CV. STUDIES IN THE METABOLISM OF PROTOZOA

III. SOME PROPERTIES OF A PROTEOLYTIC EXTRACT OBTAINED FROM *GLAUCOMA PIRIFORMIS*

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THE study of protozoal enzymes has been facilitated by the methods recently developed for the culture of protozoa in the absence of bacteria or other material containing enzymes; whilst the extensive data bearing upon the nutrition of the protozoa [A. Lwoff, 1932; Sandon, 1932] suggest the value of knowledge of the enzymic equipment of the protozoal cell for their interpretation.

A. Lwoff & Roukhelman [1926] reported that *Glaucoma piriformis* produces an enzyme which diffuses into the culture medium and liquifies gelatin. M. Lwoff [1929] found that growing cultures of *Leptomonas ctenocephali* secrete an enzyme which liquifies gelatin. A. Lwoff [1932] found that cultures of *Glaucoma piriformis*, grown upon nutrient gelatin slopes, liquified the medium. Elliott [1933] showed that growing cultures of *Colpidium striatum* liquified gelatin, clotted milk and subsequently peptonized the curd. For earlier work along these lines the reader is referred to a previous paper [Lawrie, 1935], in which also it is shown that a suspension of *Glaucoma piriformis*, saturated with chloroform, can carry out proteolysis at *p*H values ranging from 2·2 to 9·6. A cell-free material showing proteolytic activity has now been obtained from *G. piriformis*. The method used for its preparation and certain of its properties are described below.

EXPERIMENTAL

The protozoan used in these experiments is *G. piriformis*, isolated by Dr Muriel Robertson, and is the same as that used in work described in the previous paper in this series [Lawrie, 1935]. The conditions of culture also were the same as those used in that work, except that, towards the end of the work, upon the suggestion of Dr A. Lwoff, the protozoa were grown upon a medium prepared by autoclaving about 5 g. of sheep's brain with 200 ml. of distilled water.

Estimation of proteolysis. The digestion of protein was followed by means of estimations of the non-protein-nitrogen, soluble in a suitable protein precipitant, before and after incubation of protease preparation with protein in buffered solution. A method of this type offers advantages, chiefly in respect of applicability to the earliest stages of protein digestion, over methods involving the estimation of free amino-groups [Farber & Wynne, 1935]. When casein, egg albumin or α-glutelin were used as substrates, 1 ml. of the digest was precipitated with 2 ml. of 10 % trichloroacetic acid. The precipitated protein was filtered off and 2 ml. of the filtrate incinerated with 0·5 ml. of a mixture of H_2SO_4 3 vol., H_3PO_4 1 vol., selenium oxychloride 1 % in H_2SO_4 0·4 vol., in a test-tube of known weight according to the technique used by Borsook [1935] to prevent loss of material by spurting. When incineration was complete 2 ml. of water were added to each tube followed by 1 drop of 0·1 % phenol red and sufficient 40 %

NaOH to give a yellow end-point. The contents of the tube were then heated to boiling to expel the drop of incinerated material in the Folin anti-bumping device and cooled. The tube and contents were then weighed, giving the weight of incinerated and neutralized material, and a gravimetrically determined aliquot was distilled into 0·01 *N* H_2SO_4 in the Conway distillation apparatus [Conway & Byrne, 1933]. An aliquot portion of the distillate was then pipetted into a 50 ml. conical flask and the ammonia determined colorimetrically by Borsook's modification of Van Slyke's & Hiller's procedure [Borsook, 1935; Van Slyke & Hiller, 1933]. In the case of the digests containing less than 10 mg. of non-protein-nitrogen per 100 ml., the non-protein-nitrogen may be determined by this method with a difference between duplicates not greater than 0·4 mg. per 100 ml. In the case of digests containing more non-protein-nitrogen than this the difference between duplicates is not greater than 4 % of their mean.

