Examination of the phospholipids from *C. butyricum* cells indicates that this organism has reduced acylation specificity and the trend is opposite to that of organisms. That is, there is a preference for the placement of saturated acids in the β position, and unsaturated acids in the γ position.

Because the cyclopropane fatty acids show a distribution similar to that of the unsaturated acids in the phosphatidylethanolamine of *C. butyricum*, it is not possible from examination of the lipids to decide whether this organism has a real methylation specificity. Fortunately, this question can be settled by examination of the enzymatic reaction *in vitro*. When a phosphatidylethanolamine of known fatty acid content and distribution is used as the substrate for the cyclopropane fatty acid synthetase of *C. butyricum*, one can predict the distribution of the cyclopropane fatty acids on the basis of a nonspecific reaction. The distribution of cyclopropane fatty acids in the product would parallel that of unsaturated fatty acids in the substrate. Table II shows that the distribution is not in accord with such a nonspecific reaction, however. That is, although the substrate has less unsaturated fatty acids in the γ position, the isotopically labeled cyclopropane fatty acids are found in greater amounts in that position. It can be inferred, therefore, that the methylation specificity as well as the acylation specificity for unsaturated acids in this organism favors the γ position rather than the β position.

ACKNOWLEDGMENTS

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Metal-catalyzed Oxidation of 3,5-di-*t*-Butyl Pyrocatechol, and Its Significance in the Mechanism of Pyrocatechase Action*

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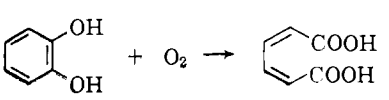
Oxidation of 3,5-di-*t*-butyl pyrocatechol by O_2 was carried out in the presence of metal salts in slightly alkaline aqueous methanol. The main product was 3,5-di-*t*-butyl *o*-benzoquinone (II). The yields, which reached as high as 96%, based on starting material, could be correlated roughly with oxidation potential of the metal ion. The oxidation of the pyrocatechol with O_2 was also studied under more alkaline conditions, leading to ring fission. The primary product appears to be 2,4-di-*t*-butyl-4,5-dihydroxy-α-hydromuconic acid, γ-lactone (III), together with the *o*-quinone (II). It was also possible to prepare compound III by oxidizing the quinone with H_2O_2. These results lead to the suggestion that pyrocatechase-type enzymes may function by promoting a series of four 1-electron steps, initiated by the reaction of O_2 with a metal ion complexed by the catechol substrate. Succeeding steps would involve two adjacent oxidation states of the metal ion, proceeding through the *o*-quinone and H_2O_2 as nonisolable intermediates, and eading to a ring-fission compound as the final product.

Oxidases or enzymes catalyzing reactions of oxygen are a particularly intriguing area of the field of reaction mechanisms, since the reduction of an oxygen molecule to water, or to combined oxygen, in which the oxidation state is formally -2, involves formal transfer of four electrons to the two oxygen atoms. Some of the most interesting oxidases are in the group exemplified by the enzyme pyrocatechase, in which the entire oxygen molecule is incorporated into a molecule in what appears to be a single reaction (Hayaishi and Hashimoto, 1950):



(1)

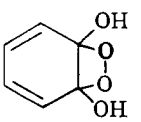
* Presented at the 145th National Meeting of the American Chemical Society, New York, N. Y., September 8–13, 1963.

The additional oxygen atoms in the product, muconic acid, can be shown by isotopic labeling of oxygen to have originated, one in each carboxyl group, in the oxygen molecule (Hayaishi *et al.*, 1957).

A number of enzymes have been reported with similar characteristics, and have been discussed in several reviews (Mehler, 1962; Crandall, 1955). In general they are characterized by the following major features: (1) the direct insertion of oxygen into the product, as mentioned; (2) the dependence of enzyme activity on the presence of ferrous ion and thiol groups; and (3) failure to demonstrate the existence of any intermediates, including quinones and hydrogen peroxide.

The substrates here all have the common characteristic of possessing two electron-donating groups (OH or NH_2), at least one of which is a phenolic hydroxyl group, in the aromatic ring. Where two ortho-hydroxyls are present, fission usually occurs between them, although some exceptions to this have been reported recently (Mehler, 1962).

