CLXXXVII. THE OXYTOCIC HORMONE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND.

IV. THE ACTION OF PREPARATIONS OF ANIMAL PROTEOLYTIC ENZYMES, AND SOME OBSERVATIONS ON THE NATURE OF THE HORMONE.

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IN Part III of this series Gulland and Macrae [1933] described the action of preparations of some plant proteolytic enzymes on the oxytocic hormone. They showed that preparations of yeast dipeptidase, aminopolypeptidase and proteinase, and of papain, contained an inactivating enzyme which had its p_H optimum at 7.4. This enzyme, which was not identified, reduced the oxytocic activity to 0.7 % of its initial value.

In continuing the investigation of the action of proteolytic enzymes, attention was then turned to those of animal origin. It was stated by Dudley [1919] and by Thorpe [1926] that pancreatic trypsin destroys the hormone. Dale and Dudley [1921] also recorded a destruction by intestinal erepsin. Freudenberg *et al.* [1932] found that pepsin and erepsin did not attack the hormone, but that it was destroyed by trypsin² and also by papain. No details of the nature of the erepsin used were given by these latter authors, but in view of the date of the investigation it may be presumed to have been more nearly enzymically homogeneous than that of Dale and Dudley.

Apparently on the basis of these results, Freudenberg and his collaborators concluded that the hormone has either a high molecular weight or is bound to a high-molecular carrier of the protein group. They also stated that the hormone is evidently of the same type as insulin. These views are in opposition to the conclusions of Smith and McClosky [1924] and of Kamm [1928] which were based on comparisons of rates of dialysis and on the relative permeability of the membranes used to the hormone, adrenaline and dyestuffs. Smith and McClosky observed that the diffusibility of the hormone was about the same as that of methylene blue, and Kamm suggested 600 as the molecular weight. He also concluded that the oxytocic hormone is more complicated than adrenaline, but simpler than insulin or the parathyroid hormone.

Unpublished experiments of our own on the pressure dialysis through pyroxylin thimbles of solutions of the hormone which had undergone a purification by charcoal adsorption [Gulland and Newton, 1932] led to the view that the hormone is probably not proteinoid in character. Consequently the destruction of the hormone by trypsin preparations was probably not due to trypsin

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² It is assumed that activated trypsin was intended, since Waldschmidt-Leitz and Purr [1929] have shown that trypsinogen (unactivated trypsin) has no proteolytic activity.

itself, as believed by the investigators mentioned above, but was the result of one or more accompanying enzymes. In arriving at this conclusion we were also influenced by the observation in Part III that the inactivation by papain preparations, which was quoted by Freudenberg *et al.* [1932] in support of the protein nature of the hormone, is in fact due to another enzyme, which is not a papainase and has no action on gelatin.

When this conclusion was investigated experimentally, it was found that the hormone was extremely rapidly inactivated by a trypsin preparation supplied by British Drug Houses, Ltd. The shape of the p_H -activity curve and the position of the p_H optimum (Fig. 1) resembled in general those which characterise the action of activated trypsin on proteins. Markedly different results,

Fig. 1. p_H -activity curve of inactivation by trypsin preparation.

however, were obtained with the same weight of an 11-year old Kahlbaum preparation of trypsin under the same conditions; the hormone was inactivated, but only very slowly. The possession of these widely different trypsin preparations offered the opportunity of comparing directly their tryptic activity with their power of inactivating the hormone, and thus of determining whether activated trypsin itself has any action on the hormone.

By following with the titration method the early stages of the hydrolysis of gelatin by these enzyme preparations, it was shown (Table III) that the tryptic activity of the B.D.H. preparation was about 50 times stronger than that of the Kahlbaum preparation; by considering only the early stages of the hydrolysis it was permissible to discount for practical purposes the effects due to ereptic activity, which also was considerably higher in the B.D.H. preparation.

