(gramme-molecules); and 22·4 = value of $P\ (V - v)$ when P and $n = 1$ and $T = 273$ (0° C.).

With the help of this equation new quantitative insight is obtained into diffusion pressure, the osmotic pressures, vapour pressures, boiling points, and melting points of solutions, and into other phenomena connected with gases and liquids. It can be shown that:

(1) Diffusion pressure of any one substance between one liquid or gas and another is proportional to the values of its partial pressure p in the one liquid or gas, and $1 - v$ in the other.

(2) The intermolecular pressure is the same for all solutions in the same solvent, up to high concentrations and with wide variations of temperature.

(3) Osmotic pressure is simply the increased intermolecular pressure required to neutralise the excess of diffusion pressure of a pure solvent inwards over that of a diluted solvent outwards, through a membrane permeable to the solvent but not to the solute which dilutes it. This excess is given, up to high concentrations, by the equation $p_0 = \frac{n}{N_1 - n} \cdot 082NT$, where p_0 = osmotic pressure, and n, N, N_1 = gramme-molecules of solute, pure solvent, and solution per litre.

(4) Diminution of vapour-pressure of the solvent and rise of vapour pressure of the solute are proportional to $\frac{n}{N_1}$.

(5) Elevation of boiling point and depression of melting point of the solvent are proportional to $\frac{n}{N_1 - n}$, and can be calculated respectively if the latent heats of boiling or melting of solvent are known.

(6) When the ratio $\frac{n}{N_1}$ is the same in different solutions with the same solvent the substances of which n denotes gramme-molecules per litre (including the solvent) are in diffusion equilibrium, and their mutual diffusion pressure is $\frac{n}{N_1} \cdot 082NT$. This diffusion pressure is of fundamental importance in physiology and physical chemistry, and has often, hitherto, been confused with osmotic pressure.

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XXXVI. STUDIES IN THE ANTITRYPTIC ACTION OF BLOOD SERUM.

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Since Hahn [1897] first definitely observed that the blood serum of normal animals produced an inhibitory action upon the digestive power of trypsin, many investigations have been made into this property, both as regards the nature of the action, and of the particular constituents of the serum which cause it, and as the result several theories have been advanced.

One represents this action as due to the presence in the serum of a true antibody in Ehrlich's sense, analogous to an antitoxin. In support of this numerous statements have been made by Achalme [1901], Jochmann and Kantorowicz [1908], Meyer [1909, 1, 2] and others, that the antitryptic power of the serum of an animal is increased as the consequence of the injection of trypsin into the animal. Meyer [1911] even goes so far as to attribute the presence of antitrypsin in serum to the proteoclastic ferments in the tissue cells acting as antigens, a view which has been reasserted more recently by Stawraky [1914]. The literature however contains much contradictory evidence upon this point, many workers having failed to confirm this increase in antitryptic power after injecting trypsin [Landsteiner, 1907; Doblin, 1909; Rosenthal, 1910, etc.]. Another explanation was given by Rosenthal [1910], who considered that the inhibition could be entirely accounted for by the presence of the products of proteoclastic action. This conclusion, however, was based upon experiments which were not very convincing, and may be dismissed as extremely unlikely; for although the products of a trypsin digest do hinder the action of the enzyme [Bayliss, 1904], yet it is only to a slight extent [Walters, 1912], whilst a very small quantity of serum is capable of producing a very marked inhibition, far too

large to be accounted for in this way. Moreover, the antitryptic power of serum is not removed by dialysis, as would be expected if it were due to amino-acids and other products of protein hydrolysis [Meyer, 1909, 1, 2; Cathcart, 1904; Stawraky, 1914].

The view which has been most generally accepted was put forward by [Hedin 1905; 1906, 1, 2; 1907], who attributed the antitryptic action to the adsorption of the enzyme by some constituent of the serum, and pointed out the similarity of the action of serum upon trypsin to the inhibition of tryptic digestion produced by the addition of animal charcoal.

With regard to the nature of the antitryptic substance in the serum, it was shown by Cathcart [1904] that this property was associated with the albumin fraction of the serum, since on fractionating the serum with ammonium sulphate the antitryptic power was retained by the albumin fraction alone, the other serum proteins being quite inactive. Later investigators have attributed the action to lipoids [Schwarz, 1909] on account of the loss of this power when serum is extracted with chloroform. Jobling and Peterson [1914] consider that it is due to the presence of unsaturated fatty acids which may be extracted from the serum by ether or chloroform, by which means they obtained an extract which possessed an actively antitryptic action. They also found that soaps of unsaturated fatty acids obtained from various sources possessed the property of inhibiting antitryptic digestion.