No detailed mechanism for the action of the enzymes has yet been proposed. Hayaishi (1955) proposed at one time a cyclic peroxide of the type



as an intermediate, but without indicating how this might be formed. Mason (1957), on the other hand, has discussed the reaction in terms of a possible ferrous ion–oxygen complex, $Fe_p \cdot O_2$ (where Fe_p represents protein-bound iron), or a higher oxidation state of iron. Mehler (1962) has discussed a similar proposal in more detail. While the evidence does not preclude such a possibility, the known properties of the oxygen carriers hemoglobin and myoglobin indicate that they are not catalysts for oxidations, but are merely storage sites for oxygen. Higher oxidation states of iron are known, but are not known to be accessible with oxygen as the oxidant. At any rate neither proposal provides a clear insight into the detailed enzyme mechanism.

The work reported here on the oxidation of 3,5-di-*t*-butyl pyrocatechol grew out of an interest in the mechanism of action of these enzymes. While some work was done with pyrocatechol itself, this was abandoned due to the difficulty of observing a single clean reaction, and this study is concerned with the use of 3,5-di-*t*-butyl pyrocatechol, in which most of the ring sites except those bearing the hydroxyl groups are blocked off. This approach was based on the work of Campbell and co-workers (Campbell, 1951; Stitt *et al.*, 1954), who have used a similar device for reducing the complexity of the oxidation of pyrogallol by oxygen. Based on the data here a proposal for a detailed mechanism for pyrocatechase-type enzymes is presented.

Experimental

Materials.—3,5-di-*t*-Butyl pyrocatechol (I) was prepared by the method of Schulze and Flaig (1952), wherein pyrocatechol was alkylated in *t*-butyl alcohol in the presence of sulfuric acid. Since 4-*t*-butyl pyrocatechol was readily available commercially, it was found more convenient to use this compound as the starting material. After recrystallizing from heptane, the dibutyl compound generally melted at 96–97° (lit., 99°).

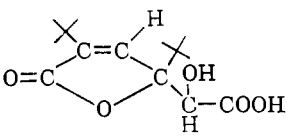
Oxidation of 3,5-di-t-Butyl Pyrocatechol by Oxygen.—Oxidations were carried out in a stirred constant-volume system, in 75% aqueous methanol. With moderately alkaline solution, oxygen uptake usually ceased after 2 or 3 hours, but occasionally continued for 24 hours in the less-alkaline solutions. The reaction mixture was prepared by dilution about 3- or 4-fold with water and addition of 10–20 g NaCl. Extraction with ether was carried out from this alkaline solution, again at a *p*H of 8–9 (HCO_3^- solution), and again after adding an excess of HCl. In some cases, one or more of these extractions were omitted.

From the first extraction was obtained a red crystalline solid, identified as 3,5-di-*t*-butyl *o*-benzoquinone (II). When recrystallized from *i*-octane it gave an mp of 112–114° (lit., 113–114°, Flaig *et al.*, 1955). The infrared spectrum of this material was identical to that reported by Ley and Muller (1956).

The main identifiable product was a white carboxylic acid (III), which was obtained in the final extraction. After two recrystallizations from a 4:1 mixture of *i*-octane and benzene compound III melted at 140–142°. It was an acid of equivalent weight 278 and *pK* of ~4.

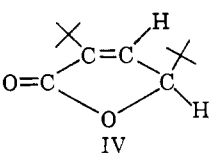
Anal. Calcd for $C_{14}H_{22}O_5$: C, 62.3; H, 8.15. Found: C, 62.9; H, 7.94; mw, 270.

