INFLUENCE OF SERUM ON ENZYMES

WITH SPECIAL REFERENCE TO ITS ACTION ON TRYPSIN.

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

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1. INTRODUCTION.

The influence of serum on enzymes may be considered kinetically as falling into one of two categories: either the enzyme is influenced by virtue of serum also containing such enzyme - 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 in itself constitutes incomplete evidence of the nature of its 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, ereptase, 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 (according to Luers & Albrecht, 1926, whose findings I have not attempted to confirm). Lipase I have also found in man, sheep, cow, rat and guinea-pig. Maltase was not found in guinea-pig serum (Blacklock, Gordon & 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 intra-peritoneal 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.

When serum influences an enzyme without itself containing this enzyme, then the influence is either one of true acceleration or of inhibition. 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 by 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 partly is 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". In a recent study of "enzyme-accelerators in serum,"however, I have shown (1930) that in the case of invertase the influence exerted by serum was entirely a function of p_H, the serum acting as an accelerator over certain ranges of p_H, and as an inhibitor over others.

The one certain conclusion to be drawn from these experiments is that as far as invertase is concerned serum contains neither accelerator nor anti-enzyme, but simply exerts a variable influence not due to specific substances present, but rather to specific physical conditions, such as those of p_H, whose variations determine the final behaviour of the serum.

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 what holds good for influence of serum on invertase holds good for its influence on most other enzymes also: that is to say, true acceleration or inhibition by serum is not due to specific accelerators or anti-enzymes, but to various physical conditions such as p_H. P_H however is not the only factor of importance: in the mechanism of enzyme-inhibition the adsorption of the enzyme by serum may play the most important part, as 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: possible p_H may not so completely determine the action of serum on lipase as on invertase.

In the ensuing part of this paper I have confined myself to an examination of the present state of our knowledge of serum as an inhibitor of enzymes. I have however given special consideration to serum antitrypsin, and have found it necessary, before arriving at a conclusion as to its nature, to consider in some detail its properties, its relation to other organic antitryptic bodies, and its variations in clinical and experimental conditions. This detailed examination of serum antitrypsin is justified because of its importance clinically, because of the inaccurate ideas still held generally as to its nature - the result of insufficient

acquaintance with its properties: and finally, because I have been able to throw new light, to however modest an extent, on its mechanism.

2. 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, and adopting the language of immunology termed these hypothetical substances anti-emulsin, antitrypsin, and antirennin respectively. The assumption underlying this terminology - namely that serum contains substances capable of specifically neutralising enzymes in a manner analogous to the neutralisation of toxins by antitoxins - has lead to a great mass of research which in the main has failed to justify the assumption. Very early, 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, while opinion differed as to the power of serum to increase its inhibition of an enzyme following injections of the enzyme 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.

So reluctant have certain workers been to relinquish the antibody theory of enzyme-inhibition by serum that an endeavour has been made to explain the limited rise in trypsin-inhibition (antitrypsin) following trypsin injections by suggesting that the accumulation of antitrypsin rapidly produces an anti-antitrypsin as a defence against paralysis of protein metabolism. Although such an explanation is theoretically adequate, there is actually as little evidence of the existence of this alleged substance anti-antitrypsin as of the existence of the chemical entity "antitrypsin" whose limitations it was invented to explain.

At various times eminent physiologists have endeavoured to expose the fallacy underlying the current conception of anti-enzymes. Weil (1910) pointed out that while serum can inhibit the haemolytic action of saponin, it does not necessarily follow that serum contains an anti-saponin. Bayliss (1919) in his characteristic manner pointed out that if a substance which inhibits an enzyme is entitled to be called an anti-enzyme, then NaOH is anti-pepsin.

3. THE ACTION OF SERUM ON ENZYMES IS LARGELY A FUNCTION OF pH

As Wells pointed out (1925), much work on enzymes has been utterly invalidated by the failure of the worker to regulate the pH of his enzyme mixtures. As is well recognised, enzymes are very sensitive to pH changes, and although they may retain activity over a fairly wide range of pH, quantitative studies are quite valueless unless the pH is fixed throughout the investigations.

The earlier workers seem to have quite ignored this principle. 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 pH to produce a corresponding fall in activity of the emulsin.

Bensley & Harvey (1912) showed that the absence of free HCl in the cells of the gastric mucose formed the best protection against the secreted pepsin: in other words, the pH of the cell substance was in itself a quite adequate "antipepsin". Dragsted & 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 not the presence of "anti-pepsin" in the exposed organs, but simply the buffer action of the blood and the absorption of the HCl and pepsin into the blood stream. Thaysen (1915) found that "antirennet" could also be accounted for by the change in pH when serum is added to a rennet mixture: he also found that adsorption of rennet by the serum proteins played an additional part.

The danger of postulating a definite chemical entity to account for a given property of serum is uniquely illustrated in the results of a study I recently made (1930) of the properties of the invertase-accelerator of serum reported by Blacklock, Gordon & Fine (1930). I examined the behaviour of guinea-pig serum to invertase solutions adjusted to different values of pH, and found that while serum accelerated invertase at the optimum pH of the latter (3 & 4), at pH 5 the action was nil, and at pH 6 there was a definite inhibition: at pH 7 & 8 serum accelerated again.

This behaviour of serum might be explained by saying that the invertase-accelerator has an inhibitory action at certain values of pH. It would however be equally logical to infer that an inhibitor was present which could accelerate at certain values of pH: sheep serum, in fact, behaves more as an inhibitor than as an accelerator. It might even be inferred that serum contains both accelerator and inhibitor, each acting only at certain pH values.

All these hypotheses, however, are essentially unsatisfactory, because they involve the assumption that influence of serum on an enzyme is necessarily due to a definite chemical substance - an invidious assumption for which no evidence has been offered by the army of workers reporting serum anti-enzymes to almost every enzyme known.

When the importance of p_H in relation to enzymes is fully recognised, it will be found that in many instances the mechanism whereby the body reacts to an excessive formation of any enzyme is not by the development of a hypothetical anti-enzyme but by the local development of a p_H unfavourable to the enzyme in question.

This mechanism undoubtedly accounts for the absence of peptic digestion of the stomach wall: no serum or tissue "anti-pepsin" need be invoked.

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, which I failed to recover from the urine during experiments in which invertase was injected in doses so large that it was found in serum: adsorption by tissue or serum proteins in the case of trypsin.

<u>In vivo and in vitro action.</u> The evidence available clearly shows that each enzyme is dealt with in an individual manner by the animal body, one or more factors being employed to check excessive enzyme action.

A careful examination of the evidence has left little doubt in my mind that the conception of "anti-enzymes" as chemical antibodies should be replaced by that of "an enzyme-regulating mechanism" in which excretion, adsorption, p_H

regulation and actual destruction play a part.

A distinction must be clearly made between the action of serum on an enzyme in vitro and the fate of the enzyme when injected in the body: in the latter case, although the action of serum may be the same as it is in vitro, the fate of the enzyme is not necessarily related to this action, since in the body there are other influences besides that of serum in play.

4. ON "ANTI-ENZYMES" REPORTED IN SERUM.

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.

This action was stated to be either normally present, or if not, could be elicited by injections of the corresponding enzymes.

The following table, showing the optimum pH of the above enzymes, is of considerable importance in interpreting the phenomena recorded.

Enzyme	Optimum pH
Trypsin (pancreatic)	8.3
Pepsin (gastric)	1.4
Rennin	5
Papain	5
Autolytic	4.5
Lipase (duodenal)	8.5
" (serum)	8 - 8.6
Amylase (pancreatic)	7
Invertase (yeast)	4.2
Laccase	6.7
Catalase	7
Tyrosinase (potato)	6.5 - 8
Emulsin	4.4
Urease	7

It would follow almost a priori that the addition of serum which has a pH of 7.45 would greatly reduce the activity of active but unbuffered solutions of pepsin, rennin, papain, autolytic enzymes, emulsin, and invertase. Even invertase, which is accelerated by serum when buffered at its optimum pH, is totally inhibited, as I more recently observed, when serum is added to

an active but unbuffered solution of invertase. In fact, a worker who did not protect his enzyme mixtures against change of pH (by using buffer solutions) would inevitably find that most enzymes were inhibited by serum, provided the optimum pH of the enzyme were not the pH of the serum.

The generally admitted discouraging effect that serum exerts on unbuffered or insufficiently buffered enzymes is in some cases to be sufficiently explained by the pH changes produced by the serum. In the case of trypsin however this factor does not appear to play any part. The influence of serum on trypsin stands apart from its action on most other enzymes, since, as I will show presently, the inhibition of trypsin by serum is 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 pH effect and partly an adsorption effect.

I will now proceed to examine briefly the evidence hitherto adduced of the existence in serum of substances inhibiting the enzymes above mentioned.

(1) Inhibition of pepsin. The evidence already submitted seems to clearly show that the inhibiting action of serum is here entirely a function of pH.

(2) Inhibition of rennin. This was first shown by Morgenroth (1899) and later by Czapek. The former claims that anti-rennin is greatly increased following rennin injections. Eisner (1909) did not find serum to have an inhibitory action on rennin, but he appears to have been equally unsuccessful with pepsin. Korschun (1902) suggested that rennin is inhibited by the formation in serum of "zymoid", i.e. a substance which resembles the enzyme in being able to combine with the substrate, but is unable to hydrolyse it. Certain

enzymes (e.g. trypsin) when heated to certain temperatures become zymoids. Bayliss (1904) and Bearn & Cramer (1907) showed this for trypsin. If rennin injections were followed by the formation in serum of rennin zymoid, the latter would produce the effect of an "antirennin" by "deviating" the caseinogen in any mixture containing rennet, so that there would subsequently be no available substrate for rennin to act upon. Korschun's theory, however plausible, has not been proved experimentally. Fuld & Spiro (1900) found rennin inhibition in horse serum associated with the pseudo-globulin fraction: This observation justifies the conclusion of Thaysen (1915), who attributed rennet-inhibition by serum partly to adsorption of rennin by serum proteins: partly also to pH change induced on adding serum to a rennin mixture. Hedin (1911) does not consider the inhibition of rennin by serum to be due to a true antibody. He found that injections of rennin did produce a rise in rennin-inhibition by serum, but a greater rise was obtained following injections of zymogen added to an actively rennin-inhibiting serum. If rennin injections produced antibodies to rennin, it is improbable that the injection of those antibodies would produce more "antirennin" than would the rennin itself. Thaysen's conclusions seem to most adequately account for the behaviour of serum to rennin.

(3) Inhibition of autolysis. Baer & Loeb (1905) and H.G.Wells (1906) early showed that serum retarded autolytic changes. Although autolysis is a well-defined phenomenon, there does not seem any advantage in accounting for it by a special set of enzymes solely concerned in it. The action of serum on autolysing tissue is merely the sum of its actions on the individual enzymes present in the tissues - notably proteolytic. It has not been shown, for example, that serum discourages any amylolytic or lipolytic changes incidental to autolysis, or that in fact any special action takes place beyond the normal protease-inhibiting action of serum, which is discussed later.

(4) Inhibition of emulsin. This was the first phenomenon of enzyme-inhi ition by serum to be reported (Hildebrandt, 1893). Ohta (1913) found that this inhibition was increased following emulsin injections. Abderhalden and Wertheimer (1922) however, in an important series of experiments dealing with the antigenic properties of a number of enzymes, failed to confirm this.

The optimum pH of emulsin (4.4) renders it probably that the action of serum is essentially due to its pH - a view taken by Bayliss (1912) after a critical examination of the work of Hildebrandt.

(5) Inhibition of other enzymes. Although amylase, lipase, and catalase are known to occur constantly in most sera, they fluctuate in value, and these fluctuations have been considered by some workers as evidence of the existence of corresponding anti-enzymes, which would then be considered to vary inversely with the enzyme: such a view has little following, and has even less evidence to support it.

It is well known clinically that a rise in serum amylase is followed by a great increase in urinary amylase, the fluctuations of which can be readily correlated with the variations of serum amylase.

It must be mentioned however that rabbit serum, which, according to Luers & Albrecht (1926) contains no amylase, has been rendered anti-amylolytic by these workers by means of amylase injections.

On the other hand, Abderhalden & Wertheimer (1922) failed to obtain anti-enzymatic serum following injections of amylase, lipase, catalase emulsin, invertase, phenolase, urease, tyrosinase, laccase, and fibrin ferment.

Bach & Engelhardt (1923) have described the inhibition of laccase by serum. Weil (1910) showed that papain is inhibited by serum as readily as is trypsin.

Among others, Blacklock, Gordon & Fine (1930) observed the inhibition of tyrosinase by serum: They failed to increase

the inhibition by tyrosinase injections, or to produce any significant variation in serum amylase by repeated injections of that enzyme. In no instance has a body been isolated from serum to account for the inhibition of an enzyme. Most workers seemed to have automatically inferred the presence of an "anti-enzyme" on finding that the addition of serum retarded an enzyme. The result is that a system of nomenclature has grown up around the action of serum on enzymes which is unfortunate in that it implies quite unproved assumptions as to the nature of this action. Such terms as anti-rennin, anti-tyrosinase, etc. - which are now common currency - imply two unproved and inaccurate ideas: namely that definite substances are present, and that they are of the nature of specific antibodies of the Ehrlich type. Although therefore these terms are misleading if interpreted immunologically, and although their use might with advantage be dispensed with generally, yet as a shorthand description they are sometimes useful, particularly in the case of so well established a term as serum antitrypsin. In using the latter term, as I shall have occasion to do - faute de mieux - none of the unwarranted implications referred to are intended.

5. ORGANIC TRYPSIN INHIBITORS.

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. It was soon found that very many substances possessing diverse properties inhibited trypsin, and if Hahn's example were to be followed, it would become necessary to postulate not a few different antitrypsins.

The following is a list of substances of organic nature, which have been reported to inhibit trypsin.

(1) Serum (Hahn, 1897). The properties of serum in relation to its inhibition of trypsin will be discussed later.

(2) Worm Extract. Weinland (1903) and Dastre & Stassano (1903) reported that extract of intestinal worms effectively inhibited tryptic action. Dastre (1903) first made the interesting observation that in proteolytic mixtures to which worm extract is added, the trypsin gradually recovers and finally acts as if quite unimpaired. He inferred that the inhibiting principle of worm extract is gradually destroyed. This behaviour of worm extract is identical with that of serum.

Hamill (1906) more fully worked out the properties of worm extract in relation to its inhibition of trypsin. He found that

a) boiling the extract in neutral or acid solution had no effect on its power to inhibit trypsin.

b) boiling in alkaline solution destroyed this action.

c) on dialysis the inhibiting property could be recovered from the dialysate only. The inhibiting principle appeared to be a crystalloid.

d) the inhibiting principle was soluble in alcohol of less than 85% strength only.

From these facts it is quite evident that the causes of trypsin-inhibition by worm extract and by serum are not identical.

Boiling and dialysis have different effects on serum, although the Dastre phenomenon is common to both.

Hamill's work suggests that a definite crystalloid substance is present as the inhibiting principle.

Burge (1915) believes that intestinal worms are protected from the host's trypsin by absorbing and oxidising the latter in their own tissues.

The mechanism of worm extract however does not appear to be one of destruction but rather one of paralysis of trypsin.

(3) Egg-white. Vernon (1904) first showed that egg-white was antitryptic, and this has been repeatedly confirmed, by Sugimoto (1913) and others. Heating for ½ hr. at 70°C.

was found destroyed the antitryptic property of egg-white.

By comparing the digestion of heated and unheated egg-white by trypsin, Bayliss (1923) found that egg-white showed the Dastre phenomenon, i.e., although little digestion took place in the first few hours in the digesting mixture containing unheated egg-white, this mixture showed a gradually increasing activity of its trypsin so that after 48 hrs. the amount of digestion was just as great as in the mixture containing heated egg-white. I have frequently elicited this phenomenon for egg-white, and in fig. 1 it is illustrated in a curve based on one of my experiments.

Considering the chemical and physical similarities between egg-white and serum, as well as the effect of heat and the presence of the Dastre phenomenon, it is highly probable that the cause of trypsin-inhibition is the same in both substances.

(4) Bacteria. Czapek (1903) first observed that bacteria resisted tryptic digestion, and Jobling (1914)[6] investigating the mechanism of this resistance found that boiling did not affect it in the case of gram positive organisms, and only slightly impaired it in gram negative ones. He also showed that bacteria lose this resistance when extracted with chloroform, and came to the conclusion that the phenomenon was due to a lipoidal envelope around the bacteria.

(5) Yeast. Buchner & Haehn (1910) found that the endotryptase of yeast did not hydrolyse certain substrates, such as gelatin and casein: according to these workers, this inaction was due to "yeast antitrypsin" a substance which adsorbed or combined with the substrates, and so rendered them unavailable to the enzyme. This mechanism, if true, is virtually a zymoid one, and not analogous to that of inhibition by serum.

(6) Animal tissues and secretions. Levene & Stookey (1903) found that body tissues generally were antitryptic. Blum & Fuld (1906) found antitryptic properties in the cells of the gastric mucosa: Langenskiold (1914) confirmed this, having previously

(1907) found similar properties in mucous secretion. Czapek (1903) found that erythrocytes were antitryptic.

