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The Cross-Linking of Tyrosine by Treatment with Tetranitromethane

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1. Tyrosine was treated with tetranitromethane. 2. Approx. 10% of the tyrosine was converted into 3-nitrotyrosine. 3. Three fluorescent compounds were also formed. They appear to be a dimer, trimer and tetramer in which tyrosine units are linked by biphenyl bonds. 4. The dimer and trimer have also been isolated from some proteins after treatment with tetranitromethane. 5. The yield of 3-nitrotyrosine from ovotransferrin after treatment with tetranitromethane was much smaller than the loss of tyrosine. 6. Several unidentified compounds were also formed by the reaction between tyrosine and tetranitromethane.

Tetranitromethane is often used to convert the free tyrosine residues of proteins into 3-nitrotyrosine (Sokolovsky, Riordan & Vallee, 1966). For example, Atassi & Habeeb (1969) found that treatment of lysozyme with tetranitromethane modified two of the three tyrosine residues and that both were recovered as 3-nitrotyrosine. They also reported the complete conversion of tyrosine and glycyltyrosine into the nitro derivatives upon treatment with tetranitromethane. Boesel & Carpenter (1970), on the other hand, treated glycyltyrosine and insulin with tetranitromethane and recovered only 24 and 62.5% respectively of the expected amounts of 3-nitrotyrosine. The rest of the tyrosine could not be accounted for and gel filtration showed that both glycyltyrosine and insulin had undergone intermolecular cross-linking. Vincent, Lazdunski & Delaage (1970) found that on treatment of trypsin and trypsinogen with tetranitromethane polymerization occurred and the sum of 3-nitrotyrosine and tyrosine in acid hydrolysates was less than the tyrosine content of the native proteins. Doyle, Bello & Roholt (1968) were the first to report that polymerization of some proteins occurred on treatment with tetranitromethane and they suggested that covalent cross-links were formed between tyrosine residues.

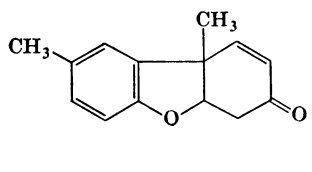
Bruice, Gregory & Walters (1968) treated several substituted phenols with tetranitromethane and found that the yield of nitrophenol was less than 30%. The tetranitromethane, however, was converted completely into trinitromethane and those nitro groups that were not recovered in nitrophenol were accounted for as nitrite. From *p*-cresol they obtained 1,2,10,11-tetrahydro-6,11-dimethyl-2-oxodibenzofuran (Pummerer's ketone, I) in 30% yield together with unidentified polymers.

The coupling of *para*-substituted phenols on treatment with a variety of oxidizing agents has been intensively studied since Pummerer, Melamed & Puttfarcken (1922) first indicated the role of phenoxide free radicals as intermediates (review by Scott, 1965). The oxidation of *p*-cresol has yielded several coupled products including Pummerer's ketone, an aromatic ether (II) and derivatives of biphenyl (III, $R = CH_3$) and terphenyl (IV, $R = CH_3$). The yields of these products vary with different oxidizing agents (Johnston, Jacobson & Williams, 1969).

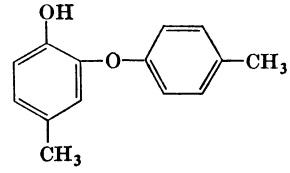
It will be shown below that treatment of tyrosine with tetranitromethane gives, in addition to 3-nitrotyrosine, fluorescent compounds that appear to be identical with the 'dityrosine' [2,2′-dihydroxy-5,5′-di-(2-carboxy-2-aminoethyl)biphenyl] and 'trityrosine' [2,2′,2″-trihydroxy-5,5′,5″-tri(2-carboxy-2-aminoethyl)terphenyl] which have been obtained previously by oxidation of tyrosine with peroxidase (Gross & Sizer, 1959) and by hydrolysis of the insect protein resilin (Andersen, 1966). The suggested formulae of these compounds are shown as (III) and (IV) [$R = CH_2–CH(NH_2)–CO_2H$] respectively. Preliminary experiments indicate that the tyrosine residues of proteins may also give these compounds on treatment with tetranitromethane.

