LXXV. ON THE INFLUENCE OF SERUM ON ENZYMES, WITH SPECIAL REFERENCE TO ITS ACTION ON TRYPSIN.

By JOSEPH FINE.

*From the Sir Alfred Lewis Jones Laboratory, Freetown,
Sierra Leone, West Africa.*

(Received January 21st, 1931.)

Introduction.

THE influence of serum on enzymes may be considered kinetically as falling into one of two categories. Either the enzyme is influenced by the presence of a similar enzyme in serum, in which case an apparent acceleration is produced, or else the action is associated with an absence from serum of the enzyme in question, in which case the action is one of true acceleration, or inhibition, as the case may be; the mere fact that serum accelerates or inhibits an enzyme constitutes in itself incomplete evidence of the nature of the action. In a recent publication [1930], I have shown how failure to recognise this principle has already vitiated work done on the subject.

It is usually a simple matter to determine whether an apparent acceleration is due to the presence of the enzyme in serum, or whether the acceleration is a true one; greater discrimination however is necessary to establish the presence of both factors if present simultaneously in a case of acceleration, and to determine to what extent each is responsible for the effect observed.

Serum-enzymes. In some instances the action of serum is wholly explained by the presence of a serum-enzyme, as in the case of serum-diastase; in other cases the action is only partially explained by the presence of an enzyme in serum, an example of which is human serum acting on lipase.

The enzymes recorded in serum include protease, peptidase, diastase, lipase, catalase, invertase, and maltase. It is important to recognise that the distribution of enzymes in serum is by no means identical in different species.

Thus, while diastase is fairly widespread, occurring in man, sheep, cow, rat, and guinea-pig, it is absent from rabbit [Luers and Albrecht, 1926]. Lipase I have also found in man, sheep, cow, rat and guinea-pig. Maltase was not found in guinea-pig serum [Blacklock, Gordon and Fine, 1930] but has been recorded in pig serum. Invertase I have failed to find in a number of animals, including man, but under experimental conditions (*e.g.* following intraperitoneal injections of a commercial preparation of invertase) I have

found it in guinea-pig serum, where it persisted for over 30 hours in one instance [Fine, 1930].

The influence of serum on enzymes not ascribable to the presence of serum enzyme. It is this category of the influence of serum on enzymes that is the subject of the present paper.

Even when serum does contain an enzyme, its action may be greater than is to be accounted for by the presence of the enzyme; for example, human serum will increase the activity of lipase to a degree exceeding what would be expected from the mere addition of a lipase of the strength of serum-lipase. The total acceleration is, in this case, partly due to the presence of serum-lipase (apparent acceleration), and is partly a true acceleration.

It has been customary to explain the effect of acceleration by serum as due to "an accelerator," and the effect of inhibition as due to an "inhibitor," or, more commonly, an "anti-enzyme." I have shown however [1930] that in the case of invertase the influence exerted by serum is entirely a function of p_H , the serum acting as an accelerator over certain ranges of p_H and as an inhibitor over others.

In the following pages I have brought together evidence both from my own experiments and from the work of others, which justifies the conclusion that with most other enzymes also true acceleration or inhibition by serum is not due to specific accelerators or anti-enzymes, but to various physical conditions such as p_H or the adsorption of the enzyme by serum, the latter being particularly significant in the case of the antitryptic action of serum.

In the previous publication referred to [1930] I have already dealt with the enzyme-accelerating properties of serum, and there pointed out that serum has been known to accelerate only two enzymes—invertase and lipase. The same serum which accelerates invertase will also inhibit it if the p_H is suitably altered. The influence of p_H on the lipase-accelerating power of serum has not been worked out.

Anti-enzymes reported in serum.

The origin and significance of the term "anti-enzyme." The first observation of the phenomenon of enzyme-inhibition by serum was made by Hildebrandt [1893], who discovered that serum inhibited the action of emulsin. Hahn [1897] followed with the observation that serum inhibited trypsin, and Morgenroth [1899] found that it inhibited rennin.

Although none of these workers isolated any substances from serum which might account for the inhibition observed, they all explained the phenomenon observed by the presence of corresponding anti-enzymes in normal serum. Very early, however, Landsteiner [1900] showed, and Cathcart [1904] and others confirmed, that trypsin inhibition was a property of the albumin fraction of serum rather than of the globulin fraction, whereas it was well established that the immunological antibodies resided in the globulin fraction.

Furthermore no one could demonstrate a material increase of the enzyme-inhibiting action of serum following enzyme injections. The consensus of opinion appears to be that trypsin is the only enzyme that can evoke the response of an increased enzyme-inhibition in serum, and even so, the increase never exceeds three or four times the original amount of inhibition. Such response is to be sharply differentiated from the effects of toxin injection on antibody formation in serum: true antibodies can invariably develop to the amount of thousands of times the original antibody content.

Weil [1910] pointed out that while serum can inhibit the haemolytic action of saponin, it does not necessarily follow that serum contains an antisaponin, and, indeed, as Bayliss [1919] says, if a substance which inhibits an enzyme is entitled to be called an anti-enzyme, then sodium hydroxide is antipepsin.

The action of serum on enzymes is largely a function of p_H . The earlier workers, as Wells [1925] has pointed out, seem to have quite ignored the effect of p_H . Bayliss [1912] in examining the evidence for Hildebrandt's anti-emulsin found that the mere addition of serum to an emulsin mixture sufficiently altered the p_H to produce a corresponding fall in activity of the emulsin. Bensley and Harvey [1912] showed that the absence of free HCl from the cells of the gastric mucosa formed the best protection against the secreted pepsin: in other words, the p_H of the cell substance was in itself an adequate "antipepsin." Dragsted and Vaughan [1924] sewed the spleen and kidney into openings made in the stomach wall, and found that they were unaffected by the consequent exposure to pepsin, provided the blood supply were kept intact. In their view the mechanism of this immunity was simply the buffer action of the blood and the absorption of the HCl and pepsin into the bloodstream. Thaysen [1915] found that "antirennet" could also be accounted for by the change in p_H when serum is added to a rennet mixture: he also found that adsorption of rennet by the serum-proteins played an additional part. The previously mentioned observations of the present author [1930] on invertase afford strong evidence in the same direction.

In the case of other enzymes however the influence of serum under different conditions of p_H has not hitherto been worked out, and although p_H regulation must play some part, it is probable that a number of factors are involved in the defence of the organism against the action of excessive amounts of enzyme. Excretion is one of the chief factors, as in the case of amylase; destruction in the body probably occurs in the case of invertase [Fine, 1930]; adsorption by tissue- or serum-proteins in the case of trypsin.

At various times serum has been reported to possess an inhibitory action towards trypsin, pepsin, rennin, papain, "autolytic enzymes," lipase, amylase, invertase, laccase, catalase, tyrosinase, emulsin, urease and fibrin-ferment. Consideration of the p_H optima of these enzymes shows however that the mere addition of serum which has a p_H of 7.45 would greatly reduce the activity of unbuffered solutions of pepsin, rennin, papain, autolytic enzymes, emulsin, and invertase. Even invertase, which is accelerated by serum when buffered

at its optimum p_H , is totally inhibited when serum is added to its unbuffered solution.