Preparation of cell-free material containing protease. Cultures containing brain tissue were first filtered through wool. Cultures in peptone required no filtration. The culture was then well shaken and a sample taken for enumeration of the protozoa present. The cells were then separated from the culture medium by centrifuging. It was soon found that prolonged centrifuging resulted in breaking up of the cells and diminished yield of protease preparation. For this reason those cells separated by centrifuging for 15 min. were at once worked up and those in the supernatant fluid discarded. In the earlier experiments the separated cells were broken up by suspension in buffer at *p*H 5·5 and freezing and thawing 12 times. Later it was found more convenient to cytolyse the protozoa by saturating the suspension in buffer with chloroform and incubating at 37° for 2 hr. After cytolysis the insoluble material was removed by centrifuging, leaving a faintly opalescent supernatant fluid. To this extract was added 1 g. of $MgSO_4, 7H_2O$ per 1 ml. to produce approximately full saturation at room temperature. When solution of the $MgSO_4, 7H_2O$ was almost complete the turbid supernatant fluid was poured off and centrifuged. The resulting clear supernatant fluid was poured off as completely as possible and the deposit transferred to a watch-glass with the aid of about 2 ml. of distilled water. The preparation was then placed in a vacuum desiccator. After standing overnight the preparation was then obtained as a white, feathery material, easily removed from the watch-glass and readily soluble in buffer at *p*H 7·0. If evaporation in the desiccator were insufficiently rapid to freeze the preparation a much less tractable material was obtained. The *p*H at which extraction of the enzyme from the cells was carried out had little effect upon the proteolytic activity of the extract. A *p*H of 5·5 was finally adopted for extraction since at this *p*H the clearest extract was obtained.

The precipitation of protease by magnesium sulphate. Proteolytic extracts were prepared by the technique described above, using freezing and thawing to bring about cytolysis. The precipitates produced by the addition of various amounts of magnesium sulphate to the extracts were separated by centrifuging and dissolved in 5 ml. of 0·05 *M* phosphate buffer at *p*H 7·0. A sample of this preparation was then taken for estimation of nitrogen content. The protease activity was then estimated by adding enough sodium caseinate to give an initial substrate concentration of 1 %, and a few drops of chloroform to prevent bacterial contamination. An initial sample was removed for estimation of non-protein-nitrogen and the remainder incubated at 37° for 24 hr. At the end of this period the final non-protein-nitrogen was estimated and the increase in non-protein-nitrogen was regarded as indicating the amount of protease precipitated by any given addition of magnesium sulphate, whilst the ratio of the

increase in non-protein-nitrogen to the total nitrogen in the protease preparation was regarded as indicating the degree to which purification of the protease, relative to the total nitrogen of the extract, had been obtained. Whilst these experiments give a reliable indication of the amount of protease precipitated under the conditions compared, yet it must be borne in mind that the indication of the degree of purification of the protease has been influenced to an unknown and possibly great extent by the variations in enzyme concentration in the different digests. The results of these experiments are given in Table I and they

Table I

Source of protease	Nitrogen in protease preparation (mg. per 100 ml.)	Casein digested (mg. N per 100 ml. of digest)	Casein digested (in mg. N) per 1 mg. of N in protease preparation
Fraction precipitated by 50% saturation	1·0	7·2	7·2
Fraction precipitated by 70% saturation	3·5	10·3	1·2
Fraction precipitated between 50 and 75% saturation	1·4	6·1	4·4
Fraction precipitated between 70 and 100% saturation	8·6	10·5	1·2

indicate that the protease is only gradually precipitated by increasing magnesium sulphate concentration and that the material precipitated at the lower concentrations probably has a higher ratio of protease to total nitrogen than that precipitated at higher concentrations.

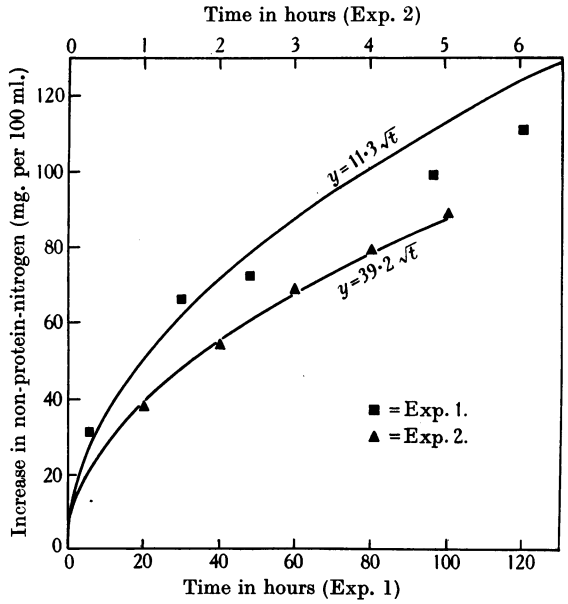

Fig. 1. Rate of proteolysis of casein.