MATERIALS AND METHODS

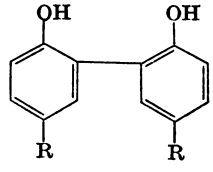
Reagents and proteins. Tetranitromethane was obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Crystalline ox insulin, L-tyrosine and *p*-cresol were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Glycyl-L-tyrosine and 3-nitro-L-tyrosine were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. 3,5-Dinitro-L-tyrosine was obtained from K & K Laboratories Inc., Plainview, N.Y., U.S.A., and



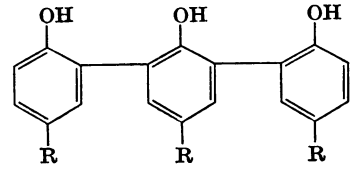
(I)



(II)



(III)



(IV)

Hollywood, Calif., U.S.A. *N*-Acetyl-L-tyrosine was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Bovine trypsin type XI (diphenylcarbamoyl chloride-treated) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Hen ovotransferrin was prepared as described by Williams (1968) and was made metal-free by the method of Warner & Weber (1951). Horseradish peroxidase was a chromatographically purified preparation of isoenzyme C and was a gift from Dr C. F. Phelps. Elastic tendons from the fore and hind wings of the dragonfly (*Aeshna cyanea*) were given by Dr H. R. Hepburn.

Treatment of tyrosine with tetranitromethane. Method (*a*). The pH of a solution of tyrosine in water (1mM) was adjusted to 9.0 with 0.1M-NaOH. It was stirred continuously and undiluted tetranitromethane was added: 1 molar equivalent of it was added at 30min intervals until the desired amount was reached. The reaction was carried out for 4h and 0.1M-NaOH was added to maintain the pH value. Samples removed during the reaction were acidified, dried by rotary evaporation and redissolved in water. In one experiment tyrosine was treated with a tenfold excess of tetranitromethane. The solution was subjected to paper electrophoresis at pH1.9 and after elution of the 3-nitrotyrosine band with 0.1M-NaOH the E_{428} was measured. A standard curve was prepared by subjecting known amounts of 3-nitrotyrosine to paper electrophoresis and elution.

Method (*b*). A solution of tyrosine (2.5mM) in 1% (w/v) NH_4HCO_3 was adjusted to pH9.2 with drops of 33% (w/v) NH_3. Tetranitromethane was added as a 10% (v/v) solution in 95% (v/v) ethanol to give the desired ratio of tetranitromethane to tyrosine. The mixture was left for 3h at room temperature and then acidified and dried by rotary evaporation. The residue was dissolved in water. In quantitative experiments tyrosine was treated with a tenfold excess of tetranitromethane and 3-nitrotyrosine was determined with a Technicon amino acid analyser (model TSM-1).

Method (*a*) was also used with glycyltyrosine, *N*-acetyltyrosine and *p*-cresol. In these experiments 100mg of each substance was treated with a single addition of 50μl of tetranitromethane and the reaction was allowed to proceed for 1h. The mixtures were then acidified, dried by rotary evaporation and dissolved in water. In the case of *p*-cresol the dry residue was extracted with 50% (v/v) ethanol and the solution was extracted with ether. The ether solution gave an intensely fluorescent spot on paper.

Enzymic oxidation. Tyrosine, glycyltyrosine and *N*-acetyltyrosine were oxidized with peroxidase and H_2O_2 (Gross & Sizer, 1959). After incubation the mixtures were dried by rotary evaporation and the residues dissolved in water. The fluorescent derivatives of biphenyl and terphenyl were purified by paper electrophoresis and paper chromatography.

