OBSERVATIONS ON LEUCOPROTEASE AND "ANTI-LEUCOPROTEASE."

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2 Figures.

According to Kossel (1888) Fritz Müller discovered that phthisical sputum digests fibrin and coagulated albumen in weakly alkaline media, but the fact that antiseptic pus digests proteins seems to have been first observed by Leber.

Achalme (1899) separated proteolytic ferments from pus from various sources and Erben (1903) and Schumm (1904) drew attention to the notable quantity of albumoses in blood from cases of myologenous leukaemia which had been incubated, and the relative absence of this proteolysis in the incubated blood from cases of lymphatic leukaemia. Ascoli and Mareschi (1901) showed that the peritoneal exudate produced by aleuronat underwent autolysis under aseptic conditions.

More recently the subject of a proteolytic ferment in leucocytes has been studied by a number of authors amongst which the principal are Opie (1905 and 1906), Müller and Jochmann (1906 I. and II.), Jochmann and Lockermann (1908), and Fiessinger and Marie (1909).

All agree as to the presence of a tryptic ferment in the polynuclears but the existence of another protease acting in feebly acid solutions in lymphocytes described by Opie is questioned by Müller and Jochmann and Fiessinger and Marie.

https://doi.org/10.1017/S0022172400042935 Published online by Cambridge University Press

Opie working with sterile exudates obtained by injection of aleuronat into pleural cavities of dogs, as well as with pus from turpentine-produced abscesses in various animals, found that in the leucocytes collected from such artificially produced exudates by centrifugalisation two ferments may be demonstrated. The main point of the proof of the separateness of these ferments is that in an alcohol ether dried preparation only one remains. He calls the two ferments *lymphoprotease* and *leucoprotease.*

Lymphoprotease is associated with mononuclear leucocytes and resembles pepsin in acting at low degrees of acidity but it is not pepsin as it fails to act in 0·2 % HCl though it will do so in feebler dilutions. It is inhibited by alkalinity but not by the serum as such.

Leucoprotease is associated with the polymorpho-nucleated cells and is a tryptic-like ferment acting best in faintly alkaline but well also in neutral media; Opie does not think this is trypsin for it is very weak compared with the latter. It is held in check by normal sera. It is not destroyed by treatment with alcohol and ether and drying. The various results of Opie and others with regard to other points will be found in their respective sections.

Lymphoprotease was also obtained by Opie and Barker (1907) by injecting tubercle bacilli into the pleural cavity of dogs. It has been previously indicated that its origin from lymphocytes has been disputed but as my own experiments deal only with leucoprotease from human pus the question will not be entered into.

I was anxious to inquire into certain points as regards the working of the so-called "anti-body" manifested in normal sera, and especially to see if one could increase this anti-proteolytic power by immunisation and if there could be found any evidence in favour of or against the idea that any anti-effect is due to a true anti-body similar to those concerned in production of immunity to various toxins and so forth. This work is not intended to be complete—the subject is in itself rather complicated and it has been difficult for me even with such facilities as I have had at the Lister Institute to get sufficient material—hence I have merely been able to cover part of the ground, the remainder of which I hope to attempt on a future occasion. Two difficulties which I have found to render accurate work on certain points so far almost impossible are, firstly the ferment in my preparations is very weak, and secondly it is always accompanied by large amounts of proteid, and I have not so far been able to devise a satisfactory method, that is to say necessarily a conservative method, by which I can at all purify the preparation.

Methods.

The material I have examined was derived from thirteen specimens of empyema pus. In the majority of experiments the material was, after screening off as much blood clot, fibrin, etc. as possible, centrifuged and washed with normal saline three or four times. The final debris was shaken well with methylated spirit, this either filtered or centrifuged, and pipetted off and then absolute alcohol and finally alcohol-free dried ether used in the same way. The debris from this process was collected in flat trays, fanned vigorously for a while and powdered, then placed in a desiccator, till apparently completely dry, if necessary re-powdered, and then kept in the desiccator till required. Here apparently it is little affected as regards its activity by lapse of time. The final product is a very fine dry powder which has a colour varying from white to pink, according to the amount of blood clot present.

For estimating the activity of the enzyme the following process, which is similar to that used by Hedin in his experiments with trypsin, was employed. This only differs in detail and the form of precipitation of the proteid from that of Opie. Measured amounts of ferment preparation, either by weighing quantities dried of the powder or pipetting suspensions or extracts, were taken into suitable stoppered bottles of about 150 c.c. capacity. To this was added the substrate used, either 25 % boiled solution of casein with toluene, or boiled fibrin, or gelatin, or egg albumen. In testing the effect of serum this was also added. Controls were made with the enzyme preparation heated in moist condition above 90° C. for several minutes. To each flask a definite amount of toluene was added as preservative and the flasks well shaken and incubated in a hot room, where they were kept in motion by an electric shaker. At the end of the digestion period the flasks were taken off, cooled to the room temperature, and precipitated by equal amounts of the tannic acid solution. The mixture was well shaken and filtered. The filter papers should be all of one kind and the same size, as also should be the filter funnels. The temperature at which the precipitation was carried out seemed to be important, as also did the time the materials were left in contact before filtering. A feature of interest noticed was that a perfectly clear filtrate, which subsequent estimation proved to contain large amounts of proteid, usually shows after some minutes a faint cloudiness, very hard to separate. This does not occur in the control flasks and the amount of proteid in

this precipitate is negligible, as whether one refilters to clearness or not, the results were within my experimental error.

