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Nitration of Human Serum Albumin and Bovine and Human Goiter Thyroglobulins with Tetranitromethane*

P. Gordon Malan† and Harold Edelhoch

ABSTRACT: Tetranitromethane was used as a chemical probe to examine the physical and chemical behavior of tyrosyl residues of human serum albumin and of bovine and human goiter thyroglobulins. Only 50% of the tyrosyl residues of human serum albumin could be nitrated using excess reagent at pH 8.0. About 70% of the 125 tyrosyl residues of both thyroglobulins were nitrated at high levels of tetranitromethane. Sedimentation velocity ultracentrifugation indicated little structural change in the proteins until high levels of nitration were reached. Human goiter thyroglobulin (containing 0.06% iodine) was chemically iodinated with tracer ^{131}I. Subsequent nitration with tetranitromethane released about 20% of the iodine. Spectrophotometric titration data of nitrophenol and phenol groups in nitrated serum albumin were analyzed by the Linderstrøm-Lang theory. The plot showed two regions where the electrostatic interaction factor was about four and two times the value obtained by other workers for the ionization behavior of native serum albumin over the same pH range. The nitrotyrosyl groups in human serum albumin appear to be represented by a range of pK values.

Ultraviolet difference spectroscopy, using 8 M urea as perturbant, showed red-shifted nitrotyrosyl difference peaks in nitrated thyroglobulin and serum albumin. In contrast to iodotyrosyl residues which show blue-shifted spectra, nitrotyrosyl residues are exposed to the solvent. This difference in behavior presumably reflects differences in polarity between nitro- and iodophenol groups. Nitration of free tyrosine was also examined and the results indicated that nitrotyrosine and other by-products were formed.

This report is part of a continuing study to characterize the chemical and physical behavior of the tyrosyl residues of thyroglobulin (Edelhoch and Perlman, 1968). The function of thyroglobulin is to synthesize (and store) the thyroid hormones, triiodothyronine and thyroxine. Since the hormones are formed in the protein by iodination and coupling of tyrosyl residues, the latter constitute what could be referred to as the active sites of this protein. In bovine thyroglobulin containing about 1% iodine (distributed in about 10 monoiodotyrosyl, 10 diiodotyrosyl, 5 thyroxyl, and less than 1 triiodothyronyl residues) about 35% of the iodinated tyrosyl residues have been coupled to form iodothyronyl residues. This coupling of iodotyrosyl residues, which occurs when only a relatively small fraction (~20%) of the tyrosyl groups have been iodinated, results in a high efficiency of conversion of iodine from precursor to hormonal forms, *i.e.*, about 45% (de Crombrugghe *et al.*, 1967).

The present study is an evaluation of the reactivity of the tyrosyl residues in thyroglobulin with tetranitromethane,[1] a reagent which attacks the same sites as iodine. Since the reaction mechanism of nitration of TNM is different from iodination (Bruice *et al.*, 1968), the influence of the environment of the tyrosyl residues in controlling their reactivity is evaluated. This reagent should also be useful in future studies designed to distinguish between those tyrosyl residues which can couple to form hormone and those which cannot. If the sequence of thyroglobulin becomes known it should then be possible to identify the residues which form hormones on iodination but are prevented by nitration.

It has been shown in several enzymes that tyrosyl residues which constitute part of the active site are preferentially nitrated with TNM (Riordan *et al.*, 1967; Cuatrecasas *et al.*, 1968; Skov *et al.*, 1969). Similar studies with iodination have revealed greater reactivity of tyrosyl residues in active site regions than those that are not involved in the function of the

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[1] Abbreviations used are: TNM, tetranitromethane: HSA, human serum albumin; Tg, thyroglobulin; NO_2Tyr, 3-nitrotyrosine.

protein or enzyme (Herriott, 1937; Simpson and Vallee, 1966; Wachsmuth, 1967; Schwartz and Reed, 1968; Wassarman and Kaplan, 1968).

Thyroglobulin is not soluble in aqueous solution below pH ~5. It is not possible, therefore, to study the properties of its nitrated derivatives throughout pH regions where nitrophenolic groups ionize. Consequently in order to evaluate the properties of nitrated tyrosyl residues in a protein throughout its ionization range, parallel studies have been performed on human serum albumin. The latter was selected because it is very soluble at all pH values and its structural and ionization properties have been extensively characterized.

Materials and Methods

Materials. Bovine thyroglobulin was prepared by the differential centrifugation procedure described previously (Edelhoch and Lippoldt, 1964), except that 0.1 M KCl was substituted for 0.1 M KNO_3. This preparation contained about 95% of 19S thyroglobulin and less than 5% of the 27S species as measured by velocity sedimentation. The iodine content was 1.1% (analysis was performed by Boston Medical Laboratories).

Human thyroglobulin, obtained from patients with simple goiter, was a gift from Prof. M. Andreoli, Rome, and contained only traces of 12S and 27S species. The iodine content was 0.06%. A molecular weight of 660,000 for both bovine (Edelhoch, 1960) and human goiter (Andreoli *et al.*, 1969) thyroglobulins was used in calculations.

Crystallized human serum albumin was obtained from Pentex, Inc. A single symmetrical peak was observed by velocity sedimentation, and a monomer molecular weight of 66,000 was assumed (Low, 1952).

Tyrosine was a product of Mann Research Laboratories, New York, N. Y.; 3-nitrotyrosine was obtained from K & K Labs, New York, N. Y.; and 3,5-dinitrotyrosine was purchased from Sigma Chemical Company, St. Louis, Missouri. Glycylnitrotyrosyl methyl ester and glycylnitrotyrosyldiketopiperazine were prepared by Dr. M. Wilchek (Weizmann Institute, Rehovoth, Israel) and were chromatographically pure. Glass distilled water was used throughout, and all chemicals were reagent grade.

Methods. NITRATION. 1. PROTEINS. The conditions of Sokolovsky *et al.* (1966) were followed. Tetranitromethane (Aldrich Chemical Co.) was diluted to 83 mM with absolute ethanol immediately before use. A protein concentration of about 1% was used routinely when nitration was performed in 0.10 M Tris–0.10 M KCl buffer, pH 8.0. The protein concentration was reduced to below 0.5% when nitration was carried out in the same buffer containing 8 M urea since at higher protein concentration an insoluble gel formed. A small volume of TNM in ethanol was added to the protein solution at room temperature to give the required ratio of moles of TNM per mole of protein (hereafter referred to as molar ratio of TNM). After 60 min the solution was dialyzed exhaustively at room temperature against several changes of either 0.10 M Tris–0.10 M KCl buffer, pH 8.0, or the same buffer containing 8 M urea, to remove nitroformate and unreacted TNM.

