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Preparation and Properties of 3-Nitrotyrosine Insulins*

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ABSTRACT: Bovine insulin at 2.5 mg/ml, pH 7.4, was nitrated by a 3- to 12-fold excess of tetranitromethane. The products were separated by ion-exchange chromatography (Sephadex A-25) at pH 8.4 into unreacted insulin, mono-3-nitrotyrosine insulin, and di-3-nitrotyrosine insulin. The positions of substitution were determined by oxidative sulfitolysis, followed by ion-exchange chromatography to separate the A and B chains. Substitution occurred almost entirely on the A chain, establishing the diconjugated derivative as di-(A14, A19)-3-nitrotyrosine insulin. Chymotryptic hydrolysis of the monoconjugated derivative established that it was predominantly mono-(A14)-3-nitrotyrosine insulin. The nitrated derivatives were used to confirm Tyr A14 as an anomolously ionizing residue. It is shown that the presence of a Tyr A14-carboxylate hydrogen bond explains the chemical, optical, and titration data on insulin. The results of bioassays and immunoassays are presented.

Many techniques have been developed to study the disposition of tyrosyl residues of proteins in solution. These techniques can be divided into two classes: spectroscopic (near-ultraviolet difference spectrophotometry, spectrophotometric titration, near-ultraviolet circular dichroism, and solvent perturbation spectrophotometry) and chemical modification with reagents that are more or less specific for tyrosyl residues. These different techniques give consistant results with many proteins. Ribonuclease, for example, has three of its six tyrosyl residues buried by the criterion of spectrophotometric titration (Shugar, 1952). Only the three exposed residues are reactive to iodination (Donovan, 1963) and *N*-acetylimidazole (Riordan *et al.*, 1965). These findings have been confirmed by X-ray crystallography (Wyckoff *et al.*, 1967). However, the identification of those tyrosyl residues that contribute to the near-ultraviolet circular dichroism has not yet been accomplished (Simmons and Glazer, 1967; Simpson and Vallee, 1966; Pflumm and Beychok, 1969; Beaven and Gratzer, 1968).

The situation with insulin is much more complex. One (Inada, 1961) or two (Shugar, 1952) of the four tyrosyl residues is assumed to be buried because of an abnormally high p*K*. However, reactivity toward iodination indicates that Tyr A14 and Tyr A19 are "exposed" (de Zoeten and Havinga, 1961). Reactivity toward cyanuric fluoride indicates that Tyr A19 and Tyr B16 are "exposed" (Aoyama *et al.*, 1965), while limited titration data of desoctapeptide insulin indicates that Tyr B26 ionizes normally (Aoyama *et al.*, 1965). Moreover, difference spectrophotometry indicates that Tyr B26 is strongly interacting (Laskowski *et al.*, 1960), while circular dichroism suggests that this residue may be involved in the monomer–dimer aggregation site (Morris *et al.*, 1968). These two techniques also suggest that the environment of another tyrosyl residue, likely A14, is dependent on an anomolous carboxyl group (Laskowski *et al.*, 1960; Morris *et al.*, 1968). However, all the data to date do not permit an unambiguous assignment of the four tyrosyl residues of insulin, nor allow identification of those residues that are "exposed" and "buried" by the criterion of solvent perturbation spectrophotometry (Weil *et al.*, 1965; Menendez *et al.*, 1969).

Recently, tetranitromethane has been added to the available tyrosine reagents (Riordan *et al.*, 1966). Because more data on the tyrosyl residues of insulin are clearly needed, and because the tyrosyl residues of insulin have been implicated in the expression of biological and immunological activity (Arquilla *et al.*, 1968), we have prepared and characterized the 3-nitrotyrosine derivatives of insulin.

Material and Methods

We used two bovine zinc insulin preparations (Lilly, lot 2842 and Novo, lot 016666). Tetranitromethane was obtained from Baker, sodium tetrathionate from K & K Labs, α-chymotrypsin from Calbiochem, and 3-nitrotyrosine and 3,5-dinitrotyrosine from Nutritional Biochemicals. All other chemicals were reagent grade.

Nitration. To limit the extent of reactions, nitration was performed for short times on highly aggregated insulin solutions. Zinc insulin at 2.5 mg/ml in 0.11 M phosphate buffer, pH 7.4, was treated at 23° for 15–50 min with a 3- to 12-fold excess (with respect to tyrosyl residues) of tetranitromethane.

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The reaction was terminated by adjusting the pH, 5.2, and the precipitate collected by centrifugation. Nitration was also carried out in homogeneous solutions of 10% ethanol; we noted no significant difference between the two methods.

Purification. To separate the nitrated derivatives we took advantage of the drop of the phenolic pK from 10 to 7 that accompanies introduction of a nitro group in the ortho position of tyrosine (Sokolovsky *et al.*, 1967). Accordingly, the dialyzed reaction mixtures were separated on a 100 × 1.5 cm DEAE-Sephadex (A25) column at 4°. The column was equilibrated with a buffer containing 7 M urea–0.14 M NaCl–0.01 M Tris-HCl, pH 8.4. Elution at 15 ml/hr was effected with a NaCl gradient. Fractions (5 ml) were collected and the absorbance determined. The corresponding peaks were promptly pooled and dialyzed at 4° against 0.01 M sodium acetate buffer, pH 5.2. The resulting precipitates were washed three times with minimal amounts of H_2O and dried *in vacuo*.

