Tetrahedron. Vol. 27, pp. 607 to 614. Pergamon Press 1971. Printed in Great Britain

OXIDATION OF TYROSINE AND ITS PEPTIDES WITH POTASSIUM NITROSODISULPHONATE

S. DUKLER, M. WILCHEK and D. LAVIE
Department of Chemistry, The Weizmann Institute of Science, Rehovot, Israel

(*Received in the UK for publication 7 August* 1970)

Abstract—The chemical oxidation of tyrosine with potassium nitrosodisulphonate (Fremy salt) was found to follow step by step the enzymatic pathways, i.e. —NH_2 terminal tyrosine is oxidized to 2-carboxy-5,6-dihydroxy-indole through the intermediate of "dopachrome", while tyrosine in the middle of a peptide chain, or with —COOH terminal follows the "dopaquinone" pattern yielding 3,4-dihydroxy-phenylalanine derivatives. The spectroscopic data have been analysed and the method is proposed as a sensitive assay replacing the enzymatic method.

THE group of compounds known as aminochromes has attracted considerable attention from organic chemists and biochemists due to their physiological importance.[1] Their formation by enzymatic and chemical means has extensively been studied and is still of current interest.[2] Within the framework of our studies on the oxidation of phenolic systems with potassium nitrosodisulphonate $(SO_3K)_2NO$ (Fremy salt)[3] we have investigated its action on tyrosine.

The enzymatic oxidation of tyrosine and of tyrosine-containing peptides in the presence of tyrosinase has been studied by spectroscopic methods and two basic mechanisms have been proposed:[4] for NH_2-terminal tyrosine peptides, a "dopachrome" pathway has to be followed which is indicated by the formation, at the initial stages of the reaction, of intermediate products showing absorption maxima at 305 mμ and 475 mμ characteristic for "dopachrome". The single absorption max. at 325 mμ, which appears only subsequently, is related to the expected 2-carboxy-5,6-dihydroxy-indole, being the end product formed by a Michael type addition of the free amine to the dienone system. In the case of COOH-terminal tyrosin peptides and tyrosine placed in the middle of the chain, the oxidation takes place via "dopaquinone", a process indicated by the characteristic o-quinone absorption at 390 mμ which is recorded from the reaction mixture.

Recently it has been shown that the oxidation of tyrosine derivatives using N-bromosuccinimide[5] also follows two different pathways which are not the same as those for the enzymatic oxidation, the NH_2-terminal tyrosine afforded the indole derivative showing the absorption maximum at 325 mμ obtained through the dopachrome pattern, while tyrosine in the middle of a peptide chain is cleaved through a process involving usually the formation of a spirodienone-lactone having an absorption of 270 mμ.

In this paper we report the chemical oxidation of tyrosine and some tyrosine derivatives with potassium nitrosodisulphonate which was found to follow the enzymatic pathway step by step, which means that NH_2-terminal tyrosine is oxidized through the "dopachrome" pattern while tyrosine in the middle of a peptide chain and with COOH-terminal, follows the "dopaquinone" pattern.

When tyrosine methylester (I) was treated in a buffer solution (pH 8) with a 5 fold excess of potassium nitrosodisulphonate and the ultraviolet absorption spectrum

