INVESTIGATIONS ON THE PROTEOLYTIC ENZYMES OF THE SPLEEN OF THE OX.

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EXCEPT the enzymes that act in the alimentary canal no proteolytic enzymes were known to occur in the animal body till E. Salkowski found that the liver, muscles and suprarenal glands, when crushed and kept together with chloroform at blood heat for a long time, undergo an alteration, in consequence of which the nitrogenous substances soluble in water increased[1]. This effect was attributed to proteolytic enzymes, which have been further studied in Salkowski's laboratory by Schwiening[2] and Biondi[3]. As regards the products of the activity of these enzymes Salkowski found leucin, tyrosin and purin bases in the digested liver mass, and Schwiening found leucin and tyrosin in muscles which had been digested for one year.

Jacoby succeeded in precipitating the liver-enzyme by filtering the digested liver mass and saturating the filtrate with Am_2SO_4[4]. Jacoby[5] also observed a proteolytic enzyme in the thymus, and Kutscher[6] has investigated the products of its activity.

Our means of studying the activity of the cells have been very much advanced by the important method of E. Buchner[7], of obtaining the contents of yeast-cells in a form suited for chemical investigation. Buchner's idea has already in some laboratories been applied to other kinds of cells. By testing juices pressed from the disintegrated cells of different animal organs Hedin and Rowland[8] found that the spleen, lymphatic glands, kidneys and liver contain proteolytic enzymes acting only or most strongly in an acid medium. As to the action of juice prepared

[1] *Zeitschr. f. klin. Med.*, 1890, Suppl.
[2] *Virchow's Archiv*, CXXXVI. p. 444. 1894.
[3] *Virchow's Archiv*, CXLIV. p. 373. 1896.
[4] *Zeitschr. f. physiol. Chem.* XXX. p. 149. 1900.
[5] *Beitr. z. chem. Physiol. u. Pathol.* I. p. 147. 1902.
[6] *Zeitschr. f. physiol. Chem.* XXXIV. p. 114. 1901.
[7] *Berl. Ber.* XXX. pp. 117, 2670. 1897.
[8] *Zeitschr. f. physiol. Chem.* XXXII. pp. 341, 531. 1901.

from skeletal muscles no marked difference was found between the action in alkaline and acid mediums, whilst the heart showed a somewhat stronger activity in an acid reaction. The animals used in our investigations were the ox, horse, sheep, pig, and dog.

I have continued these investigations in the first place on the spleen, because this organ shows the strongest proteolytic activity of all the organs tried by Rowland and myself.

I have found that the spleen of the ox contains at least two different proteolytic enzymes, the one acting only or principally in an alkaline medium, and the other, which was previously found by Rowland and myself, acting only or mainly in an acid fluid. For the former I propose the name *Lieno-α-protease,* and for the latter *Lieno-β-protease.*

The methods adopted for proving the presence of proteolytic activity in a fluid have been as follows:

1. Digesting the proteids present in the fluid itself, testing the degree of digestion by precipitating with an equal volume of tannic acid solution and estimating the nitrogen in a known volume (5 c.c.) of the filtrate[1]. The tannic acid when in excess does not precipitate peptones or lower digestion products. If the estimation of nitrogen is carried out after and before digestion, the difference between the two results therefore corresponds to the peptones and lower products formed in the course of the digestion. Since the enzyme solution was diluted with an equal volume of tannic acid solution, the amount of nitrogen found should be doubled in order to correspond to the digestion that has taken place in the original fluid.

2. Digesting fibrin, in which case the digestion can be followed to some extent by inspection. To 10 c.c. fluid I added 2 grams of hand-pressed fibrin. Where the action is only a weak one this method is not very good, because the fibrin itself contains a very weak proteolytic enzyme. Therefore I have always compared the action with the digestion in a control sample, to which an enzyme was added, that had been boiled before the digestion. In important cases the degree of digestion was determined by estimating the nitrogen soluble in weak acetic acid. Therefore acetic acid to 0·2% was added to those samples which did not already contain that amount of acid and a volume of water equal to that of the enzyme solution. After some hours the fluid was filtered and the nitrogen in 5 c.c. of the filtrate was estimated. The difference between the amount of nitrogen obtained from the specimen containing

[1] The tannic acid solution used in my investigations contains 70 grms. tannic acid, 50 c.c. glacial acetic acid, and 100 grms. sodium chloride in one litre.

the active enzyme and the one containing the boiled enzyme then apparently corresponds to the fibrin dissolved. In other cases the analysis was carried out by adding a volume of tannic acid solution equal to the enzyme solution, filtering and determining the nitrogen in 5 c.c. of the filtrate. The difference between the nitrogen obtained in the sample with active enzyme and that in the sample with boiled enzyme then corresponds to the amount of peptones and lower digestion products formed. Therefore the nitrogen obtained after precipitating with tannic acid is always considerably less than the amount obtained after filtering the acidified fluids.

3. In a few of my analyses I have made the enzymes digest coagulated serum. 5 c.c. of serum were heated in the water-bath for half-an-hour and then digested with 5 c.c. of the enzyme solution. The analyses in all these cases was carried out by precipitating with 10 c.c. tannic acid solution.

Method of obtaining the Lieno-α-protease.

