IDENTIFICATION OF MONO-IODOTYROSINE FROM IODINATED PEPsin

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In an earlier paper (1) the writer reported the isolation of l-mono-iodotyrosine from an alkaline hydrolysate of very slightly iodinated pepsin. Since then Harington and Pitt Rivers (2) have synthesized l-mono-iodotyrosine and dl-mono-iodotyrosine and a comparison of the properties of their compounds with those reported by us led them to conclude that for the compound from pepsin “the nature remains in doubt.”

Differences were noted by them in the following properties:

1. Specific Optical Rotation.—The \([\alpha]_D^{per cent}\) of synthetic l-mono-iodotyrosine was 4.4 in 1 N HCl whereas I had obtained 8.8.

2. They found that the dl-mono-iodotyrosine crystallized from water with 1 mol of water as revealed by elementary analyses. The writer’s analyses agreed with those calculated for a non-hydrated product.

3. In contrast to our positive test they failed to obtain a red color with nitrous acid and ammonia (3).

In view of the previous demonstrations (4–6) of the relationship of pepsin tyrosine and protease activity, and of the possibility that our product from pepsin might be an isomer of 3-iodotyrosine, the isolation from an alkaline hydrolysate of mildly iodinated pepsin was performed as previously described (1). dl-mono-iodotyrosine was also synthesized as described by Harington and Pitt Rivers (2). After each had been recrystallized four times their properties were determined and the results compared. The detailed results are reported below but may be summarized here. The specific optical rotation of the freshly isolated product from pepsin was found to be so much less than previously reported that we have some doubts of its being optically active. Nearly all other properties, however, were the same as reported earlier. In addition to the above properties, cross-solubility, cross-partition experiments between immiscible solvents, and the ultraviolet absorption spectra at various pH from which \(pK\) were calculated leave little doubt but that the product from pepsin is 3-mono-iodotyrosine. Our synthetic product and that from pepsin both responded positively to the nitrous acid-ammonia color test, but it was possible to free the synthetic product of its color-giving component.

From the analyses of successive extractions with butyl alcohol of an acid hydrolysate of iodinated pepsin, a calculation was made of the quantity...
of mono-iodotyrosine originally present. This calculation indicated that 73 per cent of the total iodine was mono-iodotyrosine and the bulk of the remainder of iodine behaved with respect to partition experiments like di-iodotyrosine. This is evidence that only tyrosine in pepsin and not some other amino acid or structure reacts with iodine.

RESULTS

Optical Rotation.—Harington and Pitt Rivers (2) expressed surprise, not wholly unwarranted, that our product possessed optical activity after extensive alkaline hydrolysis. However, Dakin (7) has shown that "end" amino acids are much more resistant to racemization during alkaline hydrolysis, and it was felt that since only one or possibly two tyrosine residues in each pepsin molecule was iodinated, it might be an end amino acid of a side chain and hence resist the alkaline racemization.

The earlier report contained an $[\alpha]_D^0$ per cent $\mathrm{HCl} = 8.8$, whereas Harington and Pitt Rivers found 4.4. Our present results using 35 mg. of five times recrystallized material dissolved in 1.5 ml. N HCl showed a negative rotation of 0.03° or $[\alpha] = 1.3$. This result casts considerable doubt on the earlier value and suggests that the product may be optically inactive. Unfortunately, insufficient material prevented an unequivocal answer on this point.

Water of Crystallization or Combination.—Ludwig and von Mützenbacher (8), studying mono-iodotyrosine isolated from iodinated casein, and Harington and Pitt Rivers with their synthetic $dl$-iodotyrosine, note that the crystals contained 1 mol of water per mol amino acid. Whereas Ludwig and von Mützenbacher dried their product at 100° in vacuo for 20 hours and made their determination by means of the Karl Fischer reagent, Harington and Pitt Rivers apparently relied on the elementary analyses. The latter workers state that the water could not be removed without decomposition and loss of iodine.

The elementary analyses of our previously isolated product showed no evidence of any water of combination. This was confirmed in the present results shown in Table I. In this respect the product from pepsin resembles Harington's optically active $l$-mono-iodotyrosine which does not contain water of combination.

