THE FREE RADICAL NATURE OF AROMATIC HYDROXYLATION EFFECTED BY OXYGEN, ASCORBIC ACID AND FERROUS IONS

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SUMMARY

The hydroxylation of benzoic acid by oxygen in the presence of ascorbic acid, ferrous sulphate and ethylenediaminetetraacetic acid in a phosphate buffer is shown to give a mixture of mono- and di-hydroxybenzoic acids and the approximate proportions have been estimated. They are consistent only with a homolytic substitution. The presence of free radicals in the hydroxylation mixture has been confirmed by polymerisation experiments.

INTRODUCTION

In 1952 UDENFRIEND *et al.*[1] showed that a water soluble system obtained from rat liver could, in the presence of diphosphopyridine nucleotide and oxygen, catalyse the conversion of L-, but not D-phenylalanine to tyrosine. Subsequent studies showed that ascorbic acid increased the efficiency of the conversion of tyramine to hydroxytyramine by adrenal medula homogenates. UDENFRIEND *et al.*[2] then found that the adrenal tissue was unnecessary, and that the hydroxylation of tyramine, and a number of other compounds[3] could be effected at 37° by oxygen in the presence of ferrous sulphate, ascorbic acid, and ethylenediaminetetraacetic acid in phosphate buffer. This system has been used to hydroxylate the compounds listed in Table I, and it has been suggested that both compounds normal to living systems and unusual injested materials may be hydroxylated in this manner *in vivo*.

The mechanism of the hydroxylation has been subject to speculation. UDENFRIEND *et al.*, from their own results and those of WILLIAMS[4] suggested that the hydroxyl cation (HO^+) was involved as the attack always appeared to take place at positions of high electron density. They ruled out the possibility that a free radical reaction was involved on the grounds that substitution was not sufficiently random. Nevertheless on the same data, DALGLIESH[5] later suggested that free radicals were involved as they can attack electronegative sites. While this is correct it has been thoroughly established for some time[6] that free radicals also attack electropositive positions and that homolytic attack on aromatic systems generally takes place predominately at the *ortho* and *para* positions to a single substituent irrespective of whether this substituent is electron donating or attracting in nature.

TABLE I

COMPOUNDS HYDROXYLATED BY UDENFRIEND'S PROCEDURE

Substrate	*Products*	*Yield* %	*Identification*	*Reference*
Acetanilide	2-hydroxyacetanilide	12	DU	3
	4-hydroxyacetanilide	17	I	3
Aniline	4-aminophenol	9	D	3
Anthranilic acid	3-hydroxyanthranilic acid	?	C	5
	5-hydroxyanthranilic acid	?	C	5
Antipyrine	4-hydroxyantipyrine	43	I	3
Benzoic acid	3-hydroxybenzoic acid	?	?	4
Cyanobenzene	3-cyanophenol	?	?	4
1,3-dimethylxanthine	1,3-dimethyluric acid	13	DU	3
4-ethoxyacetanilide	4-hydroxyacetanilide	7	I	3
Hippuric acid	2-hydroxyhippuric acid	?	C	9
2-hydroxyphenylalanine	2,3-dihydroxyphenylalanine	?	C	5
	2,5-dihydroxyphenylalanine	?	C	5
	Catechol?	?	C	5
Indole-3-acetic acid	5-hydroxyindole-3-acetic acid	?	C	5
	7(?)-hydroxyindole-3-acetic acid	?	—	5
Kynurenine	3-hydroxykynurenine	?	C	5
	5-hydroxykynurenine	?	C	5
Nitrobenzene	2-nitrophenol	?	?	4
Phenylacetic acid	Not clearly indentified	—	C	5
Phenylalanine	Tyrosine	?	C	5
	2-hydroxyphenylalanine	?	C	5
Phenylethylamine	Tyramine?	?	C	5
Quinoline	3-hydroxyquinoline	15	I	3
Salicylic acid	Gentisic acid	55	I	3
Tryptamine	5-hydroxytryptamine	?	?	3
	7-hydroxytryptamine	?	?	3
Tryptophane	5-hydroxytryptophane	?	C	5
	7(?)-hydroxytryptophane	?	—	5
Tyramine	3,4-dihydroxyphenylethylamine	25	C	3
Tyrosine	3,4-dihydroxyphenylalanine	?	C	5
	Hydroquinone?	?	C	5

C, paper chromatographic comparison with authentic material; D, distribution comparison with authentic material; I, isolation and mixed m.p.; U, u.v. absorption comparison with authentic material.