According to the results of Hedin and Rowland, the juice pressed from disintegrated spleen cells shows a very strong proteolytic action in an acid medium, whilst there is no action or only a very slight one in an alkaline medium. This does not necessarily imply that there is no enzyme acting in an alkaline medium present in the spleen; there may be one which is kept back in the press cake. Having failed to find any enzyme acting in alkaline solution in the juice, I therefore experimented with the mixture of spleen mass and silver sand, obtained when the spleen is ground and not pressed, and later on with the minced spleen simply without any grinding process. As a fact I have not been able to find any difference between the ground and the unground spleen mass so far as the amount of proteolytic enzymes is concerned. When the spleen mass is mixed with water and digested, it behaves very much like the pressed juice in so far as it shows a strong digestion with 0·2% acetic acid, but the action in the presence of 0·25% Na_2CO_3 seems to be more marked than in the juice, as borne out by the following rough estimation.

20 grams of a mixture of ground spleen mass and sand were mixed with 20 c.c. of water. The nitrogen not precipitable by tannic acid was determined in different specimens, before and after digestion, by adding 20 c.c. of tannic acid solution and estimating N in 5 c.c. filtrate. The N corresponded

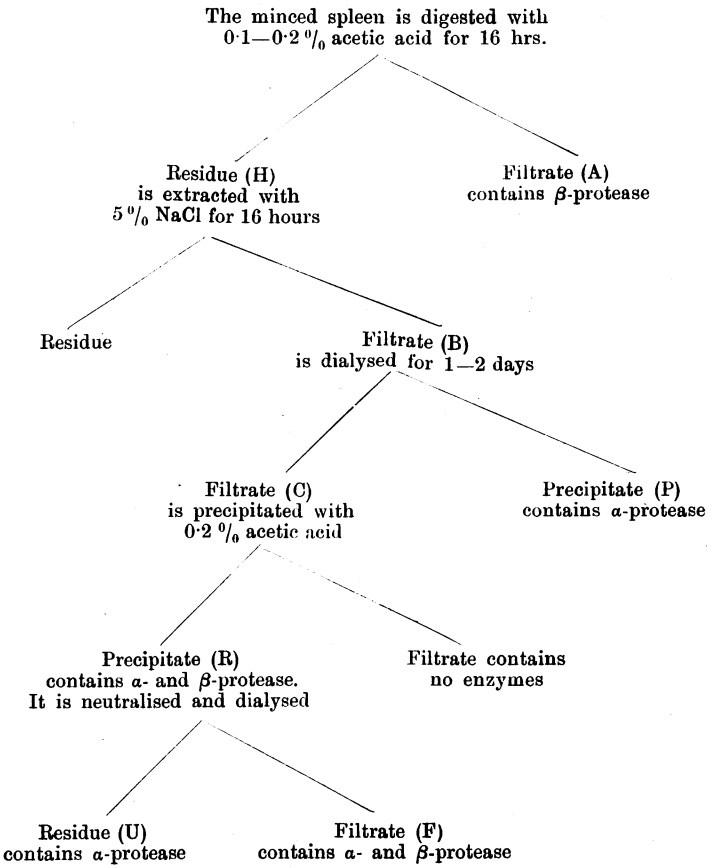
before digestion to	7·1	c.c $\frac{1}{10}$	n. acid.
after 16 hours, digestion with 0·2 % acetic acid to	14·2	,,	,,
,, ,, ,, 0·25 % Na_2CO_3 to	9·0	,,	,,

The addition of 3—6 % NaCl did not markedly increase the digestion in an alkaline fluid. The figures obtained with NaCl were:

Digestion with 0·25 % Na_2CO_3 + 3 % NaCl ... 9·3 c.c. acid.
" " " 6 % " ... 9·4 "

That the digestion in alkaline reaction is due to a special enzyme, I have been able to prove by separating it from the enzyme acting in an acid medium. The method, finally adopted for this purpose was as follows, see table I.

TABLE I.



One minced ox spleen is digested for 16 hours with 2 litres water and 10—20 c.c. of 20 % acetic acid. Chloroform and toluol are used for preventing fermentation. During this digestion most of the soluble

proteids present are digested by means of the β-protease and the solution can easily be filtered off. The filtrate (A) contains β-protease, which can be precipitated by saturating with Am_2SO_4, and after dialysing away the salt can be made to act in the presence of weak acetic acid, there being no action in an alkaline medium or only a very weak one. The residue H of the spleen mass is washed with water till the washings show no acid reaction, whereupon it is extracted with 2 litres 5% NaCl solution for 16 hours at 37°. At the end of that time the fluid is filtered and the filtrate (B) is dialysed for 24 hours. A very scanty precipitate (P) formed during the dialysis acts rather strongly in an alkaline medium and much less in an acid. It therefore contains α-protease.

EXP. 1. Showing the activity of precipitate P.

The precipitate was dissolved in 0·25% Na_2CO_3 solution. 10 c.c. of the resulting fluid was digested with 2 gr. fibrin. To another similar sample 0·2 c.c. 20% acetic acid was added, the resulting acidity corresponding to about 0·2% acetic acid. Thus the digestion was performed in alkaline and in acid medium. Control specimens contained boiled enzyme. Analyses were carried out by determining the nitrogen not precipitable by tannic acid as indicated above.

1. With Na_2CO_3.	N corresp. to	10·6	c.c. $\frac{1}{10}$	n. acid.
	Control	2·0	,,	,,
		8·6	,,	,,
2. With acid.	N corresp. to	3·0	,,	,,
	Control	1·6	,,	,,
		1·4	,,	,,

The bulk of the α-protease together with much β-protease remains in the solution on dialysing. From this the enzymes can be completely precipitated by adding acetic acid up to 0·2%. The precipitate (R) sometimes acts more strongly in an alkaline sometimes in an acid medium. When it is not required to effect a separation of the two enzymes this precipitate is the most convenient and strongest form in which to investigate the α-protease.

