INACTIVATION OF PEPSIN BY IODINE AND THE ISOLATION OF DIIODO-TYROSINE FROM IODINATED PEPSIN

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The previous work of the writer on the acetylation of pepsin (1, 2) points to a close relationship between the tyrosine of pepsin and the proteolytic activity of the protein molecule. An acetyl derivative of pepsin was prepared which had 60 per cent of the activity of pepsin, contained three labile acetyl groups, and gave a tyrosine-phenol color value lower by three tyrosine equivalents than that of pepsin. For these and other reasons it seemed probable that the labile acetyl groups were attached to the phenolic hydroxyl groups of tyrosine in pepsin. Attempts to isolate an acetylated tyrosine were unsuccessful due probably to the lability of the acetyl radical. In the hope of obtaining direct and decisive evidence concerning the rôle played by tyrosine in pepsin it was decided to study the action of iodine on pepsin. Iodine is supposed to react with the benzenoid part of tyrosine in proteins (3, 4) and since this type of iodine linkage is known to be relatively stable, it seemed likely that an iodine-tyrosine compound could be isolated from iodinated pepsin.

Under certain conditions solutions of native pepsin readily absorb iodine and the proteolytic activity decreases gradually. Pepsin is practically inactive when the number of iodine atoms per molecule of pepsin is 35–40. There is no appreciable oxidation of pepsin or of glycyl tyrosine by iodine under the conditions used for iodination. The rates of iodination of pepsin and of glycyl tyrosine are affected by a variation of pH in a like manner. The effect of pH is nearly identical with that already noted for acetylation of these two materials.

Completely iodinated pepsin was hydrolyzed and the products of hydrolysis containing iodine were fractionated. A solution containing

82 per cent of the total iodine was obtained from the iodinated pepsin. The analyses of this solution were similar to those of a solution of diiodo-tyrosine. Also present in this solution was a dark material which prevented crystallization of the diiodo-tyrosine and which could be removed only with difficulty and at the expense of the yield of crystalline diiodo-tyrosine. The yield of crystalline diiodo-tyrosine finally obtained represented 53 per cent of the original iodine.

That the iodine is attached to the tyrosine in the intact iodinated pepsin protein is further indicated from the titration curve of the iodinated protein. The groups in pepsin which titrate between pH 10.0 and 12.0, in the iodinated pepsin titrate between pH 8.0 and 10.0. These are probably the phenolic hydroxyl groups of tyrosine.

EXPERIMENTAL RESULTS

Conditions for Iodination of Pepsin

Many experiments were performed to determine the effect of the numerous variables on the iodination of pepsin. The results of some of these experiments are dealt with in detail in this paper but a large part of this work has been omitted. It will suffice, however, to say that the most reproducible and apparently uncomplicated results were obtained by observing the following conditions: the concentration of free or molecular iodine in the reaction medium should not be over one-tenth normal; the protein concentration is optimal at 2–5 mg. protein nitrogen per ml. and the pH kept at 5.0–6.0. The temperature seems to affect only the rate of iodination. Alcoholic iodine can be used in place of iodine-potassium iodide solution without any apparent difference in the result unless the alcohol content of the final solution rises above 10–15 per cent in which case the temperature should be kept nearly 0°C. and the time the protein is in contact with this medium kept at a minimum.

Effect of Iodine on the Proteolytic Activity

Under the conditions outlined above for the iodination of pepsin, the specific activity of pepsin gradually decreases as iodine is introduced into the protein molecule. Fig. 1 shows the effect graphically. The specific activity is reduced practically to zero when the iodine content is 35–40 atoms of iodine per molecule of pepsin. Of course

the solutions analyzed were reaction mixtures and in all probability not solutions of single compounds, nevertheless, the general effect of introduced iodine on the enzymatic activity of pepsin is indicated.

Experimental Details

The procedure used in the experiment indicated in Fig. 1, by the open circles o is as follows: to 65 ml. of a dialyzed 2 × crystallized Parke, Davis pepsin solution in 0.2 M pH 6.0 acetate buffer was added 4 ml. of 1 N iodine in potassium iodide solution. The final concentration free iodine equals 0.06 N and protein nitrogen per ml. equals 4 mg. The experiment was carried out at 5°C. At suitable intervals of time samples were removed, the free iodine destroyed by adding