Aliquot parts of these filtrates were Kjeldahled in the usual way and the ammonia estimated. The index of activity is furnished by the amount of ammonia in the distillates. The figures are expressed as ammonia represented by c.c. N/10 acid.

The potency of my preparations was not great and the following two experiments which are given in detail illustrate the order of activity obtained.

Powder No. 4. Time of digestion 5 days. Substrate casein 2·5 % in 0·25 % sodium carbonate solution.

		Nitrogen as c.c. of deci-normal sulph. acid in 100 c.c. of filtrate
A. Substrate 100 c.c. + powder 0·04 gm.	=	16·9
B. Substrate 100 c.c. + powder 0·04 gm. (heated)	=	4·1

Each flask precipitated with 100 c.c. tannic acid solution. 100 c.c. of filtrate taken for estimation.

Powder No. 6. Time of digestion 5 days. Substrate casein 2·5 % in 0·25 % sodium carbonate solution.

		Nitrogen as c.c. of deci-normal sulph. acid in 50 c.c. of filtrate
A. Substrate 50 c.c. + powder 0·4 gm.	=	30·6
B. Substrate 50 c.c. + powder 0·4 gm. (heated)	=	3·0

Each flask precipitated with 50 c.c. tannic acid solution. 50 c.c. of filtrate taken for estimation.

Influence of Amount of Enzyme upon the Rate of Action.

The following experiments were undertaken with a view to inquire into the rate of change brought about by the enzyme in the alcohol ether dried preparation. Bottles containing varying amounts of the powder with equal amounts of substrate were allowed to digest for varying lengths of time. In the first chart the abscissa represents time in days and the vertical ordinates thereto activity measured as cubic centimetres of deci-normal sulphuric acid. There are four curves of which the lowest represents the results obtained by allowing 0·05 gram of the powder to act on equal amounts of substrate for varying times. Four bottles containing identical amounts of substrate and powder were put into the hot room at the same time. One of these was taken out on the expiration of the times mentioned in the chart and the nitrogen as ammonia in the tannic acid filtrate estimated. The other three curves similarly represent activity at varying times of 0·1 gram, 0·2 gram and

0·4 gram respectively. They were obtained in the same way except that the curve for 0·2 gram was not continued so long and the estimations were made at different times.

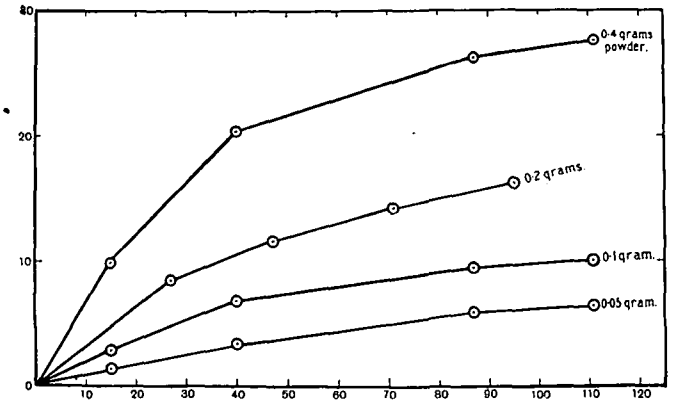

Chart 1.

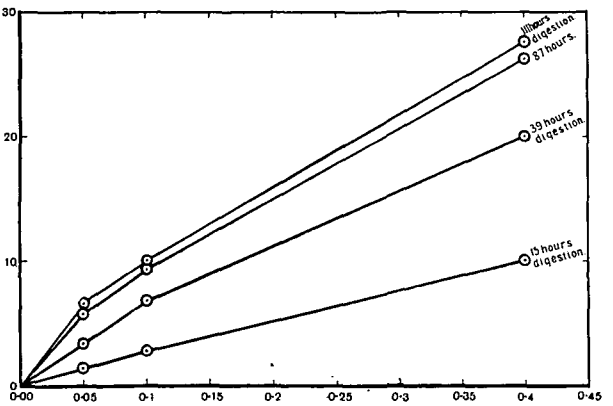

Chart 2.

The second chart is compiled from the same figures and shows certain points more clearly. In it the abscissa represents the amounts of enzyme while the vertical ordinates represent activity as cubic centimetres of deci-normal acid representing ammonia in filtrate. There are four curves at 15, 39, 87, 111 hours respectively showing effect of increasing amounts of enzyme.

The figures following the charts are the protocols of experiments from which the charts are compiled.

https://doi.org/10.1017/S0022172400042935 Published online by Cambridge University Press

Experiment 5.

Powder No. 6. Time 2½ days. Substrate, casein 2·5 % sol. in sodium carbonate solution 0·25 %.