The antitryptic power of serum has been stated to be increased in certain diseases [Brieger and Trebing, 1908; von Bergmann and Meyer, 1908; Hort, 1909; Golla, 1909, etc.], notably in cancer, in certain acute infections such as pneumonia and typhoid fever, in certain chronic infections such as tuberculosis and syphilis, and in severe anaemias, and suggestions have been made to employ this power as a clinical test. This possibility, with the additional hope, no doubt, of a clue to the cancer problem, has inspired a large amount of research in this direction, and methods have been devised by which the antitryptic power of different sera may be compared quantitatively, the comparison with a normal serum being termed the antitryptic index of the serum.

The methods generally used for obtaining this index are the Loeffler plate method, and the Fuld-Gross method. In the former an increasing series of drops of serum are added to a drop of trypsin placed on a surface of coagulated protein, the whole is incubated and the antitryptic index determined from the relative number of drops required just to prevent the trypsin from digesting away and pitting the underlying protein surface. It is obvious

that this method is very inaccurate since it is almost impossible to judge whether the surface is indented or not.

In the Fuld-Gross method a definite quantity of caseinogen is employed as substrate, and to this is added such an amount of trypsin as will in a given time digest it to such an extent that the addition of a mixture of alcohol and acetic acid just fails to produce a precipitate. To this mixture of caseinogen and trypsin, a definite amount of serum is added and the antitryptic index is obtained by finding how much more trypsin has to be added to digest the caseinogen to the same degree as before in a given time. This method is not capable of any great accuracy since it depends upon an end point reaction, so that when the quantity of substrate (caseinogen) falls below a certain level the rate of digestion is diminished.

To overcome this Golla [1909] employed a method in which equal ferment strengths were allowed to act on a substrate (caseinogen) to which equal units of normal serum and the serum under examination had been added, and continuous observations were made of the amount of caseinogen digested, either by measuring the increase in electrical conductivity, or the loss in viscosity of the mixtures. This method, however, can only give approximately quantitative results when the two sera under comparison have almost the same action, since Hedin [1906, 1] has shown that small quantities of serum produce a relatively larger effect than large quantities, and therefore when two widely different sera are compared the result will depend upon the quantity of the sera employed for the comparison.

In the course of some experiments upon the antitryptic action of serum it became necessary to compare the action of various sera on trypsin and the unsatisfactory methods usually employed made it desirable to reinvestigate the question of the comparison of two sera using more accurate methods of measuring the antitryptic action than those generally employed.

Comparison of the antitryptic action of sera.

Methods employed.

In these experiments equal quantities of a solution of caseinogen (3 per cent. in 0·2 per cent. sodium carbonate) were warmed in a water thermostat at 37° until the temperature of the bath was attained, and the trypsin and serum were then added in the required quantities. After a definite time the quantity of caseinogen digested was estimated by the method used by Hedin [1905] in which a known volume of tannic acid solution was added, the mixture allowed to stand for twelve hours, and the nitrogen determined in a

known volume of the filtrate by the Kjeldahl method. The difference between the nitrogen which escapes precipitation with tannic acid before and after the incubation gives a measure of the digestion. As all results are comparative the numbers given in the experiments detailed below represent cc. of $N/10$ acid corresponding to the ammonia distilled off in the Kjeldahl. All the digestions were carried out in the presence of 0·5 cc. of toluene. In each case care was taken to have such an excess of caseinogen present that the action was directly proportional to the amount of active enzyme, whilst the digestions were only extended over short intervals of time, so that the caseinogen available should not fall sufficiently low to affect the rate of hydrolysis. The enzyme solutions employed were a commercial sample made by Fairchild Brothers and Forster, and preparations made by digesting minced ox or sheep pancreas at 37° in the presence of toluene and chloroform for two or three days, filtering, further digesting the filtrate, and dialysing away the products of digestion against running water. These solutions gave only a faint cloudiness with tannic acid, and only a very faint biuret reaction.

Effects of varying the amount of serum.