If the precipitate obtained from one ox spleen is dissolved in 100 c.c. 0·25% Na_2CO_3 solution, then 5 c.c. of the obtained solution generally breaks up one gram pressed fibrin in one night at 37°.

The separation of the two enzymes I have not been able to effect without a considerable loss in activity and even then it is uncertain whether the separation is complete.

The α-protease can be freed from the β-protease—at any rate to a

certain extent—by suspending the precipitate R in water and adding Na_2CO_3 till the fluid no longer shows a reaction acid to litmus. By the action of the Na_2CO_3 added part of the precipitate is dissolved. The mass is dialysed for 16 hours and filtered. The residue (U) on the filter when thoroughly washed shows a much stronger activity in an alkaline medium than in an acid, whilst the filtrate (F) in most cases acts both at alkaline and at acid reaction with about equal strength like the precipitate R.

The following experiments show the activity of the residue U and the filtrate F compared with precipitate R.

Exp. 2. Digestion with fibrin. N-determination in the filtrates from the acidified fluids after 3 days.

	Precipitate R		Residue U	Filtrate F
1.	With 0·25 % Na_2CO_3. N corresp. to 25·6 c.c. $\frac{1}{10}$ n. acid		20·8 c.c.	7·2 c.c.
	Control	11·4 ,, ,,	2·0	3·4
		14·2 ,, ,,	18·8	3·8
2.	With 0·2 % acetic acid	31·8 ,, ,,	7·6	20·4
	Control	10·2 ,, ,,	2·0	3·4
		21·6 ,, ,,	5·6	17·0

Exp. 3. Digestion with coagulated serum (5 c.c. coag. serum + 5 c.c. enzymesol). N-determination in the tannic acid filtrate after 3 days.

	Precipitate R		Residue U	Filtrate F
1.	With Na_2CO_3	21·0 c.c. $\frac{1}{10}$ n. acid	9·8 c.c.	5·8 c.c.
	Control	2·0 ,,	1·2	1·6
		19·0 ,,	8·6	4·2
2.	With acetic acid	17·0 ,,	3·0	3·6
	Control	2·0 ,,	1·2	1·6
		15·0 ,,	1·8	2·0

Exp. 4. This exp. was performed exactly like Exp. 3.

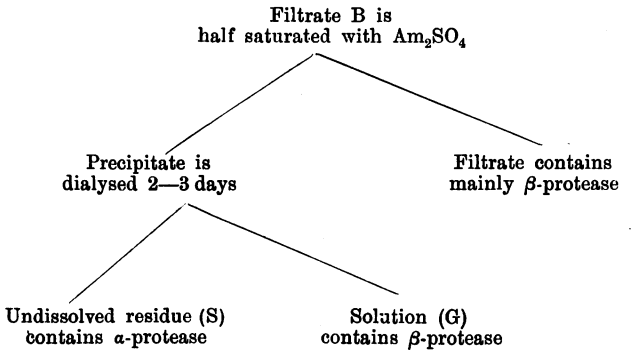
	Precipitate R		Residue U	Filtrate F
1.	With Na_2CO_3	11·0 c.c. $\frac{1}{10}$ n. acid	8·2 c.c.	4·6 c.c.
	Control	1·6 ,,	0·8	1·2
		9·4 ,,	7·4	3·4
2.	With acetic acid	7·2 ,,	1·8	4·4
	Control	1·6 ,,	0·8	1·2
		5·6 ,,	1·0	3·2

The residue U therefore contains α-protease with either no or very little β-protease, whilst filtrate F contains both enzymes.

A more complete separation of the two enzymes although with a considerable loss in activity can be performed by aid of Am_2SO_4. In this case the filtrate B is treated in a way different from that described above. It is not dialysed but about half saturated with Am_2SO_4 by adding about 300 grams salt to one litre fluid (see table II.).

The precipitate formed is dialysed with a little water for 2—3 days till the Am_2SO_4 has disappeared. The residue (S) remaining undissolved after dialysing contains the α-protease, and the solution (G) the β-protease. On full saturation with Am_2SO_4 much more β-protease is obtained but very little increase in the amount of α-protease. In the following two experiments the effect of the precipitate R, obtained from part of the filtrate B, is compared with the effect of the residue S and of the solution G.

TABLE II.



EXP. 5. Digestion with fibrin. N-determination in the "acid filtrates" after 5 days.

	Precipitate R		Residue S	Solution G
1. With Na_2CO_3	14·2 c.c.	$\frac{1}{10}$ n. acid	8·0 c.c.	3·8 c.c.
Control	8·2	,,	2·4	2·2
	6·0	,,	5·6	1·6
2. Neutral reaction	18·7	,,		4·0
Control	7·7	,,		1·6
	11·0	,,		2·4
3. With acetic acid	24·8	,,	2·5	21·2
Control	6·0	,,	1·7	2·0
	18·8	,,	0·8	19·2

Exp. 6. Digestion with fibrin. Analysis after 5 days with tannic acid.

	Precipitate R		Residue S	Solution G
1. With Na_2CO_3	22·8 c.c.	$\frac{1}{10}$ n. acid	8·0 c.c.	3·0 c.c.
Control	8·0	,,	1·4	1·0
	14·8	,,	6·6	2·0
2. Neutral reaction	21·0	,,	4·2	5·4
Control	7·2	,,	2·6	2·0
	13·8	,,	1·6	3·4
3. With acetic acid	19·0	,,	2·6	6·2
Control	7·4	,,	1·2	0·8
	11·6	,,	1·4	5·4

With this method the α-protease (residue S) is obtained free or nearly free from β-protease, whilst the β-protease (solution G) seems to have contained some α-protease—at any rate in Exp. 6.