Oxidation with alkaline ferricyanide. Oxidation of *p*-cresol was performed as described by Pummerer *et al.* (1922). After 4h the precipitate was collected, dissolved in ether and the solution extracted with 8% (w/v) NaOH. The alkaline extract was acidified and dried by rotary evaporation. The resulting mass of salt was extracted with 70% (v/v) ethanol and this solution was applied to paper and chromatographed with water as solvent. Two intensely fluorescent bands were seen, corresponding to 2,2′-dihydroxy-5,5′-dimethylbiphenyl (R_F 0.72) and 2,2′,2″-trihydroxy-5,5′,5″-trimethylterphenyl (R_F 0.30). These bands were eluted with water and rechromatographed with solvent 3 (see below), giving R_F values of 0.50 and 0.16 respectively. They were eluted with 95% (v/v) ethanol. The fluorescent material resulting from the treatment of *p*-cresol with tetranitromethane was also purified by subjecting the ether solution to paper chromatography in water and in solvent 3.

Tyrosine was oxidized in a similar manner: 1.81g of tyrosine and 2.25g of anhydrous Na_2CO_3 were dissolved in 500ml of water and 6.6g of potassium ferricyanide was added in the form of a saturated solution. The mixture rapidly became dark red and after 4h samples were subjected to paper electrophoresis at pH1.9. The main fluorescent band was eluted with water.

Treatment of proteins with tetranitromethane. Insulin hydrochloride (Carpenter, 1958), trypsin and hen ovotransferrin were dissolved in 1% NH_4HCO_3 solutions and the pH was adjusted to 9.2 with conc. NH_3. Tetranitromethane was added as a 10% solution in ethanol to

give a reagent/tyrosine molar ratio of either 1 or 10. After 3–4h the material was dialysed against water and freeze-dried. The samples were not acidified. Ovotransferrin was also reduced and aminoethylated, as described by Cole (1967), and was treated with tetranitromethane in 8M-urea–1% NH_4HCO_3, pH9.2, or in 5M-guanidinium hydrochloride, pH8.6, as solvents. Protein concentrations were 1–10mg/ml. The freeze-dried nitrated proteins were hydrolysed with 5.7M-HCl containing 1% (v/v) mercaptoethanol at 105°C for 24h. The hydrolysates were dried over NaOH. The same hydrolysis procedure was used for the elastic tendons of the dragonfly.

The recoveries of 3-nitrotyrosine and tyrosine after treatment of ovotransferrin with tetranitromethane were also measured. Samples of metal-free ovotransferrin, iron-saturated ovotransferrin and aminoethylated ovotransferrin were treated with a tenfold excess of tetranitromethane. After dialysis and freeze-drying the samples were hydrolysed. Mercaptoethanol was not added but the tubes were flushed with nitrogen several times before being sealed under vacuum. Amino acid analyses were performed with a Beckman 120C amino acid analyser.

Purification of the fluorescent compounds. Samples of the reaction mixtures were subjected to paper electrophoresis at pH6.5 and 1.9. Descending paper chromatography was carried out on Whatman 3MM paper with the following solvents: (1) butan-1-ol–acetic acid–water (3:1:1, by vol.), (2) butan-1-ol–acetic acid–water (4:1:1, by vol.) and (3) 10% (w/v) $(NH_4)_2SO_4$. After drying the papers were exposed to either HCl fumes or to NH_3 fumes and examined under u.v. light (Chromatovue viewing box obtained from Ultraviolet Products Inc., San Gabriel, Calif., U.S.A.). Papers were also stained with cadmium acetate–ninhydrin (Heilmann, Barrolier & Watzke, 1957), 1-nitroso-2-naphthol (Jepson & Smith, 1953) or with 0.4% (w/v) 2,4-dinitrophenylhydrazine in 2M-HCl. Electrophoretic mobilities at pH6.5 relative to that of aspartic acid were measured and the sign quoted shows the nature of the charge carried. Offord's (1966) charts were used to determine the relationship of charge to molecular weight.

Esterification and acetylation. The main fluorescent substance from tyrosine was esterified by overnight treatment with saturated HCl in dry methanol. The mixture was dried first by rotary evaporation and was then evacuated over NaOH. Acetylation was carried out by two methods. (*a*) Dried material was dissolved in 2.5ml of pyridine and 0.25ml of acetic anhydride was added. After 1h at room temperature the mixture was dried by rotary evaporation. (*b*) Dried material was dissolved in 1ml of 25% (v/v) acetic acid and treated with 0.25ml of acetic anhydride for 1h. The mixture was then dried as before.