Powder		Casein sol.	Time		Nitrogen as c.c. of deci-normal sulph. acid in 50 c.c. of filtrate
0·05 gm.	+	50 c.c.	15 hrs.	=	4·4
0·05	+	50	39	=	6·4
0·05	+	50	87	=	8·8
0·05	+	50	111	=	9·4
0·05 (heated)	+	50	41	=	3·0
0·1 gm.	+	50 c.c.	15 hrs.	=	5·9
0·1	+	50	39	=	9·8
0·1	+	50	87	=	12·5
0·1	+	50	111	=	13·0
0·1 (heated)	+	50	111	=	3·0
0·2 gm.	+	50 c.c.	27 hrs.	=	11·6
0·2	+	50	47	=	14·5
0·2	+	50	71	=	17·1
0·2	+	50	95	=	19·2
0·2 (heated)	+	50	47	=	3·0
0·4 gm.	+	50 c.c.	15 hrs.	=	12·9
0·4	+	50	39	=	23·2
0·4	+	50	87	=	29·3
0·4	+	50	111	=	30·6
0·4 (heated)	+	50	111	=	? about 3

As precipitant 50 c.c. tannic acid sol. was used, 50 c.c. of the filtrate after this addition was taken in each case. The flasks were well shaken during the first ten hours and for short spells afterwards.

The points brought out by the above results with regard to the action of the enzyme under the described conditions, *i.e.* acting in closed flasks on a large excess of proteid, may be summarised as follows:

Influence of amount of enzyme time constant. Chart 1.

1. There is an increase in the total action with the time up to the point of termination in these experiments. From the charts it seems that the maximum was nearly but not quite reached.

2. The amount of change per unit time is relatively greater in the earlier hours, falling off with the progress of time.

3. In the early hours and with small doses the rate of change is nearly proportional to the time.

https://doi.org/10.1017/S0022172400042935 Published online by Cambridge University Press

Influence of amount of enzyme time constant. Chart 2.

1. The total action increases with the amount of enzyme (even the largest dose in these experiments is small relatively to the total proteid to be changed, as similar experiments with 0·8 gram showed a great increase of action).

2. With amounts of enzyme up to 0·1 gram per 50 c.c. and digestion time not exceeding 39 hours the rate of hydrolysis is proportional to enzyme added.

3. On increasing the amount of enzyme or the interval for digestion the rate falls more and more short of direct proportionality.

Effect of Reaction.

The following experiments show the effect of reaction on the activity of the powder.

Experiment 6.

Powder No. 3. Substrate, fibrin 0·5 gm.

		Nitrogen as c.c. of deci-normal sulph. acid in 20 c.c. of filtrate
A. Boiled fibrin 0·5 gm. + powder 0·02 gm. + acetic acid 0·2 % 15 c.c. ...	=	0·8
B. Boiled fibrin 0·5 gm. + powder 0·02 gm. + sodium carbonate sol. 0·25 % 15 c.c.	=	2·0
C. Boiled fibrin 0·5 gm. + powder 0·02 gm. + water 15 c.c.	=	1·15
D. Boiled fibrin 0·5 gm. + powder 0·02 gm. + water 15 c.c. } (boiled)	=	0·5

Added 15 c.c. tannic acid solution. Took 2 c.c. of filtrate for estimation.

This experiment displays the absence of action in weakly acid media while there is action in approximately neutral or alkaline media.

The following experiment conducted by a different method also gives us the same results. Here the experiment is merely qualitative. Small and equal quantities of the powder were allowed to act on gelatin at incubator temperature (37° C.) under the conditions of reactions shown, and examined as to power of solidifying by cooling in running water.

Experiment 7.

Powder No. 4*a* was used and toluene as antiseptic. These were added to a large test tube with 2½ inches of 10 % gelatin.

Reaction	14 hrs.	38 hrs.
Just alkaline to Lachmoid	fluid	fluid
Just acid to Lachmoid	solid	solid
Neutral to Lachmoid	fluid	fluid

Another qualitative experiment shows the same results. Here the method employed as a test of activity was the biuret test. Equal amounts of the powder No. 6 were allowed to act on boiled dried fibrin powder in the presence of acid alkali and neutral diluents. After 24 hours they were examined with the following results:

Experiment 8.

	Biuret reaction
1. Powder + fibrin + acetic acid 0·2 %	−
2. Fibrin + acetic acid 0·2 %	−
3. Powder + fibrin + sod. carb. sol. 0·25 %	+ +
4. Fibrin + sod. carb. sol. 0·25 %	−
5. Powder + fibrin + normal saline	+ +
6. Fibrin + normal saline	−

N.B. A solution of the powder itself does not give the reaction.

The above results show that the enzyme contained in the dried preparation from human pus acts best in weak alkaline solutions, also well in approximately neutral solutions but not in the presence of 0·20 % acetic acid.

Effect of Heat.