The safest way of preparing β-protease from the precipitate R free from α-protease is to digest the precipitate R with 0·2—0·4 % acetic acid at 37° for about 16 hours. The effect of the acetic acid is to call the β-protease into action; the proteids combined with it are digested and part of the enzyme becomes soluble in weak acetic acid, whilst the α-protease remains undissolved. The filtrate therefore practically acts only in an acid medium, whereas the residue acts both in alkaline and acid medium, as borne out by the following experiments.

Exp. 7. Digestion with fibrin. N-estimation in the "acid filtrates" after 5 days.

	Precipitate R		Residue after digest. with acetic acid	Filtrate after digest. with acetic acid
1. With Na_2CO_3	14·2 c.c.	$\frac{1}{10}$ n. acid	11·5 c.c.	3·0 c.c.
Control	8·2	,,	2·4	2·2
	6·0	,,	9·1	0·8
2. With acetic acid	24·8	,,	17·6	16·4
	6·0	,,	1·6	2·8
	18·8	,,	16·0	13·6

Exp. 8. Digestion with fibrin. N-determination was performed in the "acid filtrates" and in the tannic acid filtrates after 4 days.

Precipitate R		Filtrate after digest. with acetic acid
Digestion with Na_2CO_3:		
1. Acid filtrate	30 c.c. $\frac{1}{10}$ n. acid	15·4 c.c.
Control	10 ,,	14·6
	20 ,,	0·8
2. Tannic acid filtrate	18·8 ,,	12·8
Control	7·0 ,,	12·4
	11·8 ,,	0·4
Digestion with acetic acid:		
3. Acid filtrate	31·6 ,,	23·6
Control	10·5 ,,	14·2
	21·1 ,,	9·4
4. Tannic acid filtrate	19·7 ,,	15·0
Control	7·2 ,,	12·6
	12·5 ,,	2·4

Remarks upon the above method.

Since no α-protease has been found in the acid extract (filtrate A) of the spleen, we must conclude that the enzyme is present in such a form that it is not soluble in weak acetic acid. Neutral salt solutions on the contrary dissolve it, and after removing the salt by dialysis the enzyme can be completely precipitated with very weak acetic acid. This precipitate always contains phosphorus but no purin bases, and since it is soluble in 0·25 % Na_2CO_3 and can be precipitated again with acetic acid, it must be or at any rate contain a nucleoalbumin. But if acetic acid is added to the 5 % NaCl extract of the spleen (filtrate B) without removing the salt, then a nucleoalbumin comes down without containing any α-protease. In this case I have been able to show its presence in the filtrate by dialysing away the salt, and adding 0·2 % acetic acid, which precipitates some more nucleoalbumin together with some enzyme. The rest of the enzyme can be obtained from the filtrate by dialysing away the acid, adding a solution of the nucleoalbumin free from enzyme and precipitating again with 0·2 % acetic acid, in which case the precipitate contains the enzyme.

These experiments seem to prove that the enzyme in absence of salt readily combines with nucleoalbumin; if sufficient nucleoalbumin is present, then the enzyme is quantitatively precipitated by acetic acid, but if the amount of nucleoalbumin is not sufficient to bind the enzyme, then part of the enzyme is found in the filtrate from the precipitate with

acetic acid. According to the above facts the enzyme may occur in two forms:

I. Combined with a nucleoalbumin. In this case it is not soluble in weak acetic acid but soluble in weak Na_2CO_3.

II. Not combined with nucleoalbumin, in which case it is soluble in weak acetic acid.

The first of these forms can be converted into the second by removing the nucleoalbumin with acetic acid in presence of salt; the second can be transformed into the first by adding nucleoalbumin and acetic acid.

Other properties of the α-protease.

Concerning the products of the activity of the α-protease as yet no conclusive investigations have been carried out. After 3—4 months' digestion of fibrin a distinct biuret reaction is obtained, even if fresh enzyme is added at intervals.

If an alkaline solution of the combination of α-protease and nucleoalbumin is shaken with fibrin, then a large amount of enzyme is taken up by the fibrin.

The enzyme seems to be very stable. A solution of the nucleoalbumin-enzyme in weak Na_2CO_3 showed a distinct activity after 3 weeks at room temperature. Blood heat is more destructive, but even one night at 37° does not nearly destroy it, as can be concluded from the following experiment.

A mixture of α- and β-protease was tried by digestion with fibrin, and N-estimation was carried out in the "acid filtrates" after 3 days.

1.	With Na_2CO_3	21·4 c.c.	$\frac{1}{10}$ n. acid.
	Control	5·2	,,
		16·2	,,
2.	With acetic acid	15·0	,,
	Control	5·2	,,
		9·8	,,

Specimens of the enzyme mixture were kept at 37° for 16 hours with Na_2CO_3, at neutral reaction and with acetic acid, whereupon their activity was tested again.

	After 16 hrs. digestion with Na_2CO_3		After 16 hrs. digest. at neutral reaction	After 16 hrs. digest. with acetic acid
1.	With Na_2CO_3	21·0 c.c. $\frac{1}{10}$ n. acid	19·6 c.c.	17·4 c.c.
	Control	11·0 ,,	9·8	7·8
		10·0 ,,	9·8	9·6
2.	With acetic acid	16·4 ,,	15·8	14·6
	Control	11·0 ,,	9·8	7·8
		5·4 ,,	6·0	6·8

In this experiment the α-protease was weakened to about the same extent in alkaline, neutral and acid reaction, whilst the β-protease was most weakened in an alkaline medium.

A solution of the compound nucleoalbumin-α-protease in 0·25% Na_2CO_3 is considerably weakened by half-an-hour at 55°, but it is not completely destroyed—not even at 60°; but after half-an-hour at 65° no activity could be seen.