2. TYROSINE. Tyrosine (1.38 mM) in 0.10 M Tris–0.10 M KCl buffer, pH 8.0, was nitrated in the same manner as described above. After 60 min at room temperature, nitroformate and unreacted TNM were removed by two extractions with tri-*n*-butyl phosphate and then two extractions with ether (Beaven and Gratzer, 1968). The organic solvent extraction procedure was checked using tyrosine and nitrotyrosine. Neither of the amino acids was removed from the aqueous phase, while all the TNM and nitroformate were extracted.

Concentrations of Model Compounds. Stock solutions of known concentration in 0.10 M Tris–0.10 M KCl buffer, pH 8.0, were prepared from 3-nitrotyrosine which had been dried to constant weight. The change in molar absorbance ($\Delta\epsilon$) between fully ionized and un-ionized forms of nitrotyrosine was found to be 4300 at 428 nm in water, and 4600 at 436 nm in 8 M urea, and these values were used in the calculation of nitrotyrosyl residues in thyroglobulin and HSA. They are close to the values of $\Delta\epsilon_{440nm}$ 4760 reported by Beaven and Gratzer (1968) and ϵ_{428nm} 4600 found by Verpoorte and Lindblow (1968), but are somewhat larger than the value of ϵ_{428nm} 4100 used by Sokolovsky *et al.* (1966). Other pertinent molar extinction coefficients are shown in Table I.

TABLE I: Molar Extinction Coefficients of Tyrosine and 3-Nitrotyrosine in Water and 8 M Urea Solutions.

Sample	Solvent	pH	λ (nm)	ϵ_λ
Tyrosine	H_2O, 0.10 M Tris–0.10 M KCl	8.0	280	1,200[a]
	8 M urea, 0.10 M Tris–0.10 M KCl	8.0	280	1,400
Nitrotyrosine	H_2O, 0.10 M Tris–0.10 M KCl	10.8	280	4,300
	8 M urea, 0.10 M Tris–0.10 M KCl	10.6	280	4,500
	H_2O, 0.10 M Tris–0.10 M KCl	10.8	428	4,450
	8 M urea, 0.10 M Tris–0.10 M KCl	10.6	436	4,800
Tyrosine	H_2O, 0.10 M KCl	7.0	210	6,600
Nitrotyrosine	H_2O, 0.10 M KCl	7.0	210	11,100

[a] From Wetlaufer (1962).

The concentrations of glycylnitrotyrosyl methyl ester and glycylnitrotyrosyldiketopiperazine were calculated from the absorption of the nitrophenolate group using the molar extinction value of 3-nitrotyrosine. The concentration of tyrosine was obtained from the molar extinction coefficients shown in Table I.

Determination of Protein Concentration. A value of 10.5 was used for the $E^{1\%,\,1\,cm}_{280\,nm}$ of thyroglobulin (Edelhoch, 1960). The low-iodine human goiter thyroglobulin was *assumed* to have an $E^{1\%,\,1\,cm}_{280\,nm}$ 10.0 (van Zyl and Edelhoch, 1967). A value of $E^{1\%,\,1\,cm}_{280\,nm}$ 5.3 was used for human serum albumin (Cohn *et al.*, 1947).

Since nitrotyrosine absorbs strongly in the 280-nm region the following procedures were used to measure the protein concentration of nitrated samples.

1. EXTINCTION AT 280 nm. From Beer's law, the concentration of protein, c, in g/100 ml, using a 1-cm light path is:

$$c = A_{\lambda_1}^{c}/E_{\lambda_1} \quad (1)$$

where A is the protein absorption, $E_{\lambda_1} = E_{\lambda_1}^{1\%,\ 1cm}$, and λ_1 is the wavelength at which protein concentration is usually measured, *i.e.*, 280 nm.

The amount of nitrotyrosine present in the protein may be obtained by measuring the difference in absorption (ΔA) between the nitrotyrosyl and nitrotyrosylate chromophores at 436 nm in 8 M urea solution (wavelength λ_2). The molar concentration of nitrotyrosine (NT) in the protein (see section on spectral titrations) will then be:

$$c^{NT} = \Delta A_{\lambda_2}{}^{NT}/\Delta\epsilon_{\lambda_2}{}^{NT} \quad (2)$$

Since both tyrosine (T) and nitrotyrosine absorb at 280 nm, the protein absorption, A_{λ_1}, in eq 1 must be corrected for the absorption due to nitrotyrosine:

$$A_{\lambda_1}{}^{*} = A_{\lambda_1}{}^{\text{Measured}} - A_{\lambda_1}{}^{NT} + A_{\lambda_1}{}^{T} \quad (3)$$

where $A_{\lambda_1}{}^{*}$ is the absorbance of the unnitrated protein. Protein absorbance measurements at 280 nm were made in 8 M urea at pH 8.6, where the nitrophenolate groups in the protein are about 90% ionized (see Figure 3). The model peptide, glycylnitrotyrosyl methyl ester, in 8 M urea was found to have $\epsilon_{280\ nm}$ 4900 and 4950 at pH 10.0 and pH 8.6, respectively. It should be noted that the absorbance of the nitrophenolate group at 280 nm is less than that of its unionized form. Using the molar extinction coefficients at 280 nm for the nitrotyrosyl peptide, and that for tyrosine shown in Table I, then:

$$A_{\lambda_1}{}^{NT} = c^{NT} \times \epsilon_{\lambda_1}{}^{NT} \text{ and } A_{\lambda_1}{}^{T} = c^{NT} \times \epsilon_{\lambda_1}{}^{T} \quad (4a,b)$$

Combining eq 1–4, the concentration of protein in grams per 100 ml is:

$$c = \{A_{\lambda_1}{}^{\text{Measured}} - (\epsilon_{\lambda_1}{}^{NT} - \epsilon_{\lambda_1}{}^{T})\Delta A_{\lambda_2}{}^{NT}/\Delta\epsilon_{\lambda_2}{}^{NT}\}/E_{\lambda_1} \quad (5)$$

2. EXTINCTION AT 210 nm. The protein in 0.10 M Tris–0.10 M KCl buffer, pH 8.0, was diluted with 0.1 M KCl, and a similarly diluted buffer sample was used as the reference blank. Extinctions at 210 nm were read in a Beckman Model DU spectrophotometer: adherence to Beer's law was confirmed. The mean $E_{210\ nm}^{1\%,\ 1cm}$ values and ranges were 223 ± 8 for five samples of thyroglobulin (*cf.* Salvatore *et al.* (1965) who found a value of 201 for 19S thyroglobulin) and 185 ± 3 for six samples of HSA. A small correction was used for nitrated preparations and it was obtained by using eq 5, substituting the relevant extinction coefficients shown in Table I.

The above spectrophotometric methods were checked by refractometry on a Brice-Phoenix differential refractometer (see Salvatore *et al.*, 1965). Agreement between the refractometric and E_{210} absorption measurements for the concentrations of three samples of nitrated thyroglobulin were within the limits of experimental errors (<5%).

