LXXI. THE REACTION OF AZINE COMPOUNDS WITH PROTEOLYTIC ENZYMES.

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The formation of precipitates when various azine dyes are added to solutions of proteolytic enzymes has been remarked by a number of observers. In some cases, moreover, it has been shown that the mother liquors have lost their protease activity and that this has, in part, been transferred to the precipitates [Holzberg, 1913; Wood, 1918; Marston, 1923; Forbes, 1927]. The observations and conclusions of Marston are of particular interest. Marston found that safranine was a specific precipitant for all the protease activities he tested—pepsin, trypsin, erepsin, yeast protease, papain. The dye did not, however, remove other non-proteolytic enzymes from solution. Since, moreover, other azonium salts, azines, and eurhodines behaved similarly towards trypsin, Marston concluded that he was observing a specific interaction between the azine nucleus and the structure peculiar to a protease. He further fortified this argument by drawing an analogy between the azine ring as represented by the various dyes employed and the piperazine ring which he believes to be a dominant feature of the protein molecule. Robertson [1928], indeed, has employed Marston's argument to support the diketopiperazine structure of protein. Now the view that the azine nucleus found in these dyes resembles the heterocyclic ring of a diketopiperazine is open to serious criticism upon structural grounds and makes the acceptance of the full implications of Marston's argument difficult. On the other hand, there is no doubt that valuable opportunities are opened by the demonstration that a simple well-defined structure reacts with a group of enzymes in a specific way which parallels the catalytic specificity of that group of enzymes.

In particular, Marston's observations suggested to us an opportunity for the examination of the kinetics of protease activity by means of a quantitative study of the anticatalytic effect of azine compounds on the activity of the protease. At the same time it was hoped to assemble data which would permit the precise use of this reaction as an economical means of purifying protease preparations.

The poisoning of protease activity by azine dyes.

Protease activity was determined by the viscosity method of Northrop and Hussey [1923], where the measure of activity is the time taken to produce a definite degree of viscosity change in a solution of gelatin. Results were conveniently reproducible and a relative measure of activity accurate to within 10 % was obtained when all the necessary precautions were observed. The comparison of activities was most reliable if the different enzyme preparations were separately diluted to produce approximately similar rates of viscosity change. A dilution which produced half of the total viscosity change in 10 to 30 minutes was adopted as being most convenient. Comparison of activities determined on substrate samples of markedly different initial viscosity is not strictly valid, but is useful qualitatively.

Viscosity determinations at 30° were made in viscosimeters standardised against distilled water and, after each determination, cleaned with chromic acid overnight. The substrate solutions were 2·75 % solutions of isoelectric gelatin [Loeb, 1922] adjusted with phosphoric acid or phosphate to a p_H of 2·7 for pepsin and 7·5 for trypsin determinations. After addition of a trace of thymol, these bulk solutions were stored in small bottles in the refrigerator. For use each bottle was warmed to melt the gelatin and maintained at 30° for at least 2 hours. Then, as required, samples were removed, mixed at zero time with one-twentieth volume of the enzyme preparation, and pipetted in 7 cc. amounts to the viscosimeters, every precaution being taken to minimise cooling of the solutions below 30°. Initially, determinations were made in duplicate until the precaution was found unnecessary.

Table I. *Poisoning of protease activity by azine dyes.*

Enzyme	Azine dye	$[E]$	$[D]$	t mins.	Gelatin sample
Dog's gastric juice	Safranine	0·002	—	26	a
		0·002	0·01	27	a
		0·002	0·05	37	a
		0·002	0·2	69	a
		0·002	0·6	100	b
Benger's trypsin	Neutral red	0·0015	—	29	c
		0·0015	0·00025	26	c
		0·0015	0·0012	26	c
		0·0015	0·0063	25	c
	Safranine	0·0015	0·0063	25	c
		0·0015	—	44	d
		0·0015	0·0063	43	d
		0·0015	0·025	39	d
		0·0015	0·1	47	d
		0·0015	0·2	70	d
		0·0015	0·6	90	d

$[E]$ = concentration of enzyme in 100 cc. of the digestion mixture in terms of total solids (g.) of the original preparation.

$[D]$ = concentration of dye (g. %) in the digestion mixture.

t = time, corrected to a constant arbitrary enzyme concentration, for 50 % viscosity change in the gelatin.

p_H for pepsin experiments 2·7, for trypsin experiments 7·5.