It was stated by Hedin [1906, 1, 2] that if trypsin and serum were mixed before being added to the substrate, the neutralising effect of the antibody was larger than when they were added separately; moreover the longer the mixture was kept before being added to the substrate, the greater, up to a certain point, was the amount of trypsin neutralised. Thus the adsorption of the enzyme required a certain time to attain its maximum. From this it seemed probable that if trypsin and serum were added together to caseinogen, the rate of digestion would gradually decrease. The following experiment was done to see if such were the case, goat serum being employed.

The mixtures A, B, and C were incubated at 37°, and 50 cc. of each removed at the intervals stated, 30 cc. tannic acid added and the nitrogen estimated in 60 cc. of the filtrate; the increase in nitrogen is given in cc. 0·1 N acid.

A. 250 cc. caseinogen, 7·0 cc. water, 2 cc. trypsin (ox pancreas).
B. 250 „ 6·5 „ 2 „ 0·5 cc. serum
C. 250 „ 6·0 „ 2 „ 1·0 „

Time hours	Total			Rate per hour during preceding interval		
	A	B	C	A	B	C
1	3·4	2·2	1·6	3·4	2·2	1·6
2	6·4	4·2	2·7	3·0	2·0	1·1
4	12·8	8·0	4·7	3·2	1·9	1·0
8	—	15·6	9·0	—	1·9	1·1

The digestion of the caseinogen proceeded in each case at a rate constant within the error of experiment excepting perhaps for a slight irregularity in the first hour after mixing the solutions.

Under these conditions, therefore, a definite rate of reaction may be obtained.

Hedin found that small quantities of serum had a relatively larger inhibitory effect upon the action of trypsin than large quantities; moreover he observed that no amount of serum was able completely to prevent the action of the trypsin. In the experiments quoted however only a few different concentrations of serum were employed. The following experiments were carried out with a large number of concentrations of two sera of different origin to observe the course of the inhibition in each case, and to ascertain whether a definite quantitative comparison of the two sera could be obtained.

Sheep and dog serum were employed.

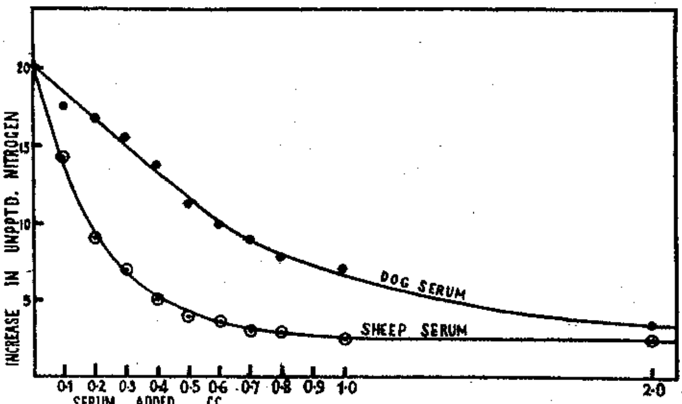
Each mixture consisted of 50 cc. caseinogen solution previously warmed to 37°, 2 cc. trypsin and N cc. serum, the total volume being made up to 55 cc. with water. After four hours 30 cc. of tannic acid were added and the nitrogen determined in 70 cc. of the filtrate. The results are given in the following table.

	Serum added in cc.	Soluble nitrogen produced	
		Sheep serum	Dog serum
1	0	20·4	20·4
2	0·1	14·5	17·8
3	0·2	9·1	17·1
4	0·3	7·0	15·6
5	0·4	5·2	13·8
6	0·5	4·1	11·5
7	0·6	3·7	10·0
8	0·7	3·3	9·0
9	0·8	3·2	8·0
10	1·0	2·6	6·1
11	2·0	2·6	3·7
12	3·0	2·7	3·9

1 cc. trypsin in the same volume in absence of serum gave 10·1 cc., showing that the rate in the absence of serum was proportional to the quantity of trypsin present, and that therefore an excess of caseinogen was present in every case.

From these results it is seen that with increasing amounts of serum the relative inhibition produced by each successive addition gradually falls off, until a point is reached beyond which further additions produce no effect and the curve runs parallel to the base line. Complete inhibition cannot be produced.

The figures are shown graphically on the accompanying diagram.



They show further that the sheep serum produced a much greater inhibitory effect than did the dog serum, both in the early stages and in the final rate. It is apparent, however, that a quantitative comparison of the actions of the two sera cannot be obtained by adding a definite amount of serum to a trypsin digest and comparing the action, since the comparison depends upon which part of the curves representing the reaction are used.