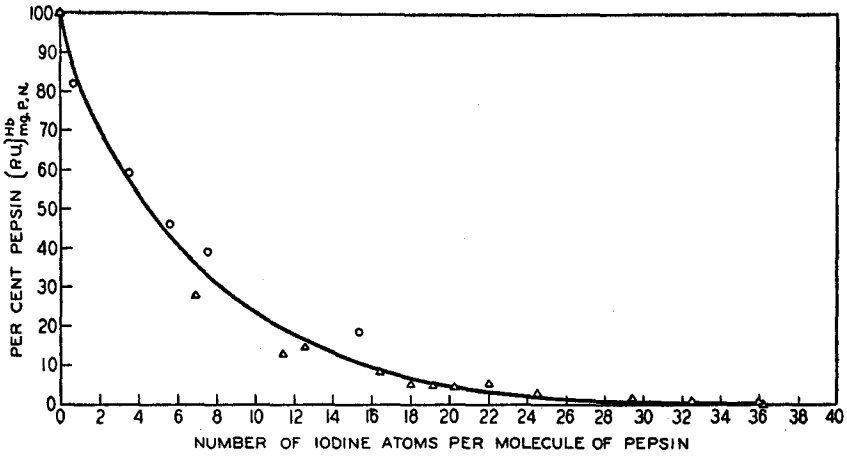

FIG. 1. Effect of bound iodine on the specific activity of pepsin.

dropwise a solution of 10 per cent sodium bisulfite. The sample was then dialyzed in a rocking dialyzer (5) for 20 hours against M/2,000 pH 5.0 acetate buffer after which it was analyzed for protein nitrogen, proteolytic activity, and total iodine by methods outlined in the section of this paper devoted to Experimental methods.

The procedure used in the experiment indicated in Fig. 1 by the triangles, △, is as follows: to a solution of dialyzed 2 × crystallized Parke, Davis pepsin containing 4 mg. of protein nitrogen per ml. was added an equal volume of 0.1 N iodine in potassium iodide solution. Aliquots were removed at intervals of time, treated, and analyzed as described above for the other part of this experiment.

Oxidation and Substitution

Thus far in this paper only the "introduced" or "bound" iodine has been considered as the cause of the effects noted with regard to

enzymatic activity. Iodine may be acting in the present instance as an oxidizing agent as well as one of substitution. There exists, therefore, the possibility that the effect noted on the specific activity of pepsin is caused, not by the bound or substituted iodine, but by a simultaneous oxidation of some group in the protein molecule. The experiments shown in Table I were designed to determine to what extent oxidation by iodine takes place during the iodination of pepsin. When iodine acts as an oxidizing agent it is itself reduced from molecular iodine I^0 to iodide ion I^-. The extent of oxidation may then be determined by estimating the production of iodide ion. It must be remembered, however, that in the iodine substitution reaction, as illustrated,

$$\text{Tyrosine} + 2\,I_2 \longrightarrow \text{diiodo-tyrosine} + 2\,H^+ + 2\,I^-$$

an amount of iodide ion is produced equal to that amount which substitutes. The iodide ion resulting from any oxidation by iodine must then be determined as that quantity of iodide ion over and above the iodide ion produced as a result of substitution. The iodide ion formation in the control experiment must, of course, be deducted.

The writer was not able, unfortunately, to determine the iodide ion directly in the presence of iodinated pepsin and was forced to resort to estimating the extent of oxidation by an indirect method. If no oxidation takes place during iodination the organically bound iodine should equal one-half of the decrease in free iodine, as may be seen from the illustrated reaction. If oxidation does occur then the organic iodine will be less than one-half of the decrease in free iodine for some of the free iodine will be consumed in the oxidation. Thus a comparison of the organic iodine determined and the organic iodine calculated as one-half of the decrease in free iodine will serve as a measure of the oxidation. Free iodine and therefore the loss in free iodine can be determined precisely by the usual iodimetric titrations. The figure for the organically bound iodine was obtained after dialyzing a sample of material free of all uncombined iodine. By making the nitrogen analysis before and after dialysis and iodine analysis after dialysis the organic iodine existing before dialysis may be calculated, assuming no loss of organically bound iodine during dialysis.

Column (f) of Table I shows the difference between the organic

iodine determined and that calculated from the loss of free iodine. The results of these experiments are not as decisive as had been hoped but the differences noted are small, indicating that little oxidation occurs, and they seem to bear no relationship to the specific activity of pepsin. It may be pointed out that using three different concentrations of iodine in Nos. 2, 4, and 10 of Table I and obtaining solutions with three quite widely different specific proteolytic activities,

TABLE I

Oxidation of Pepsin by Iodine at pH 5.6 and 5°C.

No.	Material	Original concentration of iodine	Time	$\frac{Hb}{mg.P.N.}$ [P.U.]	Determined				Calculated	Difference
					Iodine/ml.			Atoms I per mol pepsin	Decrease in free iodine / 2	(e) − (c)
					Total	Free	Organic			
					(a)	(b)	(c)	(d)	(e)	(f)
		normality	*hrs.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg./ml.*	*mg./ml.*
1	Pepsin	0.000	1	0.22						
2	Pepsin	0.005	1	0.15	0.63	0.00	0.23	5	0.32	0.09
3	Control (buffer)	0.005	1			0.65				
4	Pepsin	0.010	1	0.086	1.3	0.17	0.46	10	0.52	0.06
5	Control	0.010	1			1.2				
6	Pepsin	0.020	1	0.039	2.54	0.75	0.73	16	0.92	0.19
7	Control	0.020	1			2.6				
8	Pepsin	0.050	1	0.002	6.3	3.1	1.3	29	1.65	0.35
9	Control	0.050	1			6.4				
10	Pepsin	0.03	1	0.02	3.7	1.8	0.85	19	0.95	0.10
11	Pepsin	0.03	24	0.006	3.8	1.0	1.1	23	1.40	0.30

practically the same differences between calculated and determined organic iodine may be seen in column (f).

