625. *Di*-p-(2-*amino*-2-*carboxyethyl*)*phenyl Peroxide*: *Preparation from Tyrosine and Probable Natural Occurrence in Proteins.*

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The insolubilisation of certain proteins of barley and malt is probably due, among other causes, to the formation of a peroxide linkage between tyrosine residues of adjacent chains. In consequence 3-chlorotyrosine arises on hydrolysis of the insoluble protein by hydrochloric acid. By employing free tyrosine as a model the oxidation was simulated with hydrogen peroxide in hydrochloric acid to form di-*p*-(2-amino-2-carboxyethyl)phenyl peroxide. This compound is stable in solution only as the hydrochloride and on removal of or heating with hydrochloric acid yields 3-chlorotyrosine. Further treatment of the peroxide with hydrogen peroxide and hydrochloric acid gives successively the corresponding 3-chloro- and 3,5-dichloro-compound.

Chromatography on paper of concentrates of amino-acids obtained from brewer's wort by means of cation-exchange resins, revealed [1] the presence of an unidentified amino-acid, called compound X, occupying a position on two-dimensional chromatograms just below that of tyrosine. The same compound was observed in the hydrolysates of various proteins from malt, brewer's wort, and spent grains, and was particularly prominent in the less-soluble protein components as, for example, the deposits which separated when 70% ethanolic solutions of malt bynin (hordein) [2] were kept at 0°. The proteins in the solution then did not yield any significant amount of compound X on hydrolysis with hydrochloric acid, and the amount of the compound when it did arise was related inversely to that of tyrosine. Incidentally, the finding that compound X occurs mainly in the insoluble fractions affords an explanation of the fact that this amino-acid frequently appears as a product of the hydrolysis of the so-called protein hazes which form in malt products such as malt-extract, beer, and vinegar.

Starting with the knowledge that compound X gave, on paper chromatograms, colour reactions similar to those of tyrosine, and with the idea that its formation involved the oxidation of bound tyrosine, the oxidation of the free amino-acid was studied. Treatment of tyrosine with hydrogen peroxide or performic acid in hydrochloric acid afforded a crystalline product, m. p. 143° (decomp.), which behaved like compound X on chromatograms. However, the new product was unstable, being itself a powerful oxidising agent, and could not survive the conditions used to prepare compound X from wort or proteins. Nevertheless, on hydrolysis it yielded a stable compound occupying the same position on paper chromatograms and lacking oxidising properties. It seemed, therefore, that the primary unstable product, called compound X_1, might be a direct precursor of the stable compound X.

In similar studies, Thompson [3] identified 3-chlorotyrosine and 3,5-dichlorotyrosine among the products formed on hydrolysing, by means of hydrochloric acid, insulin which had been treated with performic acid.[4,5] The formation of these compounds was attributed to the presence, in the crude oxidation product, of hydrogen peroxide which liberated chlorine from the chloride present and so led to the chlorination of tyrosine (cf. Zeynek [6]). With this background compound X was soon identified with 3-chlorotyrosine.

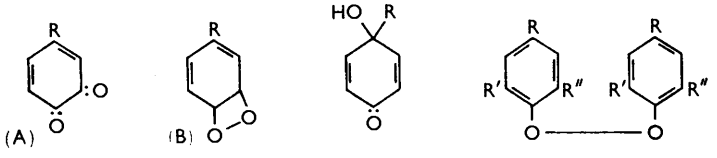
The question arises as to how this chlorinated amino-acid can arise in worts and hydrolysates when it is derived from natural proteins in the absence of added oxidising

[1] Davies, Harris, and Parsons, *J. Inst. Brewing*, 1956, **62**, 38.
[2] Scriban, "Les Protides de l'Orge, du Malt et du Moût," Lille, 1951.
[3] Thompson, *Biochim. Biophys. Acta*, 1956, **15**, 440.
[4] Sanger, *Biochem. J.*, 1949, **44**, 126.
[5] Sanger and Tuppy, *Biochem. J.*, 1951, **49**, 463.
[6] Zeynek, *Z. physiol. Chem.*, 1925, **144**, 246.

agents. It appeared that it might be formed from a precursor such as compound X_1, itself perhaps formed *via* a peroxidase known to be present in barley and malts.