Mass spectrophotometry. The major fluorescent product from tyrosine was esterified and then acetylated by method (*a*). The resulting oil was inserted directly into the ion source of an L.K.B. 9000 GC-MS instrument. The spectrum was recorded with a probe temperature of 60°C.

U.v. spectra. A Unicam SP.800 spectrophotometer was used. Spectra were measured at several pH values to determine the apparent pK of ionization of the major fluorescent product. The buffers contained 0.2M-tris, 0.5M-NaCl and acetic acid was added to give pH values between 3.2 and 11.4.

Gel filtration. Samples removed during the treatment of tyrosine with tetranitromethane by method (*a*) were subjected to gel filtration on a column (1.5cm × 95cm) of Sephadex G-25 in 1% (w/v) NH_4HCO_3. Fractions (3ml) were collected and E_{280} was measured.

RESULTS

Reaction between tyrosine and tetranitromethane

Method (a). When method (*a*) was used paper electrophoresis at pH1.9 showed several products (Fig. 1).

(i) Compounds 1, 2 and 3 showed blue fluorescence in u.v. light, in order of increasing intensity. Ammonia fumes increased the fluorescence and hydrochloric acid fumes abolished it. Compound 3 gave a weak yellow colour. All three compounds gave a pink colour with cadmium–ninhydrin reagent, that given by compound 3 being much the strongest. Nitrosonaphthol gave an intense red colour with compound 3, a weak brown colour with compound 2 and did not stain compound 1.

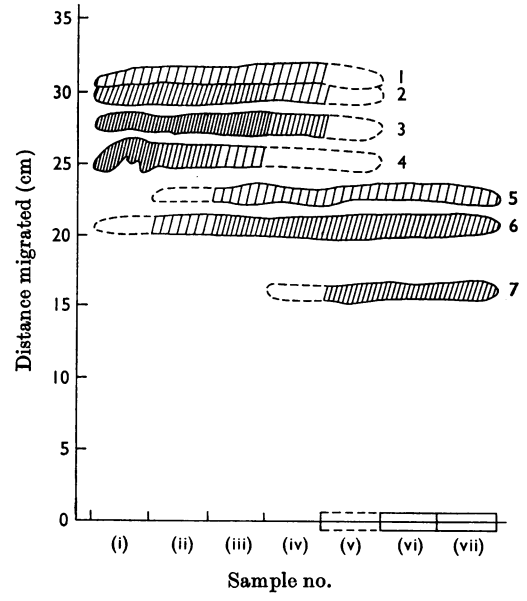


Fig. 1. Tracing of paper electrophoretogram. Tyrosine and tetranitromethane were allowed to react by method (*a*). Samples (i)–(vii) were removed at 30-min intervals and subjected to paper electrophoresis at pH1.9 (60V/cm for 75min). Bands representing compounds 1–7 are indicated. Depth of shading shows the intensity of the ninhydrin stain and the scale shows the distance in cm migrated towards the cathode.

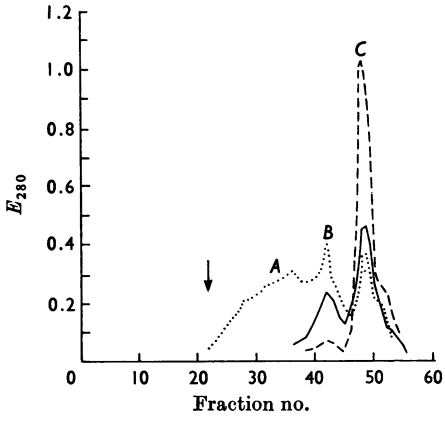


Fig. 2. Gel filtration of reaction mixture of tyrosine and tetranitromethane on Sephadex G-25. The elution profiles of samples (i), (iii) and (vii) are shown (see Fig. 1). The arrow shows the elution position of Blue Dextran. The three main peaks *A*, *B* and *C* are indicated. Sample (i) ---; sample (iii) ——; sample (vii) · · · · · · ·.