In the case of trypsin however, the p_H factor does not appear to play any part, the inhibition of trypsin by serum being, as will be shown later, purely a manifestation of the power of serum to adsorb the enzyme. Occupying an intermediate position between pepsin on the one hand, and trypsin on the other, there is a group of enzymes, of which rennin is an example, whose inhibition by serum is partly a p_H effect and partly an adsorption effect [Thaysen, 1915].

Recovery of trypsin from inhibition by serum.

Following Hahn's observation [1897] that serum contained antitrypsin, *i.e.* retarded the action of trypsin, many substances of organic nature were examined for their influence on trypsin, in the hope of some light being thrown on the phenomenon. Among these so-called inhibitors may be mentioned (1) extract of intestinal worms [Weinland, 1903; Dastre and Stassano, 1903; Hamill, 1906; Burge, 1915]; (2) egg-white [Vernon, 1904; Sugimoto, 1913; Bayliss, 1923]; (3) bacteria [Czapek, 1903; Jobling and Petersen, 1914, 4]; (4) yeast [Buchner and Haehn, 1910]; (5) body tissues generally [Levene and Stookey, 1903]; (6) cells of gastric mucosa [Blum and Fuld, 1906; Langenskiold, 1914]; (7) mucous secretion [Langenskiold, 1907]; (8) erythrocytes [Czapek, 1903]; (9) inflammatory exudates [Opie, 1905]; (10) urine [von Schoenbom, 1910; Fujimoto, 1918]; (11) unsaturated fatty acids [Jobling and Petersen, 1914, 1]; (12) lecithin [Schwartz, 1909].

An examination of the properties of these various substances showed, however, that the mechanism of their inhibition of trypsin had little in common, and that egg-white only may be considered as acting in a similar manner to serum. Among the various properties in which these two substances differed outstandingly from other trypsin-inhibiting bodies was the temporary nature of the inhibition. This property was however shared by worm extract whose behaviour generally otherwise differentiated it clearly from serum-antitrypsin.

Experimental demonstration of recovery of trypsin.

All the methods of estimating antitrypsin hitherto employed—Gross [1908] (casein precipitation); Folin and Denis [1911] (incoagulable nitrogen); viscometric method [Feldstein and Weil, 1909–10]; electro-conductivity method [Bayliss, 1923]; refractometric method [Robertson, 1918]—are subject to the criticism that when two digesting mixtures are compared, one containing serum and the other containing an equal amount of water, but otherwise the same in composition, the amount of protein in the two tubes is not the same, and therefore the amount of digestion not strictly comparable; for it must be remembered that serum is a 6 % protein solution. The technique I have used and found highly satisfactory has been as follows.

Two tubes were prepared containing equal quantities of trypsin, buffer (p_H 8), and serum; in the first the serum had been heated at 70° for half an hour to destroy antitryptic action and in the second the serum was untreated. An aliquot portion of each tube was titrated by Sørensen's method immediately the constituents were added and mixed and a fixed number of hours later. The rise in acidity indicated the amount of tryptic action that had taken place in the two tubes containing the same concentration of trypsin and substrate. If the rise in acidity in the first tube is A , and that in the second B , then the ratio $\frac{A-B}{A}$ adequately measures the antitryptic activity.

One of the chief advantages of the Sørensen method is the additional information it gives when the free and "formol" acidities are considered separately.

Using the Sørensen method and the technique I have outlined I was able to follow the course of digestion of serum-protein by trypsin, and in this way not only obtained the Dastre [1903] effect with serum, *i.e.* enzyme recovery following preliminary inhibition, but found that almost invariably the recovery of the trypsin from inhibition was such that the inhibited trypsin ultimately showed greater activity than the trypsin acting freely on heated serum.

The experiments showing this recovery were carried out as follows:

Two tubes were incubated whose contents were as below:

	Tube 1	Tube 2
	cc.	cc.
Trypsin	0.2	0.2
Serum	0.1	0.1 (inactivated)
Buffer p_H 8	0.7	0.7

(a) The source of trypsin was Liquor trypsin co. (Benger's) and before use it was neutralised with $N/10$ NaOH to p_H 8 and then further diluted to 50 % of original strength.

(b) The buffer was prepared according to Clark and Lubs.

(c) The serum in tube 2 was inactivated by heating at 70° for half an hour. It was heated together with the buffer so as to avoid the difficulty of mixing afterwards; during the heating the tube was stoppered to avoid loss of volume by evaporation.

(d) After tube 2 was cooled, the trypsin was added to the two tubes from which, after adequate mixing, 0.1 cc. was immediately withdrawn for titration of acidity with $N/100$ NaOH. A layer of toluene was added to the tubes before incubating.

The method of titration was that employed by Wigglesworth [1928] in his investigation of cockroach protease. The pipetted fluid (0.1 cc.) was added to 5 cc. of distilled water in a test-tube; 5 drops phenolphthalein solution (0.05 % in 50 % alcohol) were added, and $N/100$ NaOH was added from a 2 cc. microburette graduated in 0.01 cc. The alkali was added until a pink colour developed identical with that of a tube (of similar make) containing

Fig. 1. Digestion of 10 % guinea-pig serum by 10 % trypsin. This shows recovery of inhibited trypsin until activity is greater than that of uninhibited trypsin.

Fig. 2. Free acid produced in digestion of 10 % serum by 10 % trypsin. This shows how free acid produced by inhibited trypsin ultimately equals amount formed by uninhibited trypsin.

Fig. 3.

Fig. 4.

Fig. 3. "Formol" acid produced in digestion of 10 % serum by 10 % trypsin. This shows how inhibited trypsin ultimately forms more "formol" acid than uninhibited trypsin.

Fig. 4. Total acid curves of digestion of 10 % serum by 10 % trypsin. Recovery of inhibited trypsin is incomplete in this case: the serum was obtained from a guinea-pig which had recently had a large injection of trypsin.

5 cc. of buffer solution of p_H 9 plus 5 drops of 0.05 % phenolphthalein in 50 % alcohol. The end point was remarkably sharp.

The figure thus obtained was a measure of the "free acid" present; the "formol acid" was obtained by adding 0.5 cc. of 50 % neutralised formalin (p_H 8) and titrating further with alkali till the end point was reached again. When desired the free and "formol" acids were combined in a single figure—the "total acid."

Determinations of the acid present in 0.1 cc. of the incubated mixtures were carried out from time to time, and in this way curves of digestion were plotted.

Fig. 1 is the curve of a typical experiment, showing how the initial inhibition of trypsin is finally replaced by digestion more active than in the tube where trypsin was not initially inhibited. An analysis of Fig. 1 in the form of separate curves of free and of "formol" acid formation (Figs. 2 and 3) throws some light on the phenomenon. The latter figures show that the final superior digestion of unheated serum applies only to "formol" acid formation: on no occasion have I found more free acid ultimately formed in the presence of unheated serum (tube 1) than of heated serum (tube 2), whereas "formol" acid formation was almost invariably greater in tube 1 than in tube 2, provided digestion was sufficiently prolonged.

