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[Tetrakis(3-nitrotyrosine)]insulin†

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ABSTRACT: The reaction of Zn-free bovine insulin with a 2.5-fold excess (over the tyrosines) of tetranitromethane at pH 8 results in the modification of all four tyrosine residues in the insulin molecule. The maximum amount of 3-nitrotyrosine produced in the reaction occurs at 35 min and amounts to ~2.7 residues/molecule of insulin. The remainder of the modified tyrosines are unaccounted for by amino acid analysis and are probably involved in the formation of intermolecularly cross-linked derivatives consisting of dimers and higher polymers of insulin which compose 70% of the reaction mixture. After removal of polymeric material from the reaction mixture by gel chromatography on Sephadex G-50, the monomeric material was separated according to charge by DEAE-cellulose chromatography in 7 M urea at pH 8.0 to give a 10% yield of [tetrakis(3-nitrotyrosine)]insulin—a derivative in which each of the four tyrosines of insulin has been nitrated at the 3 position of the aromatic ring. Spectrophotometric titration indicated that each of the nitrotyrosine phenolic groups had the same pK_a value of 7.3. Sedimentation equilibrium experiments indicated that the [tetrakis(3-nitrotyrosine)]insulin did not aggregate at pH 7.4 even in the presence of Zn^{2+}. At pH 2, however, the material aggregated to a much larger extent than insulin under similar conditions. The circular dichroism spectrum of [tetrakis(3-nitrotyrosine)]insulin was similar to that of desoctapeptide-(B23–30)-insulin, an inactive derivative which also does not aggregate at pH 8. The spectrum, in contrast to that of insulin, was not perturbed by pH, protein concentration, or the presence of Zn^{2+}. [Tetrakis(3-nitrotyrosine)]insulin exhibited a hormonal response in the fat cell assay of 55% and in the mouse convulsion assay of 50–70% of that of the native hormone. Thus, [tetrakis(3-nitrotyrosine)]insulin represents a unique derivative of insulin in which the physical properties have been altered without undue effect on the hormonal activity. The inability of [tetrakis(3-nitrotyrosine)]insulin to aggregate at physiological pH values indicates that the monomeric form is the active species in the hormonal response. The fact that the introduction of the nitro group had the expected effect of lowering the pK_a values of the phenolic groups from 10.4 in insulin to 7.3 in [tetrakis(3-nitrotyrosine)]insulin but with retention of 50% of the hormonal activity implies a minor role for some if not all of the tyrosines in eliciting the hormonal response.

The study of the structural aspects of the insulin molecule that are responsible for its hormonal activity has been approached in this laboratory via the preparation of homogeneous insulin derivatives obtained through chemical or enzymatic modifications of the native hormone. Investigations of the hormonal and physical properties of the derivatives yield an insight into the significance of the changes for the function of the insulin molecule (Blundell et al., 1972). Since three of the four tyrosines in insulin are conserved in all of the species whose sequences have been determined (Dayhoff, 1976), an important functional role can be inferred for these groups. That this role may involve the hormonal action of insulin is implied by the fact that extensive iodination of the molecule leads to a loss of hormonal activity (Izzo et al., 1964; Rosa et al., 1967). The introduction of tetranitromethane as a relatively specific agent for modifying tyrosines (Riordan et al., 1966; Sokolovsky et al., 1966) stimulated our interest in

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its use on insulin. Although it is known that cysteine and tryptophan are also subject to attack by tetranitromethane (Sokolovsky et al., 1969, 1970), the absence of these residues in insulin suggested that nitration of tyrosine would be the principle reaction. In their initial paper, Sokolovsky et al. (1966) reported that only two of the tyrosines in insulin reacted with tetranitromethane as determined by the increase in absorption at 428 nm or by amino acid analysis for 3-nitrotyrosine. We found that treatment of insulin with tetranitromethane at pH 8.0 resulted in the loss of all four of the tyrosines although only 2.5–2.8 of these residues could be accounted for as 3-nitrotyrosine on amino acid analysis (Boesel & Carpenter, 1970). Furthermore, the nitration mixture was composed predominantly of intermolecularly cross-linked species consisting of dimers, trimers, and higher polymers of nitrated insulin (Boesel & Carpenter, 1970, 1972; Boesel, 1972). These results with insulin revealed another potential side reaction (intermolecular cross-linking) which must be taken into consideration during treatment of proteins with tetranitromethane. Nevertheless, under mild treatment of insulin with tetranitromethane, as reported by Morris et al. (1969, 1970), monomeric products containing nitrotyrosine residues can be obtained. These workers describe the isolation of [mono- and [bis(3-nitrotyrosine)]insulins in which nitration had taken place primarily (but not exclusively) at Tyr-A14 and Tyr-A14 and Tyr-A19, respectively. Both of these derivatives exhibited high hormonal activity. We desired to prepare a homogeneous nitrated derivative of insulin and decided to avoid the difficulties envisaged in the separation of the variety of isomers that may exist within the [mono-, [bis-, or [tris(3-nitrotyrosine)]insulins. A priori, a derivative containing four nitrotyrosine residues could exist as only one isomer, and it is this derivative, [tetrakis(3-nitrotyrosine)]-insulin (TNTI),[1] whose preparation and properties are described.