This is believed to be 2,4-di-*t*-butyl-4,5-dihydroxy-α-hydromuconic acid, γ-lactone:



III

Upon warming compound III in 2 N NaOH solution, a precipitate formed which sublimed readily from the warm solution and collected as long needles on nearby surfaces. The sublimed material showed a mp of 96°, a mw in benzene of 193, and a content of 72.85% C and 9.72% H. This compound has been identified as 2-*t*-butyl-4-hydroxy-5,5-dimethyl-2-hexenoic acid, γ-lactone:



IV

which has been reported previously (Wiberg and Hutton, 1954) to have an mp of 93.5–94.5° and for which calculated values are: mw, 196; C, 73.5%; H, 10.2%. Both compounds III and IV were prepared by Flaig *et al.* (1955) by alkaline autoxidation of compound I, but neither was identified by them. Compound IV was also isolated here from reaction mixtures when hydrogen peroxide was used to oxidize the *o*-quinone (II).

In the metal-catalyzed oxidation carried out in bicarbonate solution, a similar procedure was followed but involved only two extractions, one at the reaction *p*H, and one after addition of excess HCl.

Reaction of 3,5-di-t-Butyl o-Benzoquinone with Hydrogen Peroxide.—Aqueous 70% methanol was used as the solvent, and 30% hydrogen peroxide was added gradually over a period of at least several minutes. At 40–50° the reaction was usually over within a few minutes after all the peroxide had been added, as determined by failure of the solution to give a red color with Ti^{4+} solution. Isolation of products was accomplished as in the oxidation with oxygen. From the initial ether extraction (at the reaction *p*H) was obtained a white crystalline compound, which was identified as the methyl ester of compound III. This

compound (V), recrystallized from heptane, gave a melting point of 103–106°.

Anal. Calcd for $C_{15}H_{24}O_5$: C, 63.4; H, 8.45; mw, 284. Found: C, 62.4, H, 8.1; mw in benzene, 273.

Treatment of compound III with diazomethane to give the methyl ester gave a compound of melting point 103–105° whose infrared spectrum was identical to that of compound V. From the acidified reaction mixtures was obtained compound III, as before.

Data and Results

Uncatalyzed Reaction of Oxygen with 3,5-di-t-Butyl Pyrocatechol.—In Table I are shown data for experiments in which the main variable with the alkalinity of the system. The principal feature is that the quinone II could be isolated only in those experiments where conditions were relatively mild, i.e., low alkalinity, absence of heating, or short reaction time. The yields of the acid III behave to some extent in an opposite manner, the two highest yields being observed when the reaction mixtures were warmed in dilute NaOH media. The absence of any yield of acid in stronger NaOH is attributable to the fact, previously mentioned, that compound III decomposes on warming in NaOH to give compound IV. The quinone II thus acts very much like an intermediate product, reacting further under more vigorous conditions to give compound III.

Table I
Oxidation of 3,5-di-*t*-Butyl Pyrocatechol by O_2[a]

Expt	(NaOH) (M)	Yield of Acid III	Yield of Quinone II	Remarks
1	[b]	0	20	
2	0.06	<10	40	
3	0.12	6	25	
4	0.25	<10	0	
5	0.06	<10	0	Heated 1 hr at 100° in 1 N NaOH after reaction
6	0.06	20	0	Heated 1 hr at 100° after reaction
7	0.12	25	0	Heated 1 hr at 50° after reaction
8	2.5	0	0	
9	2.5	0	25	Stopped run after absorption of 1 mole O_2 per mole pyrocatechol (1/2 of amt. in no. 8)

[a] 0.12 M pyrocatechol in 75% aqueous methanol; room temperature. [b] No NaOH; 0.1 M $KHCO_3$ medium.

Metal-catalyzed Oxidation of 3,5-di-t-Butyl Pyrocatechol by Oxygen.—The oxidation of the pyrocatechol under mild conditions was explored further by carrying out the reaction in slightly alkaline (bicarbonate) media. Under these conditions a strong catalysis by certain metal ions was found, the only isolable product being the orthoquinone II. In Table II is shown the variation of yield with metal ion. In the case of manganous ion the quinone product was a beautiful red crystalline solid, melting without recrystallization at 109–111°. In experiments with the manganous ion on a larger scale, yields of up to 96% have been obtained. The products from the iron- and cobalt-catalyzed reactions were also crystalline, while the other products, though possessing the characteristic quinone color, were viscous reddish liquids, which crystallized on standing to tacky solids.

