ACETYLATION OF TYROSINE IN PEPSIN

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In an earlier paper (1) the writer described the preparation and isolation of three crystalline acetyl derivatives of pepsin. They were, "100 per cent active" acetyl pepsin in which the 3 or 4 primary amino groups of pepsin had been acetylated with less than 15 per cent change in the specific activity; "60 per cent active" acetyl pepsin which contained 6–11 acetyl groups per molecule; and "10 per cent active" acetyl pepsin which had 20–30 acetyl groups per molecule of protein. Reversion of the 60 per cent active enzyme into the 100 per cent active was effected by treatment with normal sulfuric acid at 5°C.1 It was pointed out in this earlier work that in all probability the primary amino groups belong to the lysine part of the protein molecule and that since acetylation of these groups failed to produce any appreciable effect on the activity of pepsin that the rôle played by the lysine in the enzymatic activity of the molecule is probably relatively small.

Since the introduction of a few acetyl radicals into other groups of the protein molecule definitely diminished the specific activity, it was reasoned that these groups of the protein must be more closely related to the seat of the enzymatic activity.

The present work was undertaken to determine the structural position of these few acetyl groups which have such a pronounced effect on the activity of pepsin. Though the evidence is somewhat indirect it nevertheless seems probable that those acetyl groups in the 60 per cent active acetyl pepsin which are responsible for the decrease in specific enzymatic activity are attached to the phenolic hydroxyl groups of some of the tyrosine components of the protein.

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1 In the previous paper, J. Gen. Physiol., 1934, 18, 53, 54, the temperature is given as 10°C. This should read 5°C.
In the 60 per cent active preparation there are three acetyl groups which are not in the 100 per cent active acetyl pepsin. These three additional acetyl groups are rapidly hydrolyzed by molar acid or by alkali at pH 10.0, whereas the acetyl groups on the amino groups are not hydrolyzed under the same conditions. With this property in mind these easily hydrolyzed acetyl groups have been designated as “pH 10.0 labile” acetyl groups. There are three less tyrosine phenol groups in the 60 per cent active acetyl pepsin as measured colorimetrically with the Folin phenol reagent under conditions which will not hydrolyze an acetylated phenol.

When the 60 per cent active material is changed back into 100 per cent active acetyl pepsin by the above mentioned acid treatment there is an accompanying loss of the pH 10.0 labile acetyl groups and the number of tyrosine phenol groups returns to that of the original pepsin.

EXPERIMENTAL RESULTS

Preparation.—A slight modification in the method of preparation of the 60 per cent active acetyl pepsin has led to more uniform and reproducible results. Table I contains the analyses of several different acetyl derivatives and of pepsin. In this table the 100 per cent active acetyl pepsin was prepared by acid hydrolysis of the 60 per cent active materials. A discussion of the procedures and the interpretation of the analyses will be found later in this paper but it might be noted at this time that the acetyl group figures are more significant than the tyrosine phenol group figures for the determination of the former is subject to less error and the interpretation of the figures is on a more sound chemical basis.

From Table I it appears that with the change from pepsin to 60 per cent active acetyl pepsin there is an increase of three pH 10.0 labile acetyl groups (besides the increase of 3 or 4 acetyl groups on the primary amino groups). There is also a decrease in the tyrosine-tryptophane value of the protein as measured by the colorimetric “pH 8.0 method,” equivalent to 3 tyrosine phenol groups.

Experimental Procedure

In general the materials, the analyses of which appear in Table I, were prepared by the methods described previously (1). In the present instance, however, the
60 per cent active acetyl pepsin was prepared in the same way that was previously described for the preparation of 10 per cent active acetyl pepsin except that the acetylation by ketene was stopped when the specific activity was approximately 60 per cent of the original pepsin. This point was determined by analyzing samples taken from time to time. The material was then precipitated from solution by acidification and half saturation with magnesium sulfate, fractionated, crystallized, and finally dialyzed in collodion bags for 24 hours on a dialyzer (2).