Spectrophotometric Titrations of Proteins. All titrations were carried out on protein or amino acid solutions containing either 0.10 M or 0.15 M KCl, and 0.01 M lysine–0.005 M K_2HPO_4. Where 8 M urea was used, the solution contained 8 M urea–0.10 M KCl–0.01 M lysine–0.005 M K_2HPO_4. Small volumes of concentrated KOH or HCl were added from an Agla syringe to the solution in a 1-cm² cuvet, and corrections were applied for the small volume changes. Absorbance was measured in a Beckman Model DU spectrophotometer, and spectra were obtained in a Cary Model 14 recording spectrophotometer. Measurements of pH were made on a Radiometer pH meter (PHM 25) equipped with a scale expander (PHA 925) and a small diameter composite electrode (4858-L60, purchased from A. H. Thomas Co., Philadelphia, Pa.).

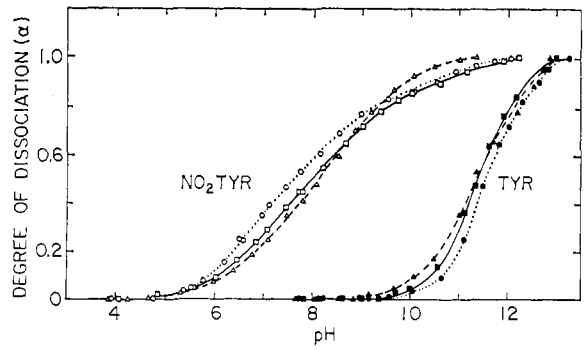

FIGURE 1: Spectral titration curves of phenolic and nitrophenolic groups followed at 295 and 428 nm, respectively, in three preparations of HSA nitrated with 5 (○, ●), 13 (□, ■), and 99 (△, ▲) molar ratios of TNM: solvent, 0.15 M KCl–0.01 M lysine–0.005 M K_2HPO_4.

Protein Tyrosyl and Nitrotyrosyl Analysis. A. NITROTYROSYL CONTENT. The amount of nitrotyrosine in proteins was obtained by spectrophotometric titration at 436 nm in 8 M urea (measured between pH limits of 3.0 and 10.6) using a $\Delta\epsilon$ value of 4600.

B. TYROSYL CONTENT. The tyrosyl content of proteins was obtained from absorbance changes at 295 nm in 8 M urea between pH values of 8.5 and 12.8 ($\Delta\epsilon$ 2550) as described by Edelhoch (1962). In 8 M urea the tyrosyl and nitrotyrosyl ionization curves of thyroglobulin and HSA, measured at 295 nm and 436 nm, respectively, overlap only slightly, *i.e.*, between pH 9 and 10, whereas they overlap significantly in water solutions (see Figure 1). Since nitrotyrosine absorbance changes were found to be trivial between pH 8 and 11 at 295 nm in 8 M urea no correction for nitrotyrosine contribution to the tyrosyl absorption was necessary.

Ultraviolet Difference Spectra. In nitrated thyroglobulin and HSA, nitrophenolic ionization is almost completely eliminated by about pH 3 in water, and by about pH 4 in 8 M urea. To obtain difference spectra that were not complicated by nitrotyrosyl ionization it was necessary to make measurements near pH 3. Difference spectra were obtained on a Cary Model 14 recording spectrophotometer equipped with a 0–0.1 slide wire.

A. HSA. Absorption spectra of solutions of nitrated HSA containing 2.7 mg/ml in 0.1 M sodium acetate, pH 3.0 or 2.0, were compared with the same solutions in 8 M urea.

B. THYROGLOBULIN. Solutions of thyroglobulin become turbid below about pH 4.8, but remain clear down to pH 3 if they contain 2 M urea. This level of urea has almost no effect on the structure of thyroglobulin as neither the viscosity

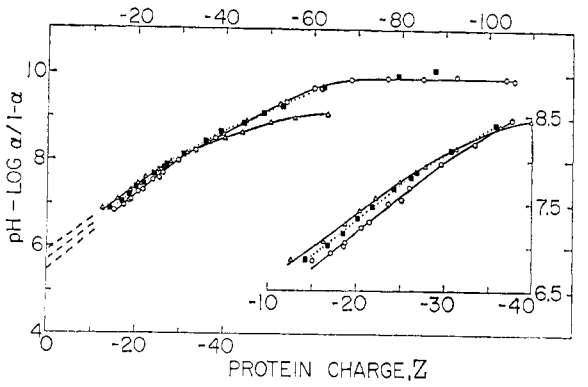

FIGURE 2: Linderstrøm-Lang plots of the nitrophenolic titration data shown in Figure 1. HSA was nitrated with 5 (○), 13 (■), and 99 (△) molar ratios of TNM. The insert is an expansion of the first part of the curves.

nor optical rotation of thyroglobulin changes at pH 7.0 in 2 M urea (Edelhoch and Lippoldt, 1964). Difference spectra were obtained on nitrated thyroglobulin (1.6 mg/ml) in 2 and 8 M urea containing 0.02 M glycylglycine buffer, pH 3.00.

Sedimentation Velocity. The HSA preparations were all diluted to a protein concentration of 0.50% in 0.10 M Tris–0.10 M KCl buffer, pH 7.0 or 9.0; thyroglobulin concentrations were at 0.60% in the same solvent at pH 8.0. Sedimentation analysis was performed on paired samples (pH 7.0 and 9.0) in double sector and wedge cells at 56,000 rpm in a Spinco Model E ultracentrifuge at room temperature. Sedimentation coefficients were calculated by standard procedures and were corrected to water at 20° ($s_{20,w}$).

Iodination Followed by Nitration. Human goiter thyroglobulin was iodinated with a 100-fold molar excess of iodine containing tracer ^{131}I. The protein (0.9%) was iodinated with 0.04 M I_2 in 0.16 M KI (plus tracer ^{131}I) under conditions described by de Crombrugghe *et al.* (1967). Unbound radioiodide was removed by exhaustive dialysis against 125 volumes of 0.10 M Tris–0.10 M KCl buffer, pH 8.0, at 2° with five changes in 3 days.

Identical samples of iodinated thyroglobulin were nitrated with 50-, 100-, 200-, 300-, and 600-fold molar ratios of TNM. A sample of iodinated thyroglobulin and the nitrated proteins were dialyzed for 24 hr against distilled water adjusted to pH 9 with ammonia (ratio of volume of protein solution to diffusate was 1:200). Samples of each dialyzed protein and of each diffusate were assayed for ^{131}I (counting error of less than 2%).

Thin-Layer Chromatography. Nitrated tyrosine derivatives were separated on silica gel thin-layer plates (Quantum Industries; Q1-F plates, 20 × 20 cm) in *t*-amyl alcohol–dioxane–1 N ammonia (2:2:1, vol/vol) (Ouellette and Balcius, 1966) for 5 hr. Tyrosine, 3-nitrotyrosine, and 3,5-dinitrotyrosine were run as markers, and the plates were sprayed with ninhydrin to detect the amino acids.