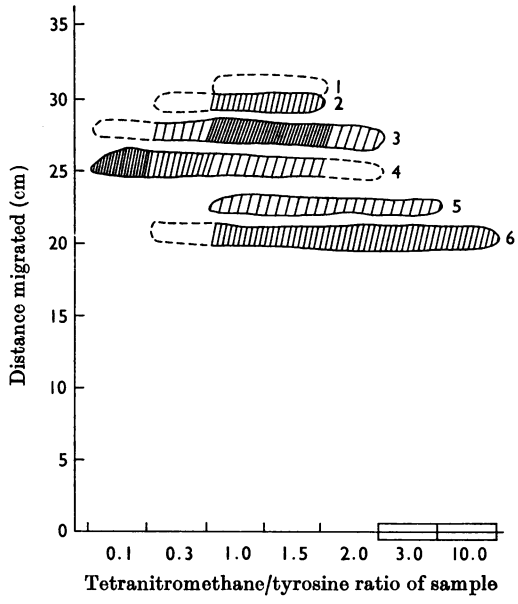


Fig. 3. Tracing of paper electrophoretogram. Tyrosine and tetranitromethane were allowed to react by method (*b*). Seven samples in which the tetranitromethane/tyrosine molar ratio ranged from 0.1 to 10.0 were subjected to paper electrophoresis at pH 1.9 (60 V/cm for 75 min). Bands representing compounds 1–6 are shown.

(ii) Compound 4 was tyrosine.

(iii) Compound 5 stained pink with cadmium–ninhydrin reagent but did not fluoresce in u.v. light. It did not stain with nitrosonaphthol.

(iv) Compound 6 was 3-nitrotyrosine.

(v) Compound 7 gave a green–blue fluorescence in u.v. light and was stained pink with cadmium–ninhydrin reagent but it gave no colour with nitrosonaphthol or with 2,4-dinitrophenylhydrazine. The last three samples showed a faint brown streaking of the background and a brown insoluble material which remained on the origin. There was no sign of 3,5-dinitrotyrosine in any sample. When the concentration of tyrosine was 1 mM and a tenfold excess of tetranitromethane was used the yield of 3-nitrotyrosine was 7.4% of the expected value and when the tyrosine was decreased to 0.1 mM the yield of 3-nitrotyrosine rose to 12.4%.

Gel filtration was performed on these samples (Fig. 2) and showed three peaks. Peak *A* began to emerge at the same elution volume as Blue Dextran and appeared to be a heterogeneous collection of polymers. On electrophoresis at pH 1.9 it gave only a faint streaking of the background and a brown insoluble material on the origin. Paper electrophoresis of peak *B* showed compound 5 and also a new compound, which ran with the same mobility as 3-nitrotyrosine. Paper electrophoresis of peak *C* showed compounds 1, 2, 3, tyrosine and 3-nitrotyrosine.

Method (b). Except for the absence of compound 7, the same products were formed when method (*b*) was used (Fig. 3). When low tetranitromethane/tyrosine ratios were used the fluorescent compounds 1, 2 and 3 were the main products of the reaction as judged by the intensity of the ninhydrin staining, but when a threefold excess of tetranitromethane was used they were no longer present. A twofold excess of tetranitromethane was needed to cause complete loss of tyrosine. Compound 5 was not formed when low concentrations of tetranitromethane were used and it was destroyed by a tenfold excess. Background streaking and insoluble material at the origin were seen when tetranitromethane was used at a greater molar excess than 3. The 3-nitrotyrosine band did not become more intense when the molar excess of tetranitromethane was increased beyond 1.5 and a tenfold excess of reagent yielded 7.2% of the expected amount of nitrotyrosine.