In some cases, even after weeks of incubation, the total acid in tube 1 remained below the value in tube 2. An example of this type of incomplete recovery is shown in Fig. 4 the curve of which was obtained from the serum of a guinea-pig which 7 days previously had received a large injection of Liquor trypsin co. An analysis of the total acid curve, in Figs. 5 and 6, shows that the incompleteness of recovery of the trypsin affects only the free acid formation, the "formol" acid curves intersecting in the same way as in Fig. 3.

Figs. 7, 8 and 9 are the total, free, and "formol" acid curves of digesting mixtures containing

10 % gelatin ...	...	...	0.5 cc.
Trypsin 50 %, neutral	...	...	0.5 cc.
Buffer p_H 8.5...	...	...	0.5 cc.
Serum...	...	...	0.2 cc.
Saline 0.88 %	...	...	0.3 cc.

Here also the greater digestion of unheated serum affects the "formol" acid curve only. The addition of the gelatin appears to hasten the recovery of the trypsin.

Interpretation of foregoing results. (a) It would appear on examining Fig. 1 that serum can not only retard the action of trypsin, but can at a later stage enhance it, after passing through an intermediate stage during which trypsin becomes gradually released from its bonds. In the Fig. 4 type of experiment the stage of enhancement is not reached, though the trypsin has recovered considerably from the inhibiting action of serum.

(b) An examination of the free and "formol" acid curves separately shows very strikingly that the transition from inhibition to enhancement applies

Fig. 5.

Fig. 6.

Fig. 5. Free acid curves of digestion of 10 % serum by 10 % trypsin. This experiment is the same as in Fig. 4, the free acid only being considered.

Fig. 6. "Formol" acid curves of digestion of 10 % serum by 10 % trypsin. This experiment is the same as in Fig. 4, "formol" acid only being considered.

only to the "formol" acid formation; the extent of formation of free acid with unheated serum never exceeds that with heated serum although, provided

Fig. 7.

Fig. 8.

Fig. 7. This shows accelerated recovery of inhibited trypsin in presence of gelatin. The dotted part of the curves is hypothetical, as no estimation of acidity was made during that interval.

Fig. 8. Free acid curves (2.5 % gelatin present). This shows the same relation between the free acid formed by inhibited and by uninhibited trypsin as in Fig. 2.

Fig. 9. "Formol" acid curve (2.5 % gelatin present). This shows the same relation between "formol" acid formed by inhibited and uninhibited trypsin as in Fig. 3.

digestion is sufficiently prolonged and the trypsin is fairly active, the same amount of free acid is finally produced in the two tubes: Fig. 5 shows a marked but not complete free acid recovery in the unheated serum tube.

(c) Serum therefore possesses the property, destroyed by heat, of temporarily retarding formation of free and "formol" acid by trypsin, and of subsequently accelerating "formol" acid formation only.

How is this behaviour of serum to be explained? The explanation would prove a difficult matter if it were assumed that pancreatic trypsin, which is the basis of the commercial preparation used (Liquor trypsin co.) were a pure enzyme. The pancreatic secretion however contains two proteolytic enzymes, protease breaking proteins down to polypeptides and increasing predominantly the free acidity of the digest, and peptidase, hydrolysing the polypeptides to amino-acids and producing a rise in "formol" acidity.

If this view is correct, the behaviour of serum becomes more easy to explain. It does not exert its action against trypsin as a whole, but only against the protease portion of it. The inhibition of protease will, by preventing the liberation from the proteins of a suitable substrate for peptidase, produce an apparent inhibition of the latter. There does not, however, appear to be any necessity for assuming that the initial depression of "formol" acid formation is due to an antipeptidase action of serum rather than to a shortage of substrate consequent on protease inhibition.

But why should "formol" acid production in the unheated serum tubes exceed that in the heated tubes? There are two possible explanations. Serum contains either a peptidase or a peptidase-accelerator. The most likely explanation is the existence of a serum-peptidase, which has been reported by various workers, including Jobling *et al.* [1912] who report that serum-peptidase is destroyed by heating at 70° and [1915, 2] that "serum-peptidase is not influenced by antitrypsin." Hence, when protease has recovered from the inhibition by serum, the peptidase of the unheated serum will reinforce that of the pancreatic trypsin, and so produce "formol" acid at a rate which will outstrip the "formol" acid production in the tube containing heated serum (where pancreatic peptidase acts alone, the serum-peptidase being destroyed by heat).

(d) In a word, the behaviour of serum to pancreatic trypsin can be explained by the presence of two factors, firstly, an antiprotease factor, and secondly, peptidase.

This conclusion is of some importance for at least two reasons. In the first place the evidence justifying it confirms in a novel yet definite manner the dual nature of trypsin; secondly, this evidence, by showing that it is the protease only (the portion forming free acid) which serum inhibits, directs serious criticism against those methods of measuring "antitrypsin" which estimate together the products of both protease and of peptidase actions. The methods thus open to criticism include those of Bayliss (electrometric), and Robertson (refractometric) though not of Folin (uncoagulated N). The error involved will depend on the period of incubation used as well as the strength of serum-peptidase present; the extent of the error will be appreciated in the following example.

Course of digestion of heated and unheated sera									
	8 hrs.			27½ hrs.			95 hrs.		
	F	For	T	F	For	T	F	For	T
Unheated sera	0.03	0.06	0.09	0.13	0.19	0.32	0.23	0.41	0.64
Heated sera	0.22	0.10	0.32	0.27	0.17	0.44	0.27	0.28	0.55
F=Free acid.			For="Formol" acid.			T=Total acid.			

Figures are cc. N/100 acid in 0.1 cc. of incubated fluid.

It will be readily seen that there is a considerable discrepancy between the degree of inhibition as measured from the free acid and that measured from the total acid. Thus:

	% inhibition (or "antitryptic index")		
	8 hrs.	27½ hrs.	95 hrs.
From free acid figs.	86	52	15
From total acid figs.	72	27	-16*

* i.e. 16 % acceleration.

The methods criticised will therefore give too low a value for the index of inhibition.

The kinetics of serum antitrypsin.

(a) *The kinetics of recovery.* The Dastre phenomenon was only obtained when the concentration of serum was suitably adjusted to the concentration of trypsin. On the other hand, it was found that (1) when the concentration of trypsin was greatly in excess of the serum the latter failed to produce any appreciable inhibition; (2) when the concentration of serum was greatly in excess of the trypsin, the latter completely failed to recover from the inhibition. The correlation of the relative trypsin-serum concentration with the manifestation of the Dastre phenomenon is illustrated by Fig. 10. The curves were

Fig. 10. Inhibition of trypsin (from 2 % to 20 %) by 10 % sheep serum. This illustrates the kinetics of recovery of trypsin from inhibition. The figure suggests a division of the curves into four trypsin zones.

