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# Studies on the Chemical Modification of the Tyrosine Residue in Bovine Neurophysin-II

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1. Bovine neurophysin-II contains 1mol of tyrosine residue/10000g of protein. This residue could be readily nitrated with tetranitromethane. On hydrolysis and amino acid analysis 1mol of 3-nitrotyrosine was found/10000g of protein. Starch-gel electrophoresis at pH 8.5 showed that nitration had converted the native protein into a single, more acidic species. The increase in acidity was consistent with the observed fall in p$K$ of the tyrosine hydroxyl from 9.2 in native neurophysin to 7.3 in the nitrated protein. Further, the absence of any intermediate species, even under conditions of minimum substitution, confirmed that the molecular weight of the monomer is 10000. 2. *O*-Acetylation of the tyrosine residue was carried out with *N*-acetylimidazole, in conjunction with the reversible blocking of amino groups by citraconylation. The degree of *O*-acetylation, determined spectroscopically, was 0.9mol of *O*-acetyltyrosine/10000g of protein. 3. The hormone-binding ability of modified protein was tested by equilibrium dialysis and was found to be unchanged by either nitration or *O*-acetylation of the tyrosine residue. 4. Interaction of neurophysin-II and [8-arginine]-vasopressin gave rise to a characteristic difference spectrum with a peak at 286.8nm and shoulder at 279.6nm. Part of this hyperchromicity is thought to result from entry of the tyrosine residue at position 2 in the hormone into the hydrophobic environment of the binding site. With nitrated neurophysin-II a second peak appeared at 436nm, showing that the tyrosine of the protein is also perturbed. The very large red shift (84nm) in this region suggests that the 3-nitrotyrosyl residue not only enters a more hydrophobic environment on protein–hormone interaction, but is caused to ionize more fully by the approach of some positively charged group.

The polypeptide hormones oxytocin and vasopressin are stored in association with the neurophysins in neurosecretory granules of the neurohypophysis. These proteins are thought to act as carriers for the hormones in the hypothalamo-neurohypophysial system (Bargmann, 1957; Sawyer, 1961), and may also represent the remains of precursors from which the hormones are split off during synthesis.

A protein–hormone complex can be precipitated by salt from extracts of acetone-desiccated pituitary posterior lobes. This complex consists mainly of three constituents, oxytocin, vasopressin and neurophysin and if the extraction of acetone-desiccated powder is performed at pH1.5 the protein moiety is identical with that found in a lysate of purified neurosecretory granules (Dean, Hollenberg & Hope, 1967). Further purification of the protein moiety separates a number of components of which at least three (two major and one minor constituent) possess the ability to bind hormones (Hollenberg & Hope, 1968; Rauch, Hollenberg & Hope, 1968, 1969). One of the two major components, neurophysin-II, was studied. Although, like the other neurophysins, it binds both oxytocin and vasopressin, there is evidence that *in vivo* it is specifically located in the neurosecretory granules with [8-arginine]-vasopressin (Dean, Hope & Kazić, 1968).

The bonds responsible for protein–hormone interaction are not covalent, the hormone can be dissociated from the complex by a number of mild procedures such as dialysis against dilute acetic acid (Acher, Chauvet & Olivry, 1956). However, the binding is very specific, and is restricted to oxytocin, vasopressin and a few structural analogues (Breslow & Abrash, 1966; Hollenberg & Hope, 1967). There must be a well-defined binding site, like that of an antibody or the catalytic site of an enzyme protein, and it seems unlikely that there should be more than one such site/polypeptide chain. Yet previous calculations from work on a crystalline complex of neurophysin-II and [8-arginine]-vasopressin gave two

binding sites/molecule of protein (Hollenberg & Hope, 1968). This was based on a molecular weight of 20000 and part of the difficulty is to determine whether this is the true molecular weight of the protein monomer.

In the present study this problem was approached by Craig's method of partial substitution (Battersby & Craig, 1951). A given residue on the protein was allowed to react with a deliberate insufficiency of reagent. This resulted in a partially substituted mixture in which the number of components depended on the number of mol of substitutable residues/mol of protein. Apart from the classical work on insulin (Harfenist & Craig, 1952) the method has been restricted mainly to peptides of molecular weight about 1500 (see, e.g., Hettinger, Kurylo-Borowska & Craig, 1968). The only difficulty in extending it to larger peptides or proteins is that the number of substitutable groups of any one kind becomes very large. The partially substituted mixture is then complex and difficult to interpret. The present approach was therefore aimed at a residue occurring in only small amounts, namely tyrosine. This is present in all three bovine neurophysins to the extent of 1mol of residue/10000g of protein. In neurophysin-II it was in an exposed position and could be readily modified by nitration. The number of components in a partially nitrated mixture was then examined by electrophoresis.