Two parallel experiments under identical conditions were then carried out to compare the powers of inactivating the hormone of the B.D.H. trypsin preparation on the one hand and of 50 times its weight of the Kahlbaum preparation on the other. If activated trypsin is responsible for inactivating the hormone, the inactivation in these two experiments should have been about the same. In actual fact, however, the inactivation by the B.D.H. preparation was many times more rapid than by the Kahlbaum preparation (Fig. 2). Thus activated trypsin itself is not responsible for the inactivation of the hormone by trypsin preparations, but this is caused by an enzyme with a p_H optimum at 8.8 (Fig. 1). This enzyme is not identical with the inactivating enzyme of yeast

and papain (Part III), and it is improbable, though not quite excluded, that they are the plant and animal variants of the same type of enzyme (identical substrates), because the enzyme of the trypsin preparation reduced the oxytomic value to 0.3 % (Table I) as compared with the value of 0.7 % in the experiments with the yeast-papain enzyme. As in the case of the activity remaining after the action of the latter enzyme, our present opinion is that the residual activity of 0.3 % is due to a derivative of the hormone produced by enzymic action.

It has thus been shown in this publication and in Part III that the apparent inactivation of the hormone by enzymes of which the characteristic substrates are proteins is not brought about by those enzymes themselves but by unidentified enzymes which accompany them. These facts seem to dispose of the contention that the hormone is proteinoid in character.

Fig. 2. Comparison of inactivation by weights of Kahlbaum trypsin (A) and B.D.H. trypsin (B) preparations having the same tryptic activity.

From the same experiments it is apparent that the sole support for the view advanced by others that the hormone is peptide in character lies in the fact that the enzymes which inactivate it accompany proteolytic enzymes. We are not opposed to the conception that the hormone molecule contains peptide linkages, but at present there is no evidence on this point.

Freudenberg *et al.* [1932] observed that pepsin did not attack the hormone. Presumably, although this is not stated, their experiments were made at or about the strongly acid p_H optimum of pepsin. As the beginning of a general study of the effect on the hormone of the enzymes of animal tissues, a proteolytically potent specimen of pepsin was examined at p_H 4.4 and p_H 7.2 for the presence of other enzymes which might attack the hormone. In neither case was any diminution in oxytomic strength observed. A bone phosphatase preparation also was tested at p_H 7.8 and found to be quite inactive towards the hormone.

From what has already been stated and from a general consideration of the problem, it is evident that a series of favourable results in a definite and somewhat prolonged sequence of investigations is required for the complete experimental demonstration that any given enzyme does inactivate the hormone. In the proof that an enzyme does not inactivate the hormone, a part or even most

of this sequence may be omitted. Apart from haphazard guesswork, the suggestion that a particular well-defined enzyme inactivates the hormone must be formulated as a result of facts observed in one or more of the following modes of attack: from a systematic examination of the effects of enzymes in general; from predictions of the presence of chemical groups as a result of the study of the action of chemical reagents; from the similarity of the p_H -activity curves of the inactivating enzyme acting on the hormone and of the suspected enzyme¹ acting on its known substrate. The last-mentioned source suffers from the disadvantage that the shape and optimum of a p_H -activity curve may vary with change of substrate. The stages of the demonstration are tabulated below.

(1) The discovery of the rate at which the hormone is inactivated by the preparation of the inactivating enzyme, and the determination of the p_H -activity curve.

(2) The examination of the action of the inactivating enzyme on the substrate of the suspected enzyme. If the degree of activity permits, the determination of the p_H -activity curve.

(3) The examination of the action of an authentic preparation of the suspected enzyme on the hormone, including the determination of the rate of action and of the p_H -activity curve for comparison with those in (1).

(4) Careful estimations of the activities of (a) an authentic preparation of the suspected enzyme, and (b) of the inactivating enzyme preparation towards the substrate of the suspected enzyme. This will permit a numerical comparison of the amounts of the suspected enzyme present in its authentic preparation and in the preparation of the inactivating enzyme.

(5) The demonstration of equal inactivation of the hormone under the same conditions by weights of preparations of the suspected enzyme and inactivating enzyme, which it is estimated would cause the same degree of inactivation if the suspected enzyme and inactivating enzyme were identical.

It was shown in Part II [Gulland, 1933] that two out of the three well-defined inactivations of the hormone which occur in presence of nitrous acid are probably due to nitrous acid itself. The rates of these inactivations and the conditions under which they occurred were not inconsistent with the view that the slower, which required a large excess of the reagent, was due to the interaction of nitrous acid with a guanidine group, and that the more rapid was the result of its interaction with an amino- or basic imino-group. A preliminary consideration from the enzymic aspect was not opposed to this conception, which was therefore examined more fully.