The formation of hydroxyl cations (HO^+) at pH 6–7 is so unlikely that it can be discounted. In any case electrophilic substitution of the highly deactivated aromatic rings of nitrobenzene, cyanobenzene and benzoic acid is difficult to effect and only occurs under very vigorous anhydrous conditions. It is highly improbable therefore that UDENFRIEND's reaction proceeds through the intermediary of a hydroxyl cation. The other possibility, a reaction involving free radicals, is consistent with the positions of hydroxylation of all the compounds listed in Table I which possess activated aromatic rings; hydroxylation takes place at the *ortho* and *para* positions to the existing substituents.

The situation is not so clear with the compounds possessing deactivated rings. In the case of nitrobenzene 2-nitrophenol is formed, in excellent agreement with the observation that phenyl radicals also attack nitrobenzene predominately at position 2 and that hydroxylation also takes place at this position (Table IV); the isomer ratios obtained for these hydroxylations are only approximate. Free radical hydroxylation of quinoline would not be expected to occur only at position 3 as claimed because

homolytic phenylation gives[7] 8-phenyl- (30 %) and 4-phenyl-quinoline (20 %) as major products along with 14 % of 3-phenylquinoline and some of all the other phenylquinolines. Both 2- and 4-hydroxyquinoline ("quinolones"), which are neither phenolic nor basic, would have been discarded in the isolation procedure used[3] in working up the quinoline oxidation and the formation of 8-hydroxyquinoline might also have been missed. Electrophilic substitution of quinoline normally takes place at positions 5 and 8 although some reactions of uncertain mechanism[8] can lead to substitution at position 3. Cyanobenzene and benzoic acid are attacked by phenyl and hydroxyl radicals predominately at positions 2 and 4 (Table IV). The suggestion (Table I) therefore that these compounds hydroxylate largely or solely at position 3 is the only one wholely inconsistent with the postulate of a free radical hydroxylation mechanism and a reinvestigation of the benzoic acid hydroxylation has been carried out. It has been shown that 3-hydroxybenzoic acid is in fact a minor product of the reaction and that the isomer ratio of the hydroxybenzoic acids formed is consistent only with a free radical substitution.

METHODS

The u.v. absorption spectra were measured on a Carey recording spectrophotometer.

The chromatograms were all done on Whatman No. 1 paper in descending isopropanol–ammonia (d 0.880)–water (8:1:1, v/v, solvent A), or ascending[9] in the organic phase of benzene–propionic acid–water (2:2:1, v/v, solvent B) unless otherwise specified. They were examined under a "Hanovia" u.v. lamp emitting 90 % of its u.v. radiation at 2537 Å, and sprayed with diazotised 4-nitroaniline[10] (DNA), diazotised sulphanilic acid (DSA), or ammoniacal silver nitrate.

Qualitative hydroxylation experiments

A phosphate buffer (250 ml) containing disodium hydrogen phosphate (0.947 g) and potassium dihydrogen phosphate (1.36 g) was shaken with ethylenediaminetetraacetic acid (1.0 g) until saturated, and after filtration ferrous sulphate (0.20 g), ascorbic acid (3.0 g) and the substrate (0.3 g) were added. The pH was then determined, and adjusted where desired and the mixture was shaken under oxygen (2 lbs./in^2) in a 500-ml flask for the appropriate time at room temperature. The solution was then strongly acidified (H_2SO_4), saturated with ammonium sulphate and extracted with ether (4 × 100 ml). The ether extracts were combined, evaporated, and chromatographed alongside authentic samples on the same papers.