**TABLE I**

*Acetyl and Tyrosine Analyses of Pepsin and Its Acetyl Derivatives*

<table>
<thead>
<tr>
<th>Name</th>
<th>No.</th>
<th>pH 7.0 P. N.</th>
<th>pH 8.0 pH 9.0 P. N.</th>
<th>pH 11.0 pH 12.0 P. N.</th>
<th>Tyrosine-tryptophane content by colorimetric method*</th>
<th>No. of acetyl groups per mol pepsin</th>
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</thead>
<tbody>
<tr>
<td>3 x cryst. P. D. pepsin</td>
<td>1</td>
<td>0.21</td>
<td>11.8</td>
<td>11.8</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>5 x</td>
<td></td>
<td>0.17</td>
<td>11.8</td>
<td>11.8</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>100 per cent active acetyl pepsin†</td>
<td>20</td>
<td>0.17</td>
<td>11.0</td>
<td>11.5</td>
<td>24</td>
<td>23</td>
</tr>
</tbody>
</table>
| " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " " 

* See discussion under Experimental methods for the determination and calculation of these figures.
† Crystallized and fractionated.
‡ Reaction mixture; i.e., not fractionated.
§ Fractionated but not crystallized.

at 5°C against m/2000 pH 4.65 acetate buffer. All preparations and samples were dialyzed as just described before final analysis. The 100 per cent active acetyl pepsin preparations were made by subjecting the 60 per cent active preparations to 1.25 m sulfuric acid at 5°C. for 75-100 hours followed by concentration, fractionation, crystallization, and dialysis. The activity estimation was made by the hemoglobin method of Anson and Mirsky (3). The other analyses were carried out as described under Experimental methods.

On treatment of the 60 per cent active acetyl pepsin with normal sulfuric acid at 5°C. the specific enzymatic activity of the protein
ACETYLATION OF TYROSINE IN PEPsin

rises to that of pepsin. The pH 10.0 labile acetyl groups are no longer detectable and the tyrosine-tryptophane value has increased to that of pepsin. It will be shown later that it is highly probable that these changes in tyrosine-tryptophane value are due to the coupling or hydrolysis of acetyl groups on tyrosine phenol groups of the protein. It is concluded, therefore, that the change in specific activity from 100 per cent active acetyl pepsin to 60 per cent active acetyl pepsin can be attributed to the acetylation of 3 tyrosine phenol groups in the protein. It is possible that not all 3 of these tyrosine phenol groups are involved in the effect produced on the activity but the writer has endeavored without success to obtain decisive evidence on this point.

In the change from 60 per cent active acetyl pepsin to the 10 per cent active acetyl pepsin there is a further increase in pH 10.0 labile acetyl groups and a decrease in tyrosine-tryptophane value of the protein. The change in the number of acetyl groups, however, is not equivalent to the decrease in number of tyrosine phenol groups calculated from the chromogenic value so that some of these acetyl groups may be attached to other than tyrosine phenol groups.

Tyrosine Content of Pepsin

It was pointed out by Wu (4) that the color produced by the phenol reagent in the presence of proteins is largely due to the tyrosine in the protein. Since two other amino acids, tryptophane (5) and cysteine (6) produce the characteristic blue color with the phenol reagent they must be considered as possible sources of color when a protein is treated with alkali and the phenol reagent. There is also the possibility that there exists in some proteins a component other than the amino acids and that this component will reduce the phenol reagent. Heme of hemoglobin is such a component and is known to reduce the reagent (6). Amino acids which in the pure state do not produce the color with the reagents may, when in combination with

The figures given for the specific activity of the 100 per cent active acetyl pepsin are some 10–20 per cent below that of pepsin. Preparations have been obtained with a specific activity more nearly that of pepsin ([P.U.]Hb mg. P.N. = 0.20–0.22) but a small fraction of these preparations seems to be unstable with respect to activity and is lost on standing; the result being a lowering in the specific activity of the total material.
other amino acids in the protein, then have the property of reducing the color reagents. There is no evidence for these possibilities in the case of pepsin except for one observation, and there seems to be no correlation of this with the present work. Pepsin gives a negative nitro-prusside test for free S H groups. Cysteine is, therefore, probably not present and the color giving property of pepsin is due to the tyrosine and tryptophane.

The estimation of the tyrosine-tryptophane content of pepsin was carried out in the present instance by two related colorimetric methods in which Folin's phenol reagent (7) was used. The conditions of one method, designated as the pH 8.0 method, are so arranged that free tyrosine phenol groups may be determined in the presence of, but without the hydrolysis of, acetylated phenol groups. The conditions of the other method, designated as the "pH 11.0 method," are such as to hydrolyze the acetylated phenols and then measure the total number of phenol groups with the same reagents and under the same conditions as the pH 8.0 method employs. From these two methods the total (free plus acetylated) phenol groups and the free phenol groups are determined. The difference between the two designates the number of acetylated groups. A discussion and an outline of the procedures are to be found in the section devoted to Experimental methods.