Since it might be suggested that the lieno-α-protease may be identical with trypsin, I have carried out some investigations on the trypsin as regards its activity in presence of Na_2CO_3 and of acetic acid and concerning its combination with nucleoalbumin prepared from the spleen.

Ox pancreas was treated in the way described above for obtaining the lieno-α-protease and with Am_2SO_4 products were separated corresponding to α- and β-protease. Both of them digested more strongly in alkaline than in acid solution, although the difference was not nearly so large as with the lieno-α-protease.

Digestion with fibrin. Testing with tannic acid after 2 days.

	Trypsin corresponding to α-protease		Trypsin corresponding to β-protease
1. With Na_2CO_3	26·4 c.c.	$\frac{1}{10}$ n. acid	25·4 c.c.
Control	5·0	,,	2·4
	21·1	,,	23·0
2. With 0·2% acetic acid	24·5	,,	23·2
Control	5·0	,,	2·4
	19·5	,,	20·8

In the activity of the two products there was therefore no difference as far as the reaction of the medium was concerned, and both in all probability contained the same enzyme, which in the case corresponding to the α-protease was combined with some nuclein substance and therefore was not soluble in water.

If a solution containing both spleen enzymes and not giving any precipitate with weak acetic acid is added to a solution of nucleoalbumin, prepared from ox spleen and heated to 65°, and the mixture is precipitated with weak acetic acid, then the nucleoalbumin takes down the enzymes. When this precipitate is digested with weak acetic acid, only the β-enzyme is set free and is found in the solution. This solution therefore acts only in an acid medium and not in an alkaline (see p. 162).

If the trypsin is treated with spleen-nucleoalbumin in the same ways, then a precipitate is obtained which contains trypsin. On digesting

with 0·3% acetic acid a solution is obtained, which acts in alkaline and in acid solution like the original trypsin.

5 c.c. of coagulated serum was digested with 5 c.c. of the trypsin solution so obtained. Analysis after 3 days' digestion with tannic acid.

1. With 0·25% Na_2CO_3	10·2 c.c.	$\frac{1}{10}$ n. acid.
Control	0·7	,,
	9·5	,,
2. With 0·3% acetic acid	4·4	,,
Control	0·7	,,
	3·7	,,

The lieno-α-protease therefore differs from trypsin in the following respects.

α-protease

Acts very slightly upon fibrin in an acid medium.

On acting upon the combination of α-protease and spleen-nucleoalbumin with 0·3% acetic acid at 37° no enzyme is set free.

The products of digestion give an evident biuret reaction even after weeks of digestion.

Trypsin

Acts upon fibrin nearly as strongly in 0·2% acetic acid as in 0·25% Na_2CO_3.

On acting upon the combination of trypsin and spleen-nucleoalbumin with 0·3% acetic acid at 37° trypsin is set free.

During digestion the biuret reaction eventually disappears.

Anti-enzymes of the α-protease.

If the α-protease is made to act upon ox serum, then either no digestion or only a very slight one takes place. The action is considerably stronger if the serum is coagulated by heating upon the water-bath for half-an-hour before the digestion but not if the serum is heated to 55° for half-an-hour.

Exp. 1. Analysis with tannic acid.

5 c.c. α-protease + 5 c.c. serum. Before digestion	1·0 c.c.	$\frac{1}{10}$ n. acid.
After 10 days' digestion:		
5 c.c. α-protease + 5 c.c. serum heated to 55°	1·1	,,
,, ,, boiled serum	7·5	,,

Exp. 2. Analysis with tannic acid after 3 days' digestion.

5 c.c. α-protease + 5 c.c. serum	1·8 c.c.	$\frac{1}{10}$ n. acid.
,, ,, boiled serum	7·0	,,

Exp. 3. Analysis with tannic acid after one week.

5 c.c. α-protease + 5 c.c. serum + 2 gr. fibrin	4·0 c.c.	$\frac{1}{10}$ n. acid.
,, ,, 0·25% Na_2CO_3 + 2 gr. fibrin	10·0	,,

From these experiments we must I think draw the inference that ox serum contains an antibody (anti-enzyme), which is not destroyed when the serum is heated to 55°, but is destroyed when it is heated to 100°.

I have tried to make out which fraction of the serum proteids contains the antibody by fractionating the proteids with Am_2SO_4. According to Spiro[1] the fraction coming down on about one-third saturation of the dilute serum is termed *euglobulin*, the one obtained between one-third and half saturation *pseudoglobulin*, and the portion precipitated between half and full saturation *albumin*. All fractions were dialysed till Am_2SO_4 had disappeared and then 0·25% Na_2CO_3 was added. Their influence upon the digesting power of the α-protease was tried in the following way.

Exp. 4. Analysis with tannic acid after 10 days' digestion with fibrin.

5 c.c. α-protease + 5 c.c.	0·25% Na_2CO_3 solution	9·0 c.c.	$\frac{1}{10}$ n. acid.
,, ,,	euglobulin solution	11·4	,,
,, ,,	pseudoglobulin solution	5·8	,,
,, ,,	boiled pseudoglobulin	10·6	,,
,, ,,	albumin solution	3·2	,,
,, ,,	boiled albumin solution	10·4	,,

Exp. 5. Digestion of 5 c.c. coagulated serum with the solutions as indicated below. Analysis with tannic acid. Before the digestion:

5 c.c. α-protease + 5 c.c.	euglobulin solution	2·2 c.c.	$\frac{1}{10}$ n. acid.
,, ,,	pseudoglobulin solution	2·2	,,
,, ,,	albumin solution	2·4	,,
After 2 days' digestion.			
5 c.c. α-protease + 5 c.c.	euglobulin solution	4·8	,,
,, ,,	boiled euglobulin solution	6·0	,,
,, ,,	pseudoglobulin solution	2·8	,,
,, ,,	boiled ,, ,,	6·8	,,
,, ,,	albumin solution	2·6	,,
,, ,,	boiled albumin solution	6·5	,,

In this case the proteid fractions were prepared from 400 c.c. of blood and the volumes of the obtained fractions were: euglobulin fraction 50 c.c., pseudoglobulin fraction 100 c.c., albumin fraction 400 c.c.