In Table I and Fig. 1 are summarised the results of experiments on gastric juice from a dog's Pavlov fistula (0·42 % solids) and upon a highly active

pancreatic extract (6·1 % solids) kindly prepared for us by Benger's Food Products Ltd.

It is obvious that dye concentrations of even 0·05 to 0·1 % in no way retard the hydrolysis of the gelatin by the enzyme. Moreover, it should be pointed out that the concentration of dye at which retardation does definitely occur is the critical concentration for immediate precipitation of the enzyme complex (though precipitation occurs in 24 hours with all dye concentrations above 0·006 %). Therefore, not only is there no poisoning with small concentrations of dye, but such diminution in activity as is observed with high concentrations occurs only upon visible separation of a gelatinous precipitate in the viscosimeter. The artificial effect of such a precipitate is obvious, and appears in the rather erratic viscosity curves at these concentrations (Fig. 1). Hence the indicated decrease in activity may well be merely apparent.

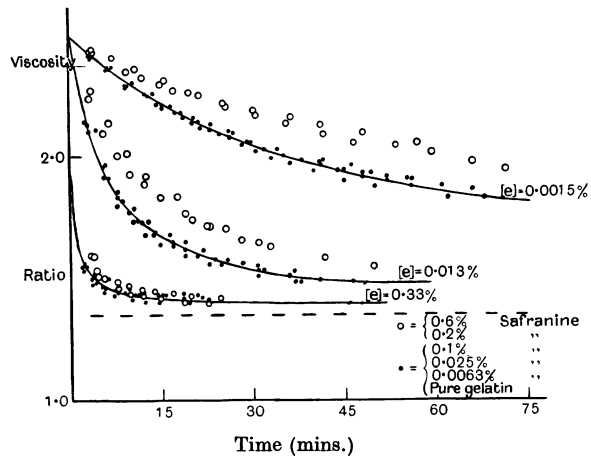

Fig. 1. Poisoning test (safranine on Benger's trypsin) $p_H = 7·5$.

To test this point the diastase activity of the Benger's pancreas extract was investigated with starch in the presence of varying concentrations of dye. Though results were more erratic, an effect running quite parallel to the above was obtained, so that, since diastase activity is not precipitated by azine dyes [Marston, 1923], any retardation would appear to be artificial and dependent at least predominantly on the gelatinous nature of the precipitate. Complete confirmation of this view was received, at least for the trypsin experiments, when it was discovered later that the tryptic activity of the Benger's extract is not significantly precipitated by azine dyes.

These results do not deny a specific chemical reaction between the dye and the catalytically active grouping of the protease. They do suggest, however, that the hypothetical complex formed is so largely dissociated in solution that the active mass of free enzyme is unaffected by such concentrations of dye as will cause an ultimate separation of the insoluble complex.

Further information was sought by direct studies of the protease activities of azine precipitates and of the mother liquors from which these precipitates have been separated.

The precipitation of pepsin and trypsin by neutral red.

The factor which has limited former studies of these precipitates has been the difficulty of redissolving the active material. Only in the case of pepsin had this been found possible [Marston, 1923; Forbes, 1927], advantage being taken of the fact that in dilute solutions of strong acids the precipitate redissolves and the dye may be extracted by such solvents as amyl alcohol. Even in this case, however, emulsification during extraction rendered quantitative recovery of the enzyme difficult.

Now Marston found that the leuco-azine bases do not form precipitates with enzymes. We find further that a reducing agent such as sodium hyposulphite will redissolve an existing precipitate. Moreover, the reducing agent does not destroy the activity of the protease present. A simple method of regaining the precipitated enzyme in solution at once suggests itself. The precipitate is removed from the reaction mixture by centrifuging, washed with 0·1 % safranine and suspended in an appropriate buffer. Sodium hyposulphite is added and the leuco-base extracted with benzene in a vessel protected from oxygen. A useful extraction device which avoids any emulsification consists of a shallow cylinder in which a thin layer of redissolved precipitate covered with a layer of benzene is gently and slowly rocked. Two changes of solvent give efficient extraction, the whole process being complete in an hour.