The rate of proteolysis of casein. Exp. 1. A protease preparation obtained from a culture containing 20×10^6 cells was dissolved in 4 ml. of a 1% solution of sodium caseinate in 0·05*M* phosphate buffer at *p*H 7·0 and incubated at 37°. The non-protein-nitrogen of this preparation was estimated at the start of

incubation and at intervals afterwards for 120 hr. Bacterial contamination was prevented by saturation of the digest with chloroform. The result of this experiment is shown in Fig. 1. The curve shown fits the well-known approximation $y = k\sqrt{t}$ relating the quantity of substrate digested (y) with the duration of enzyme action (t) where k is given the value 11·3. The observed points are seen to be in agreement with this curve.

Exp. 2. A similar experiment to the above was carried out, using a stronger enzyme preparation and confining the observations to the first 5 hr. of digestion. The initial concentrations in the digest were 1 % casein, 0·025 *M* phosphate buffer and 0·3 % enzyme preparation. The digest was saturated with chloroform and its *p*H maintained at 7·0 by the phosphate buffer. As in the previous experiment the amount of substrate digested varied with the duration of digestion according to the equation $y = k\sqrt{t}$. The results obtained are shown in Fig. 1 and are seen to agree within the limits of experimental error with such a curve when k is given the value 39·2.

The proteolysis of gelatin. The proteolysis of gelatin was measured by estimation of the non-protein-nitrogen in the digest as in the case of casein, except that 2 % tannic acid solution was used as precipitant.

Exp. 3. A digest was set up containing 1 % of gelatin, 0·025 *M* phosphate buffer at *p*H 7·0, 0·1 % of enzyme preparation and saturated with chloroform. Incubation was carried out for 24 hr. at 37°. Controls with heat-inactivated enzyme and without substrate were incubated simultaneously with the test digest and the increases in non-protein-nitrogen observed in the control digests deducted from those observed in the test digests. 29 mg. of gelatin nitrogen were digested per 100 ml. of digest.

Exp. 4. A digest was set up under exactly similar conditions to those of Exp. 3 except that a fresh enzyme preparation was used. In this case 38 mg. of gelatin nitrogen were digested per 100 ml. of digest. Thus this protease can break down gelatin to compounds soluble in tannic acid and therefore presumably of simpler constitution than peptone.

Comparison of the proteolyses of egg albumin and casein. Digests were set up containing 1 % of substrate, 0·025 *M* phosphate buffer at *p*H 7·0 and 0·1 % of enzyme preparation and incubated at 37° for 24 hr. Undigested protein was precipitated by means of trichloroacetic acid. The results of two such experiments are shown in Table II. The egg albumin used was prepared from a

Table II. *Comparison of proteolyses of egg albumin and casein. Increase in non-protein-nitrogen (mg. per 100 ml.)*

		Test digest	Heat-inactivated control	Control without substrate	Result of proteolysis
Exp. 5	Casein	81·1	2·3	0·6	78·2
	Egg albumin	6·4	1·3	0·6	4·5
Exp. 6	Casein	78·4	2·5	1·0	74·9
	Egg albumin	7·6	1·4	1·0	5·2

sample, purchased from the British Drug Houses, Ltd., by suspension in distilled water, removal of some insoluble material by filtration and evaporation of the filtrate *in vacuo* over sulphuric acid. This protein is seen to be comparatively resistant to digestion by the protease extracted from *G. piriformis*.

The hydrolysis of native and heat-coagulated egg albumin was studied in experiments similar to the above, but no consistent difference was found between the susceptibilities of these preparations to hydrolysis.