Analyses. Cellulose acetate electrophoresis was performed at pH 8.4, 0.01 M Tris-HCl–7 M urea, as previously described (Bromer *et al.*, 1967), in a Beckman Microzone electrophoresis apparatus. Oxidative sulfitolysis (Bromer *et al.*, 1967) was done in 8 M urea at a protein concentration of 10 mg/ml. Separation of the S-sulfonated A and B chains was effected by cellulose acetate electrophoresis at pH 3.2 as previously described (Bromer *et al.*, 1967) and by ion-exchange chromatography (Leggett-Bailey, 1967). The sulfitolysis reaction mixture (0.50 ml) was applied to a 5 × 50 mm column of Dowex 50W-X2 equilibrated with 0.2 M sodium citrate buffer, pH 2.8, 8 M urea. The column was developed with the starting buffer; the first 5 ml was collected in a volumetric flask. The column was then washed with 5 ml of 0.4 M sodium phosphate buffer, pH 7.6, 8 M urea, the effluent being similarly collected. The identity of A chain in the first fraction, and B chain in the second, was established by electrophoretic mobility at pH 3.2 (Bromer *et al.*, 1967) and by absorption spectra (B chain contains three phenylalanyl residues; A chain, none). Recovery from the column was 95% or better.

Chymotryptic hydrolysis of insulin and insulin derivatives was done according to Aoyama *et al.* (1965). α-Chymotrypsin was assayed by spectrophotometric monitoring of the hydrolysis of benzoyl-L-tyrosine ethyl ester (Hummel, 1959). Absorption spectra were recorded on a Cary 15 spectrophotometer. Amino acid analyses of the nitrated insulins were done on duplicate 24-hr acid hydrolysates on a Spinco 120 AutoAnalyzer. The hydrolyses were done in parallel with samples of crystalline insulin, which served as an internal standard for hydrolytic loss. Results were calculated by assuming that Gly + Ala = 7 for each preparation.

Gel filtration (Sephadex G-50) was performed in 7 M urea–0.01 M NaCl–0.05 M Tris-HCl, pH 7.5 (Boesel and Carpenter, 1970).

Bioassay. The nitrated derivatives were bioassayed by measuring their ability to lower the blood glucose of chronically diabetic mice. C57 Bl 6 mice were made diabetic by intravenous injection of alloxan (2.5 mg/25 g of body weight). Diabetic mice which had been injected with alloxan at least 10 days previously and had blood glucose levels above 300 mg % were used. They were fasted overnight and then fed standard mouse chow for 1 hr. One hour after feeding a baseline blood sugar sample was taken, followed by intraperitoneal injection (0.25 ml) of insulin, nitrotyrosine insulin, or diluent (0.15% bovine serum albumin in phosphate-buffered saline, pH 7.4). Dose ranges per 25-g mouse were: insulin, 0.25–0.60 μg; mono-3-nitrotyrosine insulin, 0.20–0.38 μg; and di-3-nitrotyrosine insulin, 0.31–0.50 μg. A second blood sample was taken 60 min later. Blood glucose levels were measured by the glucose oxidase technique (Saifer and Gerstenfeld, 1958).

Biological activity of the nitrated derivatives was determined by comparing the mean of the blood sugar depression per log (dose in micrograms) for each derivative with the corresponding result for insulin. Corrections were made for the slight differences in molecular weight. This bioassay has been shown to give excellent correlation (r = 0.99) with the mouse convulsion assay (K. M. Meadows and E. R. Arquilla, unpublished data). Further details of this assay will be published elsewhere.

Immunoassay. The ability of mono- and di-3-nitrotyrosine insulins to combine with insulin antibodies was measured by passive immune hemolysis inhibition (Arquilla *et al.*, 1969). This assay compares the amount of insulin derivative needed to neutralize 50% of a preset concentration of insulin antibodies with the amount of insulin needed to neutralize the same amount of antibodies. Insulin antibodies (0.1 ml) are added to 0.1 ml of a suspension of insulin-coated erythrocytes (6×10^8 cells/ml). To this, varying amounts of insulin, insulin derivative, or buffer are added in a volume of 0.1 ml and the mixture incubated while shaking for 1 hr at 4°. Complement, three 50% hemolytic units in 0.1 ml, is then added, and the incubation continued at 37° for 30 min. In the absence of added insulin or insulin derivative, antibodies combine with the insulin coupled to the erythrocytes, activating the complement system, which in turn lyses the cells. With increasing amounts of added insulin or insulin derivative, antibodies are neutralized and the extent of hemolysis correspondingly decreases.