Rate of Hydrolysis of pH 10.0 Labile Acetyl Groups and Diacetyl Tyrosine in Acid

It was brought out in our earlier work (1) that there is a difference in some of the acetyl groups of 60 per cent active acetylated pepsin with respect to acid hydrolysis. Those which are attached to the primary amino groups are hydrolyzed by acid with comparative difficu-

* When to solutions of pepsin or other proteins of such a concentration as is used in measuring the chromogenic value by phenol reagents, is added 1 ml. of 0.002-0.0005 \( \mu \) CuSO\(_4\) the chromogenic value is increased from 1-3 times. This increase in color value is not demonstrable with acid hydrolysates of pepsin though it is with enzymatic hydrolysates. The increase is very noticeable on purified gelatin which, according to the accepted analyses, contains little or no tyrosine and tryptophane. Proline or a pyrrole type component is suspected though in the pure state and in the presence of CuSO\(_4\) proline and hydroxy proline show no action toward the phenol reagents.
Fig. 1 shows graphically the results of an experiment in which a solution of diacetyl tyrosine, prepared as directed by Bergmann and Stern (8), and a solution of 60 per cent active acetyl pepsin were hydrolyzed by 1.25 N sulfuric acid at 5°C. Experimental difficulties prevented measurement of the tyrosine-tryptophane color value and the acetyl estimation during the hydrolysis of the enzyme solution. The pH 10.0 labile acetyl analysis was, however, performed on the initial and final products and is included in Fig. 1. The experiment confirms the experiments of our previous paper in which it was demonstrated that in strong acid the specific activity of the enzyme returns to approximately that of pepsin and the acetylated protein loses some of its acetyl groups. In addition it shows that the rate at which this reactivation takes place is very close to the rate at which the acetyl group on the phenol group of diacetyl tyrosine is hydrolyzed under identical conditions.
Experimental Procedure

**Enzyme.**—250 ml. of a dialyzed preparation of 60 per cent active acetyl pepsin containing 3.0 mg. P.N./ml. was cooled to 5°C., added to 250 ml. of cooled 2.5 N sulfuric acid, and the suspension stirred continuously. Under these conditions a large part of the protein is insoluble. Samples were taken from time to time, the precipitate filtered off, and the acid filtrate neutralized with an equal volume of 1.5 N sodium acetate. Analyses for protein nitrogen and enzymatic activity were made on this neutralized filtrate. When the specific activity had reached that of pepsin the total protein was precipitated, fractionated, crystallized, and analyzed for specific activity, pH 10.0 labile, and total acetyl groups. A sample of the original 60 per cent active pepsin was analyzed at the same time.

**Diacetyl Tyrosine.**—0.5 gm. of crystalline diacetyl tyrosine was dissolved in 79 ml. water with the aid of 1 ml. of M/1 pH 5.0 citrate buffer. 70 ml. of this solution was cooled to 5°C. and added with stirring to 70 ml. of 2.5 N sulfuric acid at 5°C. 20 ml. samples were taken from time to time and analyzed for free acetic acid by distillation from a 3 molar citrate buffer pH 4.0 and subsequent titration of the distillate. Samples were also analyzed for free phenol groups by the pH 8.0 method. In the early part of the hydrolysis the measurement of free phenol groups was made possible by adding known quantities of tyrosine to the aliquot of reaction mixture being analyzed by the pH 8.0 method and then corrected for in the calculation. This reduced the error due to comparison of widely different colorimeter readings.