Experimental Procedures

Materials

Tetranitromethane was obtained from Aldrich Chemical Co. and was washed 3 times with distilled water before use. 3-Nitrotyrosine (K and K Laboratories) was recrystallized from water to a constant melting point. Urea (Aldrich Chemical Co.) solutions for column chromatography were deionized just before use by stirring with a mixed-bed ion exchanger [Bio-Rad, AG-501-X8(D)] until the conductivity of the solution was 1 $\mu\Omega^{-1}$ or less. Sephadex G-25 (medium) and Sephadex G-50 (fine) were from Pharmacia. CM-cellulose and DEAE-cellulose were Whatman products (CM-32 and DE-32). All reagents for gel electrophoresis were from Bio-Rad. Bovine insulin (Eli Lilly and Co., Lot OLVOO or Brunnengräber, West Germany, Lot 1-8-73) was freed of desamidoinsulin by ion-exchange chromatography on CM-cellulose using a buffer consisting of 7 M urea, 0.1 M acetic acid, and 0.09 M NaCl (Busse & Carpenter, 1976).

Methods

Amino Acid Analysis. Samples were hydrolyzed for 6 h at 120 °C (Carpenter & Chrambach, 1962) in 6 N HCl–0.1% phenol contained in evacuated, sealed tubes. The analyses were performed according to Spackman et al. (1958) on a Beckman/Spinco Model 120B analyzer with column temperature of 53 °C and a flow rate of 70 mL/h. The color value for 3-nitrotyrosine was determined to be 89.1% of that of leucine, and the 3-nitrotyrosine eluted 16 min after phenylalanine on the 50-cm column.

Polyacrylamide Gel Electrophoresis. A vertical slab gel apparatus similar to that described by Ferro-Luzzi Ames (1974) was used. The discontinuous buffer system at pH 8.8 as described by Laemmli (1970) with a running gel of 15% and a stacking gel of 5% acrylamide was used in the presence of 0.1% sodium dodecyl sulfate (system A) or in the absence of sodium dodecyl sulfate (system B).

Sedimentation Equilibrium. Experiments were performed on a Spinco Model E analytical ultracentrifuge equipped with ultraviolet absorption optics and a photoelectric scanner recording system. Experiments were performed at three pH values (pH 8.0 in 0.169 M Tris-HCl, pH 7.4 in 0.05 M potassium phosphate, and pH 2.0 in 0.1 M glycine hydrochloride) and at various concentrations of protein. The scans were made between 390 and 510 nm. The spectrum of TNTI was recorded in each of the three buffers used, and a sample of each solution was subjected to amino acid analysis to determine the actual protein concentration. Extinction coefficients were then calculated for various wavelengths to be used to calculate the protein concentrations from the scans. Base line scans were made of the cells as soon as the rotor had attained the desired speed, and from those, the initial concentrations were calculated. Equilibrium scans were made at 22–26 h.

Circular Dichroism Measurements. Protein samples were dissolved in deionized water by the addition of a small amount of HCl. The solution was then adjusted to the desired pH by the addition of 1 N NaOH. In experiments where zinc was present, it was added in a very small volume so as not to alter the pH or protein concentration significantly. The CD measurements were obtained with a Cary Model 60 spectropolarimeter fitted with a Model 6002 circular dichroism accessory. All experiments were performed at 27 °C. Cells with path lengths from 0.01 to 50 mm were used, depending on protein concentration and wavelength. The data is expressed in terms of molecular ellipticity which is defined as

$$[\Theta] = \frac{\Theta M}{10(lC)}$$

where $[\Theta]$ is molecular ellipticity in deg cm^2 dmol^{-1}, M is the gram molecular weight of the sample, C is the concentration of the sample in g cm^{-3}, l is the optical pathlength in centimeters, and Θ is the observed ellipticity in degrees.