As regards the influence of the euglobulin fraction these experiments are not conclusive, Exp. 4 giving an increase and Exp. 5 a slight diminution in the activity of the enzyme, but the pseudoglobulin

[1] Fuld u. Spiro, *Zeitschr. f. physiol. Chem.* xxxi. p. 132. 1900; Porges u. Spiro, *Ibid.* xxxvi. p. 407. 1902.

fraction and more markedly the albumin fraction diminish the effect of the enzyme, the diminishing influence disappearing on boiling.

Since the effect of the euglobulin fraction had not been settled, I carried out one experiment, in which the precipitate coming down on dialysing ox serum was tried. This precipitate corresponds roughly to the part of the euglobulin fraction not soluble in water, and it might therefore be expected to have about the same effect as the euglobulin. It was washed and dissolved in 0·25 Na_2CO_3 solution.

Exp. 6. Digestion with fibrin. Analysis after one week.

5 c.c. α-protease + 5 c.c. 0·25 % Na_2CO_3 solution	5·2 c.c. $\frac{1}{10}$ n. acid.	
,, ,, precipitate solution	9·6	,,
,, ,, boiled precipitate solution	5·6	,,

The experiment shows an increasing influence of the globulin, coming down on dialysing the serum, upon the effect of the α-protease, this influence disappearing on boiling. Further investigations are being carried out as to the nature of the substance causing the increase.

The substance or substances in ox serum checking the activity of the α-protease are mainly contained in the albumin (and pseudoglobulin) fractions, whereas the euglobulin fraction as well as the globulin, precipitated on dialysing, in some cases increases the activity.

Since the fresh spleen always contains some serum, and this ought to be found in the pressed juice, we must expect that the pressed juice should check the activity of the α-protease. In fact this has been found to be the case, as borne out by the following experiment.

Exp. 7. To pressed spleen juice Na_2CO_3 was added to 0·25 %. The juice was digested with α-protease and the effect tested with tannic acid. Before digestion:

5 c.c. α-protease + 5 c.c. spleen juice	6·4 c.c. $\frac{1}{10}$ n. acid.	
After 2 days' digestion:		
5 c.c. α-protease + 5 c.c. juice	13·0	,,
,, ,, juice heated ½ hour to 60°	13·5	,,
,, ,, juice heated ½ hour to 100°	15·5	,,

The experiment shows that the substance which checks the action is not affected by a temp. of 60° but is destroyed at 100°.

Not only the ox serum but also pig and horse serum diminish the effect of α-protease, obtained from the ox spleen, as can be seen from the following experiments.

Exp. 8. Before digestion:	
5 c.c. α-protease + 5 c.c. pig serum	1·5 c.c. $\frac{1}{10}$ n. acid.
After 3 days' digestion:	
5 c.c. α-protease + 5 c.c. pig serum	3·7 ,,
,, ,, serum heated to 100°	9·4 ,,
Exp. 9. Before digestion:	
5 c.c. α-protease + 5 c.c. horse serum	1·6 c.c. $\frac{1}{10}$ n. acid.
After 3 days' digestion:	
5 c.c. α-protease + 5 c.c. serum	3·7 ,,
,, ,, serum heated to 100°	9·1 ,,

Antibodies of enzymes have been found before in normal serum and other animal tissues in several cases. Hammarsten and Rödén[1] observed that horse serum prevents the coagulation of milk, which property Fuld and Spiro[2] found to be attached to the *pseudoglobulin*, whilst the *euglobulin* accelerates the coagulation. The anti-tryptic action of normal serum has been demonstrated by Hahn[3], Camus and Gley[4], Landsteiner[5], who found the anti-tryptic activity to be bound to the albumin fraction, and by Glaessner[6], who found it in the euglobulin fraction. Weinland[7] showed an anti-tryptic activity of extracts of the small intestine and an antipeptic action in the wall of the stomach.

Lieno-β-protease.

The spleen enzyme acting in an acid medium can be obtained in different ways. These are as follows:

1. The easiest way is to digest the minced spleen with water containing 0·2—0·3% acetic acid, filter after 2—3 days, digest the filtrate for 3—4 days more, filter again and saturate with Am_2SO_4. After removing the salt from the precipitate by dialysing, the resulting solution can be used for digesting proteids.

[1] *Upsala läkareförenings förh.* XXII. p. 546.
[2] *Zeitschr. f. physiol. Chem.* XXXI. p. 132. 1900.
[3] *Berl. klin. Wochenschr.* 1897.
[4] *Compt. rend. de la Soc. de Biol.* No. 5. 1900.
[5] *Centralbl. f. Bacteriol.* 1900.
[6] *Beitr. z. ch. Physiol. u. Path.* IV. p. 79. 1903.
[7] *Zeitschr. f. Biol.* XXVI. 1902.

Exp. 1. Digestion with fibrin. Control specimens contained boiled enzyme. Digestion was carried out with 0·25 % Na_2CO_3 in neutral solution and with 0·2 % acetic acid. After 4 days' digestion N was estimated in the "acid filtrates."

