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Oxidation of Salicylate by the Model Peroxidase Catalyst Iron–Ethylenediaminetetraacetato-iron(III) Acid

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In the presence of ethylenediaminetetraacetato-iron(III) and ascorbic acid (model peroxidase system) salicylate is oxidized in aqueous solution by O_2 and H_2O_2. The principal products are 2,3- and 2,5-dihydroxybenzoic acids. Yields, based on salicylate consumed, ranged from 50–70%, and the ratio of 2,3- to 2,5-products varied from 1.1 to 3.2. H_2O_2 appears to be an intermediate when O_2 is the oxidant. Benzoic acid was oxidized to a mixture of *o*-, *m*- and *p*-hydroxybenzoic acids. The data are discussed in terms of a free radical mechanism, wherein initiation occurs by reaction of H_2O_2 or O_2 with the ferrous chelate, producing $HO\cdot$ or $HO_2\cdot$ radicals. Reaction of these radicals with salicylate produces an aryl radical which continues the chain and leads ultimately to the dihydroxy acid.

The "model" peroxidase system[1,2] is of interest because of the close similarity between its behavior and that of the enzyme peroxidase. In the presence of oxygen and a reducing agent possessing an ene-diol structure, $\begin{matrix}-COH\\ \|\\ -COH\end{matrix}$ both systems hydroxylate aromatic compounds at specific sites on the ring,[2–5] and in both cases the entering hydroxyl is derived from the oxygen molecule.[5,6] This type of activity is not peculiar to peroxidase, but is exhibited by a number of other enzyme systems, both *in vitro* and *in vivo*. An extensive review of this subject has been given by Mason.[7] Because of its greater simplicity, a knowledge of mechanism of action of the model system is important, not only because of the light which might be shed on the enzyme mechanism, but also because of the potential utility of the model as a preparative method for phenolic compounds. Because of the apparent participation of hydrogen peroxide as an intermediate in this reaction, the mechanism of the H_2O_2–ascorbic acid reaction was studied first. In the preceding paper,[8] the kinetics of this reaction were examined, and a free radical mechanism was proposed which accounts for the observed behavior. In this paper, the behavior of salicylic acid in this system (and in similar systems utilizing oxygen as the oxidant) is presented. This aspect of the work was confined mainly to isolation and identification of the products formed under various conditions.

Experimental

Materials.—Salicylic acid (USP) was made up as a solution with the buffer prior to use. The ascorbic acid used was USP grade. Other chemicals were reagent grade. Reactions were carried out by mixing the appropriate solutions, adding the H_2O_2 last. Oxygen was generally excluded from the system during a run except where it was specifically involved as the oxidant. Catalase was obtained as a solution of unspecified concentration from the H/M Chemical Co., Santa Monica, Calif. The activity was checked at dilutions of 12,500 and 125,000 in a solution containing 0.1 *M* phosphate, and 1.1×10^{-3} *M* H_2O_2 at a *p*H of 6.5. The initial rates of H_2O_2 decomposition were, respectively, 5×10^{-3} and 5×10^{-4} mole per liter per minute.

Procedure.—The course of an experiment was followed in the cases where H_2O_2 was the oxidant by dilution of an aliquot with 1 *N* H_2SO_4 to a known volume in 0.04 *M* Ti(IV) and determining the optical density of the solution at 410 mμ. When oxygen was used as an oxidant the reaction was carried out in a constant volume system containing pure oxygen, equipped with a manometer and a magnetic stirrer. In experiments where ascorbic acid was present, the reaction with H_2O_2 was over within a few minutes, at which time the H_2O_2 had been completely consumed. The reaction with O_2 required somewhat longer, but was complete within 3 to 4 hours and continued until the ascorbic acid had been consumed.