Table II
Metal-catalyzed Oxidation of 3,5-di-*t*-Butyl Pyrocatechol with O_2[a]

Metal Ion	Yield of Quinone (%)	Moles O_2 Absorbed per Mole Catechol
Mn^{2+}	88	0.64
Co^{2+}	82	0.63
Fe^{2+}	70	0.55
Cu^{2+}	55	0.61
Zn^{2+}	40	0.67
Ni^{2+}	<40 (estimate)	0.60
	<40 (estimate)	0.52

[a] 0.10 M catechol in 80% aqueous methanol; 0.10 M $KHCO_3$; 0.001 M metal chloride; room temperature.

Qualitatively, the rates of oxygen absorption were fastest with cobalt and manganese, the absorption of 2.5 mmoles of oxygen (about 60 ml) by 50 ml of solution being complete in about 15–20 minutes. The zinc and copper systems required 40–60 minutes for oxygen uptake to cease. The rate with nickel was about the same as the control (no metal ion), while the ferrous system was actually slower than the control. All of the latter three systems (nickel, iron, control) were still absorbing oxygen after 1 hour.

The measured values for oxygen uptake ranged from 0.60 to 0.67 moles per mole of pyrocatechol, and are not particularly significant except for the manganese and cobalt experiments. Here a comparison with yield of quinone indicated that about 0.75 mole of oxygen was consumed per mole of quinone formed, indicating that oxygen was being reduced beyond the hydrogen peroxide stage, presumably to water.

Reaction of 3,5-di-t-Butyl o-Benzoquinone with Hydrogen Peroxide.—The occurrence of this quinone in the reaction mixture, together with demonstration of a readily accessible path from pyrocatechol to quinone with oxygen, suggested the possibility that the quinone was itself oxidized by oxygen or hydrogen peroxide to the ring-fission product III. Experiments involving these two oxidants and the quinone are summarized in Table III. Here again the major variable was the alkalinity, and it can be seen that the greatest yields of ring-fission products were obtained in a moderately alkaline solution. It should be noted that those labeled "0.2 M OH^-" did not actually contain free hydroxide ion, since the initial hydrogen peroxide concentration was larger than this. The optimum *p*H range, therefore, is somewhere between the buffer regions of bicarbonate-carbonate and hydrogen peroxide–peroxide ion, or about 11–12. The significance of experiments 5 and 6 seems to be that the quinone is unstable in free NaOH, decomposing before peroxide was added in experiment 5. In experiment 6, when the same quantities were used, but the NaOH and hydrogen peroxide were mixed prior to addition, reaction apparently occurred with a good yield of product. Experiment 7 shows that oxygen also produces the aliphatic acid, but in lower yield. Experiment 8 shows that in the absence of oxidant no product is observed. In this experiment the red color of the quinone also disappeared during the reaction and no quinone was recovered, strengthening the conclusion that the quinone is unstable toward decomposition in this medium.

Discussion

Metal-catalyzed Oxidation of 3,5-di-t-Butyl Pyrocatechol by Oxygen.—The striking feature of this reaction is the ability of manganese, and to a lesser extent

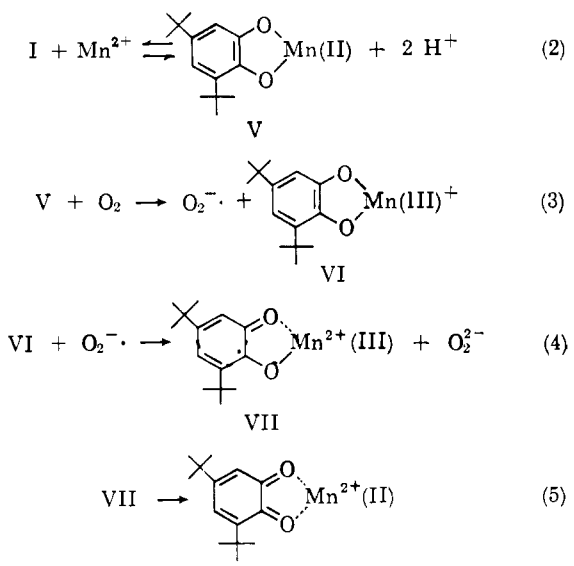
TABLE III
OXIDATION OF 3,5-DI-t-BUTYL o-BENZOQUINONE WITH H_2O_2[a]