The following experiments were performed to show the effect of heat on the enzyme, *i.e.* to determine the point at which the enzymotic activity is no longer manifested. On investigation it becomes apparent in all such research that a factor equally important to the temperature to which the substance is raised, is the time taken to do so. It is not long before one realises the futility of saying that the death point is such and such a temperature. This will be found to differ considerably with the method used. If for instance the dried powder is simply heated in that dry condition in a hot air stove, it will resist for many minutes, up to a quarter of an hour at least, a temperature well over 100° C. Whereas,

if the powder is moistened, it is killed at a far lower point; but besides this the time taken in heating is very important. Conditions are entirely different if we start heating the ferment from room temperature and bring it up to the required point than if we drop the ferment into a fluid heated to that point. Likewise if we keep the ferment less or greater time at any point so the result will differ. Two methods which I have tried give results showing what I mean, in Experiment 9 the heating was done in similar test tubes in a water bath already heated to the required amount. In this series the ferment is markedly affected by one minute at 65° to 67° C., that is to say one minute after the ferment reached 65° C. In Experiment 10, where the ferment is merely dropped into the water when that has reached the required temperature and is retained there the requisite time, it is not much affected by one minute between 69° and 71° C.

Other considerations, too, render this question I think one which gives very unprofitable results unless some standard method be adopted by all observers. Opie (1906 (1)) gives 67° C. as the thermal destruction point; but unfortunately gives little information as to his method. When it is an obvious fact that such factors as amount of proteid in solution, rate of rise of temperature, and others such as are stated above, so affect the results, it is hardly likely that any two observers will agree. Here I merely give the results of a few experiments which were really done as a means to know how I could most effectively kill the enzyme for controls.

Experiment 9.

Method. The heating was done by dropping weighed amounts of powder No. 9 into 20 c.c. of water raised to the required temperature in a water bath, taken off as required and cooled quickly. The 20 c.c. of water were heated in the bottles used for digestion and subsequently 50 c.c. of casein solution were added with 5 c.c. of toluene to each flask. After two days' digestion 50 c.c. of tannic acid solution were added and 50 c.c. of the filtrate from this taken for estimation. The figures represent nitrogen as deci-normal sulph. acid in 50 c.c. of the filtrate.

Heated to	62° C.	and	cooled immediately	= 12·8.
,,	65° C.	,,	,,	= 12·8.
,,	70° C.	,,	,,	= 12·7.
,,	75° C.	,,	,,	= 10·9.
,,	80° C.	,,	,,	= 6·7.

Heated for one minute		between	69 —71° C.	= 12·2.
,,	,,	,,	69·5—71·5° C.	= 12·15.
,,	,,	,,	72 —73° C.	= 11·55.
,,	,,	,,	74·5—75° C.	= 6·45.

Experiment 10.

Method. 0·2 grams of powder No. 5 were heated in 0·25 % sod. carb. sol. to various temperatures and kept at these temperatures for one minute. Then to each flask 20 c.c. of 10 % gelatin were added and the flasks put in the hot room (37° C.) to digest. Observations were made as to digestion by cooling the flasks under the water tap for the same time and noting the state of the gelatin.

	After 4 days	After 6 days
Powder not heated	fluid	fluid
1 minute at 65—67° C.	semi-solid	fluid
½ minute at 70—73° C.	solid	solid
1 minute at 73—75° C.	solid	solid
1 minute at 84—85° C.	solid	solid

Experiment 11.

Method. Powder was heated *dry* in watch glasses in a hot water jacketed stove to a temperature varying between 88—92° C. Then 0·1 gm. was allowed to act for three days on 15 c.c. 10 % gelatin with 15 c.c. sod. carb. sol. 0·25 %.

	Time left in stove	Nitrogen as deci-normal acid 20 c.c. of filtrate
A.	0	2·9
B.	5 minutes	3·0
C.	10 ,,	3·1
D.	15 ,,	3·3
E.	20 ,,	3·2
Control heated at 110—120° C. for several minutes in hot air stove		1·3

40 c.c. tannic acid added. 20 c.c. of filtrate used for estimation.

The Anti-Effect of Sera.

The anti-tryptic action of normal serum was noted by Hahn (1897) and Achalme (1901). Achalme succeeded in increasing the anti-tryptic effect of guinea-pig serum by immunisation. Hedin (1905 and 1906 I.) has very fully investigated the neutralising action of normal serum upon trypsin. This effect he finds is larger if the serum be added to the trypsin before adding the substrate and there is an increase in anti-action, up to a certain point, with the time for which they are left together before adding the substrate. The combination occurs more readily the higher the temperature, up to a certain point. The amount of trypsin neutralised is independent of dilution. The anti-power of the serum can be completely neutralised, but trypsin cannot be completely rendered inert by serum. The amount of trypsin neutralised is greater

relatively for small amounts than for large amounts of serum. The anti-body is completely destroyed by 0·1 to 0·2 % of acetic acid at 37° C. for 8 hours and is markedly affected by acid in a very short time. He found it impossible to separate anti-body and enzyme in a neutral mixture, *i.e.* to free the trypsin. In a further paper Hedin (1906 II.) compared the results obtained with serum with those obtained by the addition of charcoal to a tryptic digest. He found that this substance had a powerful effect in neutralising trypsin and that the neutralisation probably consisted of two consecutive stages. Firstly, a taking up or absorption of trypsin: in this stage the trypsin can be freed by the addition of more substrate. Secondly, a stage in which the trypsin is fixed. The amount fixed is larger the more charcoal used, the higher the temperature and the longer the time of interaction. He found this effect of charcoal to agree in every point as far as he could see with the effect of serum and concluded the latter to be a similar phenomenon.