Analysis of Products.—Unreacted salicylic acid was removed from the reaction by acidification and extraction with chloroform. Further extraction with ether gave a mixture of the dihydroxybenzoic acids. These were not separated further, but were identified by three different methods. The infrared spectra of the mixtures were recorded and compared with known samples of the various suspected products. Besides serving to identify the products, it could be shown also that salicylic acid was effectively removed by the preliminary chloroform extraction.

As a check on the infrared scans, the ether residues were paper chromatographed on Whatman No. 1 filter paper, using 4:1 (by volume) *i*-propyl alcohol–7 *N* NH_4OH as the eluant,[9] and developing with 1% ferric ferricyanide solution. R_f values determined in this system are

Acid	R_f value
2,6-Dihydroxybenzoic	0.71
2,5-Dihydroxybenzoic	.45
2,4-Dihydroxybenzoic	.22
2,3-Dihydroxybenzoic	.33
o-Hydroxybenzoic	.68
m-Hydroxybenzoic	.30
p-Hydroxybenzoic	.16

Actual analyses of the ether fraction were made by two methods. The 2,3-dihydroxybenzoic acid was determined by the colorimetric method given by Snell and Snell,[10] involving the color developed by alkaline ferrous tartrate and catechols. The total *o*-hydroxybenzoic content was determined by a second colorimetric procedure[11] utilizing the color developed with ferric chloride. Because only two constituents were present, these two values allowed a determination of the composition of the product to be made.

In one experiment benzoic acid was used as the substrate. The separation was carried out in a similar manner. The chloroform residue, which was mostly unreacted benzoic acid, also contained some salicylic acid, which was a

(1) S. Udenfried, C. T. Clark, J. Axelrod and B. B. Brodie, *J. Biol. Chem.*, **208**, 731 (1954).

(2) B. B. Brodie, J. Axelrod, P. A. Shore and S. Udenfriend, *ibid.*, **208**, 741 (1954).

(3) C. E. Dalgliesh, *Arch. Biochem. & Biophys.*, **58**, 214 (1955).

(4) H. S. Mason, I. Onoprienko and D. Buhler, *Biochim. et Biophys. Acta*, **24**, 225 (1957).

(5) H. S. Mason, I. Onoprienko, K. Yasunobu and D. Buhler, THIS JOURNAL, **79**, 5578 (1957).

(6) H. S. Mason and I. Onoprienko, *Federation Proc.*, **15**, 310 (1956).

(7) H. S. Mason, *Adv. in Enzymol.*, **19**, 76 (1957).

(8) R. R. Grinstead, THIS JOURNAL, **82**, 3464 (1960).

(9) R. J. Black, E. L. Durrum and G. Zweig, "Paper Chromatography and Paper Electrophoresis," 2nd ed., Academic Press, Inc., New York, N. Y., 1958, p. 307.

(10) F. D. Snell and C. T. Snell, "Colorimetric Methods of Analysis," Vol. III, 3rd ed., Van Nostrand Co., Inc., New York, N. Y., 1953, p. 127.

(11) Reference 10, p. 424.

TABLE I

MODEL PEROXIDASE SYSTEM. OXIDATION OF SALICYLIC ACID BY H_2O_2 AND O_2

[Phosphate] = 0.10 M, [salicylate] = 0.10 M, [Fe] = 1.0×10^{-3} M, [EDTA] = 1.0×10^{-2} M, [ascorbic acid] = 0.10 M

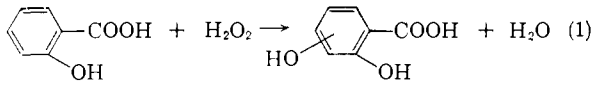
Expt.	pH	$[H_2O_2]$, M	Conversion of salicylate, %[a]	Yield of dihydroxys, % based on Salicylate[b]	based on H_2O_2[c]	based on O_2[d]	Ratio 2,3:2,5	Product composition, % (ether residue) 2, 3	2, 5	Total
76-62	4.3	0.10	30	55	16		3.2	70	22	92
76-92	6.5	.10	27	60	17		2.2	65	29	94
71	6.5	0[e]	28	61		14	1.8	60	34	94
98[f]	6.5	0[e]	30	67		15	1.1	51	45	96

