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ACTIVATION AND TRANSFER OF OXYGEN—IX

NONENZYMIC HYDROXYLATION OF PHENYLALANINE BY MODEL SYSTEMS OF DIHYDROALLOXAZINE/O_2, DIHYDROALLOXAZINE/H_2O_2 AND ALLOXAZINIUM CATION/H_2O_2

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Abstract—Efficient hydroxylations were effected without addition of metal compounds. In the dihydroalloxazine system HO· radicals were the hydroxylating species according to the stoichiometry and the distribution of the hydroxyphenylalanine isomers. The OH radicals were generated in one-electron reductions of A^R-OOH or H_2O_2, in which a dihydroalloxazine or a semiquinone acted as the reducing agent. The yield of hydroxylation varied in dependence on the further oxidation of the hydroxycyclohexadienyl radicals. Quantitative disproportionation occurred in 6N H_2SO_4, while an attack by O_2, H_2O_2 or A^R-OOH predominated in the pH region 0-7. The influence of a HO· consuming aliphatic compound e.g. EDTA was studied.

Hydroxylating species are also formed in the attack of an alloxazinium cation by H_2O_2, depending on the acidity of the medium.

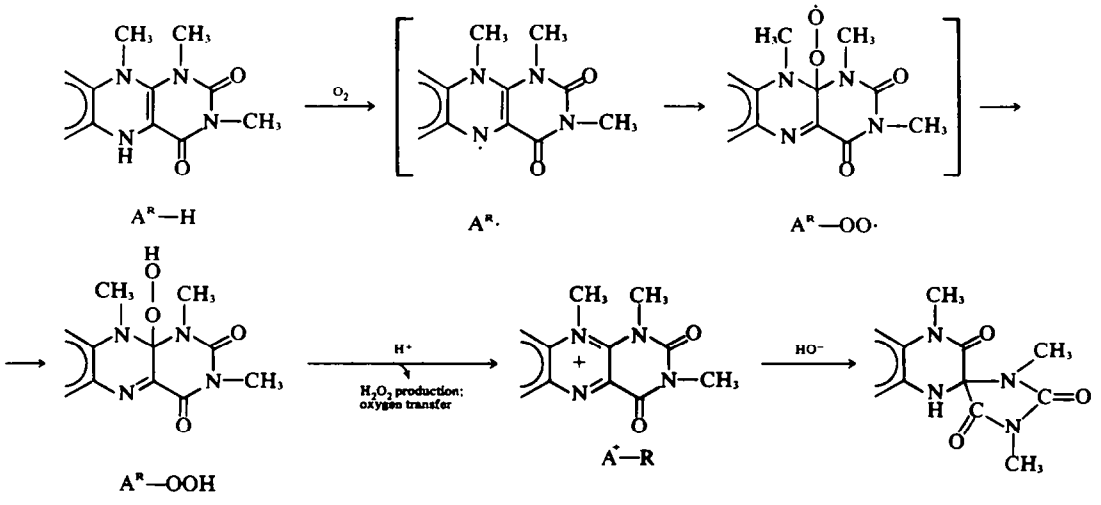
INTRODUCTION

In parts V[1] and VI[2] we discussed the autoxidative quaternization of 1, 3, 10-trimethyl-5, 10-dihydroalloxazine (A^R—H→A^+—R).

The peroxides produced (A^R—OOH and H_2O_2) could oxidize another reduced alloxazine molecule ("secondary oxidation") or an (in) organic substrate ("competitive secondary oxidation"). This peroxide consumption influences the final O_2-absorption per mole of A^R—H (n_{O_2}) and the amount of H_2O_2 to be found after autoxidation ($n_{H_2O_2}$). The relationship with the oxygen transferred in a competitive process ($n_{[O]}$) could be expressed by Eq (1).

$$n_{[O]} = 2n_{O_2} - n_{H_2O_2} - 1. \quad (1)$$

We now wish to report on the nonenzymic hydroxylation of phenylalanine without addition of metal compounds. In screening for the optimal conditions the relationship (1) was applied also in cases of negative $n_{H_2O_2}$-values (consumption of H_2O_2 added to the system).



The nature of the hydroxylating species

There is no experimental evidence to support Hamilton's[3] suggestion that a ring-opened, carbonyl oxide isomer of A^R—OOH is the actual hydroxylating agent. Instead, OH radicals are to be considered as the hydroxylating species as has now appeared from: (1) the stoichiometry under both aerobic and anaerobic conditions as summarized in Scheme 2; (2) the distribution of the hydroxyphenylalanine isomers, which is comparable with the results given by other HO· generating systems.

The stoichiometry also showed that the HO· radicals were not produced by *homolysis* of the transient A^R—OOH, but mainly by the one-electron *reduction* of A^R—OOH or H_2O_2. The dihydroalloxazine itself and its semiquinone could act as peroxide reducing agents in accordance to Eqs (2) and (3), giving the sum Eq (I) in Scheme 2. This was further confirmed by the additional finding that anaerobic hydroxylations could be effected by A^R—H and H_2O_2 in an atmosphere of argon. High yields were obtained which were practically in agreement with the amount of HO· radicals formed in the reductions (4) + (5), leading to the sum Eq (II) in Scheme 2.

$$A^R\text{—}H + A^R\text{—}OOH + H^+ \rightarrow A^{\cdot} + A^+\text{—}R + H_2O + HO\cdot \quad (2)$$

$$A^{\cdot} + A^R\text{—}OOH + 2H^+ \rightarrow 2A^+\text{—}R + H_2O + HO\cdot \quad (3)$$

$$A^R\text{—}H + H_2O_2 \rightarrow A^{\cdot} + H_2O + HO\cdot \quad (4)$$

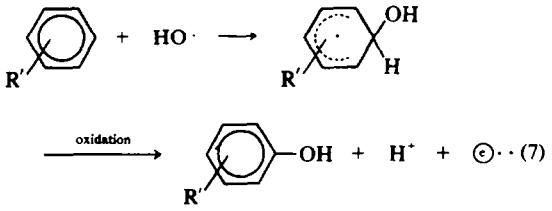
$$A^{\cdot} + H_2O_2 + H^+ \rightarrow A^+\text{—}R + H_2O + HO\cdot \quad (5)$$

H_2O_2 may arise by hydrolysis of A^R—OOH (A^R—OOH + $H^+ \rightarrow A^+$—R + H_2O_2). Therefore, the overall Eqs (2) and (3) do not give the decisive answer to the question whether A^R—OOH or H_2O_2 has been reduced in a particular autoxidative hydroxylation process. Indications could be obtained from comparative aerobic and anaerobic experiments. Differences in hydroxylation rates suggested that reduction of A^R—OOH occurred at high acid strength, while reduction of H_2O_2 became more probable on decreasing the acid strength of the medium. However, this matter will not be considered in detail in this paper. A comparative study concerning such a question whether HCl was oxidized by A^R—OOH or H_2O_2 has already been published[2].