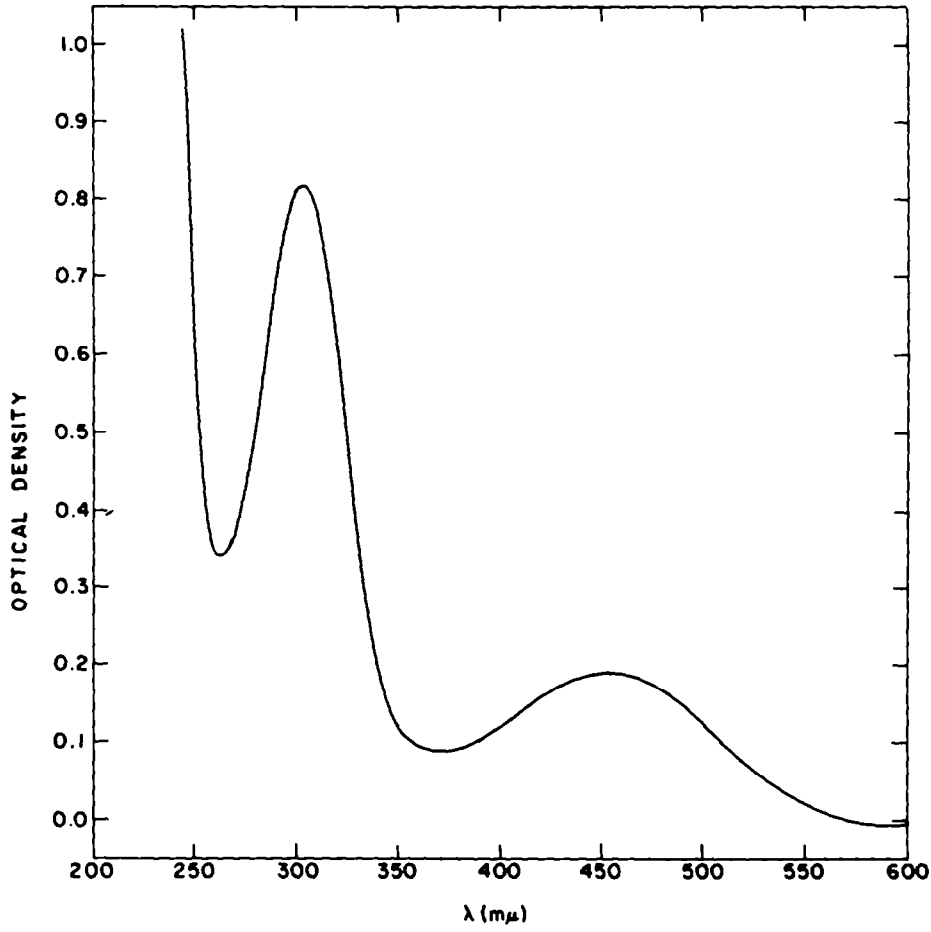


FIG. 1. Dopachrome from L-tyrosine methyl ester hydrochloride after reaction with excess potassium nitrosodisulphonate (5 folds) for 5 hr. The measurement was performed on the product following extraction with ethyl acetate (dopachrome mechanism).

scanned at intervals for several hours, a rapid appearance of a maximum at 475 mμ and a predominant end absorption at lower wave length were observed, the latter being due to the Fremy salt known to have a strong absorption in this region. In order to discover whether the product also shows absorption peaks in this lower region of the spectrum, the reaction mixture was extracted with ethyl acetate and the residue scanned. The solution showed two maxima at 305 mμ and 475 mμ (Fig. 1). This spectrum is identical with that observed during the enzymatic oxidation of an NH_2-terminal tyrosine with tyrosinase[4] and is characteristic for "dopachrome" (III). After 6 hr the 475 mμ peak started to decline, and after 24 hr it had completely disappeared. The reaction mixture was reduced with $Na_2S_2O_4$ and extracted with ethyl acetate. The isolated product absorbed at 325 mμ and is characteristic for dihydroxy-indoles. The spectra of the reaction products with Fremy salt of the peptides tyr-ala, tyr-gly, tyr-phe-ala and tyr-gly-gly which all possess free amino groups in the tyrosine were identical, and had a pattern similar to the one described above. Scanning at intervals of the latter tripetide is shown in Fig. 2.