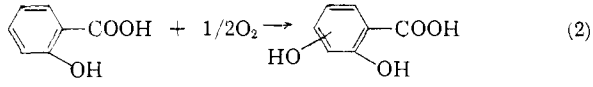
[a] % salicylate reacted. [b] Moles product found/moles salicylate reacted. [c] Moles product found/moles H_2O_2 reacted. [d] Moles product found/2 × moles O_2 used. [e] O_2 used as oxidant. [f] Catalase solution added at a dilution of 1:80.

product of the reaction. This was determined analytically as before. The ether phase was paper chromatographed in the same solvent as above, and the presence of both *m*- and *p*-hydroxybenzoic acids was established. Semi-quantitative estimates of these compounds were made by colorimetric methods. The *p*-OH isomer was estimated with Millon reagent[12] and the total phenols with *p*-nitroaniline.[13] Some dihydroxy acids were detected also by the procedure described above.

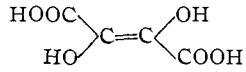
Results

The paper chromatograms of the ether fractions gave two strong spots at approximately the locations expected for the 2,3- and 2,5-dihydroxybenzoic acids. The presence of these two compounds, as well as the absence of the 2,4- and 2,6-isomers was confirmed by comparison of the infrared spectra of these samples with the spectra of the pure compounds. In no experiment was even a trace of the latter two compounds observed. The direct determination of the 2,3-isomer, together with the determination of total dihydroxybenzoates, provided the data shown in Table I. At *p*H 4.3, in the region where the previous kinetic data were obtained,[8] the yield of product is not greatly different from that at a *p*H of 6.5 where the reaction goes much faster. A noticeable difference in the ratio of *o*- to *p*-hydroxylated products is observed, the higher *p*H favoring a greater proportion of *para* product. Further oxidation of these products undoubtedly occurred, since the aqueous solution was always quite dark in color. However, no attempts were made to detect other products of this reaction. The figures for yields based on the oxidant consumption were based on the assumption that the following equations applied





By comparing experiments 71 and 92, it can be seen that the yields and product ratios were quite similar with these two oxidants. The conditions in experiment 71 are similar to those used by Brodie, *et al.*,[2] who reported only gentisic acid (2,5-dihydroxybenzoic acid) as a product. It was indicated that the presence of the 2,3- acid was not thereby ruled out, but that it was not specifically sought in those experiments.[14] It is interesting to note that the oxidation of salicylate by oxygen in the presence of the enzyme peroxidase and the ene-diol dihydroxyfumaric acid



has also been reported to give 2,3- and 2,5-dihydroxybenzoates.[5] This result further strengthens the analogy between the enzyme and the model system.

In order to obtain some information on the question of whether H_2O_2 was an intermediate in this reaction, experiment 98 was carried out. This was identical to experiment 71 except for the inclusion of the enzyme catalase. This enzyme catalyzes the decomposition of H_2O_2 to H_2O and O_2 extremely rapidly, and should alter the behavior of any set of reactions involving H_2O_2. While the results in experiments 98 and 71 were qualitatively the same, a substantial difference appeared in the product composition, confirming the participation of H_2O_2 to some extent at least in the O_2 reaction.

In a single experiment with benzoic acid as the substrate, the other conditions were the same as experiment 62, Table I. While a 46% conversion of benzoate occurred, the yield of hydroxylated products was low, about 15%. By means of the semi-quantitative analysis described above, the products gave the composition: *o*-hydroxybenzoic (salicylic) acid, 20%; *m*-hydroxybenzoic acid, 25%; *p*-hydroxybenzoic acid, 20%; dihydroxybenzoic acids, 10%, these figures are probably subject to uncertainties of several per cent. of the total composition. However, in conjunction with the evidence obtained from the paper chromatography of the samples, there appears little doubt that substantial amounts of all three hydroxybenzoic acids were formed.