Theoretical yields of hydroxylation

Peroxidation of A^R—H (Eqs 2 and 4) may terminate according to Eq (6). Addition of a substrate to the same system may lead to a competitive HO· consumption, for example, as expressed by Eq (7). It is remarkable that (7) could completely displace (6) in strongly acid solution.

$$A^{\cdot} + HO\cdot + H^+ \rightarrow A^+\text{—}R + H_2O \quad (6)$$



Maximal HO·production is represented by the sum Eqs (I) and (II) in Scheme 2. The theoretical yield of hydroxylation is considered per mole of dihydroalloxazine ($n_{[O]_{max}}$). It varies in dependence on the further oxidation of the primary substrate-radical adduct a_1. Some possibilities, based on a quantitative formation of a_1, have been summarized in scheme 2. The relationships (I^a) and (II^a) may be expected in the case of disproportionation of a_1. Higher $n_{[O]max}$-values should arise if a_1 is oxidized by A^R—OOH, H_2O_2 or O_2.

The type of the reaction is also recognizable by the $n_{[O]}/(n_{O_2} - n_{H_2O_2})$ ratio, which is extensively considered in the experimental part of this paper.

RESULTS

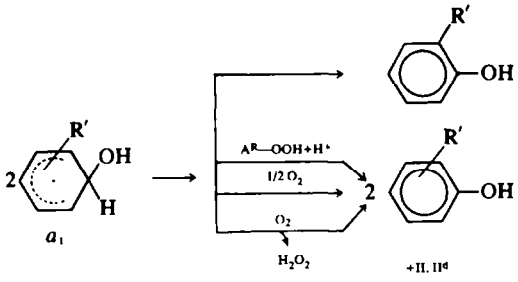
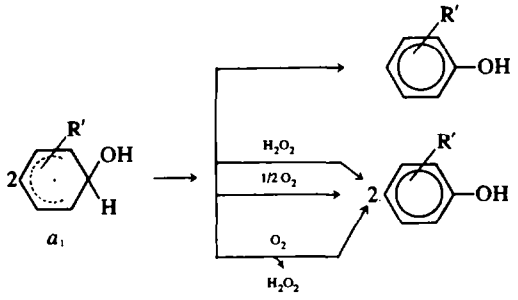
(A) The formation of dihydroxyphenylalanines and products with a converted side chain was negligible in most experiments.

The production of A^R—OOH, H_2O_2 and HO· radicals was strongly influenced by the O_2 concentration and by the nature and acid strength of the medium.[1] Optimal yields of hydroxylation were obtained in 6–0·2N H_2SO_4 and in 1–0·05N $HClO_4$ showing that the acid strength of the medium also influenced the further oxidation of the radical adduct a_1:

(1) *Quantitative disproportionation of* a_1 *in* 6N H_2SO_4. As a rule, hydroxylations in 6N H_2SO_4 were surprisingly "clean". The presence of O_2 or H_2O_2 had practically no effect on the conversion of a_1. Autoxidative hydroxylations were quite in agreement with the relationship (I^a) proving that disproportionation of a_1 was then quantitatively all-important. Autoxidative hydroxylation could simply be displaced by the anaerobic process on adding an excess of H_2O_2 to the medium. The anaerobic HO· formation was in accordance with Eq (III) differing from the anaerobic process (Eq II) in more dilute acids (*cf* conclusion 2).

(2) *Oxidation of* a_1 *by* A^R—OOH, H_2O_2 *or* O_2 *in* 1–0·2N *acid.* Disproportionation of a_1 became less important on decreasing the acid strength of the medium. It was negligible in 1–0·2N H_2SO_4 and $HClO_4$ as could be concluded from a strong increase of the yields of hydroxylation in agreement with the stoichiometry (I^c), (I^b) or (II^b). In these media a_1 was apparently attacked by O_2, A^R—OOH or H_2O_2.

The relationship (II^b) *implies that 2 moles of tyrosines could be produced per mole of dihydroal-*

		n_{O_2}	$n_{H_2O_2}$	n_{HO}	$n_{[O]max}$	$\frac{n_{[O]max}}{n_{O_2} - n_{H_2O_2}}$
I. *On autoxidation*						
$3A^R—H + 2O_2 + 3H^+ \rightarrow 3A^+—R + 2H_2O + 2HO\cdot$	I)	$\frac{2}{3}$	0	$\frac{2}{3}$		0
	a)	$\frac{2}{3}$	0	$\frac{2}{3}$	$\frac{1}{3}$	$\frac{1}{2}$
	b)	$\frac{3}{4}$	0	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{2}{3}$
	c)	$\frac{5}{6}$	0	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{4}{5}$
	d)	1	$\frac{1}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	1
	e)	1	$\rightarrow 0$	$\rightarrow 1$	$\rightarrow 1$	1
II. *On adding an excess of* H_2O_2.						
$A^R—H + 2H_2O_2 + H^+ \rightarrow A^+—R + 2H_2O + 2HO\cdot$	II)	0	-2	2		0
	a)	0	-2	2	1	$\frac{1}{2}$
	b)	0	-3	2	2	$\frac{2}{3}$
	c)	$\frac{1}{2}$	-2	2	2	$\frac{4}{5}$
	d)	1	-1	2	2	1
III. *On adding on excess of* H_2O_2 *in* 6N H_2SO_4.						
$2A^R—H + 3H_2O_2 + 2H^+ \rightarrow 2A^+—R + 4H_2O + 2HO\cdot$	III)	0	$-1\frac{1}{2}$	1		0
	a)	0	$-1\frac{1}{2}$	1	$\frac{1}{2}$	$\frac{1}{3}$

SCHEME 2.

Maximal hydroxyl radical formation and hydroxylation per mole of $A^R—H$. ($n_{H_2O_2}$: O = complete consumption of the peroxide produced; + = nett production of peroxide; − = consumption of H_2O_2 added in excess to the medium.)

loxazine ($n_{[O]_{max}} = 2$)! This result is considered to be important from both a practical and mechanistic point of view (*cf* conclusion 4). The relationship (II^b) is probably shifted into (II^c) or (II^d) by the presence of O_2. There is some uncertainty on account of the fact that the presence of O_2 also gives rise to more non-phenolic compounds.

(B) Oxidation of a_1 by O_2, H_2O_2 or $A^R—OOH$ also occurred in media of pH 2–7, but the yields of hydroxylation were not optimal. A greater part of the oxygen transfer led to non-phenolic by-products. Besides, other reactions of the transients like a decomposition of $A^R—OOH$ or a further oxidation of $A^+—R$ or the pseudobase $A^R—OH$, could have decreased the yields of hydroxylation. (An oxidative degradation of the transients is considered in the experimental part, *cf* Eqs 16 and 17).

(C) The hydroxylation of phenylalanine was further studied in the additional presence of a HO· consuming aliphatic compound and an excess of H_2O_2. We found that HO· consumption by the aliphatic substrate, leading to an aliphatic radical, did not necessarily decrease the production of tyrosines. Apparently, the aliphatic radical could be oxidized by H_2O_2 in an one-electron process to give another HO· radical, which perpetuates a chain reaction.[4*] Sometimes, an interesting stimulation of aromatic hydroxylation was observed as for exam-

*The one-electron oxidation of an organic radical by H_2O_2 according to: $R\cdot + H_2O_2 \rightarrow R^+ + HO^- + HO\cdot$ has been discussed.[4]

ple by the presence of EDTA, *also without addition of Fe^{++} or other metal ions*!

