LXXI. THE ACTION OF NITROUS ACID ON PEPSIN

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IN the search for the source of the specific activity of a protein such as pepsin one of the few available methods is to act on the protein with reagents which are known to attack certain types of group and to see whether the activity changes. Thus Herriott & Northrop [1934] and Herriott [1935-36] have acetylated pepsin with ketene, and Herriott [1937] has iodinated it. The conclusion from both researches was that tyrosine groups played a prominent part in the activity of pepsin.

The work here described was started some time ago when the conclusions would have been more novel. Nevertheless it provides further evidence of the participation of tyrosine in the activity of pepsin, and modifies the frequently quoted claim of Hugounenq & Loiseleur [1925] that the activity of pepsin is unaffected by nitrous acid. It also confirms other evidence that only a portion of the tyrosine groups in pepsin can react, at least at rates comparable with that of free tyrosine.

Preliminary experiments

The earliest experiments were done in collaboration with Miss W. Hessey and Mr B. Mainwaring.

(1) *Action of NaNO_2 and HCl .* It was found that 1.7 *N* NaNO_2 plus 1.7 *N* HCl added to a solution of crystalline pepsin (about 1%) at 25° caused rapid formation of a pale yellow precipitate. Activity determinations were uncertain owing to the insolubility of the precipitate, but very little activity could be detected. Hence the statement made by Hugounenq & Loiseleur [1925] that pepsin is not inactivated by nitrous acid "in a medium of hydrochloric acid" does not hold for pure pepsin though it may have held for the crude preparations available at that time.

(2) *Action of NaNO_2 in acetate buffer solutions.* It was found that provided that the pH was kept above 3.8 by acetate buffer no precipitate was formed, but the solution slowly became bright yellow and the activity fell off at a measurable rate to about half its original value, with a very much slower subsequent fall to zero. The yellow colour was accompanied by general absorption of light rising continuously from the blue to the short ultra-violet, and quantitative measurement showed that the band at 350-60 $\text{m}\mu$, due to NO_2^- , was not perceptibly diminished in intensity, while that at 275 $\text{m}\mu$, due chiefly to tyrosine groups in the pepsin, was diminished, although the total absorptions at both wavelengths were greatly increased by the yellow substance. This yellow substance is now known to be a diazo compound, since it shows all the colour reactions (coupling in alkaline solution with phenols, production of a red colour on irradiating in alkaline solution, colourless on irradiation in acid solution) characteristic of *o*-diazo-*p*-cresol and *o*-diazodeaminotyrosine, which we have

described in the preceding paper [1938]; some of these colour reactions have been used for its colorimetric estimation.

(3) *Liberation of N_2* . Over the pH range mentioned in the previous paragraph the concentration of undissociated HNO_2 was low, even in presence of *N* nitrite. Hence the decomposition to NO was slow enough to permit measurement of the rate of N_2 evolution from amino groups in a Barcroft or Warburg apparatus. The small amount of NO evolved was absorbed by permanganate dissolved in dilute phosphoric acid. It was essential to displace O_2 completely by purified N_2 . The final technique was still scarcely quantitative, but it showed that under the conditions used most of the N_2 was liberated in half an hour, by which time the fall in activity and production of diazo compound had hardly started. This supports the contention of Hugounenq & Loiseleur that the peptic activity does not depend on the presence of amino groups, and it also agrees with the finding of Herriott & Northrop [1934] that the amino groups can be acetylated without change of activity. The experiments on N_2 evolution were done first in a Barcroft apparatus in collaboration with (Mrs) F. J. Philpot and later in a Warburg apparatus in collaboration with Dr E. Chain.

(4) *Action of $NaNO_2$ plus acetate buffer on amino-acids*. In presence of sodium nitrite at pH about 4 (the conditions finally chosen for pepsin) a yellow colour was given slowly by tyrosine, very much more rapidly by tryptophan, but not by cystine, leucine, aspartic acid, phenylalanine, histidine or arginine.