Results

I. Spectrophotometric Titrations. A. Model nitro compounds. The apparent pK values of the nitrophenolic groups were evaluated in three nitrotyrosine compounds (*i.e.*, glycylnitrotyrosyldiketopiperazine, glycylnitrotyrosyl methyl ester, and 3-nitrotyrosine) in 0.10 M KCl and in 0.10 M KCl–8 M urea. As with the phenolic group (Donovan *et al.*, 1959), the titration curve of the nitrophenolic group in 8 M urea is displaced by almost one pH unit to higher pH values compared to its behavior in aqueous solution in 0.10 M KCl. From equilibrium theory, the degree of dissociation (α) of an ionizing group is related to the pH by the equation:

$$\text{pH} - \text{p}K = \log \alpha/(1 - \alpha) \qquad (6)$$

The apparent pK values were obtained from a plot of log $\alpha/(1-\alpha)$ *vs.* pH, and are reported in Table II along with two literature values.

TABLE II: Apparent pK Values of Several Nitrotyrosyl Compounds in Water and 8 M Urea, Obtained from Spectrophotometric Titration Data.

Compound	pK_a	Solvent
3-NO_2Tyr	6.8	0.20 M Tris–0.20 M acetate–0.50 M NaCl[a]
3-NO_2Tyr	7.5	8 M urea–0.10 M Tris–0.10 M KCl
N-AcNO_2Tyr	7.0	0.20 M Tris–0.20 M acetate–0.50 M NaCl[a]
Gly-NO_2Tyr-OMe	7.5	8 M urea–0.10 M Tris–0.10 M KCl
└Gly-NO_2Tyr┘	6.6	0.10 M KCl–0.01 M lysine–0.005 M K_2HPO_4
└Gly-NO_2Tyr┘	7.3	8 M urea–0.10 M Tris–0.10 M KCl

[a] From Riordan *et al.* (1967).

B. HSA. 1. Aqueous solution. The ionization curves of the nitrophenolic and phenolic groups of 3 samples of HSA, nitrated with 5-, 13-, and 99-fold molar ratios of TNM, giving 3.5, 5.8, and 8.7 nitrotyrosyl residues, respectively, are shown in Figure 1. The nitrophenolic dissociation curves were reversible and were measured at 428 nm, the peak of the longest wavelength absorption band of nitrotyrosine in water. The phenolic dissociation was followed simultaneously at 295 nm. It should be noted in Figure 1 that the nitrotyrosyl titration curve is spread over more than 6 pH units whereas the tyrosyl curve covers only about half of this range. The narrower range of titration of the phenolic groups is due to a change in the electrostatic interaction factor which reflects the structural change that occurs near pH 10.5 (Tanford *et al.*, 1955a,b).

The effects of molecular configurational changes on the behavior of ionizing groups are detectable when the titration data are evaluated in accord with the Linderstrøm-Lang equation:

$$\text{pH} - \log \alpha/(1 - \alpha) = \text{p}K - 0.868wZ \qquad (7)$$

A plot of pH $-$ log $\alpha/(1-\alpha)$ against Z, the net protein charge, yields a line whose slope is proportional to the electrostatic interaction parameter, w, and the intercept at zero charge

TABLE III: Apparent pK Values and Electrostatic Interaction Factors, w, for Nitrotyrosyl Residues in HSA.

Molar Ratio of TNM	Moles of NO_2Tyr/HSA	pK_1	w_1	w_2
5	3.5	5.5	0.099	0.061
13	5.8	5.7	0.093	0.054
99	8.7	5.8	0.089	0.038

gives the apparent pK of the ionizing group (see Tanford, 1962). The data in Figure 1 have been analyzed in this manner and appear in Figure 2. The net charge, Z, was obtained from Tanford's (1950) potentiometric titration curve for HSA which was corrected to molecular weight 66,000 and was adjusted for chloride binding (Scatchard *et al.*, 1957) and tyrosyl nitration. The plots give curves with three approximately linear regions with breaks at about $Z = -31$ and -65. Table III lists the apparent pK values of the nitrophenolic groups and the electrostatic interaction factors, w, of the three curves obtained graphically. Above $Z = -65$, the slopes are close to zero and occur in the pH region of the expanded form of serum albumin.

The values of pH $-$ log $\alpha/(1-\alpha)$ for the phenolic groups in the three preparations of nitrated HSA are also essentially independent of Z. It is noteworthy that the tyrosyl titration curve for the sample with 5.8 nitrotyrosyl residues is displaced by 0.2 pH unit to lower pH values than the sample with 3.5 nitrated groups. Since 2.5 tyrosyl now ionize as nitrotyrosyl residues, the shift indicates that the two preparations have comparable stability in this pH region.

2. 8 M Urea. The ionization curves of the nitrophenolic and phenolic groups of samples nitrated with 13 and 99 molar ratios of TNM was measured in 0.10 M KCl–8 M urea at 436 and 295 nm, respectively, and are shown in Figure 3. Linderstrøm-Lang plots of these data were made using Z values calculated from the potentiometric titration curve of HSA in 8 M urea obtained by Levy (1958). The nitrotyrosyl data showed only a single slope ($w = 0.028$) of much smaller magnitude than the data in water, with an apparent pK of 7.3. The tyrosyl ionization data for the two samples fell on a single curve with a very small slope ($w = 0.008$) and gave an apparent pK of 10.6. Both pK values are in agreement with those of model compounds in 8 M urea (Table II).

C. BOVINE THYROGLOBULIN. Spectrophotometric titration curves of the nitrotyrosyl and tyrosyl residues of nitrated bovine thyroglobulin could not be obtained in aqueous solution since denaturation occurs below pH ~5 where nitrophenolic groups are still partially ionized. Neither was it possible to obtain curves in 2 M urea, where thyroglobulin is soluble in acid, since back-titration from either acid or alkaline solution was not reversible. Even in 8 M urea the titration curve was not completely reversible.

II. Nitration. A. HSA. In Figure 4 is shown the number of nitrotyrosyl and tyrosyl residues in HSA as a function of the molar ratio of TNM. The two sets of points represent data obtained from two separate series of experiments. It can be seen that only half of the 17–18 tyrosyl residues were nitrated with TNM when a plateau was reached. In an experiment in

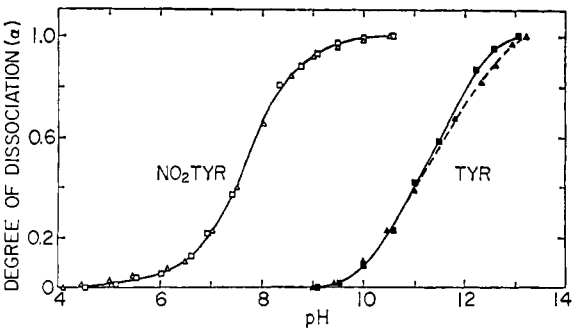

FIGURE 3: Spectral titration curves in 8 M urea of phenolic and nitrophenolic groups followed at 295 and 436 nm, respectively, in two preparations of HSA nitrated with 13 (□, ■) and 99 (△, ▲) molar ratios of TNM: solvent, 8 M urea–0.15 M KCl–0.01 M lysine–0.005 M K_2HPO_4.