Estimation of the isomer ratios in the hydroxylation of the benzoic acids

1. Benzoic acid or its 2- or 3-hydroxy derivative were hydroxylated at pH 3.1 (1 h). The ether extracts were evaporated to dryness and the residues taken up in methanol (0.5 ml). Known volumes (approx. 0.01 ml) of these solutions were chromatographed in descending isopropanol–ammonia–water alongside standard amounts of authentic hydroxybenzoic acids on the same paper. The spots were examined as regards u.v. absorption, colours were developed with DSA and ammoniacal silver nitrate, and concentrations of the standard substances were found which accurately represented the spots obtained from the hydroxylation mixtures. The final chromatograms were always done in duplicate, and the results obtained for the same compound using the different detection techniques agreed.

2. Benzoic acid was hydroxylated at pH 3.1, and 0.04 of the product was applied as a stripe (15 cm) to a Whatman 3 mm paper, and developed in isopropanol–ammonia–water until the solvent had travelled 40 cm. The bands consisting of 2-, 3-, and 4-hydroxybenzoic acids, and a band which contained benzoic and 2,5-dihydroxybenzoic acids, were located under u.v. light and by samples of the acids placed as "markers" at the edge of the chromatographic sheet. These bands were cut out, and separately eluted with methanol. The eluates were adjusted to suitable concentrations, acidified with one drop of concentrated hydrochloric acid, and then their u.v. absorption spectra were measured. Blank experiments were done with the chromatography paper only, and the recoveries of all the acids were determined by subjecting known mixtures in the phosphate buffer, at approximately the concentrations formed in the hydroxylation mixture, to the full isolation procedure. Corrections, due to impurities in the paper were comparatively large but the results were in agreement with those obtained by method (1).

Polymerisation experiments

Benzoic acid was hydroxylated (21 h) as above at pH 3.1 and 6.6 in the presence of methyl methacrylate (10 ml) and at the same time otherwise similar runs were made at the same pH's, in the absence of ascorbic acid. The reaction mixtures were acidified, extracted with chloroform, and the extracts washed with aqueous potassium bicarbonate, water and dried. The chloroform solution was then either evaporated to dryness (100° at 10 mm) to remove solvent and monomer or concentrated and diluted with methanol when polymerised methyl methacrylate was precipitated. The potassium bicarbonate washings were acidified, saturated with ammonium sulphate and extracted with ether, and the concentrated extracts chromatographed. In the absence of ascorbic acid no hydroxylation of the benzoic acid and no detectable polymerisation took place. In the presence of ascorbic acid 2-, 3- and 4-mono-, and 2,5-dihydroxybenzoic acids were formed along with poly-methyl methacrylate (approx. 0.5 g). The hydroxylation mixture, without substrate, also polymerised the ester under the above conditions (up to 4.0 g of polymer) but no detectable polymerisation occurred in the absence of ascorbic acid.

RESULTS AND DISCUSSION

The results obtained in the qualitative hydroxylations are listed in Table II. Hydroxylation proceeded much more readily at pH 6.6, than at pH 3.1, as under these conditions the formation of 3,4-dihydroxybenzoic acid from benzoic acid and of 2,4-dihydroxybenzoic acid from 4-hydroxybenzoic acid, could be detected after a reaction time of 1 h. A small number of unidentified materials, not listed, were detected after 21 h reaction in most instances, and especially from experiments carried out at pH 6.6.

It was not easy to detect 2,3-dihydroxybenzoic acid in the presence of 3-hydroxybenzoic acid by chromatography in the ammonia solvent, except by its fluorescence, as the materials travel together. Light spraying with the DSA reagent did however reveal the dihydroxy acid as a transient pink before being masked by the yellow colour due to 3-hydroxybenzoic acid; presumably 2,3-dihydroxybenzoic acid couples preferentially. The R_F's of the 2,3- and 2,5-benzoic acids formed from benzoic acid are somewhat lower than the authentic materials in the ammonia solvent because of

TABLE II

THE HYDROXYLATION PRODUCTS OBTAINED AFTER 1 H AT pH 3.1 AND IDENTIFICATION DATA

A, absorbs; b, bright; B, blue; Bk, black; Bn, brown; G, grey-brown; N, nothing observable; O, orange-brown; p, pale; P, pink; R, red; W, white.