1. With Na_2CO_3	3·4 c.c. $\frac{1}{10}$ n. acid.	
Control	2·4	,,
	1·0	,,
2. In neutral medium	8·8	,,
Control	7·0	,,
	1·8	,,
3. With acetic acid	20·2	,,
Control	2·8	,,
	17·4	,,

Exp. 2. Digestion with fibrin. After 4 days' digestion N was estimated in the "acid filtrates" and in the tannic acid filtrates.

After digestion with Na_2CO_3:

1. Acid filtrate	6·6 c.c. $\frac{1}{10}$ n. acid.	
Control	3·2	,,
	3·4	,,
2. Tannic acid filtrate	2·2	,,
Control	1·2	,,
	1·0	,,

After digestion with acetic acid:

3. Acid filtrate	26·8	,,
Control	3·0	,,
	23·8	,,
4. Tannic acid filtrate	8·6	,,
Control	1·2	,,
	7·4	,,

These experiments therefore show a very strong activity in the presence of acetic acid, although there seems to be a slight action in an alkaline medium too.

2. Another way of obtaining the β-protease is to digest the pressed spleen juice with 0·2—0·3 % acetic acid for 5—7 days, filter and saturate with Am_2SO_4 in the way just mentioned. The activity of the enzyme thus obtained is the same as of the enzyme prepared in the way set forth under 1.

EXP. 3. Analysis of the "acid filtrates" after 3 days' digestion with fibrin.

1.	With Na_2CO_3	6·6 c.c.	$\frac{1}{10}$ n. acid.
	Control	5·7	,,
		0·9	,,
2.	In neutral medium	8·6	,,
	Control	5·9	,,
		2·7	,,
3.	With acetic acid	25·5	,,
	Control	5·8	,,
		19·7	,,

3. If pressed spleen juice is diluted with 3—4 volumes of water and 0·2% acetic acid is added, then a precipitate is formed which contains β-protease. If it is filtered off and acted upon with 0·3 % acetic acid at 37° for 16 hours, then the solution obtained on filtering contains β-enzyme, as shown by the following experiments.

Digestion with fibrin. N-estimation in the "acid filtrates" after 6 days.

		Exp. 4.		Exp. 5.
1.	With Na_2CO_3	9·8 c.c.	$\frac{1}{10}$ n. acid	6·4 c.c.
	Control	9·2	,,	6·4
		0·6	,,	0·0
2.	With acetic acid	24·6	,,	21·8
	Control	9·2	,,	6·6
		15·6	,,	15·2

The residue not dissolved in 0·3% acetic acid, and obtained on filtering off the enzyme solution, can partly be dissolved in very weak (0·25%) Na_2CO_3 solution, and in this solution a precipitate is produced by weak acetic acid. This precipitate always contains phosphorus, and since it does not give any purin bases on boiling with acid, it contains a pseudo-nuclein (Hammarsten) or para-nuclein (Kossel). When an alkaline solution of this substance is added to a solution of the β-enzyme, then acetic acid, if added up to 0·2%, produces a precipitate, which contains the β-protease. If no salt is present, all of the enzyme seems to be taken down. Since the enzyme can be split off again by the action of acetic acid, this precipitate corresponds in a way to the precipitate formed by adding acetic acid to the fresh juice. In both cases it is very difficult to regenerate the whole amount of the enzyme from the precipitate. Even after digestion with acetic acid for several days

some enzyme sticks to the precipitate, and this remainder can only be obtained in solution by redissolving the nuclein in Na_2CO_3, precipitating and digesting again with acetic acid. The enzyme solution obtained with acetic acid always gives a distinct biuret reaction.

4. Finally, the β-protease can be obtained, when the α-protease is prepared. According to the table (p. 158) eventually a precipitate is obtained by adding acetic acid to the filtrate C, which precipitate (R) contains α-protease and β-protease. By acting on this precipitate with 0·3% acetic acid at 37° for 16 hours, part of the β-protease is dissolved, the α-protease remaining in the residue (see p. 161). The precipitate R being soluble in weak Na_2CO_3 and containing phosphorus suggests a comparison with the precipitate from which the β-protease was obtained, sect. 3, and in principle the two ways of obtaining the β-protease seem to be identical.

Remarks upon the above methods.

By aid of the methods given in §§ 1 and 2, pp. 169, 170, the β-protease is obtained immediately in a form soluble in water and weak acetic acid, but if the enzyme is prepared as described sects. 3 or 4, it is obtained in the first instance in a form which is not soluble in water or acetic acid, but which can be converted into the soluble form by the action of acetic acid at 37°. The insoluble form seems to be a combination between nucleoalbumins and the enzyme, from which the enzyme is liberated by digesting the nucleoalbumins. As indicated above, the soluble form of the enzyme can be converted into the insoluble by adding a nucleoalbumin and precipitating with acetic acid. Not only para-nuclein but also nucleoalbumins and nucleoproteids are capable of combining with the β-enzyme, and if no salt is present in the solution and a sufficient amount of the proteids is added, then the enzyme seems to be completely removed from the solution.

Properties of the β-protease.

The *products* of the activity of the β-protease have been studied by Dr Leathes[1]. According to his results the cleavage process effected by this enzyme is carried quite as far as by the trypsin.

Like most or possibly all proteolytic enzymes the β-protease is taken up by fibrin. As far as this enzyme is concerned the reaction of the

[1] This *Journal*, XXVIII. p. 360. 1902.

enzyme solution is very important, the enzyme combining with fibrin only in an acid medium (0·2—0·4 % acetic acid) and not in a neutral or alkaline solution.