The tyrosine residue was also substituted by *O*-acetylation and the two modified proteins were used to investigate the role of tyrosine in the hormone-binding function of the protein. Further, the nitrotyrosine chromophore made it possible to elucidate the contribution of this tyrosine residue to the difference spectrum found to be characteristic of protein–hormone interaction.

## METHODS

*Biological materials.* Protein–hormone complex was prepared from an acetone-dried powder of bovine pituitary posterior lobes (Paines and Byrne Ltd., Greenford, Middx., U.K.) by the method of Hollenberg & Hope (1968). Natural [8-arginine]-vasopressin was isolated from the complex by Sephadex G-25 chromatography in 0.1M-formic acid (Frankland, Hollenberg, Hope & Schacter, 1966).

*Preparation of purified neurophysin-II.* The hormone-free proteins resulting from Sephadex G-25 chromatography were separated from high-molecular-weight impurities by chromatography on Sephadex G-75 in 0.1M-formic acid (Hollenberg & Hope, 1968). The resulting crude neurophysin was purified by ion-exchange chromatography on CM-Sephadex in acetate buffer, *I* 0.05, by using a pH gradient from 4.4 to 5.0 as described by Hollenberg & Hope (1968). Electrophoretically homogeneous neurophysin-II was obtained from fractions eluted in the narrow pH range between 4.90 and 4.97.

*Preparation of nitrated neurophysin-II.* A sample of neurophysin-II (10mg, 1$\mu$mol assuming mol.wt. 10000) was dissolved in 2ml of 50mM-tris–HCl buffer, pH8.0, and stirred for 1h at room temperature with 100-fold molar excess of tetranitromethane. The tetranitromethane (Ralph N. Emanuel Ltd., Alperton, Middx., U.K.) was added as a solution in ethanol (10%, v/v). The nitrated protein was purified by chromatography on a column (2.0cm × 140cm) of Sephadex G-25 in 0.1M-formic acid and stored as a freeze-dried powder.

*Preparation of O-acetylneurophysin-II.* The acetylating agent was *N*-acetylimidazole (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.). The reagent was stored at 4°C as a solution in benzene over $MgSO_4$. Immediately before use a sample of the solution was evaporated in a stream of dry air and the solid *N*-acetylimidazole redissolved in the protein solution to be acetylated (Riordan & Vallee, 1967).

Acetylation was carried out in conjunction with the reversible blocking of amino groups by citraconylation (Dixon & Perham, 1968). A sample of protein (10mg, 1$\mu$mol) was dissolved in 10ml of 0.1M-sodium borate buffer, pH8.0, and 50$\mu$l of citraconic anhydride (400$\mu$mol) was added over a 20min period. The pH was maintained at 8.0 by addition of 4M-NaOH. Citraconylation was carried out for a total of 30min. The pH was lowered to 7.5 with M-HCl and acetylation carried out by addition of *N*-acetylimidazole (50$\mu$mol). After 1h the pH was lowered to 3.5 and the mixture stirred for 17h.

The *O*-acetylated protein was purified by chromatography on a column (2.0cm × 140cm) of Sephadex G-25 in 0.1M-formic acid and stored as a freeze-dried powder.

*De-O-acetylation.* A solution of hydroxylamine (2M) at pH7.5 was prepared by dissolving 2.78g of hydroxylamine hydrochloride in 20ml of 2M-NaOH. The acetylated protein was dissolved in sodium phosphate buffer, pH7.5 and *I* 0.1, and left for 10min at room temperature with an equal volume of 2M-hydroxylamine. The de-*O*-acetylated protein was dialysed exhaustively against distilled water and freeze-dried.

*Zone electrophoresis.* This was performed on starch gel at pH8.1 with a potential gradient of 26V/cm for 90min. All other details were as described by Dean & Hope (1967). For electrophoresis at pH8.5 the buffer system of Ferguson & Wallace (1961) was modified. The electrode buffer consisted of 380mM-boric acid and 115mM-LiOH, and the gel buffer consisted of a mixture of 3mM-citric acid with 28mM-tris and 80mM-boric acid with 23mM-LiOH in a 9:1 (v/v) ratio.

*Bioassays.* Pressor activity was assayed by the method of Dekanski (1952) with the modification of Dean & Hope (1967). The biological activity was assayed against the activity of solutions of synthetic [8-arginine]-vasopressin standardized against the Third International Standard (Bangham & Mussett, 1958). Results were calculated by using the (1+2) method (Gaddum, 1959).