Comparison of the proteolyses of α-glutelin and casein

α-Glutelin was prepared according to the method of Csonka & Jones [1927]. Since this protein is insoluble in buffer at *p*H 7·0, a 1 % suspension in this buffer was prepared. The comparison was carried out in exactly the same way as in the case of egg albumin and casein. The results of two experiments are given in Table III. From these results it is clear that this vegetable protein, despite its insolubility, is susceptible to hydrolysis by the protease under investigation.

Table III. *Comparison of proteolyses of α-glutelin and casein. Increase in non-protein-nitrogen (mg. per 100 ml.)*

		Test digest	Heat-inactivated control	Control without substrate	Result of proteolysis
Exp. 7	Casein	49·1	0·2	0·0	48·9
	α-Glutelin	29·2	0·3	0·0	28·9
Exp. 8	Casein	43·0	1·2	0·7	41·1
	α-Glutelin	19·6	0·5	0·7	18·4

Determination of p*H optimum for proteolysis of gelatin.* Gelatin was chosen as substrate in these experiments because of its solubility throughout the *p*H range to be studied. In order to avoid the discontinuity introduced by changing from one buffering substance to another, the sodium barbiturate buffer of Michaelis [1931] was used. When this buffer was used the proteolysis taking place at *p*H 7·0 during 24 hr. at 37° was only 38 % of that occurring in the presence of phosphate buffer at the same *p*H.

Exp. 9. A series of sodium barbiturate buffers at *p*H 3·0, 4·0, 5·0, 6·0, 7·0, 8·0 and 9·0 were prepared according to the directions of Michaelis. The buffered substrates for this experiment were prepared as follows. 2 g. of gelatin were dissolved in 100 ml. of warm water and saturated with chloroform. 5 ml. of this solution were transferred to a 10 ml. graduated cylinder and the *p*H adjusted to the required value with 0·1 *N* NaOH or 0·1 *N* sulphuric acid and the volume made up to 10 ml. with water. 2 ml. of this gelatin solution at adjusted *p*H were transferred to a test-tube and 2 ml. of sodium barbiturate buffer at the same *p*H added. 1 vol. of a solution containing 0·2 % of dry enzyme preparation was added to 1 vol. of each of the buffered substrate solutions. Initial samples were taken for estimation of non-protein-nitrogen and the digests then incubated for 24 hr. at 37°.

In order to avoid the large blank non-protein-nitrogen values which would be caused by the presence of barbiturate buffer in all the digests, each sample taken for estimation, after deproteinization with tannic acid, and before incineration, was extracted five times with successive 3 ml. vol. of ether. The efficacy of this process is shown in Table IV.

Table IV. *Extraction of barbiturate nitrogen from deproteinized 1 % gelatin solutions of approximately 2 ml. volume*

Number of extractions with 3 ml. of ether	Mg. non-protein-nitrogen per 100 ml. of solution
None	61·4
1	19·5
2	9·4
3	6·5
4	6·0

The results of Exp. 9 are shown in Fig. 2. The optimum *p*H for the digestion of gelatin is seen to be about 6·0. Subsequent experiments confirmed this value and it is also in agreement with earlier experiments in which a suspension of *G. piriformis* in presence of chloroform was used as the source of protease and caseinogen as the substrate.

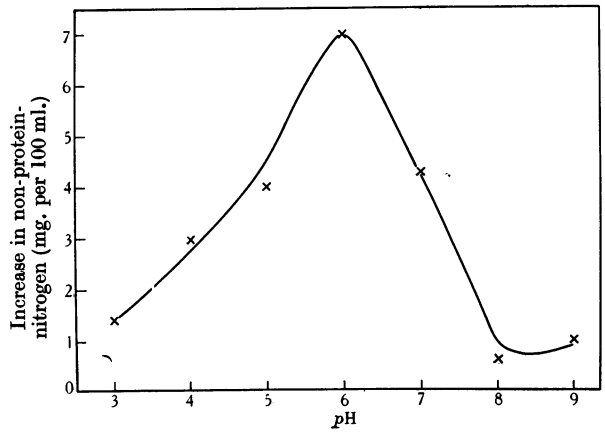

Fig. 2. Effect of *p*H upon the rate of proteolysis of gelatin.