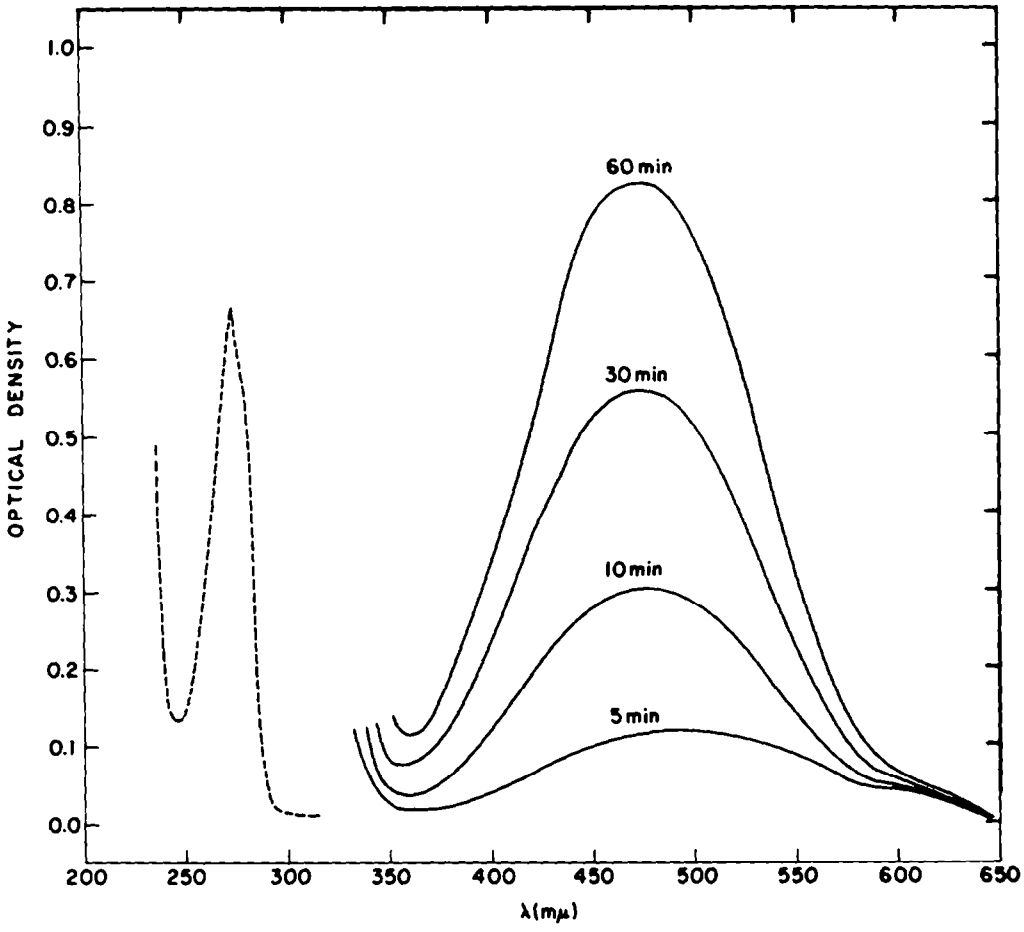
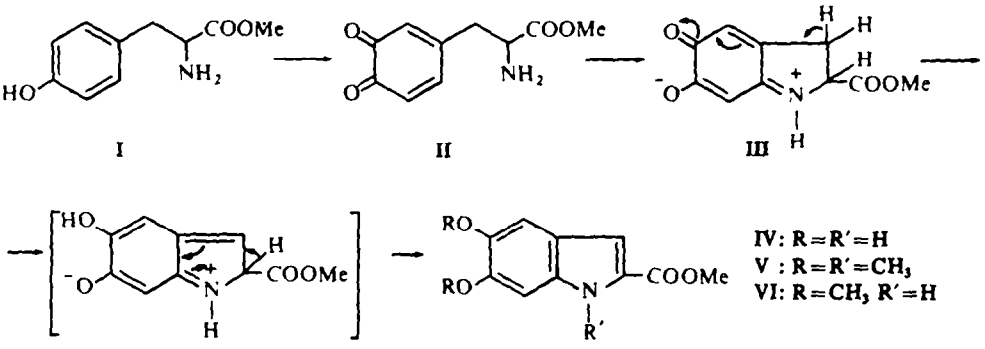


FIG. 2. Scanning at intervals of tyr-gly-gly (5×10^{-4} M solution in buffer phosphate *p*H 8) after treatment with 5 fold excess of potassium nitrosodisulphonate (dopachrome mechanism).