It appears that the material responsible for the streaking arises, at least in part, from the further action of tetranitromethane on the fluorescent substances. Compound 3 was eluted and treated with tetranitromethane at pH 9.0. The fluorescence of the solution and the reaction with nitrosonaphthol rapidly disappeared and paper electrophoresis at pH 1.9 gave only a streaky background and the brown insoluble material. Gel filtration on Sephadex G-25 showed a heterogeneous peak, which began to

appear with the void volume of the column. The nature of these polymers was not investigated further.

Nature of compound 3

U.v. spectra. In 0.1M-hydrochloric acid the spectrum showed an absorption maximum at 284nm, which in 0.1M-sodium hydroxide was shifted to 316nm. This seemed likely to be due to a phenolic ionization and the apparent pK for this was 7.2. The spectra showed an isosbestic point at 292nm. The fluorescent form of the substance is the phenoxide ion, judging from the effect of ammonia fumes on the fluorescence.

Acetylation. Compound 3 had zero electrophoretic mobility at pH6.5. After acetylation in acetic acid it gave rise to two new products: (i) compound 3*a* ($m = -0.44$) was fluorescent and stained with ninhydrin; (ii) compound 3*b* ($m = -0.80$) was fluorescent but did not react with ninhydrin. Both compounds reacted with nitrosonaphthol. The mobilities of these compounds suggest that the ratios of net charge to molecular weight are approximately 1:400 for compound 3*a* and 2:450 for compound 3*b*. Thus, the unknown substance appears to contain two amino groups and two carboxyl groups. Compound 3*a* is evidently the mono-*N*-acetyl derivative and compound 3*b* is the di-*N*-acetyl derivative. The mobilities indicate that the molecular weight of compound 3 is approximately twice that of tyrosine.

Acetylation in pyridine caused the unknown substance to lose its fluorescence and its ability to react with nitrosonaphthol. Tyrosine also failed to react with nitrosonaphthol after acetylation in pyridine whereas acetylation in acetic acid gave rise to *N*-acetyltyrosine, which stained red with nitrosonaphthol. It seems likely that acetylation in pyridine produces *NO*-diacetyltyrosine from tyrosine and the *NNOO*-tetra-acetyl derivative of compound 3.

Comparison of compound 3 *with dityrosine.* Dityrosine, which had been prepared enzymically, gave u.v.-absorption spectra in acid and alkali that were identical with those of compound 3. They were also identical on paper electrophoresis at pH 1.9 and paper chromatography in solvent 1 (R_F 0.26) and solvent 2 (R_F 0.14). Andersen (1966) gave R_F 0.18 for dityrosine in solvent 2. A compound with the same electrophoretic and chromatographic properties as compound 3 was also obtained by oxidation of tyrosine with alkaline ferricyanide and by total acid hydrolysis of the elastic tendons of the dragonfly.

Mass-spectral analysis supports the identification of compound 3 as dityrosine. The fully acetylated methyl ester of compound 3 gave a prominent molecular ion of mass 556 ($C_{28}H_{32}O_{10}N_2$ requires mol.wt. 556). The intensity of the ion at m/e 557 was 28.7% of that at m/e 556. The molecular formula requires a value of 31.8%. The fragmentation pattern was consistent with the presence of acetyl and acetate groups.

Nature of compounds 1 *and* 2

Compounds 1, 2 and 3 gave similar u.v.-absorption spectra. Acid and alkali maxima were at 286 and 316nm for compound 2 (isosbestic point 293nm) and at 287 and 316nm for compound 1 (isosbestic point 294nm). The ratio of the extinction at the alkaline maximum to that at the acid maximum was 1.06 for compound 1, 1.20 for compound 2 and 1.44 for compound 3. A comparison of these data with those given by Andersen (1966) for trityrosine suggests that compound 2 is trityrosine.