(D) The results mentioned under section A–C were given by the systems dihydroalloxazine/O_2 and dihydroalloxazine/H_2O_2. Hydroxylations could also be effected by systems consisting of oxidized species like A^+—R and H_2O_2 (*cf* Fig 1). The distribution of the hydroxyphenylalanine isomers was again consistent with the occurrence of HO· radicals. The question remains whether the addition of H_2O_2 to A^+—R indeed leads to an organic peroxide, which then provides HO· or HOO· radicals by homolysis. The HOO· radicals could be converted by H_2O_2 into O_2 and HO· radicals. On the other hand, there is some similarity between the conversion of A^+—R/H_2O_2 and a nitrile with H_2O_2. The nitrile/H_2O_2 system is already known to transfer oxygen atoms.[5]

CONCLUSIONS

The above results on the stoichiometry have some important consequences:

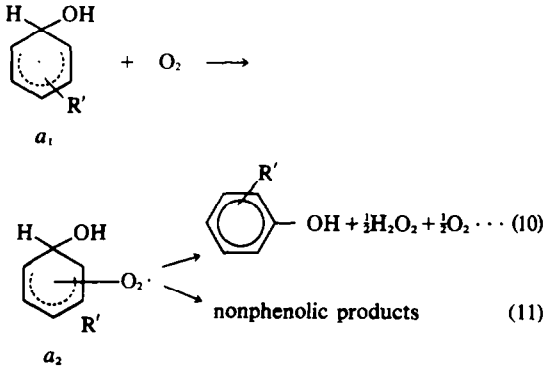
(1) The species A^R—OOH, H_2O_2, HO· and a_1 may react with inorganic anions, sometimes resulting into a complete inhibition of the tyrosine formation as in 6N $HClO_4$ and 1–6N HCl.[1,2] The efficient hydroxylations in sulfuric acid media are in striking contrast. Then, interconversions as between hydroxyl- and sulfate radicals ($HO\cdot + HSO_4^- \rightleftarrows SO_4^{\cdot-} + H_2O$) might also have played a part, but have not come to the fore in studying the overall processes.

(2) The finding of Eqs (I) and (III) in 6N H_2SO_4 implies that in the autoxidative process the HO· radicals could not have been produced by reduction of H_2O_2! In the anaerobic process (reduction of H_2O_2) relatively less HO· radicals have become available. Apparently, either the HO· formation according to Eq (4) or (5) was suppressed in 6N H_2SO_4 and replaced by reaction (8) or (9), respectively, Eq (III) is the sum of either (8) + (5) or (4) + (9). Current investigations on the semiquinone (A^R·) have confirmed the occurrence of (8) + (5) which will be considered in detail in a forthcoming paper. It has to be emphasized that the anaerobic HO· production according to Eq (II) is not suppressed any more on decreasing the acid strength of the medium (*cf* the results in 1–0·2N H_2SO_4 and $HClO_4$).

$$2A^R\text{—H} + H_2O_2 \rightarrow 2A^R\cdot + 2H_2O \qquad (8)$$

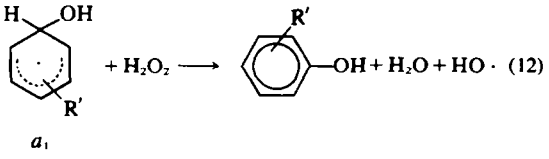
$$2A^R\cdot + H_2O_2 + 2H^+ \rightarrow 2A^+\text{—R} + 2H_2O \qquad (9)$$

(3) Since the pulse radiolytic studies of Dorfman *et al.*,[6] it has been generally accepted that an adduct like a_1 is oxidized by O_2 to a peroxy radical a_2, which decomposes into a phenol and O_2 (Eq 10). In our studies such a decomposition should result into the relationships (I^d), (I^e) or (II^d). Consequently, it has to be rejected for those experiments which showed the relationships (I^b) and (I^c). Besides



we observed an increase in non-phenolic products at higher O_2-pressures (Eq 11).

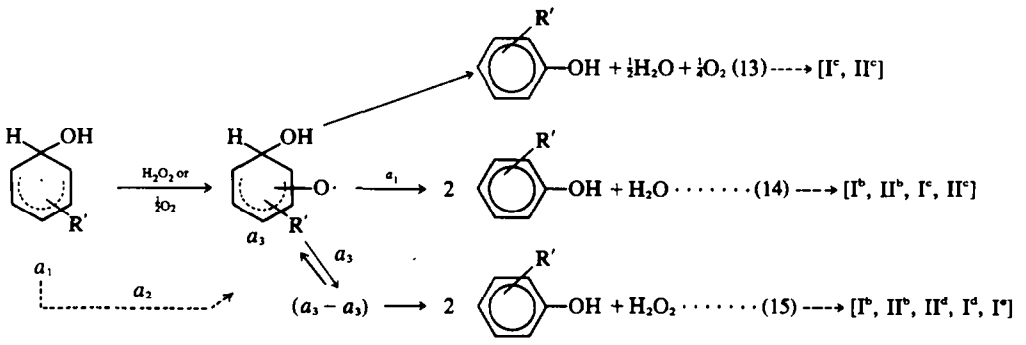
(4) The oxidation of a_1 by A^R—OOH or H_2O_2 is also questionable from a mechanistic point of view. The possibility of one-electron processes like (12) has to be considered. Such a reaction producing further HO· radicals should perpetuate aromatic hydroxylation as long as A^R—OOH or H_2O_2 are present! The yield of hydroxylation as expected from relationship (II^b) was exceeded by 5–30% in some experiments. The occurrence of (12) to a slight extent might then be one of the possible explanations. However, in general the relationships (II^b) and (I^b) were found proving that reactions like (12) were not of practical importance.



(5) As mentioned above some results are inconsistent with an occurrence of reactions (10) and (12). On the other hand, all our various experiments could be explained if we assume the forming of a transient mono-oxy radical a_3 both in the oxidation of a_1 by O_2 and H_2O_2 (A^R—OOH). (The occurrence of Dorfman's peroxy radical a_2 remains a possibility, e.g. in a self-reaction: $a_2 + a_2 \rightarrow 2a_3 + O_2$, or in a reaction with another a_1: $a_2 + a_1 \rightarrow 2a_3$). Subsequent conversions of a_3 according to the overall Eqs (13), (14) and (15) could lead to the several stoichiometric relationships. Reactions (14) and (15) are most likely on account of the various experiments which have well established Eqs (I^b), (II^b) and (I^c) and more or less (II^c) and (II^d).

EXPERIMENTAL

The experiments were carried out as mentioned before[1] in an all-glass manometric apparatus at atmospheric pressures and at an average temp of 23°. Any change of temp and atmospheric pressure was corrected by use of a thermobarometer. The dihydroalloxazine and other sub-



stances could be added to the gas-saturated solvent at any time desired, without opening the apparatus; 300 ml vessels were used in which the depth of the reaction mixture layer (50·0 ml) was about 1 cm. Stirring was accomplished by means of 4·2 cm long magnetic bars at 1200–1500 rpm. Samples from the reaction solution were analyzed[1] after 4 h on: (a) the production of monohydroxy- and dihydroxyphenylalanines; (b) the presence of $A^{\cdot}$—R in % yield (only in acid media); (c) the amount of H_2O_2, using MnO_2 in acid or neutral media or the enzyme catalase at pH = 7. (*Note*: on neutralizing a soln of $H_2O_2 + A^{\cdot}$—R, blank experiments are required to determine the H_2O_2 consumption in reaction (17), to be discussed later on in this paper). In hydroxylations experiments, the mixtures contained 4 mmoles of phenylalanine. The results are summarized in Tables 1 and 2. Experiments performed under different conditions are indicated by an asterisk (*).