**Rate of Hydrolysis of pH 10.0 Labile Acetyl Groups and of Diacetyl Tyrosine in Alkali**

Acetyl groups may be hydrolyzed by alkali as well as by acid and so an experiment was performed at pH 9.0-10.0 to see if the acetyl group on the phenol group of tyrosine is hydrolyzed under the conditions which hydrolyze those on 60 per cent active acetyl pepsin. The results are shown in Fig. 2. Unfortunately the pH of the medium was not exactly the same for the two materials, that of the protein solution being pH 9.8, compared to pH 9.0 of the diacetyl tyrosine solution (pH measurements by hydrogen electrode). Since the enzyme is immediately inactivated under these conditions the activity

4 In the medium for hydrolysis, pH 9.0-11.0, of the labile acetyl groups a glycine buffer and probably any material containing free amino groups should be avoided in high concentrations (0.2 molar or greater) for it was found with glycine that the amino group acts as an acceptor of the liberated acetyl group after hydrolysis. It is, therefore, not free to be estimated as acetic acid and is hydrolyzed from glycine only after relatively vigorous hydrolytic treatment.
could not be followed. The change in the tyrosine-tryptophane value of the protein by the pH 8.0 method was followed along with the change in acetyl groups. It seems perfectly clear from Fig. 2 that at pH 9.0–10.0 the hydrolysis of acetyl groups from 60 per cent active acetyl pepsin is accompanied by a corresponding increase in the pH 8.0 chromogenic value of the protein. Also that the hydrolysis of the oxygen acetyl linkage of diacetyl tyrosine takes place at approximately

![Graph](image)

**Fig. 2.** Rate of hydrolysis of 60 per cent active acetyl pepsin and diacetyl tyrosine at pH 9.0–10.0 and 35°C.

the same rate as does the hydrolysis of the pH 10.0 labile acetyl groups of the above mentioned 60 per cent active acetyl pepsin.

**Experimental Procedure**

*Enzyme.*—To 41 ml. of 60 per cent active acetyl pepsin containing 13 mg. P.N./ml. was added with stirring a solution of 5.2 ml. 1 N sodium hydroxide and 23.8 ml. m/10 borate buffer pH 10.5, final pH 9.8 determined by the hydrogen electrode. The solution was kept at 35.5°C. throughout the experiment. Samples were taken at varying intervals of time and analyzed for free acetic acid by distillation from 3 M citric acid with subsequent titration of the distillate. Analyses were also made for free phenol groups by the pH 8.0 method.

*Diacetyl Tyrosine.*—To 0.4 gm. of crystalline diacetyl tyrosine was added 35 ml.
of water containing 0.5 ml m/1 pH 5.0 citrate buffer. To this solution was added with stirring 34 ml m/10 pH 10.5 borate buffer and 1 ml of 1 N sodium hydroxide, the resulting mixture being pH 9.0 by the hydrogen electrode. Aliquots were analyzed from time to time for free acetic acid and free phenol groups by the above mentioned procedures. The reaction mixture was also kept at 35.5°C.

Rate of Acetylation of Tyrosine and Tryptophane

It seems highly probable from the foregoing experiments that the groups in the protein pepsin which, when acetylated, are responsible for the changes in activity and pH 8.0 chromogenic value, are tyrosine phenol groups. There exists, however, the possibility that the color producing group or structure of tryptophane in the protein pepsin may be acetylated and, when acetylated, causes the change in specific enzymatic activity. It was thought that a comparison of the rate of acetylation of tyrosine and of tryptophane would furnish evidence in this connection. It was decided to attempt acetylation under the conditions used for the acetylation of pepsin; i.e., in strong acetate buffer (pH 5.0–6.0). Since tyrosine is only slightly soluble under these conditions the glycyl derivatives of the two amino acids were used instead. Under the conditions of acetylation (in strong acetate buffer) acetyl figures would have been very difficult. Following the change in chromogenic value of the solution by both the pH 11.0 and pH 8.0 methods serves much the same purpose. By comparing the color values obtained by these two methods one may determine quantitatively the alkali reversible change in color properties of the molecule due to acetylation.

Experimental Procedure

0.5–1.0 gm. of Hoffman-La Roche preparations were dissolved or suspended in approximately 40 ml of 3 m acetate buffer at the pH indicated. Ketene from the generator previously described (9) and which was used in the preparation of the acetyl pepsin derivatives was passed in slowly with stirring. The materials, if only slightly soluble in the initial stage, were quickly converted (probably by acetylation of the amino groups) into a soluble form. Aliquots were removed at varying intervals of time, diluted, and chromogenic values determined by the pH 8.0 and pH 11.0 methods.