Discussion

In the preceding paper[8] the conclusion was drawn that in the oxidation of ascorbic acid by H_2O_2, free hydroxyl radicals participated in the reaction. These radicals disappeared mainly by reaction with ascorbic acid, producing another intermediate, the ascorbate radical. Upon adding another oxidizable substrate such as salicylic acid, it seems reasonable to expect that this compound would also be attacked by the OH· radical, leading to oxidation products of salicylate. Numerous studies of the oxidation of aromatic compounds by OH· radicals have been reported, using ferrous sulfate–H_2O_2 (Fenton reagent) and ionizing radiation to produce OH· radicals, and the oxidation products are found to include mono- or polyhydroxylated

(12) Reference 10, p. 414.
(13) Reference 10, p. 117.
(14) S. Udenfriend, personal communication.

compounds such as phenols, catechols, etc.[15] For example, benzene and toluene have been oxidized to phenol and cresol, respectively, and phenol has been oxidized to both hydroquinone and catechol. With nitrobenzene, chlorobenzene and benzoic acid, all three isomeric substituted phenols are produced in each case. Salicylic acid was reported to be oxidized to 2,5-dihydroxybenzoic acid by Fenton reagent. Of greatest interest are the experiments of Downes,[16] who irradiated solutions of salicylate with ^{60}Co γ-rays and showed that 2,3- and 2,5-dihydroxybenzoic acids were formed in the ratio 1.6:1. The correspondence of the products, as well as the similarity of their ratio to the results presented here, furnishes strong evidence that hydroxyl radicals are the active intermediate in the H_2O_2–salicylate reaction.

The question of whether this H_2O_2–salicylate reaction constitutes part of the model peroxidase system (which involves O_2 as the oxidant) was examined by means of the catalase experiment (no. 98, Table I). The change in product composition (compare experiments 71 and 98) when catalase was present shows that H_2O_2 is involved in the O_2 reaction, although the fraction of O_2 which is reduced to H_2O *via* H_2O_2 cannot be judged. While H_2O_2 participates, it is evidently not essential to the hydroxylation process, since even in the presence of catalase hydroxylation still occurs (expt. 98). Since H_2O_2 is presumably the source of OH· radicals, this experiment also suggests that OH· radicals are not essential either. However, elemental O_2 reacts readily with Fe(II)–EDTA, and one possible pair of initial products would be expected to be Fe(III)–EDTA and the perhydroxyl radical HO_2·.

$$O_2 + FeY^- + H_2O \longrightarrow FeY^- + HO_2\cdot + OH^- \quad (3)$$

While this radical is not as strong an oxidant as the OH· radical it is an oxidizing species and must be the species which initiates the hydroxylation reaction in the presence of catalase.

While further data bearing directly on the mechanism have not been obtained, it is possible on the basis of known information to propose a reasonable mechanism which will account for the gross features of the model peroxidase system. This system can be thought of as consisting of two parts, the radical generating system and the substrate oxidizing system. The former is essentially a Fenton-type reaction utilizing instead of free ferrous iron, a chelated iron species. The function of the ascorbic

$$H_2O_2 + FeY^- \longrightarrow FeY^- + OH^- + OH\cdot \quad (4)$$

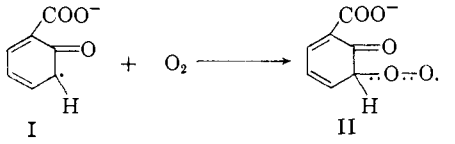
acid (or other reducing agent) is that of continually reducing the ferric chelate to the ferrous in which form it readily reacts with H_2O_2 to produce a continuous supply of OH· radicals. This type of system has been used previously to provide a continuous supply of radicals for initiation of polymerizations. Such systems include a metallic salt such as iron, a reducing agent and an organic peroxide, and produce instead of hydroxyl radicals, alkoxy radicals.[17,18] The analogy between the model peroxidase system and these polymerization initiators has already been pointed out by Krueger.[19] On the basis of this analogy, and also because the model system decarboxylates formic acid in the same manner as ionizing radiations, Krueger has also proposed that the OH· radical is the active intermediate in the model system.