Compound X_1 was an amino-acid hydrochloride, which had the empirical formula $C_9H_{14-16}ClNO_5$. Attempts to liberate the free base in solution resulted in the formation of 3-chlorotyrosine, a finding which explains the identical chromatographic behaviour of compound X and chlorotyrosine. Moreover, attempts to remove water of hydration completely brought about decomposition. Despite these difficulties the structure emerged from a consideration of the following evidence. In common with tyrosine it reacted with the formaldehyde–sulphuric acid reagent [7] and coupled with either 1-nitroso-2-naphthol or diazotised sulphanilic acid but, unlike tyrosine, gave no reaction with Millon's reagent. With the uranyl nitrate–benzene Rhodamine B reagent and with *o*-dinitrobenzene it reacted like a quinone while, unlike the original, it failed to liberate iodine from potassium iodide after treatment with *o*-phenylenediamine. Consideration was therefore given to structures such as " DOPA-3,4-quinone " (I) and toluquinol derivatives such as (II). It is interesting to observe that Earland and Stell [8] postulated the existence of a quinonoid derivative of tyrosine as an intermediate in the formation of insoluble proteins by the oxidation of silk fibroin while the formation of toluquinol [9] derivates by the oxidation of hindered phenols [10–12] is well known. However, comparison of the infrared and ultraviolet absorption spectra of compound X_1 with those of various quinones and semiquinones [7,11,13,14,15] (cf. Experimental) indicated that compound X_1 did not contain either a quinonoid or a semiquinonoid,[14,15] structure. Thus, it resembled tyrosine hydrochloride in having only a single sharp infrared absorption band (5·88 μ) in the region (5·8—6·0 μ) associated with carbonyl groupings, this band being due to the carbonyl of the undissociated carboxyl grouping, absent from the free base of tyrosine but present in the hydrochloride. The typical bands of toluquinol at 5·9—6·0 μ (conjugated carbonyl [13]) and 6·0—6·1 μ (conjugated ethylenic linkages) were absent.

In respect of its ultraviolet absorption, compound X_1 again resembled tyrosine rather than quinones or toluquinol as it had maximal absorption in aqueous acid at 280 mμ and in ethanol at 285 mμ whereas toluquinol, for example, shows absorption maxima at 277, 290, and 295 mμ.[13] In alkali it exhibited a principal absorption band at 300 mμ due to the formation of 3-chlorotyrosine. The " DOPA-quinone " structures (I) were ruled out, moreover, as compound X_1 lacks the typical 1,2-quinone infrared bands,[16] is colourless, and yields on catalytic reduction not DOPA but tyrosine. Taken in conjunction with the spectroscopic evidence, this shows that only one oxygen function is linked to the ring.



(I) (II) (III)

R = $CH_2 \cdot CH(CO_2H) \cdot NH_2, HCl$

Reaction of compound X_1 with potassium iodide showed that there was 0·5 active oxygen atom per nitrogen atom and that tyrosine was formed as the sole product rather

[7] Feigl, " Spot Tests in Organic Analysis," 5th edn., Elsevier, 1956.
[8] Earland and Stell, *Biochim. Biophys. Acta*, 1957, **23**, 97.
[9] Bamberger, *Ber.*, 1900, **33**, 3600; *Annalen*, 1912, **390**, 164.
[10] Bickel and Gersmann, *Proc. Chem. Soc.*, 1957, 231; Bickel and Kooyman, *J.*, 1953, 3211.
[11] Goodwin and Witkop, *J. Amer. chem. Soc.*, 1957, **79**, 179.
[12] Ley, *Angew. Chem.*, 1958, **70**, 74.
[13] Wessely and Sinwel, *Monatsh.*, 1950, **81**, 1055.
[14] Hathway, *J.*, 1957, 519.
[15] Hathway and Seakins, *Nature*, 1955, **176**, 218.
[16] Josien, *J. Chem. Phys.*, 1953, **21**, 331.

than chloro- or iodo-tyrosine, as might be expected from (II). Considering all the evidence, structure (III; R′ = R″ = H) best accommodates the facts. The ultraviolet absorption at 285 mμ recalls that of the analogous di-*p*-tolyl peroxide [17,18] while, in view of the lack of strong characteristic vibrations for peroxide groupings,[19] the infrared absorption is quite compatible with structure (III). The improbable formulations of compound X_1 as a simple hypochlorous acid salt, as a solvate containing hydrogen peroxide, or as a peracid can be discounted not only on the basis of the determination of active oxygen and the infrared absorption [19,20] but also because addition of 1 mole of hypochlorous acid, hydrogen peroxide, or a peracid to tyrosine failed to inhibit the Millon reaction.

