Studies with lactoperoxidase showed that a highly reactive intermediate is produced (on the enzyme) from I\(^{-}\) and H\(_2\)O\(_2\) which then diffuses from the enzyme and very rapidly and indiscriminately iodinates any Tyr or peptides containing Tyr which are in the same solution. The evidence supporting these conclusions follows. 1) iodination followed a Michaelis-Menten pattern with I\(^{-}\) and H\(_2\)O\(_2\) while the concentration of Tyr peptides had no measurable effect on the rate; 2) the rates of reaction were independent of the type of peptide in which Tyr was located; 3) the amount of iodination which had occurred after the reaction had gone to completion and the amounts of monoiiodination and diiodination after completion of the reaction were independent of the peptide type, the pH, the solvent polarity, or the ionic strength; 4) competition for reaction by two very different Tyr peptides depended only on their initial concentrations; and 5) iodination of a large protein occurred through a dialysis membrane. Free Tyr was iodinated at the same rate as Tyr peptides by lactoperoxidase, but monoiiodotyrosine and m-fluorotyrosine were iodinated at one-half that rate. The results also showed that one can choose ratios of [peptide] to [H\(_2\)O\(_2\)] such that monoiiodination is maximized relative to diiodination. It was also found that the iodination capacity of a mixture of I\(^{-}\) and H\(_2\)O\(_2\) with lactoperoxidase (when Tyr was absent) was only slowly dissipated. Finally, the results showed that lactoperoxidase can be used to brominate and chlorinate Tyr peptides at a slow rate.

The lactoperoxidase (EC 1.11.1.7)-catalyzed iodination of Tyr in peptides and proteins has become an important technique in biochemistry and molecular biology in recent years (Morrison and Schonbaum, 1976; Stanton and Hearn, 1987). With lactoperoxidase, small amounts of proteins can be labeled with \(^{125}\)I under mild conditions so that these proteins can then be readily detected (Hunter and Greenwood, 1962; Glazer et al., 1975). Iodination has also been used to determine the locations and the roles of specific Tyr residues in proteins (Seon et al., 1970; Huber et al., 1982; Edwards and Huber, 1985; Stanton and Hearn, 1987).

In order to interpret properly the results of experiments in which Tyr residues in proteins are labeled using lactoperoxidase it is important that the mechanism of iodination be known. Several authors (Dunford and Ralston, 1983; Huwiler et al., 1985; Jenzer and Kohler, 1986; Jenzer et al., 1987) have suggested that lactoperoxidase acts on I\(^{-}\) and H\(_2\)O\(_2\) to catalyze the production of an intermediate (as yet unidentified) which then reacts with Tyr, but there are conflicting reports in the literature with respect to this mechanism. The main conflict relates to whether lactoperoxidase has specificity for various classes of Tyr and, therefore, whether Tyr (or a Tyr peptide) actually binds to lactoperoxidase during the reaction. Some studies indicate that the lactoperoxidase reaction is stereospecific, preferring D-Tyr over L-Tyr, and other reports describe a specificity which depends on the environment (neighboring residues) of the Tyr in peptides (Morrison and Bayse, 1970; Bayse et al., 1972; Morrison and Schonbaum, 1976). From these reports it could be concluded that Tyr, as a free amino acid or in peptides, is bound to the lactoperoxidase during its reaction with an “enzyme-bound” intermediate formed from I\(^{-}\) and H\(_2\)O\(_2\). However, in other studies (Taurog, 1970; Huwiler et al., 1985) the results obtained show that lactoperoxidase has no stereospecificity in the case of L-Tyr and D-Tyr. It has also been shown (Geigert et al., 1983) that alkyne and cyclopropane can be reacted by lactoperoxidase even though these structures are very different from the structure of Tyr. It was suggested in those reports that Tyr and other targets may not actually bind to the enzyme.