The essential difference between the polymerization initiating systems and the model peroxidase system appears to be the fact that in the latter system O_2, as well as H_2O_2, may be utilized as the oxidizing agent. This fact undoubtedly depends upon the rather low negative value of the oxidation potential (E^0) of the iron chelate (−0.13 v.). The standard potential of the one-electron couple O_2–HO_2· is +0.13 v., and it can be calculated that a small but quite definite concentration of the HO_2· radical can be formed by reaction 3. As long as the HO_2· radicals are removed by a subsequent reaction, the initial reaction which produces them will continue. With iron chelates of greater negative oxidation potentials, or with the Fe^{++}–Fe^{+++} couple (E° = −0.77 v.), the available concentration of HO_2· radicals will be progressively less, and the subsequent reactions will be correspondingly slower. If, on the other hand, the potential of the couple is too positive, the oxidized form will not be reduced by ascorbic acid. Borsook gave for the potential of the ascorbic acid–dehydroascorbic acid system −0.17 v. at *p*H 4 and −0.08 v. at *p*H 6.4.[20] This is in the range of the iron–EDTA potential and, as was reported previously,[8] a measurable equilibrium is reached. The optimum oxidation potential for the catalyst would be expected to occur somewhere between about +0.13 v. and about −0.15 v., that is, between the E^0-values of the O_2–HO_2· and the ascorbate–dehydroascorbate couples. The second part of the model peroxidase system, that involving the reaction initiated by OH· radicals in aromatic systems, has been extensively studied by a number of authors,[15] principally Merz and Waters,[21,22] and MacKinnon and Waters.[23] The general features proposed by these authors appear to explain most of the phenomena of model peroxidase system. Reaction of the hydroxyl radical (or, presumably, a perhydroxyl radical as well) with a salicylate ion products an aromatic radical

$$HO\cdot \text{ (or } HO_2\cdot) + HOC_6H_4COO^- \longrightarrow H_2O \text{ (or } H_2O_2) + HOC_6H_3COO\cdot^- \quad (5)$$

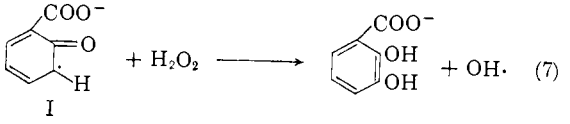
Possible reactions of such a radical are numerous, but only those reactions in which it loses another electron (*i.e.*, reacts with an oxidant) will lead to oxidation products. Aryl radicals have been found to react readily with O_2,[24] and in the presence of O_2 the following reaction would be expected to occur

(15) O. C. Dermer and M. T. Edmison, *Chem. Revs.*, **57**, 77 (1957).
(16) A. M. Downes, *Australian J. Chem.*, **11**, 154 (1958).
(17) B. A. Dolgoplosk and E. I. Tiniakova, *J. Polymer Sci.*, **30**, 315 (1950).
(18) R. G. Bacon, *Quart. Revs.*, **9**, 287 (1955).
(19) R. C. Krueger, *Federation Proc.*, **15**, 294 (1956).
(20) H. Borsook, H. W. Davenport, C. E. P. Jeffreys and R. C. Warner, *J. Biol. Chem.*, **117**, 237 (1937).
(21) J. H. Merz and W. A. Waters, *J. Chem. Soc.*, 2427 (1949).
(22) J. H. Merz and W. A. Waters, *ibid.*, S 15 (1949).
(23) D. J. MacKinnon and W. A. Waters, *ibid.*, 323 (1953).
(24) J. H. Baxendale and J. Magee, *Disc. Faraday Soc.*, **14**, 160 (1953).