A similar experiment was done with another sample of trypsin to try the effect of very small quantities of serum. The sheep serum was diluted ten times with water, and the dog serum with an equal amount of water.

In each case 2 cc. trypsin were employed and 50 cc. caseinogen, the total volume being 55 cc. The mixtures were incubated for four hours at 37°. The figures in the first column give the volumes of undiluted serum contained in the mixtures.

Serum added cc.	Soluble nitrogen produced	
	Sheep serum	Dog serum
0	11·8	11·8
0·02	12·2	—
0·04	11·6	12·5
0·05	—	12·5
0·06	10·3	—
0·08	9·5	—
0·10	8·6	12·3
0·15	6·5	—
0·20	4·9	11·8
0·25	3·6	—
0·4	—	9·2
1·0	—	4·0

1 cc. trypsin in absence of serum gave 5·5 cc.

The experiment shows that with very small quantities of serum very irregular results were obtained. In fact no definite inhibition was observed with the sheep serum until 0·06 cc. had been added, whilst with the dog serum small quantities produced a slight rise. This may be due to the reaction of the serum since a similar slight rise was produced by adding a very dilute mixture of phosphates so that the mixture when diluted to the same volume as the digest gave the same tint with a suitable indicator, as did 0·05 cc. of serum diluted to the same volume.

The action of the same sample of serum upon two different trypsin solutions of the same digestive power.

During the experiments certain discordant results seemed to point to the fact that the different solutions of trypsin were not always inhibited to the same extent by a single specimen of serum, even when the two solutions of trypsin in the absence of serum were capable of digesting the same quantity of caseinogen in the same interval of time. Experiments were carried out therefore upon this point.

I. One sample of trypsin (1) was prepared from ox pancreas, whilst the other (2) was a commercial preparation of Fairchild's trypsin.

The required volumes of the two trypsin solutions which digested the same amount of caseinogen in the same time were determined in preliminary experiments, which also showed that the quantity of caseinogen was such as not to influence the rate of reaction. In each case 50 cc. of caseinogen were employed, the total volume being made to 60 cc. Sheep serum was used and the mixtures incubated at 37° for five hours.

Serum added cc.	Digestion with	
	Trypsin 1 1·45 cc.	Trypsin 2 1 cc.
0	20·0	19·9
0·1	14·1	10·0
0·2	10·3	7·0
0·5	5·0	4·3

II. A similar experiment was carried out with two different samples of trypsin from ox-pancreas and sheep serum—five hours at 37°.

Serum added cc.	Digestion with	
	Trypsin A 2 cc.	Trypsin B 1·6 cc.
0	17·6	17·7
0·1	13·1	11·3
0·2	11·0	7·1
0·5	5·9	4·0

III. A similar experiment with goat serum and two specimens of trypsin (ox pancreas)—three hours at 37°.

Serum added cc.	Trypsin C 4 cc.	Trypsin D 1 cc.
0	12·5	12·6
0·2	8·2	7·6
0·4	5·7	5·7
0·6	4·6	5·0
2·0	3·6	3·5

IV. A similar experiment with goat serum and two more trypsin preparations from ox pancreas—three hours at 37°.

Serum added cc.	Trypsin E 0·7 cc.	Trypsin F 2·6 cc.
0	16·0	16·5
0·2	12·2	12·0
0·4	9·8	10·3
0·6	7·4	8·9
1·0	4·8	7·3
4·0	4·8	7·3

In experiments I and II the two trypsin solutions in the absence of serum digested the same quantity of caseinogen, yet the effect of adding varying quantities of the serum to each was very different. On the other hand in experiment III the effect on the two preparations was practically the same. In experiment IV, again, a considerable difference was noticed as the quantity of serum was increased.

These experiments show, therefore, that equal quantities of a serum may not always produce the same inhibitory effect upon different preparations of trypsin although the enzyme solutions have the same proteoclastic power as measured by the digestion of caseinogen.

In obtaining the antitryptic index of a serum a sample of enzyme is generally employed of such a strength that it will just digest away a given amount of caseinogen in a given time, as in the Fuld-Gross method. As shown above the effect of serum upon this may vary with the particular sample of enzyme used, and it is obvious therefore that the comparison of the antitryptic action of a different serum made at different times with different trypsin preparations, even though of standard strength, may yield erroneous results, and many of the contradictory results found in the literature may probably arise in this way.