A still simpler method of extracting the precipitate—in the case of trypsin —is based upon the observation that a neutral red precipitate will redissolve in solutions of p_H more alkaline than about 7, *i.e.* when the basic dissociation of neutral red is suppressed. It is necessary therefore only to precipitate the preparation at p_H 6, adjust the solution to p_H 9, and extract the yellow dye with benzene. The same rocking device may be employed.

The various reagents and manipulations involved in these methods have no destructive effect upon either pepsin or trypsin. This was established by comparing the activity of an enzyme solution before a precipitate was formed in it by addition of dye and after the precipitate had been redissolved in its own mother liquors and extracted by one of the methods described.

In estimating the activity remaining in the mother liquors it proved to be unnecessary to carry out a preliminary extraction of the dye since the further degree of dilution involved in carrying out the activity test reduced the concentration of dye far below that which had been found to give significant "poisoning" (Table I).

Protease activity of the enzyme precipitates and supernatant liquors.

In these investigations the enzyme solutions were buffered at p_H 3·9 (acetate buffer) for pepsin and at p_H 6·0 (phosphate buffer) for trypsin. p_H determinations were made with a Hildebrand hydrogen electrode slightly modified to eliminate frothing. Samples of the buffered preparations were then mixed with varying concentrations of dye, allowed to flocculate for 15 hours in the refrigerator, centrifuged for 20 minutes in a high speed centrifuge, and the supernatant liquors decanted from the precipitate. The precipitate was dried to constant weight in a steam-oven, and the extent of precipitation of the protease activity was estimated by the loss of activity in the supernatant liquors. In this way the loss of activity was compared with the amount of precipitate carrying it down and with the concentration of the precipitating dye. The data appear in Table II.

Table II. *Protease activity of the supernatant liquors.*

Enzyme	Dye	$[D]$	$\frac{100w}{S}$	t (supernatant) mins.	Gelatin sample
Harrington's pepsin	Neutral red	—	Not determined	11	e
2 % solution		0·024	,,	50	e
		0·045	,,	80	e
		0·083	,,	150	e
Dog's gastric juice	Safranine	—	—	6	f
$[E]=0·195$		0·005	—	6·5	f
		0·01	9	8	f
		0·02	10	16	f
		0·05	19·5	144	f
		0·1	23·5	550	f
		0·2	24	700	f
		0·5	Not determined	800	f
Harrington's trypsin	Safranine	—	,,	7	g
3 % solution		1·0	,,	10	g
Benger's trypsin	Safranine	—	—	29	h
$[E]=2·6$		0·1	5	28	h
		0·5	12	29	h
		1·0	17	28	h

$[E]$ = concentration of enzyme in 100 cc. of the precipitation mixture in terms of total solids (g.) of the original preparation.

$[D]$ = concentration of dye (g. %) in the precipitation mixture.

$\frac{100w}{S}$ = dry weight of precipitate expressed as percentage of the total solids of the original preparation.

$[E]$ and $[D]$ are diluted 20 times (or 2000 times for Benger's trypsin) in the activity tests.

A saturated aqueous solution of safranine contains just above 1 % of dye.

The data for pepsin precipitation are in general agreement with Marston's views, since loss of activity runs parallel with both the concentration of dye and the weight of precipitate. However, it is noticed that, though the critical dye concentration for removal of peptic activity from solution is 0·02 to 0·1 % with practically complete removal at 0·2 %, yet by weight 38 % of the precipitate at 0·2 % has already been precipitated in a dye concentration of

only 0·01 %. This anomaly becomes more significant when considered in the light of the trypsin results, for here we find the quite unexpected fact that, though the weight of the precipitate varies definitely with the dye concentration, the activity of the supernatant liquor is not measurably reduced by loss of this precipitate. The activity of such precipitates was quite definite, but, as Table III indicates, it varied with the origin of the preparation, and was quite a negligible fraction of the total activity.

Now Marston claims to have found 64 % of the total activity of a trypsin solution in his precipitate. This variability in extent of trypsin precipitation allows of no simple explanation other than the obvious one that the precipitation does not specifically involve the enzyme. We incline to the view that trypsin preparations may or may not contain varying amounts of a substance (probably protein) with which the enzyme tends to be closely and specifically associated, and that this substance is precipitated by azine dyes removing the enzyme with it. It is of interest, therefore, that Wood [1918] reports an active preparation of trypsin which failed to give any precipitate with safranine.