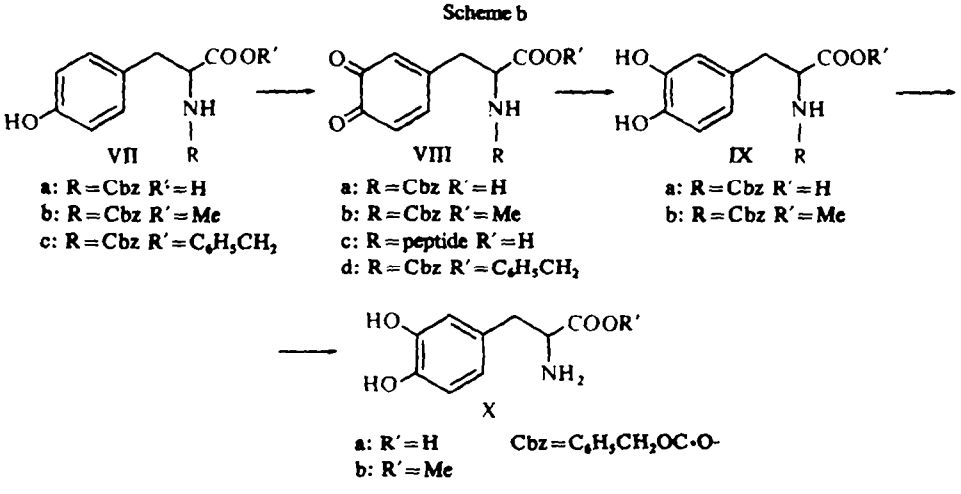
In order to identify the conversion product having the λ_{max} 325 mμ, tyrosine Me ester (I) was treated with potassium nitrosodisulphonate under the same conditions, on a preparative scale. $Na_2S_2O_4$ was added after 24 hr, to reduce the quinone which was formed, and the reaction mixture was extracted with ethyl acetate. The crude product was chromatographed on a column of silica gel, and the main fraction eluted with ethyl acetate-benzene (1 : 1). It afforded the crystalline compound IV which had an absorption maximum at 325 mμ (ε 18,000). Methylation with MeI in acetone produced the methyl N-methyl-5,6-dimethoxy-indole-2-carboxylate (V) which was identified by spectroscopic means: the ultraviolet spectrum showed an absorption maximum at 320 mμ (ε 10,500) due to the indole chromophore, and analysis of the NMR signals indicated two singlets at τ 2·95 and τ 2·80 for the C_4-H and C_7-H aromatic protons respectively, one singlet at τ 3·22 for the C_3-H vinylic proton and at high field four signals at τ 6·10, 5·99, 5·94 and 5·88 related to the protons of the four Me groups present in the molecule, namely the two MeO groupings at positions 5 and 6, the ester at C_2 and the N-Me at C_1. The mass spectrum of this compound showed the expected value M^+ 249.

When compound IV was treated with an ethereal solution of diazomethane, methyl 5,6-dimethoxyindole-2-carboxylate (VI), was obtained. This was confirmed by the NMR spectrum which showed again singlets at τ 2·90 and 2·82 for the C_4-H and C_7-H aromatic protons and τ 3·22 for the C_3-H vinylic proton, while one sharp signal at τ 6·08 for three Me groups integrated as 9 protons.

Scheme a



Scheme b



When compounds with tyrosine at the COOH—terminal is in the middle of a peptide chain were oxidised with Fremy salt, a new intermediate producing an UV absorption spectrum λ_{max} 390 mμ was obtained. This absorption which reached maximum intensity after 1 hr (Fig 3), is in good agreement with an o-benzoquinone chromophore. Peptides such as gly-tyr, carbobenzoxy-gly-gly-tyr, carbobenzoxy-glu-tyr, acetyl-tyr-amide or val-tyr-val all having blocked amino groups, when reacted with Fremy salt, also resulted in the expected λ_{max} 390 mμ absorption (cf. Fig 3).

In order to characterize the chromophore generating this absorption, carbobenzoxy-L-tyrosine benzyl ester (VIIc) was reacted with a 10 fold excess of Fremy salt in a mixture of MeOH—H_2O (2:5). The crystalline compound VIIId which was isolated showed an absorption max. at 390 mμ due to the o-quinone chromophore, and analysis of the NMR signals indicated a singlet at τ 3·86 for the C_2-H and a doublet for two

