The Formation of Diiodotyrosine in Iodinated Human Serum Albumin

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SUMMARY

Human serum albumin (HSA) has been allowed to react with varying amounts of iodine under mild conditions. Almost all of the tyrosyl residues of HSA may be converted to iodotyrosyl residues (primarily diiodotyrosyl) without producing significant changes in the conformation of the protein. Difference spectral measurements in urea show the disappearance of "buried" tyrosyl and the appearance of "buried" diiodotyrosyl residues; a difference spectral peak attributed to diiodotyrosyl residues in iodinated HSA is seen at 302 nm. It is proposed that the partly nonaqueous environment of "buried" tyrosyl residues directs their preferential conversion to diiodotyrosyl residues.

Studies of the iodination of thyroglobulin have shown that the environment of the tyrosyl residues influences the products formed by their iodination (1). Specifically, iodination of tyrosyl residues which are not fully exposed to the aqueous environment results in the formation of a greater ratio of diiodotyrosine to monoiodotyrosine than is produced by iodination of tyrosine in aqueous solution. Iodine reacts at the two ortho positions of the phenolate ion. Consequently, the reactivity of tyrosyl residues in proteins will depend on their ionization behavior and the environment of their aromatic rings. These two functions can be evaluated, respectively, by spectrophotometric titration of the phenolic ionization (2) and by ultraviolet difference spectroscopy (2). It was not possible to observe the diiodotyrosine difference spectral peak in thyroglobulin because of its overlap with the difference spectrum of tryptophan. Following iodination, KI was added to all solutions to bring them to the same iodide concentration, assuming that 1 mole of added I₂ produced 1 mole of iodide.

MATERIALS AND METHODS

Materials

HSA, four times recrystallized, was obtained from Nutritional Biochemicals. For calculation of the number of moles per mole, a monomer molecular weight of 68,000 was assumed (3). All chemicals were reagent grade. Glass-distilled water was used throughout.

Iodination

HSA was iodinated with varying amounts of I₂, ranging from 3 to 70 moles of I₂ per mole of HSA. Since HSA contains 18 tyrosyl residues (3), 36 moles of I₂ per mole of HSA are theoretically required to convert all the tyrosyl residues to diiodotyrosine. A solution containing 0.040 M I₂ in 0.16 M KI was added at a rate of 10 μl per min from an Agla syringe to a 2% solution of HSA at 25° in 0.05 M glycine-0.01 M KCl, pH 9.1, with magnetic stirring; the pH was maintained at 9.1 ± 0.1 by the addition of small amounts of KOH. Solutions which were colored after addition of I₂ were left at room temperature overnight. Lightly iodinated solutions (Preparations 2 through 5, Table II) were clear; more heavily iodinated samples remained slightly yellow. Following iodination, KI was added to all solutions to bring them to the same iodide concentration, assuming that 1 mole of added I₂ produced 1 mole of iodide.

Spectrophotometric Titrations

Measurement of Iodoamino Acid Concentrations—Iodinated HSA preparations in 8 M urea containing 0.09 M KCl, 0.013 M lysine, and 0.006 M glycine were titrated with KOH. Absorbance at selected wave lengths was measured in a Beckman model DU spectrophotometer, and the content of tyrosyl derivatives was calculated as described elsewhere (5). A modified procedure was used to calculate the diiodotyrosine and thyroxine concentrations, in which the difference in absorption between 350 and 360 μM was used. Table I shows the pH ranges, wave lengths, and molar extinction coefficients used for the calculations; preliminary experiments showed that this choice of conditions gave the best separation of the different tyrosyl derivatives. As the isosbestic point of diiodotyrosine in HSA in 8 M urea was found

1 The abbreviation used is: HSA, human serum albumin.

2 A. van Zyl and H. Edelhoch, unpublished results.
to be 390 μm, the "enhanced" values used with thyroglobulin were not used. Native HSA had a small absorption in the ultraviolet region of interest, which was subtracted from the absorption of the iodinated samples.