Abbreviations. H_2O_2 addn = mmoles of H_2O_2 present in the reaction mixture (50·0 ml) before the addition of A^R—H (1 mmole); Δ_g = nett change of the gas volume in mmoles/mmole A^R—H after 4 h: uptake(+) or evolution (−). In the experiments under argon (Table 1) a maximal generation of gas was observed varying from 0·05 to 0·1 mmole at reaction times of 15–40 min. The gas generated consisted of O_2 and/or CO_2. The O_2 was then taken up to give the final Δ_g-values mentioned in the table: $n_{O_2} = O_2$-uptake in mmoles/mmole A^R—H. As a rule, $n_{O_2} = \Delta_g$ in aerobic experiments in acid media: $n_{H_2O_2}$: nett H_2O_2 production (+), complete consumption of the peroxides produced (0) or nett consumption of H_2O_2 added to the medium (−); $n_{(O)_{calc}}$ = oxygen transfer calculated from Eq (1). For experiments under argon: $n_{(O)_{calc}} = 2\Delta_g - n_{H_2O_2} - 1$. (In this formula $n_{H_2O_2}$ is in fact corrected by twice the nett generation of O_2 and/or CO_2 ($2\Delta_g$). The correction by $2\Delta_{O_2}$ is self-evident, while the correction by $2\Delta_{CO_2}$ is consistent with the occurrence of reaction (17)); $n_{(O)_{t+d}} = n_{(O)_t}$ + twice the amount of dopa's; $n_{(O)_t}$ = o + m + p − hydroxyphenylalanines in mmoles/mmole A^R—H; o:m:p distr. = % distribution of the monohydroxyphenylalanines; the ratio's $R_o = (n_{(O)_{calc}}/(n_{O_2} - n_{H_2O_2}))$, also those obtained under non-optimal conditions, are compared with the theoretical, optimal values (*cf* Scheme 2), providing information about the way of conversion of the adduct a_1. R_o could have values in between two theoretical ratio's, if a_1 is converted in more than one way. In practice, R_o could also be lowered if the coupled oxidation (2) or the reaction (4) is terminated according to Eq (6). However, as a rule reaction (6) could be suppressed in acid media on varying the conditions e.g. by the presence of a substrate or an excess of H_2O_2 (*cf* the experiments in dilute $HClO_4$). R_o is not conclusive if a greater part of the oxygen transfer has not resulted into aromatic hydroxylation recognizable by a great discrepancy between $n_{(O)_{calc}}$ and $n_{(O)_{t+d}}$.

Hydroxylations effected by A^R—H *in* 6–0·05N *mineral acid media* (*Table* 1).

(*a*) *Series* 1–10: 6N H_2SO_4, HCl *and* $HClO_4$. Autoxidative hydroxylation in 6N H_2SO_4 became optimal on lowering the partial pressure of oxygen (expts 1 and 3; *cf* Ref 1). In spite of the non-optimal conditions expt 1 also gave a value of $R_o = \frac{1}{2}$, proving the occurrence of (I^a) and the absence of the terminating reaction (6). On the addition of H_2O_2, $n_{(O)_{t+d}}$ was increased, while n_{O_2} and R_o were lowered, indicating the displacement of (I^a) by (III^a) in series 1 and 2. Reaction type (III^a) was further confirmed by experiments under argon (series 3), although the slightly increased yields of hydroxylation might be due to 10–20% occurrence of (II^a). Expts 9 and 10 were carried out in 100 ml and 25 ml of 6N H_2SO_4, containing 8 and 4 mmoles of phenylalanine, respectively and also stirred for 4 h. In comparing with expt 8 practically no effects were observed. The additional presence of EDTA decreased the aromatic hydroxylation (*cf* expts 14 and 3); $\Delta_g = 0{\cdot}62$ was the nett result of $n_{O_2} = 0{\cdot}67$ and $\Delta_{CO_2} = -0{\cdot}05$. Expt 15 performed under argon ($\Delta_{CO_2} = -0{\cdot}09$) only gave a slight loss in aromatic hydroxylation (*cf* expt 12), in spite of the considerable oxidation of EDTA resulting in a high $n_{H_2O_2}$-value (*cf* the stimulation of aromatic hydroxylation by EDTA in series 50, Table 2). Oxidations in the absence of phenylalanine (series 6–8) gave more losses in $A^{\cdot}$—R. Apparently, the alloxazinium compound itself acted to some extent as a substrate in a competitive coupled oxidation.[1]

The oxidation of HCl in 6N acid medium (series 9) has already been discussed.[1,2] The anaerobic hydroxylation of phenylalanine was inhibited in 6N $HClO_4$ (series 10) as in the autoxidative process.[1]

(*b*) *Series* 11: 2N H_2SO_4. On decreasing the acid strength of the medium (expts 12 → 17) higher yields of hydroxylation were obtained. The anaerobic HO· production (II) was no longer suppressed, which was further confirmed by the experiments in 1–0·05N acid. The values of $R_o = 0{\cdot}55$ and $n_{(O)_{t+d}} = 1{\cdot}17$ indicated the occurrence of (II^b) in addition to either (II^a) or (6).

(*c*) *Series* 12–16: 1N H_2SO_4. Autoxidative hydroxylation in 1N H_2SO_4 was inefficient if carried out in the presence of 100%-O_2 (expt 18). It could be improved by the addition of H_2O_2 (expts 19 and 20). The increasing discrepancy between $n_{(O)_{calc}}$ and $n_{(O)_{t+d}}$ do not justify a definite conclusion, although the constant R_o-value suggests the occurrence of both (I^c) and (II^c). The yield of autoxidative hydroxylation was considerably increased on lowering the partial pressure of oxygen (expt 18 → 21). $R_o = 0{\cdot}75$ (expt

Table 1. Hydroxylation of phenylalanine (4 mmoles) by A^R—H (1·0 mmole) in various acid media (50·0 ml) at 23°, stirred at 1200–1500 rpm for 4 h (different conditions are indicated by an asterisk (*))