*Equilibrium dialysis.* The hormone-binding abilities of native and modified proteins were determined by thin-film dialysis in the 'alternate' cell of Craig & Konigsberg (1961). A solution (0.4ml) containing 3mg of protein dissolved in sodium acetate buffer, pH5.6 and *I* 0.1, was introduced on the inside of the 18/32 Visking membrane and was dialysed against three 9.0ml changes of buffer in the outer compartment of the dialysis cell. Then 9.0ml of

a solution of [8-arginine]-vasopressin (20i.u. of pressor activity/ml) was placed in the outer compartment and allowed to equilibrate with the protein for 24h at 4°C. Peptide concentrations in the inner and outer compartments were determined by bioassay for pressor activity. Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

The volume of solution in the inner compartment at the end of the experiment was determined by introducing a 20$\mu$l sample of a stock solution of Blue Dextran in buffer. This was added with the protein solution at the beginning of dialysis. After 24h 0.3ml of solution from the inner compartment was removed and diluted to 2.6ml with water and $E_{620}$ was compared with that of a suitably diluted 20$\mu$l sample of the stock solution. From this the volume of solution in the inner compartment was calculated: in a typical experiment the final volume was 0.6ml.

*Amino acid analyses.* These were performed on an automatic amino acid analyser (Evans Electroselenium Ltd., Halstead, Essex, U.K.) by the method of Spackman, Stein & Moore (1958) after hydrolysis in 6M-HCl for 17h at 110°C.

3-Nitrotyrosine was eluted on the long column after phenylalanine and gave a colour yield 1.02 times that of tyrosine. 3-Nitro-L-tyrosine used as a standard was obtained from K & K Laboratories Inc., Plainview, N.Y., U.S.A.

*Ultraviolet spectra.* These were measured on the Unicam SP. 800 recording spectrophotometer with 1cm silica cells.

*Determination of O-acetyltyrosine residues.* This was performed spectrophotometrically by the method of Riordan & Vallee (1967). A solution of *O*-acetylneurophysin-II (2mg/ml) in sodium phosphate buffer, pH7.5 and *I*0.1, was prepared. A 1ml sample was mixed with 1ml of buffer and the u.v. spectrum recorded with buffer as the reference solution. A second 1ml sample was mixed with 1ml of 2M-hydroxylamine and kept at room temperature for 5min. The u.v. spectrum was recorded with a mixture of 1ml of buffer and 1ml of 2M-hydroxylamine as the reference solution. The number of mol of *O*-acetyltyrosine residues/mol of protein ($N$) was calculated from the expression:

$$N = \Delta E_{275}/1160 \times C$$

where $\Delta E_{275}$ is the difference in extinction at 275nm between *O*-acetylated and de-*O*-acetylated proteins, 1160 is the molar difference in absorption of tyrosine at 275nm and $C$ is the molar concentration of protein. This was determined from the $E_{260}$ of the de-*O*-acetylated protein by using the molar extinction coefficient of native neurophysin-II ($3.95 \times 10^3\,cm^{-1}M^{-1}$; M. D. Hollenberg, unpublished work).

*Spectrophotometric titrations.* A solution (25ml) of protein (0.5mg/ml) in 50mM-sodium acetate buffer, pH4.6, was titrated with 0.1M-NaOH. The pH was measured on the expanded scale of a Vibron pH-meter (model 39A) and $E_{275}$ was read for native neurophysin and $E_{428}$ for nitrated neurophysin. Readings were corrected for dilution.

*Difference spectroscopy.* Spectra were recorded on a Bausch and Lomb Spectronic 505 recording spectrophotometer with a 5nm spectral slit width. A single scan from 250 to 500nm was obtained by changing from deuterium to tungsten lamps at 350nm. The instrument was calibrated by the mercury emission lines at 253.7 and 435.8nm, and errors due to stray light were measured at 280nm. Under the conditions of the experiment such errors amounted to less than 0.07% of the measured extinction and could be neglected.

Two matching pairs of 1cm silica cells (A and B; C and D) were used. To obtain the difference spectrum A and C were filled with solutions containing buffer and protein respectively plus hormone and were placed in the sample beam. Cells B and D were filled with solutions of hormone and protein respectively and placed in the reference beam. To ensure that exactly equal concentrations of protein and hormone were present in both light-paths, solutions were prepared as follows: cell C (protein plus hormone) contained equal volumes of protein solution (2mg/ml) and hormone solution (0.2mg/ml); cell B (diluted solution of hormone) contained equal volumes of the solution of hormone and of buffer; cell D (diluted solution of protein) contained equal volumes of the solution of protein and of buffer.

After each difference spectrum had been recorded the absorption spectrum of the protein solution used was obtained by reading cell D against cell A (buffer), and the absorption spectrum of the hormone solution was obtained by reading cell B against cell A. Finally all cells were filled with buffer and the three appropriate cell blanks were run to compensate for cell mismatches in difference and absorption spectra.

## RESULTS

*Preparation and analysis of nitrated neurophysin-II.* Nitration was carried out by the method of Sokolovsky, Riordan & Vallee (1966) with a 100-fold excess of tetranitromethane. The extent of nitration was determined by amino acid analysis (see Table 1). The results show that more than 90% of the tyrosine had been nitrated. The trace of cysteic acid suggests that some oxidation of cystine also occurred but otherwise the modification was specific for tyrosine.