Experimental Details

Materials.—Dialyzed 2 × crystallized Parke, Davis pepsin in M/2 sodium acetate pH 5.6; the solution contained 4 mg. protein nitrogen per ml.

Starting with 0.5 N iodine in 50 per cent ethyl alcohol, solutions of various concentrations were made up by diluting this iodine solution with 50 per cent alcohol. By dissolving the iodine in alcohol the presence of potassium iodide in the solvent was eliminated.

Procedure.—With the solutions at 5°C., 15 ml. of pepsin solution was mixed

with 15 ml. of iodine solution and allowed to remain for 1 hour at 5°C. Aliquots were then analyzed for protein nitrogen, activity by the hemoglobin method, total iodine, free iodine (direct titration), and the remainder of the solution treated with sodium bisulfite to destroy the free iodine remaining. After treatment with sodium bisulfite these solutions were dialyzed for 20 hours at 5°C. and then analyzed for total iodine and protein nitrogen. A control on the pepsin activity was made by adding 15 ml. of 50 per cent alcohol to the pepsin solution instead of the iodine solution. A control on the oxidation of iodine was made for each concentration of iodine by adding 15 ml. of iodine solution to 15 ml. of M/2 pH 5.6 acetate buffer and then analyzing after 1 hour for total and free iodine.

TABLE II

Oxidation during Iodination of Glycyl Tyrosine

No.	pH	Time	Mg. I/ml. (determined)					Mg. I/ml. (calculated)			Extent of iodination
			Total	Free	Total-free	Iodide	Organic	Organic	Iodide		
			(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)
		hrs.						$(c)_A - (d)_A$	$\frac{(c)_A - (d)_B}{2}$	$(d)_A - (d)_B$	*per cent*
1A	4.0	0.5	18.0	14.5	3.8	3.0	0.8	0.8	0.8	0.8	13
1B	—	0.5		16.5	2.3	2.2	0.0	0.1			
2A	—	72.0		8.3	10.5	6.4	4.0	4.1	3.9	3.9	65
2B	—	72.0		15.5	2.7	2.5	0.1	0.2			
3A	5.6	0.5	18.0	6.9	10.3	6.2	4.5	4.1	4.1	5.1	73
3B	—	0.5		15.7	2.2	1.1	0.2	1.1			
4A	—	72.0		2.3	13.6	7.7	4.9	5.9	4.9	5.2	84
4B	—	72.0		15.4	2.8	2.5	0.1	0.3			

In studying the iodination of glycyl tyrosine the iodide ion formed could be determined directly by a gravimetric method. It may be worth noting that even in this case with direct methods of analysis and dealing with pure simple substances the results to be found in Table II are not perfectly constant. There can be little doubt, however, that oxidation by iodine plays no part in this reaction.

Experimental Details

Materials.—1.5 gm. of 81 per cent dry weight Hoffmann-La Roche glycyl tyrosine = 1.19 gm. (1/200 mol) dissolved in water with the aid of 3 ml. N/1 sodium hydroxide and diluted to 105 ml. 0.8 gm. glycyl glycine dissolved in water plus 3 ml. N/2 sodium hydroxide and diluted to 105 ml.

Procedure.—To 50 ml. of glycyl tyrosine solution was added 25 ml. of 1.6 M acetate buffer in one case pH 4.0, in another pH 5.6, followed by 25 ml. of 0.5 N iodine in 50 per cent alcohol. As controls, equimolar solutions of glycyl glycine were substituted for glycyl tyrosine. In Table II the glycyl tyrosine is indicated as the "A" experiments, while the control, glycyl glycine experiments, are denoted by "B". The entire experiment was carried out at 5°C.

At various intervals of time samples were removed and analyzed for the following properties: (*a*) total iodine, (*b*) free iodine, (*c*) iodide ion plus organic iodine, (*d*) iodide ion, and (*e*) organic iodine.

Analysis for the total iodine (*a*) was carried out by the procedure outlined in the section of this paper devoted to Experimental methods. The free iodine (*b*) was titrated with standardized thiosulfate. The iodide ion plus organic iodine (*c*) was determined by analyzing for total iodine after extracting the free iodine out of solution by shaking with several aliquots of carbon tetrachloride. The iodide ion (*d*) was estimated gravimetrically as the silver salt which is the only material precipitated in N/10 nitric acid solution. After removal of the iodide ion as silver iodide by filtration, the organic iodine was determined on the filtrate after first removing the excess silver ion by addition of an aliquot of N/10 sodium chloride. Knowing the volumes used, calculation back to the original aliquot was made.