Hormonal Assays. We are indebted to Eli Lilly and Co. for mouse convulsion assays involving 200–300 mice per test. Stimulation of the conversion of glucose to lipids by rat epididymal fat cells was performed by the method of Moody et al. (1974).

Kinetics of Nitration of Insulin. All reactions involving tetranitromethane were performed in a fume hood. Zn-free insulin (35 mg) was dissolved in 7.0 mL of 0.05 M Tris-HCl at pH 8.0. The solution was maintained at 25 °C in a thermostated vessel and at pH 8.0 by a pH stat (Radiometer, Type TTT1C) with 0.5 N NaOH as a titrant. Tetranitromethane (35 μL) in 0.7 mL of ethanol was added with stirring. Samples (0.25 mL) were removed at various time intervals and added to 1 mL of 1 N HCl. The resulting solution was extracted with 10 mL of peroxide-free ether. The aqueous layer was placed in a hydrolysis tube with 1 mL of 12 N HCl containing 0.2% phenol and subjected to hydrolysis and amino acid analysis.

[Tetrakis(3-nitrotyrosine)]insulin (TNTI). Desamido-free insulin (200 mg, 33 μmol) was dissolved in 40 mL of 0.05 M

[1] Abbreviations used: DOI, desoctapeptide-(B23–30)-insulin; CD, circular dichroism; TNTI, [tetrakis(3-nitrotyrosine)]insulin; CM, carboxymethyl; DEAE, diethylaminoethyl.

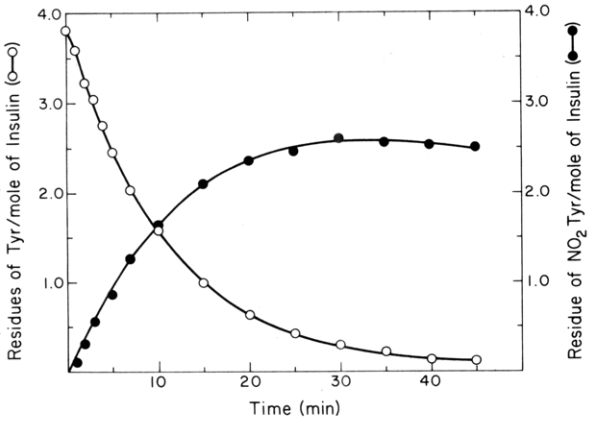

FIGURE 1: Rate of disappearance of tyrosine (O) and appearance of 3-nitrotyrosine (●) as a function of time during treatment of Zn-free insulin (5 mg/mL) with tetranitromethane at pH 8.0.

Tris-HCl at pH 8 and was maintained at this pH by the use of a pH stat with 0.5 N NaOH as titrant while 2 mL of a 2% (v/v) ethanolic solution of tetranitromethane (337 μmol) was added with stirring. After the solution had been stirred at room temperature for 35 min, 1 g of NH_4Cl (to scavenge nitrite) and 6 N HCl were added to pH 3.0. The mixture was dialyzed thoroughly against water and lyophilized. The residue, dissolved in 2 mL of 0.05 M NH_4HCO_3 (pH 8.48), was applied to a Sephadex G-50 (fine) column (2.5 × 190 cm) which was eluted with the same buffer. Fractions containing the monomer were pooled and lyophilized. The monomer fraction was dissovled in 2 mL of 0.05 M Tris-HCl (pH 8.0), 0.01 M NaCl, and 7 M urea, and the solution was applied to a DEAE-cellulose column (2 × 50 cm) which had been equilibrated with the same buffer. The column was developed with a linear salt gradient from 0.01 M NaCl (500 mL) to 0.25 M NaCl (500 mL) in the Tris–urea system. Fractions containing the TNTI were pooled and applied directly to a Sephadex G-25 (medium) column (4.5 × 54 cm) which was equilibrated and eluted with 0.05 M NH_4HCO_3. Fractions containing protein were pooled and lyophilized. The products from three to five similar runs were pooled and rechromatographed on the DEAE-cellulose column, followed by desalting on Sephadex G-25 and lyophilization. The overall yield of purified TNTI from starting insulin was 10%.