An attractive feature of the nitrated protein is the new absorption peak in the visible region (see Fig. 1). At pH8.5 the absorption maximum lies at 428nm. This shifts to lower wavelengths on acidification, as in the spectrum of 3-nitrotyrosine. At pH6.2, for example, the maximum lies at 352nm. Attempts to measure the extent of nitration quantitatively from the height of this peak gave unreliable results, as did previous attempts with other proteins (e.g. Nilsson & Lindskog, 1967; Meloun, Frič & Šorm, 1968).

*Spectrophotometric titration.* Because both tyrosine and 3-nitrotyrosine absorb much more strongly in their ionized forms, the extinction is strongly pH-dependent. Fig. 2 shows the results of spectrophotometric titration of the tyrosine hydroxyl group in native and nitrated protein. In the native protein the tyrosine has p$K$9.2, only slightly less

Table 1. *Amino acid composition of native and nitrated neurophysin-II*

Analyses were performed by the method of Spackman *et al.* (1958), after hydrolysis in 6M-HCl *in vacuo* for 17h at 110°C. Values for nitrated neurophysin-II are the means of three analyses; values for native neurophysin-II are taken from Rauch, Hollenberg & Hope (1969). Analyses are expressed as weight (g) of anhydro amino acid in 100g of protein and as no. of mol of residues of amino acid/mol of protein.

| | Nitrated neurophysin-II | | | Native neurophysin-II |
|---|---|---|---|---|
| | | No. of residues/molecule | | |
| Amino acid | Wt. (%) | (Found) | (Nearest integer) | No. of residues/molecule (Nearest integer) |
| Lysine | 2.70 | 2.0 | 2 | 2 |
| Histidine | — | 0 | 0 | 0 |
| Ammonia | 1.17 | 7.0 | 7 | 8 |
| Arginine | 10.82 | 6.6 | 7 | 6 |
| Cysteic acid | Trace | Trace | 0 | 0 |
| Aspartic acid | 6.19 | 5.2 | 5 | 5 |
| Threonine | 2.07 | 2.0 | 2 | 2 |
| Serine | 5.01 | 6.2 | 6 | 6 |
| Glutamic acid | 17.33 | 12.9 | 13 | 14 |
| Proline | 8.00 | 7.9 | 8 | 8 |
| Glycine | 8.39 | 14.6 | 15 | 15 |
| Alanine | 4.68 | 6.3 | 6 | 6 |
| Cystine (half) | 13.17 | 12.4 | 12 | 12 |
| Valine | 3.61 | 3.5 | 4 | 4 |
| Methionine | 1.10 | 0.80 | 1 | 1 |
| Isoleucine | 2.52 | 2.1 | 2 | 2 |
| Leucine | 7.17 | 6.0 | 6 | 6 |
| Tyrosine | Trace | Trace | 0 | 1 |
| Phenylalanine | 4.60 | 3.0 | 3 | 3 |
| 3-Nitrotyrosine | 1.37 | 0.93 | 1 | 0 |

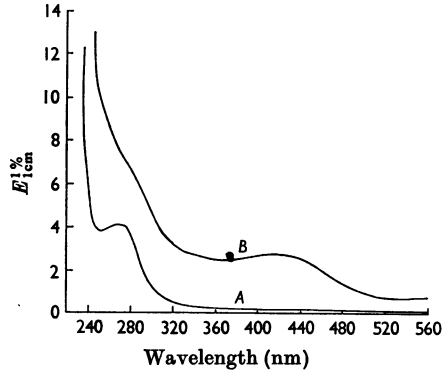


Fig. 1. Spectra of native (curve *A*) and nitrated (Curve *B*) neurophysin-II at pH8.5.

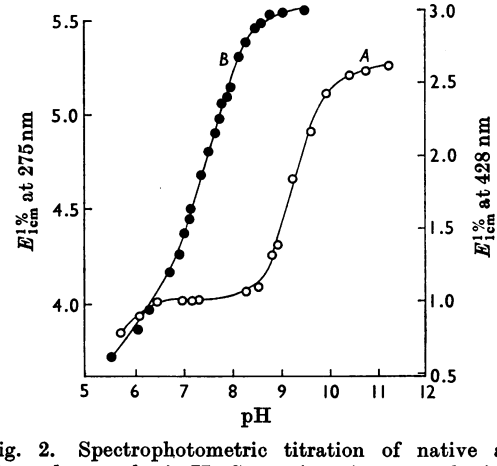


Fig. 2. Spectrophotometric titration of native and nitrated neurophysin-II. Curve *A*, native neurophysin-II (0.5mg/ml), extinction measured at 275nm; curve *B*, nitrated neurophysin-II (0.5mg/ml), extinction measured at 428nm.

than the value of 9.6 expected from studies on small molecules (Tanford, 1962). In the nitrated protein the p*K* of the tyrosine hydroxyl decreased from 9.2 to 7.3.