Structure (III) accounts well for the formation of 3-chlorotyrosine from compound X_1 on neutralisation, presumably *via* hypochlorite. Under these conditions traces of DOPA are also formed and this product arises, together with its 2,3- and 2,5-isomers,[21] 3-chlorotyrosine, 3,5-dichlorotyrosine, tyramine, and complex coloured compounds, when compound X_1 is heated with water. The formation of coloured materials recalls the oxidative transformation of tyrosine into melanins.[22] In this connection, Onslow [23] suggested that peroxide derivatives of DOPA were intermediates which are converted subsequently into quinones and hence melanins. The present findings provide a chemical analogy for this suggestion.

The formation of peroxide units as in structure (III) in a protein would account among other reasons for the insolubilisation of the protein as a result of the cross-linking of the tyrosine residues of adjacent chains. Moreover, the solubilisation of such oxidised proteins by means of reducing agents might be explained by the fission of the peroxide cross-linkage. Whether or not the formation of 3-chlorotyrosine on hydrolysis of the oxidised protein by means of hydrochloric acid is then due to the intermediate formation of chlorine or to the isomerisation [25] of a transient derivative of phenyl hypochlorite remains undecided. An alternative possibility is that polyphenols associated with the protein undergo peroxidation and that the peroxide products themselves are responsible for the chlorination of tyrosine.

The reaction of compound (III) with hydrogen peroxide in alcoholic hydrochloric acid gave two further products having oxidising properties. Both compounds, called X_2 and X_3, resembled the original in that they (*a*) liberated iodine from potassium iodide, (*b*) gave no reaction with Millon's reagent, and (*c*) were isolable only as their hydrochlorides. When heated, treated with alkali, or on chromatography, compound X_2 gave rise to 3-chlorotyrosine while compound X_3 yielded 3,5-dichlorotyrosine. Compounds X_2 and X_3 had ultraviolet absorption characteristics like those of 3-chlorotyrosine and 3,5-dichlorotyrosine, respectively. In view of their derivation, composition, and properties, structures (III; R′ = H; R″ = Cl) and (III; R′ = R″ = Cl) are suggested for compounds X_2 and X_3, respectively.

Experimental

Chromatography of Amino-acids.—This was by ascending chromatography in a Datta frame.[1] The positions occupied by the various compounds on paper chromatograms are given in Table 1.

Hydrolysis of Proteins and Amino-acids.—Hydrolysis was carried out by use of 6N-hydrochloric acid at 100° for 16 hr. in the presence of Amberlite IR120, and the products were recovered from the resin.[1] Application of the method to tyrosine, 3-chlorotyrosine, or 3,5-dichlorotyrosine caused no change in these amino-acids.

[17] Wieland and Meyer, *Annalen*, 1942, **551**, 249.
[18] Breitenbach and Derkosch, *Monatsh.*, 1950, **81**, 530.
[19] Bellamy, " Infra-red Spectra of Complex Molecules," Methuen and Son, London, 1956.
[20] Davison, *J.*, 1951, 2456.
[21] Lissitsky and Roques, *Bull. Soc. Chim. biol.*, 1957, **39**, 521.
[22] Raper, *Biochem. J.*, 1926, **20**, 735; 1927, **21**, 1932, **26**, 2000.
[23] Onslow, *Biochem. J.*, 1923, **17**, 217.
[24] Lontie, Rondelet, and Dulcino, *European Brew. Conv. Proc.*, 1953, **33**.
[25] Likhosherstov and Arkhangel'skaya, *J. Gen. Chem. U.S.S.R.*, 1937, **7**, 1914.

Protein Fractions from Barley, Malt and Spent Grains.—These were prepared by the methods described by Scriban.[2]

Compound X_1 (III; R′ = R″ = H).—Tyrosine (2·0 g.) in 2N-hydrochloric acid (10—12 ml.) and 30% hydrogen peroxide solution (8 ml.; Merck's Perhydrol) were kept at room temperature for 16—20 hr. The solution was evaporated in a vacuum by using a capillary and at below 30° until crystallisation set in. Ethanol was then added and evaporation continued with appropriate additions of ethanol until a dry residue was obtained. The solid was freed from residual