Dochez (1909) reported the absence of antitryptic properties in cerebrospinal fluid and the fluids of serous cavities generally, except when these fluids contained inflammatory exudate. Opie (1905) had previously noted the antitryptic action of inflammatory exudates.

Von Schoenbom (1910) found urine antitryptic under certain pathological conditions, but Fujimoto (1918) found this property in normal urine. Normal urine, however, loses its action if heated at 100°C for ½ hr. but not at a lower temperature (Fujimoto), and this stability sharply differentiates the antitryptic action of urine from that of serum. It is probably however that tissues as well as exudates containing plasma act in the same way as serum.

(7) Unsaturated fatty acids. Jobling (1914) first showed that the caseous material from tubercular tissue was highly antitryptic and adduced evidence to show that this was due to the unsaturated fatty acid content of the material. In a series of investigations ("Studies on Ferment action" 1912-15) into the antitryptic action of serum he showed many similarities between it and the action of unsaturated lipoids, and concluded that such lipoids were responsible for the behaviour of serum to trypsin. Jobling's theory, which has received much prominence in America, where it is regarded by many as the true explanation of the action of serum, is quite inconsistent with the essential properties of serum as an antitryptic substance. The theory will be discussed more fully later on, but I would observe here that to prove two substances identical it is essential to show that all and not merely some of their properties are identical. After showing that unsaturated fatty acids ceased to be antitryptic after saturation Jobling found that both serum and unsaturated fatty acids lost their antitryptic properties on treatment

with iodine or potassium iodide.

He inferred that the unsaturated lipoids of serum became saturated with a consequent loss of antitryptic action.

Although it is admitted that the antitryptic action of serum and unsaturated fatty acids resemble in a number of ways, there are several divergencies not to be ignored - outstandingly two:

(a) The action of serum is destroyed.by heating for ½ hr. at 70°C: unsaturated fatty acids resist such treatment.

(b) Trypsin gradually recovers from the inhibiting action of serum in a manner similar to that of egg-white or worm extract: the kinetics of inhibition by unsaturated fatty acids is totally different, as I will show.later.

(8) Lecithin. Schwartz (1909) showed that Lecithin could restore the antitryptic action of serum lost by extracting with ether.

6. THE PROPERTIES OF SERUM ANTITRYPSIN.

(1) Methods employed in estimating antitryptic action. At least six different methods have been used by various workers to determine antitryptic action: They have in the main given concordant results.

(a) Fuld & Gross method. This was one of the first employed (1907) for the purpose. It consists of adding to a number of tubes (about six) equal quantities of casein and of the serum tested. Increasing quantities of a standard solution of trypsin are then added to the tubes which are shaken and incubated for a given time (a few hours). At the end of the period of incubation equal amounts of dilute acetic acid are added to all tubes. A coagulum will appear in all tubes containing imcompletely digested casein. The tube without a coagulum containing the least amount of trypsin is noted, and the antitrypsin index of the serum is

expressed interms of the amount of trypsin in that tube: the minimum amount of trypsin being so chosen that it will just digest the casein in the given time.

This method has the disadvantage of being rather cumbersome - involving the use of a large number of tubes: it is obviously not very sensitive.

(b) Method of Folin & Dennis (1911). Jobling soon gave up the Fuld & Gross method for this one, which, like those following, is more adapted to refined quantitative measurement of antitryptic activity. Here two tubes containing equal quantities of trypsin and casein solution are incubated: to the first is added a certain volume of serum, and to the second an equal volume of water. After a given period the incoagulable nitrogen is estimated in the two tubes, and the difference between the two values divided by the second value - expressed as a percentage - gives the antitryptic index of the serum.

Young (1918) also used this method in his researches.

(c) Viscometric method. This was first used by Feldstein & Weil (1908), and more recently by O'Donovan & Davison (1927). It is based on the fact that when gelatin is hydrolysed by trypsin the viscosity of the mixture falls in proportion to the amount of tryptic action. Although Weil writes highly of this method, the application of physical changes to the measurement of chemical changes is not very satisfactory. Using ~~this~~ a viscometric method, O'Donovan & Davison failed to demonstrate the presence of amylase in human serum, although such demonstration is unequivocal by any chemical method.

(d) Electro conductivity method. Bayliss strongly advocated this method, which is based on the increase in the electrical conductivity of a protein solution when the protein molecule is split up into amino acids.

(e) Refractometric method. Brailsford Robertson (1918)

first used this method in his study of the kinetics of antitrypsin. It is based on the increase in refractive index of a casein solution in proportion to its conversion into amino acids. The measurements made are those of refractive index.

(f) Sorensen's titration. All the foregoing methods 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 - then 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 9% 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 (pH 8) and serum: in the first, the serum had been heated at 70°C for ½ hr. to destroy antitryptic action: in the second the serum was untreated.

An aliquot portion of each tube was titrated by Sorensen'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 = A, and the rise in the second = B, then the ratio $\frac{A - B}{A}$ expressed as a percentage/or as a fraction of unity adequately measures the antitryptic activity.

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

(2) The effect of Dialysis. Cathcart (1904) showed that on

dialysing serum the residue only is antitryptic. This was confirmed by Meyer (1909), Stavraky (1914), Fujimoto (1918), and others.

The reverse holds true for worm extract, the dialysate of which is antitryptic.

(3) Effect of drying. This does not affect the antitryptic properties of serum, as was early shown by Chapman (1905). Dried serum, it was further shown by Cobliner (1910) did not lose its antitryptic action when shaken up with chloroform, although undried serum is rendered much less antitryptic by such treatment.

(4) Effect of shaking. Fujimoto (1918) found that shaking did not diminish the antitryptic action of serum.

(5) The association of the various protein fractions with antitryptic action. Landsteiner (1900) was the first to observe that the precipitate formed on full stauration of serum with ammonium sulphate is highly antitryptic, and concluded the albumin fraction was the seat of antitryptic action in serum. This was confirmed by Cathcart (1904), Opie & Barker (1907) and Muller (1908). Other workers, while agreeing that the albumin fraction was the principle source of antitryptic action, also found the globulin fraction to be antitryptic: these are the findings of Doblin (1909)(2), Kammerer (1911) Kammerer & Anbry (1913) and Fujimoto (1918). Glassner (1903) found antitrypsin to reside in the euglobulin.

(6) The effect of heat. That heating at 70°C for ½ hr. will destroy the antitryptic action of serum has been well established since the work of Vandevelde (1909), Meyer (1909)(2), Jobling (1914) and others. Fujimoto (1918) has shown that there is a slight variation in the temperatures at which the sera of different animals must be exposed to destroy antitryptic action.

This influence of heat is characteristic of serum, and is not shown by either worm extract or by unsaturated fatty acids. Jobling (1914) attempts to overcome this objection to his theory by showing that although unsaturated fatty acids do not lose their antitryptic action on heating, they do lose this property when heated together with serum, at 70o C for ½ hr. Nevertheless,

as he admits, the iodine value of the mixture does not fall on heating, and therefore the loss of antitryptic action is not due to saturation of the fatty acids. Further, he states that the unheated mixture of serum and unsaturated fatty acid is less antitryptic than either of its constituents. Hence the loss of antitryptic action is not necessarily produced by heat, being initiated in the cold and merely accelerated by heat. Jobling's experiments therefore do not reconcile the action of heat on unsaturated fatty acids with that on serum.

(7). The action of chloroform and ether. Delezenne & Pozerski (1903) first showed that serum loses much of its antitryptic action when shaken up with chloroform. Cobliner (1910) noting that there is no such loss when dried serum is used concluded that the lipoids of serum are not the source of anti-tryptic action.

Kirchheim (1913) and Jobling (1914)[3] confirmed the observations of Delezenne & Pozerski.

Jobling was unable to entirely destroy the antitryptic action of serum by shaking up with chloroform and incubating for some time.

In experiments carried out with serum and egg-white I obtained in both cases marked reduction - but not entire suppression - of antitryptic action (fig. 2).

Schwartz (1909), Sugimoto (1913), and Jobling (1914)[3] found that ether acted in a similar manner to chloroform.

(8) The action of oxidising agents. Jobling (1914)[3] showed that when iodine, potassium iodide or H_2O_2 acted on serum, the latter lost in antitryptic activity. He showed that unsaturated lipoids were affected in the same way, and used this observation to argue that unsaturated lipoids were the responsible substances in serum.

(9) The action of colloids. Jobling showed that serum ceased to be antitryptic after shaking up with kaolin,

starch, agar, or a bacterial emulsinn. The mechanism of this action, it is generally agreed, is one of adsorption of certain constituents of serum by the colloid added. According to Jobling these constituents are the lipoids.

It is due to Jobling (1915) that the Abderhalden reaction has been shown to be due to the same principle of adsorption of the trypsin-inhibiting substance in serum by the introduced placenta. Very few workers now hold the original view of Abderhalden that this reaction is due to a specific enzyme.

Jobling further found that the above-mentioned colloids acted in a similar manner in vivo. An injection of kaolin for example, was followed by a great fall in serum antitrypsin together with symptoms closely resembling anaphylaxis. He was able to throw some light on the latter condition by such experiments, and formed the opinion that anaphylaxis was brought about by the adsorption of the circulating serum antitrypsin (lipoids, in his view) by the second dose of protein: "in this way the increased trypsin provoked by the first dose of protein was free to act on the circulating blood proteins and produce highly toxic products of hydrolysis." [Jobling]

(10) The Dastre phenomenon. I am applying this name to the phenomenon of an enzyme recovering in time from the action of an inhibiting substance, since it was Dastre who first showed (1903) that worm extract had an evanescent inhibiting action on trypsin. Egg-white also presents this phenomenon, as was shown by Bayliss (1923), and as I have repeatedly demonstrated myself.

The fact that serum behaved in a similar manner to egg-white was first suggested by the work of Kirchheim (1913) who demonstrated that serum merely prolonged the action of trypsin. Previously, (Bayliss & Starling, 1905, and Hedin, 1909) it had been shown that the action of serum on trypsin was not one of destruction, but one of paralysis.

using the Sorensen 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 effect with serum, 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
Trypsin	.2 cc	.2 cc
Serum	.1 cc	.1 cc (inactivated)
Buffer pH 8	.7 cc	.7 cc

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

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

(c) The serum in Tube 2 was inactivated by heating at 70oC for ½ hr. 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, .1 cc was immediately withdrawn for titration of acidity with $\frac{N}{100}$ NaOH A layer of toluol was added to the tubes before incubating.

The method of titration was that employed by Wigglesworth (1927) in his investigation on cockroach protease.

The pipetted fluid (.1 cc) was added to 5 cc distilled water in a test tube of standard size: 5 drops phenolphthalein solution (.05% in 50% ethyl alcohol)

were added, and $\frac{N}{100}$ NaOH added from a 2 cc microburette, graduated in hundredths of a cc. The alkali was added until a pink colour developed identical with that of a tube (of similar make) containing 5 ccs buffer solution of pH 9 plus 5 drops .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 .5 cc 50% neutralised formalin (pH 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 .1 cc of the incubated mixtures were carried out from time to time, and in this way curves of digestion were plotted.

Fig. 3 is the curve of a typical experiment, showing how the initial inhibition of trypsin is finally replaced by accelerated digestion more active than in the tube where trypsin was not initially inhibited. An analysis of fig. 3, in the form of separate curves of free and of formol acid formation (figs. 4 & 5) throw 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. 6, 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. 7 & 8, 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. 5.

Figs. 9, 10, and 11 are the total, free and, formol acid curves of digesting mixtures containing

10 gelatin	.5 cc
trypsin 50% neutral	.5 cc
Buffer p_H 8.5	.5 cc
Serum	.2 cc
Saline .88	.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. 3 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. 6 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 only to the formol acid formation; the extent of formation of free acid with unheated serum never exceeds that with heated serum although, provided digestion is sufficiently prolonged and the trypsin is fairly active, the same amount of free acid is finally produced in the two tubes: fig. 7 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 free and formol acid formation by trypsin, and subsequently accelerating formol acid formation only.

How is this behaviour of serum to be explained? The explanation would prove a difficult matter if it be assumed that pancreatic trypsin, which is the basis of the commercial preparation used (Liquor trypsin Co.) is a pure enzyme. There is much evidence, however, in favour of the view that the trypsin of pancreatic secretion is essentially a mixture of two enzymes- tryptase breaking proteins down to polypeptides and increasing the free acidity of the digest, and ereptase, 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 tryptase portion of it. The inhibition of tryptase will, by preventing the liberation from the proteins of a suitable substrate for ereptase (i.e. polypeptides), produce an apparent inhibition of ereptase. There does not, however, appear to be any necessity for assuming that the initial depression of formol acid formation is due to an anti-ereptase action of serum rather than to a shortage of substrate consequent on tryptase inhibition. On recovering from the inhibition, tryptase will produce polypeptides on which ereptase will act. 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 an ereptase or an ereptase-accelerator. The most likely explanation is the existence of a serum ereptase, which has been reported by various workers, including Jobling (1912) who reports that serum ereptase is destroyed by heating at 70°C and (1915)[4] that "serum ereptase is not influenced by antitrypsin". Hence, when tryptase has recovered from the inhibition by serum, the ereptase of the unheated serum will reinforce the ereptase of the

ereptase 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 ereptase acts alone, the serum ereptase 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 anti-tryptase factor, and secondly, ereptase.

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 against the tryptase portion only (the portion forming free acid) that serum directs its inhibition, and not against the ereptase moitie (the portion forming formol acid) - by showing this, it directs serious criticism against those methods of measuring "antitrypsin" which estimate toegther the products of both tryptase and of ereptase action. The methods thus open to criticism include those of Bayliss, (electrometric), and Robertson (refractometric) though not of Folin (incoagulated N). The error involved will depend on the period of incubation used as well as the strength of serum ereptase present: the extent of the error will be appreciated in the following example:

Course of digestion of heated and unheated sera.

	After 8 hrs. incub.			27½ hrs.			95		
	F	For	T	F	For	T	F	For	T
Unheated ser.	.03	.06	.09	.13	.19	.32	.23	.41	.64
heated sera.	.22	.10	.32	.27	.17	.44	.27	.28	.55

F = Free acid.
For = Formol acid.
T = total acid

Figures are cc $\frac{N}{100}$ acid in .1 cc 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")		
	In 8 hrs.	In 27½ hrs.	In 95 hrs.
From free acid figs.	86	52	15
" total " "	72	27	- 16 *

*(i.e. 16% acceleration)

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

(11) The kinetics of serum antitrypsin.

(a) The kinetics of recovery.

The Dastre phenomenon was by no means elicited by all proteolytic mixtures containing serum: it 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 in relation to the serum the serum failed to produce any appreciable inhibition. (2) When the concentration of serum was greatly in excess in relation to 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. 12. The curves were 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, pH8. 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

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

which expresses the degree of inhibition by the unheated serum. The total acid figures were taken because in this case the value of serum ereptase 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. 12).

Hence serum can influence trypsin in essentially three ways according to the relative concentration of serum and trypsin: weak trypsin is permanently inhibited: trypsin of intermediate strength is temporarily inhibited: strong trypsin is probably not inhibited.

From the continuous nature of the curves it would appear 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, who frankly regarded serum as containing an antibody to trypsin, made an important contribution to the study of the kinetics of his antibody (1906). He found that (1) a small amount of serum had a relatively greater effect on trypsin than a large amount, (2) it was impossible to completely 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. 13: this curve shows that excess of serum has no effect on the residual activity of trypsin.

Hedin thus shows that a mixture of trypsin and excess serum possesses both antitryptic and tryptic action, i.e.

trypsin and antitrypsin can co-exist when the latter is in excess, each exerting a definite action on added substrate or enzyme respectively in spite of the other. In discussing the adequacy of Ehrlich's toxin-antitoxin mechanism to explain the trypsin-antitrypsin reaction Hedin states "the fact that on further addition of antibody the fluid may contain free trypsin and free antibody at the same time this theory does not account for, unless one assumes that trypsin is made up of different constituents" (page 482).

It is evident from this statement that Hedin considered the possibility of trypsin being composed of at least two enzymes, one of which was uninfluenced by serum. My own experiments showing that serum antagonises tryptase, but not ereptase, peculiarly bear out Hedin's suggestion.

I have not however been able to confirm the common findings of Hedin & Young that excess serum will not entirely inhibit a given amount of trypsin. Fig. 12 shows that 4% and 2% trypsin were completely inhibited by 10% 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 serum. Hedin and Young combined fairly concentrated trypsin in constant amount with increasing amounts of serum: hence all flasks contained a fairly active ereptase together with a large excess of casein added as substrate. Casein, it is stated by Jobling (1915) (p. 152) is an exception to the rule that <u>ereptase does not split proteins</u>: furthermore, <u>ereptase is uninfluenced by the anti-ferment</u> (Jobling).