*Starch-gel electrophoresis of nitrated neurophysin-II.* Because the tyrosine residue appears to lie on the surface of the protein there was some chance that the lowered p*K* of this one residue might affect the electrophoretic mobility of the protein as a whole. Starch-gel electrophoresis at the usual pH of

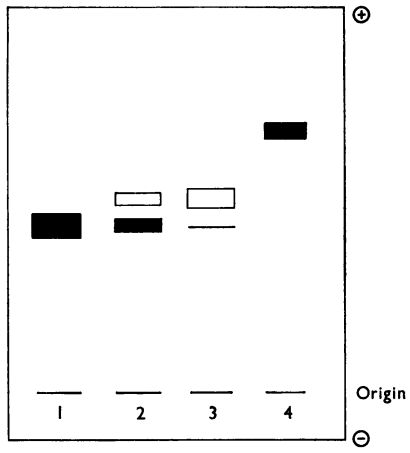


Fig. 3. Diagrammatic representation of starch-gel electrophoretograms of native and nitrated neurophysin-II. Electrophoresis was performed with horizontal starch gels by the method of Smithies (1955). Gels (starch concn. 15g/100ml of buffer) were prepared with the buffer described by Ferguson & Wallace (1961) modified to pH8.5 as described in the Methods section. Protein samples dissolved in the same buffer (5mg/ml) were applied and electrophoresis carried out for 1.5h with a constant voltage gradient of 26V/cm. Protein was detected by staining the gel with Nigrosine (0.05%, w/v) in water–methanol–acetic acid (5:5:1, by vol.). The nitrated protein was visible before staining as a yellow band. The numbers refer to: (1) native neurophysin-II; (2) partially nitrated neurophysin-II; (3) fully nitrated neurophysin-II; (4) neurophysin-I included as marker protein. The unshaded bands represent nitrated protein. These could be seen as yellow areas before staining.

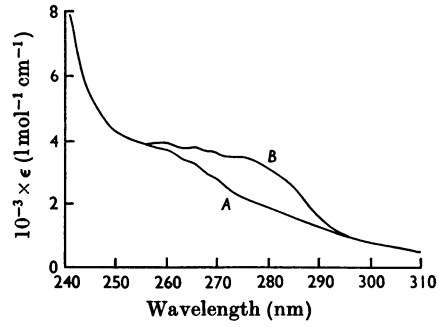


Fig. 4. Spectra of *O*-acetylneurophysin-II before (curve *A*) and after (curve *B*) treatment with hydroxylamine. The solvent was sodium phosphate buffer, pH7.5 and *I*1.0. The final concentration of protein (1.5mg/ml) was identical for the two spectra; curve *B* was obtained by making both sample and reference solutions M with respect to hydroxylamine.

8.1 did indeed give a separation of native and nitrated proteins. Greater separation was obtained by increasing the pH to 8.5, where 94% of the nitrated tyrosyl but only 5% of the native tyrosyl residues are in the ionized forms. Fig. 3 shows the electrophoresis of nitrated and native neurophysin at pH8.5. The nitrated protein gave one main band, which was yellow and corresponded to nitrated neurophysin. Traces of a slower component corresponding to unchanged starting material could also be seen. The absence of any intermediate component is significant. When the electrophoresis was repeated on a sample of partially nitrated protein (prepared by nitrating with only a tenfold molar excess of tetranitromethane) there was a more conspicuous band corresponding to unchanged protein and only 0.61mol of 3-nitrotyrosine/10000g. There was still no intermediate band, which demonstrates that during the reaction the protein was behaving as a monomer with a single nitratable tyrosine residue. Thus the molecular weight of neurophysin-II is close to 10000 and not 20000 as previously believed.

*Preparation of* O-*acetylneurophysin-II*. To study more fully the effect of tyrosine modification on the properties of neurophysin-II, a second modified protein was prepared in which the tyrosine hydroxyl group was blocked by acetylation.

Acetylation was carried out using a 50-fold molar excess of *N*-acetylimidazole and the extent of *O*-acetylation was followed spectroscopically. The reaction caused a marked decrease in $E_{275}$ owing to *O*-acetylation of the tyrosine residue (Fig. 4, curve *A*). Treatment with hydroxylamine rapidly reversed the *O*-acetylation to give a spectrum coinciding with that of native neurophysin-II (Fig. 4, curve *B*). These spectral changes were used to determine the degree of *O*-acetylation quantitatively as described in the Methods section. More than 0.9mol of *O*-acetyltyrosine was found/10000g of protein.