Effect of pH on Iodination of Pepsin and of Glycyl Tyrosine

In the studies on the acetylation of pepsin (1, 2) it was brought out that the phenolic hydroxyl group of tyrosine in pepsin and of pure glycyl tyrosine are both readily acetylated by ketene at pH 6.0 whereas at pH 4.0 acetylation is slow. Since iodine reacts with quite a different part of the tyrosine molecule than does the acetyl group it was of interest to see if the effect of pH was the same for iodination as had been observed for acetylation and the same for pepsin and glycyl tyrosine.

Experimental Details

Materials.—A dialyzed 2 × crystallized preparation of pepsin containing 6.3 mg. of protein nitrogen per ml. was used. With the exception of the buffer (citric acid) used to obtain pH 2.5, all the buffers were acetate buffers of molar concentration made up to give the indicated pH. The iodine was 0.15 normal in potassium iodide solution.

Procedures.—5 ml. pepsin solution plus 5 ml. 1 N buffer plus 5 ml. 0.15 N iodine solution let stand at room temperature for 1 hour after which 1 ml. was removed and titrated with standard thiosulfate to determine the free iodine remaining. To the remainder of the solution was added dropwise 10 per cent sodium bisulfite solution until the free iodine was destroyed. The solution was then dialyzed for 20 hours against M/2,000 pH 5.0 acetate buffer at 5°C. Appropriate controls

were run at different pH in the absence of iodine to determine the extent of inactivation of pepsin, also in the absence of pepsin to determine the extent of oxidation of iodine by products other than pepsin.

After dialysis the solutions were analyzed for protein nitrogen, activity by the hemoglobin method, and total iodine as described in the section devoted to Experimental methods.

The results recorded in column (i) of Table II and Fig. 2 show that, in general, during iodination of pepsin or of glycyl tyrosine a

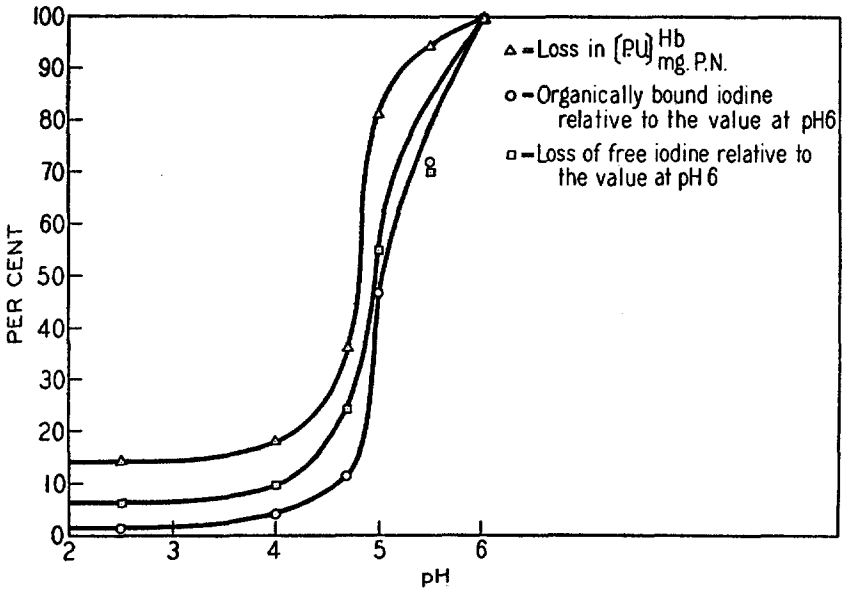

FIG. 2. Effect of pH on the iodination of pepsin.

change in pH of the medium alters the rate of iodination in much the same way as was noted for acetylation of these materials. According to the titration curve of glycyl tyrosine, determined by Greenstein (6) the carboxyl group is in the salt or zwitter ion condition at pH 5.0–7.0 but below pH 5.0 the carboxyl group ionizes as an acid.

The results in Fig. 2 bring out the fact that when free iodine is consumed iodine appears in the protein as bound iodine and the specific activity of the enzyme is depressed proportionally. If the decrease in specific activity were depressed as a result of oxidation by iodine, which cannot be completely excluded from the work of the

previous section of this paper it must then be supposed that the oxidation is affected by pH in an exactly parallel manner as is the introduction of iodine into the protein molecule. It should also be pointed out that since, as will be demonstrated later in this paper, at least 80 per cent of the iodine that is absorbed by the protein is attached to the tyrosine, to account for the depression of the proteolytic activity on the hypothesis of iodination of some component of the protein other than tyrosine, one must again assume that the union of iodine with this hypothetical component is affected by pH in a manner parallel to that of tyrosine in pepsin.

Isolation of Diiodo-Tyrosine from Iodinated Pepsin

For the most part the work carried out in the isolation of diiodotyrosine from iodinated pepsin is described in Table III. However, space would not permit inclusion of all the details and some points need explanation.

Pepsin contains approximately 10 per cent tyrosine (7) which if completely iodinated (2 atoms of iodine per molecule of tyrosine) would result in a protein with 14 per cent iodine. The material used in this isolation experiment had 13.4 per cent iodine.