(c) <u>Analogy with charcoal</u>. To Hedin 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 filtered trypsin solution after treatment with excess charcoal was quite inactive, yet an unfiltered trypsin-charcoal mixture incubated with casein was definitely and invariably active, though most of the trypsin remained inhibited. Hedin concluded that in the presence of substrate subsequently added, charcoal did not retain all the trypsin adsorbed, a small portion of it being taken up by the casein. In a similar manner might be explained the impossibility of totally inhibiting a solution of trypsin by means of serum, although it is impossible to actually demonstrate the similarity since trypsin cannot be removed with its inhibitor in a trypsin-serum mixture, in the simple manner possible with charcoal.

(d) Robertson's formula. Brailsford 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 dilute. By this method Hanson (1918) was able to show that injections of trypsin had no influence on the protein quotient of serum $\left\{\frac{\text{globulin}}{\text{albumin}}\right\}$, 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 would follow, 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. 14 illustrates this result, and fig. 15 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 trypsins, 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: Young believes that much work on antitrypsin must be invalidated by this variability of trypsins of equal activity but different source when incubated with similar sera

THE INFLUENCE ON SERUM OF INJECTIONS OF TRYPSIN.

(1) The controversy. There is a sharp division of opinion as to whether injections of trypsin will give rise to an increased power of serum to inhibit trypsin.

Achalme (1901) was the first to investigate the matter, and found a definite increase in the antitryptic power of serum following injections of trypsin. In this he was confirmed by Von Berman & Jochman & Kantarovitch (1908) Bamberg (1908), Meyer (1909), Jobling, Petersen & Eggstein (1915), Hansen (1918), and Blacklock, Gordon & Fine (1930). On the other hand negative results were obtained by Landsteiner (1907), Doblin (1909), Rosenthal (1910), Weil (1910) and Young (1918). Pozerski (1909) also found no response to injections of papain.

There is similar disagreement as to the influence of injections of tissue substances other than prepared trypsin. Thus Miller (1909) found that the injection of a mass of leucocytes (containing leuko-protease) led to a rise in antitryptic power of serum, though the rise was preceded by a fall to below normal. Braunstein & Kepinov (1910) confirmed this result using various tissue emulsions. Halpern (1911) injected pancreatic tissue from a dog into dogs with a similar result. Kepinov (1924) also obtained a rise in antitryptic value of serum of guinea-pig following intraperitoneal injections of liver. A rise was also observed by Meyer (1911) to follow oral administration of thyreoid.

On the other hand Bradley (1910) questions these results for leuco-protease, and Rosenow & Farber (1914) report that no rise in antitryptic index followed injections of Thorium X, although considerable destruction of leucocytes, with presumable liberation of leucoprotease, occurred.

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 circumstances.

(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 found a definite rise following 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, and even after injections of trypsin the rise in index by workers who did obtain a rise was comparatively slight. In Hansen's experiments the rise never exceeded three times the normal index. Consequently, if the serum was antitryptically at its peak, an injection of trypsin would fail to produce a further rise in index.

(c) Hansen 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 other conditions - physiological ones - than the results of trypsin injections.

(d) The choice of method has a very important effect on the results obtained. Elsewhere I have shown (Fine, 1930) how the use of the viscometric method applied to amylase-estimation led certain workers to conclude that serum does not contain amylase, although serum-amylase has invariably been detected by chemical methods. Weil, who failed to find a rise in index following trypsin injections, also used the physical method of viscometry for estimating tryptic action, and this must be taken to discount from the validity of his results: enzyme action is, after all, finally a chemical process, and enzyme activity should strictly be measured by

analysis of the resulting products. This being granted, purely physical methods will then possess a validity in proportion to their yielding results in agreement with chemical methods.

In methods where casein is used as a substrate another source of error is liable to arise, since casein can serve as a suitable substrate for serum ereptase, according to Jobling. Thus variations in serum ereptase will superimpose themselves on variations in antitrypsin, the nature of the latter changes being thus disguised or even distorted.

It seems to me therefore that there are so many reasons why a rise in antitryptic index following a trypsin injection might not be elicited, that a failure to obtain such a rise is only of significance as showing that under certain circumstances (serum taken without definite relationship to food: injections made when serum is at an antitryptic peak; or in an unresponsive condition following an abnormal rise) and by the use of certain methods of uncertain reliability a rise in index is not elicited. On the other hand the demonstration of a rise in antitryptic index after an injection of trypsin - provided the rise is outside the limits of normal variation - is sufficient evidence that the serum is antitryptically responsive to trypsin injections, even though response is not invariable.

(2) 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. 16, 17, and 18.

The method of obtaining the antitryptic index may be considered open to criticism since, in the technique employed (already described in a previous section) 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 in 24 hrs 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 any inhibition. Actually ofcourse 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 speciment 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 by Sørensen's method to distinguish 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 trypsin (Benger's), but rose within a few hours of a second injection of 2 cc, and remained high for some time. After a month it was swinging considerably, with a tendency to rise, which a third injection of 1 cc maintained.

More prolonged observations of T might have been desirable.

Guinea-pig U demonstrated in definitely 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, following an injection of 2.5 cc trypsin, became immobile: and although recovery set in in 20 minutes, the index fell from .6 to .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 & V responded similarly to adequate injections of trypsin, they differed in their subsequent condition, T tending to remain high, but swinging, U remaining persistently high, while V tended to return to normal in a few days.

(3) Formation of precipitins and Immune bodies.

Pozerski (1909) obtained both precipitin and immune body, but no anti-enzyme action following injections of papain. Contacuzene & Jonescu (1909) obtained analogous results with pepsin. Young, who failed to obtain an increase in serum antitrypsin following trypsin injections, was unable to demonstrate the formation of a precipitin.

These results strongly suggest that the antibodies formed in response to enzyme injections are the results of the protein impurities associated with the enzymes, the latter in themselves being incapable of giving rise to true immunity reactions.

8. CLINICAL VARIATIONS IN SERUM ANTITRYPSIN.

Many hopes were early entertained that the antitryptic index could be utilised as a test for various clinical conditions, but it soon became clear that numerous conditions, both physiological and pathological could give rise to the same kind of variation.

(1) Physiological variations.

(a) In fasting. The antitryptic index is always diminished, as was shown first by Rosenthal (1910), and later by Franz & Jarisch (1912). Jobling Petersen & Eggstein (1915)[1] found that in starvation the serum antitrypsin (rabbit) fell continuously, rising again for a short period on the ninth day, and then falling sharply till death occurred on the 13th day.

(b) After food. The same workers showed that after food the value of serum antitrypsin rose above normal [Jobling Petersen & Eggstein, 1915 (2)].

My own observations are confined to one experiment with a guinea-pig which had an index of .59 before feeding (7.30 a.m.) and .78 after feeding (3 p.m.) The following day the index was .78 after feeding (9.30 a.m.) (In all cases guinea-pigs breakfast at 8 a.m. daily.)

(c) Effect of weaning. Rheuss (1909) found an increase in the serum antitrypsin of infants when breast feeding was replaced by artificial feeding.

(d) Pregnancy and puerperium. Becker (1909) found an increase in labour and early puerperium, but not in pregnancy. Grafenberg (1909) and Franz (1914) however obtained an increased index in pregnancy, while Ecalle (1917) found that the increase commenced in the fourth month of pregnancy and continued till the second week of the puerperium.

(e) Relation to leucocytosis. Most workers find that the antitryptic index of serum follows a course parallel to the leucocyte count. This is the common conclusion of Bittorf (1907), Wiens (1907)[3], Landois (1909), Wiens & Schlect (1909)

Waelli (1912). Jürgensen (1911) failed to confirm this relationship

Since leucocytes contain a fairly active protease, a leucocytosis, if associated with a proportionate destruction of leucocytes, will produce an effect equivalent to an injection of trypsin into the blood - i.e. a rise in index. Since the rise in index following an injection of trypsin is sometimes preceded by a fall, it might be expected that a sharp increase in the leucocyte count would cause an initial fall in index. Thus Jochman (1906) found that in myeloid leukemia, when a sudden flooding of the circulation with myelocytes took place, the antitryptic index fell considerably, in some cases the serum becoming tryptic.

(2) Pathological Variations.

Conditions where the index is increased.

The majority of pathological conditions with an altered index come under this category, and of these the greater number are definitely associated with wasting: for this reason the term "cachexia reaction" has been applied to a heightened index in a wasting disease.

(a) Carcinoma. An increased index in 95% of cases of cancer was found by Brieber & Trebing (1908). Jolla (1909), Hort (1909), Landois (1909) and Waelli (1912) confirmed the presence of the "cachexia reaction" in cancer.

increased index in cancer was also found by Von Bergman & Meyer (1908).

Weil (1910) points out that although a raised index cannot be considered as an important test for cancer, yet so constant is this high index that its absence may be taken as a valid reason for excluding a diagnosis of cancer where uncertainty exists.

(b) Sarcoma. This form of malignant disease is also associated with a raised index, as has been shown by Breber & Trebing and by Waelli: the latter found the increase in index less marked than in carcinoma.

(c). Tuberculosis. The above workers found this condition also marked by a raised index to a very constant degree.

(d) Exophthalmic Goitre. Waelli found the index increased in this condition.

(e) Anaemia. Brieber & Trebing, Brenner (1909), and Grafenberg found the index increased in the more severe forms of anaemia, while Grafenberg found this increase in chlorosis also.

(f) Trauma. Zunz & Gwerts (1918) found a rise in index following traumatic injuries.

Conditions where the index is diminished.

(a) Poisoning from chloroform, phosphorus or iodine (Opie, Barker, and Dochez 1919).

(b) Post-anaphylactic shock (Pfeiffer & Jarisch).

(c) Septic conditions. (Wiens, 1907)[10]

In pneumonia the index rises from the commencement of the pyrexia until the crisis, when the index sharply falls, and after a second lesser rise assumes a normal level. (Jobling, Petersen, Eggstein, 1915)[6] in this condition therefore the fall in index is associated with a fall in temperature.

3) The application to diagnosis of the influence of serum on enzymes.

The variations of serum antitrypsin represent only one, and perhaps the least useful phase of such applications: its usefulness would no doubt be enhanced by a greater knowledge of the normal variations, as well as of the exact nature of the action of serum on trypsin. Comparatively little success has attended the use of any other enzyme test for serum. Thus the variations of serum diastase are of little significance apart from the very marked increase associated with acute pancreatic disease.

Serum lipase variations have also been claimed to be of service for diagnostic purposes. Thus Buchler (1924) states that normal serum lipase is destroyed by quinine, while lipase derived from liver or nerve tissue is quinine-stable: he was thus able to determine whether in various psychotic conditions there occurred any disintegration of liver or nerve tissue, since, when such disintegration occurred, there appeared in serum a quinine-stable lipase in addition to the normal quinine-susceptible lipase.

In this case use is made not of variation in strength of serum lipase, but of variation in properties, and this latter seems to me a much more fertile source of exploitation for diagnostic purposes.

On the whole it might be said that little diagnostic service has been rendered by the study of variations of individual enzymes or enzyme factors in serum. Thus (1) serum antitrypsin is increased in many conditions besides cancer, although the constancy of increase in cancer is very high: (2) the lipase accelerating action of serum is much depressed in cancer (Lewis & Corran, 1928), but there is little information as to the condition of the serum in other wasting diseases.

Nevertheless the presence of <u>both</u> increased antitryptic action and diminished lipase-accelerating action is not likely to recur in other diseases with the same frequency as either of these changes considered individually.

It seems to me that the study of variations (qualitative as well as quantitative) in serum enzymes and in the enzyme (i.e. "accelerators" and "inhibitors") factors of serum might yield much more fruitful results diagnostically if the variations were correlated over a sufficiently wide range of factors. In this way characteristic "enzyme pictures" of serum might be found to exist in many pathological conditions which at present have only been shown to yield definite but uncharacteristic variations.

<u>THE NATURE OF SERUM ANTITRYPSIN</u>.

There are two questions 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) <u>The antitryptic substance.</u> The three main theories as to the identify 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 & Gigon (1907) confirmed 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 & 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 to 4]. Jobling submitted evidence shewing (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 saturation of fatty acids or soaps by such means as KI, I_2, H_2O_2 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 & Zenophonton (1919): (4) that although unsaturated soaps do not lose antitryptic action when heated alone, they do lose this action if heated at 70°C for ½ hr. in the presence of serum: (5) that the chloroform extract of serum - presumably containing the lipoids - 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 lipoid exerting antitryptic action without previous saponification. After describing the formation of an antitryptic soap by the saponification of an ether extract of serum, Jobling states (p. 468) "it is not necessary to assume that they can act only as soaps: probably the esters containing unsaturated fatty acids are just as effective;" the probability, however, would have been greater had experimental evidence of it been offered. The importance of such evidence must have been clear to Jobling since he states (p. 475) that the lipoids which he considers as responsible for the antitryptic action of serum are present in the form of esters.

He does state that in unpublished experiments he found a fine olein emulsion to possess "some ferment-inhibiting action"; but no further details are given. In all the serum studies, antitryptic action is expressed in terms of the action of soap derived from serum.

(2) There is insufficient regard paid to the p_H of the enzyme mixtures; the practice described of making mixtures "a little alkaline" with $\frac{N}{10}$ NaOH creates a doubt as to the uniformity of p_H conditions throughout the experiments: this practice certainly increases the danger of assuming with the author that the serum esters are the source of antitryptic action on the ground that the "slightly alkaline" soaps prepared from the esters are antitryptic. (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.

Examination of Jobling's Theory. Jobling states [1914₁(3)] (p.460) that the discovery that sera when extracted with chloroform soon lost their antitryptic action, convinced him "that the ferment-inhibiting substrates of serum were lipoids, and that they were soluble in fat solvents".

The fact that the chloroform extract, when freed from chloroform and re-added to the extracted serum, did not restore its antitryptic action was explained by the assumption that to restore such action it was necessary to restore not only the lipoids, but also their original state of dispersion of the lipoids in the serum.

This Jobling proceeded to do by saponifying the chloroform extract and rendering it slightly alkaline!

This saponified product which Jobling found to be antitryptic cannot legitimately be considered as equivalent to the substance extracted from serum.

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 .5% 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. 19 which represents one of several experiments carried out.

It would appear that Jobling, who has demonstrated antitryptic properties in several substances such as the caseous material in tuberculosis and the envelopes of certain bacteria, felt under the necessity of proving that all such substances

contained identical trypsin-inhibitors. Actually, as I have shown, there are a number of different substances of organic origin possessing in common an antitryptic action.

(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 crystallized albumin and found it to be highly antitryptic. He admitted the possibility of some other constituent of serum entering into the composition of the srystals however, and on a 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 serum antitrypsin was due to multiple causes, of which the peculiar configuration of the protein molecule was one.

← Oppenheimer & Aron, 1903)

(1920)
Teale & Bach/came to the conclusion that the nature of the serum proteins was the cause of the antitryptic action of serum.

Although the evidence on a whole is in favour of the serum proteins being the seat of antitryptic action, there has been a feeling among workers that some other factor is involved. This has been clearly shown by the work of Beaton (1922), who found 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.

Mode of action of Proteins. The classical work of Hedin (1906) 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 contentration 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 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 to ultimately recover from the inhibition, there being no such recovery reported for other enzymes. As Bayliss points out (1923), the adsorbed trypsin is able to slowly attack the serum proteins adsorbing it until the latter are entirely hydrolysed and the trypsin 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 by enzyme action the adsorbing bodies. In my own experiments with invertase I found that inhibition was not recovered from: thus an invertase solution in unbuffered produced 10% inversion of 2% cane sugar in 24 hrs.

* See note accompanying Fig. 20.

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.

The study of the variations of serum antitrypsin in disease and experimentally led Wiens (1907)[2] to the conclusion that it was of the same nature as the immunological antibodies formed by the body in response to antigens. Meyer (1909) supported this view on the ground that serum antitrypsin is increased by trypsin injections: in his view, and in that of Stavraky (1914) normal serum antitrypsin is formed as a response to proteases liberated during the tissue destruction which normally takes place as a metabolic process.

Eisner (1909) believed antitrypsin was a true antibody because of its specificity - i.e. "it inhibited trypsin only, and not rennet, pepsin, emulsin, or cobra lipase." He evidently was not aware of the power of serum to inhibit other enzymes than trypsin.

The antibody theory, round which much controversy has raged, is not very illuminating because it refers the nature of serum antitrypsin to the nature of Ehrlich's antibodies, whatever the latter might be.

as seems probable
If/Ehrlich's bodies are protein in nature, then this theory becomes a modification of the protein theory which is discussed above.

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 antitrypsin resemble each other in that

(a) Both are protein bodies 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: antitrypsin 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, an 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 both adsorption phenomena in which the adsorbing bodies are proteins.

CONCLUSIONS.

(1) Serum can influence an enzyme in three ways:

(a) It may contain the same enzyme, by virtue of which an "apparent acceleration" is produced: examples of this

are human serum (which contains diastase) acting on diastase and guinea-pig serum (which contains lipase) acting on lipase.

(b) It may not contain the same enzyme, and therefore (1) if it increases the activity of the enzyme, the action is one of "true acceleration," and (2) if it diminishes the activity of the enzyme the action is one of inhibition. The only example known of true acceleration by serum is its action on invertase at certain values of pH.