The insulin-coated cells were prepared by covalently conjugating insulin to washed sheep erythrocytes with bisdiazobenzidine (Arquilla, 1970). The antibody preparation was anti-insulin γ-G purified from a pool of mongrel guinea pig anti-insulin serum by the method of Sela and Mozes (1966). Complement was prepared and assayed as previously described (Arquilla and Finn, 1963). All dilutions were made in Veronal-buffered saline, pH 7.4, containing 0.5 mM $MgCl_2$–1.5 mM $CaCl_2$–0.15% bovine serum albumin. To terminate the reaction, 0.5 ml of cold, buffered saline was added to the 0.4-ml reaction volume and the unlysed erythrocytes were separated by centrifugation at 800g. The supernatants were decanted and assayed for hemoglobin by spectrophotometry at 414 mμ. The preset level of anti-insulin γ-G was chosen so as to lyse approximately 70% of the 6×10^7 erythrocytes in the absence of inhibitor. This level yielded optimal sensitivity and range in the assay. Varying amounts (5–200 ng) of insulin or 3-nitrotyrosine insulins were used to inhibit hemolysis; results were plotted on probit paper to estimate the 50% end point (Figure 4).

Results

Chromatography and Electrophoresis. Figure 1 is an example of an elution profile of a lightly (*ca.* 1 equiv/mole) nitrated reaction mixture. Examination of the fractions on cellulose acetate electrophoresis (Figure 2) revealed that peak 1 (lane 1) is identical with the major fraction of crystalline insulin (lane 0). Crystalline insulin exhibits a faster moving com-

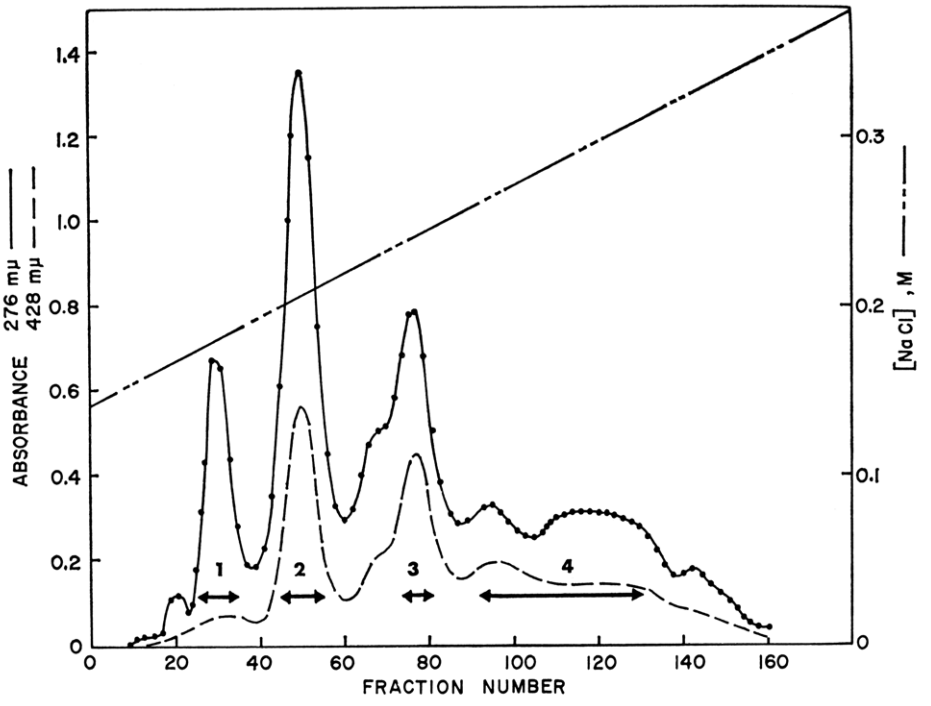

FIGURE 1: Separation of nitrotyrosine insulins on Sephadex A25. Insulin (100 mg, 17 μmoles) was nitrated for 15 min at pH 7.4 with 200 μmoles of tetranitromethane. Arrows refer to fractions pooled in peaks 1–4.

ponent desamido insulin, a minor component of commercial insulin preparations (Bromer *et al.*, 1967). Peak 2 (lane 2) is relatively homogeneous and equal in mobility to desamido insulin. Peak 3 (lane 3) migrates as a single band with a diffuse leading edge, indicating contamination with the heterogeneous trailing material from the column (lane 4). Because each ortho-substituted nitrotyrosyl residue is expected to give a charge difference of −1 relative to insulin, peaks 2 and 3 exhibit the mobility expected of mono- and di-3-nitrotyrosine insulin, respectively.

Extent of Reaction and Homogeneity of Preparations. Examination of the absorption spectra of 3-nitrotyrosine (Table I) and comparison with the spectral properties of the nitrotyrosine insulin fractions permits tentative identification of the fractions. The visible absorption spectra of fractions 2 and 3 are qualitatively similar to the spectrum of 3-nitrotyrosine. Both preparations exhibited an absorption band in acid at 353 mμ, which shifted to 428 mμ in base. Moreover, an isosbestic point near 381 mμ was obtained, and the ratio A_{353} (acid) to A_{428} (base) was characteristic of 3-nitrotyrosine. If it is assumed that each un-ionized tyrosyl residue contributes 1350 to the molar absorptivity of insulin (5950 at 276 mμ) and each un-ionized 3-nitrotyrosyl residue contributes 5520 at 276 and 2570 at 353 mμ, then the expected A_{353}/A_{276} for insulin, mono-3-nitrotyrosine insulin, and di-3-nitrotyrosine insulin are easily calculated (Table II). Based on the electrophoretic mobilities and these ratios, peak 1 is considered to be unreacted insulin, peak 2 to be mono-3-nitrotyrosine insulin, and peak 3 to be di-3-nitrotyrosine insulin.

