss of antiproteolytic activity. (This may perhaps explain the small yield of crystalline antitrypsin obtained by Schmitz's method of extraction.) Some clue to the situation may be offered by the finding that when crystalline pancreatic trypsin-inhibitor (prepared after the method of Northrop and Kunitz, 1932–33) or crystalline serum antitrypsin (prepared after the method of Schmitz, 1938) was heated at 80°C. for 10 minutes, it retained its activity, but the addition of a small amount of serum (and, to a lesser extent, of serum albumin) resulted in a marked loss of activity on heating. Experiments investigating the effect of serum and of serum albumin on the dialyzability of the above crystalline compounds were unfortunately inconclusive. However, Mělka (1932) has reported that he was able to free trypsin from the trypsin-serum antiprotease combination by dialysis of the antiprotease.

Since serum antiprotease is not dialyzable, it should not be freely diffusible in the body except when inflammation, or other pathological process, increases capillary permeability. Such has been found to be the case. Thus Fazio and Chiarolaza (1910) have reported the absence of antiprotease from normal serous fluids and from most transudates, while Weinberg and Laroche (1909) have demonstrated its presence in exudates. Muller (1907) was the first to show the absence of antiprotease from normal cerebrospinal fluid, and Dochez (1909) the first to show its presence during meningitis. The latter's findings have been more recently confirmed by Kaplan *et al.* (1939), who also found antiprotease in the cerebrospinal fluid of patients with brain tumors invading the meninges or ventricles. (According to Walter (1929) only such tumors as these increase the permeability of meningeal capillaries.) The presence of a small amount of diffusible antiproteolytic substance in normal urine has been

reported by Fujimoto (1918), while numerous workers (*e.g.*, Schippers, 1911) have reported the appearance of large amounts of antiprotease in pathological urine, especially in nephritis.

IV. SUMMARY

1. An equation is derived for the calculation of a constant which, experimental results indicate, may be a more reliable index of the antiproteolytic activity of serum than those equations hitherto used.

2. (*a*) Intramuscular administration of trypsin resulted in a slow rise in the antiproteolytic activity of the serum, followed by a lesser decline. (*b*) Intravenous administration resulted in no appreciable variation. (*c*) Oral administration resulted in a rapid rise, which was sustained during the period of administration. (*d*) Intramuscular, intravenous, or oral administration of denatured trypsin resulted in no appreciable variation. (*e*) The extent of the local necrosis following subcutaneous injection of trypsin varied inversely with the antiproteolytic activity of the serum.

3. The experimental results indicate that the products of protein hydrolysis in the intestine and parenterally are an important factor in the antiproteolytic activity of the serum. They also indicate that antibodies to trypsin are not an important factor in the antiproteolytic activity of the serum.

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