Examples of inhibition , on the other hand, are numerous, since most enzymes - with the exception of those present in normal serum (diastase, lipase, and catalase) are, to varying degrees, inhibited by serum.

(c) It may contain the same enzyme, yet increases the activity of the enzyme to a greater extent than can be accounted for by this fact. Here there is a "total acceleration" consisting of "true" and "apparent" elements. The only example of this type of influence is the action of serum (human, cow and other animals, but not rat or guinea-pig) on lipase: such serum contains both lipase and "lipase accelerator."

(2) The cause of true acceleration by serum, or at any rate one cause, has been shown - in the case of invertase - to the the pH of the final enzyme mixture: by suitable alteration of the pH the influence of serum can be changed from acceleration to inhibition

The cause of lipase-acceleration has not been shown.

(3) The causes of enzyme inhibition by serum are essentially two, and they may act together or separately. They are: (1) the pH change produced by addition of serum. This cause will operate in proportion as the pH of the serum (= 7.45) is removed from the optimum pH of the enzyme.

Thus inhibition of pepsin by serum is entirely due to this factor, while inhibition of rennin, papain, invertase, emulsin is partly due to pH and partly to the second factor, (2) adsorption. This factor is chiefly responsible for the inhibition of laccase, and tyrosinase, and solely responsible for the inhibition of trypsin.

(4) The various influences of serum on enzymes have early been attributed to definite but unspecified chemical substances, although the evidence of such entities was usually confined to the more occurrence of the phenomena of acceleration or inhibition. When inhibition was obtained it was customary to go further and state that the entity was of the nature of an Ehrlich antibody. In this way an ill-founded system of terminology arose in which the inhibiting action of serum was explained by the occurrence in serum of a system of anti-enzymes, such as anti-pepsin, anti-trypsin, anti-rennin, anti-tyrosinase, etc.

This terminology arose at a time when little was known of the nature of the action of serum on enzymes, and when very few workers took any account of the p_H of their enzyme mixtures.

(5) Other trypsin inhibitors of organic origin besides serum are

(1) Worm extract.
(2) Egg-white
(3) Bacteria
(4) Yeast
(5) Animal tissue generally
(6) Inflammatory secretions
(7) Unsaturated fatty acids.
(8) Lecithin.

An examination of the properties of these trypsin-inhibitors, while revealing varying degrees of similarity, establishes the essentially different nature of the inhibiting mechanism in serum, worm extract, yeast, bacteria and unsaturated fatty acids.

(6) Serum antitrypsin has the following essential properties:

(a) The property of inhibition is not lost on dialysis. In this it differs from worm extract, the inhibiting principle of which is in the dialysate.

(b) Heating at 70°C for ½ hr. destroys the property of inhibition. Unsaturated fatty acids and worm extract on the other hand, are unaffected by such treatment.

(c) Drying has no effect.

(d) Shaking has no effect.

(e) Mixing with $CHCl_3$ diminishes antitryptic activity in proportion to the time of contact. Ether is said to have a

similar effect.

(f) The addition of certain colloids (starch, gelatin, kaolin) diminishes the antitryptic activity.

(g) The antitryptic property is chiefly associated with the albumin fraction: the globulin fraction is also antitryptic.

(h) Serum exhibits the Dastre phenomenon, i.e. the inhibition of trypsin it causes is gradually recovered from. This phenomenon is also shown by egg albumin and worm extract.

Serum differs from these other inhibitors in that the inhibited trypsin may ultimately become more active than the trypsin which was incubated with heated serum and which therefore was uninhibited.

An analysis of these results rendered possible by the Sorensen method of estimating tryptic activity, shows that the superior activity of inhibited trypsin applies only to the "formol acid" produced, and not to the "free acid", which never exceeds, though usually ultimately equals, the free acid formed by the uninhibited trypsin.

This is explained by the hypothesis that commercial trypsin consists of a tryptase portion (producing "free acid"), and an ereptase portion (producing "Formol acid"), and it is only against the tryptase that serum exerts its inhibiting action: as a consequence, when serum also contains ereptase, the latter reinforces the ereptase of the commercial trypsin, whereas uninhibited trypsin acting on the heated serum is not so reinforced since the heating of the serum also destroys the ereptase.

Thus the ultimate superior activity of inhibited trypsin is markedly evident with guinea-pig serum which contains an active ereptase, but is not obtained with sheep serum, presumably owing to the absence of ereptase.

(i) A study of the kinetics of serum antitrypsin reveals (1) that the Dastre phenomenon is only shown when certain concentrations of serum act on certain concentrations of trypsin. In

the presence of a great excess of serum there is no recovery of trypsin. When trypsin is greatly in excess, inhibition is not obtained, or recovery is more rapid than can be detected. (2) The amount of trypsin bound by increasing quantities of serum furnishes a logarithmic relationship identical with that obtained by the binding of tryosin by colloidal charcoal. (Work of Hedin). This relationship is explained by the assumption that the [illegible] phenomenon of inhibition is due to adsorption of trypsin by the colloids of the serum.

(7) Injections of trypsin have been shown by most workers to produce a rise in the antitryptic power of serum. Failure to obtain such a result is explained by the following reasons:

(a) Unsatisfactory method used in estimating antitryptic power (e.g. viscometric method).

(b) Unsatisfactory technique (e.g. failure to control pH)

(c) Serum is subject to normal fluctuations in antitryptic index, a fact which must have a bearing on the results , since

(d) in any case the amplitude of variation of serum antitrypsin is not great, the maximum increase following injections of trypsin never exceeding three or four times the normal value.

(e) Trypsin from different sources, although of the same activity, may be differently affected by the same serum. (Young).

(8) It has not been satisfactorily proved that any other enzyme than trypsin will, on injection into the body, give rise to increased anti-enzyme action. It has been shown however that precipitins and anaboceptors have been evoked by such injections, the antigens being the protein associated with the enzyme.

(9) The chief clinical variations of antitrypsin are

(a) Physiological variations: the antitryptic index is decreased in fasting: it is increased after feeding, after weaning, and in the latter period of pregnancy and early puerperium. It runs parallel to the leucocyte count.

(b) Pathological variations.

It is increased in carcinoma, sarcoma, tuberculosis, exophthalmic goitre, anaemia and trauma.

It is diminished in poisoning by $CHCl_3$, phosphorus, or iodine, in shock following anaphylaxis and in septic conditions.

Evidently the application to diagnosis of variations of antitryptic index is not promising: nevertheless, the correlation of such variations with other enzyme changes in serum (such as the changes - quantitative and qualitative - in serum lipase) may produce more characteristic pictures.

(10) The nature of the substance in serum responsible for the antitryptic action has been the subject of many theories, the most important of which are the following three:-

(a) Amino acid, or end product theory. This is evidently untenable since the antitryptic effect of serum is at a maximum at the beginning of its action, and diminishes progressively as the end products increase.

(b) Lipoid theory, (including unsaturated fatty acid theory of Jobling). Although lipoids and serum resemble in several ways in their inhibition of trypsin, serum lipoids cannot be held responsible for the action of serum for the following reasons:

(i) The effect of heating at 70°C for ½ hr is to destroy the antitryptic property of serum whereas lipoids remain antitryptic even after boiling.

(ii) The modes of action of serum and lipoids are totally different. The inhibition of trypsin by serum is greatest at the commencement, but diminishes. Lipoids act by initial stimulation, and subsequent destruction.

(iii) Chloroform treated sera remain antitryptic.

(c) Protein theory. This only can be reconciled with the following evidence:

(i) Crystallised albumin is antitryptic.

(ii) The protein fraction of serum is antitryptic.

(iii) The proteins are the only substances in serum affected by heat.

(iv) The recovery of trypsin from inhibition is readily understood if the substance by which trypsin is adsorbed is protein, since the protein is gradually split up and trypsin released: this recovery would be difficult to understand if the adsorbing substance were other than a substrate of trypsin.

1) The mode of action of the antitryptic substance whatever be its ure is clearly one of adsorption. This is proved by the kinetic ationship between serum and trypsin as worked out by Hedin.

This mechanism explains the anomaly pointed out by Beaton, since increased antitryptic index need not be associated with an increased centration of serum albumin: it need only be associated with an rease in surface of the colloidal albumin, which is compatible with a rease in the actual amount of albumin.

2) The antibody theory of serum antitrypsin is essentially a iation of the protein theory, stated in the chemically obscure language of unology.

Immunological antibodies resemble serum antitrypsin in

(a) both being normally present in association with the protein of serum.

(b) both neutralising certain substances by the same mechanism - adsorption.

They differ, however in that -

(a) True antibodies are associated with globulin, antitrypsin with albumin (chiefly).

(b) True antibodies can be increased almost indefinitely by injections of antigen: antitrypsin only slightly responds to injections of trypsin.

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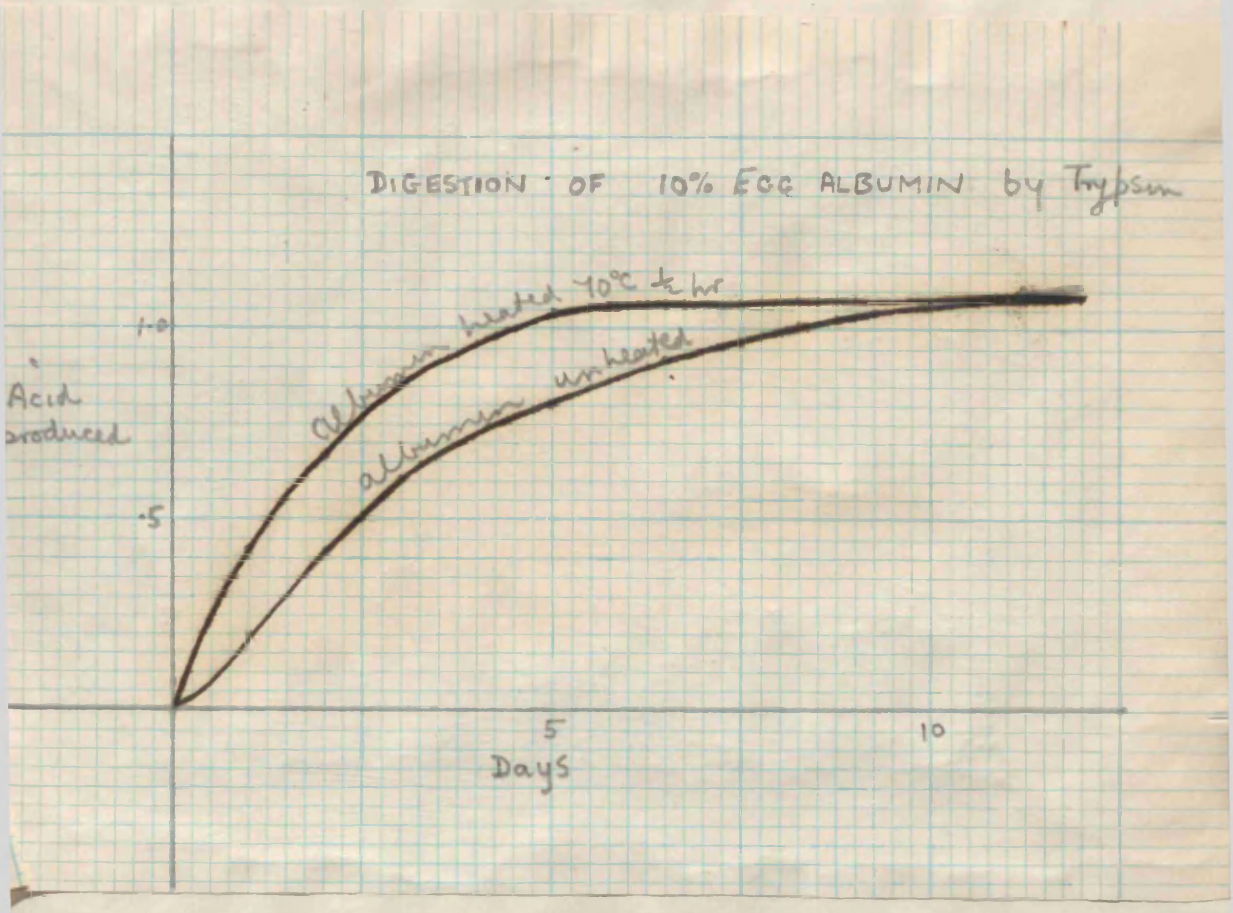


FIG. 1.

Illustrating recovery of trypsin from inhibiting action of egg albumin.

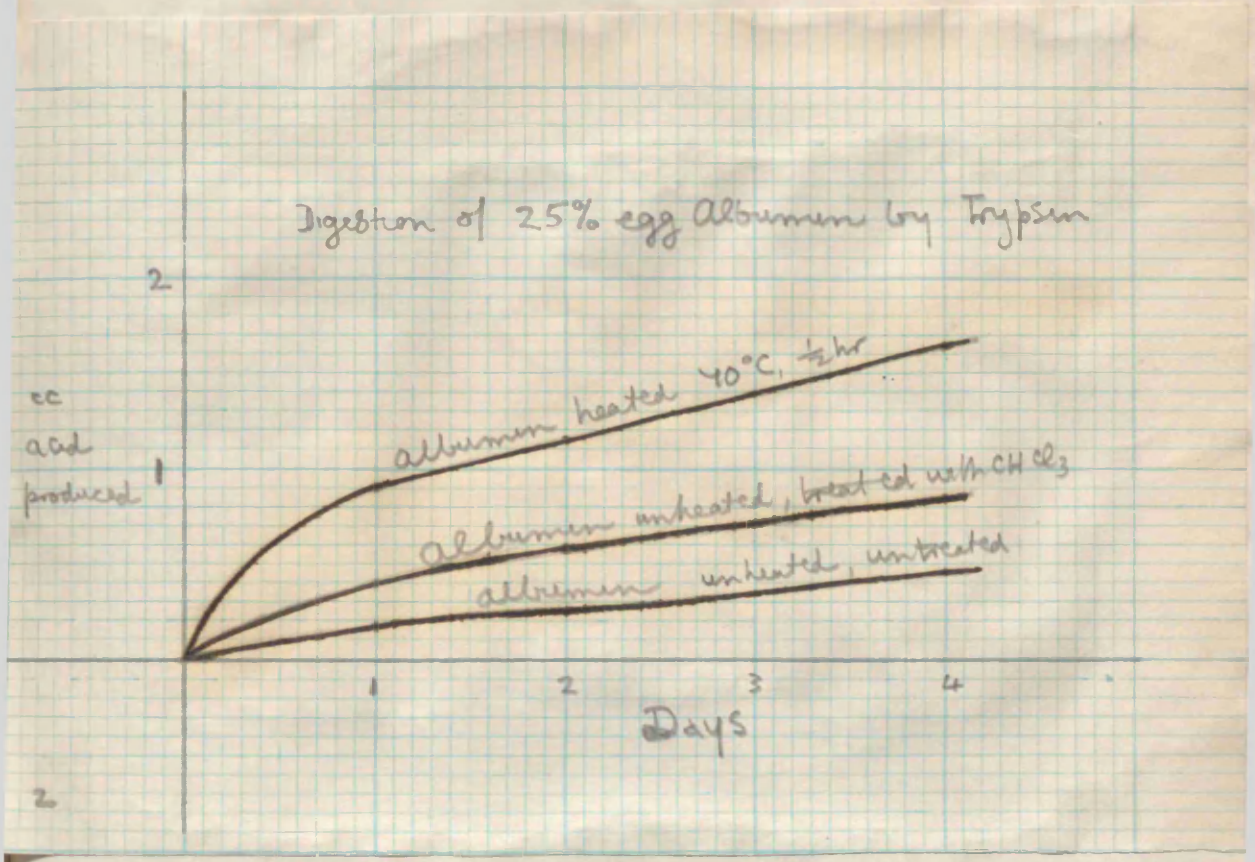


FIG. 2.

Showing partial destruction by $CHCl_3$ of inhibitory action of egg albumin.