**Ionization of Tyrosyl and Iodotyrosyl Residues in Aqueous Solution**—Two iodinated HSA preparations (Preparations 3 and 5, Table II) were titrated in water containing 0.15 M KCl after exhaustive dialysis against distilled water to remove the salts present in the iodinating system. Tyrosine ionization was measured at 290 μm since the isosbestic point for the ionization of the iodoamino acids was found to occur at this wave length. The ionization of diiodotyrosine was determined at 335 μm, where neither tyrosine nor monoiodotyrosine has significant absorption. Monoiodotyrosine ionization was estimated at 315 μm, the data being corrected for the change in absorbance due to diiodotyrosyl ionization. Between pH 4 and pH 8, where only diiodotyrosine is titrated, the ratio of the change in absorbance at 315 μm to the change at 335 μm was constant, and equal to 2.5. Above pH 8, where both monoiodotyrosine and diiodotyrosine are titrated, the contribution of diiodotyrosine to the absorbance change at 315 μm, based on the above ratio, was calculated from the absorbance change at 335 μm, and subtracted from the total absorbance change at 315 μm. The thyroxine concentration in Preparations 3 and 5 is negligible. The spectrophotometric titration data for each residue have been analyzed according to the equation of Linderstrøm-Lang,

\[
\text{pH} - \log \frac{1}{1 - \alpha} = \beta_{\text{int}} - 0.868 \, \text{pH}
\]

where \( \alpha \) is the percentage of groups of each residue dissociated, \( w \) is an electrostatic interaction factor, and \( Z \) is the net charge on the protein (6). The potentiometric titration curve reported by Tanford (7) was used to obtain the charge due to proton binding. Chloride binding, at various pH values, was taken from the data of Scatchard, Scheinberg, and Armstrong (8); the conversion of tyrosyl to monoiodotyrosyl and diiodotyrosyl residues was also accounted for in the calculation of \( Z \).

**Ultraviolet Difference Spectra**

The absorption spectra of solutions containing 2.7 mg of HSA per ml in 0.1 M acetate buffer, pH 3.9, were compared with the same solutions in 8 M urea. A Cary model 14 recording spectrophotometer with a 0.1 slide wire was used to measure the difference spectra.

**Table I**

| Molar extinction coefficients and pH intervals used to calculate concentrations of tyrosine and iodoamino acid derivatives in iodinated HSA |

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>pH range of ionization</th>
<th>Extinction coefficients in 8 M urea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>295 μm</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>9.0-12.5</td>
<td>2.55</td>
</tr>
<tr>
<td>Monoiodotyrosine</td>
<td>8.4-10.0</td>
<td></td>
</tr>
<tr>
<td>Diiodotyrosine</td>
<td>4.5-9.0</td>
<td></td>
</tr>
<tr>
<td>Thyroxine</td>
<td>4.5-9.0</td>
<td></td>
</tr>
</tbody>
</table>

The extinction coefficient of ionized thyroxine at 325 μm has been reduced by 7.7% to take account of the absorbance of the un-ionized form at this wave length. Absorbances of the un-ionized forms of the other substances are negligible.

**Table II**

| Composition and properties of iodinated HSA preparations |

<table>
<thead>
<tr>
<th>Preparations</th>
<th>1 added</th>
<th>Tyrosine (mM)</th>
<th>Monoiodotyrosine (mM)</th>
<th>Diiodotyrosine (mM)</th>
<th>Thyroxine (mM)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>17.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>15.6</td>
<td>0.9</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>14.4</td>
<td>1.7</td>
<td>0.8</td>
<td>0</td>
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<tr>
<td>4</td>
<td>12</td>
<td>12.4</td>
<td>2.3</td>
<td>2.0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>10.9</td>
<td>2.4</td>
<td>3.2</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>8.9</td>
<td>2.6</td>
<td>4.8</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>32</td>
<td>7.6</td>
<td>2.5</td>
<td>6.8</td>
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<td>8</td>
<td>40</td>
<td>5.4</td>
<td>2.3</td>
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<tr>
<td>9</td>
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<td>70</td>
<td>0.3</td>
<td>2.6</td>
<td>12.2</td>
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<table>
<thead>
<tr>
<th>pH</th>
<th>Solubility (%)</th>
<th>Immunological behavior</th>
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</thead>
<tbody>
<tr>
<td>4.5</td>
<td>-333⁻⁺</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>100</td>
<td></td>
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</table>

(-- means reaction of identity with native HSA, ± means reaction of identity, but weaker than native.)