Zone 1. 0 %–5 % trypsin. Here there is no recovery from the inhibiting action of 10 % serum.

Zone 2. 5 %–20 %. Inhibition is initially complete to partial, and recovery is partial to complete.

Zone 3. Trypsin 20 %–25 % (dotted lines); inhibition is initially small, and recovery is complete.

Zone 4. Trypsin over 25 %. Inhibition is absent. No experiments were actually carried out in Zones 3 and 4, which are merely strongly suggested by the character of the Zones 2 and 3, and by the fact established on other occasions that concentrated trypsin successfully resists the inhibiting action of serum.

obtained by plotting the digestion of a series of tubes containing (1) trypsin in concentrations from 20 % to 2 % of the commercial preparation; (2) sheep serum in constant concentration of 10 %, and (3) buffer, p_H 8. A pair of tubes was put up at each concentration of trypsin, one containing unheated serum (tube 1), the other heated (tube 2): each point on the curve was obtained by expressing as a percentage the ratio

$$\frac{\text{total acid in tube 2} - \text{total acid in tube 1}}{\text{total acid in tube 2}}$$

which expresses the degree of inhibition by the unheated serum. The total acid figures were taken because in this case the value of serum-peptidase was practically nil, so that the error involved in including "formol" acid did not affect the nature of the curves obtained, although it would affect the accuracy of the individual antitryptic indexes.

An examination of Fig. 12 shows that (1) in concentrations of 4 % and less trypsin is completely inhibited and does not recover from the inhibition even after 23 days; (2) between 4 % and 20 % trypsin is only partially inhibited and recovers to an extent proportional to its concentration; (3) at 20 % recovery is ultimately complete. From the nature of the curves it would be reasonable to infer that concentrated trypsin completely resists the inhibiting action of serum (dotted lines in Fig. 10).

From the continuous nature of the curves it appears likely that the action of serum is essentially the same at all concentrations of trypsin, varying in degree only, and that therefore the apparently permanent inhibition of weak trypsin is not due to destruction of the trypsin, any more than is the temporary inhibition of stronger trypsin.

(b) *The work of Hedin.* Hedin [1906, 1] found that (1) a small amount of serum had a relatively greater effect on trypsin than a large amount; (2) it was impossible completely to neutralise a given solution of trypsin by adding any amount of serum. This result is confirmed by Young [1918] whose results are illustrated in Fig. 11: this curve shows that excess of serum has no effect on the residual activity of trypsin.

Fig. 11. Relationship between serum and inhibited trypsin [experiment by Young, 1918, p. 503, sheep]. This illustrates: (1) that degree of inhibition is a logarithmic function of amount of inhibiting serum (thus suggesting that inhibition is an adsorption phenomenon); (2) inability of serum to achieve 100 % inhibition of trypsin, the reasons for which are discussed in the text.

Hedin thus shows that a mixture of trypsin and excess of serum possesses both antitryptic and tryptic actions, *i.e.* trypsin and antitrypsin can co-exist when the latter is in excess, each exerting an independent action on added substrate or enzyme respectively.

I have not been able to confirm the common findings of Hedin and Young that excess of serum will not entirely inhibit a given amount of trypsin. Fig. 10 shows that 4 % and 2 % trypsin were completely inhibited by 10 % of serum. The cause of the discrepancy is difficult to explain, but may be due to the difference in the methods employed in studying the kinetics of trypsin. Hedin and Young combined fairly concentrated trypsin in constant amount with increasing amounts of serum: hence all flasks contained a fairly active peptidase together with a large excess of caseinogen added as substrate. Caseinogen it is stated by Jobling and Petersen [1914, 4, p. 152] is an exception to the rule that peptidase does not split proteins; furthermore, peptidase is uninfluenced by the antiferment (Jobling).

(c) *Analogy with charcoal.* To Hedin [1906, 2] is also due the discovery that charcoal inhibits trypsin in a manner kinetically very similar to that of serum. Thus the amount of trypsin bound by charcoal follows the same law as the binding of trypsin by serum: small amounts of charcoal bind relatively more trypsin than larger amounts.

This relationship, which can be expressed by the equation $x = ky^n$ (where x = amount of trypsin bound, and y = amount of active charcoal), is a logarithmic one, and is readily accounted for by assuming that the mode of action of charcoal is one of adsorption of the trypsin. Hence Hedin on the basis of kinetic similarity concluded that serum also inhibited trypsin by the process of adsorption.

The behaviour of charcoal throws some further light on the adsorptive process. Hedin showed that although a trypsin solution filtered after treatment with excess of charcoal was quite inactive, yet an unfiltered trypsin-charcoal mixture incubated with caseinogen was definitely and invariably active, though most of the trypsin remained inhibited. Hedin concluded that in the presence of subsequently added substrate charcoal did not retain all the trypsin adsorbed, a small portion of it being taken up by the substrate. In a similar manner might be explained the impossibility of totally inhibiting a solution of trypsin by means of serum, although it is impossible actually to demonstrate the similarity since trypsin cannot be removed with its inhibitor from a trypsin-serum mixture in the simple manner possible with charcoal.

(d) *Robertson's formula.* Robertson [1918] using the refractometric method, examined quantitatively the inhibition of trypsin by serum and arrived at the formula

$$\frac{T}{A(1-T)} = C$$

where T = fraction of trypsin inhibited;

A = concentration of serum present;

C = a constant representing the antitryptic value of the serum used.

Robertson claimed that his constant was an absolute measure of the inhibiting power of serum, being independent of the concentration of serum used. An examination of his results, however, shows that the fraction $\frac{T}{A(1-T)}$ tends to be constant only for mixtures containing more concentrated serum (10 % to 33 %) and even then the variation in C is too considerable (5.87 to 6.95 for a single serum) to permit of the value of C being used as a characteristic figure for the inhibiting power of serum.

Where approximate results only are required, however, the constant is a useful expression of antitryptic power, and sufficiently accurate, provided the concentration of serum in the mixture is not too small. By this method Hanson [1918] was able to show that injections of trypsin had no influence on the globulin/albumin ratio of serum, although the antitryptic index (as measured by C) rose to three times the normal value.

(e) *The specificity of the inhibiting action of serum.* Von Eisler [1905] found that human serum did not inhibit human pancreatic trypsin any more than it inhibited pig trypsin, while Weil [1910] found that serum actively inhibited the vegetable protease papain. Serum, it follows, does not exert any biologically specific action on trypsin, but inhibits all proteases alike. Apparent specificity may be shown by serum nevertheless, as the work of Young [1918] proves. He found that whereas dilute serum equally inhibited two samples of equally active trypsin obtained from the same source, the inhibition was different when the concentration of serum was increased. Fig. 12 illustrates

Fig. 12. Inhibition of equally active samples of ox pancreas [experiment by Young, 1918, p. 506, Exp. IV]. This shows that samples of trypsin equally inhibited by weak serum may not be equally inhibited by stronger serum.

this result, and Fig. 13 shows the result of another of Young's experiments in which equally active samples of fresh and commercial trypsin are compared. Young was unable to account for the preferential action shown by serum for certain tryptins, though this preference was not shown in all the experiments carried out: he did show, however, that the presence of zymoids could not account for the phenomenon.