Although *N*-acetylimidazole is a mild acetylating agent it is not specific for tyrosine residues and a considerable degree of *N*-acetylation also took place. This did not affect the u.v. spectrum of the protein but could be detected by the change in electrophoretic mobility caused by blocking of positively charged amino groups. Starch-gel electrophoresis at pH8.1 revealed a number of new acidic components. These persisted after treatment with hydroxylamine and are thought to represent partially *N*-acetylated derivatives.

*N*-Acetylation could be eliminated by using a tenfold rather than a 50-fold molar excess of reagent but there was then only 43% *O*-acetylation. To achieve complete and specific *O*-acetylation the amino groups were blocked during the reaction by

citraconylation. The advantage of this method is that the $\epsilon$-citraconyl residues are acid-labile and can therefore be removed without hydrolysis of the *O*-acetyl group.

The protein was first citraconylated then acetylated and finally decitraconylated as described in the Methods section. The starch-gel electrophoretogram of decitraconylated protein was identical with that of the native neurophysin-II, showing that all amino groups were successfully blocked by citraconylation. The extent of *O*-acetylation determined spectroscopically was 89%.

*Hormone-binding ability of modified neurophysins.* To determine the effect of tyrosine modification on the biological activity of the protein, the hormone-binding ability was tested by equilibrium dialysis. The modified protein was always compared with a sample of native protein in the same experiment so that any variation in absolute values could be neglected. With the *O*-acetylated protein a control was included to make sure that the labile *O*-acetyl group was not hydrolysed even under the mild conditions of the experiment. Two samples of acetylated protein were taken; one was dialysed against hormone in the usual way and the control was dialysed against buffer alone. After 24h the content of *O*-acetyltyrosine in the control protein was determined spectroscopically and found to be 0.87mol/10000g.

The results given in Table 2 show that neither *O*-acetylation nor nitration led to any fall in the binding capacity of the protein.

*Difference spectroscopy.* Difference spectra were obtained by comparing a solution of protein plus hormone with protein and hormone in separate cells. Details of the procedure are given in the Methods section. Because of the low isoelectric point of the complex, spectra were obtained at pH6.2 rather than at pH5.6 used for equilibrium-dialysis experiments. The higher pH was a great advantage because it did not markedly decrease binding but it prevented concentrated solutions of the complex from becoming cloudy with precipitation.

Addition of hormone to the solution of native neurophysin-II caused a marked hyperchromicity in the region characteristic of tyrosine absorption, giving the u.v. difference peak labelled as peak I in Fig. 5. The spectral slit width was rather large (5nm) and prevented resolution of the smaller difference peak characteristic of tyrosine perturbation (Herskovits & Sorensen, 1968); this was seen as a shoulder (at 279.6nm) on the main peak at 286.8nm.

The absorption spectra of hormone and of protein are also shown. These were obtained after each experiment with solutions at the same concentration as those taken to form the complex. The absorption peak due to tyrosine, which is seen more clearly in the hormone spectrum, is at 275nm. The difference peak produced on protein–hormone interaction therefore represents a red-shift of 11.8nm.

At this point it was not clear whether the difference spectrum was a result of a change in the environment of the tyrosine residue on the hormone alone or whether the tyrosine residue of the protein

Table 2. *Effect of tyrosine modification on the hormone-binding ability of neurophysin-II*

Hormone-binding ability was tested by equilibrium dialysis against a solution of [8-arginine]-vasopressin in sodium acetate buffer, pH5.6 and *I* 0.1. In each experiment the hormone-binding ability of the native protein was tested under identical conditions and with samples of the same solution of hormone as for the modified protein. Values are the means of three bioassays; the S.D. of the results are shown in parentheses.

| | Units bound/mg of protein |
|---|---|
| *O*-Acetylated neurophysin II | 11.5 (± 0.25) |
| Native neurophysin-II | 9.1 (± 0.61) |
| Nitrated neurophysin-II | 13.3 (± 0.82) |
| Native neurophysin-II | 13.9 (± 0.92) |