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a Percentage of HSA which is soluble in the (NH₄)₂SO₄-acetate-precipitating buffer.
b + = reaction of identity with native HSA; f = reaction of identity, but weaker than native.
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[Graph showing the distribution of tyrosyl (TYR), monoiodotyrosyl (MIT), diiodotyrosyl (DIT), and thyroxyl (T₄) residues in HSA as a function of added iodine.]

The sum of the residues is shown as Total.

where B is the partial specific volume and M is the mass, the subscripts N and I referring to native and iodinated HSA; this calculation assumes that iodination caused no change in the molar volume of HSA. Sedimentation coefficients based on these estimates of B are presented in Fig. 7 and labeled as theoretical. Estimates of B from the weight average \( \bar{B} \) of protein and iodine, assuming that the incorporated iodine had a density of 4.93 (the density of I₂), gave sedimentation coefficients 1 to 2% lower.

![Graph showing the relationship between sedimentation coefficient and added iodine.]

Optical Rotatory Dispersion

Solutions containing about 1.4 mg of HSA per ml in 0.1 M KCl were examined in a Cary model 60 recording spectropolarimeter between 260 and 500 nm. The data were analyzed according to the Moffitt equation (9),

\[ [\alpha]_\lambda = \frac{3}{n^2 + 2} \frac{MRW}{100} \left( \frac{a \lambda^2}{\lambda^2 - \lambda_0^2} + \frac{b \lambda_0^2}{(\lambda^2 - \lambda_0^2)} \right) \]

where \([\alpha]_\lambda\) is the specific rotation at wave length \(\lambda\), \(MRW\) is the mean residue weight, for which a value of 115 has been assumed, and \(n\) is the refractive index of the solvent at wave length \(\lambda\). \(\lambda_0\) was assumed to be 212 nm.

Solubility

The loss in solubility of iodinated HSA samples was determined by the method of Levy and Warner (10). HSA samples containing about 1 mg per ml were diluted 1:21 with a buffer containing 2.17 M (NH₄)₂SO₄ and 0.87 M acetate, pH 5.1 (precipitating buffer); the solutions were centrifuged for 15 min at 17,000 × g, the supernatants were decanted, and their absorbances were measured at 280 nm.

Immunological Studies

Samples containing 0.1% HSA in 0.1 M NaCl-0.01 M glycine, pH 9.2, were examined by the Ouchterlony gel diffusion method against a sample of horse antihuman serum kindly provided by Dr. Henry Metzger.

Iodine Content

Iodine analyses were performed by the Boston Medical Laboratories. Samples were exhaustively dialyzed against the NaCl-glycine buffer before analysis.

RESULTS

Formation of Iodoamino Acids

The distribution of tyrosine, monoiodotyrosine, diiodotyrosine, and thyroxine in iodinated HSA was determined spectrophotometrically (see “Materials and Methods”) and is reported in Fig. 1 and Table II. The tyrosyl content decreased monotonically with increasing iodine, 70 moles of I₂ required to convert essentially all of the tyrosyl residues into iodinated derivatives. Since 2 moles of I₂ are required to form 1 mole of diiodotyrosine, almost twice the stoichiometric amount of iodine was needed to iodinate the 18 tyrosyl residues in HSA.

Below 10 moles of added iodine, monoiodotyrosine was the principal derivative formed. The monoiodotyrosyl content increased rapidly to about 3 moles per mole, and then remained constant to very high levels of added iodine. The diiodotyrosyl content increased sigmoidally, with increasing iodine, reaching a maximum of about 12 moles per mole. At high levels of iodination, small amounts of thyroxine were also apparently formed; the thyroxine content remained constant at about 0.7 mole per mole between 50 and 70 moles of added iodine. All of the tyrosyl residues were accounted for, since the sum of the unreacted tyrosyl and various iodinated derivatives remained constant, at approximately 18 to 19 moles per molecule (counting thyroxine as 2), with iodination (see Fig. 1).