Compound 2 had zero electrophoretic mobility at pH6.5. After treatment with acetic anhydride in acetic acid it gave rise to three new fluorescent products: (i) compound 2*a* ($m = -0.45$) stained pink with cadmium–ninhydrin reagent, (ii) compound 2*b* ($m = -0.73$) stained pink with cadmium–ninhydrin reagent and (iii) compound 2*c* ($m = -0.93$) gave no colour with cadmium–ninhydrin reagent. The following approximate ratios of net charge to molecular weight are indicated by the electrophoretic mobilities of these products: compound 2*a*, 1:550; compound 2*b*, 2:600; and compound 2*c*, 3:650. This suggests that compound 2 has three amino groups and three carboxyl groups. Compounds 2*a*, 2*b* and 2*c* evidently represent the mono-*N*-acetyl, di-*N*-acetyl and tri-*N*-acetyl derivatives respectively.

Acid hydrolysates of elastic tendons of the dragonfly and peroxidase treatment of tyrosine both yielded a fluorescent substance corresponding to compound 2 in electrophoretic mobility at pH 1.9 and in paper chromatography. In solvent 1 it had R_F 0.13 and in solvent 2 R_F 0.06. Andersen (1966) reported R_F 0.05 for trityrosine in solvent 2.

No substance corresponding to compound 1 was seen in acid hydrolysates of elastic tendons or after peroxidase treatment of tyrosine but the similarity of its u.v.-absorption spectrum to those of dityrosine and trityrosine suggests that compound 1 may be tetratyrosine.

Fluorescent products from glycyltyrosine, N-*acetyltyrosine,* p-*cresol and proteins*

Glycyltyrosine. After treatment of glycyltyrosine with tetranitromethane paper electrophoresis at pH 1.9 showed the following four products, in order of increasing mobility towards the cathode: (i) compound GT*a* was glycyl-3-nitrotyrosine; (ii) compound GT*b* stained yellow with cadmium–ninhydrin

reagent but did not fluoresce; (iii) compound GTc showed intense blue fluorescence under u.v. light and stained yellow with cadmium–ninhydrin; (iv) compound GT*d* gave a less intense band than compound GTc but was also fluorescent and stained yellow. Peroxidase treatment of glycyltyrosine also gave rise to compounds GTc and GT*d*.

Total acid hydrolysis of compound GTc gave glycine and dityrosine and compound GT*d* gave glycine and trityrosine. After one application of phenyl isothiocyanate degradation (Gray, 1967) paper electrophoresis at pH 1.9 gave dityrosine from compound GTc and trityrosine from compound GT*d*. Thus in these substances the dipeptide appears to be cross-linked by biphenyl and terphenyl linkages between the tyrosine residues.

N-*Acetyltyrosine*. After treatment of *N*-acetyltyrosine with tetranitromethane paper electrophoresis at pH 6.5 showed three new products: (i) compound AT*a* ($m = -1.80$) was yellow and was assumed to be *N*-acetylnitrotyrosine; (ii) compound AT*b* ($m = -0.91$), which was weakly fluorescent; and (iii) compound ATc ($m = -0.83$), which was strongly fluorescent. All the compounds were ninhydrin-negative. Compounds AT*b* and ATc were also given by peroxidase-treated *N*-acetyltyrosine and they appear to represent the *N*-acetyl derivatives of trityrosine and dityrosine respectively.

Treatment of a mixture of tyrosine and *N*-acetyltyrosine with tetranitromethane gave three main fluorescent products at pH 6.5: (i) compound TAT*a* ($m = 0$) stained with ninhydrin and was probably dityrosine; (ii) compound TAT*b* ($m = -0.55$) stained with ninhydrin and was, therefore, mono-*N*-acetyldityrosine; (iii) compound TATc ($m = -0.88$) did not stain with ninhydrin and was probably di-*N*-acetyldityrosine.

p-*Cresol*. Paper chromatography of tetranitromethane-treated *p*-cresol showed one main fluorescent product and by comparison with the fluorescent products obtained by alkaline ferricyanide treatment of *p*-cresol this material appeared to be dicresol (R_F in water 0.72, and in solvent 3 0.50). The ether extract of tetranitromethane-treated *p*-cresol, when applied to paper, also gave an intense yellow colour with 2,4-dinitrophenylhydrazine, suggesting the presence of Pummerer's ketone, since this compound has already been identified as a product of the reaction (Bruice *et al.* 1968).