TABLE 1. *$R_{Tyrosine}$ values for tyrosine derivatives.*

Compound	Solvent	$R_{Tyrosine}$
3-Chlorotyrosine (X), di-*p*-(2-amino-2-carboxyethyl)phenyl-peroxide (X_1), and di-[4-(2-amino-2-carboxyethyl)-3-chlorophenyl]peroxide (X_2)	n-Butanol–acetic acid–water (4 : 1 : 1; v/v)	1·26
	Ethanol–2N-ammonia (9 : 1; v/v)	0·78
3,5-Dichlorotyrosine and di-[4-(2-amino-2-carboxyethyl)-3,5-dichlorophenyl] peroxide	n-Butanol–acetic acid–water (4 : 1 : 1; v/v)	1·58
	Ethanol–2N-ammonia (9 : 1; v/v)	0·46

solvent and excess of hydrogen chloride in a vacuum for 48 hr. and then extracted with cold ethanol. The solution was evaporated in a vacuum; the residue of *di*-p-(*2-amino-2-carboxyethyl*)*phenyl peroxide hydrochloride* crystallised from ethanol–ether as laths (1·6—1·7 g.), m. p. 143° (decomp.), $[\alpha]_D^{20}$ +12·9° (*c* 1·64 in EtOH) (Found: C, 42·9; H, 5·6; Cl, 13·4; N, 5·2. $C_{18}H_{22}Cl_2N_2O_6,4H_2O$ requires C, 42·8; H, 5·9; Cl, 14·1; N, 5·5%). Increasing the scale of the oxidation reduced the yield considerably.

The product contained 2 equiv. of amino-nitrogen as estimated by the van Slyke procedure [26] or Yemm and Cocking's colorimetric method.[27] Heating with 6N-hydrochloric acid or treatment with 1 mole of N-sodium hydroxide or N-sodium carbonate yielded 3-chlorotyrosine, m. p. and mixed m. p. 261—263° (decomp.) (Found: C, 46·5; H, 5·3; Cl, 15·3; N, 6·3. Calc. for $C_9H_{10}ClNO_3,H_2O$: C, 46·3; H, 5·1; Cl, 15·3; N, 6·0%). The final solution in each case, and particularly after treatment with sodium hydroxide, on hydrogenation contained in addition a small amount of 3,4-dihydroxyphenylalanine as revealed by chromatography in the solvent system butanol–acetic acid–water (4 : 1 : 1, v/v), and spraying of the final chromatogram with 2,6-dichloroquinone-4-chloroimide,[28] a reagent which is specific for 1,2-dihydroxybenzene derivatives in this series.

The peroxide (III) was put on to Whatman No. 3 MM papers, and the chromatogram developed with butanol–acetic acid–water. The zone reactive to ninhydrin was eluted from the dried papers with water, the solution concentrated, and the residue crystallised from acetic acid to yield 3-chlorotyrosine, m. p. and mixed m. p. 264°.

The results of spot tests [7] on (III; R′ = R″ = H) are listed in Table 2.

3-Chlorotyrosine.—The hydrochloride, m. p. 240°, was prepared as described by Zeynek [6] and converted into the free base, m. p. 264° (decomp.). It gave a positive reaction with Millon's reagent and failed to liberate iodine from potassium iodide.

3,5-Dichlorotyrosine.—The hydrochloride,[29] m. p. 246°, gave a negative reaction with Millon's reagent but did not liberate iodine from potassium iodide.

Catalytic Reduction of (III; R′ = R″ = H).—The compound was hydrogenated in water in the presence of palladium. Chromatography of the filtrate revealed the presence of only tyrosine. Evaporation of the solution and crystallisation of the residue from ethanol–ether gave tyrosine hydrochloride, m. p. 227—230° (Found: N, 6·4. Calc. for $C_9H_{11}NO_3,HCl$: N, 6·3%). With Adams' catalyst in place of palladium, only 3-chlorotyrosine was formed.

Reaction of (III; R′ = R″ = H) *with Potassium Iodide.*—The compound (8·74 mg.) was set aside for 10 min. with excess of potassium iodide in hydrochloric acid. The liberated iodine was titrated with sodium thiosulphate and corresponded to one active oxygen per 2 nitrogen atoms (Found: 3·2, 3·3; required 3·2%). Chromatography of the solution revealed the presence of tyrosine, which was worked up by means of Amberlite IR120. Corresponding

[26] Van Slyke, McFadyen, and Hamilton, *J. Biol. Chem.*, 1941, **141**, 671.
[27] Yemm and Cocking, *Analyst*, 1955, **80**, 209.
[28] Harris, *J. Inst. Brewing*, 1956, **43**, 390.
[29] Bouchilloux, *Bull. Soc. Chim. biol.*, 1955, **37**, 255.

solutions which had not been treated with potassium iodide yielded only 3-chlorotyrosine when worked up in the same way.