It was found in preliminary experiments that pancreatic extract would digest the iodinated pepsin. Since this is a much less violent treatment than boiling in alkali it was decided to carry out the enzymatic digestion before treatment with alkali. It might be noted that the Bristol pancreatic extract used carries the digestion of the iodinated pepsin only one-third as far as it does pepsin, as measured by the increase in Van Slyke amino nitrogen.

Refluxing for 30 hours with barium hydroxide was necessary to complete hydrolysis even after the preliminary enzymatic hydrolysis. By cooling to 5°C. a large part of the barium hydroxide crystallized out and was separated by filtration.

The butyl alcohol extraction proved to be an efficient and satisfactory method of purification. The writer is indebted to Dr. G. L. Foster for this suggestion.

In No. 11 of Table III the basic lead acetate precipitate was suspended in about 75–100 ml. of water and glacial acetic acid added. The suspension was warmed to 50°C. to aid in the solution of the

TABLE III

Isolation of Diiodo-Tyrosine from Iodinated Pepsin

Materials and procedures	Solution No.	Volume	Total		Original iodine	Phenol color-iodine ratio*
			Nitrogen	Iodine		
		ml.	*mg.*	*mg.*	*per cent*	
Dialyzed iodinated pepsin No. 13 of 8/2/35 13.4 per cent iodine	1	515	2800	2400	100	0.57
Dialyzed Bristol pancreatic extract	2	5	27			
No. 1 + No. 2 + alkali to pH 9.0–10.0 at 37°C. for 48 hrs. 104 gm. crystalline $Ba(OH)_2$ (making a 20 per cent solution) added and refluxed for 30 hrs.; placed at 5°C. for 24 hrs.; residue filtered and washed with cold water; residue discarded; combined filtrates and washings	3	720	2200	2300	96	0.58
No. 3 + 70 ml. concentrated HCl (pH = approximately 0.7); shaken in separatory funnel seven times with 100 ml. quantities of butyl alcohol (reagent grade)	4					
Acid-water solution of No. 4 after last extraction with butyl alcohol	5	710		113	5	1.3
Butyl alcohol solutions of No. 4 extracted two times with 150 ml. quantities of 0.25 N NaOH and once with 0.1 N NaOH	6					
Butyl alcohol solution of No. 6 after last extraction with alkali	7	380		38	1.6	
Alkali-water solution of No. 6 after separation from the butyl alcohol	8	858	515	2400	100	0.46
No. 8 + 20 ml. of 50 per cent (by weight) $PbAc_2$ + 5 ml. glacial acetic acid; left at 5°C. for 20 hrs.; filtered and residue washed on funnel with 0.1 N acetic acid	9					
Filtrates + washings of No. 9	10	1370		2250	94	0.40
No. 10 + concentrated NH_4OH to pH 8.0–9.0; let settle 24 hrs.; filtered; residue washed by grinding in a mortar with 0.1 per cent NH_4OH and filtered. Residue stirred with H_2O and 12 ml. glacial acetic acid; warmed to 50°C. stirred, filtered, residue washed with 0.1 N acetic acid	11†					
Residue No. 11 (crystals) dissolved in dilute NH_4OH.	12	300		780	33	0.25

* See section on Experimental methods for a discussion of these figures.

† See text for discussion of the procedures from No. 11 on.

TABLE III—*Concluded*

Materials and procedures	Solution No.	Volume	Total		Original iodine	Phenol color-iodine ratio*
			Nitrogen	Iodine		
		ml.	*mg.*	*mg.*	*per cent*	
Solution No. 11 + No. 12 + NH_4OH to pH 8.0–9.0; allowed to stand 24 hrs.; filtered, residue washed with water. Residue suspended in 4–6 liters water at 40°C. H_2S bubbled in till completely saturated. Suspension stirred 6–10 hrs.; filtered; residue resuspended in water; H_2S bubbled in as before, filtered; filtrates combined; evaporated under reduced pressure at 40°C.	13	800		1980	82	0.29
Solution No. 13 evaporated to 50 ml.; very poor crystallization; solution very dark—became black on addition of NH_4OH to pH 8.0.	14					
Precipitated with $PbAc_2$ at pH 8.0 and dissolved by acetic acid or by decomposition by H_2S, repeated several times. Fractional precipitation from dilute NH_4OH solution by addition of dilute acetic acid resulted in a tan colored crystalline product. A yield of 2.1 gm. of dried material was obtained.	15				53	0.25–0.29
No. 15 on recrystallization came down as bundles of needles at room temperature and as whet stones when the mother liquor was placed in the ice box. From 50 per cent acetic acid the crystals were platelets or flat prisms. After drying in vacuo at 100°C. the crystals melted 204°C.	16					