The experiments described here were designed to study in detail the specificity of lactoperoxidase with respect to Tyr in order to determine whether the iodination occurs while Tyr (or peptides and proteins with Tyr) is bound to the enzyme during iodination. In addition, a variety of conditions was used in order to study the effects of these conditions on the rate and overall reactivity of the enzyme. Iodination when lactoperoxidase and a large protein were separated by a semipermeable dialysis membrane was also studied as was the loss in iodinating capacity in the absence of Tyr. A practical aspect is also presented in the form of conditions under which one is likely to obtain chosen amounts of monoiiodinated and diiodinated product. Finally, the enzyme was tested for its ability to catalyze bromination and chlorination of Tyr.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lactoperoxidase and the test peptides which were used were obtained from Sigma. Sodium iodide and 30% H\(_2\)O\(_2\) ("ACS certified") were from Fisher. The Na\(^{131}\)I was obtained from Amersham Corp. Other chemicals were obtained from Fisher or similar sources and were the purest grades available.

\(\beta\)-Galactosidase (Escherichia coli) was purified to homogeneity by methods similar to those reported by Brake et al. (1978).

**Assay**—Iodination reactions were normally carried out in 50 mM potassium phosphate buffer at pH 7.0 and 25 °C with varying amounts of NaI, peptides, and H\(_2\)O\(_2\). H\(_2\)O\(_2\) was added last in order to ensure that it would not decompose before the experiment started, but there was no evidence of significant decomposition during the experiments. Fresh H\(_2\)O\(_2\) was purchased frequently. The concentration of the H\(_2\)O\(_2\) was assumed to be that given by the manufacturer, and the very close 1:1 stoichiometry observed for the reactions confirmed the validity of...
that assumption. In some cases the pH and the buffer concentrations were varied in order to determine their effects on the reaction. Experiments were also carried out in varying concentrations of added methanol to see the effect of solvent polarity.

For experiments in which the rate of the reaction was being determined, 0.25 μg of lactoperoxidase was added per ml of reaction mixture. Aliquots (200 μl) for HPLC analysis were removed at 30 and 60 s, and the reaction was stopped by adding these 200-μl aliquots to 50 μl of 60 mM 2-mercaptoethanol (which chemically reduced the H₂O₂).

For experiments in which the extent of the iodination reaction and the amounts of monoiodinated and diiodinated product were to be determined upon depletion of one of the substrates (usually H₂O₂), 25 μg of lactoperoxidase was added to the reaction mixture, and the aliquots were removed at 30 or 60 min (the reaction had stopped due to the depletion of one of the reactants, but the aliquots were nevertheless added to 2-mercaptoethanol as described above). Studies were carried out which showed that under these conditions the nonenzymatic reaction was negligible.

For analysis, samples of the aliquots with the 2-mercaptoethanol were chromatographed on a Hewlett-Packard Co. model 1084B HPLC equipped with a Vydac "protein and peptide" C18 column (0.25 × 25 cm). The peptides were eluted with a flow rate of 1 ml/min and a gradient (Carne et al., 1986) of acetonitrile and water (each containing 0.1% trifluoroacetic acid). The product peaks in every case were clearly resolved from each other and from the reactant peaks. The Tyr, monoiodotyrosine, and diiodotyrosine or peptides containing these amino acids were detected by the absorbance at 275 nm. These compounds were quantitated by comparing the integrated areas under the peaks with areas of peaks from standards of Tyr, monoiodotyrosine, and diiodotyrosine or the peptides containing these amino acids.

One unit was defined as 1 μmol of iodine incorporation/min.

**Iodination through Dialysis Membranes**—One-ml aliquots of 0.05 M potassium phosphate buffer (pH 7.0) with 400 μM NaI, 60 μM H₂O₂, trace amounts of Na⁺⁺ and 5 mg of β-galactosidase were placed into each of two dialysis bags. Dialysis was carried out in two 10-ml beakers using as the dialyzing medium 5 ml of the above solution without the β-galactosidase. The contents of the beakers were stirred with magnetic stirring bars. Lactoperoxidase (25 μg) was added to the dialyzing medium in one of the beakers but not to the other. After 1 h, the dialysis bags were removed and washed. The contents of the tubes were then added to 250 μl of 60 mM 2-mercaptoethanol to stop the reaction. Aliquots (500 μl) were eluted through a Biotechnology Inc. FPLC fast desalting column, and 1-ml fractions were collected. In each case over 95% of the β-galactosidase was in one eluted fraction. The contents of that tube were counted, and the concentration of β-galactosidase in each of the two dialyzed reactions was determined.