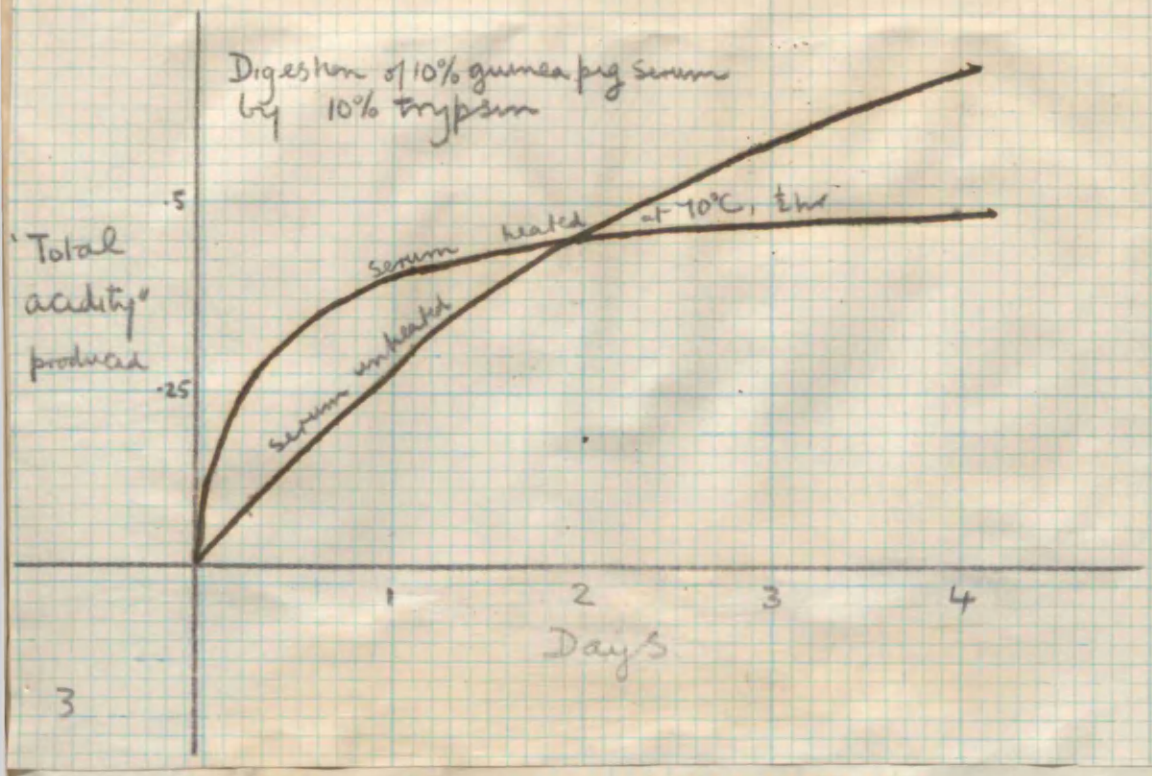


FIG. 3.

Showing recovery of inhibited trypsin until activity is greater than that of uninhibited trypsin.

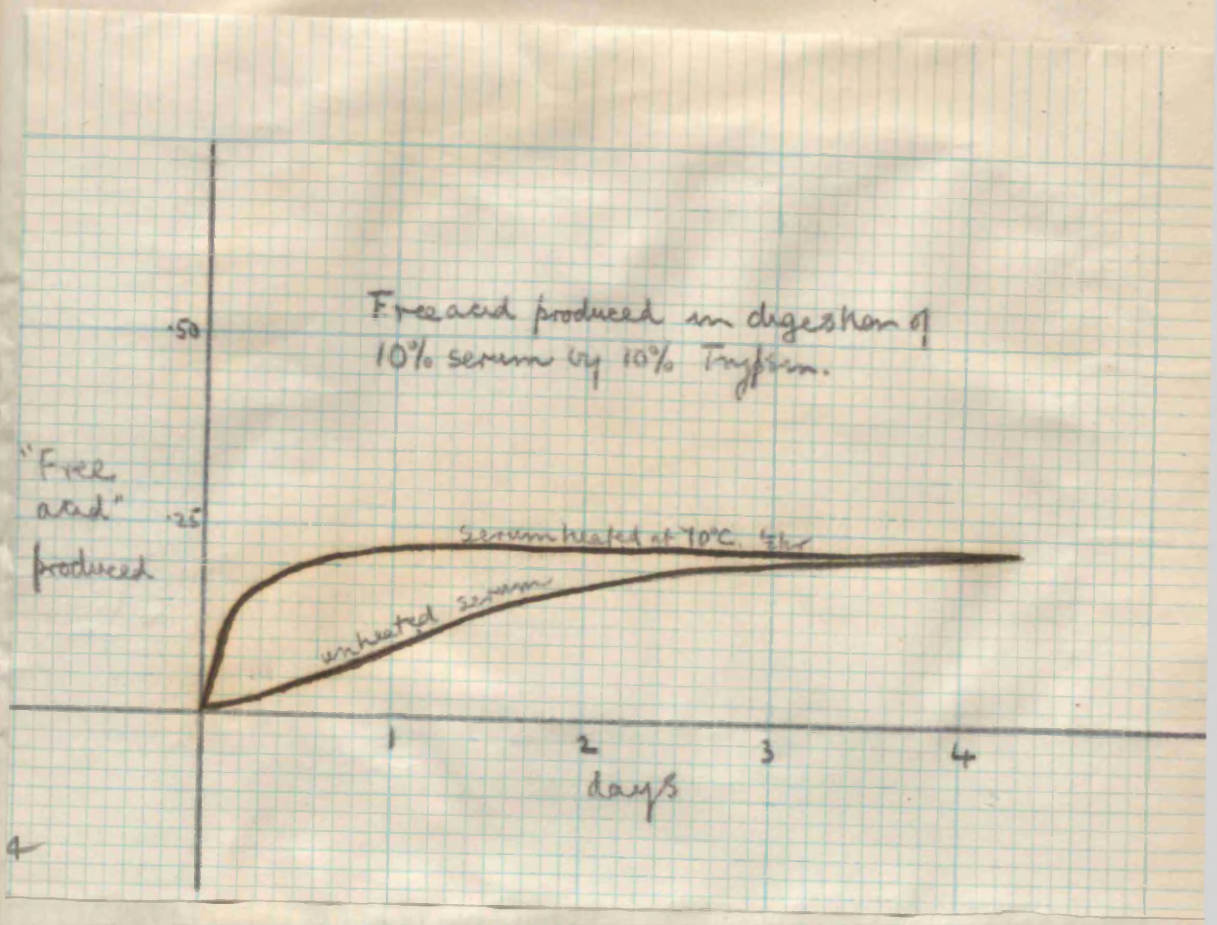


FIG. 4.

Showing how free acid produced by inhibited trypsin ultimately equals amount formed by uninhibited trypsin.

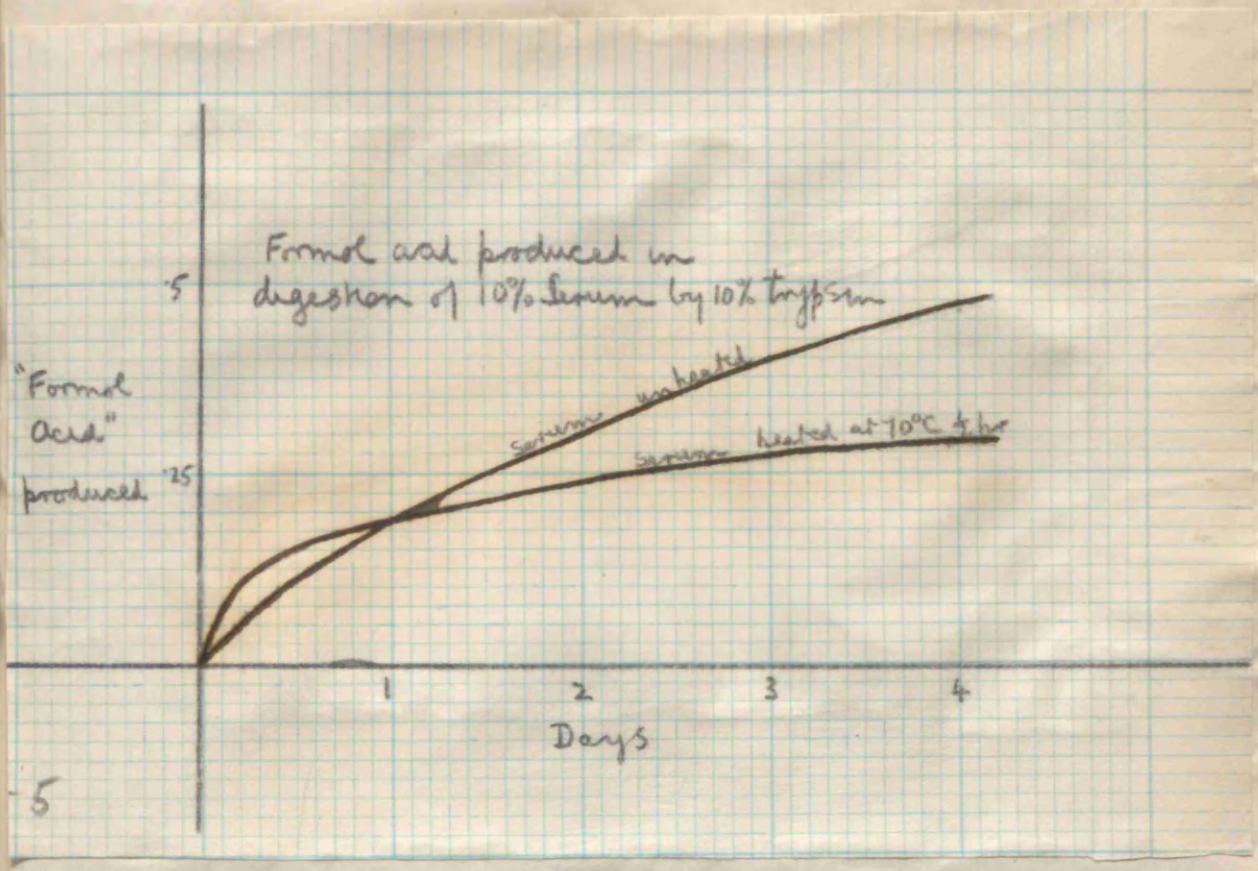


FIG. 5.

Showing how inhibited trypsin ultimately forms more formol acid than uninhibited trypsin.

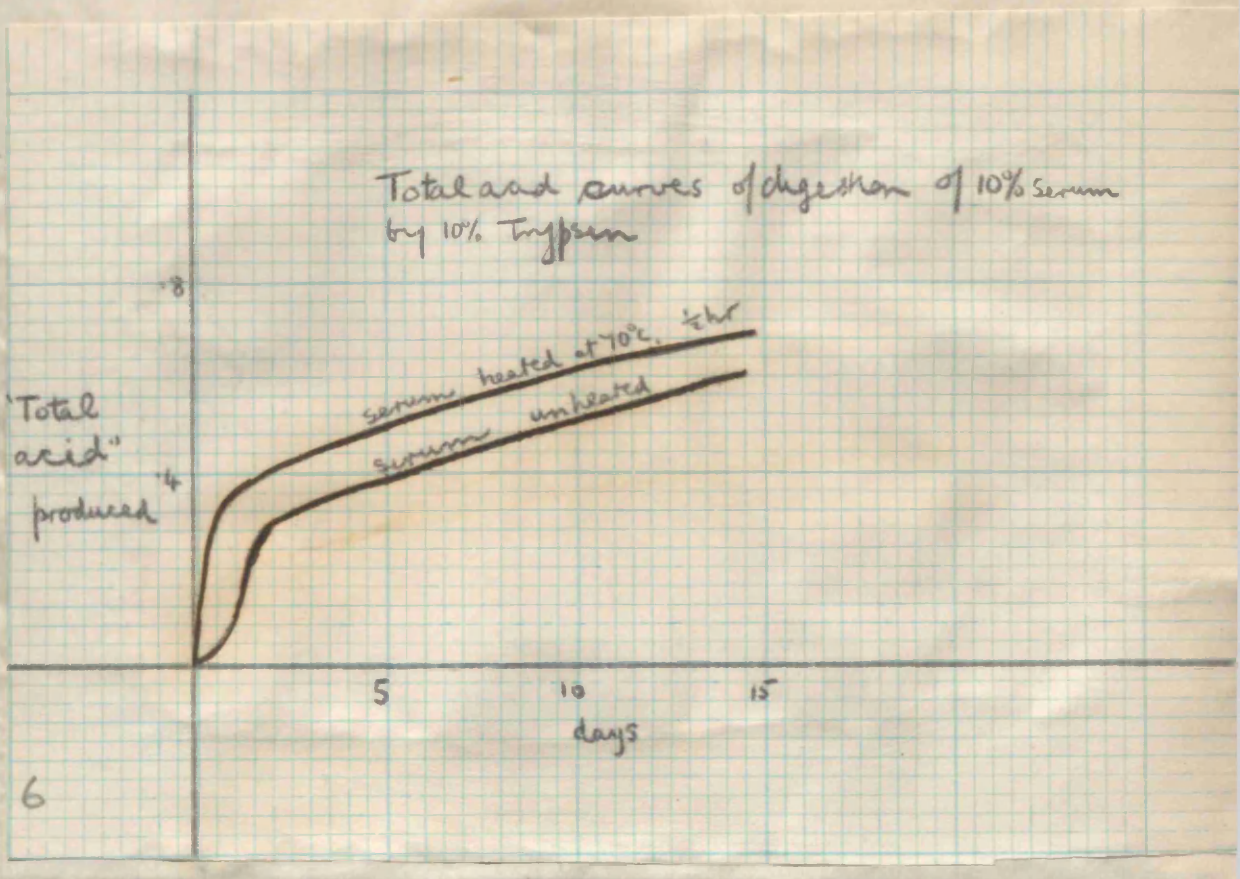


FIG. 6.

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.

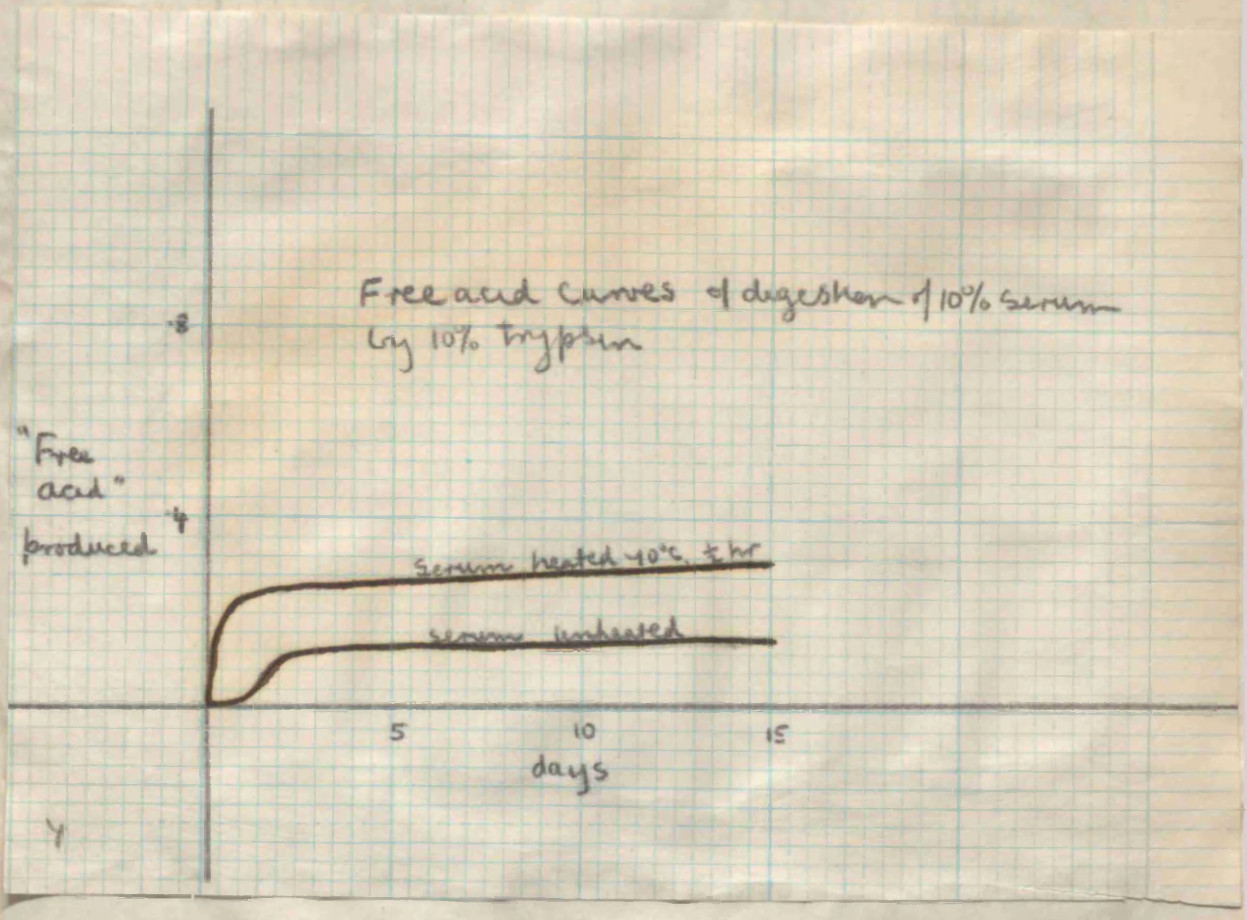


FIG. 7

Experiment is the same as in Fig. 6, the free acid only being considered.

