Reaction of Tetranitromethane with Lutropin, Oxytocin, and Vasopressin*

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Tetranitromethane reaction with intact ovine lutropin and its isolated subunits was studied using spectrophotometric measurements, amino acid analysis, and isolation of tyrosyl peptides. Tyrosyl residues in the \( \beta \) subunit (387, 359) did not react with tetranitromethane in the intact hormone, but were nitrated in the isolated subunit. The sequence and extent of reaction of tetranitromethane with the tyrosyl residues in the \( \alpha \) subunit was \( \alpha_{21} = \alpha_{22} = \alpha_{39} \) (in intact hormone or isolated subunit) > \( \alpha_{41} \) (reacted in isolated subunit only) > \( \alpha_{30} \) (reacted in isolated subunit in 8 M urea only).

Polymerization was observed as a side reaction in agreement with previous studies. The degree of polymerization appeared to be related to both primary sequence and tertiary structure, and for lutropin had the relation: \( \alpha \) subunit (93% polymerized) > intact hormone > \( \beta \) subunit (less than 40%). Polymerization observed with vasopressin was significantly greater than with oxytocin; for these peptides the tyrosine residues in the monomeric product were converted to 3-nitrotyrosine. Neither 3-nitrotyrosine nor tyrosine was detected in the polymerized by-products.

In the tetranitromethane reaction with intact ovine lutropin, other reaction products characterized by absorption spectra were found. Peptides isolated from these products lacked the characteristic 428 nm absorption maxima of 3-nitrotyrosyl peptides and showed instead absorption in the 310 to 350 nm region. Similar products from tetranitromethane reactions with di- and tripeptides containing tyrosine have been observed previously (Boyd, N. D., and Smith, D. B. (1971) Can. J. Biochem. 49, 154-161), but they have not been studied in proteins. A possible relationship to the polymerization side reaction is suggested.

Reaction of tyrosyl side chains with tetranitromethane has been used widely as a selective modification procedure (1, 2). Despite its general use a number of complicating side reactions, including inter- and intramolecular cross-linking (3) and the formation of monomeric products other than the expected 3-nitrotyrosyl products (4), have been observed. The present study examines the products of the tetranitromethane reaction using ovine lutropin, its separated subunits, oxytocin, and arginine vasopressin. In \( \alpha \) LH, tyrosyl residues have been modified in the intact hormone and its separated subunits. Reactivity of tyrosyl residues has been determined by isolation and analysis of tryptic peptides. The extent of cross-linking and the nature of tyrosine modification in \( \alpha \) LH and in purified oxytocin and arginine vasopressin are also examined under various reaction conditions.

Two previous reports concerning this reaction with lutropin have been published (5, 6). These studies used ovine and bovine LH, which are identical in amino acid sequence (7-10), but the authors arrived at different conclusions concerning surface accessibility of tyrosyl side chains in the intact molecule. Effects of these modifications on specific binding to the hormone receptor and biological activity of the hormone are reported in the accompanying paper (11).

EXPERIMENTAL METHODS AND MATERIALS

Hormone Preparations—LH was isolated from fresh frozen ovine pituitary glands (12). Oxytocin and vasopressin were prepared from posterior lobes of bovine pituitaries (13, 14).

Chemicals—Chemicals used were reagent grade or best grade available. Reagents used in the Edman degradation were prepared by standard methods for that purpose or purchased from Pierce. Fluorescamine was obtained from Roche.

Preparation of Trypsin—Trypsin used for LH digestion was purified using an affinity column prepared by attaching soybean trypsin inhibitor (Sigma) to a Sepharose 4B support using cyanogen bromide activation (15). The column was equilibrated with 0.1 M Tris-HCl, pH
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TABLE I