The position at a relatively alkaline reaction of the p_H optimum of the inactivation of the hormone by the B.D.H. trypsin preparation was not far removed from that of arginase. Moreover, this preparation contained a small, but definite, arginase activity, which was estimated by a modification of the arginase-urease method of Edlbacher and Röthler [1925]. In accordance with the procedure outlined above for the examination of a suspected enzyme, a highly potent glycerol solution of arginase was prepared from fresh liver [Edlbacher and Röthler, 1925]. The amounts of this arginase solution and of the B.D.H. trypsin preparation were then calculated from their arginase activities, so that, if arginase were the inactivating enzyme, the arginase solution should have caused approximately twice as much inactivation of the hormone as the trypsin preparation—twice as much, rather than the same, inactivation,

¹ This term denotes the well-defined enzyme with known substrate which is suspected of attacking the hormone. The term "inactivating enzyme" denotes the unknown enzyme in a preparation which attacks the hormone.

in order to emphasise the result. When these enzymes were examined under identical conditions for their action on the hormone, the trypsin preparation reduced the activity to 7 % of its initial value in 30 minutes, whereas with the liver extract no inactivation was detectable after 4 hours. Thus the inactivating enzyme of the B.D.H. trypsin preparation is not arginase. The papain preparation used in the earlier experiments exhibited no arginase activity, and hence the hormone does not contain the group I, although the group II or another guanidine derivative is not excluded [Felix *et al.*, 1928; Edlbacher and Burchard, 1931].

The shape and position of the optimum of the p_{H} -activity curve of the inactivating enzyme of the yeast and papain preparations (Part III) closely resembled those (Fig. 3) of prolinase, of which the simplest substrate is prolylglycine [Grassmann *et al.*, 1932]; the substrates of this enzyme all contain the

Fig. 3. p_{H} -activity curves of inactivating enzyme of yeast proteinase solutions (A) and of Grassmann's prolinase (B).

basic imino-group of proline. When the yeast dipeptidase, yeast proteinase and papain preparations used in Part III were tested for prolinase activity, all contained traces of this enzyme. It seemed probable that these amounts were too small for prolinase to be the inactivator, but, on the other hand, the affinity of the inactivating enzyme for the hormone is unknown and may be very great. The routine examination already described was therefore undertaken. A solution of prolinase, prepared from a neutral autolysate of yeast, contained more than 50 times the prolinase activity of the yeast proteinase solution. From this figure, a calculation was made of the amounts of the two enzymes needed to compare their power of inactivating the hormone; as before, however, only half the requisite amount of proteinase preparation was used, in order to emphasise the result. The enzyme of the proteinase solution reduced the oxytomic value to 38 % in 30 minutes, whereas with the yeast autolysate it had only become 90 % in that time, and after 4 hours was still 73 %. Thus, the inactivating enzyme of the yeast and papain preparations is not prolinase.

The B.D.H. trypsin preparation contained moderate amounts of an enzyme which hydrolysed prolylglycine. The dependence of the activity of this enzyme

on hydrogen ion concentration resembled that of prolinase [Grassmann *et al.*, 1932] in having an optimum just below p_H 8. It was therefore improbable that the inactivating enzyme (optimum at p_H 8.8) of the B.D.H. trypsin preparation was prolinase.

EXPERIMENTAL.

As in previous communications, the oxytocic activity of solutions was assayed by the degree of contraction they produced in the isolated uterus of the guinea-pig. The technique described in Part III was adopted in the present experiments.

Action of B.D.H. trypsin preparation at p_H 7.5.

A hormone solution (2.0 cc., containing 156 oxytocic units per cc.), $M/3$ sodium phosphate at p_H 7.5 (3 cc.) and a solution of B.D.H. trypsin preparation (10 mg. in 2 cc. of water) were mixed, and the mixture diluted to 10.0 cc. at p_H 7.5 by means of water and a trace of N ammonia. A control sample (1.0 cc.) was immediately removed into a 2.5 cc. flask, heated for 3 minutes in a boiling water-bath and placed in a thermostat at 40°. The remainder of the mixture had meantime been placed in the thermostat. Further samples were removed at appropriate intervals, and finally all were diluted to 2.5 cc. at p_H 3.5 with 2.5 N acetic acid (0.5 cc.) and water. Each sample was assayed against the control solution, 0.2 cc. being diluted to 10 cc. at p_H 7.5; these dilutions would therefore have contained 0.25 unit per cc. if no inactivation had occurred. The results are given in Table I.

Table I.