precipitate. The precipitate was almost completely dissolved when a fine white precipitate began to form and became quite heavy. A microscopic examination showed this precipitate to be very small crystals—too small to identify. The crystals were filtered off, washed several times with N/10 acetic acid, and a sample of the residue analyzed. It gave the color with the phenol reagent and iodine analysis expected of diiodo-tyrosine and dissolved instantly in dilute ammonia. The ammoniacal solution was combined with the mother liquor in solution No. 11 in the hope of crystallizing the entire amount of iodine compound in the next step. This, however, was not to be

the case. After the second precipitation with lead acetate at pH 8.0 and decomposition of the precipitate with hydrogen sulfide the solution was evaporated to 50 ml. Crystallization did not take place as had been hoped. The solution was cooled and even seeded with a few crystals of diiodo-tyrosine. A small precipitate appeared in 24 hours but the amount was insignificant. Precipitation as the lead salt and subsequent solution was repeated several times. Crystals or precipitates came out but they were always incomplete and dark brown or black in color. It soon became evident that there was some extraneous black material present in the solution which precipitated under the conditions for crystallization of diiodo-tyrosine and that its removal was difficult. That this black material prevented crystallization was proven to the writer's satisfaction when on adding acetic acid slowly to a dark ammoniacal solution of the unknown there appeared at first a black flocculent precipitate which settled leaving the supernatant liquor water clear. Almost immediately after the black material settled crystals formed in the supernatant liquor. In a few moments the container was nearly solid with a white crystalline product. Separation of the flocculent black material from the crystalline product was not so easily accomplished as might be supposed, for solution and precipitation conditions of both materials were almost identical. The separation was, therefore, for the most part fractional precipitation from dilute ammoniacal solutions by the addition of acetic acid followed by rapid filtration before crystallization occurred. These procedures were very costly to the yield of crystalline product as may be seen from Table III. Although the solution No. 13, just before the final fractionations were performed, contained 82 per cent of the original iodine and gave a color-iodine ratio nearly that of known diiodo-tyrosine, yet only 53 per cent of the original iodine was finally obtained in crystalline form, the difference being lost for reasons already stated. The black flocculent material contained no iodine and gave no color with the phenol reagent. No other analyses were made on it.

The iodine product after recrystallization from dilute ammonia by the addition of a few drops of acetic acid and drying *in vacuo* had a melting point of 203°C. uncorrected. Hoffmann-La Roche diiodotyrosine dried in a similar fashion melted at 204°C. while a mixed

melting point of the two materials was 204°C. The phenol reagent color-iodine ratio of the crystalline product was the same as that for the Hoffmann-La Roche diiodo-tyrosine.

Titration Curve of Iodinated Pepsin

Although a large part of the iodine in iodinated pepsin was identified as diiodo-tyrosine this in itself is not proof that the iodine was attached to the tyrosine in the intact iodinated pepsin. The vigorous treatment to which the protein was subjected in the process of isolation of diiodo-tyrosine could conceivably cause the rearrangement or migration of iodine from some other position to the tyrosine.

From the work of Neuberger (3) and others it is believed that when the tyrosine of proteins is iodinated the titration of the phenolic hydroxyl group of the tyrosine is shifted toward the acid region. If then tyrosine in pepsin is iodinated by the methods used, the titration curve of iodinated pepsin should be quite different from that of pepsin. It is to be expected that the curve of iodinated pepsin will show a rise in the curve between pH 8.0 and 10.0 instead of pH 10.0–12.0 as in pepsin. As may be seen in Fig. 3 this is exactly as expected.

Experimental Procedure

Materials.—Iodinated pepsin: 150 ml. dialyzed 2 × crystallized Parke, Davis pepsin, 14 mg. protein nitrogen per ml. plus 50 ml. 4 M sodium acetate plus 300 ml. water plus 500 ml. N/10 iodine solution. Mixture left 72 hours at 37°C. Protein precipitated completely by acidification to pH 3.0, filtered, and residue dissolved with the aid of sodium hydroxide. This solution was then dialyzed 36 hours against pH 6.0 acetate buffer at 5°C. The solution contained 4.0 mg. protein nitrogen per ml. and the protein was 16 per cent iodine.

Pepsin: Dialyzed 2 × crystallized Parke, Davis pepsin dialyzed 36 hours against pH 6.0 acetate buffer at 5°C. The solution contained 4.3 mg. protein nitrogen per ml.

Procedure.—The hydrogen and calomel electrodes were standardized before and after each experiment with pH 4.0 acetate buffer, prepared as recommended by Clark (8). To 50 ml. of protein solution was added 1 ml. saturated potassium chloride and 1 drop of octyl alcohol and the hydrogen electrode saturated with hydrogen introduced. The solution was stirred throughout. 0.05 ml. quantities of 3.05 N alkali were run in through a capillary tube leading underneath the surface of the solution. A total of approximately 1 ml. of the alkali was added. The E.M.F. was read from the bridge of the Leeds and Northrup potentiometer and the pH calculated. Correction was made for the quantity of alkali required to produce the same pH in a similar volume of distilled water.