Summary of oLH and oLH subunit reactions with tetranitromethane (TNM)

| Preparation Sample | Protein concentration (mg/ml) | Reaction temperature (°C) | TNM/Tyr | Yields | Tyrosine analyses on isolated monomer | Preparation designation |
|--------------------|-------------------------------|---------------------------|---------|--------|-----------------------------------|-------------------------|
|                    |                               |                           |         |        | Tyrine | 3-Nitrotyrosine | Total Yield | |
|                    | Pref | mol/mol | Yield | Amount | mg | % | mg | % | mg | % | |
| 1                  | a    | 2      | 4*    | 1/1   | 14   | 47 | 11 | 37 | 5.6 | 0.6 | 6.2 | 89 | Monostyrosyl |
| 2                  | a    | 2      | 25    | 1/1   | 17   | 57 | 10 | 33 | 6.0 | 0.6 | 6.6 | 94 | oLH |
| 3                  | a    | 2      | 4     | 5/1   | 16   | 53 | 11 | 37 | 4.5 | 1.1 | 5.6 | 80 | Diatyrosyl |
| 4                  | a    | 2      | 10    | 25/1  | 17   | 57 | 11 | 37 | 5.0 | 1.2 | 6.2 | 89 | oLH |
| 5                  | a    | 2      | 4     | 10/1  | 15   | 45 | 11 | 33 | 4.6 | 0.7 | 5.3 | 76 | Triatyrosyl |
| 6                  | a    | 2      | 4     | 25/1  | 15   | 50 | 8  | 27 | 3.7 | 1.5 | 5.2 | 74 | oLH |
| 7                  | a    | 2      | 4     | 25/1  | 20   | 67 | 6  | 20 | 3.6 | 1.6 | 5.2 | 74 | oLH |
| 8                  | a    | 2      | 4     | 30/1  | 17   | 57 | 7  | 43 | 3.7 | 1.7 | 5.4 | 77 | oLH |
| 9                  | a    | 2      | 4     | 50/1  | 16   | 53 | 7  | 23 | 3.8 | 2.2 | 6.0 | 60 | oLH |
| 10                 | a    | 5      | 4     | 10/1  | 25   | 83 | 2  | 7  | 0.8 | 2.1 | 3.0 | 60 | oLH |
| 11                 | a    | 2      | 4     | 15/1  | 22   | 73 | 3  | 10 | 0.7 | 2.8 | 3.5 | 70 | oLH |
| 12                 | a    | 2      | 4     | 25/1  | 22   | 73 | 3  | 10 | 0.7 | 2.8 | 3.5 | 70 | oLH |
| 13                 | a    | 2      | 25    | 25/1  | 23   | 77 | 1.5 | 5  | 0.8 | 2.1 | 3.0 | 60 | oLH |
| 14                 | a    | 1      | 4     | 25/1  | 24   | 80 | 1.5 | 5  | 0.6 | 2.6 | 3.2 | 64 | oLH |
| 15                 | a    | 2      | 25    | 25/1  | 18   | 60 | 9  | 30 | 0.0 | 4.1 | 4.1 | 82 | Pentatryrosyl oLH |
| 16                 | a    | 2      | 50    | 50/1  | —    | —  | —  | —  | —  | 0.0 | 1.8 | 1.8 | 90 | oLH |

* *a*, o, and / separated subunit as starting material for the tetranitromethane reaction.

* Starting material for each reaction was 30 mg dry weight. Values calculated from amino acid analyses.

* Values expressed as moles per mol of polypeptides as determined by amino acid analysis.

* Not determined.

* This reaction carried out in 0.05 M Tris-Cl, 8.0 M urea, pH 8.1.

7.1/20 mM CaCl₂ at 25°. Trypsin (Worthington) was dissolved in this buffer and adsorbed to the column. The column was eluted, first with the initial buffer, second with 5 mM sodium acetate, 20 mM CaCl₂, pH 4.5, and third with 10 mM HCl. 20 mM CaCl₂, Elution of proteins was followed by measuring the absorbance at 280 nm. Trypsin of the desired specificity is eluted with the third buffer.

**Amino Acid Analysis**—Analyzes were done using Durrum resin (DC-2a) with a single column buffer system (16). Samples were hydrolyzed for 24 hours at 105° in vacuum-sealed tubes with 6 N HCl containing 0.1% phenol.

**Spectral Studies**—Absorbance measurements were made using a Cary spectrophotometer (model 15) or a Gilford spectrophotometer (model 240).

**Edman Degradation**—The Edman degradation and identification of dansyl amino acids were done using micro adaptations of procedures reviewed by Hartley (17).