Results

Kinetics of Nitration of Insulin. Figure 1 shows the rates of disappearance of tyrosine and the production of nitrotyrosine during treatment of insulin with tetranitromethane. Although nearly all of the four tyrosine residues of insulin have disappeared after a 30-min reaction, only ~2.7 of these residues appear as nitrotyrosine. Evidently ~33% of the tyrosine residues have undergone some reaction which does not yield nitrotyrosine upon hydrolysis of the nitrated insulin. This missing tyrosine could not be accounted for as 3,5-dinitrotyrosine which was present in <1% yield. The rate of disappearance of the tyrosine approaches first-order kinetics with a $t_{1/2}$ of 7.5 min which is approximately the same time observed for the production of 50% of the nitrotyrosine.

Chromatography and Gel Electrophoresis. We have previously reported (Boesel & Carpenter, 1970) that treatment of insulin with tetranitromethane gives rise to a number of cross-linked insulin derivatives which were demonstrated by gel chromatography in solutions containing 7 M urea. As shown in Figure 2, chromatography of the nitrated insulin on Sephadex G-50 in 0.05 M NH_4HCO_3 results in a good separation of monomers from dimers and higher polymers in the

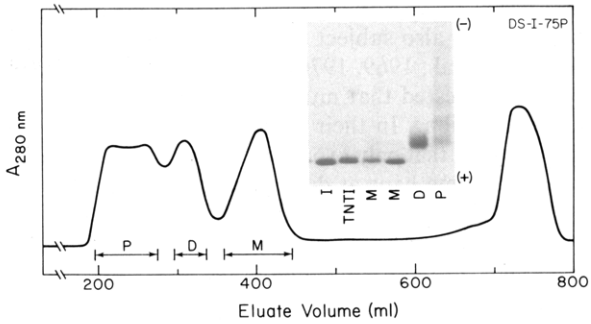

FIGURE 2: Chromatography of nitrated insulin (200 mg) on Sephadex G-50 (fine) (2.5 × 190 cm) eluted with 0.05 M NH_4HCO_3 at pH 8.48. Fractions P (polymers), D (dimers), and M (monomers) were pooled and lyophilized. (Insert) Polyacrylamide gel electrophoresis at pH 8.8 in presence of sodium dodecyl sulfate (system A) of insulin (I), purified [tetrakis(3-nitrotyrosine]insulin (TNTI), monomer (M), dimer (D), and polymer (P) fractions.

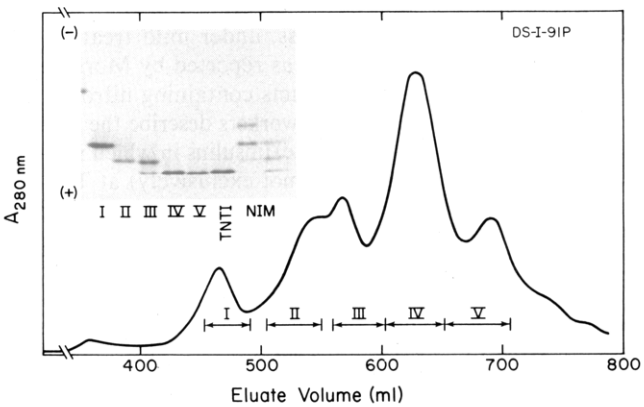

FIGURE 3: Chromatography of the monomer fraction (M of the Figure 2) of nitrated insulin on DEAE-cellulose (2 × 45 cm) in 0.05 M Tris-HCl (pH 8.0) and 7 M urea with a linear gradient from 0.01 M NaCl (500 mL) to 0.25 M NaCl (500 mL). Fractions I–V were desalted and lyophilized. Fraction IV was used for the isolation of TNTI. (Insert) Polyacrylamide gel electrophoresis at pH 8.8 (system B) of fractions I–V, of purified [tetrakis(3-nitrotyrosine)]insulin (TNTI), and of the monomeric fractions of incompletely nitrated insulin (NIM) isolated as in Figure 2.