In Fig. 3 the curve for iodinated pepsin does not meet the pepsin curve beyond pH 12.0 and there are several rises in the curve beyond pH 9.0. This was very disconcerting until it was remembered that hydrogenation of diiodo-tyrosine to give tyrosine is carried out under conditions (9) somewhat similar to those occurring during measurement of pH by the hydrogen electrode. That there was actual liberation of iodine from iodinated pepsin was shown by the fact that the nitrogen content of the titrated solution remained practically unchanged during dialysis whereas the iodine content was lower after

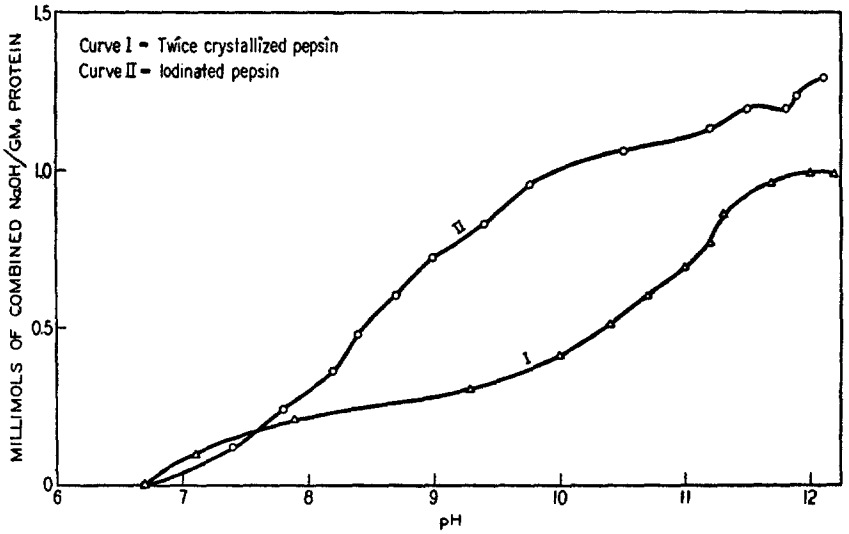

FIG. 3. Titration curves of pepsin and iodinated pepsin.

dialysis. The liberated iodine would be in the form of hydriodic acid and would therefore require additional alkali and make the total alkali per gram of protein appear to be greater in the case of iodinated pepsin than with pepsin.

Neuberger (3) titrating zein and iodinated zein with a hydrogen electrode obtained curves which, although separated between pH 8.0 and 12.0 in the same way as herein demonstrated for pepsin and iodinated pepsin, nevertheless found that the two curves converged at pH 12.0. The time allowed for establishing equilibrium is probably responsible for the difference in the two cases. The writer

without considering the possibility of hydrogenation of the combined iodine consumed at least 2 hours in the measurements of each curve.

It is interesting to note that from Fig. 3 the amount of tyrosine iodinated in pepsin may be estimated. The difference between the pepsin curve I and the iodinated pepsin curve II at pH 10.0 is, roughly, 0.6 millimols of alkali per gram of pepsin or, in terms of tyrosine, is 11 per cent of the protein. The figure is remarkably close to the amount of tyrosine found (7) to exist in pepsin.

DISCUSSION

The work on the acetylation of pepsin showed that the free amino groups of pepsin, which are probably the ϵ amino groups of lysine, could be acetylated without a detectable change in the enzymatic activity. On the other hand, acetylation of two or three tyrosine phenol groups caused a loss of 40–50 per cent of the enzymatic activity. The present work further indicates that alteration of the tyrosine of pepsin results in a change of the proteolytic activity. Whereas the acetyl group was attached to the phenolic hydroxyl group of the tyrosine in pepsin, the iodine substitutes in the benzenoid part of tyrosine, a quite different position, yet the effect of both additions is to reduce the peptic activity of the protein. It appears, therefore, that at least part of the tyrosine of pepsin is so arranged in the molecule as to be essential for normal activity. The writer does not mean to imply that any part or all of the tyrosine is the hypothetical active group of the enzyme.

Other proteins contain tyrosine and the other amino acids which have been found in pepsin and yet no other protein has the properties peculiar to pepsin. Since there is no evidence for the existence of a prosthetic group which, when separated from the rest of the molecule retains the proteolytic activity, the peptic action cannot then be attributed exclusively to any particular part of the protein molecule but must be due to the arrangement of its component parts. The work thus far reported by this writer indicates that in the arrangement of component parts the tyrosine plays an important rôle.

Experimental Methods

Pepsin Activity.—Measurement of the peptic activity was made by the hemoglobin method of Anson and Mirsky (10).

Protein Nitrogen.—This estimation was made as the difference between the total nitrogen and the non-protein nitrogen, the details being described in a previous paper (1).

pH.—pH determinations were for the most part made colorimetrically using the indicators recommended by Clark (8).

Total Iodine.—The total iodine estimations were made by a method which for the most part is the procedure of Kendall (11) with some additions or modifications. In order that the changes may be scrutinized the entire scheme is included here.