**Nitration Reactions**—The nitration reaction was carried out by variations of procedures described by Sokolovsky et al. (1). The molar ratio of tetranitromethane to tyrosine varied from 1 to 50 (see Table I). Tetranitromethane was dissolved in 1.5 ml of 95% ethanol and added dropwise to the protein solution with rapid stirring. Reaction rate was followed by measuring the increase in absorbance in 428 nm. The reaction was terminated when the absorbance approached a constant value. The reaction mixture was lyophilized, dissolved in 0.05 M Tris-Cl, 8.0 M urea, pH 8.1, and applied to a Sephadex G-100 column, eluting with the same buffer. The reaction with oLH used a tetranitromethane/tyrosine ratio of 25, but after 1 hour an equal solutions on elution with the basic buffer.

**Isolation and Identification of Peptides From Tetranitromethane-modified LH Preparations**—Identification of the specific modified tyrosine residues in the oLH sequence, peptides were isolated from the tryptic digest using two-dimensional separation procedures. Both reagents were made up immediately before use. After spraying with the developing solvent, the paper was dried in warm air for about 10 min and a developing solvent consisting of 0.5% volume of volume pyridine in acetone was applied. Both reagents were made up immediately before use. After spraying with the developing solvent, the paper was dried in warm air for about 10 min and a developing solvent consisting of 0.5% volume of pyridine in acetone was applied. Both reagents were made up immediately before use. After spraying with the developing solvent, the paper was dried in warm air for about 10 min and a developing solvent consisting of 0.5% volume of pyridine in acetone was applied. Both reagents were made up immediately before use.

**Nitration of LH and Its Subunits**—Fig. 1 summarizes the nitration of LH and its subunits. The modified subunits were oxidized with performic acid (19) and the oxidized sample digested with trypsin. Digestion was carried out at 25° for 16 hours in 0.1 M NH₂HCO₃ at a trypsin/substrate ratio at 1/100 (w/w) and a final substrate concentration of 1 mg/ml. The digest was frozen and lyophilized.

Peptides were isolated from the tryptic digest using two-dimensional maps on Whatman No. 3MM paper with combinations of high voltage electrophoresis and descending chromatography as indicated. The electrophoresis buffer systems were those described by Offord (20).

**Results**

Nitration of LH and Its Subunits—Table I summarizes the rates of reaction of tetranitromethane with LH and its subunits. The customary use of absorbance at 428 nm to calculate the formation of 3-nitrotyrosyl products is only...
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Fig. 1. Tetranitromethane (TNM) reaction with oLH and its subunits as followed by the increase in absorbance at 428 nm (A428). Conditions of reaction for various preparations are given in Table I. a, reaction with intact oLH: Curve A, Preparation 6; Curve B, Preparation 5; Curve C, Preparation 1; b, reaction with oLHa: Curve A, Preparation 15 (in 8 M urea); Curve B, Preparation 12; c, reaction with oLHβ: Preparation 16.

approximate since such side products as nitroformate absorb at 428 nm. Moreover, as shown below, other side products with absorbance in this region are sometimes formed.

Table I shows that the extent of modification in the purified monomer products was more dependent upon the ratio of tetranitromethane to tyrosine than upon protein concentration or reaction temperature, even though these factors affected the relative amounts of monomeric product and cross-linked products obtained. Tyrosyl modification in the purified monomer was largely independent of protein concentration and temperature. The monomeric products were separated from other reaction products by chromatography on Sephadex G-100 (Fig. 2). In the reaction of intact oLH at a tetranitromethane/tyrosine molar ratio of 1, a monomeric form lacking 1 residue of tyrosine was found (Table I). Somewhat higher tetranitromethane/tyrosine ratios gave monomers in which about 2 tyrosine residues were modified, and still higher ratios led to the modification of approximately 3 residues.

Separation of the subunits from the isolated monomers in the oLH Preparations 1 to 9, Table I, by countercurrent distribution (18) gave patterns such as that in Fig. 3. Measurement of the 310 to 350 nm absorbance indicated that modification was confined to the α subunit. This was also confirmed by amino acid analysis (Table II).

Tetranitromethane treatment of the separated subunits gave quite different results. Reaction of oLHa using tetranitromethane/tyrosine ratios between 5 and 50 gave varying yields of isolated monomeric products in which 4 of the 5 tyrosyl residues were modified. Modification of all 5 tyrosyl residues in oLHβ was attained only by using similar reaction conditions in the presence of 8 M urea.