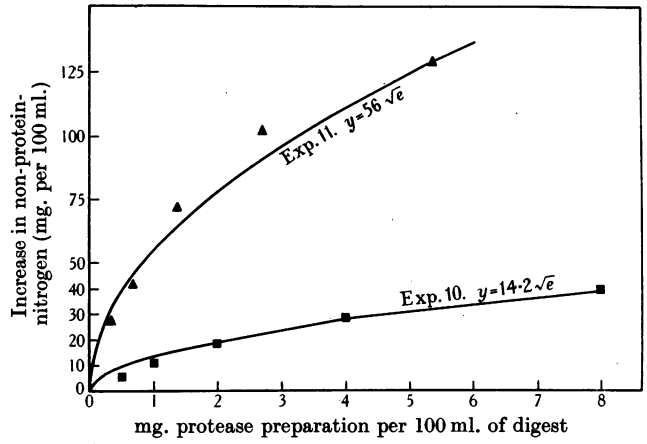

Fig. 3. Effect of protease concentration upon rate of proteolysis.

Effect of enzyme concentration upon the rate of proteolysis of casein. Exp. 10. A 1% solution of caseinogen in 0·025 *M* phthalate buffer at *p*H 5·5 was prepared. Digests were set up with this substrate containing 0·5, 1·0, 2·0, 4·0 and 8·0 mg. of enzyme preparation per 1 ml. and saturated with chloroform. Initial samples were taken for estimation of non-protein-nitrogen at once and final samples after incubation for 6 hr. at 37°. Within a few minutes of adding substrate to the enzyme preparations coagulation of protein was observed in the digest containing 8·0 mg. of enzyme preparation per 1 ml. and deep opalescence in the digest containing one-half of this enzyme concentration. On account of the liability of the casein to precipitate at a *p*H of 5·5 and so become less

accessible to the enzyme and prevent accurate sampling of the digests, this experiment was repeated at *p*H 7·0. The concentrations of enzyme preparation used were 0·33, 0·67, 1·33, 2·66 and 5·32 mg. per 1 ml., whilst the other conditions of digestion and analysis were the same as in the previous experiment. No precipitation of substrate occurred.

The results of these two experiments are given in Fig. 3. It will be seen that the results in both experiments follow Schutz's rule fairly closely. In Exp. 10 the observations lie close to the curve $y = 14{\cdot}2\sqrt{e}$, and in Exp. 11 they are in fairly close agreement with the curve $y = 56\sqrt{e}$. In both cases "y" is the rate of proteolysis measured over the first 6 hr. of digestion and "e" the corresponding enzyme concentration. The less active proteolysis of casein in Exp. 10 was probably due to the precipitation of substrate which occurred very soon after adding substrate to the enzyme preparation.

The effect of substrate concentration upon the rate of proteolysis. In experiments carried out in order to determine this relationship for the case of the protease under investigation gelatin was used as substrate, the *p*H was maintained at 6·0 by means of 0·05 *M* phosphate buffer and the temperature of incubation was 37°.

Exp. 12. Gelatin concentrations of 10, 5, 2·5 and 1 % were employed. The concentration of enzyme preparation in the digests was 0·2 % and the duration of incubation 6 hr. The tannic acid solutions used to precipitate the undigested gelatin contained $2n$ % of tannic acid where "n" is the percentage of gelatin in the digests at the commencement of incubation. The greatest increase in non-protein-nitrogen occurred in the digest containing 1 % of gelatin; higher concentrations causing slight inhibition.