FIGURE 2: Cellulose acetate electrophoresis of nitrated insulins. Samples (about 5 μg) were electrophoresed for 2 hr at 15 V/cm, and stained with ponceau S: lane 0, authentic insulin; lanes 1–4, peaks 1–4 of figure 1, respectively; lane 5, mixture of peaks 1–4. Origin is at bottom of figure.

In view of the expected contamination of peak 2 with desamido insulin and the uncertainty in the estimation of expected absorbancies, the agreement of the calculated and experimental absorbancy ratios is considered to be quite satisfactory.[1] The discrepancy between the calculated and experimental absorbancy ratios for mono-3-nitrotyrosine insulin can be explained by a 15% contamination with desamido insulin. Similarly, the discrepancy between the calculated and observed values for di-3-nitrotyrosine insulin may be attributed in part to contamination with monodesamido, mono-3-nitrotyrosine insulin. The degree of nitration was supported by amino acid analysis (Table III). As can be seen the two homogeneous fractions gave no evidence of substitution of residues other than tyrosyl.

In all reaction mixtures considerable heterogeneity was

[1] Mono-3-nitrotyrosine insulin isolated from heavily nitrated reaction mixtures (*i.e.*, with very little unreacted insulin) had A_{353}/A_{276} of 0.250–0.252.

TABLE I: Molar Absorptivities of 3-Nitrotyrosine.

Wavelength (mμ)	428	381	353	283	276
A_M	3800[a]	2000	2570	3800	5520
Solvent	Base[b]	c	Acid[d]	Base	Acid

[a] Riordan *et al.* (1966). [b] Base: 0.01 N NaOH. [c] Isosbestic point. [d] Acid: 0.01 N HCl.

TABLE II: Spectral Properties of 3-Nitrotyrosine Insulin Fractions. Expected Absorbancy Ratios Were Calculated as Described in Text. Peak Numbers Refer to Figure 1. Measurements Were Made in 0.01 N HCl.

	A_{353}/A_{276}			
		Found		
Preparation	Calcd	Peak 1	Peak 2	Peak 3
Insulin	0.000	0.059		
Mono-3-nitrotyrosine Insulin	0.254		0.229	
Di-3-nitrotyrosine Insulin	0.359			0.349

TABLE III: Amino Acid Analyses of Nitrotyrosine Insulins.

	Residues in Insulin	Mono-3-nitrotyrosine Insulin	Di-3-nitrotyrosine Insulin
Lys	1	1.00	0.98
His	2	1.52	2.00
Arg	1	1.02	1.06
Asp	3	2.95	3.14
Ser	3	3.19	2.36
Thr	1	1.03	1.13
Glu	7	6.92	7.12
Pro	1	0.92	1.11
Gly	4	4.00	4.01
Ala	3	3.00	2.99
Cys/2	6	5.81[a]	6.17[a]
Val	5	5.06	5.25
Ile	1	1.08	1.10
Leu	6	6.00	6.35
Tyr	4	2.81	2.36
Phe	3	2.77	2.75
3-Nitrotyrosine		0.69[b]	1.36[b]

[a] Includes cysteic acid. [b] Uncorrected for hydrolytic loss and based on the same ninhydrin value as tyrosine.

found in the trailing peaks (*e.g.*, Figure 1); this heterogeneity was also seen on cellulose acetate electrophoresis. Amino acid analysis of this material was unexceptional, except that there was a considerable loss of half-cystine and a concomitant increase in cysteic acid. The altered charge properties, absorption spectra, and abnormally large consumption of tetranitromethane during the reaction with insulin (see below) demonstrate that the reaction of insulin with tetranitromethane is not specific for ortho nitration of tyrosyl residues.

Gel filtration of the purified monoconjugated derivative gave no indication of intermolecular cross-linking, as has been noted in unseparated reaction mixtures of nitrated insulins (Boesel and Carpenter, 1970). The monoconjugated derivative crystallized when acidic solutions were dialyzed at 25° against 0.001 M $ZnSO_4$–5% NaCl–0.01 M sodium acetate buffer, pH 5.9.

When nitration was performed for 50 min with a 12-fold excess of tetranitromethane the major products were di-3-nitrotyrosine insulin and trailing material. However, no fraction was observed with an A_{353}/A_{276} greater than that expected of di-3-nitrotyrosine insulin. This is in agreement with the results of Sokolovsky *et al.* (1966), which demonstrated that only two tyrosyl residues in insulin are reactive with tetranitromethane.

Localization of Sites of Substitution. Mono- and di-3-nitrotyrosine insulins were subjected to oxidative sulfitolysis. Visual inspection of the electrophoretically separated S-sulfonated chains revealed the presence of 3-nitrotyrosine only in the A chain. When the S-sulfonated chains were separated on Dowex, absorption spectra of the effluates revealed that, for both derivatives, 90–92% of the 3-nitrotyrosine was recovered in the A chain fraction, the remainder being found in the B chain fraction. In view of the results of Sokolovsky *et al.* (confirmed here), the presence of nitrotyrosine in the B chain fraction was regarded as a contaminant arising from either incomplete sulfitolysis or incomplete separation on the column.[2] Therefore, the identity of peak 3 is established to be di-(A14, A19)-3-nitrotyrosine insulin.