Of the amino-acids shown by Calvery *et al.* [1936] to be present in pepsin, the only ones not tried were lysine and glutamic acid, neither of which is likely to give a colour. The yellow product from pepsin gives the colour reactions of diazotyrosine as described in the preceding paper, and can therefore be considered as due to tyrosine, and possibly in part to tryptophan, though we have no definite evidence on the latter point.

FINAL EXPERIMENTS

The preliminary experiments and the work of Herriott and Northrop on other reactions suggested that the partial inactivation of pepsin by HNO_2 was due to the action on tyrosine groups in the molecule. The final experiments were designed to prove this as rigidly as was practicable by showing not only that the kinetics of the partial inactivation of pepsin were identical with those of colour development from pepsin and free tyrosine, but that other amino-acids (tryptophan) and even other *p*-substituted phenols (*p*-cresol) react at quite different rates.

This is important for two reasons. First, even though tyrosine is concerned in the peptic activity of the protein, it is conceivable that this activity might depend on the presence of intact groups of both tyrosine and some other amino-acid, e.g. tryptophan. Since tryptophan is attacked by HNO_2 much faster than pepsin is inactivated, we conclude that it is not concerned in the activity of pepsin. Secondly, in order to exclude the possibility that the activity is due to some other *p*-substituted phenol, not yet isolated from pepsin, it is necessary to show that the velocity constants obtained are highly specific.

Runs were done with the following *p*-substituted phenols: *p*-cresol, *p*-hydroxy-phenylacetic acid, tyrosol and α -bromopropionyltyrosine, to investigate the effect of the side chain on the reaction rate.

Materials

Pepsin was prepared by the following modification of the Northrop method: 100 g. Parke Davis 1:10,000 pepsin were dissolved in 100 ml. H_2O and 100 ml. $N H_2SO_4$ were added. The solution was filtered through Whatman No. 44 filter paper and 200 ml. saturated $MgSO_4$ were then dropped in from a capillary with mechanical stirring over a period of $\frac{1}{2}$ hr. The finely granular precipitate was centrifuged and redissolved in 70 ml. acetate buffer of pH 4.6 ($AcONa$, 0.5 N ; $AcOH$, 0.548 N). The solution was dialysed with gentle stirring at 1 atm. excess pressure in cellophane, reinforced with netting, against a solution containing 0.3 N $NaCl$, 0.0144 N $AcONa$ and 0.186 N $AcOH$, pH about 3.5. Crystallization was complete after 5 days at room temperature, yield 18 g. of moist crystals. The product was recrystallized by dissolving in the pH 4.6 acetate buffer and repeating the dialysis; yield 60 %.

The specimens used were generally twice crystallized. For storage the crystals were dissolved as before, pressure-dialysed against distilled water, and the concentrated nearly salt-free solution was left in a vacuum desiccator. The product consisted of brownish glassy flakes resembling the starting material in appearance. It was easily denatured by grinding, but otherwise would dissolve to a clear solution which possessed undiminished activity, was homogeneous in the ultracentrifuge, and as far as could be found contained no more non-protein material than the original solution of crystals. Great difficulty was experienced in determining the activity in absolute units, owing to variations in sensitivity in different preparations of haemoglobin.

As a result of earlier fruitless attempts to crystallize by dialysis it was observed that crystallization only occurred within a critical range of salt concentration, which was more restricted the lower the pH. Outside the critical range, only amorphous precipitates could be obtained. Below about pH 3.3 no salt concentration would yield crystals, while above pH 4.0 the pepsin became too soluble and crystallization was very slow and incomplete. When the salt concentration was at the limit of the critical range the edges of the crystals were rounded.

The amino-acids and phenols were the purest available, but no special precautions were taken as to their purity, since it was not likely to affect any of the conclusions of this paper.

Technique

Peptic activity was measured by the method of Anson & Mirsky [1932-33]. Control experiments showed that the method was unaffected by small amounts of HNO_2 added to the haemoglobin, and that the lower apparent activity of the pepsin derivative was not due to its instability under the conditions of the activity determination.