Time (hours)	0.5	1	6	27
Activity present (%)	0.7	0.5	0.3	0.3

Action of Kahlbaum's trypsin at p_H 7.5.

The amounts of hormone, enzyme and buffer were those of the preceding experiment, and the procedure was exactly the same (Table II).

Table II.

Time (hours)	1	6	24
Activity present (%)	100	76	40

Comparison of the action of B.D.H. and Kahlbaum trypsin preparations.

A. *Proteolytic activity.* The proteolytic activities of the two preparations were compared by the method of titration in 90 % alcohol with alkali, gelatin being used as substrate. Gelatin solution (6.0 cc. of 10 %), $M/3$ sodium phosphate buffer at p_H 7.5 (1.0 cc.) and varied amounts of the solid enzyme preparations were mixed and diluted to 10.0 cc. at p_H 7.5, the p_H being adjusted with ammonia or acetic acid. In each case a sample (1.0 cc.) was immediately removed into 9 cc. of absolute alcohol at 60°, this mixture being titrated with $N/20$ alcoholic KOH whilst being shaken. The reaction mixtures were incubated at 40°, and further samples were taken at the times stated in Table III.

It is evident (see especially columns marked †) that the proteinase activity of the B.D.H. preparation was about 50 times greater than that of the Kahlbaum preparation. By comparing only the increases in acidity in the early stages of the hydrolysis, the effects due to ereptic action may be disregarded or estimated. Of the two preparations, the B.D.H. one is richer in ereptic activity; this is shown by the greater increase in acidity in the later stages of the experiments in which it is used.

Table III.

The figures denote increases in acidity of 1 cc. portions of the reaction mixtures, expressed as cc. of $N/20$ KOH. The weight of the enzyme preparation used is shown above each column.

Kahlbaum trypsin prep. (Dec. 1922)				Time hours	B.D.H. trypsin prep. (May 1931)			
10 mg.*	20 mg.	100 mg.	500 mg.†		1 mg.	2 mg.	10 mg.	10 mg.†
—	—	—	0.38	$\frac{1}{4}$	—	—	—	0.38
—	—	—	0.68	$\frac{1}{2}$	—	—	0.65	0.74
—	—	0.38	0.76	1	0.23	0.45	—	0.96
0.05	—	0.46	—	2	0.40	0.53	—	—
—	—	—	—	5	—	—	1.14	—
0.15	—	—	—	$5\frac{1}{2}$	—	—	—	—
—	—	0.60	—	6	0.82	0.88	—	—
—	0.47	—	—	17	—	—	—	—
—	—	—	—	20	—	—	1.48	—
0.52	—	—	—	23	—	—	—	—
—	0.51	—	—	41	—	—	—	—
0.60	—	—	—	47	—	—	—	—

* The reaction mixture contained 1.0 cc. of $N/2.5$ ammonium chloride in addition to the other components.

B. *Inactivation of the hormone.* The experiments with both enzymes were made under identical conditions. A hormone solution (1.0 cc., containing 156 units), $M/3$ sodium phosphate buffer at p_H 7.5 (1.5 cc.) and a solution of the enzyme preparation (125 mg. of Kahlbaum trypsin, or 2.5 mg. of B.D.H. trypsin) in water (0.5 cc.) were mixed, adjusted to p_H 7.5 with N ammonia and diluted to 5.0 cc. with water. The customary procedure was observed, the samples measuring 1.0 cc. and being taken at the same intervals. The samples were assayed against their control samples, 0.2 cc. in each case being diluted to 10 cc. at p_H 7.5. The results are given in Table IV and Fig. 2.

Table IV.

Kahlbaum prep., activity present (%)	Time hours	B.D.H. prep., activity present (%)
86	$\frac{1}{2}$	19
71	1	2.7
59	2	0.6

These results showed that the inactivation of the hormone was not caused by trypsin, assuming that the different rates of inactivation were not due to a more rapid destruction of the trypsin in one preparation than in the other. It was shown that if destruction did occur, it was approximately equal in the case of both enzymes. At the end of the incubations the residual proteinase activity was determined as follows. Each enzyme-hormone-buffer mixture (1 cc.) was mixed with 10 % gelatin solution (3 cc.), and the mixture was adjusted to p_H 7.5, diluted to 5 cc. with water and incubated at 40°. The rate of liquefaction of the gelatin was then followed. After 1 hour the gelatin of both mixtures solidified with great difficulty when cooled under the tap, the times required for solidification being approximately the same. After 2 hours the gelatin of both experiments did not solidify.