Expt No.	H_2O_2 addn.	Δ_g	n_{O_2}	$n_{H_2O_2}$	$n_{[O]_{calc}}$	$n_{[O]_{red}}$	$n_{[O]_t}$	o:m:p distr.	A·—R %	$\frac{n_{[O]_{calc}}}{n_{O_2}-n_{H_2O_2}}$
Series 1: In 6N H_2SO_4 + 100% − O_2 + phenylalanine:										
1	0		0·82	0·45	0·19	0·16	0·15	44:30:26	97	0·51
2	5·54		0·30	−1·01	0·61	0·42	0·41	44:30:26	100	0·46
Series 2: In 6N H_2SO_4 + air + phenylalanine:										
3	0		0·67	0	0·34	0·32	0·31	44:32:24	100	0·51
4	1·10		0·29	−0·84	0·42	0·43	0·42	45:30:25	93	0·37
5	2·75		0·13	−1·32	0·58	0·48	0·47	46:30:24	95	0·40
6	5·55		0·06	−1·50	0·62	0·49	0·48	47:29:24	96	0·40
7	22·00		0·04	−1·35	0·43	0·29	0·28	46:30:24	100	0·31
Series 3: In 6N H_2SO_4 + argon + phenylalanine:										
8	2·60	0		−1·58	0·58	0·59	0·58	45:31:24	96	0·37
9*	2·60	−0·01		−1·59	0·57	0·55	0·54	44:30:26	92	0·36
10*	2·60	−0·01		−1·59	0·57	0·56	0·55	45:32:23	97	0·36
11	5·24	0		−1·60	0·60	0·50	0·49	46:32:22	99	0·37
12	10·50	0		−1·60	0·60	0·49	0·48	45:30:25	97	0·37
13	21·05	0		−1·50	0·50	0·31	0·30	47:30:23	98	0·33
Series 4: In 6N H_2SO_4 + air + phenylalanine + EDTA (4 mmoles):										
14	0	+0·62	0·67	0	0·34	0·26	0·25	45:30:25	95	0·51
Series 5: In 6N H_2SO_4 + argon + phenylalanine + EDTA (4 mmoles):										
15	10·56	−0·09		−3·52	2·34	0·44	0·42	44:33:23	96	0·70
Series 6: In 6N H_2SO_4 + 100% − O_2, in the absence of phenylalanine:										
1[b]	0		0·78	0·56	0				96	0
2[b]	5·54		0·23	−0·78	0·24				88	0·24
Series 7: In 6N H_2SO_4 + air, in the absence of phenylalanine:										
3[b]	0		0·56	0·06	0·06				90	0·10
4[b]	1·10		0·17	−0·82	0·16				82	0·16
Series 8: In 6N H_2SO_4 + argon, in the absence of phenylalanine:										
8[b]	2·60	−0·02		−1·40	0·36				84	0·26
12[b]	10·50	−0·02		−1·32	0·28				81	0·22
Series 9: In 6N HCl → oxidation of $HCl^{1,2}$.										
Series 10: In 6N $HClO_4$ + argon + phenylalanine:										
16	5·54	0		−1·16	0·16	0·06	0·06	44:32:24	87	0·14
Series 11: In 2N H_2SO_4 + argon + phenylalanine:										
17	10·96	−0·01		−2·25	1·23	1·17	1·16	46:29:25	95	0·55
Series 12: In 1N H_2SO_4 + 100% − O_2 + phenylalanine:										
18	0		0·93	0·86	0	0·05	0·04		95	0
19	5·52		0·90	0·30	0·50	0·34	0·28	42:30:28	98	0·83
20	16·48		0·76	−0·76	1·28	0·51	0·46	45:29:26	98	0·84
Series 13: In 1N H_2SO_4 + air + phenylalanine:										
21	0		0·90	0·50	0·30	0·32	0·31	45:30:25	93	0·75
22	2·70		0·67	−0·53	0·87	0·59	0·57	46:31:23	95	0·73
23	5·40		0·51	−1·25	1·27	0·94	0·92	44:30:26	90	0·72
24	11·05		0·40	−1·50	1·30	0·90	0·87	47:30:23	88	0·68
25	16·24		0·36	−1·85	1·57	1·03	1·00	45:30:25	93	0·71
26*	2·78		0·32	−1·54	1·18	1·17	1·14	45:30:25	91	0·63
27*	2·80		0·21	−1·82	1·24	1·22	1·16	44:32:24	86	0·61
Series 14: In 1N H_2SO_4 + argon + phenylalanine:										
28	5·24	−0·02		−2·82	1·78	1·57	1·55	44:30:26	91	0·64
29	10·80	−0·02		−2·70	1·66	1·54	1·50	44:32:24	88	0·62
30	16·40	−0·02		−2·68	1·64	1·50	1·48	45:30:25	92	0·62
31	22·40	−0·02		−2·60	1·56	1·24	1·21	45:32:23	88	0·61

Table 1—*Continued*

Expt. No.	H_2O_2 addn.	Δ_s	n_{O_2}	$n_{H_2O_2}$	$n_{(O)_{calc}}$	$n_{(O)_{f+d}}$	$n_{(O)_f}$	o:m:p distr.	A^*—R %	$\frac{n_{(O)_{calc}}}{n_{O_2}-n_{H_2O_2}}$
Series 15: In 1N H_2SO_4 + air, in the absence of phenylalanine:										
21[b]	0		0·89	0·81	−0·03				88	0
23[b]	5·40		0·42	−0·40	0·24				77	0·29
Series 16: In 1N H_2SO_4 + argon, in the absence of phenylalanine:										
28[b]	5·24	−0·05		−1·70	0·60				70	0·37
30[b]	16·38	−0·03		−1·60	0·54				69	0·35
Series 17: In 1N HCl + air + phenylalanine:										
32	0		0·67	0·33	0·01	0·02	0·02		100	0·03
33	10·24		0·05	−0·96	0·06	0·07	0·07	40:36:24	99	0·06
Series 18: In 1N HCl + argon + phenylalanine:										
34	10·84		0	−1·04	0·04	0·09	0·08	43:32:25	98	0·04
Series 19: In 1N HCl + air, in the absence of phenylalanine:										
32[b]	0		0·73	0·46	0				100	0
33[b]	10·24		0·10	−0·80	0				100	0
Series 20: In 1N HCl + argon, in the absence of phenylalanine:										
34[b]	10·84		0	−1·00	0				100	0
Series 21: In 1N $HClO_4$ + air + Phenylalanine:										
35	0		0·75	0·21	0·29	0·35	0·34	40:35:25	93	0·54
Series 22: In 1N $HClO_4$ + argon + phenylalanine:										
36	10·84	−0·01		−2·62	1·60	1·63	1·62	44:32:24	90	0·61
Series 23: In 0·5N H_2SO_4 + 100% − O_2 + phenylalanine:										
37	8·16		0·84	0·24	0·44	0·22	0·20	42:32:24	95	0·73
38	16·08		0·83	−1·04	1·70	0·48	0·34	41:33:26	95	0·91
39	32·32		0·80	−0·80	1·40	0·38	0·34	43:30:27	98	0·87
Series 24: In 0·5N H_2SO_4 + air + phenylalanine:										
40	0		0·86	0·41	0·31	0·32	0·31	44:30:26	95	0·69
41	8·18		0·61	−1·32	1·54	0·82	0·81	43:30:27	94	0·80
42*	8·18		0·45	−1·84	1·74	1·15	1·11	44:29:27	93	0·76
43	16·28		0·52	−1·75	1·79	1·00	0·96	45:31:24	92	0·79
Series 25: In 0·5N H_2SO_4 + argon + phenylalanine:										
44	5·60	−0·02		−2·98	1·96	1·62	1·56	46:30:24	86	0·66
45	8·18	0		−3·15	2·15	1·84	1·78	46:30:24	86	0·68
46	11·20	0		−2·90	1·90	1·61	1·55	46:29:25	88	0·65
47	16·40	0		−3·10	2·10	1·66	1·62	44:30:26	87	0·68
Series 26: In 0·5N H_2SO_4 + argon + phenylalanine + EDTA (4 mmoles):										
48	11·20	−0·07		−3·02	1·88	1·62	1·58	47:29:24	84	0·65
Series 27: In 0·5N H_2SO_4 + air, in the absence of phenylalanine:										
40[b]	0		0·90	0·73	0·07				85	0·41
43[b]	16·30		0·23	−1·12	0·58				67	0·43
Series 28: In 0·5N $HClO_4$ + 100% − O_2 + phenylalanine:										
49	16·16		0·77	−0·08	0·62	0·48	0·40	40:30:30	94	0·73
Series 29: In 0·5N $HClO_4$ + air + phenylalanine:										
50	0		0·75	0·19	0·31	0·34	0·32	39:35:26	90	0·55
51	16·24		0·30	−1·52	1·12	1·23	1·13	43:32:25	95	0·62
Series 30: In 0·2N H_2SO_4 + air + phenylalanine:										
52	0		0·94	0·72	0·16	0·16	0·15	42:34:24	90	0·73
53*	0		0·89	0·10	0·68	0·52	0·48	41:35:24	58	0·86
54	11·28		0·74	−0·99	1·47	0·75	0·72	44:32:24	77	0·85
Series 31: In 0·2N H_2SO_4 + argon + phenylalanine:										
55	11·28	−0·06		−3·04	1·92	1·71	1·49	43:32:25	78	0·66