His findings are important in showing the danger of attributing all variation in antitryptic action to the serum alone without first determining the uniformity of the trypsin solutions used.

The influence on serum of injections of trypsin.

A careful examination of the properties of serum-antitrypsin and of the various methods employed in measuring its value has led me to the conclusion that the failure of workers to obtain a rise in antitryptic value of serum following injections of trypsin could adequately be accounted for by one or more of the following circumstances¹.

Fig. 13. Inhibition of equally active samples of fresh and commercially prepared trypsin [experiment by Young, 1918, p. 505, Exp. I]. Here weak serum inhibits the two samples differently, the difference diminishing as the serum becomes stronger.

(a) The antitryptic power of serum is not constant in value, but follows a swinging course which in the normal animal is often determined by definite physiological conditions. Thus Jobling *et al.* [1915, 1] found a definite rise a few hours after a meal, a result which I have confirmed.

(b) It is generally agreed that the variations in antitryptic value are not very great. In Hanson's experiments [1918] the rise never exceeded three times the normal index. Consequently if the serum were antitryptically at its peak, an injection of trypsin would fail to produce a further rise in index.

(c) Hanson records that when the index returns to normal after a rise due to a trypsin injection, further repeated injections fail to produce a second rise. It is probable that such a refractory condition of serum might arise from physiological conditions other than the results of trypsin injections.

(d) The choice of method has a very important effect on the results obtained [Fine, 1930].

Experimental demonstration of the rise in antitryptic index following injections of trypsin. The results of experiments carried out with three guinea-pigs are graphically represented in Figs. 14, 15 and 16.

The method of obtaining the antitryptic index may be considered open to criticism since, in the technique employed, the substrate used is the serum itself and must therefore vary with each specimen taken: secondly, it may be objected that since substrate is not present in excess, the extent of hydrolysis

¹ Among those who failed were Doblin [1909], Rosenthal [1910], Weil [1910], Young [1918], and Pozerski [1909] (using papain instead of trypsin). Positive results, on the other hand, were obtained by Achalmé [1901], von Berman and Bamberg [1908], Jochman and Kantarovitch [1908], Meyer [1909], Jobling, Petersen and Eggstein [1915, 2] and Hanson [1918].

in 24 hours cannot be considered as representing the activity of the enzyme present.

I would answer these objections as follows.

(a) The index is calculated from the ratio of digestion of a given amount of serum-protein to the digestion of the same amount but in the absence of

Fig. 14. Influence of trypsin injections on antitryptic index of serum. Guinea-pig *T*. This shows a rise in index immediately or shortly after each injection of trypsin. The guinea-pig was bled daily without definite relationship to the time of feeding, this leading to apparently spontaneous fluctuations in the curve.

Fig. 15. Influence of trypsin injections on antitryptic index of serum. Guinea-pig *U*. After the first injection the index rises and remains high throughout the experiment.

Fig. 16. Influence of trypsin injections on antitryptic index of serum. Guinea-pig *V*. Both injections are definitely associated with a subsequent rise in index. The very large first injection produced a preliminary fall.

any inhibition. Actually of course the index is the difference between this ratio and unity. Hence the actual amount of protein present is not of consequence provided it does not vary greatly from specimen to specimen: in practice the variation was not found to be great.

(b) It is true that the acidity measured is not a true measure of the activity of enzyme present: it must be realised, however, that in the method used it is not the activity of the inhibited enzyme that is measured, but the extent to which it has in a given time recovered from the inhibiting action, this degree of recovery being directly related to the degree of inhibition.

An important advantage of the method used is the possibility of distinguishing between the free acid and "formol" acid produced: since the formation of free acid only is opposed by serum, the index is in all cases calculated with reference to free acid formation only.

The three guinea-pigs experimented on were kept under observation for over a month: in each case there was a gap of 14 days during which, as can be seen, no examinations were made owing to an interruption in the investigation.

Guinea-pig *T* did not respond to any extent to the first injection of 1 cc. of trypsin (Benger's), but showed a rise within a few hours of a second injection of 2 cc., which persisted for some time. After a month the index was swinging considerably, with a tendency to rise, which a third injection of 1 cc. maintained. Guinea-pig *U* demonstrated in unequivocal fashion the rise in index in response to an injection of 1 cc. trypsin. The rise was maintained even after 20 days, a second injection having no observable effect. Guinea-pig *V* also responded to a first injection, although the rise was here preceded by a definite fall in index, accompanied by symptoms of collapse (immobility of many hours' duration). In 17 days the index had returned to a lower level, and again responded to an injection (of half the first dose): the rise was maintained for a week. A fall in index immediately following a large injection of trypsin seemed to be associated with symptoms of collapse. Thus guinea-pig *Q* became immobile, following an injection of 2.5 cc. of trypsin, and, although recovery set in in 20 minutes, the index fell from 0.6 to 0.4 in 5 hours, and death ensued in 22 hours. In this case the trypsin was not neutralised, though in all other cases it was brought to a p_H of 8.3 before injection: the route in all cases was intraperitoneal. It will be seen that although guinea-pigs *T*, *U* and *V* responded similarly to adequate injections of trypsin, they differed in their subsequent condition, the index of *T* tending to remain high, but swinging, that of *U* remaining persistently high, while that of *V* tended to return to normal in a few days.

The nature of serum-antitrypsin.

There are two questions which it is necessary to answer if the nature of serum-antitrypsin is to be known: firstly, what is the substance in serum responsible for the inhibition of trypsin? secondly, what is the mode of action of this substance on trypsin?

(1) *The antitryptic substance.* The three main theories as to identification of the antitryptic substance are described below, and their relative merits then discussed: the antibody theory is treated as a variation of the protein theory.

(a) *Amino-acid theory.* Bayliss first showed [1904] that the end-products of tryptic action retard the action of trypsin: Abderhalden and Gigon [1907] confirmed the fact that trypsin was retarded by amino-acids. On the strength of such observations Rosenthal [1910] suggested that serum-antitrypsin owed its action to the amino-acid content of serum. Walters [1912] pointed out that the action of amino-acids is too slight to account for the behaviour of serum. More recently Hussey and Northrop [1923] suggested that the polypeptides formed by tryptic action are the source of serum-inhibition. No serious proof has been offered for either of these theories, and the occurrence of the Dastre phenomenon contradicts them emphatically: end-products of tryptic action cannot reasonably account for an antitryptic action which is at its maximum at the commencement and progressively diminishes.