Preparation of Model Quinones, Semiquinones, and Peroxides.—*p*-Benzoquinone and *p*-toluquinone,[30] 2,2-diacetoxy-4-methylcyclohexa-3,5-dien-1-one, 4-acetoxy-4-methylcyclohexa-2,5-dien-1-one, *p*-toluquinol [11] and di-*p*-tolyl peroxide [17] were made according to literature methods.

Oxidation [30] *of Compound* (III; R′ = R″ = H).—The compound (0·38 g.) in 10% sulphuric

TABLE 2. *Results of spot tests* [7] *on compound* X_1.

Reagent	Test for	Result: Tyrosine	Result: Compound X_1
Formaldehyde–sulphuric acid	Aromatic nucleus	+	+
Ehrlich reagent	Coupling	+	+
Uranyl nitrate–benzene Rhodamine B	–CO·CO– or CH_2·CO–	—	+
Potassium iodide	–CO– or	—	+
Potassium iodide after *o*-phenylenediamine	–CO·CO–	—	—
o-Dinitrobenzene	–CO·CO– and quinones	Indef.	+
Pyrolysis		Odour of burned hair	Odour of chlorine
Millon reagent	*p*-Substituted phenols	+	—
Sodium nitroprusside–aldehyde	–NH–	—	—
p-Dimethylaminobenzaldehyde	Indole	—	—
Sodium hypochlorite–Fuchsin–sulphurous acid	R·CH(NH_2)·CO_2H	+	+
Thionyl chloride–hydroxylamine–sodium hydroxide	–CO_2H	+	+
Ferric chloride–potassium ferricyanide	Phenol	+	+

acid (10 ml.) was treated with potassium permanganate (0·75 g.), and the mixture warmed for 10 min. Sodium hydrogen sulphite was added to discharge the colour, the mixture was extracted with ether, and the ethereal solution evaporated to dryness. The product was identified by chromatography as *p*-coumaric acid.[31]

Action of Hot Water on Compound (III; R′ = R″ = H).—The peroxide (0·8 g.) in water (50 ml.) was heated under reflux for 0·5 hr. The solution was concentrated in a vacuum and the product chromatographed in butanol–acetic acid–water to reveal 3-chlorotyrosine, tyrosine, tyramine, and dihydroxyphenylalanines. Similar heating of tyrosine, monochlorotyrosine, and dichlorotyrosine yielded only starting materials.

Compound X_2.—Compound (III; R′ = R″ = H) (5·36 g.) was dissolved in ethanol (100 ml.) and sufficient 2N-hydrochloric acid to complete solution. Perhydrol (20 ml.) was added and the mixture worked up as described above for the starting material. Crystallisation from ethanol–ether yielded *di*-[4-(2-*amino*-2-*carboxyethyl*)-3-*chlorophenyl*] *peroxide dihydrochloride* (III; R′ = H, R″ = Cl) as needles, m. p. 130° (decomp.), $[\alpha]_D^{20}$ +12·6° (*c* 1·288 in ethanol) (Found: C, 39·5; H, 4·9; Cl, 25·6; N, 5·2. $C_{18}H_{20}Cl_4N_2O_6,2H_2O$ requires C, 40·1; H, 4·5; Cl, 26·4; N, 5·2%). Treatment of the ground solid with 1 mole of ethereal diazomethane yielded 3-chlorotyrosine.

Compound X_3.—Compound (III; R′ = R″ = H) (1·05 g.) was dissolved in ethanol (10 ml.) and 2N-hydrochloric acid (12 ml.), and the solution treated with perhydrol (8 ml.). After 24 hr. the solution was evaporated as above. The orange solid was washed with ethanol–ether and dried in a vacuum to give *di*-[4-(2-*amino*-2-*carboxyethyl*)-3,5-*dichlorophenyl*] *peroxide dihydrochloride* (III; R′ = R″ = Cl), m. p. 98° (Found: C, 35·9; H, 3·9; Cl, 35·6; N, 4·3. $C_{18}H_{18}Cl_6N_2O_6,2H_2O$ requires C, 35·6; H, 3·6; Cl, 35·1; N, 4·6%).

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[30] Fieser, " Experiments in Organic Chemistry," D.C. Heath & Co., Chicago, 1935.
[31] Bengough and Harris, *J. Inst. Brewing*, 1955, **61**, 134.