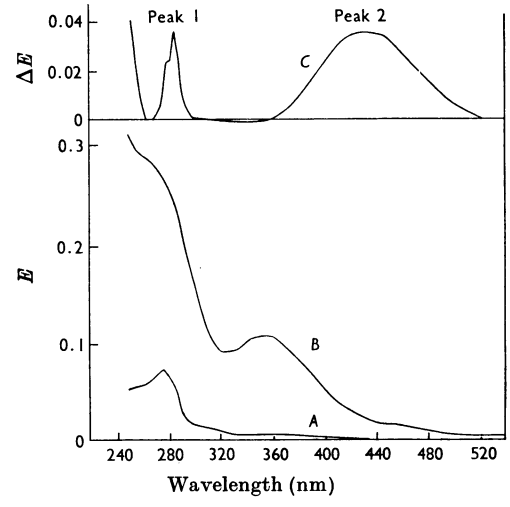


Fig. 5. Difference spectrum obtained on the interaction between nitrated neurophysin-II and [8-arginine]-vasopressin. The solvent was cacodylate buffer, pH6.2 and *I* 0.1; the spectral slit width was 5nm. Curve *A*, absorption spectrum of [8-arginine]-vasopressin; curve *B*, absorption spectrum of nitrated neurophysin-II; curve *C*, difference spectrum obtained by comparing a solution of protein plus hormone placed in the sample beam with solutions of protein and of hormone placed in separate cells in the reference beam. Peak I is thought to represent perturbation of chromophores of both protein and hormone; peak II represents perturbation of the protein nitrotyrosine alone.

was also perturbed by the interaction. The experiment was repeated with *O*-acetylneurophysin-II with the same concentrations of protein and hormone as before. The same difference spectrum was obtained as with the native protein but the height of the peak was very much lower. Equilibrium dialysis confirmed that the binding ability was not decreased so it is possible that the protein-tyrosine residue now blocked by acetylation had previously contributed to the difference peak in this region.

More decisive results were obtained with the nitrated protein. At pH 6.2 the nitrotyrosine peak is at 352nm, which is far enough from the u.v.-absorption bands to assign any change close to 350nm unambiguously to the tyrosine of the protein. The difference spectrum obtained with nitrated neurophysin-II is shown in Fig. 5. A second peak appeared with a broad maximum at about 436nm. This represented a red-shift of 84nm and shows that protein–hormone interaction produces a change in the environment of the tyrosine residues of the protein as well as that of the hormone.

## DISCUSSION

Specific modification of the tyrosine residue in neurophysin-II was part of a project to elucidate the minimum chemical molecular weight of the protein monomer by using Craig's method of partial substitution (Battersby & Craig, 1951). Previous calculations based on results from sedimentation-equilibrium experiments give a value of 20000 for native neurophysin-II, whereas oxidized neurophysin-II in which cystine residues had been oxidized by performic acid to cysteic acid residues, appeared to have a molecular weight close to 10000 (D. B. Hope, M. D. Hollenberg & W. B. Watkins, unpublished work). The simplest interpretation of this is that the unit of molecular weight 20000 is a dimer composed of two polypeptide chains of molecular weight 10000; the results of partial substitution described here support this conclusion. If two residues of tyrosine were associated with a single polypeptide chain of molecular weight 20000 it should be possible to nitrate partially and obtain a mixture of three species: disubstituted, monosubstituted and unchanged protein (Battersby & Craig, 1951). This situation occurs with pancreatic trypsin inhibitor where one of the two unburied tyrosines can be preferentially nitrated. Mono- and di-substituted proteins can then be separated by ion-exchange chromatography (Meloun *et al.* 1968). In contrast, nitration of neurophysin gave only one product. The protein was converted into a single electrophoretic component and amino acid analysis indicated the presence of 0.9mol of 3-nitrotyrosine/10000g of protein or 1.8mol/20000g. Traces of unchanged protein were visible but no evidence was obtained for the presence of an intermediate corresponding to partially nitrated material even when nitration was repeated under conditions giving only 60% reaction. It seems clear that the monomer contains only one tyrosine residue and must therefore have a molecular weight of 10000 rather than 20000. The results of previous hormone-binding experiments (e.g. Breslow & Abrash, 1966; Hollenberg & Hope, 1968) can now be recalculated, and give one binding site/polypeptide chain.

As well as providing information about the molecular weight, the nitrotyrosine residue was a good example of the 'reporter group' envisaged by Burr & Koshland (1964). It did not interfere with the hormone-binding function of the protein (see Table 2) but was strategically placed where it could reflect environmental changes induced by protein–hormone interaction. This interaction gave rise to a difference spectrum (Fig. 5) reflecting perturbations of two different tyrosines, that of the protein and that of the hormone. Studies with structural analogues of the hormone show that the first three residues are the most critical in binding (Breslow & Abrash, 1966), so that the molecule can be pictured as entering the binding site 'head first'. The resulting change from aqueous to hydrophobic environment might be expected to give rise to a difference spectrum characteristic of tyrosine entering a region of lower dielectric constant. Peak I of the observed difference spectrum fits very well with this prediction. The wavelength maximum shifts from 275nm to give a peak at 286.8nm with a shoulder at 279.6nm. This is very close to the difference peaks at 286.0 and 279.0nm found on perturbation of the model compound *N*-acetyltyrosine ethyl ester in 20% glycerol (Herskovits & Sorensen, 1968).