An aliquot of material to be analyzed containing 0.1–2.0 mg. of iodine was introduced into a 6 × 6 cm. nickel crucible. 5 ml. of saturated sodium hydroxide and approximately 5 gm. of solid sodium hydroxide and a few alundum chips are added and the mixture heated on an electric hot plate, care being taken in the heating to prevent spattering of the boiling solution. When a crust begins to form on the hot alkali mixture the crucible is placed into a 7 × 7 cm. nickel crucible over a Bunsen burner. A tripod is a convenient support for the larger crucible and the bottoms of the two crucibles should be separated by a few millimeters of fine sand. If the larger or outside crucible is then heated by the Bunsen burner until the lower third of it is red, the inner crucible will be about the right temperature. A gas (probably carbon dioxide) is given off from the alkali solution as small bubbles and upon their ceasing to appear a few small crystals of potassium nitrate are added and usually the evolution of gas commences again. Addition of a few crystals of potassium nitrate is repeated several times or until no gas appears on addition of the potassium nitrate. By means of crucible tongs the crucible is then lifted out and the melt poured onto a crucible cover. The crucible is held over the cover until that melt clinging to the crucible has solidified; this is usually only a moment. The solidified melt on the crucible cover is then put into a 500 ml. wide mouth Erlenmeyer flask and the cover and crucible washed free of the melt, the washings all being introduced into the flask. The volume of the solution in the flask should be between 200–300 ml. When the melt is entirely dissolved 2 drops of 0.1 per cent methyl orange and 1 ml. of 10 per cent sodium bisulfite are added to the flask and the solution titrated to a pink color with 85 per cent reagent grade phosphoric acid. A few drops in excess should be added. With the introduction of a few alundum chips the solution is then boiled on a hot plate for 2–3 minutes. The flask is removed from the hot plate and while still hot but not boiling 3 ml. of saturated bromine water is added. The solution should remain yellow after mixing if enough bromine water has been added. 3 ml. is sufficient for up to 3 mg. of iodine in the original sample. If the solution is not yellow more bromine water should be added after which the solution is boiled until colorless and then about 2 minutes longer. The flask is then set in running cold water and 4 drops of a 10 per cent solution of sodium salicylate are added to the solution. When cooled to room temperature a drop or two of 85 per cent phosphoric acid is added followed by 5 ml. of a 10 per cent potassium iodide solution. It was found desirable to then add another drop or two of 85 per cent phosphoric acid to bring the full and rapid liberation of iodine. This solution was then immediately titrated with standard

0.01 N thiosulfate, each ml. of which is equivalent to 0.21 mg. of iodine in the original sample due to the nature of the reaction. 3 ml. of 0.5 per cent starch solution aids in determining the end point. The thiosulfate was prepared as recommended by Leland and Foster (12) in 0.005 N alkali. This solution was found to be perfectly stable for at least a month.

Phenol Color Value.—The phenol color value of solutions was obtained by use of the following procedure: an amount of material which gave approximately the color produced by 0.15 mg. of tyrosine was diluted with water to 19 ml. 3 ml. of 1.28 N sodium hydroxide was added followed by 3 ml. of a 1:3 dilution of Folin's phenol reagent. The solution was allowed to stand 5 minutes for the color to develop after which it was compared in a colorimeter against 0.15 mg. of tyrosine treated in a like manner.

Since the reference or standard was tyrosine the values of all solutions analyzed were calculated in terms of tyrosine.

The ratio found in Table III was obtained by dividing the color value of a solution, in terms of milligrams of tyrosine, by the milligrams of iodine of the same solution.

SUMMARY

In the presence of iodine at pH 5.0–6.0 a solution of pepsin absorbs iodine and the specific proteolytic activity of the solution decreases. The activity is less than 1 per cent of the original activity when the number of iodine atoms per mol of pepsin is 35–40.

If the pH is 4.5 or less, iodine reacts very slowly and there is a correspondingly slower loss in activity. Glycyl tyrosine reacts with iodine in a manner similar to pepsin.

Experiments were performed to determine the extent to which oxidation of pepsin by iodine occurs during iodination, and if such oxidation were responsible for the loss in enzymatic activity. Although the results were not absolutely decisive, there seems to be no appreciable oxidation taking place during iodination and no relationship between the slight oxidation and loss in peptic activity.

From a dialyzed preparation of completely iodinated pepsin which was inactive and contained 13.4 per cent bound iodine, 82 per cent of the iodine was obtained in a solution which analyzed as a solution of diiodo-tyrosine. Because of the presence of a material which contained no iodine and prevented quantitative crystallization, only 53 per cent of the iodine containing substance could be crystallized. This 53 per cent was, however, identified as diiodo-tyrosine.

The part of the titration curve which in pepsin and most proteins

represents the phenolic group of tyrosine was, in the curve for iodinated pepsin, shifted toward the acid region as expected.

From these results, it appears that the loss in proteolytic activity of pepsin, when treated with iodine under the specified conditions, is due to the reaction of the iodine with the tyrosine in pepsin.

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