The ratio of iodine incorporated into protein (based on iodine analysis) to added iodine was constant, comprising 75%, in samples iodinated between 0 and 40 moles of I₂. The formation of iodotyrosyl residues in these samples, however, accounted for only about 50% of the added iodine. Since more iodine is bound than appears in tyrosyl derivatives, other amino acids must also bind iodine; histidyl residues have recently been shown to form iodinated derivatives and may account for part of the difference (11).
Ultraviolet Difference Spectra

The environment of the aromatic amino acid residues of proteins may be evaluated from changes in their absorption resulting from changes in solvent composition (2). Since these absorption changes are small, they are most easily followed by difference measurements. Fig. 2 shows the ultraviolet difference spectra of HSA in 0.1 M acetate buffer, pH 3.9, comparing aqueous with 8 M urea solutions. The pH had to be reduced to 3.9 to avoid absorbance changes due to ionization of the iodinated amino acids. Native HSA has a major negative difference spectral peak at 286 μm and a small positive peak at 302 μm, which presumably are due to the tyrosyl residues and 1 tryptophanyl residue, respectively (2). With increasing iodination, the 286 μm peak decreases, and a new negative peak appears at 302 μm. This latter peak can be assigned to the diiodotyrosyl residues, since difference spectra of the free amino acids, tyrosine and diiodotyrosine, comparing aqueous with 8 M urea solutions, show peaks at 285 μm and 300 μm, respectively (Fig. 3). The magnitude of the two difference spectral peaks are plotted in Fig. 4 as a function of the number of moles of tyrosine and diiodotyrosine per mole. The tyrosyl difference spectral peak (286 μm) shows a sharp decline with iodination of the first 8 residues; then little further change takes place with iodination of the remaining 10 residues. The diiodotyrosine difference peak (302 μm) also changes more rapidly at first, but continues to increase linearly between 4 and 12 moles of diiodotyrosine per mole. The data suggest that the first tyrosyl residue which react and the first diiodotyrosyl residues which are formed contribute more importantly to the difference spectra; however, at higher levels of iodination, the molecular structure is altered (see below), and a decrease in the difference spectral increment (per diiodotyrosyl residue) may result as a consequence.

Ionization of Tyrosine, Monoiodotyrosine, and Diiodotyrosine

Since iodinated HSA is soluble and monodisperse between pH 4 and 12, it is possible to evaluate the ionization constants of the iodoamino acids. Fig. 5 presents spectrophotometric titration data for the tyrosyl and iodotyrosyl residues of Preparation 5. The curves were reversible when back-titrated with acid from the highest pH values shown. In Fig. 6 the titration data for Preparations 3 and 5 are plotted according to the Linderstrom-Lang equation. The evaluation of the degree of ionization, α, as well as the net charge, Z, is indicated under "Materials and

Fig. 2. Difference absorbance spectra (blue shift) of HSA in 8 M urea with respect to H₂O. pH 3.9, 0.1 M acetate. Protein concentration, 2.7%. Samples: 1, —; 2, ···· ; 3, ····· ; 4, ····· ; 7, ···· ; 8, ···· ; 9, ···· ; 11, ···· . The samples are in numerical order, from bottom to top, at 302 μm. See Table II for the degree of iodination of these samples.

Fig. 3. Difference absorbance spectra (red shift) of tyrosine (TYR) and diiodotyrosine (DIT) in 8 M urea with respect to H₂O, pH 1. The ordinate indicates the difference absorbance per mole of amino acid.

Fig. 4. Magnitude of difference absorbance peaks of tyrosine (TYR) (A) and diiodotyrosine (DIT) (B) as a function of their concentration in HSA. In B, the difference absorbance of native HSA at 302 μm is taken as 0. Data taken from Figs. 1 and 2.
Iodination of Human Serum Albumin

**Molecular Properties of Iodinated Human Serum Albumin Preparations**

**Sedimentation Velocity**—Interpretation of the difference spectra and ionization curves depends on knowing the effect of iodination on the molecular form of HSA. The sedimentation rate of HSA increased with iodination. Fig. 7 shows the dependence of the sedimentation coefficient of 0.8% solutions of HSA on the content of iodine as determined by iodine analyses. The $s_{20, w}$ of native HSA was slightly above 4.0. Incorporation of approximately 5% iodine resulted in an increase of almost 20% in the $s_{20, w}$. A "theoretical" curve of $s_{20, w}$ is also plotted in Fig. 7 and will be discussed later.