8 M urea at pH 8.0 approximately the same number of tyrosyl residues were nitrated when the molar ratio of TNM was 73.

B. BOVINE THYROGLOBULIN. The number of nitrotyrosyl and tyrosyl residues in bovine thyroglobulin as a function of TNM is reported in Figure 5. The nitrotyrosyl curve approaches a plateau at a molar ratio of TNM to protein of 600 where about 95 tyrosyl residues are nitrated. In contrast to the data with HSA, the curve depicting the sum of the nitrated and unreacted tyrosyl residues increases by about 20 residues with nitration. The increase could be due to deiodination of iodotyrosyl residues in native bovine thyroglobulin by TNM. This sample contained 1.1% iodine or about 57 atoms per molecule. A second possible explanation is that the synthesis of a few dinitrotyrosyl residues would lead to an overestimation of the mononitrotyrosyl residues due to the greater molar absorption of the former at 436 nm. Nitration in 8 M urea at pH 8.0 at a molar ratio of 600 gave fewer nitrotyrosyl residues, *i.e.*, 75, than in water.

C. HUMAN THYROGLOBULIN. A sample of human thyroglobulin, containing only 0.06% iodine, was nitrated under the same conditions as were used for the bovine protein. The data shown in Figure 6 are similar to those obtained

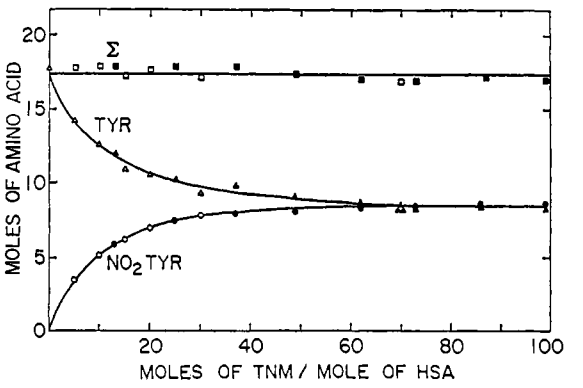

FIGURE 4: The distribution of tyrosyl (△, ▲) and nitrotyrosyl (○, ●) residues in native and nitrated HSA as a function of the molar ratio of TNM. Spectral analysis was carried out in 8 M urea. The data from two separate experiments are indicated by open and filled symbols respectively. The uppermost curve labeled Σ (□, ■) represents the sum of tyrosyl and nitrotyrosyl residues.

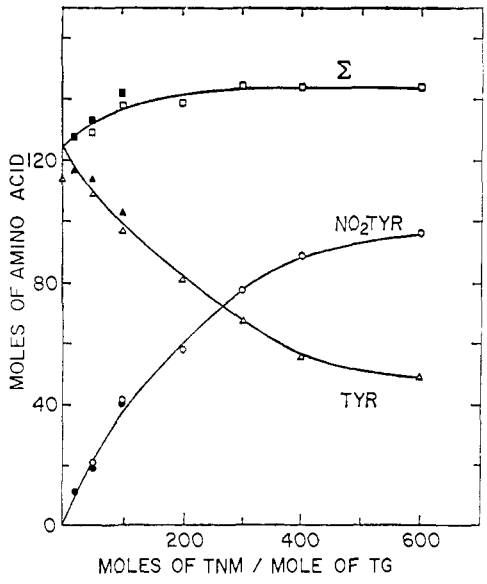

FIGURE 5: The distribution of tyrosyl and nitrotyrosyl residues in native and nitrated bovine thyroglobulin as a function of the molar ratio of TNM. Conditions and symbols are the same as those described in Figure 4. The data were obtained from two series of experiments.

with the higher iodine bovine sample. However, with the human low-iodine protein, the curve for the sum of nitrotyrosyl and tyrosyl residues remains constant at all levels of nitration. These data give indirect support to the deiodination mechanism suggested in the preceding section, since bovine and human thyroglobulins contain approximately the same number of tyrosyl residues (Rolland *et al.*, 1966). Nitration in 8 M urea gave almost the same nitrotyrosine curve as was obtained in water.

D. IODINATED HUMAN THYROGLOBULIN. The effect of nitration on the stability of the iodoamino acids was studied by first labeling human thyroglobulin with stable iodine containing tracer radioiodine. Iodination was performed with a molar ratio of iodine to protein of 100. The final iodo-

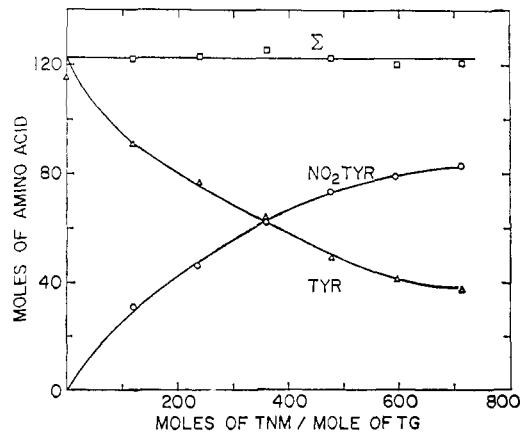

FIGURE 6: The distribution of tyrosyl and nitrotyrosyl residues in native and nitrated human goiter thyroglobulin as a function of the molar ratio of TNM. Conditions and symbols are the same as those described in Figure 4.

TABLE IV: Per Cent of Total Radioiodine Found in the Diffusate after ^{131}I-Labeled Human Goiter Thyroglobulin Was Nitrated with Different Levels of TNM.

TNM molar ratio	0	50	100	200	300	600
Per cent of total radioactivity in diffusate	1.5	13.5	12.8	19.1	20.5	19.9

amino acid content, as determined spectrally (Edelhoch, 1962), was: monoiodotyrosine, 12.7; diiodotyrosine, 18.3; thyroxine, 2.7; and tyrosine, 88. The calculated iodine content was therefore 1.16% (see de Crombrugghe *et al.*, 1967).

Samples of ^{131}I-labeled human thyroglobulin were nitrated at various levels with TNM and then dialyzed as described in the Methods section. Table IV gives the percentage of radioiodine that appeared in the diffusate at different molar ratios of TNM. The recovered radioiodine rose rapidly to a maximum of about 20% of the radioactivity originally present. This percentage corresponds to either deiodination of all the monoiodinated residues or complete deiodination of 6 of the 18 diiodotyrosyl residues.