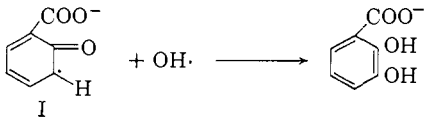
(6)

Organoperoxide radicals are common intermediates in oxidation reactions, and generally decompose to give alcohol or carbonyl compounds. Thus decomposition of II would lead eventually to the dihydroxybenzoate and would incidentally not involve H_2O_2 as an intermediate. In the presence of H_2O_2(O_2 absent) the occurrence of a reaction with H_2O_2 corresponding to (6) is doubtful.



(7)

Merz and Waters concluded after studying the oxidation of a number of aromatic compounds that the aryl radicals of the type represented by I did not react with H_2O_2, but instead underwent dimerization or reaction with OH· radicals to give the phenol.[22] In the latter case, the dihydroxybenzoate would again be produced.



(8)

The hydroxylation of salicylate exclusively in the positions *ortho* and *para* to the phenol group is evidently characteristic of the ring substituents.[25] Thus hydroxylation of phenol, like salicylic acid, gives substitution only *ortho* and *para* to the hydroxyl group.[26] Chlorobenzene,[27] benzoic acid[16,26] and nitrobenzene[28] give substantial amounts of all three isomers. The single experiment reported here with benzoic acid confirms the latter results. In none of these cases, however, has the *meta* product been found to predominate.[15] Quantum mechanical interpretations have been published which agree with the experimental results, and have led to the conclusion that in homolytic reactions of the aromatic nucleus all substituent groups are *ortho–para* directing.[25] This is accounted for by the fact that the most stable resonance forms of the intermediate aromatic free radical are those with the odd electron at the positions *ortho* and *para* to the substituent.

At this point it is pertinent to consider the mechanism proposed by Mason[7] to account for the action of the enzyme peroxidase and the model system. Mason postulated the existence of a ferryl species, Fe_pO^{++} as the active intermediate, where the subscript refers to the fact that the iron is complexed by a chelating structure which may occupy up to five of the six available coordination positions. Such a structure would normally be considered to contain +4 iron, and indeed is accepted as one of the forms of the enzyme peroxidase in some of its reactions.[29] The basis for the ferryl intermediate consists of two main types of evidence: (1) the appearance of the hydroxyl group at specific sites, generally the most electronegative ones on the ring, and (2) the fact that tracer oxygen experiments show that the entering oxygen atom is always derived from the O_2 molecule, never from H_2O.[5,6] The participation of OH· radicals would, in Mason's view, allow exchange with water to occur with accompanying loss of labeling, and might also result in random attack of the ring due to the extreme activity in the radical. The Fe_pO^{++} species, besides providing a method of transferring a specific O atom, would possibly act as an electrophilic agent and thereby account for the product distribution.

With respect to (1) there appears to be no serious conflict of the available information with the expected behavior of the hydroxyl radicals. In the preceding paragraph it was pointed out that both the predicted and the observed behavior of hydroxyl radicals was that of *ortho–para* hydroxylation. The only cases cited by Mason which appeared to be in conflict involved the hydroxylation of nitrobenzene, benzoic acid and cyanobenzene. Here only *meta* products were reported, thus suggesting a "normal" attack by a nucleophilic agent. It is not clear, however, whether the corresponding *ortho* and *para* products were unequivocally shown to be absent.