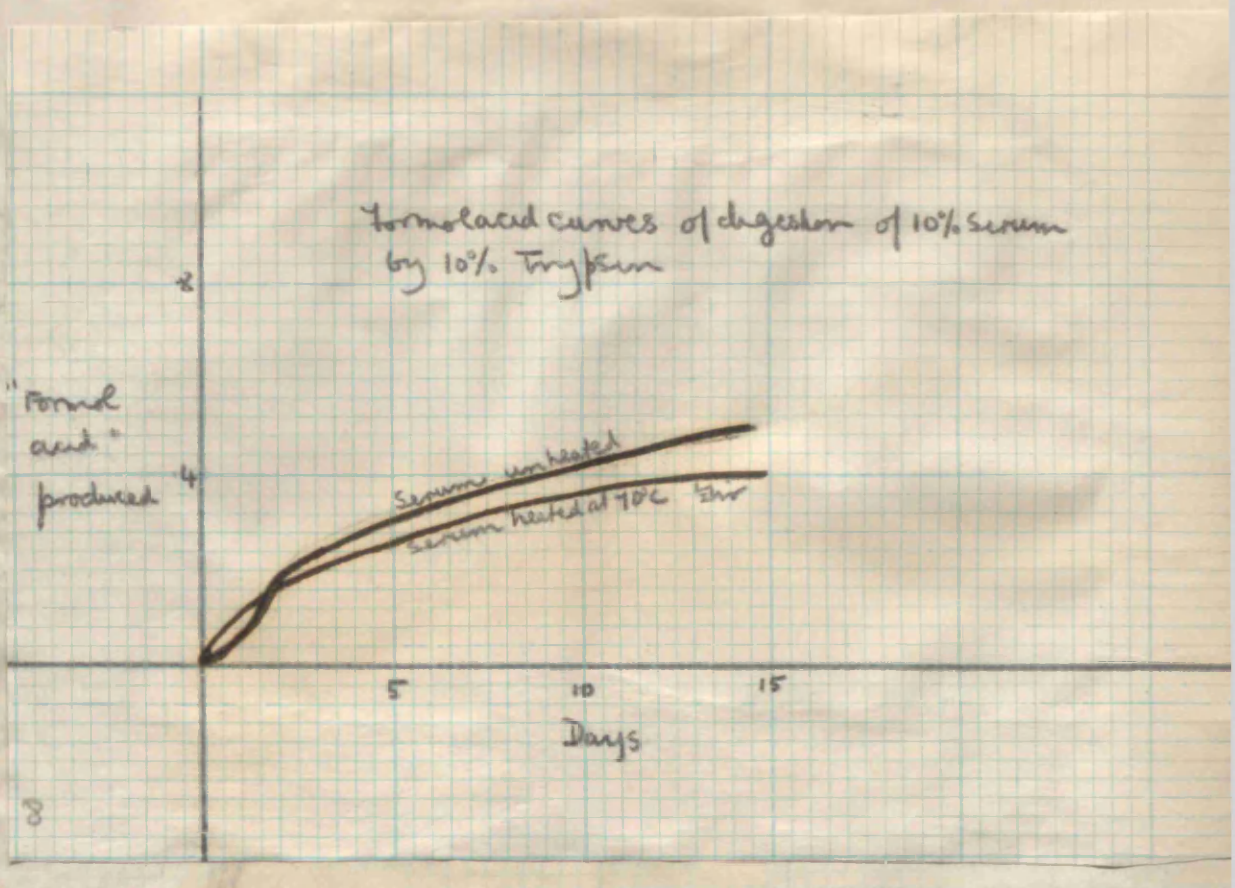


FIG. 8.

Experiment is same as in Fig. 6, formol acid only being considered.

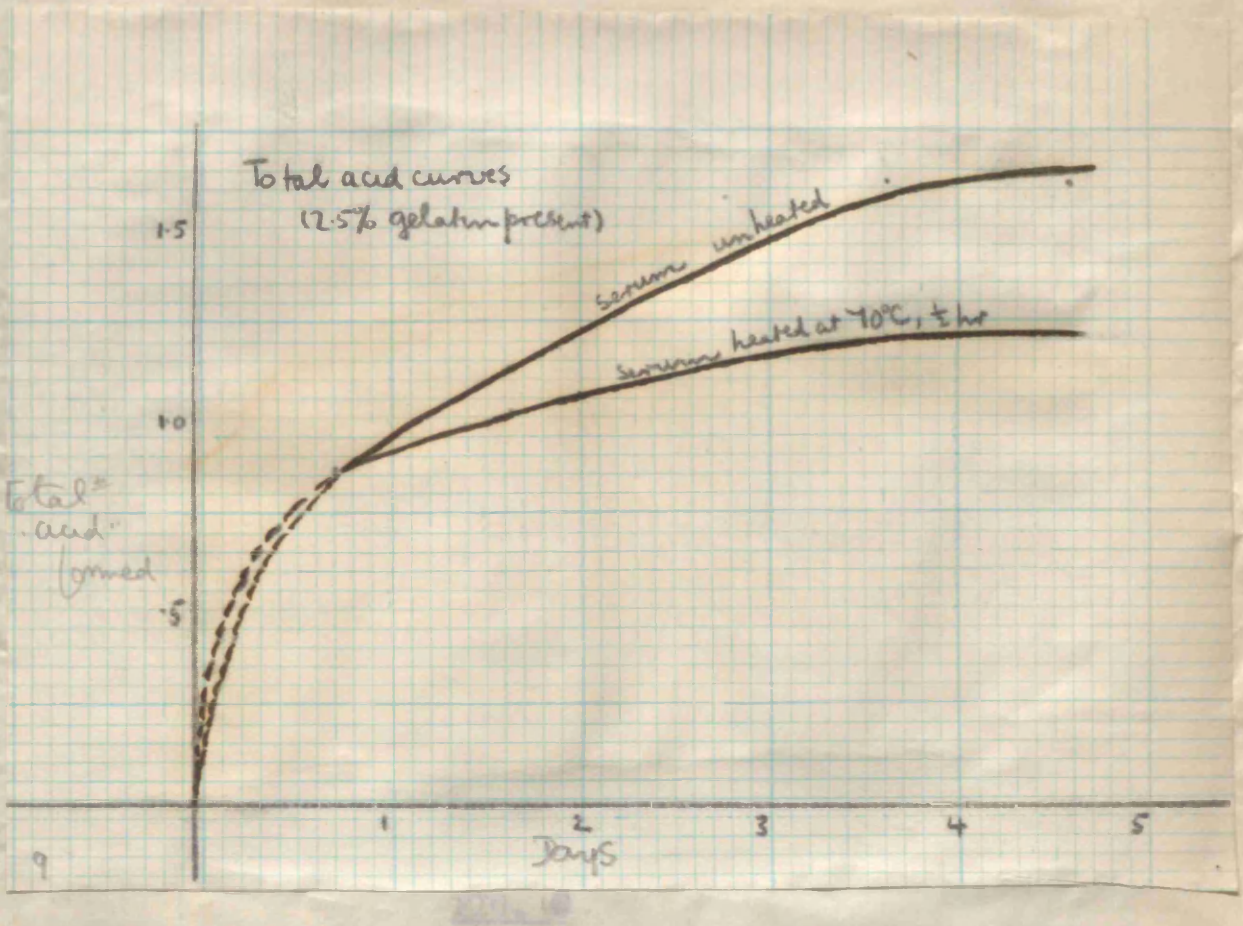


FIG. 9.

Showing 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.

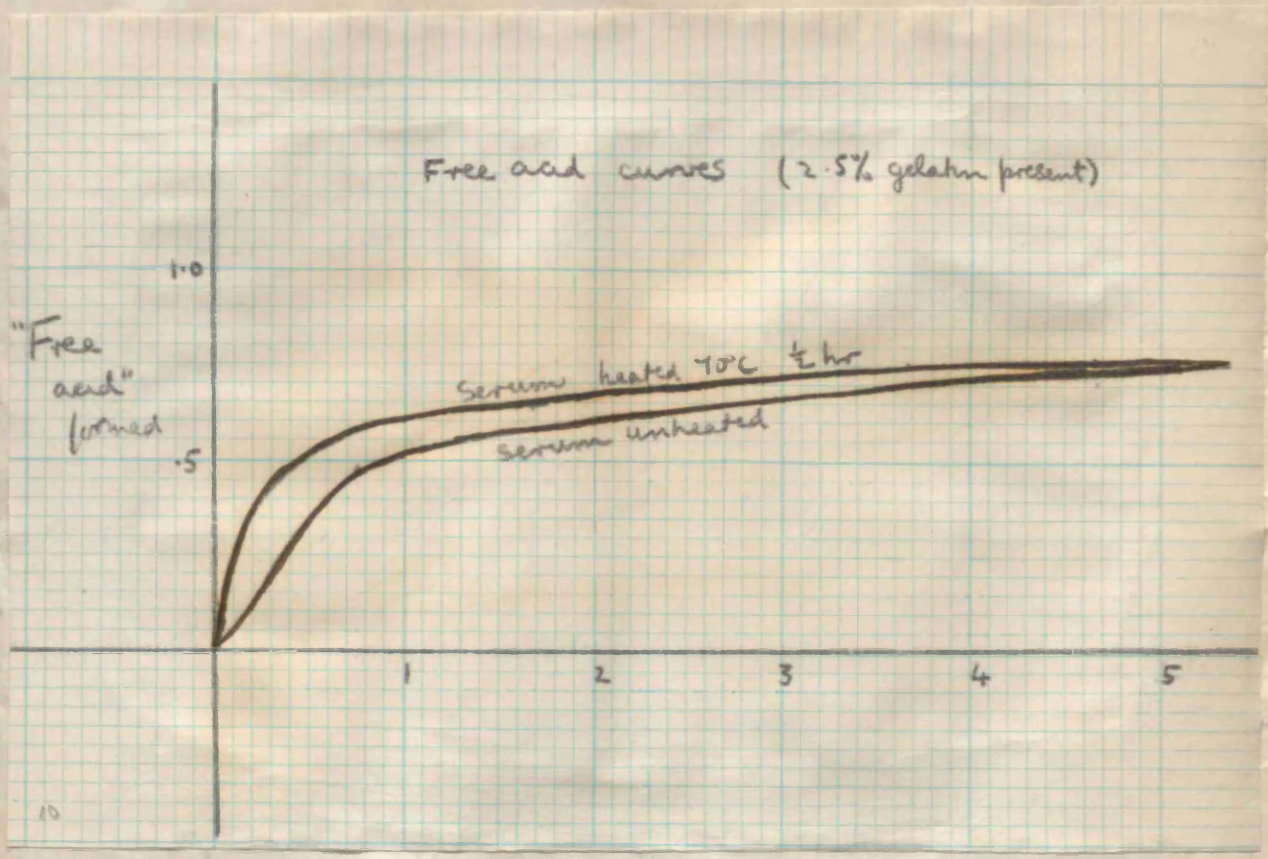


FIG. 10

Showing the same relation between the free acid formed by inhibited and by uninhibited trypsin as in Fig. 4.

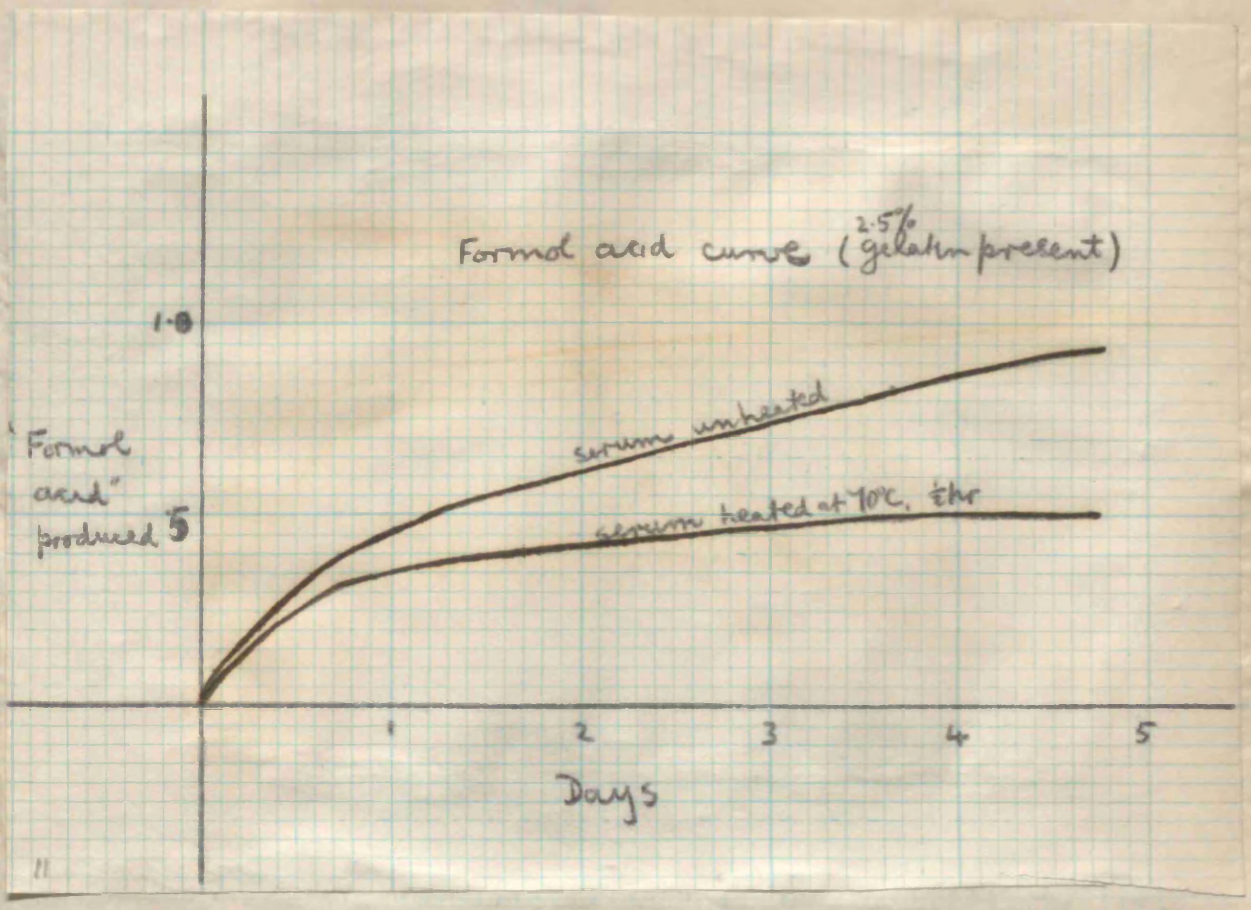


FIG. 11.

Showing same relation between formol acid formed by inhibited and uninhibited trypsin as in Fig. 5.

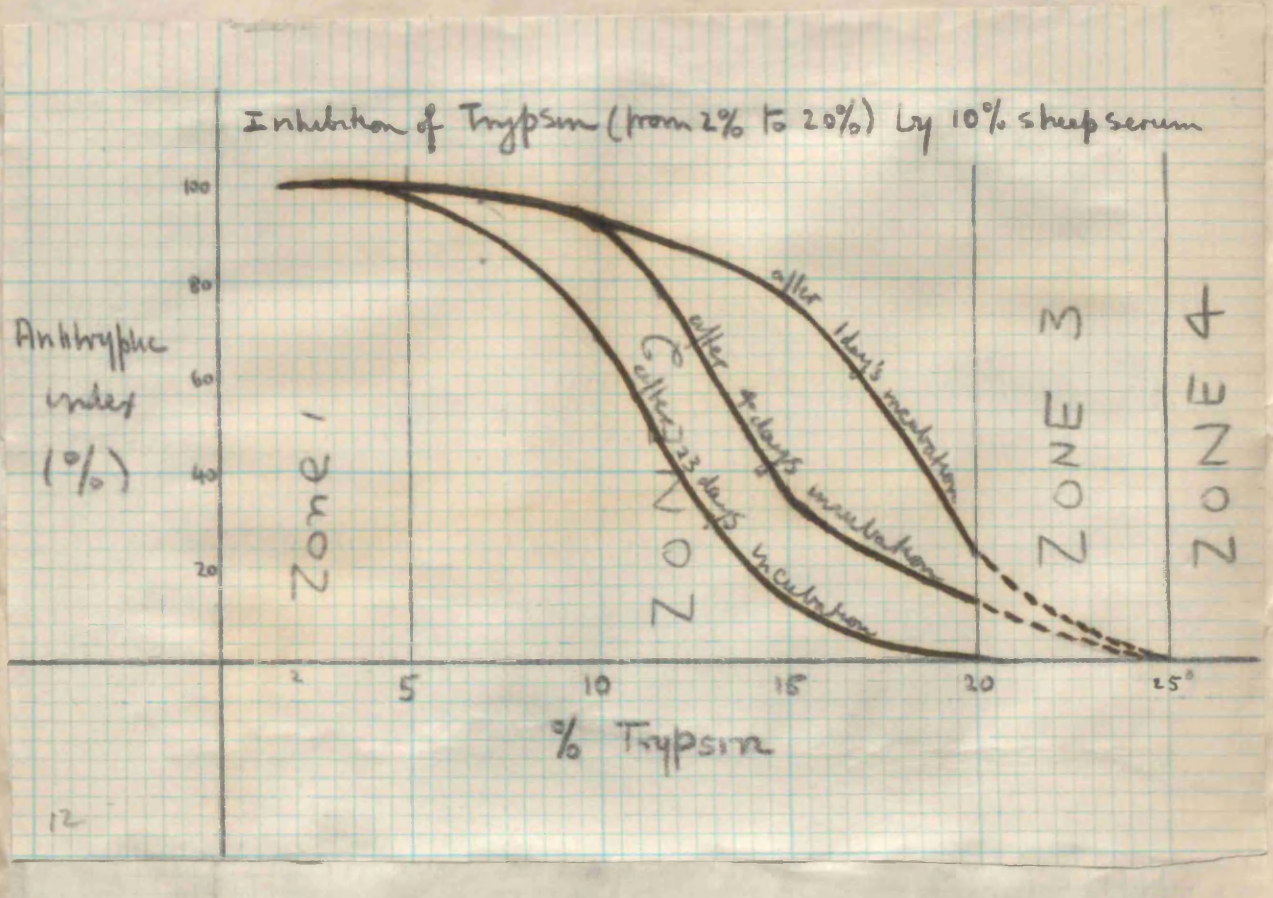


FIG. 19.