The reaction rate for oLHa was considerably slower under equivalent conditions than that of oLH or oLHβ. A second addition of tetranitromethane was made after 1 hour of reaction, giving an overall tetranitromethane/tyrosine ratio of 50/1. Under these conditions, both tyrosyl residues in oLHβ were modified, giving high yields of the monomeric preparation.

Analyses of the purified monomers for tyrosine and 3-nitrotyrosine are shown in Table I. In none of the preparations was 3,5-dinitrotyrosine detected. In many of the preparations the amount of 3-nitrotyrosine formed was not equal to the amount of tyrosine lost during the modification procedure. Cheng and Pierce (5) observed a similar discrepancy, but Sairam et al. (6), who used a normalized calculation, did not report such differences. The discrepancy may be explained at least partially by tyrosyl side reactions which give rise to products other than 3-nitrotyrosine. Such products have been partially characterized in the present study by spectral studies and analysis of modified tyrosyl peptides.
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Table II

Analysis of oLHa and oLHβ subunits obtained from mono-, di-, and triatyrosyl LH derivatives by counter current distribution

Values are expressed as residues per mol of protein (15,000 Mr) based on a 22-hour acid hydrolysis without corrections for loss during hydrolysis.

| Amino acid residue | Preparation 1 “Monoatryrosyl” LH | Preparation 3 “Triatryrosyl” LH | Preparation 6 “Triatryrosyl” LH |
|--------------------|----------------------------------|----------------------------------|----------------------------------|
| oLHa               | oLHβ                             | oLHa                             | oLHβ                             |
| Lysine             | 10.3                             | 2.2                              | 10.0                             |
| Histidine          | 2.9                              | 2.9                              | 2.1                              |
| Arginine           | 3.3                              | 7.2                              | 3.8                              |
| Aspartic acid      | 6.0                              | 5.0                              | 6.1                              |
| Threonine          | 8.9                              | 7.2                              | 8.0                              |
| Serine             | 5.6                              | 6.8                              | 6.1                              |
| Glutamic acid      | 8.6                              | 5.9                              | 8.3                              |
| Proline            | 0.8                              | 19.4                             | 19.5                             |
| Glycine            | 4.3                              | 6.5                              | 6.2                              |
| Alanine            | 7.5                              | 7.6                              | 7.0                              |
| 1/2Cystine         | 10.0                             | 11.8                             | 11.5                             |
| Valine             | 4.8                              | 7.5                              | 6.0                              |
| Met(OH)            | 5.8                              | 2.7                              | 3.4                              |
| Isoleucine         | 2.0                              | 3.6                              | 2.1                              |
| Leucine            | 2.2                              | 10.0                             | 2.4                              |
| Tyrosine           | 3.2                              | 10.0                             | 2.4                              |
| Phenylalanine      | 3.6                              | 2.9                              | 2.9                              |
| Tyr(3NO2)          | 0.6                              | 0.9                              | 0.9                              |

*See text.

Isolation and Analysis of Tetranitromethane-modified Tyrosine Peptides—The countercurrent distribution separations indicated that no modification of the β subunit occurred in the intact hormone. Since both tyrosine residues in this subunit were nitrated completely in the isolated subunit no further study of the tryptic peptides from the nitrated β subunit was undertaken. Isolation of nitrated peptides was confined to those of the α subunit in various preparations. Fig. 4 shows the sequence of oLHa as determined by Liu et al. (8) with the theoretical tryptic peptides enumerated from the NH2 terminus as T1 through T14, in which T4-T5, T6, and T13 are the theoretical tryptic peptides in good yield from these preparations, the absence of cross-linked peptides detectable on the peptide maps, and the expected separation of the subunits from the monomeric oLH with countercurrent distribution indicated that very little interchain or intrasubunit cross-linking occurred in these monomeric products. Analyses for tyrosine and 3-nitrotyrosine in these peptides from several of the preparations are given in Table III. These data show that 1 of the tyrosyl residues in T4-T5 and tyrosyl 41 in T6 are protected from tetranitromethane modification in the intact molecule, but tyrosyl 41 is readily accessible to modification in reactions using the separated oLHa subunit.