As a control, oxidative sulfitolysis of mono- and di-FTC[3] insulin (Bromer *et al.*, 1967) was performed in parallel with the nitrotyrosine insulins. Mono-FTC insulin was found to be substituted predominantly on the B chain, and di-FTC insulin labeled equally on the A and B chains. This agrees with the position of labeling determined previously (mono-FTC insulin is substituted predominantly at B1, and di-FTC insulin is substituted equally at B1 and A1) (Bromer *et al.*, 1967).

To locate the site(s) of substitution of the mononitrated derivative, it was subjected to chymotryptic digestion. Aliquots were removed at intervals over a 10-hr period and the chymotryptic core precipitated with trichloroacetic acid. According to Aoyama *et al.* (1965) the presence of 2 equiv of tyrosine in the trichloroacetic acid precipitate is a result of the cleavage of two peptide bonds, A14-A15 and B16-B17; the soluble peptides contain tyrosyl residues A19 and B26, whereas the precipitate contains tyrosyl residues A14 and B16. As can be seen in Figure 3a, chymotryptic hydrolysis of insulin leads to the release of two tyrosyl residues, in agreement with the expected result. Somewhat surprisingly, the reaction appears to be first order in substrate (Figure 3b). Apparently, the hy-

[2] A slight degree of crosscontamination of the A and B chain fractions is to be expected (Leggett-Bailey, 1967).

[3] Abbreviation used is: FTC, fluorescein thiocarbamyl.

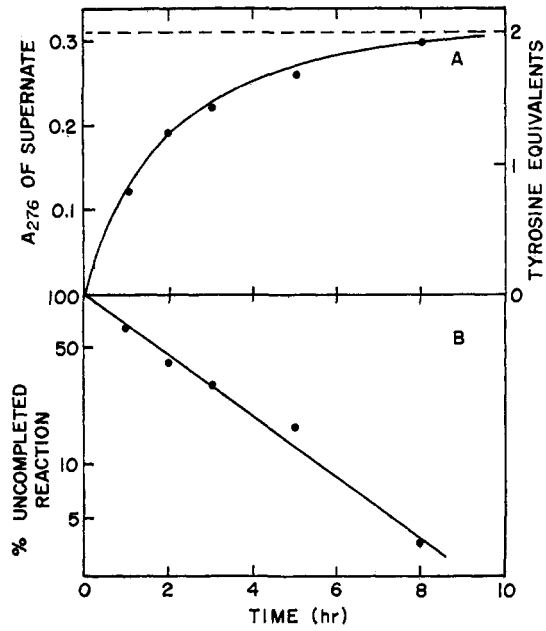

FIGURE 3: Chymotryptic hydrolysis of insulin: (a) the release of 2 equiv of trichloroacetic acid soluble tyrosyl residues; (b) apparent first-order behavior of the reaction. Complete solubility of insulin in trichloroacetic acid would correspond to A_{276} 0.622.

drolysis of one peptide bond is strongly rate limiting. The identical conclusion has been reached on the basis of ultracentrifugal studies (Ginsburg and Schachman, 1960). The activity of solutions of α-chymotrypsin was found to be undiminished over the 10-hr course of the experiments.

The chymotryptic digestion of mono-3-nitrotyrosine insulin was monitored at 5 and 8 hr. The trichloroacetic acid supernatants were assayed spectrophotometrically for both tyrosine and 3-nitrotyrosine as described above. At 5 hr, 0.062 equiv of nitrotyrosyl residue and 1.00 equiv of tyrosyl residue were soluble in trichloroacetic acid. At 8 hr, the results were 0.083 and 1.31 equiv, respectively. These results demonstrate that mono-3-nitrotyrosine insulin is substituted predominantly at A14. For this preparation (peak 2 of Figure 1) the ratio of substitution at A14 to that at A19 was calculated to be 86:14, assuming 15% contamination with desamido insulin. Other monosubstituted preparations yielded ratios of 89:11 and 83:17.

As control, the distribution of substitution in tri-FTC insulin was determined by chymotryptic digestion. Over a 10-hr period, 0.32 of the fluorescein appeared in the trichloroacetic acid supernatant. This is in complete agreement with previous results (Bromer *et al.*, 1967), since two-thirds of the fluorescein substitution sites (A1 and B1) is in the chymotryptic core, while the third (B29) would be expected in the trichloroacetic acid supernatant. The production of trichloroacetic acid soluble fluorescein peptides also followed first-order kinetics, with a first-order rate constant (corrected for enzyme concentration) roughly equal to that found for the digestion of insulin. When di-3-nitrotyrosine insulin was hydrolyzed with α-chymotrypsin, half (47%) of the 3-nitrotyrosine appeared in the trichloroacetic acid supernatant.