**RESULTS**

**Effects of NaI and H₂O₂ Concentrations on the Rate of Iodination**—Fig. 1a shows a Hofstee (1959) plot of the rate of the lactoperoxidase reaction at various NaI concentrations at 60 μM H₂O₂ and 100 μM Gly-Leu-Tyr (one of the test peptides used in these studies). There was inhibition at high NaI concentrations (Huwiler et al., 1985) also reported that high NaI concentrations inhibit the enzyme), but the points from the lower NaI levels could be extrapolated to give a value for the apparent V of 208 units/mg and a value of 4.8 mM NaI for the apparent Kᵣ. Fig. 1b shows the plot of the rate with various [H₂O₂] concentrations at 400 μM NaI and 100 μM Gly-Leu-Tyr. In the case of H₂O₂ variation, there was no inhibition (Fig. 1b) at high H₂O₂ concentrations (at least not in the concentration range that was used). Calculation showed that the apparent V when the H₂O₂ concentration was varied was 102 units/mg and the apparent Kᵣ was 25.8 μM H₂O₂. The difference in the apparent V values in the two studies (Fig. 1, a and b) is due to the fact that the H₂O₂ study was done at a low NaI concentration to ensure that there was no inhibition, while the apparent V for the NaI study represents the rate at

![Fig. 1. a, Hofstee (1959) plot of the rate of the iodination reaction as a function of the NaI (mM) concentrations (at 60 μM H₂O₂ and 100 μM Gly-Leu-Tyr). The extrapolated line represents the theoretical line which would be obtained if there were not any substrate inhibition. b, Hofstee (1959) plot of the rate of the iodination reaction as a function of the H₂O₂ (μM) concentration (at 400 μM NaI and 100 μM Gly-Leu-Tyr).](image-url)

the theoretical saturating concentration of NaI (the extrapolated value if there were no inhibition by NaI) and a relatively high concentration of H₂O₂. Because of inhibition by NaI it was impossible to obtain a true V value or true Kᵣ values. This, however, was really not important because the main objective of this particular experiment was to see whether these substrates (NaI and H₂O₂) follow Michaelis-Menten kinetics and, except for the inhibition at high NaI concentrations, that was obviously the case.

**Rate as a Function of Peptide Concentrations**—Fig. 2 shows that the rate of iodination was independent of the concentration of Gly-Leu-Tyr. Reactions with peptide concentrations below 4 μM were too small to be measured practically with the instrument sensitivity available.

**Effect of pH**—Fig. 3 shows how the rate of the lactoperoxidase reaction varied with pH. The rate changed in a normal bell-shaped fashion with a pH optimum near 6.0. The amounts of monoiodotyrosine and diiodotyrosine produced when the reaction was allowed to go to completion (as also shown in Fig. 3) were, however, essentially independent of pH (the small drop in the amount of monoiodinated product at high pH is probably not significant; it may be related to a difference in absorption properties).

**Rate as a Function of Peptide Composition**—The rates of iodination of several Tyr-containing peptides were measured. Peptides were present at 100 μM, NaI at 400 μM, H₂O₂ at 60 μM, and 0.25 μg/ml of lactoperoxidase was added. Under these conditions, the rates of reaction were: Gly-Leu-Tyr, 37.6 units/mg; Gly-Gly-Tyr-Arg, 34.0 units/mg; Val-Tyr-Val, 39.2
When large amounts of enzyme were added and the reaction was allowed to go to completion (30- or 60-min readings were taken, but each gave essentially the same result because the reaction was complete after 30 min), the final amount of iodination depended only on the limiting amount of either the \( \text{H}_2\text{O}_2 \) or the Tyr peptide present. This is shown by the fact that the overall amount of iodination which occurred was dependent only on the ratio of [peptide] to \( \text{H}_2\text{O}_2 \). Calculations using data plotted in Fig. 4 showed that each \( \text{H}_2\text{O}_2 \) unit which was added caused the addition of 1 iodine unit. This means that the reaction is very efficient (stoichiometric) and is essentially irreversible.