When our synthetic $dl$-mono-iodotyrosine was tested for water content using the Karl Fischer reagent in a micro apparatus (9), less than 1 mol of water per mol amino acid was found. Using varying amounts of iodoamino acid dried either over drierite, or at 60° in vacuo overnight and varying considerably the time it was allowed to stand in contact with the absolute methanol, we obtained 1.4 to 1.8 per cent water while 1 mol of water calls for 5.5 per cent.

Ultraviolet Absorption Spectra.—The absorption spectrum of a resonating compound such as the one under discussion is a highly specific property and
as such is very useful for identification purposes. From the work on somewhat similar compounds (10) it was expected that ortho- and meta-iodotyrosines would have detectably different absorption spectra in the ultraviolet region.

If the iodotyrosine from pepsin was an isomer of 3-iodotyrosine, then its absorption spectrum would be expected to be different from that of the synthetic product at some pH, particularly in the region where the phenol group of one is 50 per cent ionized.

Fig. 1 contains the absorption curves of equivalent amounts of tyrosine, mono-iodotyrosine (synthetic and from pepsin), and di-iodotyrosine at pH 1.1, 7.6, and 13. Also included are the curves of the two mono-iodotyrosines at pH 8.12 where the ionization is very close to 50 per cent.

### TABLE I

**Elementary Analyses**

<table>
<thead>
<tr>
<th>Material</th>
<th>Drying process</th>
<th>Dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>per cent</td>
</tr>
<tr>
<td>C₆H₅O₂NI (calculated)</td>
<td>36.1</td>
<td>3.7</td>
</tr>
<tr>
<td>C₆H₅O₂NI·H₂O (calculated)</td>
<td>33.2</td>
<td>3.7</td>
</tr>
<tr>
<td>3-Iodo L-tyrosine (Harlington and Pitt Rivers)</td>
<td>35.6</td>
<td>3.8</td>
</tr>
<tr>
<td>3-Iodo D,L-tyrosine (Harlington and Pitt Rivers)</td>
<td>33.7</td>
<td>3.9</td>
</tr>
<tr>
<td>4 times crystallized</td>
<td>37.0</td>
<td>3.8</td>
</tr>
<tr>
<td>3-iodo-D,L-tyrosine (Herriott)</td>
<td>36.6</td>
<td>3.5</td>
</tr>
<tr>
<td>Repeat analysis*</td>
<td>35.3</td>
<td>3.5</td>
</tr>
<tr>
<td>5 times crystallized material isolated from iodinated pepsin*</td>
<td>36.6</td>
<td>3.5</td>
</tr>
<tr>
<td>65° C. in vacuo for 18 hrs.</td>
<td>39.2</td>
<td>38.8</td>
</tr>
<tr>
<td>65° C. in vacuo for 18 hrs.</td>
<td>40.8</td>
<td>41.0</td>
</tr>
</tbody>
</table>

*Analyses made by A. Elek, Rockefeller Institute, micro analyst.
It may be seen that the spectra of the product from pepsin are very similar to those of synthetic 3-iodo dl-tyrosine and are very different from those of either tyrosine or di-iodotyrosine. The difference of 10 to 15 per cent in the mono-iodotyrosine curves determined at pH 13 is probably due to differences in concentrations through some error, for in other experiments no such differences were observed.

![Figure 1. Ultraviolet absorption spectra of equivalent amounts (5.0 µg N/ml.) of tyrosine (T) indicated by ●, mono-iodotyrosine (IT) from pepsin marked ○, synthetic mono-iodotyrosine marked X, and di-iodotyrosine (IT) marked △, in solutions of different pH.](image)

$pK$—Determinations of the ionization constants of phenols by observing changes in the absorption spectra with changes in pH were described by Stenstrom and Goldsmith (11). Other have found this method useful (12, 13). Using this method the $pK$ of the phenolic group of mono-iodotyrosine has been calculated. A value of $pK = 8.2$ was obtained for both the synthetic derivative and the product from pepsin.

**Solubility Experiment.**—A solubility curve of five times crystallized synthetic 3-iodo-dl-tyrosine in water at 9°C. is shown in Fig. 2. The solid line is the expected curve of a single substance. The experimental points fall close to this curve.

It would have been desirable to compare the complete solubility curves of
both the mono-iodotyrosines, but owing to the small amount of material from pepsin a slightly different experiment was performed. The results are shown in Table II. In general, if two substances are different, a saturated solution of one material will be expected to dissolve some of the second substance. This concept has served as the basis of the present experiments.