From these experiments the conclusion must be drawn that the antitryptic action of the two sera may be compared only by observing the effect of varying quantities of each upon the same sample of trypsin, and that only a qualitative comparison is then possible.

Is this action due to the presence of zymoid in the enzyme preparations?

The different inhibitory powers of the same serum upon different preparations of trypsin might be explained by the presence in the solution of enzyme so altered that it is incapable of digesting caseinogen but is yet able to enter into association with the inhibitory substance in the serum. Evidence of the existence of a modified enzyme which would still combine with antibody but had lost the power of clotting milk, has been put forward by Korschun [1902] in the case of rennet, whilst Bayliss [1904] obtained facts which suggested the production of a similar modification of trypsin by warming to 25° for a day or two. For these modifications Bayliss suggested the name of zymoid. Furthermore Bearn and Cramer [1907] stated that when trypsin was heated to about 50° or 60° the presence of a zymoid was apparent. Experiments were carried out therefore, to see if trypsin could be altered in this manner by heating.

A solution of the trypsin was heated for two hours at 60°, and thus rendered inactive; the addition of this to a mixture of caseinogen, trypsin and serum was without effect upon the quantity of caseinogen digested. Solutions of trypsin were kept at different temperatures, between 30° and 40° for varying times so as to lessen their activity, and quantities of each of similar digestive power to a given volume of the original trypsin, were tested upon caseinogen in the presence of varying quantities of the same serum. In every case it was found that the power of associating with the serum was lost in proportion to the loss of activity. No evidence therefore could be obtained of the presence of zymoid.

Variations in the antitryptic action of serum.

As already stated the antitryptic action of serum has been proposed for diagnostic purposes in disease, a proposal which assumes that the sera of normal animals of the same species have the same inhibitory action upon trypsin. On the other hand it has been contended that considerable variations do occur in the action of normal serum even in the same animal.

In the following experiment the inhibitory action of the serum from three dogs was compared to see if naturally occurring differences could be found. To avoid errors due to different trypsin preparations, the digestions were carried out at the same time using the same trypsin solution. Parallel series of mixtures were incubated for three hours at 37°, and the extent of digestion of the caseinogen measured as before. Each mixture contained 50 cc. casein-

ogen, 5 cc. trypsin and varying amounts of fresh serum, the total volume being constant. The results are given in the table.

Two of the dogs (1 and 2) were young dogs, about four months of age, and the third was a fully grown female. For comparison also the action of the serum of a goat is given.

Serum added	Soluble nitrogen produced			
cc.	Dog 1	Dog 2	Dog 3	Goat
0	12·6	12·6	12·6	12·6
0·2	10·4	9·6	10·2	8·2
0·4	7·6	7·2	7·6	5·7
1·0	5·1	5·2	5·5	4·0
2·0	4·2	4·2	4·4	3·4
3·0	4·1	4·0	4·2	3·2

The inhibitions produced by the sera from the three dogs were thus substantially the same in every case; the goat serum, however, showed a more marked difference from the others; in an earlier experiment we have already seen that serum from dog and sheep were different in their behaviour.

Attempted immunisation against trypsin.

The question whether the injection of enzyme into an experimental animal is followed by the production of true antibodies has given rise to a considerable amount of work. It has been pointed out by Bayliss [1914, p. 127], that the evidence tends to show that enzymes are not proteins, and as it is very doubtful whether any other substances are capable of acting as antigens and producing antibodies when injected into living organisms, these are *prima facie* grounds for doubting the capacity of enzymes to act as antigens.

The first "anti-enzyme" was described by Hildebrandt [1893], who stated that the injection of emulsin into animals was followed by the appearance in the serum of the power of inhibiting the action of emulsin on glucosides. Bayliss [1912], however, found that this action was due to the diminution of acidity of the mixture, and he showed that when the hydrogen ion concentration was brought back to its original value the effect disappeared, whilst the same inhibitory effect could be produced by making the emulsin to the same hydrogen ion concentration as that produced by adding the immune serum. In the case of the "anti-rennet" produced by immunising animals with rennet, Hedin [1911, 1912] states that it is not a true antibody, whilst Thaysen [1915] says that it may be completely accounted for partly by adsorption of the enzyme, and partly by the effect of change of hydrogen ion concentration.