**Viscosity**—The effect of iodination on the molecular shape of HSA was evaluated by viscosity measurements. The reduced specific viscosity of 1% solutions of HSA in 0.1 M NaCl-0.01 M glycine, pH 9.2, did not vary with the degree of iodination. A value between 0.040 and 0.044 dl per g was found for all preparations. Oncley, Scatchard, and Brown (12) have reported that the intrinsic viscosity of human serum albumin is 0.042 dl per g.

**Optical Rotatory Dispersion**—Optical rotatory dispersion measurements of HSA were made in order to determine the effect of iodination on the helical content, and to look for Cotton effects in the region of tyrosyl and iodotyrosyl absorption. The Moffitt constant, $b_0$, determined from the dispersion data for several samples of iodinated HSA at two pH values, is listed in Table II. A $b_0$ of −330 was found for native HSA, which is in close agreement with the value reported by Jirgensons (13). Lightly iodinated samples had essentially the same dispersion, indicating that mild iodination does not affect the helical structure. In a more highly iodinated sample, No. 8, $b_0$ decreased to −250, indicating a small loss of helical structures. No Cotton effects were observed between 260 and 300 nm in any of the samples examined.

**Solubility**—Solubility studies of native and iodinated HSA showed no loss in solubility in Samples 2 through 6 (Table II). In these preparations the optical rotatory dispersion was hardly affected. At higher levels of iodination, however, HSA lost its characteristic solubility. Preparations after reaction with 50 or more moles of I$_2$ became insoluble at approximately pH 5 in the precipitating buffer.

**Immunological Behavior** The immunological behavior of several preparations of HSA is also noted in Table II. All samples tested gave reactions of identity with native HSA against horse antihuman serum. The reaction of the most heavily iodinated sample (No. 11) was weaker than that of the native; the reactions of all of the other samples were indistinguishable from the native preparation.

**DISCUSSION**

**Molecular Conformation**

In order to interpret the difference spectra and sedimentation data of iodinated albumin, it was necessary to determine whether...
iodination produced changes in the internal organization or molecular form of the protein. In Samples 2 through 6, in which as many as 2.5 residues of monoiodotyrosine and 4 to 5 residues of diiodotyrosine were formed by iodination, little or no change was observed in viscosity, optical rotatory dispersion, solubility, or immunological behavior. The lack of significant molecular change is consistent with the results of Hughes and Stassie (14), who were able to crystallize HSA containing up to 12 atoms of iodine per mole. Moreover, Baldwin, Laughton, and Alberty (15) found only a minor increase in the electrophoretic heterogeneity constant, $\beta$, in HSA containing 7.2 atoms of iodine. In preparations containing 18 atoms of iodine, a larger increase in $\beta$ was observed (15). At higher levels of iodination, the optical rotatory and solubility properties of HSA were modified, whereas the viscosity and antigenicity remained unaltered. Evidently the former two methods are more sensitive than the latter two to minor structural changes in iodinated HSA.

The expected change in sedimentation coefficient of HSA with iodination can be calculated by the Svedberg equation from the increase in mass and density, assuming that the frictional coefficient remains constant. This is a reasonable assumption since the viscosity did not change with iodination. Assuming that iodination caused no change in the molecular form of HSA, the calculated sedimentation coefficient, based on the total amount of iodine incorporated, agrees very well with the experimental value up to 3% iodine (about 15 atoms per mole), but diverges at higher levels of iodination (Fig. 7). The failure to account for other changes in the chemical composition of HSA resulting either in changes in density or in minor structural alterations may account for the divergence at higher levels of iodination.