From Table II it is readily seen that there is a gradual decrease in pH 8.0 color value of glycyl tyrosine solution during acetylation, whereas the pH 11.0 color value remains constant. This is inter-
preted as acetylation of the phenol group of glycyl tyrosine. The acetyl group is hydrolyzed yielding the full value in the pH 11.0 method, whereas the pH 8.0 method measures only the free or unacetylated phenol groups. In the case of tryptophane and glycyl tryptophane there is a decrease in color values as measured by both

<table>
<thead>
<tr>
<th>Material</th>
<th>pH</th>
<th>Time</th>
<th>Calculated from color value by the pH 11.0 method</th>
<th>Ratio pH 8.0 value to pH 11.0 value</th>
<th>Acetylated (calculated) per cent</th>
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<tr>
<td>Glycyl tyrosine</td>
<td>5-6</td>
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<td>1.6 1.5 0.94 0</td>
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<td></td>
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<td>0.5</td>
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<td></td>
<td></td>
<td>1.0</td>
<td>1.6 0.92 0.57 39</td>
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<tr>
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<td></td>
<td>1.5</td>
<td>1.5 0.57 0.38 60</td>
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<td></td>
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<td>1.4 0.28 0.20 79</td>
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<tr>
<td>Glycyl tryptophane</td>
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<td>2.1 2.0 1.0 0</td>
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<td>Glycyl tyrosine</td>
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<td>1.7 1.6 0.94 0</td>
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<td>4.0</td>
<td>1.6 1.0 0.65 33</td>
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</table>

methods. The ratio of pH 8.0 color to pH 11.0 color is practically constant throughout although there is a considerable loss in total color value. The tryptophane solution after 4 hours of acetylation was heated with alkali and yet the change in color value occurring during acetylation did not revert to its original value. The change in color of tryptophane and glycyl tryptophane, whether caused by
acetylation or some other factor, is therefore entirely different from the change which takes place on acetylation of glycyl tyrosine or of pepsin.

In the previous paper (1) it was pointed out that the specific activity of pepsin drops much more slowly when acetylation is carried out at pH 4.0–4.5 than when carried out at pH 5.0–6.0. If, then, the change in specific activity during acetylation of pepsin is due to acetylation of the tyrosine phenol group, it might be expected that the phenol group of pure tyrosine or glycyl tyrosine would acetylate more slowly at pH 4.0–4.5 than at pH 5.0–6.0. This was found to be the case as may be seen in Table II. The rate at pH 4.3 is less than one-half that at pH 5.6. The results of these two experiments tend to eliminate the possibility of tryptophane and point definitely to tyrosine as being the component of pepsin which, when acetylated, results in a marked decrease in specific activity.

Action of Hydrolytic Enzymes on Acetyl Derivatives of Pepsin

All of the foregoing proofs for the existence of acetylated phenol groups in acetylated derivatives of pepsin have been indirect or by analogy. In hope for a more direct proof an attempt was made to isolate the acetylated tyrosine from the acetyl pepsin derivatives. Since acid and alkali will hydrolyze acetylated phenols it was decided to use enzyme solutions as the hydrolytic agents. Solutions of 10 per cent active, 60 per cent active acetyl pepsins, and pepsin (control) as substrates were hydrolyzed at pH 7.0–7.5 with Fairchild’s crude trypsin, Wilson’s commercial steapsin, crystalline trypsin and chymotrypsin; with the same enzyme solutions at pH 6.0 after heat denaturation of the pepsins; and with Parke Davis 1:10,000 pepsin and crystalline pepsin at pH 2.0 after denaturation; and with the preceding enzymes at pH 6.0 in the native state. During the experiments the chromogenic values of the solutions were followed by the pH 11.0 and pH 8.0 methods and in the last instance (“native” pepsin and acetyl derivatives at pH 6.0) the specific enzymatic activity was followed. Hydrolysis by Fairchild’s trypsin of the three different enzyme preparations was attempted at 5°C., 20–25°C. (room temperature), and 35°C.

The results were completely negative with regard to isolating any
acetyl tyrosine from the acetylated pepsins. Fairchild's trypsin carries the digestion far enough to release the tyrosine molecule but it also contains some material which brings about the hydrolysis of the acetyl group of the oxy-acetyl tyrosine. The action of the tryptic enzymes on native acetyl pepsins in no case produced an increase in specific activity of the enzyme and no increase in pH 8.0 chromogenic value of the proteins although there was destruction of a large part of the total protein. It seems probable, therefore, that the agent which brings about hydrolysis of oxy-acetyl tyrosine in the decomposition products of the acetyl derivatives of pepsin will not act similarly on the native active protein.