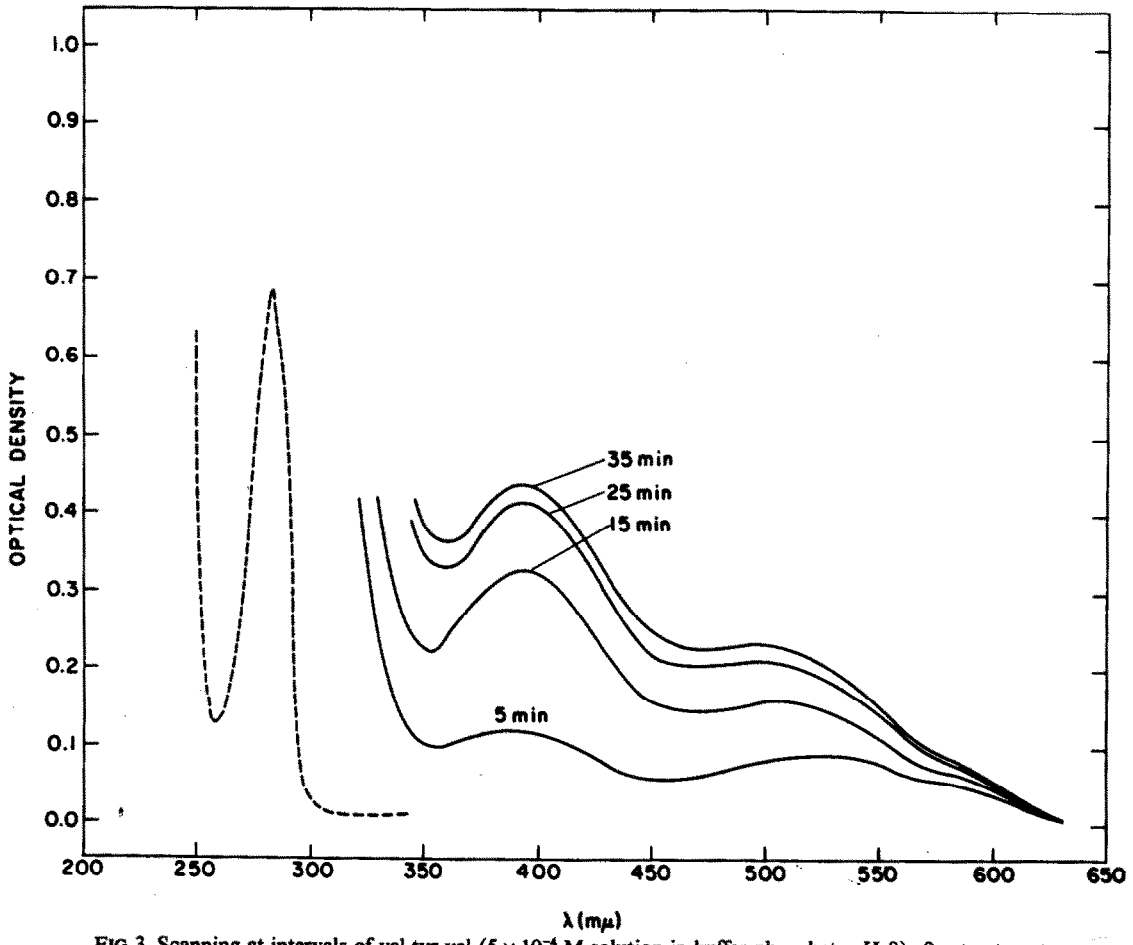


FIG 3. Scanning at intervals of val-tyr-val (5×10^{-4} M solution in buffer phosphate *p*H 8) after treatment with 5 fold excess of potassium nitrosodisulphonate (dopaquinone mechanism).

protons at 4·43 (H-10 Hz) for C_5-H and C_6-H. The same reaction was performed on carbobenzoxy-L-tyrosine (VIIa), after 10 min $Na_2S_2O_4$ was added and the reaction mixture extracted with ethyl acetate and treated with HBr in acetic acid to cleave off the carbobenzoxy moiety. The mixture consisted of 3,4-dihydroxy-L-phenyl-alanine (Xa) and unreacted tyrosine as shown by the amino acid analyzer using authentic samples for reference and unidentified melanine like polymers. Though the λ_{max} 390 mμ, due to compound VIIIa, reached its maximum intensity only after 2 hr, the reaction had to be stopped after 10 min since otherwise no dihydroxy-L-phenylalanine could be identified but instead polymeric oxidation products of tyrosine were formed. In a separate experiment, 3,4-dihydroxy-L-phenyl alanine Me ester (Xb) was obtained by first esterifying the acid and then cleaving the carbobenzoxy moiety thereby enabling its easy separation from the reaction mixture for complete characterization.

DISCUSSION

The present study enables the chemical oxidation of tyrosine with Fremy salt to be classified on the same basis as the two basic pathways one involving "dopachrome" (type III) as intermediate, which is characteristic when an NH_2-terminal group is present, and the second connected with the "dopaquinone" (type VIIIc) intermediate when tyrosine is present in the middle of a peptide chain or bears a terminal—COOH. For the former process scheme **a** is suggested, while for the latter scheme **b** has to be considered.

Considering scheme **a** for the cases connected with tyrosine having a terminal —NH_2, one has to assume that the Fremy salt introduces first an oxygen atom *ortho* to the OH group according to a procedure already described,[6] forming an intermediate with an o-quinonoid structure (II), this is then followed by an intramolecular cyclisation through a Michael type addition reaction producing a species shown by III. The combination of these products is responsible for the ultraviolet spectrum of 305 and 475 mμ. These absorptions are displayed in the aminochromes by "dopachrome" itself.[2] The free amino group in this system enables the cyclisation reaction to take place producing after the reduction with sodium hydrosulphite the dihydroxy-indole derivative (IV) having the ultra-violet maximum at 325 mμ. in the present oxidation reaction carried out with Fremy salt, the excess salt in the reaction mixture has a strong end absorption (in the lower region of the spectrum) masking the 325 mμ maximum usually observable in the enzymatic reaction, it is therefore seen only after isolation of the product.