absence of urea. The nature of the various fractions revealed by gel chromatography is confirmed by their behavior in gel electrophoresis in the presence of sodium dodecyl sulfate (insert to Figure 2). Nitration of the tyrosines of insulin lowers the pK_a of the phenolic group to ~7.3. At pH 8.5 these phenolic groups would all possess a negative charge. The extensive negative charge appears to prevent the aggregation of insulin and permits the elimination of urea in the sizing columns. The monomer fraction from the sizing column was separated according to charge on DEAE-cellulose as shown in Figure 3. At pH 8.0, where these columns were run, each nitrotyrosine adds a fractional negative charge to the molecule resulting in a progressive retardation in the elution from the DEAE-cellulose with increasing numbers of nitrotyrosine residues. In order to avoid possible contamination of TNTI by [tris(nitrotyrosine)]desamidoinsulin, we freed the starting insulin of the desamido form before nitration. The nature of the various fractions isolated from the DEAE-cellulose column was confirmed by their behavior on gel electrophoresis at pH 8.8 (insert to Figure 3). The major component (fraction IV) contained the TNTI which, upon rechromatography on DEAE-cellulose, was isolated in pure form. The nature of fraction V, which behaves as if it had one more negative charge than TNTI on DEAE-cellulose but not on gel electrophoresis, is unknown.

Table I: Amino Acid Composition of [Tetrakis(3-nitrotyrosine)]insulin (TNTI)

amino acid	residues in insulin	TNTI	amino acid	residues in insulin	TNTI
Asp	3.0	3.12	Ile	1.0	0.52
Thr	1.0	0.90	Leu	6.0	5.78
Ser	3.0	2.86	Tyr	4.0	0.03
Glu	7.0	7.00	Phe	3.0	2.77
Gly	4.0	3.90	NO_2Tyr		3.96
Ala	3.0	3.08	Lys	1.0	1.08
$^1/_2$-Cys	6.0	4.96	His	2.0	1.94
Val	5.0	4.29	Arg	1.0	0.95

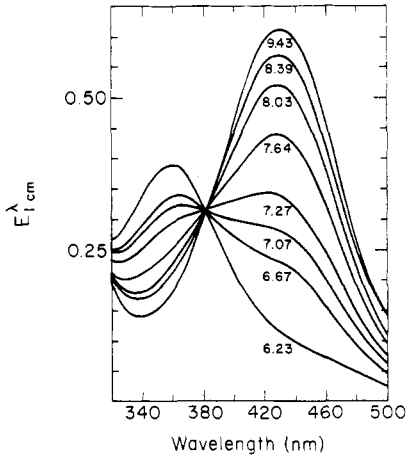

FIGURE 4: Spectrophotometric titration of [tetrakis(3-nitrotyrosine)]insulin in 0.1 M NaCl and 0.01 M Tris with glacial acetic acid. The numbers on the curve indicate the pH of the solution.

Properties of TNTI. The amino acid composition of TNTI is shown in Table I. The derivative was virtually free of tyrosine and contained four nitrotyrosine residues. This indicates that each one of the four tyrosines in insulin has been nitrated. None of the other amino acids showed any significant change from the values reported for insulin which had been hydrolyzed under similar conditions (Carpenter & Chrambach, 1962; Africa & Carpenter, 1970). The near-ultraviolet spectra of TNTI at various pH values from 6.23 to 9.43 are shown in Figure 4. In acidic solution there was an absorption maximum at 360 nm which was shifted in basic solution to 428 nm with an isosbestic point at 381 nm. From these data a plot of the log of the fraction ionized to unionized vs. pH gave a straight line with a slope of 1 and an intercept at 0 of 7.27 which indicates that all of the nitrotyrosines have the same pK_a value of 7.27. Equilibrium sedimentation experiments were performed on TNTI at pH 8.0 at concentrations from 0.09 to 2.2 mg/mL. When the data were plotted as the log of absorbance vs. the square of the distance from the center of rotation, a straight line was obtained in all experiments. The molecular weights calculated from the slopes gave values of 5000–6980 with an average value of 5800 (Boesel, 1972). The same was true when the sedimentation was performed at pH 7.4 in the presence of 1 mM Zn^{2+} (Figure 5). However, when the sedimentation equilibrium experiments were performed at pH 2.0, there was a pronounced curvature in the plot of log of absorbance vs. the square of the distance from the center of rotation (Boesel, 1972). This behavior is typical of an aggregating system. From a study of this behavior as a function of concentration of TNTI, weight average molecular weights from 12000 at 0.1 mg/mL to 100000 at 3 mg/mL were estimated (Boesel, 1972).