Effect of pH on Tyrosine Reactivity. The effect of pH on nitroform production was determined in 10% ethanol so that the tetranitromethane was soluble. We found that more tetranitromethane was consumed (as measured by nitroform production at 350 mμ) than could be accounted for by the production of 3-nitrotyrosine. This phenomenon has been noted with other proteins (Sokolovsky *et al.*, 1966). At pH 7.4, only 50% of the consumed tetranitromethane was incorporated into 3-nitrotyrosine. Moreover, when nitration was performed at higher pH values (9 to 10), the reaction mixture exhibited several anomolies.

1. We were unable to achieve satisfactory separation on DEAE-Sephadex or cellulose acetate electrophoresis.

2. Chymotryptic hydrolysis of the mixture did not proceed to completion; only 60–70% reaction occurred after 24 hr.

3. The absorption spectra were anomolous. Absorption at 340 mμ was higher than expected, and greater in base than in acid. This result is similar to that observed with the nitration of ribonuclease (Beaven and Gratzer, 1968).

With these difficulties in mind, we estimated the distribution of 3-nitrotyrosine between Tyr A14 and A19 as a function of pH. We assumed that nitration at the two sites was statistically independent and computed the ratio of monoconjugation at Tyr A14 to that at A19 from the incomplete chymotryptic hydrolyses of the unseparated reaction mixtures. Estimation of 3-nitrotyrosyl residues was performed at 428 mμ in base, because of anomolous absorption in the vicinity of 350 mμ. At pH 7.4, the extent of substitution at Tyr A14 was calculated to be 89%. This value fell to 63% at pH 9.0, and to 45% at pH 10.0. The value calculated for pH 7.4 is close to that obtained from the purified monoconjugated derivative.

Titration of Nitrotyrosine Insulins. Spectrophotometric titrations studies have shown that one (Inada, 1961) or two (Crammer and Neuberger, 1943) of the four tyrosyl residues of insulin ionize with an abnormally high pK. Titration of the nitrated derivatives yielded a value of 7.1 for the apparent pK of the nitrotyrosyl residues. This is a normal value (Sokolovsky *et al.*, 1967). Since this pK is far removed from the pH region of tyrosine ionization the nitrated derivatives permit a partial assignment of the anomously ionizing residue(s). Our results with insulin (D. A. Bihler, J. W. S. Morris, and E. R. Arquilla, unpublished results) indicate that two residues ionize with an apparent pK of 10.2, and two residues ionize with an apparent pK of about 11.7, in agreement with the results of Crammer and Neuberger (*cf.* Shugar, 1952). We have not been able to verify the results of Inada, although we have observed (Morris *et al.*, 1968) a time-dependent increase in absorbance at high pH. We recorded absorption spectra of freshly prepared solutions of insulin and the nitrated derivatives in KCl–KOH (μ = 0.10) at pH 11.7 and 12.8. The value of 2325 for ΔA_{295} per ionized tyrosyl residue was used. The absorption spectrum of insulin at pH 11.7 indicated the ionization of 3.0 tyrosyl residues; *i.e.*, the two anomolous residues were 50% ionized. At pH 12.8, an additional 0.96 tyrosyl residue was ionized, indicating that all 4 tyrosyl residues were now ionized. When the procedure was repeated with mono- and di-3-nitrotyrosine insulins, raising the pH from 11.7 to 12.8 resulted in the ionization of 0.46 and 0.38 tyrosyl residue, respectively. This is consistent with the identification of Tyr A14 as an anomolously ionizing residue. Since substitution at Tyr A19 had little additional effect, the second anomolous tyrosyl residue is probably located on the B chain.