Direct evidence for the formation of an o-quinone intermediate of type II was obtained by carrying out the reaction with tyrosine having the amino group blocked by carbobenzoxy or acetamido groups. A maximum at 390 mμ was then generated due to the "dopaquinone" chromophore, and the product of this reaction was, after cleaving the carbobenzoxy moiety, 3,4-dihydroxy-L-phenylalanine. The latter reaction indicates the second pathway described in scheme **b** which is connected with tyrosine occurring in the middle of a peptide chain or with a terminal and free—COOH. In these cases the amino group is bound, and indeed the solution always displays a λ_{max} 390 mμ due to the o-benzoquinone formed in the reaction mixture while no cyclization can take place under these conditions. Reaction of N-bromosuccinimide with blocked tyrosine peptides usually results in cleavage of the peptide chain;[5] no such cleavage was observed with Fremy salt. This was confirmed by treating phlorethylglycine having one peptide link, with a 50 fold excess of Fremy salt, and only 0·5% cleavage could be detected. By carrying out the reaction with Fremy salt, a "dopachrome" or "dopaquinone" pattern of oxidation can be detected by spectroscopic means distinguishing thereby between peptides having a terminal —NH_2 or terminal —COOH tyrosine.

EXPERIMENTAL

M.ps were taken on Fisher-Johns apparatus and are uncorrected. TLC were run on chromatoplates of silica gel G (Merck). UV absorption spectra were recorded on a Cary 14 spectrophotometer. IR spectra were recorded on a Perkin–Elmer Infracord model 137 spectrophotometer with a NaCl prism. NMR spectra were recorded on a Varian A-60 spectrometer, for 25% solns in $CDCl_3$, containing TMS as internal standard. The mol wts were determined by mass spectrometry on an Atlas CH4 instrument. Analyses were performed by the Microanalytical laboratory of our Institute, under the direction of Mr. R. Heller.

Methyl 5-6-dihydroxy-indole-2-carboxylate (IV). A solution of L-tyrosine methyl ester (I) (1 g) in MeOH

(30 ml) was added to a stirred solution of potassium nitrosodisulphonate (6 g) in buffer phosphate, pH·8 (250 ml) at room temperature. After 30 min the soln turned red: the ultraviolet spectrum of this solution showed the two amino-chrome bands at λ_{max} 305 and λ_{max} 475 mμ, and after 2 hr a new peak at λ_{max} 325 mμ appeared. The solution was allowed to stand overnight, then $Na_2S_2O_4$ was added to reduce the amino-chrome, and the mixture extracted with ethyl acetate several times. The ethyl acetate extract was washed with H_2O, dried over Na_2SO_4 and the solvent removed in vacuo. The crude material (600 mg) showed several spots on a chromatoplate. This mixture was chromatographed on silica gel (0·05–0·2) and elution with benzene-ethyl acetate (9:1) afforded the methyl 5-6-dihydroxy-indole-2-carboxylate (450 mg). Recrystallization from ethyl acetate gave the pure product (419 mg, 40%), m.p. 235–238° dec. λ_{max}^{EtOH} 325 mμ (ε 18,500); ν_{max}^{KBr} 3350 (OH), 1650 (C═O), 1295 (C—N stretching), 1245 (C—O—C) cm^{-1} (found: C. 57·78; H, 4·58; N, 6·91. $C_{10}H_9O_4N$ requires: C, 57·97; H, 4·38; N, 6·76%).

The *diacetate* was obtained by acetylating IV (150 mg) in the usual manner to yield methyl 5-6-diacetoxy-indole-2-carboxylate (120 mg), m.p. 177–178°, ν_{max}^{KBr} 3200 (N—H), 1750, 1645, 1270 and 1185 cm^{-1}. (Found: C, 58·05; H, 4·46; N, 4·67. $C_{14}H_{13}O_6N$ requires: C, 57·73; H, 4·50; N, 4·82%).