The anti-tryptic action of normal sera was found by Landsteiner (1900) to be associated with the albumin fraction of the serum (Pick). This was confirmed by Cathcart (1904). Opie (1905, 1906) and Opie and Barker (1907), working with leucoprotease, found that serum either from purulent exudates or from blood inhibits the proteolytic effect of leucoprotease. This property is destroyed by acids and by heating to 75° C. for half an hour. A certain amount of serum is only capable of controlling a limited amount of enzyme. As in the case for trypsin the inhibiting action was associated with the albumin fraction of the serum and not with the globulin.

Müller and Jochmann (1906) also made observations on the anti-effect of serum. The anti-leucoprotease is not specific. The serum of one animal is equally effective against leucoprotease from all sources (Opie and Barker 1907).

The anti-body in normal sera neutralises both trypsin and leucoprotease (Jochmann and Lockermann 1908).

Variations in the anti-tryptic power of human serum have been found to occur in patients suffering from diseases involving cell destruction (Bittorf 1907) or associated with leucocytosis (Wiens 1907), tuberculosis (Wiens) and cancer (Brieger and Trebing 1908). Eisner (1909), however, finds that it is only in the cachectic state of cancer that the anti-tryptic power is modified.

In this country the subject has been exploited with a view to its possible utility in diagnosis by Golla (1909), Hort (1909) and Bayly

(1909). Golla has introduced means of considerable accuracy for measuring the anti-tryptic power of different sera under clinical conditions.

Before detailing my experiments with sera it will be useful to say a few words as to methods.

So as to make the total amount of proteid equal in each case, it was necessary in performing experiments with different amounts of fresh serum to make up the total serum content to the same figure by the addition of heated serum. Heated serum must also be used for maximum and minimum control. By suitable dilution we can arrive at a satisfactory method of getting a serum sufficiently heated and still in a liquid state. For this purpose I have found the dilution of 1 serum to 4 water the best, 1 serum to 4 normal saline is likely at times to precipitate before a sufficient temperature for use in controls is reached. For heating purposes I used a water bath already boiling; in this the dilute serum is immersed in a thin flask and kept moving briskly until the temperature as registered by a thermometer inside the flask reaches the required point. I have employed 90° C. as a useful temperature for heating all controls from the experience of such an experiment as No. 12. With regard to the effect of heat on the anti-power of serum, Opie found that heating equal quantities of serum diluted with equal amounts of salt solution to 75° C. for half an hour sufficed to destroy the anti-power. Lower temperatures seemed to afford a slight increase in the anti-power; he does not state how the heating was done. In the following experiment equal quantities, 10 c.c. of serum in the dilution of 1 serum to 4 water, were heated in the water bath from the water supply temperature to the required temperature in test tubes of equal thickness and calibre. Thus the time taken in heating to the required temperature should be in each case the same. The test tubes were immediately cooled under the water tap. This heated serum was allowed to act on a mixture of enzyme and substrate. In this case a fluid preparation $\frac{1}{2}$ % of the powder in 0·25 % sod. carb. sol. was used in a dose of 20 c.c. in each flask. Of the casein substrate 50 c.c. were used in each flask. After $2\frac{1}{2}$ days' incubation 50 c.c. of tannic acid solution were added as precipitant and 50 c.c. of the filtrate from this taken for estimation. The results below show that it has begun to be affected at 72° C. and apparently is not much more affected by 76° C. But at 90° C. for several minutes it is completely destroyed so that this is a sufficient temperature to heat the serum for the controls.

Experiment 12.

Powder No. 5.

	Nitrogen as deci-normal acid in 50 c.c. of filtrate
With fresh serum	3·05
With serum heated to 50° C.	3·15
,, ,, ,, 55	3·2
,, ,, ,, 60	3·0
,, ,, ,, 70	3·2
,, ,, ,, 72	4·30
,, ,, ,, 74	4·25
,, ,, ,, 76	4·25
Control at 90° C. for several minutes	4·95
Control with enzyme sol. heated to 90° C. for several minutes	1·95

The following experiments show the anti-power of the normal sera of ox and goats for leucoprotease.

Experiment 13.

Powder No. 5. Time 7 days. Ox serum collected two days before and kept on ice.

		Nitrogen as deci-normal acid in 100 c.c. of filtrate
A.	Powder 0·2 gm. + casein sol. 50 c.c. + serum (1 to saline 2) 50 c.c. (heated) =	36·6
B.	Powder 0·2 gm. + casein sol. 50 c.c. + serum (1 to saline 2) 50 c.c. (fresh) =	23·8
C.	Powder 0·2 gm. heated + casein sol. 50 c.c. + serum (1 to saline 2) 50 c.c. (heated) =	6·4

Heated in water bath to dryness from wet state. 100 c.c. tannic acid added. Took 100 c.c. filtrate.

Experiment 14.

Powder No. 5. Time 4 days under sol. 5 c.c. Serum Ox collected previous day. Dil. serum 1, normal saline 3, a total of 50 c.c. of diluted serum in each. The heated serum was raised to 91° C. Substrate, casein sol. 2·5 % in 0·25 % sod. carb. sol.