(b) *Lipoid theory.* Schwartz first showed [1909] that extraction with ether lessened the antitryptic power of serum, and considered this as proof that serum owed its antitryptic action to its lipoid content. His findings were confirmed by Sugimoto [1913], and the lipoid theory in a modified form was strongly urged by Jobling and his colleagues in a series of papers entitled "Studies in ferment action" [1914, 1-4]. Jobling submitted evidence showing (1) that unsaturated fatty acids and soaps possessed definite antitryptic action; (2) that saturated fatty acids and soaps possess no such antitryptic action; (3) that the iodination or hydroxylation of unsaturated fatty acids or soaps leads to the loss of antitryptic action: similar treatment of serum leads to the loss of antitryptic action of serum. This is confirmed by Slovzov and Zenophentova [1919]; (4) that although unsaturated soaps do not lose antitryptic action when heated alone, they do lose this action if heated at 70° for half an hour in the presence of serum; (5) that the chloroform extract of serum will exert an antitryptic action if saponified, although without this treatment the extract will not exert such action.

A more careful scrutiny of Jobling's experimental results, however, reveals the following facts: (1) On no occasion is there any record of an unsaturated lipid exerting antitryptic action without previous saponification. (2) Insufficient regard is paid to the p_H of the enzyme mixtures. (3) The attempt is made to bridge the gap between antitryptic lipoids and serum as regards the effect of heat by showing that unsaturated soaps do lose antitryptic power when heated in the presence of serum. From Jobling's figures, however, it is clear that loss of antitryptic power of the unsaturated soap takes place on mixing with serum in the cold, and that heat merely increases the loss: acidity of the soap-serum mixture also increased the loss.

That the lipoids of serum have any connection with the antitryptic action of the latter is contradicted by the work of Cobliner [1910], who found that dry serum retains its antitryptic action after extraction with chloroform or other fat solvents.

Finally, I have shown that the nature of the inhibition produced by serum is essentially different from that produced by unsaturated soaps. The initial

effect of 0.5 % of sodium oleate is to stimulate trypsin, which after 24 hours shows lessening of activity. Serum, on the other hand, initially exerts its maximum inhibition on trypsin, which may ultimately recover completely. This contrast is illustrated in Fig. 17 which represents one of several experiments carried out.

Fig. 17. Comparison between inhibition of trypsin by unsaturated soap and inhibition by serum. This shows that sodium oleate initially stimulates and ultimately depresses trypsin, whereas serum exerts an initial inhibition from which trypsin progressively recovers.

These curves were obtained by plotting the course of digestion of the following four tubes, the usual Sørensen's technique being followed.

	Tube 1	Tube 2	Tube 3	Tube 4
Serum	0.2	0.2	0.2	0.2
		inactivated	inactivated	inactivated
Trypsin 50 % (neutralised)	0.4	0.4	0.4	0.4
Buffer (p_H 8)	0.9	0.9	0.9	0.9
Sodium oleate 2 %	0.0	0.5	0.2	0.0
Distilled water	0.5	0.0	0.3	0.5

Figures represent cc.

(c) *The protein theory.* It has been definitely shown that the antitryptic action of serum is associated with the protein fraction [Landsteiner, 1900; Cathcart, 1904; and others], the albumin fraction being more antitryptic than the globulin.

Fujimoto [1918] obtained crystallised albumin and found it to be highly antitryptic. He admitted the possibility of some other constituent of serum entering into the composition of the crystals however, and on the whole considered that the antitryptic action was due to the serum-proteins and possibly some other undetermined factors in addition. Oppenheimer [1913] came to the similar conclusion that antitryptic action of serum was due to multiple causes, of which the peculiar configuration of the protein molecule was one [Oppenheimer and Aron, 1903]. Teale and Bach [1920] concluded that the nature of the serum-proteins was the cause of the antitryptic action of serum.

Beaton [1922] found however that the antitryptic index may rise without any change in the albumin content of serum, and in some cases the rise may even be accompanied by a fall in serum-albumin.

There is therefore some additional factor in the action of serum besides

the presence of protein, and this factor is in all probability a physical one, namely the degree of dispersion of the proteins.

(2) *Mode of action of proteins.* The classical work of Hedin [1906, 1, 2] confirmed by Hata [1909], Bayliss [1923], Young [1918] and others, has shown that the mechanism of inhibition by serum is one of adsorption of trypsin by the serum-proteins. Such a mechanism is perfectly reconcilable with Beaton's findings since the amount of adsorption depends not only on the amount of the adsorbing colloids, but also on their degree of dispersion. Thus the antitryptic index of serum might increase without the concentration of proteins necessarily increasing, for the existing amount of circulating protein, by an increase in its state of dispersion, can give rise to a greatly increased surface for adsorption, and therefore to a heightened capacity to adsorb trypsin. It can be shown by a simple calculation that when any particle undergoes division into n smaller particles, the total surface of the smaller particles is $\sqrt[3]{n}$ times the surface of the parent particle. Hence, if the degree of trypsin inhibition can be considered as directly proportional to the surface presented by the colloidal particles of the serum-protein, then the antitryptic index can be doubled either by doubling the amount of protein without altering the average size of colloidal particle, or by the formation of eight colloidal particles from each original one without altering the total amount of circulating protein.

The mechanism of adsorption would also serve to explain one important difference between trypsin inhibition and the inhibition of other enzymes by serum—namely the capacity of trypsin ultimately to recover from the inhibition, there being no such recovery reported for other enzymes. As Bayliss points out [1923], the adsorbed trypsin is able slowly to attack the serum-proteins adsorbing it until the latter are entirely hydrolysed and the trypsin is thus liberated.

When another enzyme such as invertase is inhibited by serum then, on the assumption that the inhibition is also due to adsorption by serum-proteins, it would not be expected that recovery should take place, since invertase would be unable to hydrolyse the adsorbing bodies. In my own experiments with invertase I found that inhibition was not recovered from; thus an invertase solution when unbuffered produced 10 % inversion of 2 % cane sugar in 24 hours and 30 % inversion in 3 days, while with a similar unbuffered solution in the presence of 10 % serum no inversion took place after 3 days.

Adsorption however is not a complete explanation of serum-antitrypsin; since all enzymes before exerting their specific action must be adsorbed by their respective substrates it still remains to be explained why trypsin should be inhibited when it is adsorbed by serum-proteins, although it acts freely when adsorbed by other protein substrates.

Discussion of the antibody theory.

There is no doubt that kinetically the action of serum on trypsin resembles that of antiserum on toxin: in both cases the amount of serum required is not proportional to the amount of substance neutralised, the ratio of serum required increasing very considerably as neutralisation of the substance is approached. The relationship is represented by a logarithmic curve such as satisfies the requirements of an adsorption phenomenon.

It may therefore be said that immunological antibodies and serum anti-trypsin resemble each other in the following respects.

(a) Both are proteins acting by means of adsorption.

(b) They can be increased in amount by injections of the substances they antagonise.

(c) Both are normally present in serum.

On the other hand the following differences between them exist.

(a) Immune antibodies are associated with the globulin of serum: anti-trypsin with the albumin chiefly.

(b) The injection of antigens can lead to increase of immune bodies to the extent of thousands of times the normal amount: whereas even an active response to trypsin injections will only treble the normal antitrypsin content.