Modification of tyrosyl residues in peptides T4-T5 and T13 occurred in varying degrees in all of these preparations, and the extent of modification of these residues in the intact molecule depended on the tetranitromethane/tyrosine ratio. Table III shows that the appearance of 3-nitrotyrosine did not equal the loss of tyrosine, and thus the loss of tyrosine on amino acid analysis was the best measure of tyrosyl residue modification. The present experiments do not allow a determination of the difference in accessibility between the 2 adjacent tyrosyl residues in T13, a92 and 93, if such a difference exists. However, the even increase in the amount of tyrosine modification in peptides T13 and T13' with increasing tetranitromethane concentration suggests that these residues may be equally accessible to tetranitromethane modification. Analysis of the modification of tyrosyl residues a21 and a23 in peptide T4-T5 was readily evaluated in all of the preparations analyzed because Tyr a21 was the NH2 terminus of the peptide. Analyses of this peptide from various preparations before and after a single Edman degradation afforded a determination of the extent of modification of the separate residues (see also Ref. 5). Preparation 1 showed that Tyr a21 was accessible to tetranitromethane modification in the intact molecule, while Tyr a23 was inaccessible under the same conditions. The data for Preparation 11 (Table III) showed that this residue is also inaccessible in the separated oLHa subunit. Tyr a21 and Tyr...
Fig. 6. Maps of oLHa tryptic peptides: pH 6.5 high voltage electrophoresis (HVE) mobilities normalized with respect to aspartate (M<sub>60</sub>); pH 2.1 high voltage electrophoresis mobilities normalized with respect to N<sup>α</sup>-DNS-Arg(A<sub>α-DNS</sub>); specific conditions as indicated. Descending chromatography as indicated in 1-butanol/acetate acid/water/pyridine 15/3/12/10 (BAWP). a, isolation of basic peptides, including T6; high voltage electrophoresis, pH 6.5, 3 kv, 50 min versus isolation of T13 and T13'; high voltage electrophoresis, pH 6.5, 3 kv, 180 min, strip removed from b after chromatography versus high voltage electrophoresis, pH 2.1, 2 kv, 180 min.

### TABLE III

| Amino Acid | Preparation 1 | Preparation 3 | Preparation 6 | Preparation 11 | Preparation 15 |
|------------|---------------|---------------|---------------|---------------|---------------|
| Lys        | 1.2           | 0.7           | 0.9           | 1.1           | 1.2           |
| His        | 0.9           | 1.0           | 1.0           | 0.8           | 1.0           |
| Arg        | 1.1           | 1.0           | 1.2           | 1.1           | 1.2           |
| Cys        | 1.1           | 1.0           | 1.0           | 1.1           | 1.0           |
| Thr        | 1.1           | 1.2           | 1.4           | 1.3           | 1.2           |
| Ser        | 1.1           | 1.0           | 1.0           | 1.1           | 1.0           |
| Glu        | 1.1           | 1.0           | 1.0           | 1.1           | 1.0           |
| Pro        | 1.1           | 1.0           | 1.0           | 1.1           | 1.0           |
| Gly        | 1.1           | 1.0           | 1.0           | 1.1           | 1.0           |
| Ala        | 1.1           | 1.0           | 1.0           | 1.1           | 1.0           |
| Val        | 1.1           | 1.0           | 1.0           | 1.1           | 1.0           |
| MetSO<sub>2</sub> | 1.0         | 0.8           | 0.9           | 0.8           | 0.9           |
| Ile        | 1.1           | 0.8           | 0.9           | 0.8           | 0.9           |
| Leu        | 1.1           | 0.8           | 0.9           | 0.8           | 0.9           |
| Tyr        | 1.1           | 0.8           | 0.9           | 0.8           | 0.9           |
| Phe        | 1.7           | 1.5           | 2.0           | 1.6           | 1.5           |

**Footnotes:**

* No 3-nitrotyrosine detected; peptide spot strongly absorbed UV light, turned yellow when exposed to ammonium vapor, and was fluorescamine and ninhydrin negative, i.e. did not show typical reactions of the primary amino group.

** Small amount of 3-nitrotyrosine detected; data did not permit accurate quantitation.

Analysis of Modified T4-T5 peptides following a single Edman degradation.