The circular dichroism (CD) spectra of Zn-free TNTI, insulin, and desoctapeptide-insulin (DOI) at pH 8.0 and at a concentration of 5×10^{-4} M (3 mg/mL) are given in Figure 6. All three compounds exhibit a negative trough at 207 nm. The spectrum of insulin possesses another trough at 222 nm which is greatly attenuated in the spectra of DOI and TNTI. In the near-ultraviolet region, both TNTI and DOI have a small trough at 265 nm. In contrast, the spectrum of insulin in this region exhibits a negative shoulder at 265 nm and a larger minimum at 273 nm as well as other fine structure (Goldman & Carpenter, 1974). The CD spectra of TNTI at

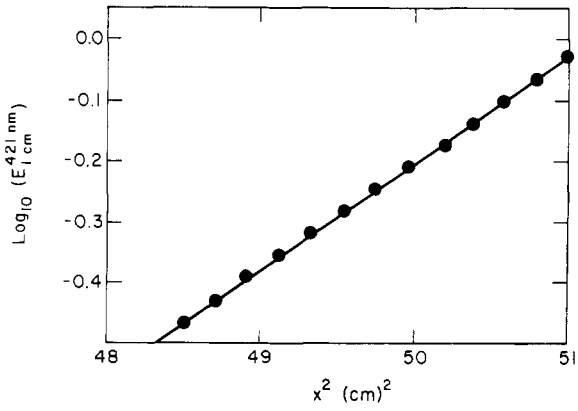

FIGURE 5: Equilibrium sedimentation of [tetrakis(3-nitrotyrosine)]insulin (0.3 mg/mL) in 0.05 M potassium phosphate buffer at pH 7.4 and 1 mM $ZnCl_2$ performed at 34000 rpm at 20 °C. The molecular weight calculated from the slope was 5580.

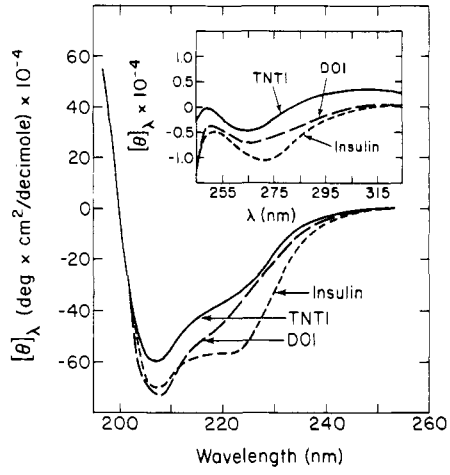

FIGURE 6: Circular dichroism spectra of insulin (---), desoctapeptide-insulin (DOI) (– – –), and [tetrakis(3-nitrotyrosine)]insulin (TNTI) (—), all Zn-free at 5×10^{-4} M and pH 8.0.

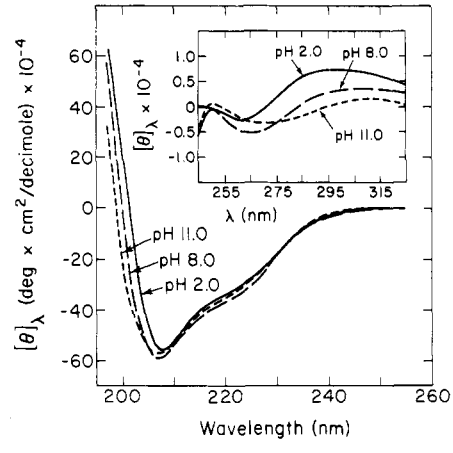

FIGURE 7: Circular dichroism spectra of Zn-free [tetrakis(3-nitrotyrosine)]insulin at pH 2.0 (—), pH 8.0 (– – –), and pH 11.0 (---) at a concentration of 5×10^{-4} M.

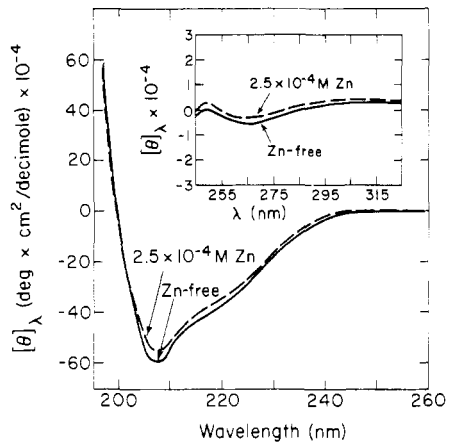

FIGURE 8: Circular dichroism spectra of [tetrakis(3-nitrotyrosine)]insulin at pH 8.0 and a concentration of 5×10^{-4} M in the absence (—) and presence of 2.5×10^{-4} M Zn^{2+} (– – –).