*Effect of hydrogen ion concentration on the rate of inactivation of the hormone
by the B.D.H. trypsin preparation.*

An aqueous solution of B.D.H. trypsin (1.25 mg. in 0.25 cc.) was added to an ice-cold mixture of a hormone solution (0.5 cc., containing 156 units per cc.) and *M*/3 sodium phosphate buffer at the required p_H (1.75 cc.). A control sample was immediately removed into a 2.5 cc. flask, heated for 3 minutes at 100° and placed in the thermostat at 40°. The remainder of the solution was kept in the thermostat for 30 minutes, when a second sample was taken as described above. The p_H of the remainder of the solution was determined and taken as the p_H of the experiment; determinations at p_H 9.3 and 10.2 were made with a hydrogen electrode, the others colorimetrically.

The experimental solutions were assayed against the control solutions, 0.2 cc. in each case being diluted to 10 cc. at p_H 7.5. The results are given in Table V and Fig. 1.

Table V.

p_H	5.2	6.0	7.0	7.5	8.0	8.8	9.3	10.2
Inactivation (%)	0	26	67	82.5	90	95	92.5	81

Action of B.D.H. pepsin preparation.

A. At p_H 7.2. A hormone solution (6.7 cc., containing 3 units per cc.), *M*/3 sodium phosphate solution at p_H 7.5 (1 cc.) and a solution of active B.D.H. pepsin (100 mg. in 2 cc. of water) were mixed and diluted to 10 cc. at p_H 7.2. A control sample (2.0 cc.) was immediately removed, and other samples were taken at $\frac{1}{2}$, 2, and 25 hours. The usual technique was followed.

B. At p_H 4.4. The procedure was exactly as described above, except that *N* acetate buffer was used. The p_H of the mixture was 4.4. In both series of experiments the samples were assayed against the controls, 1 cc. being diluted to 10 cc. In no case could any inactivation be detected.

Action of bone phosphatase. A hormone solution (0.25 cc., containing 312 units per cc.), *M*/5 sodium phosphate at p_H 7.8 (1 cc.) and a solution of a bone phosphatase preparation (10 mg. of $A/W=0.23$ [Martland and Robison, 1929]) in water (1 cc.) were mixed and diluted to 5 cc. with water. The p_H was 7.8. The usual technique was followed, samples (1 cc.) being removed at $\frac{1}{2}$, $1\frac{1}{2}$, and 26 hours and assayed against the control sample. No inactivation of the hormone was detected.

Consideration of the possibility that arginase inactivates the hormone.

A. *Estimation of arginase in B.D.H. trypsin preparation.* A solution of trypsin (100 mg.) in water (2 cc.), *M*/10 glycine-sodium hydroxide buffer at p_H 9.5 (2 cc.) and a 1 % *D*-arginine solution (2 cc.) were mixed, and the mixture was adjusted to p_H 9.5, incubated for 1 hour at 40° and heated for 4 minutes at 100°. The p_H of the mixture was then adjusted to 7, and a suspension of one powdered tablet of B.D.H. urease¹ in *M*/3 phosphate buffer at p_H 7 (1 cc.) was added. The mixture, in a closed tube, was incubated at 40° for 1 hour, cooled and transferred to a micro-Kjeldahl apparatus containing saturated sodium carbonate solution (2 cc.) and some medicinal paraffin (to prevent frothing). Steam was passed into the apparatus in the usual way², and the

¹ One tablet hydrolyses 50 mg. urea in 1 hour at 35°.

² This is a modification of the method of Edlbacher and Röthler [1925]. They aspirated the ammonia from the warm solution, but we obtained inconsistent results owing to incomplete removal of ammonia.

distillate was collected in $N/50$ hydrochloric acid (10.0 cc.); after actual distillation had proceeded for 3 minutes, the contents of the receiver were titrated to methyl red with $N/50$ NaOH. Control experiments were also carried out following the same procedure, except that the trypsin-buffer-arginase mixture was heated for 4 minutes before incubation instead of afterwards. The experiments were repeated several times, and in all cases a small but definite arginase activity was detected. It was estimated that under the conditions of the experiment 100 mg. of the trypsin preparation yielded 0.10–0.15 cc. of $N/50$ ammonia.