(c) The immune antibodies are highly specific, increased capacity to neutralise a particular toxin being attended by a low neutralising power for other toxins: there is never any marked variation in the power of serum to inhibit the various enzymes normally inhibited by it.

The antibody theory of trypsin therefore amounts to little more than an expression of the fact that both antitoxic action and antitryptic action are adsorption phenomena in which the adsorbing bodies are proteins.

On the occurrence of non-amide linkages in the protein molecule.

In examining the meaning of the Dastre phenomenon, the newer knowledge of the heterogeneous nature of trypsin and erepsin was ignored in order to avoid complication of the issue: in any case it still remains true that the proteolytic enzymes can broadly be divided into two groups—one attacking chiefly the protein molecules and the larger polypeptides, and the other attacking mainly the smaller molecules.

The conclusion that serum behaved as an antiprotease was an expression of the fact that one phase only of the action of trypsin was inhibited. If the action of trypsin on a protein be examined by Sørensen's method it will be found that at the commencement free acid production predominates over formol acid, but that ultimately "formol" acid overtakes free acid.

Since the first action of trypsin is to hydrolyse the protein molecule into polypeptides, the predominance of free acid formation may be correlated with the power to attack the protein molecule, while predominance of "formol" acid may denote hydrolysis of the earlier products of activity. This correlation, which has hitherto been assumed, is borne out by the behaviour of pepsin,

the hydrolysis of proteins by which to the peptone stage only is correlated with the preponderance of free over "formol" acid throughout the period of digestion.

Strictly speaking however it cannot be assumed that free acid formation is the sole activity of the enzyme attacking the protein molecule since, as Haldane points out [1930], the specificity of the proteolytic enzymes is not absolutely fixed, each enzyme being capable of effecting a number of allied hydrolytic reactions. Consequently the inhibition of free acid formation does not necessarily prove the inhibition of protease, but denotes the suppression of one form of activity of protease.

The significance of free acid formation. Plimmer pointed out [1908] that in addition to the carbamic linkage —CONH— there may be present in the protein molecule (1) the arginine linkage —CH—NH—C— ; (2) the diketopiperazine linkage; (3) among the hydroxy-acids, the ether, ester, and anhydride linkages.

He did not consider it proved that such linkages did exist, however, and even quite recently Gränacher [1929], who believes the presence of the ester linkage highly probable in proteins, considers definite proof of this wanting.

It seems to me that the formation of free acid during hydrolysis of serum-proteins is proof of the existence of non-amide linkages. Clearly the hydrolysis of —CONH— by forming carboxyl- and amino-groups, cannot increase free acidity, but will increase "formol" acidity.

Free acid could, however, be produced by hydrolysis of esters; since this is usually the function of special enzymes (lipase and phosphatase) it is necessary to eliminate the possibility of free acid formation being due to them. In my own experiments this can readily be done.

(1) The commercial trypsin used was kept in the ice chest, and at p_H 2, which is destructive of lipase. Even if lipase were present in the trypsin, and were responsible for free acidity by action on serum-fats, the action would have been greater on unheated serum than on heated, since guinea-pig serum possesses a very active lipase [Fine, 1930].

(2) The action of phosphatase can be eliminated if the amount of available phosphate in serum be considered. On the basis for the figure for human serum (0.005 % phosphorus) the maximum amount of free phosphoric acid obtainable from 0.2 cc. of a 10 % solution of serum (the amount used in my titrations) is equivalent to 0.01 cc. $N/100$ NaOH—a negligible value within the range of error of the experiments.

That the function of pepsin is to hydrolyse linkages other than —CONH— is proved by the large proportion of free acid to the total acid developed during hydrolysis of proteins [Wigglesworth, 1928]. There is also more definite evidence of this function. Thus Harris showed [1923] that —SH groups were unmasked during peptic digestion, and Abderhalden and Schwab [1930] showed that pepsin completely hydrolysed dileucylthyroxine, although one of the leucine molecules was combined as an ester. Haldane suggests that in

the latter instance the ester linkage was split by a lipase present, but it is unlikely that lipase would act at the optimum p_H of pepsin.

It is very significant that in a list of 53 polypeptides, of which 29 were hydrolysed by trypsin and 24 were not, all those containing tyrosine (four), cystine (two) and *isoserine* (one), *i.e.* the only amino-acids with potentialities for other than —CONH— linkages, were in the hydrolysable group [Plimmer, 1908]. There is no mention however as to whether free acid increased during any of the hydrolyses, and therefore the nature of the linkages is not proved.

Serum antitrypsin as a resistance of the non-amide linkages of serum-proteins. If free acid formation is admitted to be the result of hydrolysis of other than —CONH— linkages, then the inhibition by serum-proteins of free acid formation must be due to a special resistance of these other linkages, a resistance which is overcome by heating at 70° for half an hour. This effect of heat is not due to coagulation, since serum heated with buffer at p_H 8 became only faintly turbid, yet lost its antitryptic action. Possibly the molecules adjoining the special linkages are stereochemically antagonistic to the action of trypsin, but are sterically altered by heat. Such stereochemical specificity is met with throughout enzyme reactions, one of the simplest examples being the resistance of glycyl-*d*-leucine, but not glycyl-*l*-leucine to hydrolysis by yeast-crepsin [Abderhalden and Handovsky, 1921]. That proteins can become less hydrolysable by trypsin as a result of stereochemical alteration has been shown by Dakin and Dudley [1913] who found that partial racemisation of caseinogen by alkali (the rotary power falling to 60 %) made it resistant to trypsin; Lin, Wu and Chen [1928] however found that resistance, although marked, was not complete.

But serum not only resists trypsin: it adsorbs it also, rendering it unavailable for other substrates present. The complete mechanism of serum-antitrypsin might therefore be conceived as

- (a) an adsorption, at the ester and other non-amide linkages, of those constituents of trypsin capable of attacking them;
- (b) a resistance to hydrolysis on account of neighbouring stereochemical influences;
- (c) a breakdown of the resistance, whether through stereochemical changes or other causes.

CONCLUSIONS.

1. The influence of serum on enzymes can be divided into the following categories.

- (a) *Serum contains the enzyme.* Here an apparent acceleration is produced (*e.g.* diastase).
- (b) *Serum accelerates an enzyme, although it does not contain it.* This is true acceleration (*e.g.* invertase at p_H 4).
- (c) *Serum contains the enzyme, but accelerates it more than is accounted for by this fact* (*e.g.* lipase).
- (d) *Serum inhibits the enzyme.*

2. The effect on invertase varies uniformly with p_H , at certain values of which inhibition takes place.

3. The action of serum is sufficiently accounted for by the change of p_H produced by the serum (*e.g.* pepsin) or by the adsorption of the enzyme by the serum proteins (*e.g.* trypsin), or by both factors (most other enzymes).

4. A comparison between serum-antitrypsin and a typical Ehrlich antibody such as antitoxin shows that they resemble each other in the way they act on trypsin and toxin respectively—*i.e.* by adsorbing them—and in their increase following injections into the animal body of the respective “antigens”: nevertheless the increase in antitrypsin is trivial and difficult to obtain compared with the great and constant increase in antitoxin.