Concerning (2), direct information on the relative rates of exchange of OH· radicals with H_2O as compared with reactions of the type 8 does not appear to exist. Undoubtedly the specific rate of (8) is much greater than that of the OH·–H_2O exchange, but the predominance of H_2O over I renders it difficult to draw any conclusions concerning the relative over-all rates. However, in the presence of oxygen, the principal means of attachment of an oxygen atom to the ring is probably reaction 6, in which case the source of the entering oxygen atom is clearly the O_2 molecule. Actually the use of labeled O_2 would assure the labeling of both H_2O_2 and HO_2· as well, so that the existence of labeled oxygen in the ring means that either the exchange with water or reaction 8 is not important in this system. Tracer experiments with labeled H_2O_2 in the absence of O_2, which might shed further light on this aspect, have not been done.

While the above discussion applies mainly to the model peroxidase system, similar remarks apply to the enzyme system as well. A similarity of products points to the participation of OH· radicals as the active oxidizing intermediate. With respect to the oxidation states of the iron atom in peroxidase, the situation is not completely clear. Mason[3] has pointed out that the inhibition of this reaction by CO[30] gives direct evidence of participation of the ferroperoxidase (+2 iron) form of the enzyme, which extends the analogy with the model system. The specificity of dihydroxyfumaric acid as the reducing agent in this

(25) D. R. Augood, D. H. Hey and A. Nechvatal, *Nature*, **167**, 725 (1959).

(26) H. Loebl, G. Stein and J. Weiss, *J. Chem. Soc.*, 405 (1951).

(27) G. R. A. Johnson, G. Stein and J. Weiss, *ibid.*, 3275 (1951).

(28) H. Loebl, G. Stein and J. Weiss, *ibid.*, 2074 (1949); 2704 (1950).

(29) P. George in "Currents in Biochemical Research," D. Green, Ed., Interscience Publishers, Inc., New York, N. Y., 1956, p. 338.

(30) B. Chance, *J. Biol. Chem.*, **197**, 577 (1952).

system may be related to the oxidation potential of the ferro–ferriperoxidase couple, which may be too positive to be reduced by ascorbic acid. Whether or not a higher oxidation state of the peroxidase iron atom may participate is apparently open to question. Mason suggested that compound III of peroxidase, which predominates in systems containing peroxidase and dihydroxyfumaric acid, is a ferrous form of the enzyme. Chance, however, was able to reduce compound III with $Na_2S_2O_4$ to a form which reacted with CO,[30] suggesting that this species contains iron in a higher oxidation state than +2. Spectral evidence also indicated the presence of compound II, the +4 iron form of the enzyme. On the basis of the available information, the behavior of the model system, at least, appears to be satisfactorily explained by a free radical mechanism. Stated in another way, there appears to be no good reason for accepting a mechanism involving +4 iron species as long as the data are compatible with the radical mechanism involving conventional intermediate species. This is not to exclude any participation by a ferryl species which may actually be involved to some extent. In the case of the enzyme itself, some evidence of the +4 iron species exists, although the reaction products are again compatible with the free radical mechanism as the principal one.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF STANFORD UNIVERSITY]

Terpenoids. XLII.[1] The Absolute Configuration of (−)-Methylisopulegone[2]

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The stereochemistry of the direct alkylation product of (+)-pulegone (I)—(−)-methylisopulegone (II)—has been established by determining the absolute configuration of the quaternary carbon atom and this was found to be contrary to earlier expectation (III). The stereochemical assignment was based on a multi-stage degradation of (−)-methylisopulegone (II) to (+)-α-methyl-α-isopropylglutaric acid (XII), which was related by Fredga's quasi-racemate method to the known (+)-α-isopropylglutaric acid (XVI). Alternatively, (−)-methylisopulegone (II) was transformed to (−)-2-methyl-2-isopropylbutane-1,4-diol (IX), also derivable from the known (−)-α-methyl-α-isopropylsuccinic acid.