ααα were both modified extensively when the reaction of the oLHa subunit was carried out in 8 M urea, as shown in Preparation 15.

**Spectral Studies**—Comparison of the absorbance spectra of purified monomer oLH derivatives, of the modified separated subunits, and of the isolated modified peptides with that given by 3-nitrotyrosine confirmed the conclusion that tetranitromethane treatment results in the formation of other products in addition to 3-nitrotyrosine. Fig. 6 shows the spectrum of T6 from Preparation 11 compared with spectra of 3-nitrotyrosine and the unmodified peptide. The spectrum of the modified peptide shows significant absorbance in the 310 to 350 nm region which correlated well with the amount of 3-nitrotyrosine found by amino acid analysis. High absorbance in the 310 to 350 nm region correlated with the disappearance of tyrosine but not with the appearance of 3-nitrotyrosine. The possibility that the products absorbing in the 310 to 350 nm region are generated by performic acid oxidation of the nitrated protein was eliminated by examination of the visible spectrum of Preparation 15 before and after treatment, as shown in Fig. 7b. In addition, analyses for tyrosine and 3-nitrotyrosine before and after performic acid treatment were virtually the same. It thus appears that these products are not the result of subsequent treatment, and must be formed by different reactions of the tyrosyl residues.

While the amounts of the differently absorbing products...
were variable even for repeated preparations at the same tetranitromethane/tyrosine ratio, relatively more of the 310 to 350 nm absorbing products were formed when low tetranitromethane/tyrosine ratios were used, while the yield of 3-nitrotyrosine increased in the α subunit when the structure was unfolded in 8.0 M urea. High yields of 3-nitrotyrosyl derivatives were also obtained in the reaction of tetranitromethane with the β subunit without urea denaturation, Fig. 7b. The spectrum of peptide T4-T5 isolated from “monoatyrosyl” Preparation 1 is shown in Fig. 8a, together with a spectrum made after a single Edman degradation. Analyses of these peptides (Table III) revealed 1 intact tyrosyl residue both before and after the Edman degradation. Dansylation showed no identifiable NH₂-terminal amino acid in the intact peptide. Dansyl phenylalanine appears in good yield after one Edman degradation. The yellow color shown by this peptide on the map upon exposure to ammonia vapor was less intense than that shown by similar amounts of other peptides containing true 3-nitrotyrosyl residues, and the peptide also gave an atypical reaction with fluorescamine or ninhydrin. It was revealed on fluorescamine-treated maps by a strong absorbance of ultraviolet light, instead of the yellow-green fluorescent spot characteristic of the fluorescamine reaction products.
Similar products have been reported in studies using tyrosyl-containing di- and tripeptides (4).

In contrast to the above peptide isolated from Preparation 1, the T4-T5 peptide isolated from Preparation 15 (carried out in 8 M urea) gave the visible spectrum shown in Fig. 8b, which also shows the spectrum after a single Edman degradation. Yields of the 3-nitrotyrosyl derivatives in the same peptides were much higher in Preparation 15 than in Preparation 1 (Table III), and the relative amount of 310 to 350 nm absorbance was correspondingly less. This suggests that relaxation of the tertiary structure in the presence of urea may favor the production of the 3-nitrotyrosyl derivative.

Studies on Oxytocin and Vasopressin—Effects of tertiary structure on the course of the reaction of tetranitromethane with the tyrosyl residues in oLH suggested the examination of the reaction with small peptides where tertiary structure may not exert as much influence. Oxytocin and vasopressin are cyclic, disulfide-containing octapeptides, each of which contains a tyrosine residue in position 2. Sequences around the tyrosine are: H-Cys-Tyr-Ile- and H-Cys-Tyr-Phe- for oxytocin and vasopressin, respectively. Their reaction with tetranitromethane permitted three observations:

1. The range and yield of polymeric and monomeric products were very different for these two structurally similar peptides.
2. The size distribution of products produced from either peptide, as measured by gel chromatography, was relatively independent of the tetranitromethane/tyrosine ratio employed.
3. The production of 3-nitrotyrosine derivatives was limited to the monomeric forms.