III. Sedimentation Velocity. A. NITRATED HSA. The effect of nitration on the conformation of HSA was evaluated by measuring its sedimentation rate. The sedimentation coefficients of two nitrated samples were determined at Z values which fell in the middle of the first two linear portions of the Linderstrøm-Lang plot of the titration data (Figure 2). The selected Z values were about -21 and -39 which correspond to pH values of 7.0 and 9.0, respectively. Measurements at the two pH values on one sample were made in a single experiment, thereby eliminating the factors which depend on temperature in the comparison of their sedimentation rates. In Table V the sedimentation coefficients of native and two nitrated samples of HSA are reported, all measured at 0.50% protein concentration. There was no difference between the $s_{20,w}$ values of the native molecule between pH 7.0 and 9.0. The nitrated sample with 7.5 nitrotyrosyl

TABLE V: Sedimentation Coefficients of Native and Nitrated HSA in 0.10 M Tris–0.10 M KCl buffer, pH 7.0 and 9.0. Protein Concentration was 0.50%.

TNM Molar Ratio	Number of NO_2Tyr/HSA	pH	$s_{20,w}$
0	0	7.0	4.08
		9.0	4.05
25	7.5	7.0	4.11
		9.0	3.99
86	8.7	7.0	3.85
		9.0	3.65

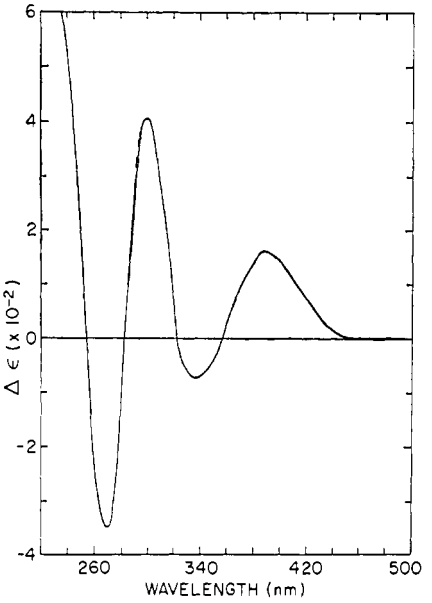

FIGURE 7: Ultraviolet difference spectrum of glycylnitrotyrosyl methyl ester in 8 M urea with respect to water (reference cell), pH 3.0. The *ordinate* values indicate the absorbance difference per mole.

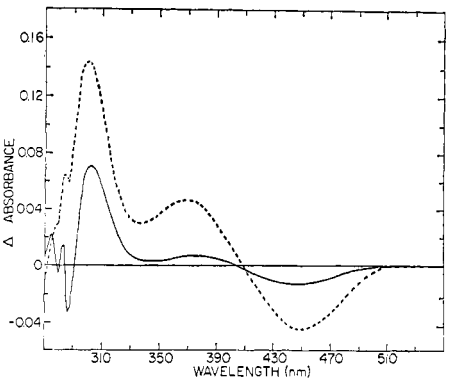

FIGURE 8: Ultraviolet difference spectra of nitrated HSA in 8 M urea with respect to water, pH 3.0. Nitration with 5 (——) and 15 (----) molar ratios of TNM in water at pH 8.0. Protein concentration was 0.27%, and solutions were 0.1 M in acetate.

residues per molecule showed a normal value at pH 7.0 and a marginal decrease at pH 9.0. The more highly nitrated sample (8.7 residues) sedimented significantly more slowly at both pH values. It should be noted that iodinated HSA showed greater sedimentation rates than the native molecule and these results could be accounted for by the increase in protein density due to the incorporated iodine (Perlman and Edelhoch, 1967).

B. NITRATED BOVINE THYROGLOBULIN. A series of six solutions of bovine thyroglobulin, nitrated with between 50 and 600 molar ratios of TNM, were examined by sedimentation velocity in 0.10 M Tris–0.10 M KCl buffer at pH 8.0 and at 0.60% protein concentration. About 10% of the subunit species, *i.e.*, 12S, appeared in the samples nitrated with molar ratios of 400 and 600. The sedimentation coefficients were 17.4 ± 0.3 at all levels of nitration between 0 and 400 molar ratios of TNM. At a molar ratio of 600, the sedimentation coefficient was significantly lower, *i.e.*, 15.8. There was also evidence of considerable broadening of the schlieren boundary at 400 and 600 molar ratios.

IV. Ultraviolet Difference Spectra. A. NITROTYROSINE. The effect of 8 M urea on the absorption spectrum of glycylnitrotyrosyl methyl ester in 0.10 M acetate, pH 3.0, is shown in Figure 7 as a difference spectrum. Two red-shifted bands are seen with peaks at 300 and 390 nm; a third peak evidently occurs below 240 nm.

B. NITRATED HSA. Figure 8 shows the effect of 8 M urea at pH 3.0, 0.10 M acetate, on the difference spectra of HSA nitrated with 5 and 15 molar ratios of TNM (3.5 and 6.2 nitrotyrosyl residues, respectively). The major positive difference peak occurs at 300 nm and corresponds exactly to the wavelength value observed in the model compound reported in Figure 7. The intensities of the peaks at 300 nm are approximately proportional to the number of nitrotyrosyl residues in the two samples. A second, minor positive peak occurs at a slightly lower wavelength, *i.e.*, 370 nm, than appears in the model. The lower wavelength peak may result from its being cut off by the neighboring longer wavelength band of opposite sign.

A negative difference peak is also found at about 450 nm in the two nitrated samples. The wavelength minimum of the difference peak was shifted from 450 to 455 nm and the magnitude was reduced by 50% when the pH of another nitrated HSA solution, containing 7.0 nitrotyrosyl residues, was reduced from pH 3.0 to 2.0. The wavelength of the absorption peak of ionized mononitrotyrosine is at 436 nm in 8 M urea (or at 428 nm in water), whereas that of ionized dinitrotyrosine in 8 M urea is at 454 nm. The pK values for mono- and dinitrotyrosine are 7.5 and 3.8, respectively, in 8 M urea. The 456-nm peak observed at pH 2 and part of the 450 peak at pH 3 are therefore probably due to a small amount of ionized dinitrotyrosine. Since the magnitude of this peak represents the ionization of only a trivial fraction of the nitrophenol groups, it is not apparent in the spectrophotometric titration curves illustrated in Figure 1.

It is noteworthy that in 8 M urea the nitrophenolic groups are red-shifted at pH 3.0 whereas iodophenolic groups in iodinated HSA at pH 3.9 are blue-shifted (Perlman and Edelhoch, 1967).

Two distinct negative difference peaks are evident at 286 and 279 nm, wavelength peaks characteristic of tyrosine difference spectra (Wetlaufer, 1962).

C. NITRATED BOVINE THYROGLOBULIN. Thyroglobulin nitrated with a 400-fold excess of TNM gave results similar to those found with HSA. A difference spectrum of the nitrated thyroglobulin at pH 3.0 was obtained by comparing the protein dissolved in 8 M urea with an identical sample in 2 M urea, since thyroglobulin is soluble in 2 M urea but not in water at this pH. A broad negative band was centered around 456 nm which is probably due to ionized dinitrophenol groups. Two red-shifted, positive peaks occurred at 305 and 375 nm, respectively. A negative band also occurred at 286 nm, representing blue-shifted unmodified phenol groups.