	Powder		Casein sol.		Dilute serum: Heated		Dilute serum: Unheated		Nitrogen as deci-normal acid in 100 c.c. of filtrate
(*a*)	0·2 gm.	+	50 c.c.	+	50 c.c.	+	0 c.c.	=	37·0
(*b*)	0·2	+	50	+	40	+	10	=	29·0
(*c*)	0·2	+	50	+	25	+	25	=	22·3
(*d*)	0·2	+	50	+	0	+	50	=	18·0
(*e*)	0·2	+	50	+	50	+	0	=	5·1 control

Tannic acid 100 c.c. 100 c.c. of filtrate taken for estimation.

Experiment 15.

Same conditions with same serum. Time 2 days.

	Powder		Casein sol.		Dilute serum: Heated		Dilute serum: Unheated		Nitrogen as deci-normal acid in 100 c.c. of filtrate
(*a*)	0·2 gm.	+	50 c.c.	+	50 c.c.	+	0 c.c.	=	26·1
(*b*)	0·2	+	50	+	25	+	25	=	16·3
(*c*)	0·2	+	50	+	0	+	50	=	11·0

Tannic acid 100 c.c. 100 c.c. of filtrate taken for estimation.

https://doi.org/10.1017/S0022172400042935 Published online by Cambridge University Press

Experiment 16.

Powder No. 6. Time 2½ days. Substrate, casein sol. 50 c.c. Goat serum, diluted, serum 1, normal saline 4. Total of 20 c.c. serum in each case.

	Powder		Substrate		Dilute serum: Heated		Dilute serum: Unheated		Nitrogen as deci-normal acid in 50 c.c. of filtrate
(*a*)	0·2 gm.	+	50 c.c.	+	20 c.c.	+	0 c.c.	=	19·3
(*b*)	0·2	+	50	+	15	+	5	=	17·6
(*c*)	0·2	+	50	+	10	+	10	=	15·2
(*d*)	0·2	+	50	+	5	+	15	=	15·0
(*e*)	0·2	+	50	+	0	+	20	=	13·3
(*f*)	0·2 (heated)	+	50	+	20	+	0	=	2·8

Tannic acid 50 c.c. added. Took 50 c.c. of filtrate.

Experiment 17.

Powder No. 6. Time 2½ days. Substrate, casein 50 c.c. Serum Ox, dil. serum 1, normal saline 4. Total of 20 c.c. dil. serum in each.

	Powder		Substrate		Dilute serum: Heated		Dilute serum: Unheated		Nitrogen as deci-normal acid in 50 c.c. of filtrate
(*a*)	0·2 gm.	+	50 c.c.	+	20 c.c.	+	0 c.c.	=	19·0
(*b*)	0·2	+	50	+	10	+	10	=	15·5
(*c*)	0·2	+	50	+	0	+	20	=	13·0
(*d*)	0·2 (heated)	+	50	+	20	+	0	=	2·9

Tannic acid 50 c.c. added. 50 c.c. of filtrate taken for estimation.

Experiment 18.

Powder No. 8. Substrate, casein 50 c.c. Time 2½ days. Serum Goat, dil. serum 1, normal saline 4. Total of 20 c.c. dil. serum in each.

	Powder		Substrate		Dilute serum: Heated		Dilute serum: Unheated		Nitrogen as deci-normal acid in 50 c.c. of filtrate
(*a*)	0·2 gm.	+	50 c.c.	+	20 c.c.	+	0 c.c.	=	21·0
(*b*)	0·2	+	50	+	10	+	10	=	17·4
(*c*)	0·2	+	50	+	0	+	20	=	14·6
(*d*)	0·2	+	50	+	20	+	0	=	2·8

Tannic acid 50 c.c. added. 100 c.c. of filtrate taken for estimation.

Comparative inhibitory effect of Sera from Different Animals.

With regard to the degree of inhibition manifested by normal sera Opie and Barker (1907) find it is not more marked with the serum of the species of animal from which the leucoprotease is taken than with other sera.

Pappenheim's results (quoted by Eisner 1909) do not agree altogether with Opie's. Pappenheim found the serum of the rabbit to be *less* active than that of the dog versus leucoprotease. He however agrees that birds' sera are little, if at all, active.

Delezenne using trypsin as ferment found considerable differences between various animal sera in their anti-power towards this ferment.

Mesnil (1903) working with actinodiastase as ferment found that sera of different species varied in their anti-power towards it and gives the following order of activity: sheep, goat, rabbit, birds.

As the evidence is at present then, normal sera from different animals show varying degrees of activity towards proteolytic ferments. This variability is thought by various observers to be a specific one but as regards leucoprotease at any rate, where two observers have worked on the same animals with the same ferment, they have not infrequently arrived at contradictory results, thus indicating that the differences between sera of individuals of the same species is as great as that between different species.

In my experiments I failed to find any very material difference between the activity of the sera of the various mammals on which I have experimented. In each case controls of maximum digestion, *i.e.* with all serum heated, and also two or more experiments with the fresh serum, one with a larger quantity than the other, were made. This enables one to see that one is not using such a dose of serum that it will give a maximum effect under the given conditions. The amount of serum added in each case is rendered equal by making up to constant amount with heated serum. The following experiments show some of my results:

Experiment 19.