The amount of the product which was monoiodinated (relative to the total iodination) was also dependent only on the [peptide]:[\( \text{H}_2\text{O}_2 \)] ratio (Fig. 4). The curves on the figure were independent of the starting amounts of either the peptide or the \( \text{H}_2\text{O}_2 \). The important parameter was only the ratio of [peptide] to \( \text{H}_2\text{O}_2 \). Also significant was the fact (Fig. 4) that when other Tyr-containing peptides (Gly-Gly-Tyr-Arg, Val-Tyr-Val, Tyr-Ala, or Tyr-Glu) were substituted for Gly-Leu-Tyr, the same results were found. The amount of iodination after depletion of one of the reactants and the ratio of the diiodinated product to the total product were, therefore, also independent of the type of residues around Tyr.

Direct Competition for the Iodination Reaction—Two very different test peptides (Gly-Leu-Tyr and Gly-Gly-Tyr-Arg) were mixed in varying ratios before lactoperoxidase was added. The rates of the iodinations and the amount of iodination that had occurred with each of the peptides (upon depletion of \( \text{H}_2\text{O}_2 \)) were determined. Fig. 5 shows that both the amount of iodination and the rate of the reaction were dependent only upon the ratio of the peptides at the beginning of the reaction, and neither reactant was iodinated in preference to the other.

Iodination through Dialysis Membranes—Calculations showed that <0.5 pmol of iodine were incorporated per nmol of \( \beta \)-galactosidase monomer inside the dialysis bag (less than...
without a dialysis bag (Huber et al., 1982), but it is to be determined. For the rate studies, 0.25 μg/ml lactoperoxidase was added. For the studies to determine the amount of tyrosine and diiodotyrosine at the depletion of H₂O₂ were compared to the total amount of product which was iodinated Gly-Gly-Tyr-Arg when the reaction was allowed to go to completion (because of depletion of a reaction component) was measured and compared to the total amount of iodination.

![Graph](image)

**TABLE I**

**Effects of methanol and of buffer strength on the rate of the lactoperoxidase reaction and on the amounts of products produced at the end of the reaction (upon depletion of H₂O₂)**

<table>
<thead>
<tr>
<th>Reaction condition</th>
<th>Rate</th>
<th>I-Tyr</th>
<th>l-Tyr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol (v/v)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>30.4</td>
<td>39.9</td>
<td>12.8</td>
</tr>
<tr>
<td>25%</td>
<td>18.2</td>
<td>40.9</td>
<td>10.6</td>
</tr>
<tr>
<td>50%</td>
<td>7.7</td>
<td>39.9</td>
<td>8.7</td>
</tr>
<tr>
<td>Buffer (ionic) strength</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM</td>
<td>18.0</td>
<td>42.1</td>
<td>13.2</td>
</tr>
<tr>
<td>50 mM</td>
<td>29.0</td>
<td>42.2</td>
<td>13.1</td>
</tr>
<tr>
<td>150 mM</td>
<td>32.1</td>
<td>38.6</td>
<td>11.3</td>
</tr>
<tr>
<td>500 mM</td>
<td>50.0</td>
<td>38.8</td>
<td>11.2</td>
</tr>
</tbody>
</table>

the lower limit of detection) in the absence of lactoperoxidase in the dialyzing medium. With lactoperoxidase added outside of the dialysis bag, 40 pmol of iodine were incorporated per nmol of β-galactosidase monomer. The amount of iodination here was quite low relative to the amount which occurred without a dialysis bag (Huber et al., 1982), but it is to be expected that reactions through a dialysis bag would be quite inefficient.

**Effect of Methanol on the Reaction**—At high concentrations of methanol, the rate of the lactoperoxidase reaction decreased, but the amount of reaction and the proportions of monooiodotyrosine and diiodotyrosine at the end of the reaction were independent of the amount of methanol present (Table I).

**Effect of Ionic Strength**—Increasing the ionic strength (see Table I) caused an increase in the rate of the iodination reaction.