V. Nitration of Tyrosine. Tyrosine in 0.10 M Tris–0.10 M KCl buffer, pH 8.0, was nitrated with 1–10 moles of TNM per mole of tyrosine. Excess reagent and nitroformate were removed by solvent extraction as described above.

Spectrophotometric titration in 8 M urea was performed

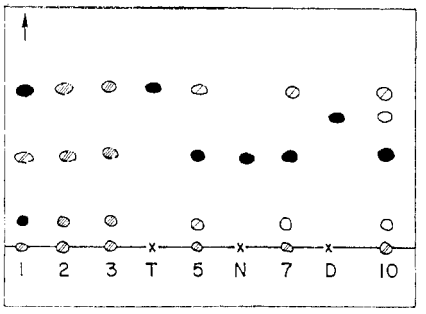

FIGURE 9: Sketch of thin-layer chromatogram of samples of tyrosine which had been nitrated with TNM. Molar ratios of TNM are indicated by numbers, and color intensity of spots is represented approximately by the degree of cross-hatching. The silica gel plate was developed for 5.0 hr in *t*-amyl alcohol–dioxane–1 N NH_4OH (2:2:1). Solvent was run to the top of the plate (not shown). R_F values of marker compounds were: tyrosine (T), 0.38; 3-nitrotyrosine (N), 0.23; 3,5-dinitrotyrosine (D), 0.31.

TABLE VI: Concentrations of Tyrosine and Nitrotyrosine Obtained by Spectral Titration after Nitration of Tyrosine in 0.10 M Tris–0.10 M KCl buffer, pH 8.0, with Increasing Molar Ratios of TNM.

TNM Molar Ratio	Concentration (mM)		
	Tyr	NO_2Tyr	(Tyr + NO_2Tyr)
0	1.38	—	1.38
1	0.93	0.13	1.06
2	0.47	0.22	0.69
3	0.32	0.29	0.61
5	0.22	0.36	0.58
7	0.21	0.41	0.62
10	0.21	0.43	0.64

to determine the amounts of tyrosine and nitrotyrosine present in each sample, and the results are shown in Table VI. The initial tyrosine concentration was 1.38 mM. The nitrotyrosine increased with TNM and reached a limiting value of 0.43 mM at a molar ratio of 10. Tyrosine decreased rapidly to reach a value of 0.21 mM when the TNM molar ratio was greater than 5. The sum of tyrosine and nitrotyrosine also fell sharply and reached a plateau in the same region as tyrosine. Some product other than nitrotyrosine must therefore be formed to account for the apparent decrease in the sum of tyrosine and nitrotyrosine observed by spectral titration. This compound is probably a tyrosyl derivative which interferes with the spectral titration, since the apparent level of tyrosine is constant while the nitrotyrosine concentration continues to increase (see below).

Each of the nitrated tyrosine samples shown in Table VI was examined by thin-layer chromatography. The results are illustrated in Figure 9. The ninhydrin spot corresponding to tyrosine decreased rapidly at first, and only a small amount was present in the 5 through 10 molar ratio samples. Nitrotyrosine increased throughout the series, and a trace of dinitrotyrosine was visible *only* in the experiment where the molar ratio of TNM to tyrosine was 10. Ninhydrin-positive spots were present at the origin of all the nitrated samples, but none was present at the origin of the marker tyrosine or nitrotyrosine compounds. A second spot, which was slightly removed from the origin, was maximal in the 1 to 2 molar ratio experiments but decreased in the remaining experiments with increasing levels of TNM.

The identity of the origin and near-origin spots is not known, though they may be polyphenol derivatives of tyrosine (see Andersen, 1966), whose formation might be mediated by free radicals derived from TNM. Bruice *et al.* (1968) postulated a free radical mechanism to explain the reaction kinetics of TNM with several phenols in water, where the products of reaction included nitrophenol and polyphenol compounds. Doyle *et al.* (1968) suggested that TNM caused cross-linking in collagen and γ-globulin when the nitrated proteins were examined by sedimentation velocity, but the nature of the cross-linkages was not determined.

Nitration of thyroglobulin or HSA in 8 M urea yielded approximately the same number of nitrotyrosyl residues as were found when nitration was performed in water. The effect of urea, at concentrations of 2, 4, 5, 7, and 8 M, on the nitration of tyrosine was therefore investigated. The nitrotyrosine and tyrosine concentrations were 0.40 ± 0.005 mM and 0.18 ± 0.015 mM, respectively, in all the urea solutions when the molar ratio of TNM to tyrosine was six. These are the same values as were obtained when tyrosine was nitrated in water with a sixfold molar ratio of TNM (*cf.* Table VI).

Discussion

Since the tyrosyl residues are the functional groups of thyroglobulin, their reactivity toward different reagents may indicate those properties which facilitate their iodination and coupling to form the thyroid hormones. This study is a parallel to the previous reports describing the reaction of iodine with thyroglobulin (van Zyl and Edelhoch, 1967) and HSA (Perlman and Edelhoch, 1967). As iodination *in vitro* leads to a similar distribution of iodoamino acids as occurs *in vivo* (de Crombrugghe *et al.*, 1967; Cavalieri *et al.*, 1970), it would appear that the structure of this protein controls the products that are formed by iodination.

Nitration or iodination of the tyrosyl residues of proteins with TNM or I_2, respectively, occurs by reaction with the phenolate anion (Bruice *et al.*, 1968; Sokolovsky *et al.*, 1966; Mayberry *et al.*, 1964). The rate of reaction of TNM increases with pH, but reaction with hydroxyl ions limits the alkalinity at which the reaction is usually performed (Schmidt, 1919).

The ionization of the nitrophenolic groups was followed spectrophotometrically at wavelengths where they are the only groups that absorb. The analysis of data obtained in this way is less ambiguous than potentiometric data where the division of the hydrogen binding curve between the various ionizable side chains can be somewhat arbitrary. Titration curves were obtained only on nitrated HSA, since those on nitrated thyroglobulin were not reversible. In 0.15 M KCl, the ionization of nitrophenolic groups in HSA preparations was spread over a wider pH range, *i.e.*, pH 4 to 12, than in the model compounds which ionized between pH 4.5 and 8.5.

The Linderstrøm-Lang curves of all three nitrated HSA preparations exhibit three distinctive slopes. The interaction term, w, of the first two regions, each of which covers about 40% of the total ionization, is about 4 and 2 times the magnitude of the value of w reported by Tanford *et al.* (1955b) in the corresponding pH range in native HSA (Table III). The third region, representing the final 20% ionization of the nitrophenolic groups, has zero slope and indicates that there are no longer any electrostatic interactions. The latter fraction of the nitrophenolic groups behaves similarly to that of the phenolic groups which ionize in the same pH range. The disappearance of electrostatic interactions is due to the molecular expansion of HSA in this pH region (Tanford *et al.*, 1955a).