Table 1—*Continued*

Expt. No.	H_2O_2 addn.	Δ_s	n_{O_2}	$n_{H_2O_2}$	$n_{[O]_{calc}}$	$n_{[O]_{t+d}}$	$n_{[O]_t}$	o:m:p distr.	A^+—R %	$\frac{n_{[O]_{calc}}}{n_{O_2}-n_{H_2O_2}}$
Series 32: In 0·2N $HClO_4$ + 100% − O_2 + phenylalanine:										
56	16·60		0·89	0	0·78	0·42	0·30	38:32:30	92	0·88
Series 33: In 0·2N $HClO_4$ + air + phenylalanine:										
57	0		0·77	0·26	0·28	0·30	0·29	38:33:29	88	0·55
58*	0		0·76	0·06	0·46	0·41	0·40	40:35:25	80	0·66
59	16·40		0·62	−0·96	1·20	1·09	0·96	41:31:28	91	0·76
Series 34: In 0·2N $HClO_4$ + argon + phenylalanine:										
60	11·00	−0·06		−3·20	2·08	2·13	1·93	44:29:27	83	0·67
61*	10·72	−0·13		−3·88	2·62	2·61	2·13	45:32:23	77	0·72
Series 35: In 0·05N $HClO_4$ + argon + phenylalanine:										
62*	11·04	−0·03		−3·80	2·74	2·25	1·83	45:30:25	0	0·73

21) is consistent with a main occurrence of (I^c). The R_o-value was slightly decreased by H_2O_2 in the expts 22–25, but considerably in expt 26 (stirred at 100 rpm for 3 h, followed by 1200–1500 rpm for 1 h) and in expt 27 (non-stirred, and analyzed after standing overnight). High yields of hydroxylation were obtained under argon (series 14). R_o-values in between 0·50 and 0·67 imply the oxidation of a_1 by H_2O_2 in addition to disproportionation of a_1 or to some occurrence of the terminating reaction (6). The loss in A^+—R in the absence of phenylalanine appears from the series 15 and 16.

(*d*) *Series* 17–20: 1N HCl (*cf* Ref 1). The hydroxylation of phenylalanine was considerably inhibited. In the absence of a substrate, A^-—R was also quantitatively recovered which is in contrast with series 15 and 16.

(*e*) *Series* 21–22: 1N $HClO_4$. The excellent hydroxylations in dilute $HClO_4$ e.g. expts 35 and 36 are in contrast with the inhibition in 6N $HClO_4$.[1,2] The R_o-values in 1N $HClO_4$ and 1N H_2SO_4 are comparable for the anaerobic expts 36 and 29, but differ for the aerobic expts 35 and 21. Hydroxylations in sulfuric acid proceeded in clear solutions, but those in perchloric acid occurred in suspen-

Table 2. Hydroxylation of phenylalanine (4 mmoles) by A^R—H (1·0 mmole) in aqueous media (50·0 ml; pH 2–7) at 23°, stirred at 1200–1500 rpm for 4 h (different conditions are indicated by an asterisk (*))

Expt No.	pH	Δ_s	n_{O_2}	$n_{H_2O_2}$	$n_{[O]_{calc}}$	$n_{[O]_{t+d}}$	$n_{[O]_t}$	o:m:p distr.	R_o
Series 36: In 1NAcOH + air + phenylalanine:									
63	2·50		0·99	0·68	0·30	0·13	0·11	46:30:24	0·97
64*	2·50		0·84	0·08	0·60	0·31	0·29	47:30:23	0·79
Series 37: In 1N AcOH + H_2O_2 (20 mmoles) + argon + phenylalanine:									
65	2·78	−0·07		−3·59	2·45	1·50	1·40	45:29:26	0·71
Series 38: In 1N AcOH + air, in the absence of phenylalanine:									
63[b]	2·50		0·94	0·87	0·01	—	—	—	0·14
Series 39: In 1N AcOH + H_2O_2 (20 mmoles) + argon, in the absence of ph.al.:									
65[b]	2·20	−0·25		−2·56	1·06	—	—	—	0·51
Series 40: In water + 100%-O_2 + phenylalanine:									
66*	4–5		0·96	0·40	0·52	0·12	0·11	41:30:29	0·93
Series 41: In water + air + phenylalanine:									
67	4–5		0·78	0·57	−0·01	0·01	0·01	—	−0·05
68*	4–5		0·84	0·09	0·59	0·20	0·18	42:33:25	0·79
69*	4–5		0·48	0	−0·04	0·02	0·02	—	−0·08
70*	4·50		0·81	0·22	0·40	0·16	0·14	40:30:30	0·68
71*	5·70		0·79	0·16	0·42	0·16	0·14	40:33:27	0·67
72*	6·70		0·75	0·08	0·42	0·15	0·14	41:30:29	0·63
Series 42: In water + H_2O_2 (10 and 20 mmoles) + air + phenylalanine:									
73	4·50	−0·06		−1·28	0·16	0·11	0·09	41:33:26	—
74	4·45	−0·08		−1·36	0·20	0·19	0·15	40:33:27	—
Series 43: In water + H_2O_2 (20 mmoles) + air, in the absence of ph.al.:									
74[b]	6·5→6·2	−0·11		−1·17	−0·05				—