EXPERIMENTAL METHODS

In general the technique in handling the proteins, measuring enzymatic activity, nitrogen, pH, etc. was the same as that used and described in the previous work (1). Any deviations or new procedures are described below or in the experimental procedures found in the main body of this paper.

Acetyl Estimation

_Total Acetyle._—The procedure for estimating acetyl groups has been improved over that described in the previous paper so that a detailed account of the procedure will be given. All protein solutions to be analyzed for acetyl were dialyzed in a Kunitz and Simms (2) dialyzer for at least 20 hours at 5°C. against \( \frac{1}{2000} \) pH 4.65 acetate buffer. This reduces the acetate ion of a protein solution to approximately \( \frac{1}{2000} \) which is negligibly small. Dialysis also removes any buffers, salts, and most of the non-protein nitrogen. From a nitrogen analysis of this dialyzed solution the protein content is calculated, assuming the pepsin to contain 15.4 per cent nitrogen. To a volume containing approximately 0.5 gm. of protein is then added 1 ml. of 4.2 N sodium hydroxide and the volume, if less than 12 ml., is diluted with water to 12 ml. and placed at 35.5°C. for 5 days. After this time the entire volume is mixed with 5 ml. of 3.5 M citric acid and 0.1-0.2 ml. octyl alcohol in a 150 ml. modified Claissen distilling flask and distilled for approximately 15 minutes at 50°C. ± 5°C. and 20 mm. pressure. After the distillation a drop of 0.5 per cent phenolphthalein is added to the receiving flask and the distillate titrated with N/50 sodium hydroxide and the titration checked by adding a drop of 0.1 per cent brom cresol green and back titrating to pH 4.7 with N/50 hydrochloric acid. The latter or back, titration will be half the first titration if the acid in the distillate is acetic acid. After this titration the receiving flask is rinsed out with distilled water, 10-15 ml. of distilled water and 0.1-0.2 ml. octyl alcohol added to the distilling flask, and the distillation and titration repeated. This procedure is repeated three or four times or until the titration of the distillate is
less than 0.25 ml. of N/50 sodium hydroxide. A blank titration of 0.10 ml. N/50 sodium hydroxide is subtracted from each titration and the acetyl's calculated from the sum of the titrations.

**pH 10.0 Labile Acetyl's.**—The protein solutions were first freed of soluble non-protein nitrogen, acetate ion, salts, etc. by dialysis just as in the above procedure. From a nitrogen analysis the protein/ml. is calculated and a volume is then taken which contains approximately 0.5 gm. of protein. To this is added with stirring a mixture of 5 ml. of saturated borax and 1 ml. of N/1 sodium hydroxide. If 0.5 gm. of pepsin protein is taken and if the non-protein nitrogen content is not above 10 per cent of the total nitrogen the pH of the solution resulting from the above mixture will be pH 9.8 ± 0.2. This solution is allowed to stand at 35.5°C. for approximately 20 hours after which time the acetic acid is distilled and estimated just as described in the estimation of total acetyl.

**Determination of Tyrosine Phenol Groups**

The tyrosine phenol groups of pepsin and of its acetyl derivatives were estimated colorimetrically by means of Folin's phenol reagent (7) under particular conditions with reference to pH. The alkali reagent is made up so that the final solution in which the color develops is pH 8.0. At this pH tyrosine phenol groups give rise to color with the phenol reagent but acetylated tyrosine phenol groups do not. This procedure has been called the pH 8.0 method and the details of it are to be found later in this section.

The procedure designated as the pH 11.0 method differs from the pH 8.0 method only in that the sample to be analyzed is made alkaline to pH 11.0-12.0 then adjusted to pH 8.0 for estimation of the color value. This treatment hydrolyzes all acetylated tyrosine phenol groups and gives, therefore, the total tyrosine color value of the sample.