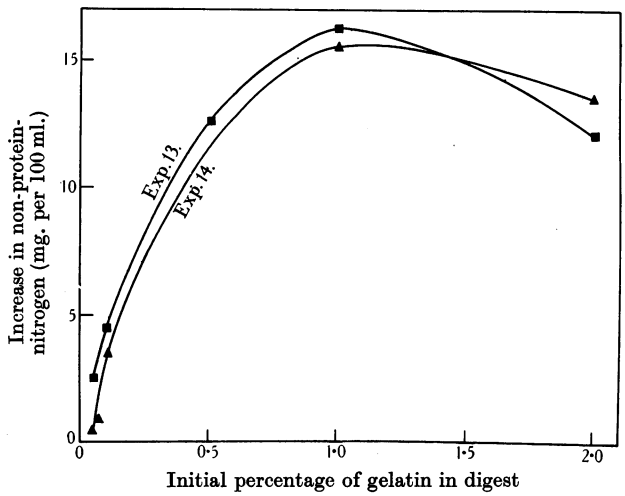

Fig. 4. Effect of substrate concentration upon the rate of proteolysis of gelatin.

Exp. 13. In view of the results obtained in the previous experiments gelatin concentrations of 2, 1, 0·5, 0·1 and 0·05 % were used. A control without substrate was also set up. The concentration of enzyme preparation was 0·2 % and the duration of incubation 2 hr.

Exp. 14. The gelatin concentrations used in this experiment were 2, 1, 0·1, 0·07, 0·04 % and nil. The concentration of enzyme preparation was 0·096 % and the duration of incubation 6 hr.

In Exps. 13 and 14 4 % tannic acid was used to precipitate the digests set up to contain 2 % of gelatin, and 2 % tannic acid solution to precipitate all other digests.

The results of Exps. 13 and 14 are shown in Fig. 4. These curves were examined to see whether they conformed to the equation $k = x\left(\frac{V}{v} - 1\right)$, where V = the maximum velocity of enzyme action, v = the observed velocity at substrate concentration = x, and k = the "Michaelis constant" for the enzyme and substrate concerned. This relation clearly cannot hold good for velocities of enzyme action approximating to the maximum velocity, but in the system under consideration it was not found to apply to the region of relatively low velocities either.

The possibility of activation of the protease by cyanide

Exp. 15. In the first attempts to activate the protease with cyanide a technique taken from early work on the activation of papain by HCN was adopted. A 0·1 *M* solution of HCN, adjusted to a *p*H of approximately 5·0, was prepared according to the method used by Frankel [1917]. 4·3 ml. of a solution containing 0·36 % of protease preparation were prepared and two 2 ml. samples withdrawn. One of these was inactivated by boiling for 5 min. and restoring the volume to 2 ml. with distilled water. This heat-inactivated control was subsequently treated in exactly the same way as the active protease preparations and served to guard against errors due to non-enzymic hydrolysis of substrate or bacterial contamination of digests. To one 0·9 ml. sample of protease solution was then added 0·9 ml. of 0·1 *M* HCN at *p*H 5·0, and to a second 0·9 ml. sample 0·9 ml. of water. These preparations were now incubated at 37° for 2 hr. The *p*H of the preparations containing HCN was then adjusted to 7·0 with 0·1 *N* NaOH and a volume of water equal to that of the alkali required added to the corresponding control. To each preparation was then added an equal volume of 2 % sodium caseinate in 0·025 *M* buffer at *p*H 7·0, initial samples were taken for estimation of non-protein-nitrogen and the digests then incubated for 20 hr. at 37°. The results of this experiment are given in Table V. Within the limits of

Table V. *The effect of cyanide at* p*H 5·0 upon the activity of protease*

Protease preparation	Increase in non-protein-nitrogen
(1) Untreated	44·6
(2) Heat-inactivated control corresponding to (1)	0·0
(3) Treated with cyanide preparation	44·9
(4) Heat-inactivated control corresponding to (3)	0·6

Activation by cyanide preparation at *p*H 5·0 = 0·7 %.

experimental error no activation or inhibition was observed, and a second experiment carried out in the same way with a fresh enzyme preparation gave the same result.

Exp. 16. The effect of free HCN acting upon the protease in unbuffered solution was next studied. The procedure in this experiment was the same as that in Exp. 15 except that a 0·04 *M* solution of HCN, obtained by dilution of a stock 2 % solution, was used as activator. The results of this experiment are shown in Table VI. It is seen that a strong inhibition was obtained. In a subsequent confirmatory experiment an inactivation of 70 % was obtained.