Table 2—*Continued*

Expt No.	pH	Δ_a	n_{O_2}	$n_{H_2O_2}$	$n_{[O]_{calc}}$	$n_{[O]_{h+d}}$	$n_{[O]_h}$	o:m:p distr.	R_o
Series 44: In water + H_2O_2 (20 mmoles) + argon + phenylalanine:									
75	2·90	−0·06		−3·00	1·88	1·29	1·19	46:30:24	0·65
76	5·70	−0·43		−2·26	0·40	0·44	0·41	42:30:28	0·28
77	6·70	−0·55		−2·40	0·30	0·30	0·29	43:30:27	0·23
Series 45: In water + H_2O_2 (20 mmoles) + argon, in the absence of ph.al.:									
77[b]	6·5→5·7	−0·50		−2·00	0				0
Series 46: In 0·2M acetate + H_2O_2 (20 mmoles) + argon + phenylalanine:									
78	4·48	−0·09		−3·20	2·02	0·74	0·71	44:29:27	0·67
79	5·00	−0·18		−2·96	1·60	0·65	0·55	45:28:27	0·61
80	5·76	−0·46		−2·48	0·56	0·30	0·28	40:30:30	0·36
81	6·00	−0·52		−2·75	0·71	0·26	0·23	40:33:27	0·41
82	6·70	−0·54		−2·72	0·64	0·16	0·15	40:30:30	0·39
Series 47: In 0·2M acetate + H_2O_2 (20 mmoles) + argon, in the absence of ph. al.:									
81[b]	6·00	−0·50		−2·44	0·44				0·30
Series 48: In 0·2M phosphate + H_2O_2 (20 mmoles) + argon + phenylalanine:									
83	4·52	−0·06		−2·96	1·84	0·83	0·79	40:31:29	0·65
84	5·02	−0·14		−2·80	1·52	0·67	0·64	41:31:28	0·60
85	5·77	−0·40		−2·32	0·52	0·38	0·35	41:31:28	0·34
86	6·00	−0·52		−2·72	0·68	0·29	0·26	41:32:27	0·40
87	6·70	−0·49		−2·30	0·32	0·23	0·22	40:30:30	0·24
Series 49: In 0·2M phosphate + H_2O_2 (20 mmoles) + argon, in the absence of phenylalanine:									
88	3·00	−0·09		−1·76	0·58				0·37
83[b]	4·25	−0·21		−2·01	0·59				0·37
85[b]	5·72	−0·49		−2·08	0·10				0·09
87[b]	6·73	−0·49		−2·00	0·02				0·02
Series 50: In water + H_2O_2 (20 mmoles) + argon + phenylalanine + EDTA (2, 4 and 8 mmoles, respectively):									
89	4·48	−0·22		−5·05	3·61	1·63	1·52	46:29:25	0·78
90	4·50	−0·32		−5·22	3·58	1·64	1·50	44:31:25	0·78
91	4·46	−0·38		−5·84	4·08	1·62	1·47	44:30:26	0·80
Series 51: In water + H_2O_2 (20 mmoles) + argon + ph. al. + lactic acid (15 mmoles):									
92	5·75	−0·70		−3·28	0·88	0·52	0·50	42:33:25	0·47
Series 52: In water + H_2O_2 (20 mmoles) + argon + phenylalanine + Methyl pyruvate (22 mmoles):									
93	5·7→3·3	< −1·40		< −10·0		0·15	0·14	42:33:25	
Series 53: In water + H_2O_2 (20 mmoles) + argon + phenylalanine + EtOH (34 mmoles):									
94	5·65	−0·55		−2·56	0·46	0·21	0·18	41:33:26	0·31

sions, probably allowing reaction (6) to take place and to decrease the R_o-values in the aerobic experiments. Apparently, in the anaerobic experiments reactions (6) was displaced by (5) on account of the excess of H_2O_2.

(*f*) *Series* 23–27: 0·5N H_2SO_4. In comparison with expt 41, expt 42 (stirred moderately at 300–500 rpm) gave a higher $n_{[O]_{h-d}}$, a lower percentage discrepancy between $n_{[O]_{calc}}$ and $n_{[O]_{h+d}}$ (47% and 34%), but comparable R_o's. The results from expts 41–43 suggest the occurrence of (II[c]). The R_o's of expts 38 and 39 are in between those for (II[c]) and (II[d]) but are also not conclusive.

Series 25 carried out under argon nicely illustrates the occurrence of (II[b]). There was no effect of EDTA (*cf* expts 48 and 46).

(*g*) *Series* 28–29: 0·5N $HClO_4$. The R_o's of expts 49, 50 and 51 are lower than of the corresponding expts 38, 40 and 43 in 0·5N H_2SO_4, probably caused by some occurrence of (6) as already mentioned under series 21–22.

(*h*) *Series* 30–31: 0·2N H_2SO_4. The yield of hydroxlation (expt 52) could be increased on keeping the reaction mixture unstirred for 3 days (expt 53).[1] $R_o = 0·86$ is consistent with the main oxidation of a_1 by O_2 (I[c]). Addition of an excess of H_2O_2 (expt 54) did not change R_o, but led to a greater discrepancy between $n_{[O]_{calc}}$ and $n_{[O]_{h+d}}$.

Reaction type (II[b]) is nicely demonstrated by expt 55 performed under argon.

(*i*) *Series* 32–34: 0·2N $HClO_4$. Expt 57 gave a better hydroxylation but a lower R_o than the corresponding expt 52 in 0·2N H_2SO_4. Expt 58 (not stirred for 3 days) also showed a lower R_o than expt 53. As already mentioned under series 21–22 and 28–29, the adduct a_1 was probably oxidized by O_2, but the R_o was lowered by some occurrence of (6). Although performed in the presence of O_2 and H_2O_2, expt 59 did not give a discrepancy between $n_{[O]_{calc}}$ and $n_{[O]_{h+d}}$. The data are consistent with the 1:1 occurrence of (I[c]) and (II[c]).

Oxidation of a_1 by H_2O_2 according to (II[b]) is all-important in 0·2N $HClO_4$ under argon, but the theoretical yields as expected from (II[b]) could be exceeded by 5–30%. The mixture of expt 60 was analyzed as usual after 4 h

stirring. However, expt 61 (stirred overnight) showed that anaerobic hydroxylations in suspensions of 0·2N $HClO_4$ required a longer reaction time. The data found for $n_{H_2O_2}$, $n_{(O)_{calc}}$ and $n_{(O)_{t+d}}$ are 30% higher than the theoretical values to be expected from (II^b).

(*j*) *Series* 35: 0·05N $HClO_4$. Expt 62 (stirred overnight) gave comparable results. However, spirohydantoin was produced instead of A^+—R, which is a matter of pH.

Oxidation of A^+—R by H_2O_2

(*a*) *In mineral acid media.* In connection with the hydroxylations summarized in Table 1 various blanks were carried out. For example, a soln of A^+—R, HSO_4^- (1·0 mmole), H_2O_2 (15 mmoles) and phenylalanine (4·0 mmoles) in 0·5N H_2SO_4 (50·0 ml) was stirred for 4 h and then analyzed to give a negligible $n_{(O)_{t+d}}$. However, the surprising thing is that in the long run the same mixture produced a considerable amount of hydroxyphenylalanines (0·4 mmole; *cf* Fig 1). Apparently, hydroxylating species are formed in a slow conversion of A^+—R by H_2O_2 giving about the same isomer distributions as mentioned in Table 1.

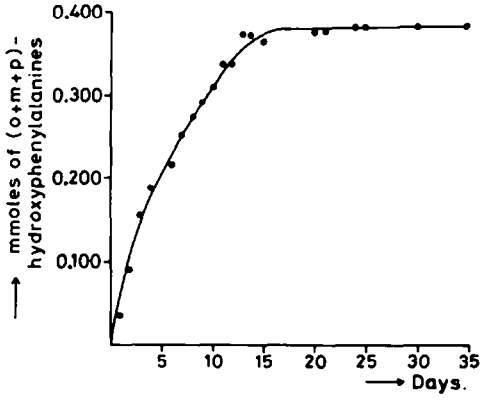


Fig 1. Slow hydroxylation of phenylalanine (4 mmoles) effected bij A^+—R, HSO_4^- (1·0 mmole) + H_2O_2 (15 mmoles) in 0·5N H_2SO_4 (50·0 ml) at 23°.