The pH 8.0 method was standardized by determining the tyrosine and tryptophane content of several different proteins by the colorimetric method at pH 8.0 and comparing these values with tyrosine and tryptophane analyses obtained by other methods. These determinations are to be found in Table III. Unfortunately many proteins are insoluble under the conditions necessary for this measurement but Table III includes analyses of four proteins with different tyrosine-tryptophane values which are soluble at pH 8.0 in the presence of the phenol reagents. On the average 59 per cent of the color expected from the tyrosine and tryptophane content of these four proteins was found by the pH 8.0 method. From this fact it appears that the tyrosine and tryptophane groups of proteins do not give their full color value by the pH 8.0 method. It is possible, of course, that

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5 This property is not a peculiarity of the tyrosine phenol group for almost any phenol group gives rise to the characteristic blue color with the phenol reagent (10). The writer has found that no color was obtained by the pH 8.0 method when phenyl acetate or acetyl salicylic acid were used whereas they give the expected quantity of color by the pH 11.0 method.
only 59 per cent of the total number of these groups in protein react at pH 8.0
but with several proteins containing different amounts of tyrosine and tryptophane
this interpretation seems unlikely. As yet the writer has no decisive experimental
evidence on the question. The interpretation, however, affects only the exact
value and does not change the order of magnitude of the final figure.

Amino acid analyses of crystalline pepsin show about 10.3 per cent tyrosine and
2.2 per cent tryptophane. In order to have a common basis for color giving
groups the tryptophane value may be expressed in terms of tyrosine. Thus, 2.2
per cent tryptophane is equivalent to about 2.0 per cent tyrosine. The tyrosine-
tryptophane content of pepsin, expressed in terms of tyrosine is, therefore, 12.3

<table>
<thead>
<tr>
<th>Protein</th>
<th>Analysis after acid hydrolysis</th>
<th>Ratio: Tyrosine + tryptophane content by pH 8.0 method</th>
<th>Tyrosine - tryptophane expressed as tyrosine</th>
<th>Tyrosine + tryptophane content after hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 8.0 method</td>
<td>Tyrosine</td>
<td>Tryptophane</td>
<td>Tyrosine + tryptophane expressed as tyrosine</td>
</tr>
</tbody>
</table>
| Dialyzed 5 x cryst. P.D. pepsin | 7.9 | 10.3* | 2.2* | 12.3 | 0.64
| Dialyzed 3 x cryst. egg albumin | 2.9 | 4.0† | 1.2† | 5.1 | 0.57
| Kahlbaum-Hammarsten casein | 4.1 | 6.4† | 1.4† | 7.6 | 0.54
| Horse serum albumin | 3.0 | 4.7† | 0.5† | 5.1 | 0.59
|                          | Average | 5.9 ± 0.03 |

* Personal communication of Dr. H. O. Calvery.
† Analyses by Polin and associates.

per cent or 25 tyrosine groups per mole of pepsin. The tyrosine-tryptophane
content of pepsin, as determined by the pH 8.0 method is about 7.7 per cent.
Assuming that all the groups react under these conditions, one mol of tyrosine or
tryptophane in pepsin gives the color equivalent of \( \frac{7.7}{12.3} = 0.64 \) mols of tyrosine.
In determining the tyrosine and tryptophane content of pepsin at pH 8.0, therefore,
the total tyrosine-tryptophane is calculated as \( \frac{1.0}{0.64} = 1.6 \) times the quantity

6 A personal communication from Dr. H. O. Calvery of the Department of
Physiological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan.
of tyrosine which develops the same color as the pepsin. The results are shown in Table I.

It may be seen in Table I that the 10 per cent active and 60 per cent active acetyl pepsins have tyrosine-tryptophane values by the pH 8.0 method less than that of pepsin. If these materials are all titrated to pH 11.0-12.0, left for a moment, and then acidified to pH 8.0 followed by an estimation of the color by the pH 8.0 method they will all show the same value as pepsin. This scheme has been used in this work and has been designated as the pH 11.0 method. The alkali

![Graph of color development](image_url)

**Fig. 3.** Rate of color development of pepsin and pure tyrosine by pH 8.0 method.

at pH 11.0-12.0 quickly hydrolyzes the acetylated tyrosine phenol groups thus returning the number of pH 8.0 color giving groups to that of pepsin.