Some of the hyperchromicity in the region of peak I must also be due to perturbation of the tyrosine on the protein. This follows from work on *O*-acetylneurophysin whose absorption spectrum contrasts sharply with that of the native protein, and shows the beginnings of a trough in the 275nm region (Fig. 4, curve *A*). Interaction of *O*-acetylneurophysin with hormone still gave a difference peak in the position of peak 1, but this was very much lower than that obtained with native protein even though equilibrium dialysis confirmed that the binding ability was not impaired (Table 2). This would be expected if the protein tyrosine residue, now blocked by acetylation, had previously contributed to the difference peak in this region.

By using nitrated neurophysin-II we distinguished very convincingly between perturbations of residues on the protein and those on the hormone. A second peak (peak 2 in Fig. 5) appeared in the difference spectrum in the region corresponding to absorption of the 3-nitrotyrosine residue.

The wavelength maximum underwent a red-shift of 84nm (5380cm$^{-1}$), which is a much greater change in terms of energy than the 11.8nm (15000cm$^{-1}$) shift in the region of peak 1. This suggests that some additional factor is involved besides a change in hydrophobicity. One possible explanation is that protein–hormone interaction also increases the degree of ionization of the 3-nitrotyrosine chromophore. At pH 6.2 the wavelength maximum is at about 352nm in the spectrum of both 3-nitrotyrosine and nitrated neurophysin-II (Fig. 6 in Sokolovsky *et al.* 1966; Fig. 5, curve *B*, in this paper). At pH 8.5 the chromophore is fully ionized (Fig. 2) and the wavelength maximum has shifted to 428nm (Fig. 1). In the observed difference spectrum, however, the wavelength maximum shifted rather further than this to 436nm. This is almost identical with the 435nm wavelength maximum found when 3-nitrotyrosine at pH 8.1 is perturbed by 50% dioxan (Cuatrecasas, Fuchs & Anfinsen, 1968) and suggests that the hyperchromicity of peak 2 is a dual effect. The chromophore becomes fully ionized even at pH 6.2 and at the same time enters a more hydrophobic environment.

There are a number of ways in which interaction with the hormone could bring about these changes in environment. The increased degree of ionization indicates the approach of some highly charged positive group, possible candidates being the amino group of residue 1 of the hormone, the arginine residue in position 8 of the hormone, or some unspecified group on the protein, that is brought nearer to the tyrosine residue by a conformational change induced by interaction with the hormone. At present it is not possible to eliminate any of these three possibilities, but it seems unlikely that residue 1 of the hormone is involved because the tyrosine residue of the protein would then have to be located deep in the binding site where it would come close to the tightly bound residues at the *N*-terminal end of the hormone. Such a position seems unlikely because tyrosine substitution causes no decrease in hormone-binding ability. The residue may still be located where it can interact with the arginine at position 8 of the hormone, and it might be of interest to look for hyperchromicity on interaction with oxytocin which has no such positively charged group near the *C*-terminal end.

Two tyrosine residues of ribonuclease S-protein are perturbed by the interaction with S-peptide to give a sharp difference peak at 287nm (Richards & Logue, 1962). Data from X-ray crystallography show that, with the possible exception of tyrosine-25, there is no likelihood of direct interaction between S-peptide and any of the S-protein tyrosines (Wyckoff *et al.* 1967), although useful kinetic data were obtained by using the height of the difference peak at 287nm as a measure of protein–peptide interaction (Richards & Logue, 1962). Therefore whatever the origin of the difference spectrum found here, the possibility now exists of using it to study the interaction of neurophysin-II and hormone.

The retention of full binding activity by *O*-acetylneurophysin-II is significant not only for the information it gives about the tyrosine residue but also because it was prepared with the use of citraconic anhydride. This reagent allowed specific reaction at a single amino acid site by reversibly blocking amino groups during the acetylation. Unlike many other blocking groups, the $\epsilon$-citraconyl residues can be removed by mild acid hydrolysis (Dixon & Perham, 1968). After decitraconylation the protein closely resembled native neurophysin as far as could be judged by comparing the patterns on gel electrophoresis, the u.v. spectra and the hormone-binding abilities. Citraconic anhydride has been used successfully as a blocking agent before tryptic digestion, where changes in conformation are not important, and the present work on neurophysin suggests that it may also be useful in cases where the tertiary structure of the protein has to be preserved.

*Note added in proof.* Dr Lyman C. Craig kindly read the manuscript of this paper and reported to us the results of an experiment done in 1961 which bears on the question of the molecular weight of neurophysin. In an experiment done on a preparation of neurophysin (3.0mg in 0.3ml of water) little protein escaped through a 20/32 membrane at 25°C in 28min. However, when the diffusate was changed to 0.01M-acetic acid there was a half escape time of 1.5h compared with 3h for ribonuclease in the same membrane. This indicated a diffusional size about half that expected for a molecular weight of 20000. The findings reported in the present work seem to explain this result.

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