(*b*) *In aqueous media of pH* 2–7. In the pH region 2–7 the alloxazinium ion is rapidly converted into the spirohydantoin via the transient 10°-pseudobase A^R—OH.[1,7]

We have now found that this reaction (16) can be partly or completely displaced by the CO_2-generating reaction (17) in dependence on the excess of H_2O_2 and the pH.

$$A^R\text{—OH} \longrightarrow \text{spirohydantoin} \qquad (16)$$

$$A^+\text{—R} \xrightarrow[HO^-]{HOO^-} A^R\text{—OOH} \xrightarrow{H_2O_2} X + CO_2 \qquad (17)$$

Under the same conditions the spirohydantoin itself is quite inert towards H_2O_2. The product X, of which the structure is not yet quite elucidated, can be detected by TLC on silicagel GF_{254} with ethylacetate as solvent (10 cm; $R_f = 0{\cdot}25$). The experiments were performed in a manometric apparatus e.g.: A^+—R, ClO_4^- (1·0 mmole) was added to well-stirred, gas-saturated solutions of H_2O_2 (3–20 mmoles) in buffered or non-buffered water (50·0 ml) at various pH. Some results obtained in the presence of 20 mmoles of H_2O_2, are: Δ_{CO_2} (pH) = −0·06 (2·50); −0·43 (5·10); −0·60 (5·50); −0·75 (6·00); −1·00 (6–7). The gas generation was rapid: 50% of the final Δ_{CO_2} in less than 1 min, 75% in 3–8 min. Afterwards, the nett consumptions of H_2O_2 were determined, proving the relationship: $n_{H_2O_2} = 2\Delta_{CO_2}$. The gas was generated in the same rates in 0·1–0·5M solutions of acetate or phosphate. Some aromatic hydroxylation ($n_{(O)_{t+d}} = 0{\cdot}05$–$0{\cdot}06$) took place on adding phenylalanine (4 mmoles) to the above systems of A^+—R/H_2O_2 at pH 2·5–7. (Solutions of phenylalanine/H_2O_2 then served as blanks). Systems of phenylalanine/A^+—R/H_2O_2 were also studied as blanks for hydroxylations effected by A^R—H at pH 2–7 (Table 2).

Conclusion. The 10°-position can be competitively attacked by the nucleophile HOO^- to give probably A^R—OOH. A subsequent reaction with a second molecule of H_2O_2 leads to the forming of hydroxylating species with degradation of the alloxazine ringsystem.

Hydroxylations effected by A^R—H in aqueous media at pH 2–7 (Table 2)

On increasing the pH lower yields of hydroxylation were obtained on account of either: (1) reaction (6) which could not be displaced as completely as in acid media; (2) oxidation of the substrate into non-phenolic products; (3) oxidation of other components of the medium; (4) degradation of alloxazine transients; (5) a dominating peroxide formation under autoxidative conditions if no care is given to factors influencing the concentration and diffusion of oxygen.[1]

(a) *Series* 36–39: *In* 1N *acetic acid.* The yield of autoxidative hydroxylation (expt 63) was improved if the mixture was not stirred for one week (expt 64). Some substrate was also converted into non-phenolic products as could be concluded from the $n_{(O)_{calc}}$- and $n_{(O)_{t+d}}$-values in comparison with those from e.g. expt 63^b. Good results were obtained from stirring experiments under anaerobic conditions (expt 65) although e.g. expt. 65^b showed that some acetic acid or alloxazine transients had also been oxidized.

(*b*) *Series* 40: *In non-buffered water* + 100%-O_2. H_2O_2 formation was predominant in normally stirred reaction mixtures. Oxygen transfer took place if the reaction mixture was not stirred for 6 days (expt 66), although the greater part did not lead to aromatic hydroxylation (*cf* $n_{(O)_{calc}}$ and $n_{(O)_{t+d}}$).

(*c*) *Series* 41–43: *In non-buffered and buffered water + air.* Expts 67, 68, 69, 73 and 74 were carried out in non-buffered reaction mixtures; expts 70, 71 and 72 in: 1M, 0·2M and 0·1M sodium phosphate, respectively, adjusted to various pH by adding some H_2SO_4 or NaOH. The normally stirred expt 67 showed practically no hydroxylation in contrast with expt 68 (non-stirred for 2 days). Oxygen transfer was inhibited in the additional presence of the *enzyme catalase* (expt 69, non-stirred for 2 days). Hydroxylation occurred in phosphate solns (expts 70–72) which, however, had to be kept non-stirred for about 10 days (*cf* the retarding effect of phosphate on the CO_2 evolution rate, illustrated in Fig 2). The R_o-values of series 41 are not conclusive on account of the discrepancies between the $n_{(O)_{calc}}$- and $n_{(O)_{t+d}}$-data. Hydroxylation was found in the normally stirred expts 73 and 74, but reaction (6) was not efficiently displaced as could be concluded from the data mentioned. The Δ_s-values in series 42 and 43 are nett results from a simultaneous O_2 uptake and a CO_2 evolution. (The amount of CO_2 has to be determined e.g. by absorption and titration in order to calculate n_{O_2} and R_o).

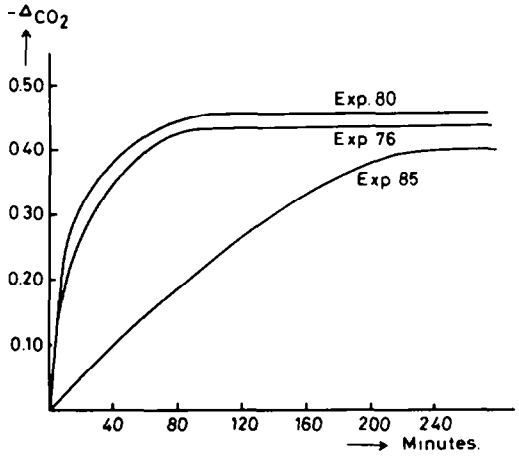


Fig 2. The inhibiting effect of phosphate on the CO_2 evolution rate.

(*d*) *Series* 44–49: *In non-buffered and buffered water* + H_2O_2 + *argon.*

0·2M sodium acetate and phosphate solns were adjusted to various pH by adding H_2SO_4 or NaOH. There were no additional effects on varying the acetate or phosphate concentrations from 0·1 to 1M. Spirohydantoin was inert under the same conditions. The Δ_o-values are nett CO_2 evolutions. The series showed that on increasing the pH the CO_2 evolution increases while oxygen transfer decreases. The low R_o-values (e.g. expts 76 and 77) are consistent with a considerable occurrence of reaction (6). Aromatic hydroxylation ($n_{(O)_{H+d}}$) was slightly lowered by the presence of acetate or phosphate anions. Phosphate has also an inhibiting effect on the CO_2 evolution rate (series 48 and 49; illustrated by a few examples in Fig 2). This is due to an influence of phosphate on either reaction (4) or (5), considering the fact that reaction (17) was not inhibited at all by phosphate.

(*e*) *Series* 50–53: *the additional presence of an aliphatic substrate.* Only a few aliphatic compounds chosen arbitrarily will be mentioned in this paper. The forming of H_2O_2-consuming aliphatic radicals appears from the increased $n_{H_2O_2}$-values (e.g. series 50 in comparison with expts 78 and 83). The stimulation of aromatic hydroxylation in series 50 comes to the fore on comparing the $n_{(O)_{H+d}}$-values. No further stimulation was effected on increasing the EDTA concentration (expts 89→91). Stock solutions containing phenylalanine + H_2O_2 + EDTA served as blanks, proving that the stimulation of the aromatic hydroxylation could not have been caused by *traces of metal ions acting as a "masked" Fenton's reagent*! A slight stimulation was given by lactic acid (expt 92). Methyl pyruvate is rather stable towards H_2O_2 in contrast with pyruvic acid which rapidly decarboxylates also in the absence of A^R—H. On adding A^R—H to a solution of methyl pyruvate and H_2O_2 (expt 93) an increased CO_2 production and H_2O_2 consumption were found which had not yet come to an end after 4 h. Aromatic hydroxylation was then inhibited as in the presence of e.g. ethanol (expt 94).

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