Attempts were now made to encourage removal of the dye by inducing formation of some foreign precipitate in the solution. Robertson mentions the precipitation of phosphates and nucleic acid by azine dyes, so experiments were conducted in which Benger's trypsin was mixed with varying concentrations of nucleic acid and then precipitated in the usual fashion. The results were entirely negative and appear in Table III. Precipitations were performed in 0·1 % neutral red at p_H 6. Activities were determined in the supernatant liquors after extraction of the dye with benzene at p_H 9.

Table III.

(*a*) *Protease activity of the precipitates.*

(*b*) *Effect of induced precipitation.*

$[D]=0{\cdot}1$ %.

Enzyme	Nucleic acid conc. %	t mins.
10 % Harrington's trypsin	—	1·5
,, ,, extracted precipitate	—	36
Benger's trypsin, 3 % total solids	—	15
,, etc., extracted precipitate	—	6×10^4
,, ,, extracted supernatant	—	12
,, ,, ,,	0·016	13
,, ,, ,,	0·08	11·5
,, ,, ,,	0·4	12·5

The values of t given for the extracted precipitates have been corrected so as to define the activity of these extracts when diluted to the same volume as that of the enzyme solution from which the precipitates were obtained. In the case of Benger's trypsin this leads to a calculation value which has only qualitative significance indicating virtual absence of activity in the precipitate.

It is obvious that no trypsin activity has been incorporated within the bulky precipitate formed between neutral red and nucleic acid. It does not appear therefore that the presence of nucleic acid in the enzyme preparations of Marston was responsible for his results.

Effect of p_H *on precipitation of pepsin.*

Aliquots of a sample of mixed gastric juice were titrated to definite p_H values with a citric acid-citrate buffer of p_H 6, being at the same time adjusted to equal dilution. Safranine (p_H 2·6) was added, the final concentration being 0·2 % dye and 0·175 % pepsin (total solids). The solutions were left for 15 hours for flocculation, the precipitates separated in the centrifuge, and the mother liquors decanted and tested for activity. The dry weights of the precipitates were determined. The results appear in Fig. 2 and in Table IV.

Table IV. *Effect of* p_H *on precipitation of gastric juice by safranine.*

$[D]=0{\cdot}2\ \%$. $[E]=0{\cdot}175\ \%$.

p_H	t mins.	$\frac{100T}{t}$	$\frac{100w}{S}$	$100\left(1-\frac{T}{t}\right)$
1·0 (dye-free)	7·5	100	—	—
2·45 (dye-free)	8	95	—	—
4·0 (dye-free)	5·5	135	—	—
1·0	11	68	16	32
1·95	14·5	52	18	48
2·2	18	42	19	58
2·45	30	25	21	75
2·75	60	12·5	22	87·5
2·95	160	4·7	27	95·3
3·97	1300	0·6	65	99·4
4·85	1300	0·6	91	99·4
(From Table II)				
3·9	700 (× 1·1)	0·9	24	99·1
3·9 (dye-free)	6 (× 1·1)	109	—	—

$\frac{100T}{t}$ = the percentage of the original protease activity remaining in the supernatant liquors after precipitation at the given p_H ($T=7{\cdot}5$).

$100\left(1-\frac{T}{t}\right)$ = the percentage of the original protease activity removed with the precipitate by precipitation at the given p_H.

It will be observed that the degree of removal of activity and the weight of precipitate both increase as the p_H rises above 1. At the same time these two factors are not closely parallel, since 88 % of the enzyme has been removed at p_H 2·75 although the weight of precipitate is only 24 % of that found at p_H 4·85. Indeed, the actual separation of insoluble matter is spread over a range of about four p_H units while the precipitation of activity is a phenomenon localised fairly definitely about p_H 2 (Fig. 3). This is of interest in view of the fact that a number of observers have reported a critical point for pepsin—isoelectric point, point of optimum adsorption, etc.—within the region of p_H 2·5 [Michaelis and Davidsohn, 1910; Northrop, 1920; Kikawa, 1926; Forbes, 1927; Fenger and Andrew, 1927]. The curve reproduced in Fig. 3 is that of a hypothetical hydrion dissociation of pepsin of pK 1·9, and

the alignment with the observed degree of precipitation of the activity may be taken to suggest that this reaction of pepsin with safranine is conditioned by the acid-base relations of the reactants. It is of course quite probable that this pK is a characteristic of the inert material with which the enzyme is