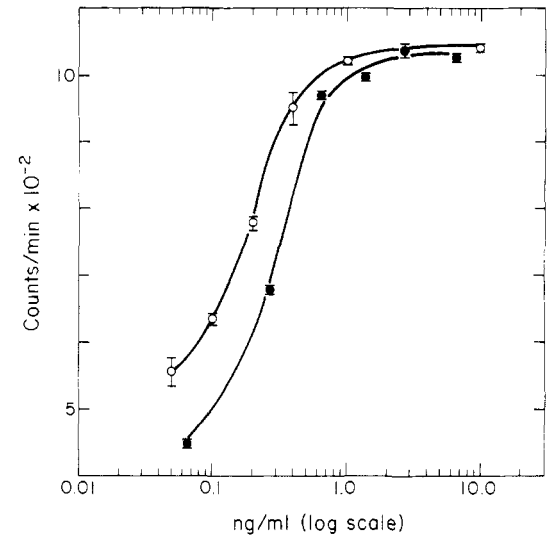

FIGURE 9: Stimulation of the conversion of [3-^{3}H]glucose to lipids in isolated fat cells (Moody et al., 1974) by bovine insulin (○) and by [tetrakis(3-nitrotyrosine)]insulin (●). The vertical brackets indicate the range of triplicate samples.

several different pH values are given in Figure 7. There are small differences in the near-ultraviolet range, but, on the whole, the spectra are remarkably similar over the pH range from 2 to 11. In contrast to the situation with insulin where the CD spectrum at pH 8.0 is substantially different between Zn–insulin and Zn-free insulin (Goldman & Carpenter, 1974), the CD spectrum of TNTI at pH 8.0 is little effected by the presence of zinc ions (Figure 8).

Hormonal assays were performed by Eli Lilly and Co. by the mouse convulsion assay on two preparations of TNTI. One of these gave a value of 50 ± 7%, the other 70 ± 7% of the value of insulin. The preparation of TNTI which had 50% hormonal activity in the mouse convulsion assay was tested for stimulation of lipogenesis in the rat fat cell assay of Moody et al. (1974). The results (Figure 9) indicate a specific activity corresponding to 55% of that of bovine insulin and further show that TNTI is a complete agonist in that full stimulation was attained at high concentrations of TNTI. The good agreement between the results obtained in assays on the whole animal as compared with those on the isolated fat cells indicates that the absorption and transport of TNTI is not substantially different from insulin. Two other preparations of TNTI gave values of 59 and 61% of the hormonal activity of bovine insulin in the lipogeneis assay of Moody et al. (1974).

Discussion

In order to obtain the maximum yield of nitrated insulin, we used nitration conditions which yielded the maximum amount of 3-nitrotyrosine. The kinetic experiments indicated that the amount of nitrotyrosine reached a peak of ~70% of theory after 35 min of nitration under the conditions depicted in Figure 1. Other conditions involving variation in the insulin concentration and pH were investigated without substantial improvement in this yield (Boesel, 1972). Comparison of the results in Figure 1 with those in Figure 2 reveals that the conditions which yielded the maximum amount of nitrotyrosine resulted in products in which the monomeric form of nitrated insulin was a minor component of the reaction mixture. The major components consisted of dimer and higher polymeric forms of nitrated insulin. These latter compounds were readily removed by gel chromatography. Subjection of the monomeric fraction to ion-exchange chromatography at pH 8 separated the more highly charged TNTI from the less highly charged, partially nitrated monomers, albeit the overall yield (10%) from starting insulin was quite low.

The amino acid analysis of TNTI showed the presence of four tyrosine residues which had been nitrated on the 3 position of the aromatic ring. The derivative was homogeneous in size and charge as revealed by gel electrophoresis and ion-exchange chromatography. The spectrophotometric titration of TNTI yielded a sharp isosbestic point which indicated that nitrotyrosine was the only ionizing chromophore present which absorbed light at 381 nm. The introduction of a nitro group at the 3 position of tyrosine lowers the pK_a value of the phenolic group from 10 to ~7.2 (Sokolovsky et al., 1967). The pK_a values of the tyrosine phenolic groups of insulin range from 10.4 to 11.4 (Inada, 1961), whereas in TNTI all four nitrotyrosine residues exhibited the same pK_a value of 7.3.