In the case of trypsin, as stated earlier, many statements have been made to show that an antitrypsin can be produced by the injection of the enzyme, whilst on the other hand there are recorded numerous failures to obtain it. In the case of trypsin the difficulty lies in the presence of the antitryptic property in normal serum. In reviewing the literature it appeared probable that many of the contradictory results might be accounted for by several causes. In many cases the "immune" sera from the experimental animals were tested against the serum of a normal animal of the same species, and it is possible that the antitryptic power of normal animals might vary with the individual. On the other hand, if the blood of the same animal were employed and its antitryptic activity tested before and after injection, the tests would be made usually by the Fuld-Gross method with the fresh serum and thus at different times and possibly with different specimens of trypsin. The latter although of standard activity as regards action on the substrate, might, as has already been shown, be adsorbed to a different degree by the serum, so that it might appear as if the activity of the serum had been altered. In the following experiments attempts have been made to avoid these difficulties by testing the serum of the animal before and after injection against the same trypsin solution. The solutions of trypsin used for injection were rendered as sterile as possible by keeping them for a day or two in the presence of toluene, the latter being then removed by a rapid current of air previously passed through sterile cotton wool. Before the injections were made the experimental animal was bled generally twice with an interval of a week, to obtain two samples of normal serum, which were preserved as described below. The solutions were then injected intravenously, several injections being made at suitable intervals. After a certain time the animal was again bled and the serum collected. In this way two specimens were obtained of the normal serum of the animal for comparison with the serum of the same animal after immunisation, the object being to try their inhibitory effect upon the same sample of trypsin, and to ascertain whether any difference in activity was produced and if so whether this difference was greater than such difference as might exist between the two samples of normal serum. In each experiment the antitryptic action of the serum was tested against the same trypsin preparation as was used for the injection. The sera as they were obtained were dried rapidly by the method recommended by Chapman [1905]. This was done by spreading 10 cc. over the surface of a tared flat glass dish, which was then kept in an evacuated desiccator containing sulphuric acid and placed in an incubator at a temperature of 37°. Within 24 hours the serum dried up. The dish and

contents were then weighed and the dried mass powdered and preserved in a dry atmosphere[1] in the ice chest until required, when the powder was dissolved in water and made up to the volume of the original serum from which it had come. The serum when preserved in this manner retains its activity for a long time. In every case the sera were neutralised to litmus paper by the addition of sodium dihydrogen phosphate, before being tested. The tests were made by adding an increasing series of quantities to mixtures of trypsin and caseinogen, incubating at 37°, precipitating with tannic acid, and estimating the nitrogen in the filtrate. The following experiments with the same samples of serum dried and undried show that the drying had not produced any appreciable effect upon the antitryptic activity. The undried serum was kept on ice during the time taken in the drying of the other portion.

I. Each mixture consisted of 25 cc. caseinogen, 1·2 cc. trypsin (sheep pancreas), and varying amounts of the serum, the total volume being made to 30 cc. with water. After 18 hours at 37°, 15 cc. of tannic acid were added to each and the nitrogen estimated in 20 cc. of the filtrate. In the absence of serum 30·2 cc. were obtained.

Serum added cc.	Digestion with	
	Dried serum	Undried serum
0·5	6·0	6·0
1·0	3·6	4·0
2·0	3·1	3·2

II. A similar experiment with 1·2 cc. trypsin (ox pancreas) and sheep serum. In the absence of serum the digestion corresponded to 24·5 cc.

Serum added cc.	Digestion with	
	Dried serum	Undried serum
0·05	22·7	23·1
0·10	21·7	21·3
0·30	14·1	13·9
0·50	10·1	10·1
1·00	6·1	6·4

III. A goat (I) was injected intravenously at weekly intervals with the following quantities of trypsin (commercial sample of Grübler) dissolved in

[1] On several occasions the serum powder when preserved for some time without special precautions being taken to keep it dry, was found to have become partially insoluble in water. Professor Chapman has informed me that he has noticed the same thing, and he attributes it to the effect of moisture which causes the proteins of the serum to coagulate. This was confirmed by an experiment in which two portions of the same serum powder were preserved, the one in a desiccator containing calcium chloride, and the other in moist air. In the latter case the serum soon became insoluble, whilst in the former it remained perfectly soluble. When precautions were taken to keep the powders dry this insolubility was never observed.