Methyl 5-6-dimethoxy-indole-2-carboxylate (VI). Compoind (IV) (1 g) in $CHCl_3$–MeOH (1:1) was treated with ethereal diazomethane for 24 hr at room temp. Evaporation of the solvents afforded the product which was chromatographed on a column of silica gel and eluted with hexane-ethyl acetate (7:3) to yield methyl 5-6-dimethoxy-indole-2-carboxylate, m.p. 118–120°. (Found: C, 61·62; H, 5·53; N, 6·10. $C_{12}H_{13}NO_4$ requires: C, 61·27; H, 5·57; N. 5·96%).

Methyl N-methyl-5-6-dimethoxy-2-carboxylate (V). To a stirred solution of IV (160 mg) in dry acetone (60 ml) containing anhydrous K_2CO_3 (3 g), MeI (10 ml) was added. The mixture was heated to reflux under N_2 for 12 hr, filtered and the filtrate evaporated to dryness. The residue was chromatographed on silica gel H. Elution with C_6H_{14}—$CHCl_3$ (7:3) afforded 120 mg as colourless crystals, m.p. 110—112°. Recrystallization from ethyl acetate yielded 105 mg, 134–135°; λ_{max}^{MeOH} 320 mμ (ε 10,800); $\nu_{max}^{CHCl_3}$ 1670 cm^{-1} (no bands for the phenol OH grouping and the N—H stretching). (Found: M⁺ 249; $C_{13}H_{15}O_4N$, requires: mol. wt. 249·26)

N-Carbobenzoxy-L-tyrosine benzyl ester. Tyrosine benzyl ester hydrochloride (30·7 g, 0·1 mole) was dissolved in H_2O (75 ml) and $CHCl_3$ (100 ml) was added at 0°. While stirring vigorously MgO (5·2 g, 0·13 mole) was added followed by dropwise addition of carbobenzoxy chloride (22 g, 0·13 mole) during 30 min. Stirring was continued for additional 30 min when pyridine (5 ml) was added. After 5 min the reaction mixture was acidified to congo red with 5N HCl. The $CHCl_3$ layer was separated and washed with 0·5N HCl, H_2O 5% $NaHCO_3$ solution, H_2O and dried over Na_2SO_4. The organic layer was concentrated to dryness in vacuo and the resulting precipitate crystallized from benzene, m.p. 121°; yield 35 g (86%). (Found: C, 71·01; H, 5·61; N, 3·52. $C_{24}H_{23}NO_5$ requires: C, 71·11; H, 5·67; N, 3·45%).

N-Carbobenzoxy-5,6-*dioxo-L-phenylalanine benzyl ester* (VIIId). N-Carbobenzoxy-tyrosine benzyl ester (1 g) dissolved in MeOH—H_2O 1:1 (100 ml) was added to a stirred solution of potassium nitrosodisulphonate (3·1 g) in buffer phosphate pH 8 (200 ml). After two hr at room temp. the reaction mixture was extracted with $CHCl_3$ which was washed with H_2O, dried over Na_2SO_4 and concentrated to dryness in vacuo at 40°. after several crystallizations from ethanol the pure o-quinone was obtained (500 mg); $\lambda_{max}^{CHCl_3}$ 395 mμ; ν_{max}^{KBr} 1720 (C═O) 1640 (o-quinone) 1170, 775 and 748 cm^{-1}.

3,4-*Dihydroxy-L-phenylalanine methyl ester* (Xb). To a solution of N-carbobenzoxy-L-tyrosine (VIIa) (500 mg) in CH_3OH (30 ml) potassium nitrosodisulphonate (2 g) in phosphate buffer *p*H 8 (200 ml) was added, and the soln which turned red, due to the formation of an o-quinone chromophore, had a λ_{max} 390 mμ. After 15 min $Na_2S_2O_4$ was added to reduce the o-quinone to the dihydroxy derivative. The solution was then acidified with HCl to *p*H 1-2 and then extracted with ethyl acetate. Removal of the solvent in vacuo yielded the crude product (340 mg) which was directly esterified with $SOCl_2$ in MeOH. After evaporation of the solvent and excess $SOCl_2$, the ester was isolated and purified by chromatography (silica gel column). Pure N-carbobenzoxy-3,4-dihydroxy-L-phenylalanine methyl ester (IXb) was eluted with a solvent system of benzene-ethyl acetate (7:3). Treatment with HBr in acetic acid (30%) for 15 min brought about removal of the carbobenzoxy group to yield exclusively 3,4-dihydroxy-L-phenylalanine methyl ester HBr. The structure of methyl ester DOPA was identified by comparing the UV and IR spectra with an authentic sample, both being superimposable; the mmp was undepressed.