The light absorption of the yellow products was measured by means of a small Hilger spectrograph (not the standard model) used in conjunction with a rotating logarithmic sector and a Dobson microphotometer in a manner similar to that described by Dobson *et al.* [1926]; in experiments on tryptophan, and some on tyrosine, the increase in absorption at 411.5 $m\mu$, a wave-length at which neither unchanged amino-acid nor nitrite absorbs appreciably, was taken as a measure of the extent of reaction. This method was unfortunately the only one available for use with tryptophan.

In some experiments on pepsin and tyrosine the amount of the yellow diazo compound produced was estimated colorimetrically by one of the colour reactions mentioned in the preceding paper [1938], that with β -naphthol in alkaline

solution being found most convenient; although the colour produced with resorcinol was more intense, the excess resorcinol oxidized in air after some time, so that the intensity of colour was not constant. In a kinetic run, samples were taken out at intervals and made alkaline, sufficient alkali being added to give a final concentration of N NaOH; finally 1 ml. of a 1% solution of β -naphthol in dilute alkali was added, and the colour estimated. The intensity of the red colour developed on illumination of the alkaline solution was also used to follow the reaction, but was much less satisfactory. All solutions of diazo compounds were kept in the dark.

The rate of formation of the Cu complex of the *o*-nitroso derivative from tyrosine (see previous paper) was also studied, the amount of the compound produced being estimated colorimetrically, using sodium light. The reaction was also followed in a very few instances by using Folin's reagent. The intensity of blue colour at the end of the reaction with tyrosine was about 10% of its initial value; the decrease in blue colour was taken as proportional to the extent of the reaction.

When the various methods had been shown to lead to the same velocity for tyrosine, and it had been established that they were measuring the velocity of the same reactions (i.e. $R.C_6H_4.OH + HNO_2 \rightarrow R.C_6H_3.(NO).OH + H_2O$, the rate-determining step which also controls the production of diazo compound) it was found most convenient to use the Cu nitroso compound, since it is the simplest, most convenient and most accurate.

The concentrations generally used for activity measurements were about 2.5 mg. pepsin per ml., and samples were diluted 1:50 with distilled water for activity measurements. The concentration of tyrosine was about 1 mg./ml. for measurement as diazo compound or nitroso compound. When pepsin was used for these two latter methods, its concentration was chosen so that the amount of tyrosine reacting was about the same.

The final concentrations of $NaNO_2$ used were $N/16$ at 38° and N at 0° . The two temperatures used, 38 and 0° , were obtained respectively by a gas thermostat, and by immersing in ice and distilled water kept in the cold room. Both were correct to 0.05° .

The pH was controlled by means of acetate buffers of ionic strength 0.5. Their composition was calculated from data given by Clark [1928]. Corrections were made for the buffering effect of the nitrite in calculating the concentration of free HNO_2 . The buffering effect of the pepsin was negligible. The pH of buffers was occasionally checked by means of a glass electrode (the pH of 0.5 N sodium acetate plus 0.548 N acetic acid being taken as 4.6) and agreed within 0.05 unit. Systematic errors in pH were probably the limiting factor in the accuracy of the velocity constants finally obtained.

The concentration of free HNO_2 in equilibrium with nitrite at a given pH was calculated using a value for the dissociation constant extrapolated for 38° from the data of Klemenč & Hayek [1929] for 0, 12.5 and 30° . The values so obtained are given in Table I below.

Below pH 3.8 the product from pepsin precipitated even from quite dilute solutions, causing technical inconveniences, while above pH 5 the rate of alkaline inactivation of pepsin was comparable with that of the reaction under investigation. The final experiments were therefore confined to pH 3.8–4.6. Control experiments showed that within this range the inactivation of pepsin in absence of nitrite was negligible during the time of the experiment.