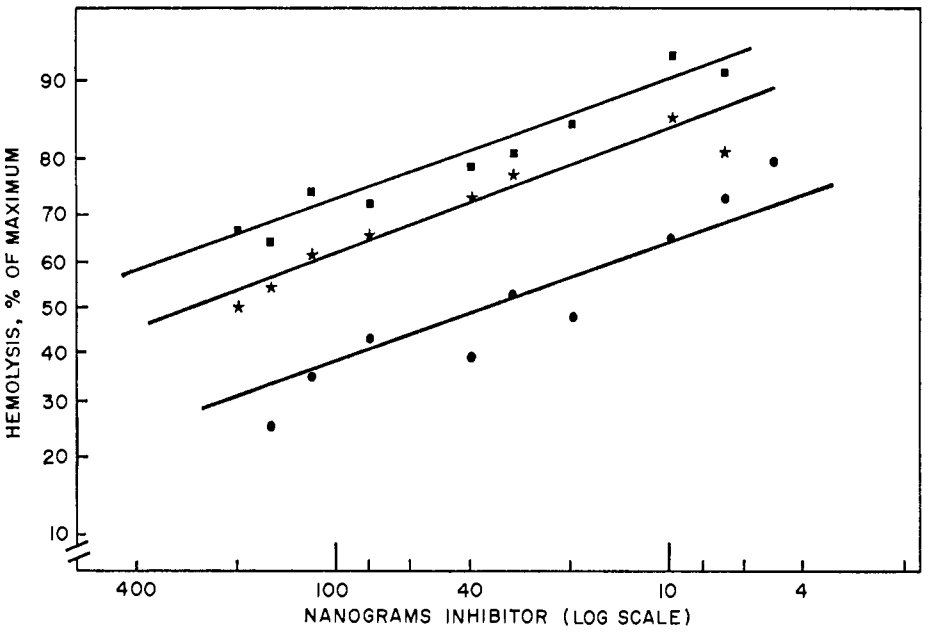

FIGURE 4: Immunoassay of nitrotyrosine insulins. See text for experimental details. Results of the inhibition of immune hemolysis at each concentration of insulin or insulin derivative are plotted here on probit paper. Estimation of the 50% end points yields values of 4 and 9% for the relative immunological activities of the mono- and dinitrated insulins. Preparations assayed here were obtained from heavily nitrated reaction mixtures, so that mono-3-nitrotyrosine insulin is relatively free from contamination, but di-3-nitrotyrosine insulin is expected to be contaminated with monodesamido, mono-3-nitrotyrosine insulin: ■, mono 3-nitrotyrosine insulin; ★, di-3-nitrotyrosine insulin; ●, insulin.

Immunological and Biological Activity. Biological activities of the mono- and dinitrated preparations of Figure 1, expressed as IU/mg ± SEM (number of animals), were, respectively: 25.8 ± 2.4 (n = 24) and 18.4 ± 2.3 (n = 18), relative to the bovine insulin standard: 24.4 ± 1.4 (n = 122). The value for mono-3-nitrotyrosine insulin has been corrected for the expected 15% contamination with desamido insulin. The immunological activities of the mono- and di-3-nitrotyrosine insulins were 4 and 9%, respectively (Figure 4). While the unreacted insulin peaks from the columns were not assayed here for biological or immunological activity, we have previously shown (Bromer *et al.*, 1967) that similar isolation and purification techniques have no effect on either the biological or immunological activity of insulin.

Discussion

Environment of Tyr A14. The anomolous ionization of A14 must be due to factors other than inaccessability to solvent since it can be iodinated and nitrated. An appealing explanation is the presence of a tyrosyl–carboxylate hydrogen bond, first implicated in proteins by Crammer and Neuberger (1943), and in insulin by Laskowski *et al.* (1960).

Salicylic acid is a simple model of an intramolecular tyrosyl–carboxylate hydrogen bond. This compound shows symmetric shifts in pK of both the phenolic and carboxylic functions (Branch and Yabroff, 1934), in accord with equations derived by Scheraga (1961).

We have previously shown (Morris *et al.*, 1968) that the environment of one tyrosyl residue of insulin, probably A14, is dependent on the ionization of a group with an apparent pK of 3.0; *i.e.*, roughly 1.5 units lower than that of a "normal" β- or δ-carboxyl group. This shift is approximately equal and opposite to the shift of the apparent pK of Tyr A14 (Inada, 1961; Crammer and Neuberger, 1943). Assuming that the model for a tyrosyl–carboxylate hydrogen bond applies to insulin, the magnitude of the pK shift in insulin yields a value of 31 for K_H, and a value of −2.0 kcal/mole for the free energy of formation of the hydrogen bond. The presence of an exposed Tyr A14-carboxylate hydrogen bond is fully consistent with the chemical and optical data. Moreover, such a hydrogen bond, by increasing the phenoxide character of the tyrosyl residue might be expected to increase the rate of substitution at pH's much lower than normal pK of tyrosine. This is in accord with our results; at pH 7.4 Tyr A14 is more reactive than Tyr A19.

However, this model has the necessary consequence that, at pH values close the pK of tyrosine, a normal tyrosyl residue will be more reactive than a hydrogen-bonded one, because the hydrogen bond sharply reduces the concentration of highly reactive phenoxide ion. Thus, the hydrogen bond provides a partial explanation for the apparent reversal in reactivities of Tyr A14 and Tyr A19 with increase in pH. It must be pointed out that other factors must be operative as well, since the results are only in qualitative agreement with the model.

Other Tyrosyl Derivatives of Insulin. Iodination of the tyrosyl residues of insulin also involves an electrophilic ring substitution (Mayberry *et al.*, 1965), and are therefore of considerable interest here. It was found (de Zoeten and Havinga, 1961) that the A chain tyrosyl residues are much more reactive than the B chain residues, in agreement with the results of nitration. At low levels of iodination, Tyr A19 is more reactive than Tyr A14. This is in accord with the model presented above, since the reaction was performed at pH 9.2. At higher levels of iodination, however, the relative incorporation of io-

dine was reversed. It was suggested that Tyr A14 is readily diiodinated, while Tyr A19, perhaps because one of the ortho positions is buried *cannot* be readily diiodinated. The conclusions of these workers is in accord with our finding that Tyr A19 is less reactive to tetranitromethane at pH 7.4 than Tyr A14.

Cyanuric fluoride normally reacts only with Tyr A19 and Tyr B16 (Aoyama *et al.*, 1965). It is clear that no direct comparison with the results of iodination and nitration is possible, since the reaction was conducted in an alkaline medium (pH 9.7) in the presence of dioxane. These factors effect both the aggregation state (Fredericq, 1956) and conformation (Goldman and Carpenter, 1969; Mercola *et al.*, 1967) of insulin. However, the nonreactivity of Tyr A14 is not surprising. It is likely that cyanuric fluoride is involved in electrophilic attack of the nonprotonated oxygen atom of the phenoxide form of tyrosine (Gorbunoff, 1967). The tyrosyl A14-carboxylate hydrogen bond, by ensuring protonation at pH 9.7, would sharply lower the reactivity of this residue.