70 cc. of normal saline in each case; 0·4, 0·4, 0·4, 0·6, 1, 1, 1, 1, 1, g. After another week a sample of serum was obtained. Two samples of normal serum were collected before the injections for comparison. The results are given in the following table. Each mixture contained 25 cc. caseinogen, 5 cc. trypsin (1 g. in 50 cc.) and the quantities of serum given below, the total volume being constant.

Serum added	Digestion in the presence of serum		
	Before 1	Before 2	After injection
0·5	17·0	17·4	18·0
1·0	10·1	10·3	10·5

In absence of serum digestion = 28·6.

IV. A goat (II) received five intravenous injections of trypsin solution prepared from ox pancreas, 5, 7·5, 10, 20, 20 cc. being injected at weekly intervals. Two samples of normal and one of "immune" serum were collected.

The action on the enzyme is given below, each mixture containing 2·5 cc. trypsin, 25 cc. caseinogen and varying amounts of the serum. The mixtures were incubated at 37° for 18 hours.

Serum added cc.	Digestion in presence of serum		
	Before 1	Before 2	After injection
0·5	9·1	8·7	9·4
5	2·5	2·4	3·1

In the absence of serum 18·8 cc. were obtained.

V. A goat (III) received three injections intravenously of 20 cc. of trypsin (ox pancreas). Only one sample of normal serum was used. To test the antitryptic activity 2·5 cc. trypsin and 25 cc. caseinogen were digested with the sera, as given below, at 37° for 18 hours.

Serum added cc.	Digestion in the presence of serum	
	Before	After injection
0·01	18·0	18·0
0·05	17·5	17·5
0·10	16·7	16·5
0·25	15·2	14·6
0·50	10·1	9·1
1·00	6·1	5·7

In the absence of serum 18·9 cc. were obtained.

VI. In this experiment a goat was injected intraperitoneally with 20 cc. of trypsin (ox pancreas) on three successive days and was bled after seven days. The injections were not followed by any untoward results in the animal. The mixtures for testing the sera contained 2·5 cc. trypsin, 25 cc. caseinogen and varying amounts of serum, and were kept at 37° for 16 hours.

Serum added cc.	Digestion in the presence of serum		
	Before 1	Before 2	After injection
0·01	18·4	18·1	18·2
0·05	16·8	16·4	17·5
0·10	15·9	15·2	16·3
0·25	10·6	11·0	13·0
0·50	6·5	6·2	6·2
1·00	5·0	4·8	4·5
2·50	4·7	4·6	4·1
5·00	3·9	3·1	3·4

In absence of serum 18·9 cc. were obtained.

Intraperitoneal injection had produced no increased antitryptic action.

The following two experiments were made upon sheep with a sample of trypsin prepared from the pancreas of an animal of the same species.

VII. A sheep (I) was injected intravenously at weekly intervals with 40, 70, 75 cc. of trypsin solution (sheep pancreas). The mixtures for testing the sera containing 1·2 cc. trypsin, 25 cc. caseinogen and varying volumes of serum were incubated at 37° for 18 hours.

Serum added cc.	Digestion in the presence of serum		
	Before 1	Before 2	After injection
0·5	16·2	17·6	16·0
1·0	3·2	3·3	4·0
2·0	2·9	3·1	3·1

In absence of serum—30·2 cc.

The animal then received two further injections of 75 and 175 cc. trypsin.

Serum added cc.	Digestion	
	Before	After injection
0·1	24·8	24·0
0·5	17·7	18·6
1·0	5·9	5·1
2·0	3·0	3·3

In the absence of serum = 29·0 cc.

VIII. A sheep (II) was injected intravenously with a solution of trypsin from sheep pancreas receiving the following doses at weekly intervals—40, 70, 75, 75 cc.

Serum added cc.	Digestion in presence of serum		
	Before 1	Before 2	After injection
0·1	26·5	26·5	26·0
0·2	20·1	24·0	24·4
0·4	19·6	20·5	21·0
1·0	10·3	10·5	11·6
2·0	5·9	6·1	6·3

In absence of serum = 30 cc.

A further injection of 200 cc. of a fresh preparation from sheep pancreas was then given and the serum collected after 14 days and tested against the original normal serum.

Serum added cc.	Digestion	
	Before 1	After injection
0·1	8·3	9·1
0·25	4·7	4·8
0·50	4·5	4·4
1·00	4·5	4·5
1·50	4·2	4·1

In absence of serum = 23·4 cc.