Table I. *Concentrations of HNO_2 in buffer solutions* $\text{NaNO}_2 = 0.0625$, Na acetate = 0.25, 38°

Buffer pH	Final pH	$[\text{HNO}_2]$	
3.7	3.727	0.0137	
3.75	3.775	0.0126	
3.8	3.823	0.0116	"Buffer pH" is the pH of the
3.9	3.920	0.00965	buffer at 18° before addition of
4.0	4.017	0.00810	nitrite.
4.1	4.115	0.00689	"Final pH" is the pH at 18°
4.2	4.213	0.00567	after addition of nitrite, calcu-
4.3	4.311	0.00462	lated allowing for the buffering
4.4	4.410	0.00372	effect of the nitrite.
4.5	4.509	0.00299	$[\text{HNO}_2]$ is the concentration of
4.6	4.608	0.00239	free nitrous acid at 38 or 0°
5.0	5.004	0.000970	respectively.

 $\text{NaNO}_2 = 1.0$, Na acetate = 0.25, 0°

3.7	4.002	0.198
3.8	4.079	0.171
3.9	4.158	0.147
4.0	4.236	0.127
4.2	4.397	0.0944

Table II. *Velocity constants at 38°* (a) $\text{NaNO}_2 = 0.0625$; ionic strength = 0.313

- Method A. As diazo compound by photo-reaction.
 „ B. As diazo compound by coupling with phenols.
 „ C. As nitroso compound in presence of copper.
 „ D. By decrease in blue colour with Folin's reagent.
 „ P. By peptic activity. Method S. Spectrographically.

Substance	Method	pH	K_u	K_b
Pepsin	P	3.8	0.00226	0.00748
	P	3.8	0.00248	0.00821
	P	3.8	0.00246	0.00815*
	P	4.0	0.00190	0.00875
	P	4.0	0.00186	0.00855†
	B	4.1	0.00161	0.00895
	P	4.1	0.00160	0.00890
	P	4.2	0.00120	0.00815
	A	4.2	0.00119	0.00809
	P	4.2	0.00120	0.00815
	P	4.3	0.00098	0.00815
	P	4.3	0.00100	0.00823
	P	4.4	0.00078	0.00808
	P	4.5	0.00073	0.00936
	P	4.5	0.00067	0.00860
			Mean	0.00839
Tyrosine	A	3.8	0.00270	0.00895
	C	3.8	0.00285	0.00943
	D	3.8	0.00223	0.00739
	C	3.8	0.00228	0.00755
	B	4.0	0.00173	0.00796
	C	4.0	0.00178	0.00821
	C	4.0	0.00178	0.00821
	A	4.2	0.00115	0.00780
	B	4.2	0.00126	0.00855
	B	4.2	0.00120	0.00815
	B	4.2	0.00115	0.00780
	C	4.2	0.00119	0.00807
	D	4.2	0.00115	0.00780
	C	4.4	0.00076	0.00792
	C	4.6	0.00050	0.00805
	D	4.6	0.00051	0.00820
	C	3.8	0.00251	0.00832
	C	3.9	0.00212	0.00844
	C	4.0	0.00178	0.00821
	C	4.1	0.00147	0.00816
			Mean	0.00816

Table II (*cont.*)

Substance	Method	pH	K_u	K_b
α -Bromopropionyl-tyrosine	C	3.75	0.00260	0.00780
	C	3.8	0.00270	0.00895
	Mean			0.00838
Tyrosol	C	4.0	0.00285	0.0131
	S	4.2	0.00150	0.0115
	S	4.6	0.00070	0.0113
	Mean			0.0120
<i>p</i> -Cresol	C	3.75	0.00625	0.0190
	C	3.8	0.00540	0.0179
	C	4.0	0.00416	0.0191
	Mean			0.0187
<i>p</i> -Hydroxyphenyl-acetic acid	C	3.8	0.00268	0.00890
	C	3.9	0.00222	0.00885
	C	4.0	0.00202	0.00925
	C	4.1	0.00164	0.00912
	Mean			0.00903
Tryptophan	S	4.2	0.019	0.15†

* Illuminated Hg vapour lamp.