**Effect of Delay in Peptide Addition on the Iodination Rate**—Table II shows that when the reaction was allowed to proceed for 50 s in the absence of a Tyr peptide, the reaction, upon addition of peptide, proceeded at essentially the same rate as when peptide was added at zero time. There was not any significant jump in the rate. This shows that if an intermediate is formed, it does not accumulate in the mixture under the conditions we used. The table also shows that if the reaction was allowed to proceed for varying periods of time before the Gly-Leu-Tyr was added, there were only small decreases in the overall extent of the iodination at depletion of H₂O₂. Thus, the iodination capacity is not rapidly dissipated in the absence of a Tyr target.

**Bromination and Chlorination**—Substituting NaBr for NaI resulted in the production of monobrominated and dibrominated Gly-Leu-Tyr, but the rate of bromination (0.93 units/mg) was much slower than the rate of iodination (30.4 units/mg). When NaCl was substituted, the reaction also occurred but was very slow (0.034 units/mg). In order to see some reaction in the case of NaCl, the incubation had to be carried out for 2 h. With NaI there would have been some nonenzymatic reaction during such a long incubation. With NaCl, however, no reaction was noted in the absence of enzyme for the 2-h period of time, and the reaction which occurred was, therefore, enzyme-catalyzed. For both the bromide and the chloride reactions, the monohalogenation to dihalogenation ratios were the same as they were with iodide.

**DISCUSSION**

The results reported here show that lactoperoxidase catalyzes iodination by binding I⁻ and H₂O₂ and producing a very reactive intermediate which diffuses from the enzyme and reacts, rapidly and indiscriminately, with any Tyr it encounters. The formation of this intermediate by the enzyme is the rate-determining step for the iodination. The following points support these arguments.

1) The rates of reaction with both NaI and H₂O₂ followed the normal Michaelis-Menten pattern (except for substrate inhibition at high NaI), indicating that both bind to the lactoperoxidase during the catalytic reaction. The concentration of Tyr peptide, on the other hand, did not affect the steady-state rate of iodination. If Tyr or Tyr-containing peptides did bind to lactoperoxidase, the steady-state rate should have followed the Michaelis-Menten pattern unless the Kₘ values for these peptides were very small relative to the peptide concentrations tested (i.e., the results obtained could
only be consistent with Tyr binding if the Tyr peptide concentrations tested were all high enough relative to their $K_{m}$ values so that they would be in the zero order range of their Michaelis-Menten concentrations). Since the rate did not decrease even when the peptide concentration was as low as 4 μM, it is unlikely that the concentration was high enough relative to the $K_{m}$ to cause the rates to be in the zero order range. Therefore, the actual iodination step must take place nonenzymatically by means of an enzyme-formed highly reactive intermediate which diffuses and then reacts rapidly with any Tyr it contacts. The rate of reaction of the reactive intermediate with Tyr must be so rapid relative to the rate of formation of the intermediate (and the reaction must be so extensive) that there are not any differences in the rate due to peptide concentrations. The rate-determining step must, therefore, be the production of the intermediate by the enzyme.

2) The rates of reaction with Tyr and with every Tyr peptide studied were very similar. The peptides tested were all very different in structure. If they did bind to lactoperoxidase they should have bound with different affinities, and thus, the rates should have been different. If somehow the different peptides did bind with similar affinities it is unlikely that in addition the $K_{m}$ values would also be the same. Therefore, this evidence again supports the suggestion that Tyr (or Tyr peptide) does not bind to lactoperoxidase.

3) Studies showing that the extent of the reaction when one of the components was depleted and studies showing that the final amount of moniodinated product formed relative to the amount of diiodinated product was only dependent on the peptide to $[H_{2}O_{2}]$ ratio under a variety of reaction conditions (different peptide types, pH, methanol content, and ionic strength) also support the conclusion that the actual iodination step is nonenzymatic and is mediated by a highly reactive intermediate. Although the reaction rates were dependent upon pH, methanol content, and ionic strength, the final extent of the reactions was independent of these conditions. If the formation of the highly reactive compound is enzyme-mediated and rate-determining, the rate of its formation would be expected to be dependent upon reaction conditions such as pH, methanol content, and ionic strength. Also, there is no reason that a diffusible or nondiffusible enzyme intermediate would not also be dependent on pH and solvent conditions. However, since neither the ionization of the Tyr (pH effects) nor the solvent polarity and ionic state of the medium resulted in changes in the relative proportion of monoiiodotyrosine to diiodotyrosine, the monoiiodotyrosine production relative to diiodotyrosine production must be a function only of the relative amounts of the target molecules present (regardless of the other conditions used). This clearly shows the high reactivity of the intermediate. It shows that initially, under any of the conditions used, only monoiiodotyrosine is formed, and then only as the population of monoiiodotyrosine is built up is a significant amount of diiodotyrosine formed. Therefore, these studies suggest that the only factor which affected the amounts of monoiiodinated and diiodinated product formed (under the conditions we used) was the relative chance of collision, and this was the same for all of the peptides studied and for all of the conditions tested, as would be expected for a nonenzymatic reaction of a highly reactive intermediate.