![Figure 2](image.png)

**Fig. 2.** A solubility curve of synthetic dl-mono-iodotyrosine in water at 9°C. The sample indicated by the open circle ○ contained no solid phase after equilibration. Samples indicated by closed circles ● contained excess solid phase at equilibrium.

<p>| TABLE II |
|------------------|------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>From iodine analyses</th>
<th>From spectrophotometric analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original saturated solution</td>
<td>6.1</td>
<td>6.4</td>
</tr>
<tr>
<td>(a) After equilibrating with iodo material from pepsin</td>
<td>6.2</td>
<td>6.2</td>
</tr>
<tr>
<td>(b) After equilibrating with synthetic iodotyrosine</td>
<td>5.8</td>
<td>6.2</td>
</tr>
</tbody>
</table>

**EXPERIMENTAL PROCEDURE**

Water was saturated by stirring overnight with a large excess of five times crystallized synthetic dl-iodotyrosine after which the solid phase was separated by filtration. To 1 ml. aliquots of the filtrate were added 3 mg. of five times crystallized (a) iodotyrosine from pepsin, (b) synthetic iodotyrosine. These suspensions were then equilibrated for 24 hours at 10°C. by rotating about its short axis a small test tube containing the suspension plus a glass bead. After equilibration and centrifugation the concentration of material in the supernatants was determined from iodine and spectrophotometric analyses at pH 7.6 and wave lengths from 285 to 310 mμ.

The iodine and spectrophotometric analyses in Table II indicate that the iodo product from pepsin did not dissolve. Thus no difference in preparations could
be detected by this extremely sensitive test. This experiment also suggests, though by itself does not prove, that the material from pepsin is probably not optically active, since the solubilities of optically active enantiomorphs are often qualitatively independent of the solubility of the racemic compound.

**Partition or Distribution Coefficient.**—The results of distribution experiments on the synthetic iodotyrosine and the iodo product from pepsin are shown in Table III. The solvents were dilute acid (pH 2) and butyl alcohol. Mixed or cross-distribution experiments were also performed. After equilibration the aqueous phases of each system were reequilibrated but with the butyl alcohol of the other system. After reequilibration the concentration of iodine in the various solutions was determined and the coefficients calculated.

The results of these experiments shown in Table III demonstrate that the distribution coefficient is the same for all these various systems. There is, therefore, no indication from these experiments that in these two compounds the position of the iodine is different.

**Nitrous Acid—Ammonia Color Test.**—Harington and Pitt Rivers (2) were unable to produce a red (i.e. positive) color when examining mono-iodotyrosine with the nitrous acid—ammonia color reaction (3). The writer obtained a positive color test with nearly all samples of four or five times crystallized synthetic or peptic products. In the case of the synthetic, however, it was found that upon fractional crystallization of an already five times crystallized preparation, the first fraction gave a strong color reaction, the second less, and

### TABLE III

**Experimental Procedure**

To 5 ml. of the solutions containing 1 mg./ml. of the iodo products in two separate tubes was added 0.15 ml. of 0.35 N HCl to pH 2.1 followed by 5 ml. of reagent grade normal butyl alcohol. These were shaken hard and after separation was complete aliquots of butyl alcohol layers were analyzed for iodine. The upper alcohol volume was 4.45 ml. while the aqueous layer was 5.7 ml. 3.5 ml. of the butyl alcohol layers were then drawn off and superimposed on the aqueous layer of the opposite system, and after reequilibration the iodine analyses were again made on the butyl alcohol of the two systems. The concentration in the aqueous layers was calculated.

<table>
<thead>
<tr>
<th>No.</th>
<th>System</th>
<th>Mg. iodine/ml.</th>
<th>Partition or distribution coefficient*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Butyl alcohol</td>
<td>Aqueous</td>
</tr>
<tr>
<td>1</td>
<td>Iodotyrosine (from pepsin)</td>
<td>0.166</td>
<td>0.22</td>
</tr>
<tr>
<td>2</td>
<td>3-iodotyrosine (synthetic)</td>
<td>0.166</td>
<td>0.22</td>
</tr>
<tr>
<td>3</td>
<td>Aqueous (1) and butyl alcohol (2)</td>
<td>0.154</td>
<td>0.23</td>
</tr>
<tr>
<td>4</td>
<td>Aqueous (2) and butyl alcohol (1)</td>
<td>0.162</td>
<td>0.225</td>
</tr>
</tbody>
</table>

*In experiments employing dilute H₂SO₄ at pH 3, in place of the above HCl at pH 2.1, the coefficient was 0.6 — 0.65 for both iodo products.
the mother liquor no color reaction, the same quantity of material being analyzed in each instance. It is clear in this case that the color arose from an impurity.