Substrate	*Positions of hydroxyl groups in reaction products*	*R_F's (× 100) of products and (authentic materials) in solvent*		*Colours observed with*			
		A	*B*	*UV*	*DSA*	*DNA*	*Ag^+/NH_3*
Benzoic acid	2-	78 (80)	86 (85)	B	pY	R	N
	3-	54 (55)	52 (51)	N	Y	P	N
	4-	44 (41)	56* (53)	A	Y	R	N
	2,3-**	52 (57)	41 (41)	bB	Pi	Bn	Bk
	2,5-	61 (68)	27 (26)	bB	W	pBn	Bk
	3,4-***	12 (13)	15 (16)	N	pP	Bn	Bk
2-hydroxybenzoic acid	2,3-	51 (57)	42 (41)	bB	pP	Bn	Bk
	2,4-	35 (35)	Not resolved	B	Bn	O	G
	2,5-	64 (65)	25 (26)	bB	W	Bn	Bk
	2,3-	57 (60)	44 (41)	bB	pP	Bn	Bk
3-hydroxybenzoic acid	2,5-	68 (68)	27 (26)	bB	W	pBn	Bk
	3,4-	14 (13)	15 (16)	N	pP	Bn	Bk
4-hydroxybenzoic acid	2,4-***	35 (32)	33 (34)	B	Bn	O	G
	3,4-	14 (12)	18 (16)	N	pP	Bn	Bk

* Detected by its u.v. absorption in this solvent as 3-hydroxybenzoic acid moves to almost the same position.

** First detected after 4-h reaction.

*** First detected after 21-h reaction.

retardation by the large amount of benzoic acid present. A small quantity of a compound (R_F approx. 0.9 in solvent A) which possessed a similar u.v. fluorescence to that of salicylic acid, was invariably formed during the hydroxylation of salicylic acid. It gave no colour with DSA, and was not identical with a specimen of 2,6-dihydroxybenzoic acid (m.p. 170°) prepared from an impure (m.p. $< 110°$) commercial specimen (Lights) by paper chromatography. No 3,5-dihydroxybenzoic acid could be detected in the products from 3-dihydroxybenzoic acid.

The fact that UDENFRIEND's hydroxylation mixture initiates the polymerisation of methyl methacrylate is consistent with a free radical mechanism. This agrees with the observation[11], which we have confirmed, that benzoic acid does not hydroxylate in aqueous ethanol as the ethanol is preferentially oxidised to acetaldehyde. The fact that DALGLIESH[5] hydroxylated a number of compounds in solutions containing a little ethanol is of no consequence as this solvent would be rapidly removed by the air stream bubbled through his apparatus.

Table III shows the results obtained from the isomer ratio experiments. These agree well with a homolytic mechanism and are quite incompatible with an electrophilic attack on the ring.

No 3,4-dihydroxybenzoic acid could be detected in the products from benzoic acid after hydroxylation for 1 h. This suggests that the 2,5-dihydroxybenzoic acid is formed by the further hydroxylation of 2-hydroxy- but not of 3-hydroxy-benzoic acid, and on this premise the ratios in Table IV have been calculated. If the 2,5-dihydroxybenzoic acid were formed by the hydroxylation of salicylic acid a corresponding amount of 2,3-dihydroxybenzoic acid should be formed. However in spite of

TABLE III

ISOMER RATIOS OBTAINED IN THE HYDROXYLATIONS AND DETERMINED CHROMATOGRAPHICALLY

Substrate	*Approximate % conversion*	*Positions of hydroxyl groups in products*							
		2-	*3-*	*4-*	*2,3-*	*2,4-*	*2,5-*	*2,6-*	*3,4-*
Benzoic acid	1	5:	1:	2:	—	—	1 ~ 2	—	—
2-hydroxybenzoic acid	20	—	—	—	7:	Trace	5	—	—
3-hydroxybenzoic acid	6	—	—	—	5:	—	4:	—	8