**Reactivity of Tyrosyl Residues**

It is necessary to evaluate both the ionization behavior of the phenolic hydroxyl groups and the accessibility of the ortho positions in order to assess the reactivity of the tyrosyl residues in proteins toward iodine, since iodination takes place at the ortho positions of the ionized form of tyrosyl residues (10). The properties of these two activities of the tyrosyl residues can be investigated independently, the phenolic ionization by spectrophotometric titration, and the exposure of the aromatic ring (to the solvent) by ultraviolet difference absorption measurements (2). Most of the tyrosyl residues in HSA ionize after a pH-dependent, reversible molecular expansion occurs near pH 10.5 (7, 17). Consequently, it is not possible to evaluate their ionization properties in the native form of the protein. However, some insight into their behavior may be afforded by the ionization constants of their iodinated derivatives.

The conversion of tyrosine to diiodotyrosine results in a reduction in $pK_a$ of about 4 units (18). Consequently, the diiodotyrosyl residues in iodinated HSA should ionize before the alkaline conformational change takes place. In accord with the Linderstrøm-Lang equation, the diiodotyrosyl ionization data show a linear dependence of $pH - \log [\alpha/(1-\alpha)]$ on $Z$ (Fig. 6). However, the electrostatic interaction factor, $\omega$, computed from the slope of the line was abnormally large. A large $\omega$ value is also apparent in the diiodotyrosyl absorbance curve (Fig. 5) since it extends over 6 pH units, i.e. from pH 4 to 10. The $\omega$ value of 0.11 computed from the curve in Fig. 5 is 4 to 5 times larger than the value determined for other residues of HSA in solutions of the same ionic strength (7, 17). This anomalously large value cannot result from changes in either molecular size or shape, since these properties remain essentially constant at low levels of iodination. Moreover, any unfolding or structural disorganization would tend to reduce $\omega$. The broad ionization curve may therefore be interpreted by assuming that the $pK_{int}$ values vary for different diiodotyrosyl residues, rather than by virtue of unusual electrostatic interaction effects, although the latter cannot be excluded.

Differences in $pK_{int}$ values are to be expected if the environments of the diiodotyrosyl residues vary. Kendrew (19) has classified the amino acid residues in myoglobin into three categories, i.e. internal, surface, and external (protruding into the solvent). If some of the phenolic hydroxyl groups of HSA are internal or surface, their $pK_{int}$ values may be modified by interactions with their neighbors. Since these interactions may be stronger for either the ionized or the un-ionized form, their $pK_{int}$ values may be either lower or higher than those of the external groups. The extended pH range of diiodotyrosyl ionization and the abnormally low $pK_{int}$ value obtained by extrapolation to $Z = 0$, i.e. 5.9, suggest that both possibilities may occur. Heterogeneity in $pK_{int}$ values may result either from variable intramolecular interaction or from molecular heterogeneity. Petersen and Foster (20) have separated crystalline HSA into fractions with similar but nonidentical physical properties. Without studying the properties of iodinated fractions it is not possible to distinguish between the two possibilities.

A Linderstrøm-Lang plot of the monoiodotyrosyl ionization data reveals a sharp break in the curve where the slope changes from a value similar to that for the diiodotyrosyl data to one close to zero. Both the break in the monoiodotyrosyl curve in iodinated HSA and the alkaline expansion of native HSA occur at approximately the same net charge, i.e. $-45$. Evidently, the formation of several residues of monoiodotyrosine and diiodotyrosine has little or no effect on the pH stability of HSA. Above this charge (pH about 10.6) the slope of the monoiodotyrosyl data is close to zero, indicating that charge interactions have been essentially eliminated. In agreement with the behavior of the monoiodotyrosyl residues at pH values above 10.6, the ionization of the tyrosyl residues (Preparations 3 and 5) also shows no dependence on $Z$.

The difference spectra clearly reveal the disappearance of internal or surface tyrosyl residues during iodination. A diiodotyrosyl difference peak developed concomitantly with the loss of the tyrosyl difference peak. Some of the diiodotyrosyl residues must therefore be classified as internal or surface. It should be noted, of course, that the spectral data were obtained at pH 3.9, whereas iodination was carried out at pH 9.1. Herskovits and Laskowski (21) have reported that about 40% of the tyrosyl residues in HSA are exposed to the aqueous solvent at neutral pH, and that an additional 20% become exposed in the acid transition between pH 5 and pH 3. At pH 3.9, therefore, the difference spectra are probably smaller than would be observed at neutral pH if measurements could be performed at neutrality.