† In presence Cu; final activity = 30 %.

‡ Only approx.

(b) Effect of varying salt concentration. Tyrosine, pH 4.4, $\text{NaNO}_2 = 0.0625$, copper nitroso method

Ionic strength μ	K_u	Salt added
0.425	0.0082	CuSO_4
0.81	0.0085	$\text{Na}_2\text{SO}_4 + \text{CuSO}_4$
0.875	0.0085	$\text{NaCl} + \text{CuSO}_4$
1.31	0.00875	$\text{NaCl} + \text{CuSO}_4$

Table III. *Velocity constants at 0°*(a) $\text{NaNO}_2 = 1.0$; total salt concentration = 1.25

Substance	Method	pH	K_u	K_b	Remarks
Pepsin	P	3.7	0.0077	0.00150	
	B	—	0.0069	0.00134	
	P	3.8	0.0045	0.00101	
	Mean			0.00128	Methods as in Table I
Tyrosine	B	3.7	0.0066	0.00128	
	C	—	0.0065	0.00126	
	C	3.8	0.0054	0.00121	
	Mean			0.00125	

(b) Effect of varying nitrite. Tyrosine, pH 3.8, method C

Nitrite	Ionic strength	K_u	K_u' $\mu = 1.25$	$[\text{HNO}_2]$	K_b
0.5	0.75	0.0025	0.0026	0.103	0.00097
1.0	1.25	0.0054	0.0054	0.171	0.00121
1.5	1.75	0.0086	0.0083	0.225	0.00142

 K_u' is K_u corrected for salt effect, assumed the same as at 38°.If $V = k_{b_0}$ (tyrosine) $(\text{HNO}_2) (1 + a[\text{NO}_2^-])$, $a = 0.66$ and $K_{b_0} = 0.000775$.Mean velocity of pepsin and tyrosine at 0°, corrected for salt effect and nitrite effect, i.e. calculated for $\mu = 0.3125$ and total nitrite = 0.0625 as used at 38°, is 0.00078.

RESULTS

(1) Under the conditions used, there being a very large excess of nitrite, all the reactions are pseudo-unimolecular. This is illustrated by the logarithmic plots for four runs in Fig. 1. A curve of peptic activity against time is also given, to illustrate that the final activity is 50 %, the mean value found for the series being $50 \pm 1\%$. This final activity is fairly stable, but further diminishes at a

Fig. 1.

very low rate, which is comparable with the rate at which HNO_2 attacks diazo compound. If the HNO_2 is removed with urea or by dialysis no further loss of activity occurs.

(2) The pseudo-unimolecular constants for the inactivation of pepsin, for the production of diazo compound from tyrosine or pepsin, and the production of copper nitroso compound from tyrosine or α -bromopropionyltyrosine are almost identical under similar conditions, and with a constant $[\text{NO}_2^-]$ are proportional to the $[\text{HNO}_2]$.

p-Cresol and tyrosol react at quite different rates from pepsin or tyrosine, but the rate of *p*-hydroxyphenylacetic acid is not very different. However, application of the "Student" *t*-test to the rates of pepsin and *p*-hydroxyphenylacetic acid shows that the probability that the figures obtained are all members of the same set is only 0.02. To eliminate the possibility that differences in technique might upset this conclusion, four runs (the four last of the tyrosine series in Table II) were done by the same method which was used for *p*-hydroxyphenylacetic acid (the copper method), two before and two after the set of experiments on the latter substance, and they agreed excellently with earlier results. By application of the *t*-test, it was shown that the probability of the rates of tyrosine and *p*-hydroxyphenylacetic acid being members of the same set is only 0.01.

The rates of reaction of different *p*-substituted phenols are therefore sufficiently specific for conclusions to be drawn from them. There is, however, one factor which is difficult to eliminate, that is the influence of the formation of peptide or similar linkages on the rate of reaction. Two runs were done with

effect of the introduction of substituents into the tyrosine group rather than to general damage of the molecule.

