CCXIV. THE ACTIONS OF IODINE AND HYPOIODOUS ACID ON PEPSIN

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THIS paper gives experimental details of the work which we reported briefly at the Royal Society Discussion Meeting on "The Protein Molecule" [Philpot & Small, 1939]. We claim that inactivation of pepsin by iodine is sometimes due to action on groups other than tyrosine, and that these groups are not any of the ordinary amino-acids. Our evidence is that pepsin which has reacted with enough iodine to inactivate it still gives the full blue colour with Folin's reagent, whereas it should give less colour if the tyrosine groups had reacted appreciably.

EXPERIMENTAL

1. General technique

Pepsin was prepared as before [Philpot & Small, 1938, 2]. The concentrations quoted refer to this material, which contains about 10 % of water and 30–50 % of impurity [cf. Tiselius, 1938]. Peptic activity was measured by the original Anson-Mirsky method [Anson & Mirsky, 1932], except that the acid haemoglobin solution was made by mixing 1 vol. of methaemoglobin solution with 4 vols. of a solution containing 0.625 *M* H_3PO_4 and 0.167 *M* NaOH. This buffer gave a more reproducible pH than the HCl usually used. Methaemoglobin was used since oxyhaemoglobin gradually turns into it on storage. It was prepared as follows, partly following Adair & Adair [1934]. 450 ml. washed ox corpuscles were mixed with 132 ml. H_2O and 132 ml. purified ether, and shaken for 3 min. Then 57 g. NaCl were added, and the mixture shaken and centrifuged. The haemoglobin was treated with 25 ml. 10 % ferricyanide and 0.5 ml. octyl alcohol, shaken well and pressure-dialysed against distilled water. The concentration was adjusted so that on 250-fold dilution the blue colour given with Folin's reagent [Folin & Ciocalteu, 1927] was equal to that given by 0.055 mg. tyrosine per ml. The stock solution was stored under octyl alcohol in the cold room. These modifications caused little or no change in the activity unit, but seemed to give rather better reproducibility. The recent modifications by Anson [1938] appeared too late to be used in this work.

Folin's reagent [Folin & Ciocalteu, 1927] was used as follows. For peptic activities 3 ml. of the trichloroacetic acid filtrate were treated with 20 ml. 0.3 *M* NaOH and 1 ml. Folin's reagent. For Folin values of pepsin itself the trichloroacetic acid etc. were not present, though the pepsin was buffered, and a corresponding adjustment was made in the amount of NaOH. The colour intensity was compared in sodium light, after 30 min., with that of a glass filter or a mixture of cobalt nitrate and copper sulphate made to match the tint. As absolute values were not necessary calibration was usually dispensed with, so the units given are arbitrary and include dilution factors. In Fig. 3, however, the units are the same for both curves, so that the slopes can be directly compared.

2. *The action of iodine on pepsin*

Experiments were done under various conditions of pH, temperature, concentration and medium. Pepsin and iodine (in potassium iodide) were mixed and samples withdrawn from time to time. The unused iodine was titrated with bisulphite since the tetrathionate formed from thiosulphate reduces Folin's reagent. The titrated samples were then used for estimations of Folin value and peptic activity, after suitable dilution. In order to obtain reasonable velocities with moderate pepsin concentrations the iodine had to be in large excess. Hence the determination of iodine uptake was not very accurate, especially with the small volumes used and with bisulphite as titrant. The Folin values were more accurate, but unfortunately the greatest expected fall in Folin value was only 18 %. This arises from the facts that according to the analysis of Calvery *et al.* [1936] 11.5 % of the Folin value of pepsin should be due to tryptophan under our conditions, that only 10 of the 20 tyrosine groups are reactive (at least towards nitrous acid (cf. Philpot & Small [1938, 2]) and that under our conditions iodinated tyrosine has 50–60 % of the Folin value of tyrosine, mole for mole. For these reasons no attempt has been made at an exact kinetic analysis, though the time-courses of four experiments are recorded in Table I.

Table I. *Action of iodine on pepsin*

Curve (Fig. 1)	A			B		C		D	
pH	4.6			5.0		5.5		6.0	
Temp. ° C.	38			19		19		19	
Pepsin % (approx.)	0.25			0.12		0.12		0.12	
I ₂ normality	0.05			0.05		0.05		0.05	
<i>t</i>	I ₂	F	P	F	P	F	P	F	P
0	0.0470	41.1	50.5	52.5	46.9	51.0	47.2	49.5	51.4
2	0.0453	41.0	31.0	—	—	—	—	—	—
4	0.0446	40.0	28.2	—	—	—	—	—	—
6	0.0443	40.2	25.8	—	—	—	—	—	—
8	0.0436	39.8	22.0	—	—	—	—	—	—
10	0.0427	39.4	17.8	—	—	—	—	—	—
15	0.0423	39.6	14.3	—	—	—	—	—	—
20	0.0419	39.0	10.0	—	—	—	—	—	—
25	0.0419	39.0	8.0	—	—	—	—	—	—
30	0.0416	38.4	7.2	51.5	41.7	50.0	19.0	48.5	12.5
60	0.0395	37.5	6.9	50.5	37.2	49.0	11.5	48.0	6.0
90	0.0385	37.8	6.5	—	—	—	—	—	—
120	—	—	—	50.0	28.0	47.9	7.2	46.8	5.8
180	—	—	—	49.0	22.1	47.0	6.2	46.2	5.7
240	—	—	—	48.0	19.0	46.6	5.7	45.8	5.8