Preparation of 3,4-dihydroxy-L-phenylalanine, DOPA (Xa). Carbobenzoxy-L-tyrosine was treated with potassium nitrosodisulphonate as described above. After extraction with ethyl acetate the solution was concentrated to dryness and treated with HBr in acetic acid (30% v:v) for 15 min. Dry ether was added and the crystalline product was isolated by filtration. On thin layer chromatography it was found to contain L-

DOPA and unreacted L-tyrosine. Quantitative amino acid analysis on Beckman amino acid analyzer Model 120 C Showed the presence of DOPA and some tyrosine.

Spectroscopic studies of the oxidation of peptides with Fremy salt. All the peptides, apart from L-tyrosyl-L-alanine the synthesis of which is given below, were synthesized according to published procedures or obtained from the sources referred to: L-glycyl-L-tyrosine[8], L-tyrosyl-L-glycyl-L-glycine, L-tyrosyl-L-glycyl-L-phenylalaine[8], L-tyrosyl-L-alanine, L-valine-L-tyrosyl-L-valine,[10] cbz-L-glycyl-L-glycyl-L-tyrosine,[9] cbz-L-glutamyl-L-tyrosine.[10]

The spectra were taken in 3 cm cells from 1 mM stock aqueous solutions of each of the peptides. Only the last two samples had to be dissolved first with a few drops of methanol and then diluted with water.

A freshly prepared solution of potassium nitrosodisulphonate 1 mM dissolved in a buffer phosphate of *p*H 8 was used for each experiment adding 0·05 ml of solution (5 folds) with immediate shaking at room temperature. The ultraviolet spectrum was recorded at intervals (Fig. 2, 3).

L-Tyrosyl-L-alanine. N-O-Dicarbobenxoxy-L-tyrosyl-L-alanine-benzyl ester was prepared according to the DCC method[7] from dicarbobenzoxy-L-tyrosine and L-alaninebenzyl ester in 83% yeild. m.p. 145°; recrystallization from EtOH (Found: C, 68·72; H, 5·40; N, 4·53. $C_{35}H_{34}N_2O_8$ requires: C, 68·84; H, 5·61; N, 4·59%).

N-O-Dicarbobenzoxy-L-tyrosyl-L-alanine-benzyl ester (5 g) was dissolved in 20 ml HBr in acetic acid solution 30% v:v). The solution was allowed to stand overnight, then absolute ether was added until an oily residue was obtained. The oil was washed several times with ether, and then dissolved in a minimum amount of EtOH. To this solution were added triethylamine (1 equiv.) and after 10 min ether producing an oily precipitate. The oil was dissolved in hot $CHCl_3$ when a crystalline compound separated; recrystallized from H_2O—EtOH (1·2 g, yield 60%) (Found: C, 57·39; H, 6·43; N, 11·00. $C_{12}H_{16}N_2O_4$ requires: C, 57·13; H, 6·39; N, 11·11%).

Acknowledgement—Thanks are due to Mrs. R. Waitman for taking part in certain experiments.

REFERENCES

[1] H. Sobotka and J. Austin, *J. Am. Chem. Soc.* **73** 3077 (1951)
[2] R. H. Heacock in *Advances in Heterocyclic Chemistry* (Edited by A. R. Katritzky) vol. 5 p. 205. Academic Press, New York and London (1965)
[3] H. J. Teuber and W. Raw, *Chem. Ber.* **86** 1036 (1953)
[4] H. S. Raper, *Biochem, J.* **21** 89 (1927); K. T. Yasunoba, E. W. Peterson and H. S. Mason, *Biological Chem.* **234** 3291 (1959); H. Wyler and J. Chiovini. *Helv. Chim. Acta* **51** 1476 (1968)
[5] M. Wilchek, T. Spande, B. Witkop, *J. Am. Chem. Soc.* **89**, 3349 (1967); M. Wilchek, T. Spande, G. Milne and B. Witkop, *Biochenistry* **7**, 1777 (1968)
[6] H. J. Teuber and G. Staiger, *Chem. Ber.* **88**, 802 (1955)
[7] J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.* **77**, 1067, (1955)
[8] M. Wilchek, T. Spande and B. Witkop, *Biochemistry* **7**, 1787 (1968)
[9] H. Edelhoch, R. L. Perlman and M. Wilchek, *Ibid.* **7**, 3893 (1968)
[10] Obtained from Yeda, Rehovoth, Israel.