Illustrating the kinetics of recovery of trypsin from inhibition. This 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 & 4, which are merely strongly suggested by the character of the Zones 2 & 3, and by the fact established on other occasions that concentrated trypsin successfully resists the inhibiting action of serum.

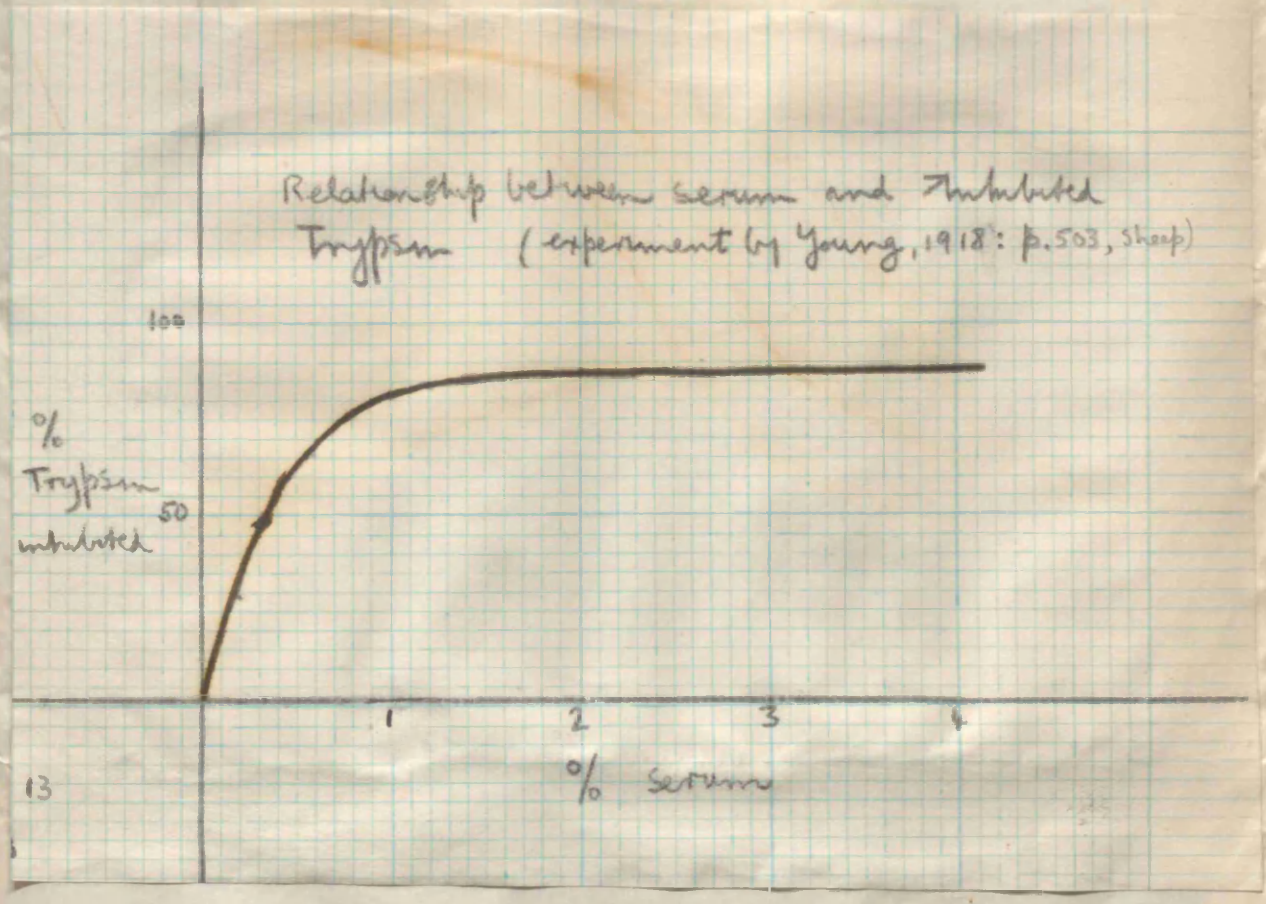


FIG. 13.

Illustrating

(1) that degree of inhibiting 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.

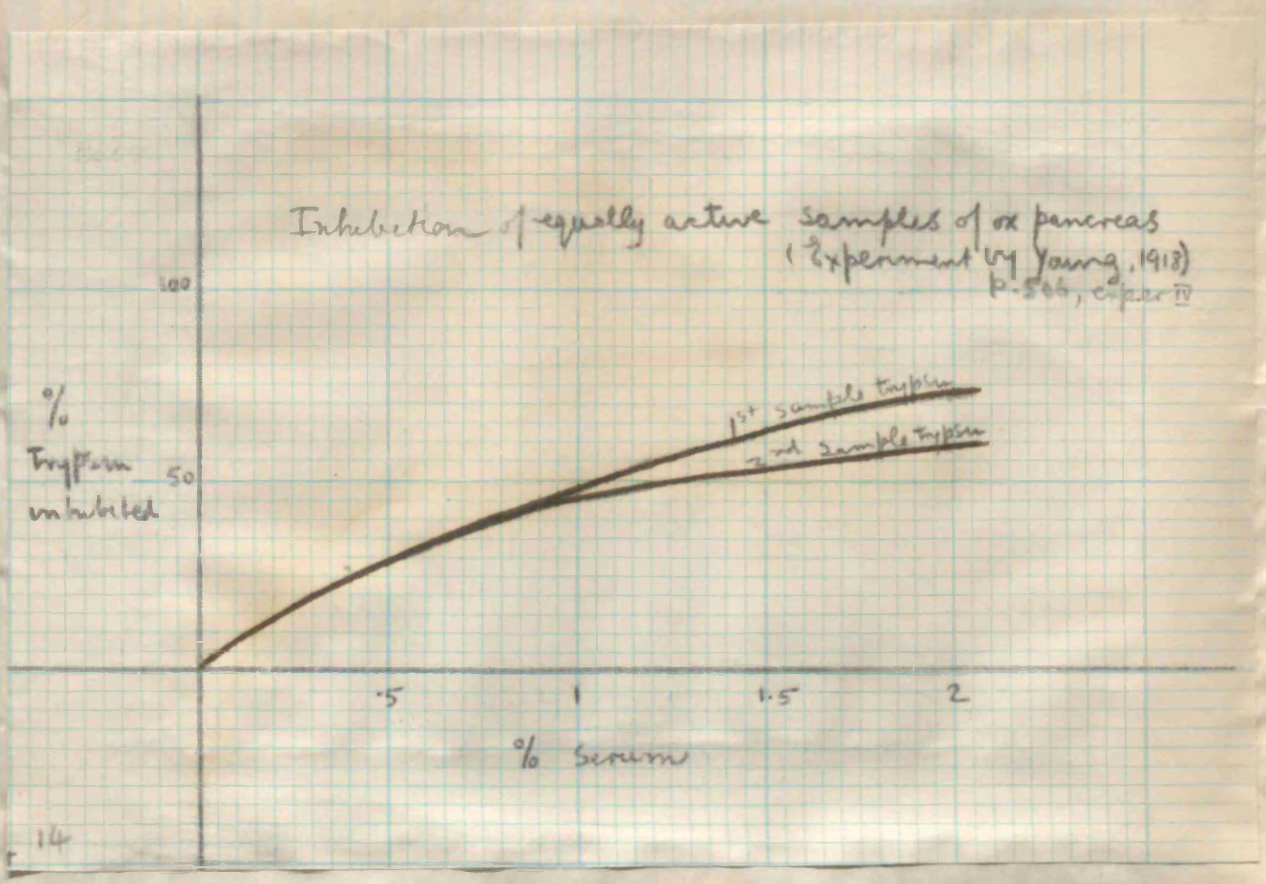


FIG. 14

Showing that samples of trypsin equally inhibited by weak serum may not be equally inhibited by stronger serum.

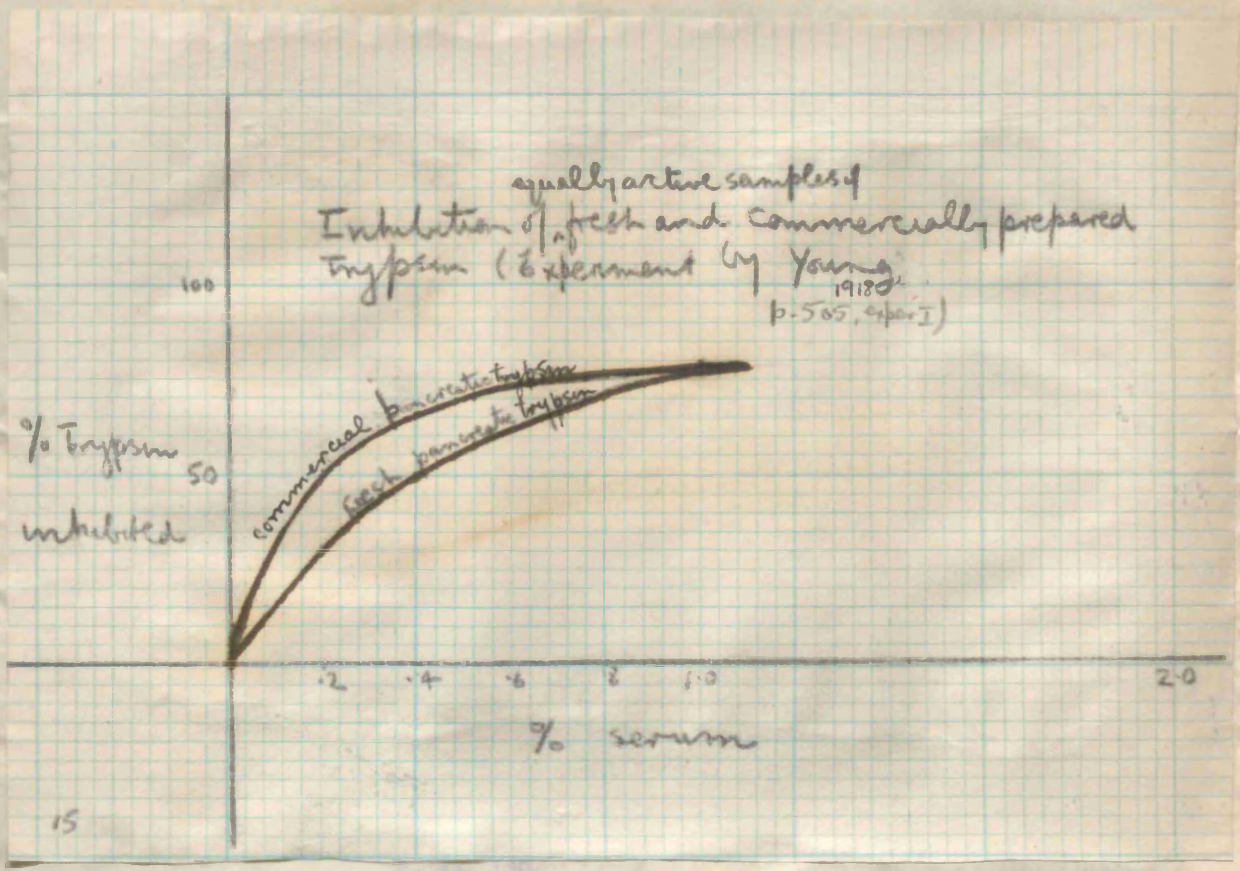


FIG. 15.

Here weak serum inhibits the two samples differently, the difference diminishing as the serum becomes stronger.

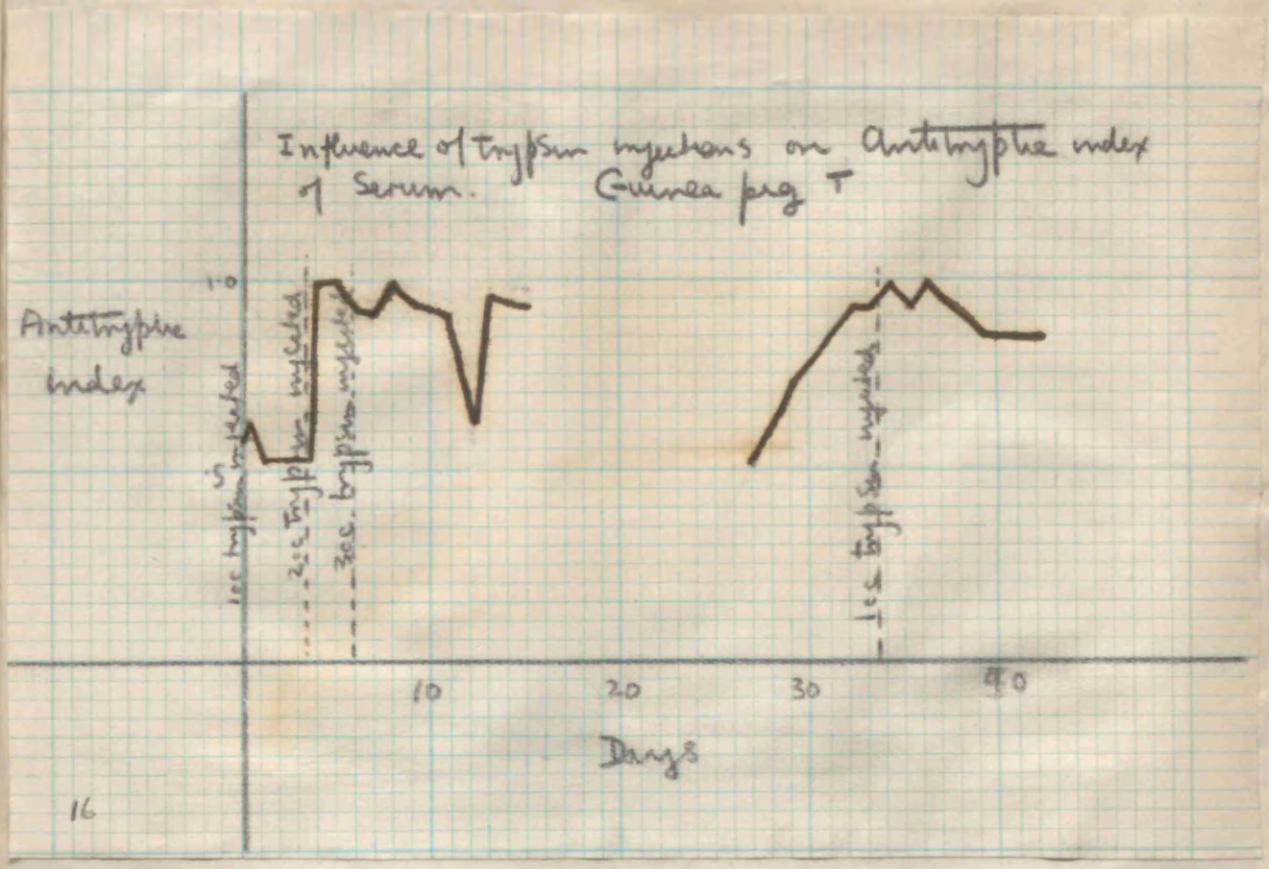


FIG. 16.

Showing 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.