TABLE IV

SOME ISOMER RATIOS OBSERVED IN HOMOLYTIC SUBSTITUTIONS

Substrate	*Attacking radical*	*Source of radical*	*Ratio of isomers formed in substitution at positions*						*References*
			2		*3*		*4*		
Benzoic acid	?	Ascorbic acid and Fe^{++}	6 ~ 7	:	1	:	2		This work
Benzoic acid	C_6H_5	Benzoyl peroxide	59	:	15	:	26		12
Benzoic acid	HO	H_2O and X-rays	5	:	2	:	10		13
Benzoic acid	HO	U.V. and Fe^{++}	Approx. 2	:	Approx. 2	:	Approx. 1		14
Benzoic acid	HO	U.V. and H_2O_2	10	:	5	:	5*		15
Nitrobenzene	HO	H_2O_2 and Fe^{++}	2.5–3	:	2–2.5	:	5–5.5		16
Nitrobenzene	HO	H_2O and X-rays	3.5	:	3.0	:	3.5		17
Nitrobenzene	C_6H_5	Benzoyl peroxide	6.2	:	1.0	:	2.8		6
Cyanobenzene	C_6H_5	Benzoyl peroxide	6	:	1	:	3		6

* 2,5- (: 2) and 3,4-dihydroxybenzoic acid (: 8) also formed.

intensive efforts no 2,3-dihydroxybenzoic acid could be detected on chromatograms of the products from several 1 h hydroxylations at pH 3.1; the 2,5-acid showed up very clearly on these chromatograms. One explanation is that the initial radical formed in the oxidation of benzoic acid is converted to 2,5-dihydroxybenzoic acid without the intermediary of 2-hydroxy-benzoic acid. It is also possible that other compounds on the chromatograms interfered with the detection. The DSA spray and the u.v. examination are about 10 times less sensitive for 2,3-dihydroxybenzoic acid than for its 2,5-isomer, but nevertheless the expected amount if, present, should have been detected readily.

The isomer ratio observed for the hydroxylation of benzoic acid in UDENFRIEND's mixture is in remarkable accord with that reported for the phenylation of benzoic acid. This latter value (Table IV) has been calculated from infrared absorption spectrum measurements and is comparatively very accurate. These results are not in such good agreement with the other, very approximate, hydroxylation data, except in so far as 2- and 4-substitution exceeds that at the 3-position.

The identity of the radical which actually attacks the substrate is a subject for speculation and further study. It must be produced in some way from the ascorbic acid, it need not be the hydroxyl radical, and the observation[2] that hydroxylation occurs after the destruction of all the ascorbic acid in the mixture is of particular relevance.

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CYTOCHROME *C* OXIDASE: THE EFFECTS OF LIPIDES AND SURFACE ACTIVE AGENTS ON ENZYMIC ACTIVITY

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SUMMARY

Cytochrome *c* oxidase prepared by fractionation of DPNH oxidase loses up to 90% of its activity when suspended in 5% sucrose. This loss of activity can be restored by surface active agents, deoxycholate and unsaturated lysolecithin. When frozen cytochrome *c* oxidase is thawed it separates into three layers which have different original activities, but which can be reactivated to the same level by these compounds. Similarly particles can be separated by centrifugation which have different initial activities, but which can be stimulated by lysolecithin to the same final activity.

Extraction of cytochrome *c* oxidase with acetone or mixtures of acetone and ethanol results in removal of some phosphorus and marked diminution of activity. This inactivation can be reversed to some degree by a number of phospholipides in the presence of deoxycholate. Much better reactivation is obtained with yeast lysolecithin. In no case is the degree of reactivation greater than the percentage of original phospholipide remaining in the preparations of enzyme.