The greater decrease in the tyrosine difference peak at 286 mp (Fig. 4) at lower compared to higher levels of iodination indicates that some of the unexposed residues are more reactive toward iodine than are the exposed residues. This unexpected result suggests that environmental interactions may enhance the reactivity of surface (or perhaps internal) residues relative to external ones.
Solvent perturbation experiments, following the procedure of Herskovits and Laskowski, designed to determine the percentage of exposed residues (21), permitted only a rough estimate. With either propylene glycol (20%) or dimethylsulfoxide (20%) as perturbant, the difference peaks of the tyrosyl and diiodotyrosyl residues in Preparations 3 and 5 were not as well defined as in the water as opposed to 8 M urea experiments. The difference peaks of both residues were about 20 to 40% as strong in water as in 5 M guanidine.

The conversion of unexposed tyrosyl to unexposed diiodotyrosyl residues may be envisioned to occur by the following pathway. Presumably, tyrosyl residues must be exposed when they are iodinated. The iodination of internal or surface tyrosyl residues suggests that an equilibrium exists between two (or more) forms of the protein, in which residues are unexposed in the native form but exposed in the reactive form (23). Consequently their environment would still be largely that of the protein, and could be quite different from the environment of the external tyrosyl residues.

Mayberry, Rall, and Bertoli (16) have determined the rate constants for the first iodination of N-acetyltirosine (k1) and the iodination of N-acetylmonoiodotirosine (k2). The ratio, k1/k2, will control the relative amounts of monoiodotirosine and diiodotirosine formed in the presence of limiting amounts of iodine. Of particular interest is their finding that the ratio of the two rate constants, k1/k2, is independent of pH, ionic strength, and base catalyst in aqueous media. However, this ratio decreased significantly in solvents which lower the dielectric constant of the medium (e.g. methyl alcohol). Conversely, in urea solutions, in which the dielectric constant is greater than that of water, the ratio k1/k2 increased. It is clear, therefore, that the relative amounts of monoiodotirosine and diiodotirosine (or analogues) that are formed will depend in part on the polarity, or the relative concentration of water, in the neighborhood of the reactive tyrosyl residues. The low yield of monoiodotirosine obtained by iodination of HSA can thereby be rationalized by assuming that iodine reacts with tyrosyl residues in the environment of the protein, which will be less polar than the aqueous solvent. The polarity at the reaction site will depend on the topology of the protein near the reactive residues.

Of the proteins in which the concentrations of monoiodotirosine and diiodotirosine have been determined as a function of the degree of iodination, several show low yields of monoiodotirosine, i.e. HSA, rabbit γ-globulin (24), and bovine thyroglobulin (1). This low yield can be explained by a low ratio of k1/k2, which, in turn, may be due to iodination taking place in a relatively nonaqueous environment. In all three proteins the monoiodotirosine formed at low levels of iodination did not disappear at higher levels. The persistence of these monoiodotirosyl residues suggests that they are strongly stabilized by interactions with other residues in the protein. In several other proteins, e.g. ribonuclease (25) and insulin (26), the yields of monoiodotirosine and diiodotirosine with increasing degrees of iodination are in closer agreement with the model studies of Mayberry et al. (16) and Roche et al. (27) in aqueous solution. In these proteins, the reactive tyrosyl residues are presumably external and freely accessible to the solvent. Only 3 of the 6 tyrosyl residues in ribonuclease react with iodine in aqueous solution; the other 3, unreactive residues are presumably well buried.

The ultraviolet difference spectral studies have shown that, in the iodination of HSA, tyrosyl residues which are shielded from the solvent are converted to similarly shielded diiodotirosyl residues. The location of tyrosyl residues in a partly nonaqueous environment within proteins directs their preferential conversion to diiodotirosyl residues.

REFERENCES