5. The inhibition of trypsin by serum is recovered from if digestion is continued. An analysis of the free and “formol” acid figures shows that the inhibition is directed against free acid formation, and that the initially low “formol” acid production is merely the result of shortage of substrate for the peptidase of trypsin.

6. This phenomenon of recovery rules out the lipoid and the amino-acid theories of serum antitrypsin.

An examination of the evidence makes it clear that the proteins are the bodies responsible for the antitryptic action of serum, and that they act by adsorption.

7. The formation of free acid during tryptic and peptic digestion can only be the result of the hydrolysis of other than —CONH— linkages when the presence of other ester-hydrolysing enzymes is eliminated. The behaviour of serum suggests that it is at the non-amide linkages that adsorption of the inhibited enzyme takes place, while the resistance itself may be due to an unfavourable stereochemical configuration.

I have pleasure in expressing my acknowledgments to Prof. D. B. Blacklock, formerly Director of the Sir A. L. Jones Laboratory, for permission to publish details of experiments carried out in his laboratory.

REFERENCES.

- Abderhalden and Gigon (1907). *Z. physiol. Chem.* **53**, 521.
— and Handovsky (1921). *Fermentforsch.* **4**, 316.
— and Schwab (1930). *Fermentforsch.* **11**, 164.
Achalme (1901). *Ann. Inst. Pasteur*, **15**, 737.
Bayliss (1904). *Arch. Sci. Biol.* **11**, Supplement 261.
— (1912). *J. Physiol.* **43**, 455.
— (1919). *General physiology* (Longmans, London).
— (1923). *Nature of enzyme action*, chap. 8 (Longmans, London).
Beaton (1922). *Brit. J. Exp. Path.* **3**, 224.
Bensley and Harvey (1912). *Biol. Bull.* **23**, 225.
von Berman and Bamberg (1908). *Klin. Woch.* **45**, 1396.
Blacklock, Gordon and Fine (1930). *Ann. Trop. Med. and Parasitol.* **24**, 1.
Blum and Fuld (1906). *Z. klin. Med.* **58**, 505.
Buchner and Haehn (1910). *Biochem. Z.* **26**, 171.
Burge (1915). *J. Parasitol.* **1**, 179.

- Cathcart (1904). *J. Physiol.* **31**, 497.
 Cobliner (1910). *Biochem. Z.* **25**, 494.
 Czapek (1903). *Ber. deutsch. bot. Ges.* **21**, 229.
 Dakin and Dudley (1913). *J. Biol. Chem.* **16**, 505.
 Dastre (1903). *Compt. Rend. Soc. Biol.* **55**, 633.
 — and Stassano (1903). *Compt. Rend. Soc. Biol.* **55**, 130, 254.
 Doblin (1909). *Z. Immunol.* **1**, 650.
 Dragsted and Vaughan (1924). *Arch. Surg.* **8**, 791.
 von Eisler (1905). *Ber. Wien. Akad.* **104**, 119.
 Feldstein and Weil (1909–10). *Proc. Soc. Exp. Biol. Med.* **7**, 61
 Fine (1930). *Biochem. J.* **24**, 1282.
 Folin and Denis (1911). *J. Biol. Chem.* **11**, 527.
 Fujimoto (1918). *J. Immunol.* **3**, 51.
 Gränacher (1929). *Handbuch der biologischen Arbeitsmethoden*: I, II, **4**, 638.
 Gross (1908). *Arch. exp. Path. Pharm.* **58**, 157.
 Hahn (1897). *Klin. Woch.* 499.
 Haldane (1930). *Enzymes* (Longmans, London).
 Hamill (1906). *J. Physiol.* **33**, 476.
 Hanson (1918). *J. Immunol.* **3**, 139.
 Harris (1923). *Proc. Roy. Soc. Lond. B* **94**, 426.
 Hata (1909). *Biochem. Z.* **17**, 156.
 Hedin (1906, 1). *Biochem. J.* **1**, 474.
 — (1906, 2). *Biochem. J.* **1**, 484.
 Hildebrandt (1893). *Virchow's Arch.* **184**, 325.
 Hussey and Northrop (1923). *J. Gen. Physiol.* **5**, 335.
 Jobling and Strouse (1912). *J. Exp. Med.* **16**, 3.
 — and Petersen (1914, 1). *J. Exp. Med.* **19**, 3.
 — (1914, 2). *J. Exp. Med.* **19**, 4.
 — (1914, 3). *J. Exp. Med.* **19**, 5.
 — (1914, 4). *J. Exp. Med.* **20**, 5.
 — and Eggstein (1915, 1). *J. Exp. Med.* **22**, 129.
 — (1915, 2). *J. Exp. Med.* **22**, 141.
 Jochman and Kantarovitch (1908). *Munch. med. Woch.* **55**, 728.
 Landsteiner (1900). *Zentr. Bakt. Par.* **1**, 27, 357.
 Langenskiöld (1907). *Arch. Int. Physiol.* **5**, 297.
 — (1914). *Scand. Arch. Physiol.* **31**, 1.
 Levene and Stookey (1903). *J. Med. Res.* **10**, 217.
 Lin, Wu and Chen (1928). *Chinese J. Physiol.* **2**, 131.
 Luers and Albrecht (1926). *Fermentforsch.* **8**, 52.
 Meyer (1909). *Klin. Woch.* 23.
 Morgenroth (1899). *Zentr. Bakt. Par.* **1**, 26, 349.
 Opie (1905). *J. Exp. Med.* **7**, 316.
 Oppenheimer (1913). *Die Fermente und ihre Wirkungen*, **1**, 478.
 — and Aron (1903). *Hofmeister's Beiträge*, **4**, 297.
 Plimmer (1908). *Chemical constitution of the proteins* (Longmans, London).
 Pozerski (1909). *Ann. Inst. Pasteur* **23**, 205.
 Robertson (1918). *J. Immunol.* **3**, 131.
 Rosenthal (1910). *Folia Serol.* **6**, 285.
 von Schoenbom (1910). *Z. Biol.* **53**, 386.
 Schwartz (1909). *Wien. klin. Woch.* **22**, 1151.
 Slovzov and Zenophentova (1919). *Russ. Physiol. J.* **2**, 261.
 Sugimoto (1913). *Arch. Exp. Path. Pharm.* **74**, 14.
 Teale and Bach (1920). *Proc. Roy. Soc. Path.* **13**, 5.
 Thaysen (1915). *Biochem. J.* **9**, 110.
 Vernon (1904). *J. Physiol.* **31**, 346.
 Walters (1912). *J. Biol. Chem.* **12**, 43.
 Weil (1910). *Amer. J. Med. Sci.* **139**, 714.
 Weinland (1903). *Z. Biol.* **44**, 1.
 Wells (1925). *Chemical pathology* (Saunders, Philadelphia).
 Wigglesworth (1928). *Biochem. J.* **22**, 150.
 Young (1918). *Biochem. J.* **12**, 499.