4) The experiment in which the reactions with two test peptides were measured at competitive concentrations adds further support to the contention that Tyr peptides do not bind to lactoperoxidase during the iodination reaction. The two test peptides were chosen specifically because they are so different. Gly-Leu-Tyr is quite hydrophobic compared to Gly-Gly-Tyr-Arg. In the case of Gly-Leu-Tyr, the Tyr is C-terminal while for Gly-Gly-Tyr-Arg, the Tyr is internal. In addition, Gly-Gly-Tyr-Arg obviously has a strong positive charge which Gly-Leu-Tyr does not have. Surely there would be different binding specificities for two such different peptides. If Tyr peptides do bind to lactoperoxidase, the binding specificity should be reflected in the comparative rates even if the $K_{m}$ values are so low that the concentrations tested are in the zero order range (as discussed above). That is, there should be differences in the relative amounts of iodination because the lactoperoxidase would be loaded preferentially with one peptide as compared to another. Instead, the rates and the final amounts of iodination were only dependent upon the starting proportions of the two peptides (Fig. 5), which means that they were probably not bound to the lactoperoxidase during the reaction and that the rates depended only on the chances of collision of the intermediate with the Tyr peptides which, in turn, would depend only on their relative ratios.

5) The fact that iodination occurred through a dialysis membrane very strongly supports the argument that the iodination reaction occurs nonenzymatically after a reactive intermediate is formed and diffuses from the lactoperoxidase. Because of the sizes of lactoperoxidase (79 kDa) and β-galactosidase (464 kDa), there is no possibility that they could have come into contact with each other through the dialysis membrane. Thus, the reaction must have taken place via a small molecule which was produced by lactoperoxidase and which then diffused off the enzyme and through the dialysis membrane to react with β-galactosidase.

The data reported here, showing a lack of specificity of lactoperoxidase for different forms of Tyr, are quite different from results reported by some other workers (Morrison and Bayse, 1970; Bayse et al., 1972; Morrison and Schonbaum, 1976) but support some studies which have shown that when L-Tyr was compared to D-Tyr as a lactoperoxidase substrate, there were no differences in rates (Taurog, 1970; Huwiler et al., 1985) and other studies which have shown that there is iodination of alkenes and cyclopropanes (Geigert et al., 1983), which are very different in structure from Tyr. It is hard to reconcile our results with those of the studies by Morrison and Bayse (1970), Bayse et al. (1972), and Morrison and Schonbaum (1976), although one possible explanation for the differences may be that in our case the data were obtained by HPLC analysis, while in the other studies the data were obtained by less specific spectrophotometric methods.

The fact that monoiiodotyrosine and m-fluorotyrosine reacted at one-half the rate of reaction with Tyr is interesting. This is the only difference in rate that we found for the different forms of Tyr and may somehow be related to the fact that only one-half of the sites available for iodination were available for the reaction. The fact that the reaction stopped after 1 iodine unit was added was expected.