The β-protease is very stable. Kept for a fortnight together with toluol at ordinary temperature it shows no marked diminution in activity, and even after $3\frac{1}{2}$ months it showed an evident effect.

The temperature at which the enzyme loses its activity depends very much upon the other substances present in the solution. Thus the fresh pressed spleen juice seems to act nearly as strongly at 60° as at 37° in an acid medium, and we must conclude that the β-enzyme is not seriously affected by a temperature of 60°, this in all probability depending upon the protecting influence of proteids present. An enzyme, obtained by precipitating with Am_2SO_4 after 16 hours' digestion, was destroyed by heating to 55° for half-an-hour in the presence of 0·2 % acetic acid or 0·25 % Na_2CO_3 but not in a neutral medium. When the enzyme is prepared from an isolated nuclein compound by digesting with acetic acid, then the solution contains only very little proteids, the biuret reaction being weak; in this case the enzyme is destroyed by 55°, but not by 50° for half-an-hour in neutral solution.

The alkaline reaction of the blood would prevent the β-protease from acting if it were present there, and therefore a particular *anti-enzyme* would be without purpose. Experiments carried out with a view to finding antibodies in ox serum have been without success, the digestion with boiled and with unboiled serum giving exactly the same result.

In order to compare the influence of acetic acid and of HCl upon the activity of the enzyme the following experiment was carried out.

2 grms. boiled fibrin were digested with 10 c.c. enzyme solution, in the presence of the following amounts of acid for one week, and N was determined in 5 c.c. of the filtrate.

0·1 %	acetic acid.	N corresponds to	10·6 c.c.	$\frac{1}{10}$ n. acid.
0·2	,,	,,	9·6	,,
0·3	,,	,,	10·0	,,
0·4	,,	,,	9·4	,,
0·5	,,	,,	8·6	,,
0·6	,,	,,	7·8	,,
0·8	HCl.	,,	6·2	,,
0·1	,,	,,	5·2	,,
0·2	,,	,,	5·4	,,

In another experiment unboiled fibrin was used and control specimens contained boiled enzyme. N was estimated both in the acid filtrate and in the filtrate from the tannic acid precipitate.

	0·3 % acetic acid:		0·08 % HCl:
1.	Acid filtrate	26·8 c.c. $\frac{1}{10}$ n. acid	14·4 c.c.
	Control	3·0 ,,	2·6
		23·8 ,,	11·8
2.	Tannic acid filtrate	8·6 ,,	5·4
	Control	1·2 ,,	1·2
		7·4 ,,	4·2

In both experiments the influence of HCl was weaker than of acetic acid.

The β-protease cannot be identical with the pepsin.

β-protease	Pepsin
Digestion goes as far as with trypsin. Biuret reaction eventually disappears.	Digestion does not go so far as with trypsin. Biuret reaction does not disappear.
Acts stronger with acetic acid than with HCl.	Acts stronger with HCl than with acetic acid.
Is not destroyed by one night's digestion with 0·25 % Na_2CO_3.	Is destroyed by Na_2CO_3.

Conclusions.

The results of the above investigations can be summed up as follows.

1. The spleen of the ox contains two proteolytic enzymes, the one (α-protease) acting only or principally in an alkaline medium, and the other (β-protease) acting only or mainly in an acid medium.

2. The β-protease can be obtained in solution by digesting the spleen with very weak acetic acid, and the α-protease (together with β-protease) by extracting the residue with sodium chloride solution, dialysing and precipitating with weak acetic acid. The precipitate contains both enzymes.

3. Both enzymes have been obtained in two forms:

a. Combined with nuclein substances, in which case they are not soluble in weak acetic acid.

b. Not combined with nuclein substances and therefore soluble in weak acetic acid.

4. The form not soluble in acetic acid can be obtained from the soluble form by adding a nuclein substance and acetic acid in absence of salt.

5. The soluble form of the α-protease can be prepared from a salt solution of the unsoluble form by removing the nuclein substance with

acetic acid, and the soluble form of the β-protease can be obtained from the unsoluble by digesting the nuclein substance in the presence of weak acetic acid.

6. Ox serum contains antibodies of the α-protease but not of the β-protease.

The spleen enzymes in all probability are contained in the leucocytes of the spleen and there they may perform their functions. Thus they may effect the metabolism of the nitrogen inside the cells, and they may also have something to do with the digesting processes, which according to the view of Metchnikoff take place inside these cells during the phagocytosis. As to the significance of the β-protease it should be remembered that the acid reaction required for its activity according to investigations carried out by Ehrlich and particularly by Metchnikoff and his pupils occurs at some places inside the leucocytes. Thus the reaction of the nuclei is considered to be acid, and the digestion of bacteria and other substances taken up by the leucocytes in most cases takes place inside the so-called digesting vacuoles, where the reaction as a rule is acid[1].

Whether the spleen enzymes may be active outside the leucocytes as well as inside is a question which cannot be settled at present. It does not seem to be impossible that the enzymes could leave the cells either by an act of secretion or perhaps by digesting some of the cells. Similar or perhaps identical enzymes seem to be present in several organs[2] although not in such amounts as in the spleen, and there may be proteolytic enzymes present in the blood although their activity may be checked by antibodies[3]. Therefore I do not think that the spleen enzymes should at once be ranked with the so-called intracellular enzymes.

[1] Metchnikoff. *L'immunité dans les maladies infectieuses*, Paris, 1901.
[2] *Zeitschr. f. physiol. Chem.* XXXII. p. 531. 1901.
[3] *Compt. rend. de la Soc. Biol.* LV. No. 23. 1903.