Because of the tendency of insulin to aggregate in solution, it has been a matter of speculation as to which form is biologically active (i.e., monomer, dimer, etc.). The aggregation constants of bovine insulin are such as to make it highly improbable for the dimer to be present at physiological concentrations (Goldman & Carpenter, 1974). Although guinea pig insulin does not aggregate (Zimmerman et al., 1972; Zimmerman & Yip, 1974), it exhibits quite low hormonal activity (5–10% of that of bovine insulin when assayed on fat cells from either the guinea pig or the rat (Zimmerman et al., 1974; Hurok et al., 1979) which could be related to the inability to aggregate. The fact that TNTI shows no tendency to aggregate at physiological pH values even in the presence of Zn^{2+} (and still possesses high hormonal activity) is further evidence that the monomer is the active hormonal species. If the ability to aggregrate, which is exhibited by most species of insulin, has a functional significance, it is probably related to the storage of insulin in granules. The reason that TNTI does not aggregate at pH values above 7.4 is probably related to the negative charges developed on the nitrotyrosine phenolic groups. In acidic solution, where the phenolic groups are not ionized, the TNTI aggregates extensively and to a greater degree than insulin under similar conditions.

The CD spectrum of TNTI is quite different from that of insulin and similar to that of DOI—a derivative with very low hormonal activity. Unlike insulin, the CD spectrum of TNTI is not perturbed by pH, concentration, or the presence of Zn^{2+} ions. The perturbation of the CD spectrum of insulin by concentration and Zn^{2+} ions has been associated with aggregation (Goldman & Carpenter, 1974). Although TNTI does not aggregate at or above pH 7.4, it does aggregate in acid solution. However, since this aggregation has no effect on the

CD spectrum, the TNTI must aggregate in a less specific fashion than that exhibited by insulin at higher pH values. In many respects the solution properties of TNTI are more similar to those of derivatives with very low hormonal activity, such as desalanine-desasparagine-insulin or desoctapeptide-insulin (Goldman & Carpenter, 1974), than they are to insulin. In this sense TNTI is a unique insulin derivative which possesses substantial hormonal activity despite dramatic differences in solution properties from those of insulin.

Although one or perhaps two of the tyrosines in insulin can be modified by iodination (Garratt, 1964; Izzo et al., 1964; Rosa et al., 1967; Sodoyez et al., 1975) or nitration (Morris et al., 1970) without substantial effect on the hormonal activity, extensive iodination leads to inactive products (Izzo et al., 1964; Rosa et al., 1967). This, when coupled with the fact that three of the four tyrosines are conserved in all insulin species for which sequences are known (Dayhoff, 1976), implies a fundamental role for some of the tyrosine phenolic groups in the hormonal response. In TNTI the pK_a values of the phenolic groups have been decreased to 7.3 from their values of 10.4 (or above) in insulin. As a consequence, a substantial portion of these groups in TNTI will be ionized at physiological pH values. Indeed the abnormal behavior (CD spectrum and failure to aggregate) of TNTI as compared with insulin in solution at physiological pH values can be attributed to the ionization of the nitrated phenolic groups. If the phenolic groups of insulin are intimately involved in the hormonal response, they must participate in the unionized form. It is unlikely that the ionized phenolic groups found in TNTI would mimic the unionized phenolic groups in insulin. Therefore the substantial hormonal activity of TNTI (over 50% of that of insulin) would argue against a fundamental role for most of the phenolic groups in the hormonal response. However, it is possible that only one or two of the tyrosine residues in insulin are essential in the hormonal response—a possibility which is supported by the hormonal activity of partially iodinated or nitrated insulins (Sodoyez et al., 1975; Morris et al., 1970). If this is so, then the pK_a values (7.3) of the TNTI are close enough to physiological conditions so that 50% of one or two of the critical nitrotyrosine residues could be in the nonionized form, especially when absorbed to a lipid membrane, and thus account for the hormonal response. This explanation will not account for the conservation of three of the four tyrosines in various species and suggests that they may play a role in some other function such as folding of proinsulin, insulin storage, or secretion.

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