Powder No. 8, 0·2 gm. Time 3½ days. Substrate, 50 c.c. casein sol. Serum, diluted serum 1, water 4. Total amount of dilute serum 20 c.c. in each case.

	Dilute serum	Nitrogen as deci-normal acid in 25 c.c. of filtrate			
		Ox	Sheep	Goat	Rabbit
1.	With 20 c.c. heated	8·0	7·9	7·65	7·4
2.	With 10 c.c. fresh	6·8	6·3	6·15	6·4
3.	With 20 c.c. fresh	—	5·25	5·3	5·6
4.	Control	1·1	1·0	1·1	1·1

50 c.c. tannic acid sol. added. 25 c.c. of filtrate taken for estimation.

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Experiment 20.

5 gm. of powder No. 10; ground with glass in sodium carbonate 0·25 % sol.; centrifuged; filtered and made up to 500 c.c.

Enzyme, solution as above 20 c.c. Time 2½ days. Substrate, casein solution 50 c.c. Dilute serum, serum 1, water 4. Total of 20 c.c. of dilute serum in each case.

	Nitrogen as deci-normal acid in 50 c.c. of filtrate			
Dilute serum	Ox	Sheep	Rabbit	Pigeon
All serum heated	7·4	7·5	7·3	—
10 c.c. dil. fresh serum	5·55	5·55	4·6	7·55
20 c.c. ,, ,,	4·6	4·4	3·4	—
Control	2·15	2·05	2·3	—

Tannic acid 50 c.c. Filtrate taken 50 c.c.

Experiment 21.

Powder No. 10, ·02 gm. Time 2½ days. Substrate, casein sol. 50 c.c. Serum, 1 serum, 4 water. Total 20 c.c. in each case.

	Nitrogen as deci-normal acid in 50 c.c. of filtrate	
Dilute serum	Ox	Sheep
Serum all heated	16·15	16·9
10 c.c. fresh	15·35	14·65
20 c.c. ,,	13·15	11·8
Control	2·5	2·75

Tannic acid added 50 c.c. Took 50 c.c. of filtrate.

Experiment 22.

Enzyme. Powder No. 5, 0·1 gm. Substrate, gelatin 10 % 10 c.c. Time 3½ days. Diluent sodium carb. sol. 0·25 % 10 c.c. Serum (1—6 water), 10 c.c. in each case.

	Nitrogen as deci-normal acid in 50 c.c. of filtrate	
Dilute serum	Ox	Sheep
All serum heated	6·0	5·75
5 unheated	4·70	4·8
10 unheated	4·2	4·3
Control	1·8	1·7

50 c.c. tannic acid sol. added. 50 c.c. of filtrate taken.

Immunity.

Achalme (1901) increased the anti-tryptic power of serum in guinea-pigs by injecting them intra-peritoneally with trypsin. Dean (1901) immunised goats and geese with trypsin but obtained only a trifling increase in anti-tryptic power.

Levene and Stookey (1903) found that the serum of trypsin-immunised rabbits was *markedly* more powerful as anti-body to tryptic

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digestion. The animals were treated for eight weeks with a dose increasing to 2 c.c. intravenously.

Landsteiner (1907) failed to increase the anti-tryptic power of serum notwithstanding a lengthy immunisation.

Jochmann and Kantorowicz (1908) immunised rabbits with both trypsin and leucoprotease and found an increase of thirtyfold in the power of the serum. The sera of the animals injected with either trypsin or leucoprotease contained an anti-body for both enzymes and saturation of the sera with the one enzyme exhausted it of its neutralising power for both.

Bergmann and Bamberg (1908) doubled the anti-tryptic power of dog's serum by two months' immunisation.

Döblin (1909) failed to increase the anti-power by injecting trypsin into rabbits whereas Meyer (1909) obtained in the same animals an increased anti-tryptic power after the injection of trypsin but not after trypsinogen or kinase.

My own results with leucoprotease are parallel with those of Dean, there being according to the figures (Ex. 23 and 24 below) a slight increase of anti-power. As however the nutrition of the goats was somewhat influenced by the course of injections, *i.e.* loss of weight was noticed, I am inclined to think that such slight variation as is shown in the course of my experiments is as likely if not more likely to be caused by such influences than by any process of true immunisation. Had I used rabbits or dogs the results might have been different.

The goats were treated by subcutaneous injection of preparations of the pus-powder, prepared as mentioned above. This was either given in the form of suspensions in normal saline or sodium carbonate 0·25 % sol. or by extracts of the powder made by grinding it with glass in saline or sod. carb. sol., centrifuging and filtering. This treatment the animals bore well. The early injections were followed by a good deal of reaction causing firm nodules to appear which were however subsequently absorbed. After the later injections although the dose was considerably increased, this was far less marked. The doses injected are shown in the table as approximate amounts of powder used. I have given the actual ammonia figures of the estimation as well as the percentages of inhibition of the serum.

In estimating this anti-tryptic action the following method was used. 0·2 gm. of the powder was allowed to act in the presence of 2 c.c. and 4 c.c. of the pure serum on 50 c.c. of 2·5 casein solution in the sodium carbonate solution 0·25 %. In each case the serum was

Experiment 23.