Fig. 9 shows elution profiles on Sephadex G-25 gel filtration chromatography of products from the reactions which were soluble in 0.1 M ammonium bicarbonate. The insoluble residue was removed by centrifugation and analyzed for amino acid content. In oxytocin preparations this residue constituted a major fraction of the material, whereas with vasopressin it was a minor fraction (Table IV).

The major product formed in the isolated monomer fraction was a peptide containing the 3-nitrotyrosyl derivative (Table V). In marked contrast, the polymeric fractions showed no detectable 3-nitrotyrosine, although solution suspension in alkaline media indicated substantial yellow-colored material (presumptive nitration). The soluble material in the high molecular weight fractions showed higher 340 to 428 nm absorbance ratios, with spectral patterns similar to the peptide shown in Fig. 8a. Neither 3-nitrotyrosine nor unmodified tyrosine were present in these fractions. This suggests that nitration via the unidentified derivative with an absorption maximum near 340 nm may favor polymerization.

The absorbance of each fraction from the soluble products was measured at three wavelengths (Fig. 9). Elution profiles are similar with tetranitromethane/tyrosine ratios of 1.5/l or 25/l for either oxytocin or vasopressin, but the difference between the two peptides is dramatic. In each preparation all fractions except C and F gave the expected analysis for oxytocin or vasopressin, except that tyrosine was absent (Table IV). Analyses of the monomeric products (Fractions C) are presented in Table V. The spectra of these fractions showed an absorbance peak at 428 nm consistent with the amount of 3-nitrotyrosine found by amino acid analysis. Fractions F are at the included volume of the column (salt peak) and their absorbance spectra showed a sharp peak at 340 nm which was identical with that of nitroformate, a side product always present in these reactions (24).

DISCUSSION

The accessibilities of oLH tyrosyl residues found in this study are in general agreement with those determined for bovine lutropin (5). They are also consistent with the ratio of iodination of specific tyrosyl residues in porcine lutropin (25).

The study of Sairam et al. (6) on nitration of ovine lutropin, in which they report the accessibility of all tyrosyl residues except a21 and b259 is in disagreement with both the present studies and those cited above. Since both studies (Ref. 6 and the present report) deal with lutropin from the same species, the difference must lie in the methods employed, or interpretation of the results, or both. Sairam et al. (6) used analytical data from peptides partially isolated from digests of the intact hormone, whereas the present studies have used data only from pure peptides isolated from the subunits.

The present studies on oLH, considered together with those of Cheng and Pierce (5) and Combarbous and Maghuin-Rogis-Register (25) on bLH and pLH, respectively, show that the topology around the specific tyrosyl residues, as revealed by their
accessibility to tetranitromethane modification or iodination, is highly conserved among these species as might be expected from the general conservation of sequence in these regions. This conservation is most likely a reflection of the importance of these regions in defining the tertiary structure and functional properties of lutropin, and is confirmed by an examination of specific receptor binding and biological activities of these modified preparations which is reported in the accompanying paper (11). A point of interest is that the Pro $\alpha$25 $\rightarrow$ Leu $\alpha$25 substitution between oLH and pLH, which would not be considered a conservative substitution, does not disturb the tertiary structure around Tyr $\alpha$21 and Tyr $\alpha$30, which exhibit similar accessibility to modification in both hormones.

The isolation of peptides which contain a tyrosyl derivative other than the 3-nitrotirosyl is in accord with the reported losses of tyrosine in previous studies (5) without the consequent appearance of 3-nitrotirosine. The present data do not permit a proposal for the structure of this implied derivative, but its inability to react with fluorescamine or ninhydrin in the usual fashion and the fact that it undergoes a normal Edman degradation (re: results with peptide T4-T5) suggest that cyclization or substitution to a secondary amino compound analogous to proline may be involved. A structural assignment will require more extensive characterization of such modified peptides. The studies of Boyd and Smith (4) demonstrated that similar derivatives were monomers of the starting peptide; thus a polymerization is not directly involved, and this appears to be the case in our own studies. The possibility that the unidentified derivative (or derivatives) are intermediates in the polymerization reactions observed with tetranitromethane is consistent with all the data obtained. Moreover, our results with the $\beta$ subunit (very little polymerization), and the differences in degree of polymerization with oxytocin and vasopressin suggest that primary amino acid sequence and tertiary structure around the tyrosyl residues are important factors in the course of this side reaction.

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