B. *Estimation of arginase activity in a preparation from liver.* The arginase content of a glycerol extract [Edlbacher and Röthler, 1925] of guinea-pig liver was estimated as described above. The 1:3 glycerol extract (1 cc.) was diluted to 10 cc., and 0.2 and 0.4 cc. of the dilution used for the estimation (Table VI).

Table VI.

Diluted glycerol extract (cc.)	Experiment cc. $N/50$ NaOH	Control cc. $N/50$ NaOH	Ammonia formed cc. $N/50$
0.2	7.48	9.69	2.21
0.4	6.08	9.65	3.57

C. *Comparison of hormonal inactivation by B.D.H. trypsin and arginase preparations.* The two experiments followed the customary procedure of assaying experimental against control solutions and need not be described in detail. The volumes of the reaction solutions were 2.5 cc., the p_H values were 9.3, and the amounts of hormone used were comparable. In one experiment the B.D.H. trypsin preparation (1.25 mg.) was used, in the other a solution containing 0.00004 cc. of the glycerol extract of guinea-pig liver. This solution thus contained about twice as much arginase activity as the amount of trypsin preparation used. The trypsin preparation effected 92.5 % of the possible inactivation in $\frac{1}{2}$ hour, whereas little or no inactivation (possibly as much as 10 %) occurred after 4 hours in the experiment with arginase solution. If arginase inactivated the hormone, the solution of the glycerol extract of arginase should have caused about twice as much inactivation as the trypsin preparation. It follows, therefore, that the inactivating enzyme of the trypsin preparation was not arginase.

The B.D.H. papain preparation contained no arginase, nor did it exhibit any urease activity, which would have interfered with the arginase estimation. The inactivating enzyme of yeast and papain preparations is not arginase.

Consideration of the possibility that prolinase inactivates the hormone.

A. *Estimation of prolinase in preparations of yeast proteolytic enzymes.* Prolinase activity was measured by titrating the increased acidity of prolylglycine with alkali in 90 % alcohol [Grassmann *et al.*, 1932]. *dl*-Prolylglycine (40 mg.) was dissolved in $M/3$ phosphate buffer at p_H 7.5 (0.25 cc.) in a 2.5 cc. flask. An aqueous solution (1.5 cc.) of a yeast dipeptidase, yeast proteinase or B.D.H. papain preparation was added, and the mixture was diluted with water and alkali to 2.5 cc. at p_H 7.5. The enzyme preparations were those used in Part III. A sample (0.5 cc.) was immediately removed into a flask containing absolute alcohol (4.5 cc.) and titrated to thymolphthalein with $N/20$ potassium hydroxide in 90 % alcohol. The remainder of the solution was incubated at 40°, and samples were taken at 4 and 23 hours. In the control experiment the enzyme solution was replaced by water. The results are given in Table VII, and it will be seen that each enzyme preparation contains traces of prolinase.

Table VII.

The figures represent the degree of hydrolysis and are the increase in acidity of 0.5 cc. samples quoted as cc. *N*/20 alcoholic KOH.

Time hours	Control	Dipeptidase 5 mg.	Papain 5 mg.	Proteinase 0.5 cc.
4	0.00	0.01	0.02	0.01
23	0.00	0.06	0.04	0.02

B. *Preparation of a potent prolinase solution from yeast.* English brewer's yeast (50 g.) was liquefied with ethyl acetate (5 cc.) and allowed to autolyse for 1.5 hours at p_H 6.5–7, ammonia being added to maintain the p_H . The yeast was centrifuged, washed and then allowed to autolyse at p_H 7.0 for 24 hours in presence of a few drops of toluene. Finally the yeast was centrifuged, and the prolinase activity of the clear solution was estimated as described above. Acting on *dl*-prolylglycine (40 mg.) for 2 hours at p_H 7.5 and 40° in a total volume of 2.5 cc., the prolinase solution (0.5 cc.) caused an increase in the acidity of 1.0 cc. portions corresponding to 0.24 cc. of *N*/20 KOH. The prolinase solution had therefore more than 50 times the prolinase activity of the proteinase solution (above).