Expt	Buffer	Mole Ratio H_2O_2-Quinone	Yield (%)[b] III	IV	V	Total	Remarks
1	0.10 M HCO_3^-	5	0			0	
2	0.05 M HCO_3^- 0.05 M CO_3^{2-}	5	70	15		85	
3	0.10 M CO_3^{2-}	2.5	50			50	
4	0.10 M CO_3^{2-}	5	50		20	70	
5	0.20 M OH^-	5	0			0	NaOH added prior to H_2O_2
6	0.20 M OH^-	5	50			50	H_2O_2 and NaOH added together
7	0.10 M CO_3^{2-}	0	0			0	No quinone recovered after expt
8	0.10 M CO_3^{2-}	0[c]	20			20	

[a] 0.10 M quinone in 70% aqueous methanol; temp., 40–50°. [b] Based on original quinone. [c] O_2 passed into solution.

cobalt, to confine the reaction to almost a single path. The oxidation of phenolic compounds, particularly by oxygen and other free-radical systems, is usually considered to involve aryl radicals of various sorts, and the tarry polymeric products apparently arise from coupling of radicals with each other or with unreacted phenolic molecules, directly or through oxygen bridges (Cosgrove and Waters, 1951; Dermer and Edmison, 1957). That such tar formation occurs here in the absence of catalysts indicates such reactions can occur here too, either through the phenolic hydroxyls or through the unsubstituted 6 position.

In this situation it seems most likely that the function of a polyvalent metal-ion catalyst is to scavenge aryl radicals, oxidizing them to quinones before side reactions can occur. This idea is embodied in the following scheme, using manganese as an example:



(2) (3) (4) (5)

Equation (2) merely involves the reversible formation of a metal chelate of the pyrocatechol. Equations (3) and (4) represent successive 1-electron transfers from the chelate to the oxygen molecule or its reduction products. Reaction (5) represents an internal redox reaction: the transfer of an electron from the organic portion of the chelate to the metal ion.

Besides the obvious requirement of possessing two adjacent oxidation states the metal ion must also meet four requirements with respect to the redox potential of these two stages: (A) the higher oxidation state of the complexed ion must be a sufficiently good oxidant to react readily with the aryl radical in equation (5); (B) the lower oxidation state of the metal ion must be stable toward oxidation by oxygen in the uncomplexed state, in order that it be available for reaction (2); (C) the lower oxidation state must be susceptible to oxidation in the complexed form V; (D) in order to provide for initiation the half-reaction

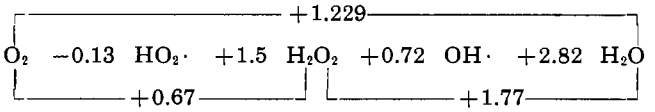


FIG. 1.—Redox potential diagram for reduction of oxygen to water. Numbers are $E°$ values in volts. (Electrode potentials in this paper are given according to the convention in which the oxidized form of the couple is written on the left-hand side of the equation for the half-cell reaction.)

TABLE IV
STANDARD REDOX POTENTIALS OF SOME METAL IONS[a]

	$E°$
Co^{2+}–Co^{3+}	+1.82
Mn^{2+}–Mn^{3+}	+1.51
Fe^{2+}–Fe^{3+}	+0.771
Cu^{+}–Cu^{2+}	+0.153

[a] From Latimer, W. M., Oxidation Potentials, New York, Prentice-Hall, 2nd ed., 1952.

$$O_2 + e^- \longrightarrow O_2^{-\cdot} \qquad (6)$$

must be capable of acting as the oxidant in either (3) or (4), and perhaps both. It is shown participating in (3), and the second half-reaction

$$O_2^{-\cdot} + e^- \longrightarrow O_2^{2-} \qquad (7)$$

is shown participating in (4). The reverse situation is possible, but since neither of the original reactants, oxygen and compound V, would then be involved together, this would require the existence of another initiating reaction.