In all these experiments there was no greater difference observed between the antitryptic action of the serum of the animals before and after the injections, than was found between the two samples of serum from the same animal before injection. There was therefore no evidence of the production of any antitrypsin by the injection of the enzyme.

These experiments were extended by examining the sera of the animals to see if the injections had given rise to the formation of a specific precipitin. The serum of goat (I) gave no precipitate when mixed with the trypsin solution used for the injection; on the other hand, after the injection of the trypsin the serum gave a precipitate with dilutions of the trypsin down to 1 in 1000.

The serum of goat (II) injected with ox pancreas trypsin before injection gave no precipitate with the trypsin solution. After injection, however, it gave a distinct precipitate when mixed with the trypsin solution in dilutions down to 1 in 100.

Similarly with goat (III) intraperitoneal injections with trypsin (ox pancreas) produced a faint precipitin reaction.

The sera of the two sheep before injection were also compared with the sera obtained after the final injection of trypsin. On mixing with the solution of trypsin employed, a distinct precipitate was noticed in the presence of 1/100 and 1/1000 respectively of enzyme solution, whereas with the normal sera the mixture remained quite clear.

The last two cases are of interest as an instance of a precipitin being produced by injecting the extract of the pancreas of an animal of the same species.

From these results it is seen that although there was no increased antitryptic power observed in the serum due to the injection, yet a precipitin was produced. They thus show a similarity to the experiments quoted by Bayliss [1912] with emulsin where a precipitin was also produced when enzyme preparations were injected, although he was able to demonstrate that

no true antibody to the enzyme was produced at the same time, and a similar conclusion must be drawn here as was done by Bayliss that the antigen to this precipitin was most probably some protein accompanying the trypsin and not the enzyme itself. These experiments contain therefore additional evidence that the enzyme trypsin is not a protein.

The results obtained do not bear out the statement of Porter [1914] that trypsin is inhibited by a specific precipitate. In her case the effect was compared of adding egg white to a mixture of trypsin and serum from a rabbit immunised with egg white, with that produced by adding egg white to a mixture of trypsin and normal serum. The differences shown were exceedingly small however, and no attempt was made to ascertain whether they could not be accounted for by changes in alkalinity of the mixture.

Summary.

The observations herein recorded confirm those of Hedin and show that when increasing amounts of blood serum are added to a mixture of trypsin and excess of caseinogen the inhibitory effect on the trypsin, produced by equal increments of serum, decreases as the total quantity of serum present gets larger, until a point is reached beyond which the further addition of serum produces no further decrease in the action of the enzyme. In no case was it found possible completely to inhibit the action of trypsin by the addition of serum.

Different preparations of trypsin of equal digestive power upon caseinogen are not always inhibited to the same extent by the addition of equal quantities of the same serum.

Experiments to ascertain whether this was due to the presence of a zymoid—*i.e.* altered enzyme which had lost its power of hydrolysing caseinogen but was still able to form an association with the antibody in the serum—were unsuccessful.

The experiments show that in making a comparison of the effect of different sera upon trypsin, it is necessary to employ the same individual preparation of trypsin, and even then only a qualitative result is possible. The so-called antitryptic index, therefore, is not a definite constant for a serum.

In the experiments quoted in this paper the sera of normal animals of the same species did not show any marked variations in "antitryptic action," nor was any great change observed in the serum drawn at different times from the same animal; on the other hand, marked differences were observed between the actions of the serum from different animals (goat, sheep, dog).

It is shown that the possible errors in the methods usually employed for

comparing the effect of sera upon trypsin may account for the contradictory results obtained by so many workers upon the question whether the injection of trypsin into experimental animals is followed by an increase in the antitryptic action of the serum.

Experiments are recorded in which attempts were made to increase the antitryptic action of the serum of an animal by injecting the enzyme intravenously or intraperitoneally, precautions being taken in testing the serum to avoid the errors suggested by the previous experiments.

The action of the "immune" serum in each case was compared with that of two samples of serum drawn from the same animal before the injections were made, and the comparison was made in parallel series of increasing amounts of serum, the same solution of trypsin being employed and the experiments being carried out at the same time. In no case was any increased action observed to follow the injections.

The injections, however, caused the production of a precipitin, which must therefore be attributed to the presence of protein accompanying the enzyme and not to the enzyme itself. These experiments, therefore, bring forward some evidence that trypsin is not a protein.

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