The activity of the diazo compound is not changed when it is acted on by light, either in presence or in the absence of HNO_2 . Reduction with $\text{Na}_2\text{S}_2\text{O}_4$ does not change the activity by more than 5%. Hence the diazo, hydroxy and several other derivatives have equal peptic activities.

(6) The purple colour developed from pepsin in presence of Cu and HNO_2 is approximately 45% of that expected from its tyrosine content. If the pepsin is broken down by autolysis at pH 2 and 38° , or hydrolysis with strong alkali at 100° , it then develops the same amount of colour as a tyrosine solution of equivalent concentration. This suggests that only a portion of the tyrosine in native pepsin is free to react, and recalls the observation of Herriott [1935-36] that several proteins, including pepsin, only give about 60% of the theoretical colour intensity with Folin's reagent at pH 8.

DISCUSSION

The chief result of the present work is to add nitrous acid to the brief list of reagents which can act in a known manner on a native protein without damaging the molecule as a whole. A more special result is to provide further evidence in support of the claim of Northrop and Herriott that tyrosine groups, or at least some phenolic groups, play a part in peptic activity. While Herriott's isolation of diiodotyrosine from iodinated pepsin showed conclusively that tyrosine groups in pepsin react with iodine, it did not exclude the possibility that the fall in peptic activity was due to one or two other phenolic groups reacting at the same rate as tyrosine. Our finding that *p*-cresol, tyrosol and *p*-hydroxyphenyl-acetic acid react at measurably different rates from tyrosine makes it increasingly probable that the phenolic group concerned with peptic activity is that of tyrosine itself. The very rapid reaction of tryptophan also excludes the possibility that unsubstituted tryptophan groups are essential for peptic activity.

The action of nitrous acid on pepsin seems to be simpler kinetically, as far as peptic activity is concerned, than that of either ketene or iodine. The unimolecular approach to a 50% active product, with only a very slow subsequent change, is unprecedented. That the 50% active product also possesses about 50% of its tyrosine groups intact is probably mere coincidence; but it is possible that those tyrosine groups which are protected from nitrous acid are nevertheless somehow able to participate in peptic activity.

In any case it is probable that those groups which do react with nitrous acid become quite inactive enzymically, since they can undergo subsequent changes, e.g. by irradiation, without causing any change in total peptic activity. The relationship between our 50% active product and the several products yielded by ketene and iodine is obscure, and can only be elucidated by further work, e.g. by successive action of the different reagents.

SUMMARY

1. The reaction between pepsin and nitrous acid has been studied up to pH 4.5 and at 0 and 38° .
2. The rate is proportional to the concentration of unchanged pepsin and of undissociated nitrous acid, and depends slightly upon the nitrite concentration.
3. The product is a yellow photosensitive diazo compound and has 50% of the original activity. Subsequent changes are very slow.

4. Comparisons with the action of nitrous acid on amino-acids and other substances support the view that the fall in activity is due to an action on the tyrosine groups in pepsin.

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REFERENCES

- Anson & Mirsky (1932-33). *J. gen. Physiol.* **16**, 59.
Calvery, Herriott & Northrop (1936). *J. biol. Chem.* **113**, 11.
Clark (1928). *The Determination of Hydrogen Ions*. (Baltimore: Williams and Wilkins.)
Dobson, Griffith & Harrison (1926). *Photographic Photometry*. (Oxford University Press.)
Herriott (1935-36). *J. gen. Physiol.* **19**, 283.
—— (1937). *J. gen. Physiol.* **20**, 335.
—— & Northrop (1934). *J. gen. Physiol.* **18**, 35.
Hugounenq & Loiseleur (1925). *Bull. Soc. Chim. biol., Paris*, **7**, 995.
Klemenč & Hayek (1929). *Mh. Chem.* **53-54**, 407.
Philpot & Small (1938). *Biochem. J.* **32**, 534.
Taylor (1929). *J. Chem. Soc.* 2052.
Veibel (1930). *Ber. dtsh. chem. Ges.* **63**, 1577