The reason for phrasing requirement (D) in terms of reaction (6) is shown in Figure 1, which is a potential diagram for the reduction of oxygen, by various successive steps, to water. The step represented by (6) is seen to be the poorest oxidizing couple, and it is therefore the most critical in terms of the reaction scheme.

This mechanism may be viewed in another manner. The oxidation of a pyrocatechol to a quinone is a 2-electron step, involving a very reactive semiquinone radical as a 1-electron intermediate. When complexed with an appropriate metal, however, the first electron

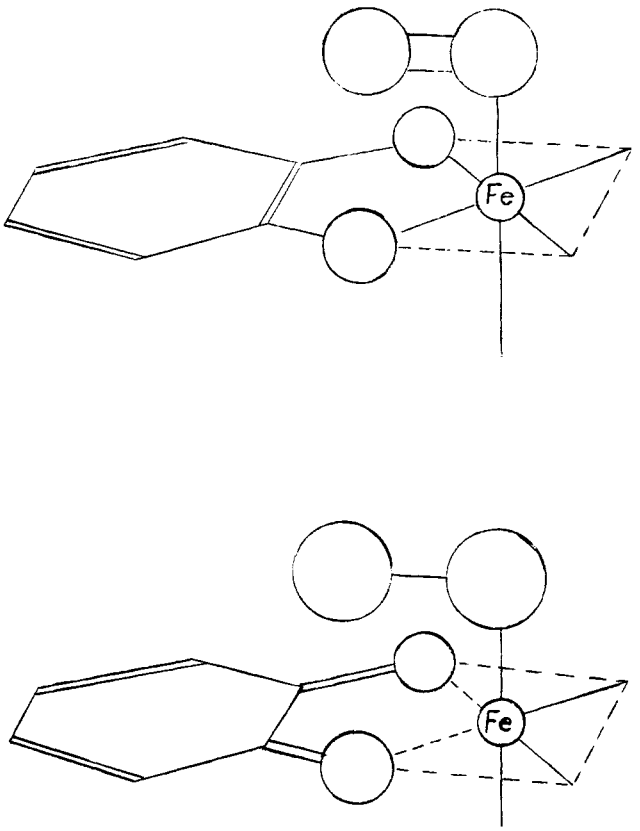


FIG. 2.—Geometry of proposed active complex. (a) (Above), iron(II)–catechol complex and O_2 molecule prior to reaction; (b) (below), peroxide anion, quinone, and iron(II) ion after transfer of two electrons.

is preferentially removed from the metal ion. The semiquinone radical is formed only upon removal of the second electron, and is almost instantaneously eliminated by reaction with the metal ion. The 1-electron intermediate is thus only a relatively unreactive catechol complex, instead of the semiquinone radical.

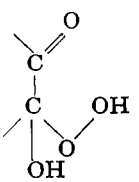
It is probably of some significance that the two best catalysts, manganese and cobalt, have the most positive redox potentials, as Table IV shows. Such a comparison is at best qualitative, since in the reaction system all the ions are highly complexed and the order of potentials might differ significantly from the order of $E°$ values. In general, complexing by ligands where double-bonding is not involved is stronger for the higher oxidation state (Graddon, 1961), and the potentials in the complexed systems are less positive. For example, while the $E°$ of the Fe(II)–Fe(III) couple is +0.77 v, it is in the range of +0.3 in the presence of many amino acids (Perrin, 1959), and is only +0.12 v in the presence of EDTA (Cheng, 1958). The Fe(II)-EDTA chelate is extremely reactive toward oxygen, while ferrous ion in noncomplexing media is not. The Co(II)-Co(III) couple is lowered from $E°$ = +1.82 v to about +0.6 v in the presence of EDTA, and to about +0.1 v in the presence of ammonia (Cheng, 1958). The $Co(NH_3)^{2+}$ ion is also readily oxidized by oxygen while the free cobaltous ion is not. The manganese system presumably behaves similarly, though equivalent data could not be located. However, while the manganous ion is stable toward oxygen, $Mn(OH)_2$ is readily oxidized by oxygen to the (III) and (IV) oxidation states.