The data in Fig. 4 show that one should be able to pick ratios of [peptide] to $[H_{2}O_{2}]$ such that the product is mainly monoiiodinated. When modifying proteins to find whether Tyr is important for the function of a protein, the interpretation of results would be simplified if one could limit the extent of the modifications so that the protein structure is perturbed as little as possible. Iodine is very large, and the addition of two iodine units to a single Tyr unit would almost certainly perturb the surrounding structure to an unacceptable extent, making it difficult to interpret whether biological activity is lost due to a change in Tyr reactivity or to a perturbation of structure. It would, therefore, be of advantage to find condi-
Iodination by Lactoperoxidase

tions which result mainly in monoiodination. It is also important to ensure that a certain threshold number of the Tyr have reacted in order to see a significant change in activity (e.g., if only 5% of the Tyr at a particular position were iodinated, they would almost all be monoiodinated, as in Fig. 4, but a 5% activity loss may be within the experimental error of the activity measurement). Using the data in Fig. 4, it is possible to select conditions to optimize the iodination in terms of the amount of Tyr reacted and the percentage of monoiodinated Tyr formed. As an example, one could set up a condition such that more than 25% of the Tyr had reacted and more than 80% of the iodinated product was monoiodotyrosine. The shaded section shown in Fig. 4 would give a range of proper starting [peptide]:[H$_2$O$_2$] ratios for this condition. Of course, one could choose any limiting condition. The data in Fig. 4 also show the limitations of the iodination reaction. For example, it is impossible to achieve 100% iodination of Tyr peptides (of simple peptides at least) without the formation of a significant amount (>80%) of diiodotyrosine (Fig. 4).

Our results indicate that diiodination only becomes significant after enough monoiodotyrosine has formed to make the monoiodotyrosine a suitable target for the highly reactive intermediate formed from I$^-$ and H$_2$O$_2$. There are, however, examples in the literature of Tyr in proteins which become mostly diiodinated and in which it is difficult to detect any monoiodotyrosine at any stage of the iodination reaction (Seon et al., 1970; Stanton and Hearn, 1987). We feel that this could occur with Tyr buried within a protein (or possibly with Tyr found in a hydrophobic area where the intermediate might be excluded). An initial iodination might only occur if a large amount of lactoperoxidase was used and the [H$_2$O$_2$]:[protein] ratio was high so that a high concentration of intermediate was formed in order to ensure that some would reach the Tyr and iodinate it. Once this initial iodination had occurred, there could be a rapid perturbation in structure due to the addition of the large iodine atom, and the monoiodotyrosine could become fully exposed for further reaction. The large [H$_2$O$_2$]:[peptide] ratio required to form enough intermediate to diffuse in and cause the first iodine to react (to form monoiodotyrosine) would then result in iodination of that monoiodotyrosine as soon as it became exposed. The results of Seon et al. (1970) support this. Those workers showed that one of the two Tyr in Bence Jones protein could be monoiodinated readily at a low iodide concentration. Only at high iodide concentrations was there a significant amount of diiodotyrosine formed. However, the other Tyr only became iodinated when a large amount of iodide was added, and in that case only small quantities of monoiodotyrosine could be detected. The most likely reason for this is that the monoiodination probably triggered a conformational change which exposed the monoiodotyrosine for a rapid second iodination.

Surprisingly, whatever the species is that brings about the iodination (Dunford and Ralston, 1983; Huwiler et al., 1985), our data indicate that it does not accumulate and it does not rapidly dissipate (at least not under the conditions we used) in the absence of a suitable Tyr acceptor (Table II). It is possible that it is only formed in small amounts (see Scheme 1) and is in rapid equilibrium with I$^-$ and H$_2$O$_2$ (i.e., possibly the intermediate is only depleted by rapid irreversible reaction with Tyr and then it is rapidly replenished to maintain the equilibrium concentration).

The results reported also showed that lactoperoxidase can be used for bromination and for chlorination. However, much more enzyme or much longer times of incubation were needed for a similar amount of Tyr modification (relative to iodination). Bromide and chloride may, however, be better halides to use for modifying proteins to check on the involvement of Tyr in a protein's activity because bromine and chlorine are smaller than iodine and thus would perturb the structure of proteins to a lesser extent.

Acknowledgment—Expert technical assistance from Joanne Simala is acknowledged.

REFERENCES

SCHEME 1. A possible reaction scheme for the iodination reaction which accounts for the fact that iodination capacity does not build up in the absence of Tyr target and is not dissipated. The scheme also shows that the actual enzyme reaction is rate-determining and is fully reversible. E, enzyme; the dots indicate that some type of complex is formed. The arrow from the reactive intermediate to Tyr indicates that the reactive intermediate is attacking the Tyr.