Direct methylation of (+)-pulegone (I)[4] is known[5] to lead in good yield to (−)-methylisopulegone (2,5-dimethyl-2-isopropenylcyclohexanone). While there exists no question about the structure of this product, its stereochemistry can be represented by either II or III and a tentative preference for the latter has been expressed recently.[6] In connection with extensive studies in this Laboratory[7] on the relationship of rotatory dispersion and conformation of monocyclic cyclohexanones, (−)-methylisopulegone (II or III), constituted a key intermediate and it was felt necessary to establish its stereochemistry by unambiguous means. The solution of this stereochemical problem also has a bearing on the mechanism of alkylation[7] of conformationally flexible cyclohexanones and no secure *a priori* predictions can be made in view of recent information[8] on the preferred conformations of (−)-menthone and (+)-isomenthone, which suggests that in the latter an axial isopropyl substituent adjacent to the ketone function may be preferred over an axial methyl group in the 3-position of a cyclohexanone.

The extensive quasi-racemate studies of Fredga[9] and his collaborators have led to the elucidation of the absolute configurations of a variety of alkylated succinic and glutaric acids. We felt, therefore, that the most straightforward solution to the stereochemistry of (−)-methylisopulegone (subsequently shown to be II) would be a systematic degradation of its reduction product, methyldihydroisopulegone (2,5-dimethyl-2-isopropylcyclohexanone) (IV), to a substituted succinic or glutaric acid which would then be amenable to interrelation with one of Fredga's reference acids.[4]

(−)-Methylisopulegone (II),[5] carefully purified by regeneration of its crystalline semicarbazone, was hydrogenated to (+)-methyldihydroisopulegone (IV)[4] and then brominated in aqueous solution. A homogeneous, crystalline monobromide, (−)-2-bromo-3,6-dimethyl-6-isopropylcyclohexanone (V), was obtained in high yield and careful, high-resolution infrared spectroscopic measurements in solvents of different polarities[10] indicated that the substance existed as a mixture of two conformational isomers, the conformer with the equatorial bromine atom[11] predominating (71%

(1) Paper XLI, D. Arigoni, D. H. R. Barton, R. Bernasconi, C. Djerassi, J. S. Mills and R. E. Wolff, *J. Chem. Soc.*, 1900 (1960).

(2) The major portion of the experimental work was performed in the Department of Chemistry of Wayne State University. Grateful acknowledgment is made to the Division of Research Grants (grant No. RG-3863 and RG-6840) of the National Institutes of Health, U. S. Public Health Service, for financial assistance.

(3) Undergraduate (sophomore) research fellow, 1958–1959.

(4) All structural formulas in the present paper imply absolute configurational representations using the steroid convention: dotted bonds denote a substituent below the plane of the paper, while a solid bond refers to one above it.

(5) G. A. R. Kon and J. H. Nutland, *J. Chem. Soc.*, 3101 (1926); J. M. Conia, *Bull. soc. chim. France*, 943 (1954).

(6) A. Melera, D. Arigoni, A. Eschenmoser, O. Jeger and L. Ruzicka, *Helv. Chim. Acta*, **39**, 441 (1956).

(7) J. Osiecki, E. J. Eisenbraun and C. Djerassi, to be published. See also paper presented by C. Djerassi, L. E. Geller, J. Osiecki and E. J. Eisenbraun at Symposium on Conformational Analysis, A.C.S. Meeting, San Francisco, Calif., April, 1958, Abstracts, p. 30-N.

(8) C. Djerassi, "Optical Rotatory Dispersion: Applications to Organic Chemistry," McGraw–Hill Book Co., Inc., New York, N. Y., 1960, Chapter 7, Section 3.

(9) See A. Fredga, *Tetrahedron*, **8**, 126 (1960), and *Svensk. Kem. Tid.*, **67**, 343 (1955) for review and leading references.

(10) J. Allinger and N. L. Allinger, *ibid.*, **2**, 64 (1958); N. L. Allinger and J. Allinger, THIS JOURNAL, **80**, 5476 (1958).

(11) In spite of the fact that the infrared measurements demonstrate the predominance of an equatorially oriented bromine atom, we are presently refraining from an absolute configurational assignment of the bromine atom in V, since this is partly dependent upon the conforma-