Protocols of estimations of anti-action of serum of immunised goats.

	Goat 1 B	Goat 2 B	Goat 1 L
	Dates:—8. 3. '09	9. 3. '09	26. 2. '09
A.	21·0	19·9	19·3
B.	17·4 (20)	16·3 (21)	15·2 (24)
C.	14·6 (35)	13·7 (36)	13·3 (36)
D.	2·8	2·8	2·8
	Dates:—26. 3. '09	26. 3. '09	13. 3. '09
A.	18·8	18·3	19·4
B.	14·55 (26)	15·05 (20)	15·6 (22)
C.	12·0 (40)	13·1 (32)	12·6 (40)
D.	2·2	2·1	—
	Dates:—14. 4. '09	14. 4. '09	26. 3. '09
A.	16·3	15·7	18·8
B.	14·25 (14)	13·5 (16)	—
C.	11·5 (33)	11·4 (31)	12·45 (38)
D.	1·8	1·9	2·2
	Dates:—24. 4. '09	24. 4. '09	—
A.	17·05	—	—
B.	14·5 (17)	14·25 (19)	—
C.	11·9 (34)	12·5 (30)	—
D.	2·3	2·15	—
	Dates:—17. 5. '09	17. 5. '09	—
A.	17·65	17·45	—
B.	14·0 (25)	14·15 (23)	—
C.	12·0 (39)	11·75 (40)	—
D.	3·25	3·1	—
	Dates:—4. 6. '09	4. 6. '09	—
A.	14·85	15·55	—
B.	11·8 (25)	11·85 (28)	—
C.	9·4 (43)	—	—
D.	2·4	2·4	—
	Dates:—15. 6. '09	15. 6. '09	—
A.	18·9	19·6	—
B.	14·4 (28)	14·45 (30)	—
C.	11·4 (46)	11·4 (48)	—
D.	2·65	(2·65)	—

First figures = nitrogen as c.c. N/10 acid in 50 c.c. of filtrate. Figures in brackets = % reductions. 50 c.c. tannic acid sol. added.

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Experiment 24.

Days	Amount of powder injected	Percentage reductions With 2 c.c.	With 4 c.c.
Goat 1 L.			
1	—	24	36
11	0·05 a	—	—
12	0·2 a	—	—
13	0·3 b	—	—
16	0·4 b	22	40
29	—	—	38
Goat 1 B.			
1	—	20	35
13	0·2 b	—	—
18	0·2 b	—	—
19	—	26	41
26	0·2 c	—	—
31	0·2 c	—	—
37	0·4 b	14	33
39	0·8 b	—	—
48	—	17	34
71	0·6 d	25	39
80	1·2 d	—	—
89	1·6 e	25	43
100	0·08 IV	28	46
Goat 2 B.			
1	—	21	36
12	0·2 b	—	—
17	0·2 b	—	—
18	—	20	32
25	0·2 c	—	—
30	0·2 c	—	—
36	0·4 b	16	31
38	1·0 b	—	—
47	—	19	30
70	0·6 d	23	40
80	1·2 d	—	—
89	1·6 e	28	—
99	0·1 IV	30	48

a—1 % extract in normal saline.
b—suspension in sodium carbonate solution 0·25 %.
c—suspension 1 % in normal saline.
d—1 % extract in sodium carbonate solution 0·25 %.
e – 4 % suspension in normal saline.
IV—1 % extract in sodium carbonate solution 0·25 % given intravenously.

https://doi.org/10.1017/S0022172400042935 Published online by Cambridge University Press

diluted in the proportion of 1 to 4 of normal saline and the total amount of this dilute serum was in each case made up to 20 c.c. by similarly diluted serum previously heated to 90° C. In the preceding table the numbers given in the horizontal columns A, B, C, D represent the following:

A. Maximum digestion with all serum, 20 of dilute, heated.

B. With 2 c.c. serum, 10 c.c. of the dilute serum, fresh.

C. With 4 c.c. of the serum, 20 c.c. of dilute, fresh.

D. Control, enzyme powder heated, serum all heated, 20 c.c. of dilute.

The figures in brackets after B and C in Expt. 23 represent percentage reduction of activity.

Before treatment all three goats gave percentage reduction figures between 20 % and 24 % for 2 c.c. of fresh serum and between 35 % and 36 % for 4 c.c. of fresh serum, while at the end of the course of injections the percentage reduction figures for goats 1 B and 2 B were between 28 % and 30 % for 2 c.c. of fresh serum and between 46 % and 48 % for 4 c.c. of fresh serum.

During the course of estimation it will be noticed that on one occasion the percentage reduction figures fell to between 14 % and 16 % for 2 c.c. of fresh serum and 33 % and 31 % for 4 c.c. of fresh serum. I think it is likely that the 14 % and 16 % are for some reason underestimations but otherwise the 2 c.c. and 4 c.c. estimations run pretty fairly parallel.

In conclusion I wish to express my thanks to Dr Martin, Director of the Institute, and Professor Leathes, Head of the Laboratories for Pathological Chemistry. To Professor Leathes I am indebted for much valuable advice and assistance throughout the progress of the work.

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