Table VI. *The effect of free HCN upon the activity of protease*

Protease preparations	Increase in non-protein-nitrogen (mg. per 100 ml.)
(1) Untreated	59·2
(2) Heat-inactivated control corresponding to (1)	0·1
(3) Treated with HCN	12·8
(4) Heat-inactivated control corresponding to (3)	0·4

Inactivation by HCN = 78%.

Exp. 17. Since the *p*H at which cyanide was allowed to act upon the protease appeared to influence the result so greatly, an attempt was made to activate the enzyme by means of incubation for 2 hr. at 37° with 0·02 *M* KCN in phosphate buffer at *p*H 7·0. After activation, substrate was added as in the previous experiments, and proteolysis allowed to proceed at 37° for 6 hr. A small degree of activation was observed, as is shown in Table VII. A subsequent experiment to confirm the above result, using a fresh protease preparation, gave an activation of 19%.

Table VII. *The effect of cyanide at* p*H 7·0 upon the activity of protease*

Protease preparation	Increase in non-protein-nitrogen (mg. per 100 ml.)
(1) Untreated	32·9
(2) Heat-inactivated control corresponding to (1)	0·0
(3) Treated with KCN at *p*H 7·0	37·5
(4) Heat-inactivated control corresponding to (3)	0·0

Activation by KCN at *p*H 7·0 = 14%.

Exp. 18. The effect of cyanide acting at an alkaline reaction was also studied. The protease preparation was incubated for 2 hr. at 17° with a 0·02 *M* solution of KCN at a *p*H which changed somewhat during incubation but lay between 9·5 and 10·0. An inhibition of 4% was observed, but this is probably just within the limits of experimental error. The results obtained are shown in Table VIII.

Table VIII. *The effect of cyanide at* p*H 9·5–10·0 upon the activity of protease*

Protease preparation	Increase in non-protein-nitrogen (mg. per 100 ml.)
(1) Untreated	71·2
(2) Heat-inactivated control corresponding to (1)	0·0
(3) Treated with KCN *p*H 9·5–10·0	68·3
(4) Heat-inactivated control corresponding to (3)	0·0

Inactivation by KCN at *p*H 9·5 to 10·0 = 4%.

Discussion of results

In the experiments described above the protease extracted from the cells of *Glaucoma piriformis* has been studied as a single proteolytic system. This procedure was necessary in order to ascertain the optimum conditions for proteolysis as a basis for further study. It is most probable, however, that the process used in preparing this protease yields a mixture of proteolytic enzymes. Using the terminology of Grassmann [1932], the protease contains at least one proteinase, since it readily hydrolyses intact proteins. No evidence is available

as to the presence or absence of peptidases in the protease. The demonstration of a single *p*H optimum at 6·0 for the hydrolysis of gelatin shows further that the protease belongs to the group of papainases and that pepsinases and tryptases are absent.

The behaviour shown by the protease when treated with HCN is unusual among the papainases. Thus whilst marked activation by HCN and H_2S is the rule with most proteases of the cathepsin type from animal tissues or of the papain type from vegetable tissues, yet the protease of the gourd (*Cucurbita pepo*) having a *p*H optimum of 7·0 is strongly inhibited by both HCN and H_2S, and that of yeast, having a *p*H optimum about 6·3, is not activated by HCN [Euler, 1927].

The proportionality of the rate of proteolysis to the square root of the enzyme concentration (Schutz's rule), which applies to the reaction between the protease under consideration and casein, is characteristic of many impure proteases. The amount of casein digested is also proportional to the square root of the duration of digestion. This relationship (Arrhenius's rule) applies to the tryptic digestion of gelatin and casein [Haldane, 1930].

Summary

A protease has been isolated from the cells of *Glaucoma piriformis* and shown to contain a proteinase of the papainase group. This protease digests casein, gelatin and α-glutelin readily and egg albumin very slowly. The *p*H optimum for the digestion of gelatin is approximately 6·0. The protease is slightly activated by cyanide at *p*H 7·0, but is inactivated by free HCN.

The effects upon the amount of substrate proteolysed of the duration of protease action, of the protease concentration and of the substrate concentration have been investigated.

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