In both the pH 8.0 and pH 11.0 methods the phenol reagent is added to the protein solution before the alkaline buffer is added to prevent the solution ever getting above pH 8.0. The color develops slowly at pH 8.0 and reaches a maximum in 10-24 hours at room temperature. As may be seen from Fig. 3 the rate of color development is the same in the protein pepsin as in pure tyrosine. One is justified, therefore, in comparing the colors produced by the protein and by tyrosine at any arbitrary time interval as long as it is the same for both materials. A time interval of 15 minutes has been used during which time the flasks containing the colored solutions were kept at 35.5°C.
Experimental Procedure

The procedure used in the experiment, shown graphically in Fig. 3 was as follows: 1.86 mg. of purified pepsin and 0.15 mg. of tyrosine respectively were added to two 50 ml. Erlenmeyer flasks and diluted with water to a volume of 17 ml., followed by 3 ml. of 1/3 diluted Folin’s phenol reagent and 5 ml. of the alkaline phosphate solution (60 ml. 0.5 M K2HPO4 + 34 ml. N/1 NaOH + 6 ml. H2O). The alkaline phosphate was added to the flask with stirring by whirling the flask. The solutions were allowed to remain at room temperature and from time to time were read in the colorimeter against a standard blue glass.

pH 8.0 Method.—An amount of material yielding a colorimeter reading approximately equal to that produced by 0.30 mg. of tyrosine under similar conditions is diluted to 11 ml. with water. To this solution is added 6 ml. of N/10 sodium chloride solution and 3 ml. of a 1:3 dilution of Folin’s phenol reagent followed by 5 ml. of an alkali phosphate. (The alkali phosphate solution is made up of 60 ml. of 0.5 M K2HPO4 + 34 ml. N/1 NaOH + 6 ml. of H2O.) This solution is placed at 35.5°C. for 15 minutes and compared to a solution of 0.30 mg. of tyrosine under similar conditions. If the amount of salts, buffers, and non-protein nitrogen content of the original material to be tested is very small the pH of the final colored solution will be pH 7.8 ± 0.2. If any buffer or alkali neutralizing material is present to any appreciable extent (which should be determined before the estimation by simply titrating an aliquot) a determined amount of alkali should be added to the 5 ml. of alkali phosphate to bring the solution to the same pH in all measurements. Approximately 3.0 mg. of pepsin protein is used in the pH 8.0 method of estimating tyrosine-tryptophane values. Aliquots of a standard solution of tyrosine were run parallel to the protein solutions using the same technique and reagents.

pH 11.0 Method.—An amount of material yielding a colorimeter reading approximately equal to that produced by 0.30 mg. of tyrosine under similar conditions is diluted to 11 ml. with water. To this solution is added 3 ml. of N/10 sodium hydroxide and the solution allowed to stand about 5 minutes and then the alkali is neutralized by 3 ml. of N/10 hydrochloric acid. 3 ml. of a 1:3 dilution of Folin’s phenol reagent is added followed by the introduction of 5 ml. of the alkali phosphate solution described in the pH 8.0 method. The 3 ml. of N/10 sodium hydroxide is sufficiently strong (unless buffers are present) to carry the pH of the solution to or beyond pH 11.0 where the acetyl groups come off of the phenol groups almost instantaneously.7

7 In some of the experiments reported in this paper the procedure of the pH 11.0 method was not identical with that described above. The color was allowed to develop at pH 11.0-12.0 rather than at pH 8.0. The results obtained in this way, although different from the results by the above described pH 11.0 method, were proportionately different for all the chromogenic materials used and so the end result was not affected. The pH 11.0 method, as above described, was later developed and is to be preferred because of its general convenience.
SUMMARY

Crystalline 60 per cent active acetyl pepsin has 7 acetyl groups per mol of pepsin, 3 of which are readily hydrolyzed in acid at pH 0.0 or in weak alkali at pH 10.0.

The tyrosine-tryptophane content of this acetylated pepsin, measured colorimetrically, is less than pepsin by three tyrosine equivalents.

Hydrolysis at pH 0.0 or pH 10.0 of the 3 acetyl groups results in a concomitant increase in the number of tyrosine equivalents. In the pH 0.0 hydrolysis experiment there is also a simultaneous increase in specific activity.

The phenol group of glycyl tyrosine is acetylated by ketene under the conditions used in the acetylation of pepsin and the effect of pH on the rate of acetylation is similar in the two cases.

It is concluded that the acetyl groups in the 60 per cent active acetyl pepsin, which are responsible for the decrease in specific enzymatic activity, are 3 in number and are attached to 3 tyrosine phenol groups of the pepsin molecule.

REFERENCES

10. Folin, O., and Denis, W., J. Biol. Chem., 1912, 12, 239.