The electrostatic interaction parameters, w, are clearly greater for the nitrophenolic groups ionizing below about pH 10.5 than those reported for any group in the native protein. The origin of the elevated values cannot be ascribed to an increase in molecular symmetry with a reduction in intercharge distances, since the smaller sedimentation coefficients of nitrated HSA suggest that the opposite effect takes place. Consequently the assumption of the Linderstrøm-Lang theory that all groups have a single pK value becomes questionable. Moreover, the extrapolated pK values (at $Z = 0$) are about one pK unit less for the three samples than is found with simple model compounds. Evidently these residues are in environments which affect their ionization behavior and result in pK values that are abnormal and distributed about an average value of 5.7. Shifts in pK values of nitrophenolic groups due to environmental effects have been reported for nitrated staphyloccal nuclease (Cuatrecasas *et al.*, 1968) and carboxypeptidase (Riordan *et al.*, 1967) upon addition of enzyme inhibitor molecules.

The abrupt change in slope at $Z = -31$ in all three samples suggests that a structural alteration takes place producing a molecular species intermediate in unfolding between that of the native and alkaline forms. The sedimentation coefficients of nitrated HSA (Table V), performed at pH values in the middle of the linear regions of the Linderstrøm-Lang plots, *i.e.*, pH 7.0 and 9.0 ($Z = -21$ and -39, respectively), show smaller values at the higher pH. In a few proteins, nitration does not appear to affect the structure or activity significantly (Beaven and Gratzer, 1968; Meloun *et al.*, 1968, 1969; Velpoorte and Lindblow, 1968). In lysozyme, however, enhanced reactivity of one disulfide group and susceptibility to tryptic digestion have been reported after nitration of two of its three tyrosyl residues (Atassi and Habeeb, 1969).

The environment of the aromatic rings of the nitrotyrosyl residues was evaluated independently by difference spectroscopy. The red shifts at 300 and 370 nm in nitrated HSA (and thyroglobulin) observed in 8 M urea at pH 3.0 suggest that these groups are in contact with solvent molecules in aqueous solution. Experiments were restricted to acid pH where the nitrophenolic groups are essentially undissociated. Thus measurements were obtained below the acid transition of native HSA, pH ~4 (Yang and Foster, 1954; Tanford *et al.*, 1955a), in which the degree of exposure of the phenolic groups increases from approximately 40 to 60% (Herskovits and Laskowski, 1962). This structural change could also affect the nature or magnitude of the red shift observed at pH 3.0. This result contrasts with that found with iodinated HSA, where strong blue shifts were observed in 8 M urea at pH 3.9 (Perlman and Edelhoch, 1967). The difference in pH between the two sets of experiments is not likely to change the difference absorption from a blue to a red shift. It appears that the nitrophenolic groups remain in contact with solvent molecules after formation, whereas the iodophenol residues are withdrawn into the protein. The difference in the degree of exposure may be due to the difference in polarity between the iodophenolic and nitrophenolic groups. A similar situation may prevail in carbonic anhydrase judging from the changes in its enzymatic and physical properties (Verpoorte and Lindblow, 1968). Loss in enzyme activity and optical activity was found with iodination whereas these properties were unaffected by nitration. One would expect greater structural disorganization by substituents that penetrate the interior of the protein compared to those that remain on the outside. Unfortunately difference absorption measurements were not reported with modified carbonic anhydrase so that the disposition of the altered residues is not known.

In addition to the difference in exposure found between nitrophenolic and iodophenolic groups, only half as many tyrosyl residues in HSA react with TNM as with iodine. In fact, the number that react with TNM is approximately equal to the number of exposed groups as measured by difference spectroscopy (Herskovits and Laskowski, 1962). It seems reasonable to assume that the groups that react with iodine and not with TNM are the unexposed ones and that the greater reactivity of iodine is due to its ability to penetrate the hydrophobic surface of the protein because of its low polarity. It has also been suggested that the unusual relation found in the monoiodotyrosine:diiodotyrosine ratios in HSA (and thyroglobulin) results from the iodination of tyrosyl residues which are in an environment which is less polar than the solvent and therefore give fewer monoiodotyrosyl residues (Perlman and Edelhoch, 1967; van Zyl and Edelhoch, 1967; Schwartz and Reed, 1968; Libor and Elodi, 1970). In harmony with these findings, it has been reported by Mayberry and Hockert (1970) that the ratio of the rate constants of the first and second iodinations of tyrosine is reduced when the polarity of aqueous solutions is lowered by the addition of organic solvents.

In both human and bovine thyroglobulin, about one-third of the total number of tyrosyl residues are unreactive since the nitration curves approach a plateau at the highest levels of TNM. The similarity in degree of nitration and iodination (van Zyl and Edelhoch, 1967; de Crombrugghe *et al.*, 1967) in thyroglobulin indicates that the unreactive groups in thyroglobulin are buried and completely inaccessible to either reagent, whereas the reactive groups do not appear to distinguish between the two reagents.

The significance of the incomplete nitration of the tyrosyl residues in 8 M urea in both HSA and thyroglobulin is unclear. In both proteins the aromatic chromophores are almost completely exposed to the solvent in 8 M urea (Edelhoch and Lippoldt, 1964; Herskovits and Laskowski, 1962). One difference between experiments in the two solvents is that the pK of the phenolic group is about 0.8 of a unit higher in 8 M urea than in water (Donovan *et al.*, 1959). This factor may affect the rate of nitration slightly, since it is the phenolate ion that is reactive with both reagents. In 8 M urea, exposure of buried methionine, cysteine, and tryptophan residues, which also react with TNM (Cuatrecasas

et al., 1968; Sokolovsky *et al.*, 1969), could not account for the incomplete nitration of all the tyrosyl residues as the nitrotyrosyl curve approaches a plateau at the highest levels of TNM. Reaction of free tyrosine with a sixfold molar ratio of TNM yielded identical results when the solvent was either water or 8 M urea. Chromatographic results suggested that a tyrosyl condensation product could have been formed (*cf.* Bruice *et al.*, 1968). Cross-linking of protein tyrosyl residues must therefore be considered as a possibility and has been observed by others (Doyle *et al.*, 1968; Boesel and Carpenter, 1970; Vincent *et al.*, 1970). Preliminary results indicate that intermolecular cross-linkages are formed when thyroglobulin or serum albumin is nitrated with large excesses of TNM in 8 M urea, but are not formed when the native protein is nitrated in water. Samples of nitrated HSA and thyroglobulin were examined by sedimentation velocity in synthetic boundary, double-sector cells in 8 M urea and 6 M guanidine. Aggregated material was observed in both proteins that were nitrated in 8 M urea, and normal patterns were found when nitration was in water.

Without further studies on model tyrosyl peptides, it is also not possible to exclude the influence of adjacent residues on nitration of the tyrosyl residues in the unfolded molecule that exists in 8 M urea.

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