It is possible that traces of di-iodotyrosine carried along with the mono-iodo derivative from pepsin account for the positive reaction of the latter. Unfortunately, when it was discovered that the synthetic product gave a negative reaction, we no longer had any of the amino acid from pepsin to fractionate and examine in the above manner.

Amount of Mono-Iodotyrosine in the Hydrolysate of Iodinated Pepsin.—In our previous communication (1) separation of the iodine-containing component was effected by precipitation with lead acetate, which involved very little loss of iodine, followed by successive extractions with butyl alcohol. A calculation of the distribution coefficient for the iodine component after each extraction (solutions 14 to 21 in Table II of a previous paper (1)) shows that there was a gradual drift in the coefficient from 1.0 to 0.6. This indicates the presence of more than one iodine component. The coefficients for the last two extractions were 0.62 and 0.61, or virtually identical with the value obtained for crystalline synthetic mono-iodotyrosine (see footnote in Table III) as well as that for the crystalline iodo product from pepsin obtained under the above conditions. Using the value of 0.6 for the coefficient, the quantity of mono-iodotyrosine in each of the preceding fractions of this extraction series was then calculated. The sum of these quantities totaled 73 per cent of the total iodine at the start. This quantity was confirmed in the present work in which no preliminary purification procedures were involved. The hydrolysate after removal of the barium hydroxide was merely titrated to pH 3 and extracted repeatedly.

A calculation was also made of the coefficient of the 27 per cent remaining iodine. A somewhat variable value in the neighborhood of 2.5 was obtained, which is close enough to the value of 2.1 for di-iodotyrosine to be very suggestive—particularly since di-iodotyrosine would from the nature of the iodination reaction be expected to be present. Thus with virtually all the iodine accounted for, it seems highly improbable that any structure other than tyrosine in pepsin is iodinated under the conditions specified.

EXPERIMENTAL DETAILS

Preparation of Iodotyrosine from Iodinated Pepsin.—Two lots of crystalline pepsin were iodinated to a slight degree (0.7 per cent or approximately 2 atoms of iodine per mol of pepsin) and the iodine compound isolated in one instance by the method previously described. In the second instance the butyl alcohol extraction preceded the precipitation with lead acetate. Four to five crystallizations were necessary to free the material of a brownish colored contaminant and other materials, to a point where the absorption spectra of the crystals and mother liquor were identical.

Preparation of Synthetic di-Mono-Iodotyrosine.—Synthesis of this product was per-
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formed according to procedure of Harington and Pitt Rivers (2). One change was made in the final steps of the preparation which appeared to improve the yield. After decomposition of the diazonium salt by boiling concentrated KI, 45 gm. (0.15 moles) of Ba (OH)$_2$·2 H$_2$O were dissolved to neutralize the H$_2$SO$_4$. Upon filtering, washing the residue, and evaporating the wash water and filtrate in vacuo, a nearly colorless product was obtained. When this neutralization was omitted appreciable quantities of dark material contaminated the crystals. Our final product was four times recrystallized from hot water at pH 5 to 6.

The absorption spectra were determined with the aid of a Beckmann D.U. ultraviolet spectrophotometer standardized at 656 m$\mu$ according to the manufacturer’s directions and in the ultraviolet against 0.02 per cent reagent grade benzene in isooctane.

The writer was assisted in this work by Mrs. Helen F. Dunk.

SUMMARY

Isolation of mono-iodotyrosine from slightly iodinated pepsin has been repeated and the properties of the product compared with those of synthetic dl-3-iodotyrosine. Ultraviolet absorption spectra, $pK$ values of the phenol group, solubility measurements, and partition coefficients were so nearly identical for the two materials that there is now no reason to doubt that the product from pepsin is 3-iodotyrosine.

BIBLIOGRAPHY