It seems reasonable to expect, therefore, that in the presence of a pyrocatechol reaction (3) occurs to some extent with those ions in Table II which exhibit two oxidation states. The fact that oxidation also occurs in the absence of metal ions, as well as with zinc and nickel ions, which do not have other readily accessible oxidation states, suggests that the pyrocatechol is sufficiently readily oxidizable that the O_2-$O_2^{\cdot-}$ half-reaction can also be utilized in reaction (4). In these cases reaction (3) cannot occur and initiation must occur by reaction (4).

The lower yields of quinone observed with iron and copper, according to this mechanism, are owing to the inability of the complexed ions of Fe(III) and Cu(II) to rapidly oxidize the semiquinone; i.e., reaction (5) does not occur readily. The lifetime of the intermediate VII is thereby increased, and the chances that it will undergo a side reaction leading to tars are increased.

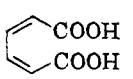
The above mechanism involves only the first two reduction products of oxygen, namely, the radical $O_2^{\cdot-}$ and the peroxide anion O_2^{2-}. The further reduction of peroxide to water should occur in a similar fashion, since the path involved is a similar one; i.e., a 2-electron path with a free-radical intermediate. As Figure 2 shows, however, the couples involved here are much better oxidants than the corresponding ones involved in the reduction of oxygen to peroxide, and the reactions should occur much more readily.

Oxidation of Quinone(II) by Hydrogen Peroxide.—The oxidation of *o*-benzoquinone by hydrogen peroxide has been studied by Witkop (Patchett and Witkop, 1957). Although the reaction is complicated by the initial dimerization of the quinone, he was able to isolate a peroxide adduct of the quinone carbonyl group containing the structure



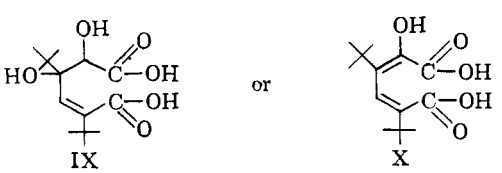
and to observe that this adduct readily decomposed with fission of the carbon-carbon bond to give a dicarboxylic acid.

It seems reasonable to suppose that the oxidation of compound II observed here proceeds by an analogous mechanism. The fact that the best yields are obtained in moderately alkaline solution suggests that the hydroperoxide ion, HOO^-, is probably the reacting species. The reaction is complicated, however, since the actual product III is not that which would be expected from fission of the ring between the carbonyl groups, which is compound VIII:



VIII

Compound III may be considered the γ-lactone derived from either



IX or X

Both of these involve either further oxidation of the quinone ring prior to fission, or subsequent oxidation of the acid VIII after it is formed. The keto form of compound X was actually reported by Flaig (Flaig

et al., 1955) as a product of the alkaline autoxidation of compound I, but was not observed here.

The precise path by which compound II is converted to compound III is obviously not clear. Nor is it clear how compound III is converted to compound IV, since the reaction is more than a simple decarboxylation. The important point to be made is the rapid conversion of the quinone to a product in which fission of the aromatic ring has occurred and two carboxyl groups appear at the terminal carbons.

Possible Mechanism of Pyrocatechase Action.—The incorporation of oxygen into organic compounds, e.g., through autoxidations, generally involves several intermediates, some of which are free radicals containing the oxygen molecule or fragments of it. In a few cases oxygen appears to be directly incorporated to give a peroxide, which further reacts to give products. Examples of this are the formation of transannular peroxides in fused-ring aromatic systems (Russell, 1959), and the reaction of certain aromatic ketones with oxygen (Fuson *et al.*, 1945); Doering and Haines, 1954). In the latter case a cyclic 1,2-peroxide was isolated, which readily decomposed by simultaneous fission of the carbon-carbon and oxygen-oxygen bonds to give products analogous to those observed in pyrocatechase-type systems. This provides some precedent for the peroxide intermediate of Hayaishi. While this is a reasonable intermediate, the steps leading to it are unknown, including the role of the ferrous ion, which is presumed to be involved in the enzyme system.