t Time from start in minutes.I₂ Normality of iodine.

F Folin value, arbitrary units.

P Peptic activity, arbitrary units.

For our present purpose the most relevant information is obtained by plotting peptic activity against Folin value, each expressed as percentage of the original. This is done in Fig. 1. If the inactivation were due to iodination of one or more of the ten reactive tyrosine groups, all ten reacting at the same rate, one would obtain the line "10" in Fig. 1. If the inactivation were due to iodination of a single tyrosine group, which for some reason reacted much more rapidly than the others, one would obtain the line "1" in Fig. 1. Similarly two such groups would give the line "2". The experimental curves A–D represent the various conditions of temperature and pH recorded in Table I. Curve B, obtained at

19° and pH 5, is near the line "10"; but curves C and D, at pH 5.5 and 6, lie between the lines "1" and "2" until the inactivation is nearly complete. Curve A shows that at 38° the simple "10" hypothesis is untenable even at

Fig. 1. Action of iodine on pepsin. For details see Table I and text.

pH 4.6. The general conclusion from these experiments is that at sufficiently high pH and temperature pepsin can be largely inactivated by iodine without iodinating more than two tyrosine groups.

3. The action of iodine on diazopepsin

The name "diazopepsin" refers to the yellow 50 %-active product of the action of nitrous acid, in which 50 % of the tyrosine groups have a diazo-group introduced *ortho* to the hydroxyl [Philpot & Small, 1938, 1, 2]. Since any further action of nitrous acid on the compound is extremely slow, and since the diazo-group can be altered in several different ways, even coupled with naphthylamine, without altering the activity (unpublished observations by P. A. Small) it was of interest to study the action of iodine.

The diazopepsin was prepared by mixing 20 ml. 2.4 % pepsin with 10 ml. 10 *M* acetic acid and 10 ml. *M* $NaNO_2$, leaving 3 days at 0° and then dialysing free of nitrite. It was stored at pH 4.6 in a darkened vessel in the cold room. It was handled with minimal exposure to light, to which it is very sensitive.

Table II gives the results of two experiments with iodine, and Fig. 2 gives the activity plotted against Folin value. The lines "1" and "10" are as in Fig. 1. The fall in activity is as rapid as with pepsin itself, but curve B, shows that at 38° much less than one tyrosine group per mol. is iodinated even when the activity has fallen to 20 %. The fall in activity must therefore be due to action on some other groups. These could be diazotyrosine groups, whose initial Folin value is practically zero; but no such action is known, and the indifference of the activity towards other changes in the diazotyrosine groups suggests that they play no part in it.

Table II. *Action of iodine on "diazopepsin"*

Curve (Fig. 2)	A		B	
pH	4.6		4.6	
Temp. ° C.	18		38	
Diazopepsin % (approx.)	0.6		0.6	
I ₂	0.05		0.05	
	F P		F P	
t				
0	40.0	42.7	33.3	40.0
4	—	—	33.5	18.1
8	—	—	33.3	12.5
15	—	—	33.3	9.0
24	—	—	33.2	7.0
30	40.0	35.5	—	—
60	40.0	28.6	—	—
120	39.5	21.2	—	—
180	38.9	16.6	—	—
240	39.2	14.0	—	—
360	38.4	11.2	—	—
480	38.1	8.3	—	—

Symbols as in Table I.

Fig. 2. Action of iodine on "diazopepsin". A at 18°; B at 38°.

4. *The action of hypiodous acid on pepsin*

It was shown by Cofman [1920] that hypiodous acid acts extremely rapidly on phenols. Since iodine solutions are in equilibrium with hypiodous acid it is probable that the latter is an intermediate when the former acts on tyrosine. It was of interest to see whether the hypothetical non-tyrosine groups were acted on even more rapidly than tyrosine by hypiodous acid, as they should be if it was an intermediate for them as well.