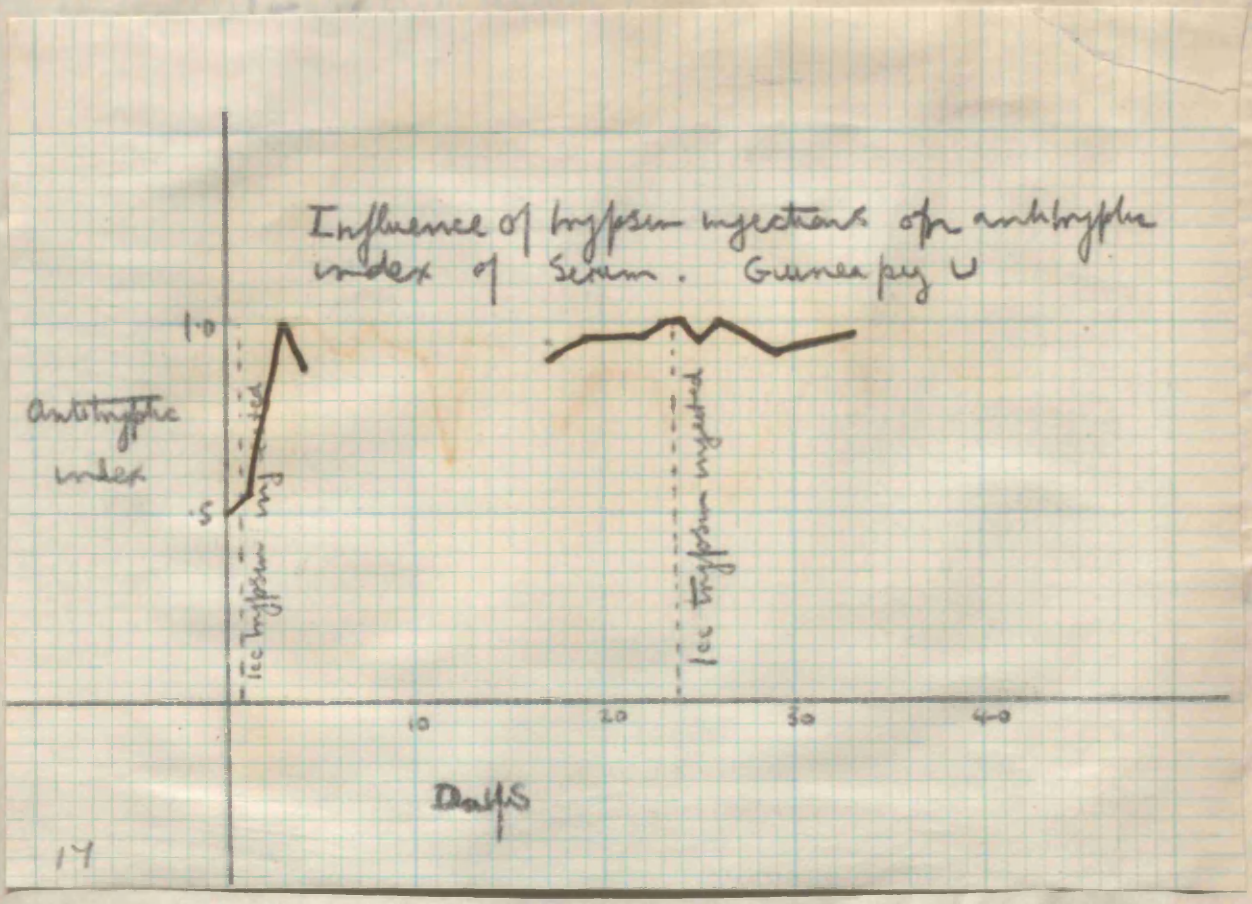


FIG. 17.

After the first injection the index rises and remains high throughout the experiment.

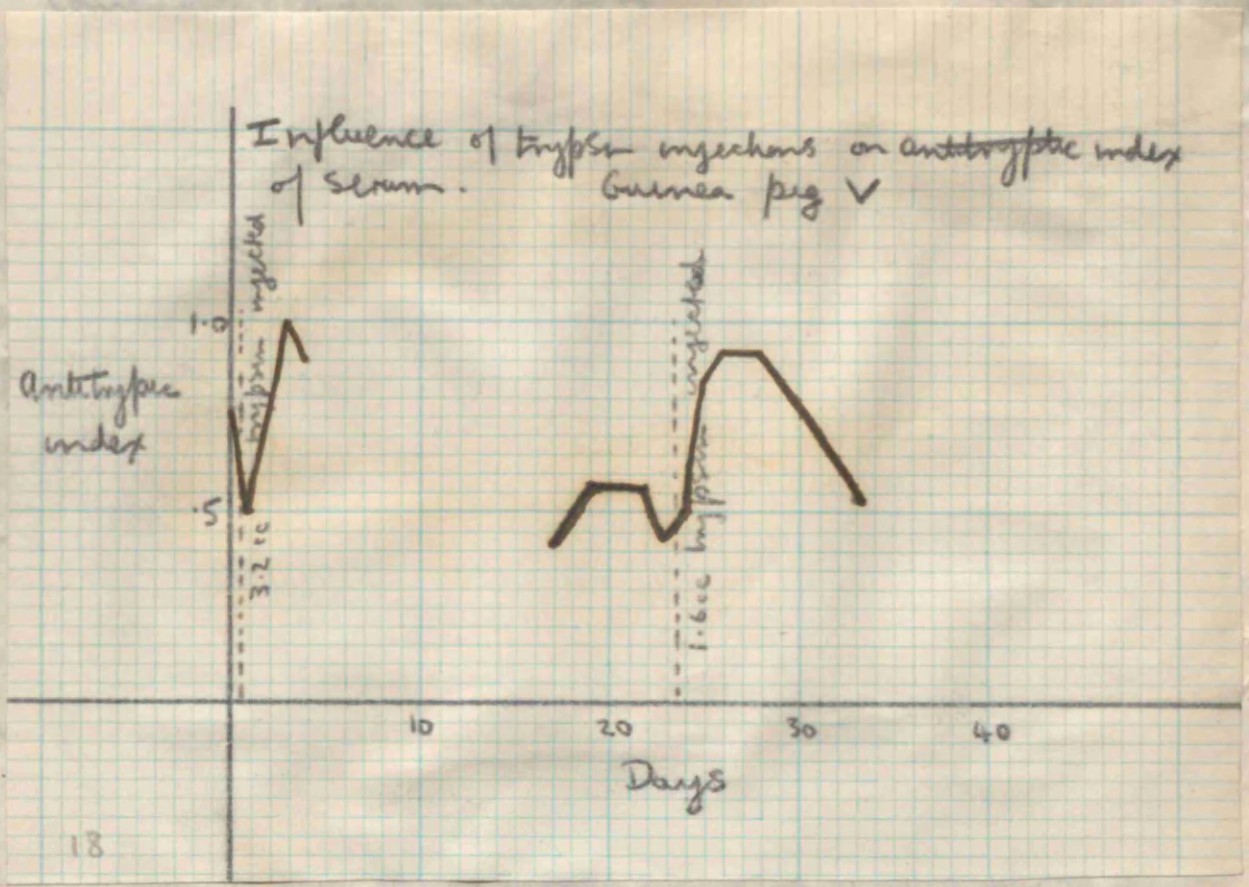


FIG. 18.

Both injections are definitely associated with a subsequent rise in index. The very large first injection produced a preliminary fall.

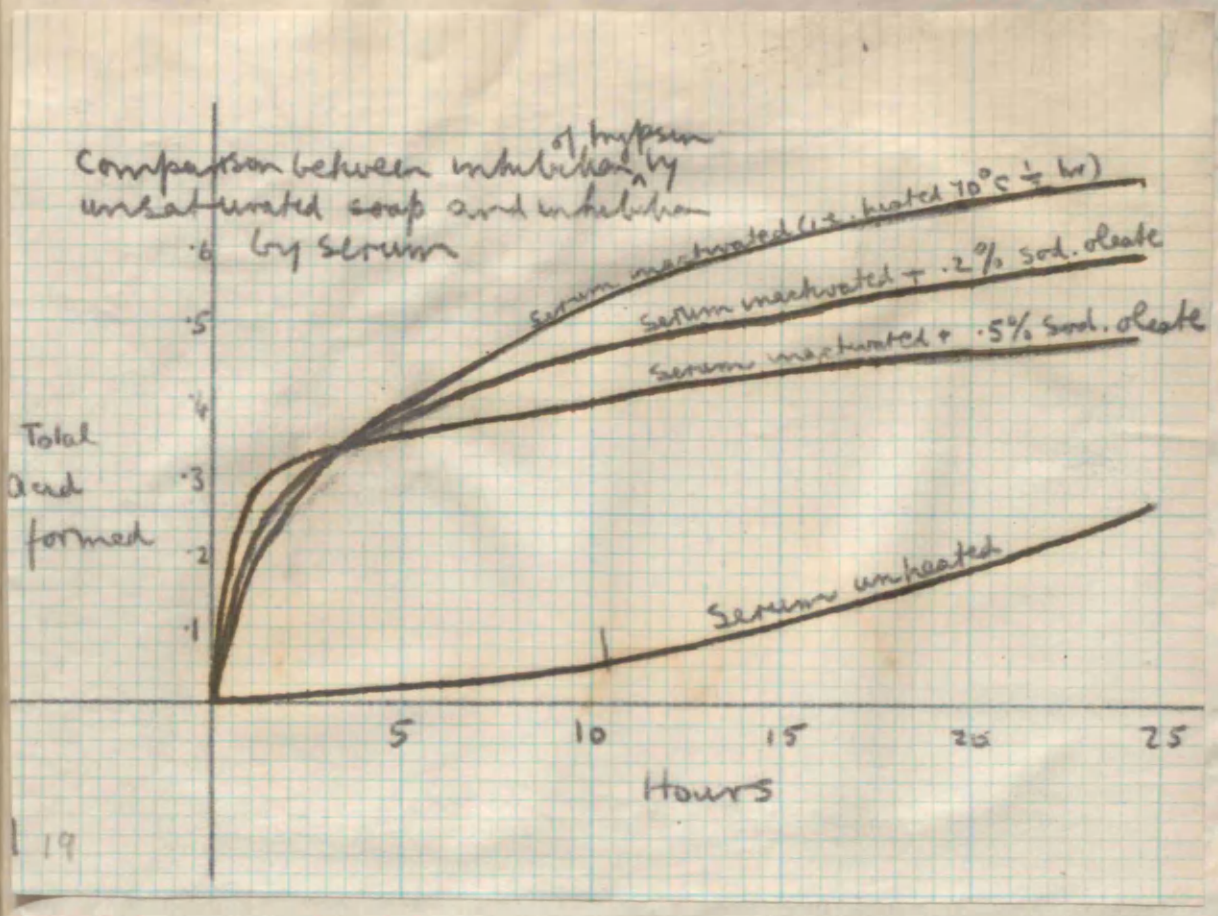


Fig. 19

Showing 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 Sorensen's technique being followed.

	Tube 1	Tube 2	Tube 3	Tube 4.
Serum	.2	.2 inactivated	.2 inactiv.	.2 inactivated
Trypsin 50% (neutralised	.4	.4	.4	.4
Buffer (pH8)	.9	.9	.9	.9
Sodium oleate 2%	0	.5	.2	0
Distilled water	.5	0	.3	.5

figures represent cc.

* If a spherical particle of volume V is dispersed into n spherical particles each of volume v, then if R = radius of large particle, and r = radius of small particle,

$$V = nv$$

$$\therefore \quad n = \frac{V}{v} = \frac{\frac{4}{3}\pi R^3}{\frac{4}{3}\pi r^3} = \left(\frac{R}{r}\right)^3$$

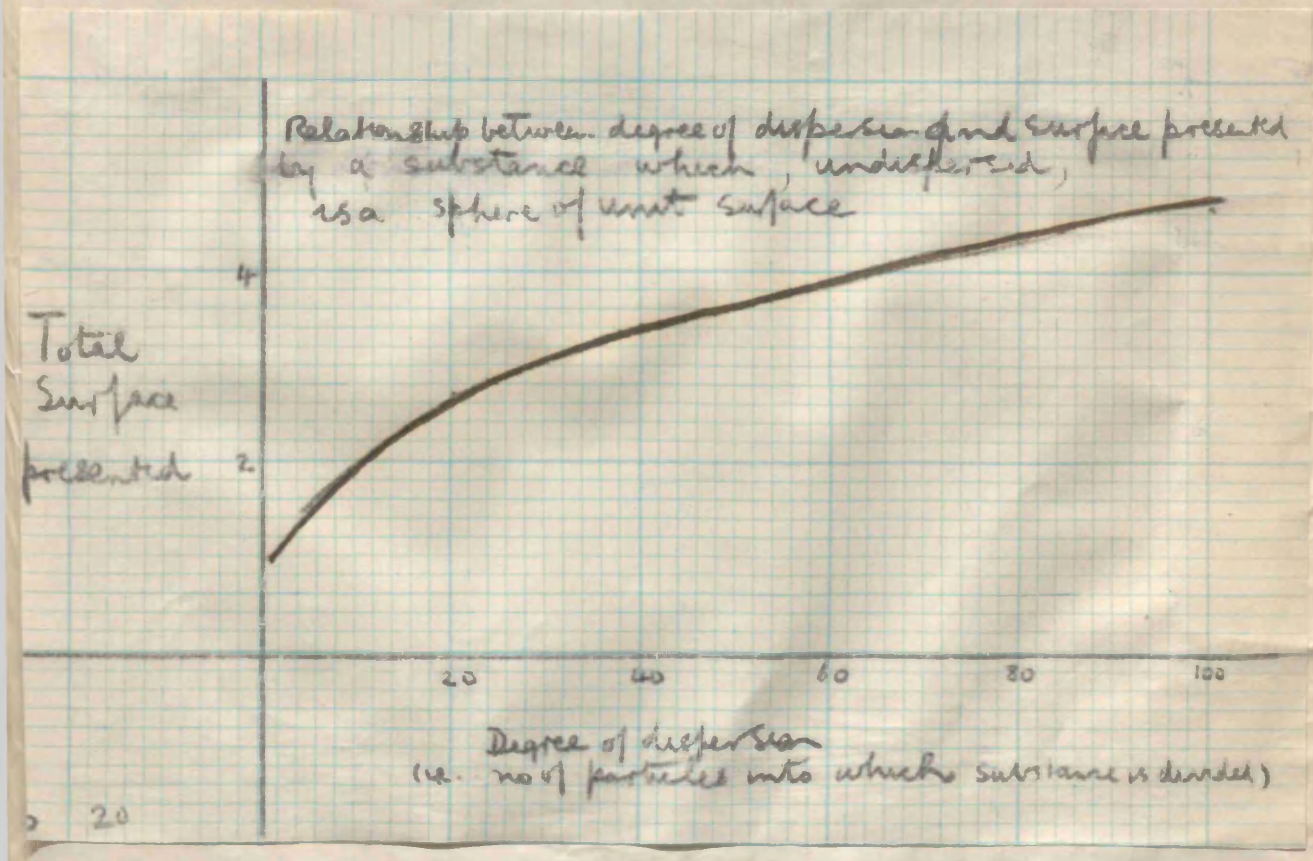
$$\frac{R}{r} = n^{\frac{1}{3}}$$

Hence $\dfrac{\text{total surface of n smaller spheres}}{\text{total surface of parent sphere}}$

$$= \frac{n.4\pi r^2}{4\pi R^2} = n\left(\frac{r}{R}\right)^2$$

$$= \frac{n}{(n^{\frac{1}{3}})^2} = \sqrt[3]{n}$$

The following Fig. (20) shows relationship between degree of dispersion of a fixed volume of a substance and the total surface presented for adsorption.



APPENDIX.

ON THE OCCURRENCE OF OTHER THAN CARBAMIC LINKAGES (CONH) 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 anti-tryptase 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 Sorensen's method it will be found that at the commencement free acid production predominates over formal acid, but that ultimately formol acid overtakes free acid.

Since the first action of trypsin is to hydrolise 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 formal acid may denote hydrolysis of the earlier products of activity.

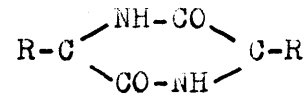
This correlation, which has hitherto been assumed, is borne out by the behaviour of pepsin, whose hydrolysis of proteins to the peptone stage only is correlated with the preponderance of free over formol acid throughout the period of digestion.

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 proteinase, but denotes the suppression of one form of activity of proteinase.

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 oxy-acids, the ether, ester, and anhydride linkages.

He did not consider it proved that such linkages did exist, however, and even quite recently Granacher (1929), who believes 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 linkages other than CONH. Clearly the hydrolysis of -CONH- by forming carboxylic and amino groups, cannot increase free acidity, but will increase formol acidity.

The linkages whose hydrolysis could produce free acid are ester and thio-ester, since on hydrolysis ester produces carboxyl and hydroxyl, and thio-ester produces carboxyl and sulphhydril. Thio-ester is an unlikely linkage, since the only sulphur amino-acid in the protein molecule is present as cystine, and not as the mercaptan cysteine.

Since hydrolysis of esters is usually the function of special enzymes (lipase and phosphates) it is necessary to eliminate the possibility of free acid formation being due to them.

In my own experiments this can readily be done, since

(1) The commefcial trypsin used was kept in the ice chest, and at a pH of 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 (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 .2 cc of a 10% solution of serum (the amount used in my titrations) is equivalent to .01 cc $\frac{N}{100}$ 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: but there is more definite evidence of this function.

Thus Harris shewed (1923) that SH groups were unmarked during peptic digestion, and Abderhalden & Schwab (1930) showed that pepsin completely hydrolysed di-leucyl-thyroxine, 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 pH of pepsin.

It is very significant that in a list of 53 polypeptides, of which 24 were hydrolysed by trypsin and 29 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 (Plummer, 1908).

This would be no coincidence if hydrolysis of other than CONH linkages were a special function of trypsin or of one of its constituents.

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 carbamic linkages of serum proteins.

If free acid formation is admitted 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°C for ½ hr.

This effect of heat is not due to coagulation, since serum heated with buffer at pH 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 glycine d leucine, but not glycine l leucine to hydrolysis by yeast erepsin (Abderhalden & Handowsky 1921). That proteins can become less hydrolysable by trypsin as a result of stereochemical alteration has been shown by Dakin & Dudley (1913) who found that partial racemisation of casein by alkali (the rotary power falling to 60%) made it resistant to trypsin: Lin Wu & 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 carbamic 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.

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