C. *Comparison of hormonal inactivation by yeast proteinase and prolinase solutions.* Hormone solutions (each containing 30 units) were mixed either with the proteinase solution (0.25 cc.) or with an aqueous dilution of the yeast autolysate corresponding with 0.007 cc. of this autolysate; the experiment with the proteinase thus contained about half the prolinase activity of that with the yeast autolysate. The mixtures were diluted to 2.5 cc. at p_H 7.0, being buffered with *M*/15 sodium phosphate, and were incubated at 40°. The usual procedure was followed, samples being removed immediately and after 30 minutes; a sample from the yeast autolysate experiment was also taken after 4 hours. In the proteinase experiment 38 % of the initial oxytocic activity remained after 30 minutes, whereas in the yeast autolysate experiment more than 90 % was present after that period, and after 4 hours 73 % still remained. The inactivating enzyme of the yeast and papain preparations is therefore not prolinase.

D. *Action of B.D.H. trypsin preparation on prolylglycine.* *dl*-Prolylglycine (40 mg.) in *M*/3 phosphate buffer at the appropriate reaction (0.25 cc.) was mixed with a solution of B.D.H. trypsin preparation (25 mg.) in water (0.5 cc.) in a 2.5 cc. flask. The mixture was adjusted to the required p_H and diluted to 2.5 cc. Samples (1.0 cc.) were removed for titration in the usual way at the beginning and after 2½ hours. The control experiments contained no substrate. The results are given in Table VIII.

Table VIII.

The figures represent increases in acidity in terms of *N*/20 KOH.

p_H	6.0	7.0	8.0	9.0
Experiment	0.16	0.27	0.44	0.29
Control	0.06	0.07	0.12	0.16
Due to prolinase	0.10	0.20	0.32	0.13

Difficulties encountered in determining the end-points increased the experimental error of these results. It is evident however that the p_H optimum of the prolinase is considerably less alkaline than p_H 9 and probably less than p_H 8. The inactivating enzyme of the trypsin preparation, having an optimum at p_H 8.8, is probably not prolinase.

An experiment similar to that described above was carried out with the Kahlbaum trypsin preparation (125 mg. in 2.5 cc.) at p_H 7.2. No increase in acidity was observed after incubation for 2.5 hours. This preparation, therefore, did not contain prolinase.

SUMMARY.

1. A B.D.H. trypsin preparation inactivated the hormone very rapidly at p_H 7.5, the p_H optimum of the inactivation being 8.8.

2. Under similar conditions a Kahlbaum trypsin preparation inactivated the hormone extremely slowly.

3. Experiments in which quantities of these preparations having equal tryptic activities acted on the hormone showed that, contrary to existing conclusions, the inactivating enzyme was not trypsin. This result, conjoined to the earlier proof that the inactivating enzyme of papain preparations is not a papainase, affords strong evidence against the contention that the hormone is proteinoid.

4. In attempts to identify the inactivating enzyme of yeast and papain preparations and also that of the B.D.H. trypsin preparation, it was shown that neither arginase nor prolinase inactivates the hormone.

5. No evidence is yet available to support the view that the hormone contains peptide linkages.

6. The hormone was not attacked by a B.D.H. pepsin preparation at p_H 4.4 or at p_H 7.2, or by a bone phosphatase preparation at p_H 7.8.

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REFERENCES.

- Dale and Dudley (1921). *J. Pharm. Exp. Ther.* **18**, 27.
 Dudley (1919). *J. Pharm. Exp. Ther.* **14**, 295.
 Edlbacher and Burchard (1931). *Z. physiol. Chem.* **194**, 69.
 — and Röthler (1925). *Z. physiol. Chem.* **148**, 264.
 Felix, Müller and Dirr (1928). *Z. physiol. Chem.* **178**, 192.
 Freudenberg, Weiss and Eyer (1932). *Naturwiss.* **20**, 658.
 Grassmann, Schoenebeck and Auerbach (1932). *Z. physiol. Chem.* **210**, 1.
 Gulland (1933). *Biochem. J.* **27**, 1218.
 — and Macrae (1933). *Biochem. J.* **27**, 1229.
 — and Newton (1932). *Biochem. J.* **26**, 337.
 Kamm (1928). *Science*, **67**, 199.
 Martland and Robison (1929). *Biochem. J.* **23**, 237.
 Smith and McClosky (1924). *J. Pharm. Exp. Ther.* **24**, 391.
 Thorpe (1926). *Biochem. J.* **20**, 374.
 Waldschmidt-Leitz and Purr (1929). *Ber. deutsch. chem. Ges.* **62**, 2217.