Proteins. When insulin and trypsin were treated with an amount of tetranitromethane equal to the tyrosine content of the protein, acid hydrolysates of the treated proteins showed distinct bands of dityrosine and trityrosine on electrophoresis at pH 1.9. With a tenfold excess of tetranitromethane the fluorescent bands were not observed. Dityrosine and trityrosine were also obtained after treating aminoethylated ovotransferrin with an equimolar amount of tetranitromethane. On the other hand, when a tenfold excess of reagent was used the solution of aminoethylated ovotransferrin in 5M-guanidinium hydrochloride rapidly formed a gel, suggesting the occurrence of extensive cross-linking, but no dityrosine or trityrosine were found after total acid hydrolysis. Neither dityrosine nor trityrosine was observed after treatment of native ovotransferrin with tetranitromethane.

Table 1. *Tyrosine and nitrotyrosine contents of proteins expressed as residues/80000 g of protein*

	Tyrosine	3-Nitrotyrosine
Ovotransferrin control	19.7	—
Nitrated ovotransferrin	—	2.5
Nitrated iron ovotransferrin	2.8	5.3
Nitrated aminoethylated ovotransferrin	0.6	4.1

Table 1 shows the contents of tyrosine and 3-nitrotyrosine in ovotransferrin, iron-saturated ovotransferrin and aminoethylated ovotransferrin after treatment with a tenfold excess of tetranitromethane.

DISCUSSION

The chemical characteristics of compounds 2 and 3 indicate that they are identical with trityrosine and dityrosine respectively, which have been isolated from acid hydrolysates of the insect protein resilin by Andersen (1966). In its action on tyrosine tetranitromethane thus resembles other oxidizing agents such as ferric salts, alkaline ferricyanide, peroxidase and *tert.*-butyl peroxide (Johnston *et al.* 1969) which can produce dehydrogeno dimers from *para*-substituted phenol units. The products of the reaction between tetranitromethane and tyrosine are complex and several unidentified compounds were observed in the present work. The biphenyl and terphenyl derivatives were only recovered when low ratios of tetranitromethane to tyrosine were used and the nature of the ultimate polymers formed with large amounts of the reagent is not known. Gross & Sizer (1959) noted that the final product of the reaction between tyrosine and peroxidase is a brown pigment, as in the present case.

The structure 2,2′-dihydroxy-5,5′-di-(2-amino-2-carboxyethyl)biphenyl was assigned to dityrosine by Gross & Sizer (1959) by analogy with the dicresol that Westerfield & Lowe (1942) obtained by treating *p*-cresol with peroxidase. This compound was shown to be 2,2′-dihydroxy-5,5′-dimethylbiphenyl by synthesis from 3-bromo-4-methoxytoluene.

For free tyrosine, coupling reactions appear to predominate over nitration, as Bruice *et al.* (1968)

have observed for several *para*-substituted phenols. Our preliminary experiments on the formation of biphenyl and terphenyl derivatives from the tyrosine residues of proteins suggest a possible mode of cross-linking that may be responsible for the polymerization of some proteins on treatment with tetranitromethane (Doyle *et al.* 1968; Boesel & Carpenter, 1970; Vincent *et al.* 1970).

Amino acid analyses of ovotransferrin treated with tetranitromethane (Table 1) show that practically complete loss of tyrosine occurs in the metal-free and aminoethylated samples. About three residues of tyrosine appear to be protected in iron-saturated ovotransferrin. The role of metal-binding in protecting some of the tyrosine residues of transferrin against reaction with iodine has been studied by Azari & Feeney (1961). The yield of nitrotyrosine was in all three cases much less than the decrease in the yield of tyrosine. Previous workers have reported inability to account quantitatively for the tyrosine contents of insulin, trypsin and trypsinogen after treatment with tetranitromethane (Boesel & Carpenter, 1970; Vincent *et al.* 1970). In those cases where the tyrosine residues of tetranitromethane-treated proteins have been quantitatively accounted for it may be suggested that nitratable tyrosine residues occupy such positions in the protein that coupling to other residues is prevented.

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