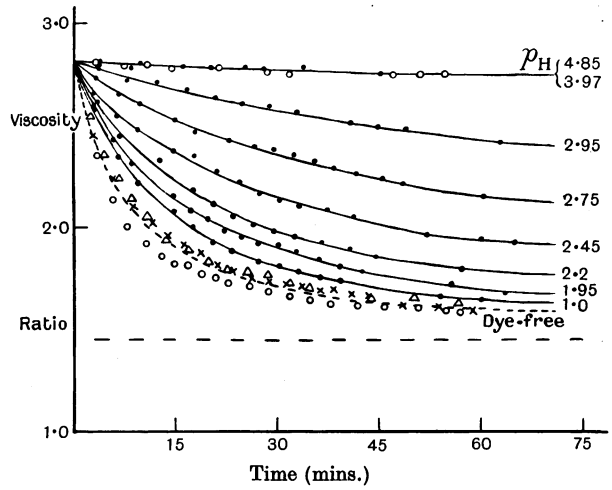

Fig. 2. Effect of p_H on precipitation of gastric juice by safranine (activity of supernatant liquors). $[D]=0{\cdot}2\,\%$. $[E]=0{\cdot}175\,\%$.

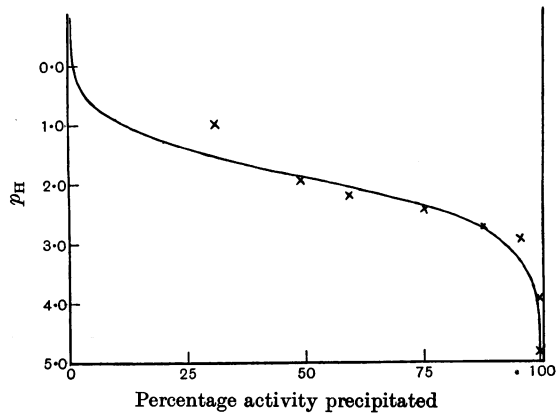

Fig. 3. Relation of precipitated activity to p_H (safranine and dog's gastric juice). The curve is that for a hypothetical hydrion dissociation of pK 1·9.

closely associated rather than of the enzyme itself. The divergence from the curve at p_H 1 may plausibly be attributed to the effect of the weak second basic group of the dye modifying the active mass of safranine at this p_H. In any case, "percentage activity" is a reciprocal term permitting considerable error below values of 50 %.

Purification of pepsin.

The following observations are of interest.

(1) An 8 % solution of a dry preparation of pepsin (Harrington and Co.) after dialysis against running water retained 95 % of its activity and lost 62 % of its solids. The neutral red precipitate from the dialysed solution contained the whole of the activity in 3 % (inclusive of dye) of the original dry weight.

(2) Neutral red precipitates from gastric juice containing the whole of the original activity weighed, according to the p_H of precipitation, from 25 to 90 % (p_H 3 to 4·8) of the dry weight of the original preparation.

(3) The minimum efficient concentration of dye was found to be 0·1 %, and the optimum p_H for purification just above 3.

(4) The weight of inactive matter precipitated from Benger's trypsin by safranine was 17 % of the total.

SUMMARY.

1. The observation of Marston and others is confirmed that the addition of safranine or neutral red to a solution of pepsin or trypsin leads to the separation of a flocculent precipitate. In the case of pepsin solutions, including gastric juice itself, the precipitate removed the peptic activity from solution. The supernatant liquors from the trypsin precipitates, on the other hand, lost no significant proportion of their activity.

2. The optimum conditions of concentration and p_H for the complete removal of pepsin from solution by this means have been determined and a method is described for the recovery of the precipitated pepsin.

3. It has been shown that azine dyes do not "poison" the catalytic activity of either pepsin or trypsin. Consequently there exists in solution under the experimental conditions no significant concentration of a specific protease-azine complex antagonistic to protein hydrolysis.

4. These results give no support to the view of Marston that the azine nucleus reacts specifically with a protease with the formation of a protease-azine complex. The formation of precipitates when azine dyes are added to protease preparations may not be used as an argument either for the piperazine structure of proteins or for any structural scheme of the mechanism of protease digestion.

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