The very high reaction rates involved here necessitated a change in technique which was advantageous for our purpose. Instead of adding a fairly large excess of iodine and titrating at various times, various small amounts of hypiodous

acid were added and allowed to react to completion. The activity and Folin values were then measured as functions of added hypoiodous acid, thereby eliminating time as a variable and avoiding the iodine titration. Under these conditions the residual concentration of the fastest reacting group present will be a linear function of the amount of added hypoiodous acid until it is low enough for more slowly reacting groups to compete.

Since HIO is fairly rapidly converted into IO_3^- and I^- it had to be generated *in situ*. The most convenient way of doing this was to add the appropriate amount of iodine and then an excess of mercuric acetate. The reaction was

After the action on pepsin was complete addition of excess iodide brought the $[\text{Hg}^{++}]$ to a very low value and prevented it from interfering with later operations. The iodide also regenerated iodine from any unchanged hypoiodous acid, and also from any iodate at *pH* below about 4, so that by testing for iodine at low *pH* it could be shown that the hypoiodous acid, if not in excess, had reacted completely in the time allowed. Controls showed that the reagents used without iodine did not affect the peptic activity but lowered the Folin value by about 4%. The 0.003 *N* I_2 used was made up of 0.0005 *M* KIO_3 , 0.004 *M* KI and 0.005 *N* H_2SO_4 . *x* ml. of it were added to 5-*x* ml. buffered pepsin or tyrosine and HIO was generated by adding 0.5 ml. of 0.03 *M* mercuric acetate. After 5 min. at room temperature 1 ml. of 0.2 *M* NaI was added, and the Folin value and activity were then determined as already described.

Fig. 3 shows the Folin values of pepsin and tyrosine plotted against amount of HIO. The point of interest is the initial slope, which is zero for pepsin but not

Fig. 3. The action of hypoiodous acid on pepsin and tyrosine at *pH* 5.4. A, pepsin 2.5 mg. B, tyrosine 0.18 mg. Beyond the vertical lines hypoiodous acid was in excess.

for tyrosine. This proves that the first action of HIO on pepsin is something other than iodination of tyrosine groups. The activity curve which is plotted with the Folin curve in our preliminary communication [Philpot & Small, 1939], shows that the same amount of HIO which leaves the Folin value unaffected causes nearly total loss of peptic activity.

5. Studies on groups other than tyrosine

In our preliminary communication a list was given of other groups which could not be concerned in the inactivation process in question. The evidence is as follows. Histidine, gelatin, asparagine and acetone reacted too slowly or not at all under our conditions. This rules out histidine, proline, hydroxyproline, amide, amino, carboxyl, peptide and simple ketone groups. Tryptophan and simple phenol groups are ruled out because if not masked they would react with nitrous acid, whereas the end-product from nitrous acid still contains the iodine-sensitive groups. Sulphydryl groups react much too rapidly with iodine, and cannot be detected in pepsin. The possible participation of disulphide groups has been difficult to eliminate. Cystine reacts fairly rapidly with iodine and very rapidly with hypiodous acid, the uptake in the latter case corresponding exactly to formation of cysteic acid. However, peptic activity is unaffected by hydrosulphite, which should reduce disulphide groups. Also, we have made various attempts to estimate the disulphide groups in pepsin before and after treatment with hypiodous acid. This involved special difficulties which have not yet been completely overcome; but the provisional conclusion is that not enough of the disulphide groups in pepsin can react at *pH* 5.4 to account for the whole of the HIO uptake involved in the inactivation process.

DISCUSSION

We do not claim that the above evidence for the existence of essential groups in pepsin other than ordinary amino-acids is complete, but we think it provides sufficient stimulus for an attempt to isolate the groups in question. It is perhaps worth noting that the optimal conditions for formation of thyroxine by an anomalous iodination of tyrosine [Ludwig & Mützenbecher, 1939] in proteins are very similar to our conditions for obtaining no initial fall in Folin value. We do not think that this can invalidate our conclusions, but the point might be investigated. Our results are nowhere in direct conflict with those of Herriott [1937], who claimed that inactivation of pepsin was due to iodination of tyrosine groups, since he did not use Folin's reagent to study the course of the reaction. Iodination of the tyrosine groups alone should certainly diminish the activity and might abolish it altogether; but we have no evidence on this point.

SUMMARY

1. When iodine acts on pepsin the initial ratio of fall in Folin blue value to fall in peptic activity decreases with increasing *pH* and temperature, reaching a very low figure at *pH* 6 and 38°.
2. The same occurs with "diazopepsin", in which the reactive tyrosine groups have previously been attacked by nitrous acid.
3. When pepsin is treated with small amounts of hypiodous acid the initial ratio of fall in Folin blue value to amount of hypiodous acid is much lower than for tyrosine, being practically nil at *pH* 5.4.
4. The hypiodous acid required to produce a just perceptible fall in Folin blue value is sufficient to destroy most of the peptic activity.
5. It is concluded that some groups other than tyrosine are concerned and reasons are given for excluding other amino-acids.

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