Assignment of the Tyrosyl Residues of Insulin. The model of the insulin tyrosyl residues developed here is: (1) Tyr A14 is exposed and hydrogen bonded to a carboxylate group; (2) Tyr A19 is at least partly exposed; (3) Tyr B16 and B26 are not exposed, at least in aggregated forms of insulin.

This is in agreement with the results of solvent perturbation spectrophotometry of zinc insulin, which indicate two exposed and two buried residues (Weil *et al.*, 1965), or two buried, one exposed, and one partly buried residues (Menendez *et al.*, 1969).

The recently obtained results of X-ray diffraction (Adams *et al.*, 1969) are in general agreement with these assignments. In the crystal, Tyr A14 appears to be hydrogen bonded to Gln A5, adjacent to the (presumed) ionic interaction between Lys B29 and Glu A4 (Adams *et al.*, 1969). It is possible that the behavior of Tyr A14 at high and low pH is a manifestation of the titration of the latter interaction.

Immunological and Biological Activity. In general, chemical modifications have been found to have parallel effects of the immunological and biological activity of insulin (Arquilla *et al.*, 1969). As shown above, the nitrated insulins are striking exceptions to the rule. The data to date indicate that, in solution, Tyr A14 is involved in side-chain interactions. These interactions are broken upon nitration, since mono-3-nitrotyrosine insulin exhibits a normal nitrotyrosyl pK. In addition, Tyr A14 acquires a partial charge at neutral pH due to lowering of the phenolic pK. We found these physical changes to have no effect on the biological activity of insulin. In contrast, the immunological activity of mono-3-nitrotyrosine insulin is only 4% of that of insulin. In the absence of widespread conformational changes, this implies that the region in the neighborhood of Tyr A14 must be an integral part of a large fraction of the multiple antigenic determinants of insulin (Arquilla *et al.*, 1969).

Our results of the nitration of insulin may be correlated with earlier results of the sulfation of insulin (Moloney *et al.*, 1964). It was found that progressive sulfation of insulin led to preparations containing a single sulfonated tyrosyl residue. The ratio of the biological activity to the immunological activity (12:1 to 40:1) of these preparations was of the same order as we found for mono-3-nitrotyrosine insulin (25:1). It is tempting to speculate that the same tyrosyl residue (A14) is involved in each case.

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Added in Proof

Refinements of the crystallographic model have led to modifications in the region of Tyr A14. The hydrogen bond of Tyr A14 to the carbonyl group of A5 no longer appears to be feasible. However, it is apparent that Tyr A14 can hydrogen bond with either the carbonyl group of B2 or the carboxylate group of A17 (G. G. Dodson, personal communication).

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Studies on Spin-Labeled Actin*

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ABSTRACT: Using the spin-labeling technique of McConnell it has been found that a paramagnetic derivative of maleimide is an excellent probe of actin–actin interactions. Incorporation of molar quantities of this spin label into the actin molecule does not impair the polymerizability of this muscle protein. Electron paramagnetic resonance spectra of spin-labeled actin reveal a strong immobilization of the label accompanies polymerization. As with the viscosity change, both the rate and the extent of the spectral change were found to depend on the actin and KCl concentrations employed in the polymerization reaction. Analysis of these data suggests that the spectral change results either from masking of label situated near the polymerization site or from a conformational change within the monomer unit brought about by linkage of monomer to the growing polymer chain. In contrast to actin–actin interactions, spin label on actin partially inhibits interaction with myosin. No spectral changes were observed to accompany the binding of spin-labeled actin to myosin.

The two characteristic properties of the muscle protein actin are (1) conversion from a monomeric state of globular subunits (G-actin) into a fibrous polymer (F-actin) in the presence of neutral salts, and (2) interaction with another muscle protein, myosin A, to provide the structural continuity and elevated ATPase activity requisite for muscle contraction. Although it is now reasonably certain that polymerization of actin is an *in vitro* phenomenon which does not accompany contraction, the finding of increased exchangeability of F-actin-bound nucleotide during the contraction-like process of superprecipitation (Szent-Györgyi and Prior, 1966; Moos *et al.*, 1967) suggests that some loosening of the actin polymer may occur during contraction. It is thus evident that knowledge of actin–myosin as well as actin–actin interactions is central to the understanding of contraction.

In studying these interactions, we have employed the spin-labeling technique of McConnell and coworkers (Stone *et al.*, 1965; Hamilton and McConnell, 1968). In this procedure 1 or more moles of a nitroxide-free radical (spin label, I–III) are covalently bound to a protein. The electron paramagnetic resonance of these compounds is sensitive to molecular motion and the restrictions imposed on the free rotation of the spin label by covalent bonding to a large, relatively motionless protein molecule produce sizable changes in the spectra. Furthermore, small changes in the rotational freedom of appropriately located spin labels such as might result from a conformational change or union of one protein with another are readily detectable in the spectra.

We have found that the maleimide spin label (I) is an excellent probe of actin–actin interaction. This spin label does not interfere with polymerization, but suffers a large immobi-

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