The possibility that quinones and hydrogen peroxide are intermediates has been considered by various investigators (Mason, 1957), who have used amine-trapping agents and the enzyme catalase to detect the participation of either of these compounds in the enzyme reaction. No positive evidence has yet been obtained, and these results have apparently deterred any strong support for a mechanism involving these intermediates. The failure of tests for hydrogen peroxide and quinones, however, does not eliminate them as intermediates of short life, which may react further before dissociating from the enzyme complex.

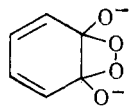
The experiments reported here have shown that quinones are readily formed intermediates in the oxidation of at least a particular pyrocatechol by oxygen, and the following mechanism is proposed as a reasonable one providing a more detailed picture of the enzyme process than has existed heretofore.

The first four steps of the process are identical to equations (2)–(5), except that the metal ion is the ferrous ion and is partly chelated by donor groups attached to the enzyme. With respect to these groups the first requirement to be satisfied is that the ferrous ion not be susceptible to oxidation by oxygen in the resting state, but that the additional complexing provided by the substrate lower the redox potential sufficiently to make the iron susceptible. In view of the effect of complexing with negative donors mentioned above, this is the direction in which the $E°$ value would be expected to shift. However, chelates of ferrous iron with simple ligands are generally readily oxidized by air, and the requirement of insensitivity to oxygen in the resting state probably requires that the iron be bound to the enzyme by unsaturated ligands, such as for example *o*-phenanthroline. These ligands generally shift the $E°$ value of the Fe(II)-Fe(III) couple to more positive values, stabilizing ferrous iron toward oxidation. Alternatively, the iron might be protected from oxidation by being fully coordinated by enzyme groups, some of which could be replaced by substrate. Since some substrates possess only one phenolic group at the site of fission, the shift in $E°$ must be accomplished in some cases by the attachment of a single ligand to the iron.

The second major requirement is that the products of reaction (5), which are the orthoquinone and the peroxide ion, O_2^{2-}, be formed in such a geometry as to be capable of immediate reaction with each other. That they will react rapidly has been demonstrated, and there seems a good chance that the additional assistance provided by a favorable steric location would allow reaction to occur prior to dissociation of the quinone from the enzyme. Actually this imposes a steric requirement only on reaction (4), since this is the reaction which forms these two species. However, it seems likely that the requirement would be met by reaction (3), i.e., by the approach of the original oxygen molecule, since it is necessary that the products of (3) be the same molecules as the reactants in (4). Otherwise free $O_2^{-\cdot}$ radicals, and probably consequently hydrogen peroxide, would be detectable in solution.

Such a geometry is shown in Figure 2a. The reacting oxygen molecule is in a *cis* position in the coordination sphere of the metal ion with respect to a phenolic hydroxyl ligand. Occurrence of reaction (3) will produce an $O_2^{-\cdot}$ radical, one end of which is favorably located to react with the aromatic portion of the chelate. Since this radical is a good oxidizing agent, reaction (3) should be followed rapidly by (4), and further by (5), resulting in the system shown in Figure 2b.

Two possibilities are available for the reaction of the quinone and peroxide ion formed in (4). One involves direct addition of first one, and then both ends of the peroxide anion to the two carbonyl carbons of the quinone, producing structure XI:



XI

Decomposition of this to the dicarboxylate ion merely involves an electronic rearrangement which, as previous work indicates (Patchett and Witkop, 1957), occurs readily.

The second possibility is that the peroxide ion can undergo a Fenton-type reaction with the ferrous ion, producing an Fe(III) species and the ion of the hydroxyl radical:

$$Fe^{2+} + O{-}O^{2-} \longrightarrow Fe{-}O{-}O \longrightarrow FeO^{+}(III) + O^{-\cdot} \quad (8)$$

The radical would unquestionably react with the quinone at once, producing an organic radical which could be oxidized further by the ferric ion. This possibility differs from the first only in breaking the oxygen-oxygen bond before, rather than after, attachment of the oxygen atoms to the quinone. The actual process is probably a hybrid, the weakening of the oxygen-oxygen bond in reaction (8) being accompanied by the formation of bonds between the oxygen atoms and the nearby carbonyl carbon atoms.

This mechanism thus provides for a sequence of four steps, which involve for the most part only electronic rearrangements, all of which by analogy with similar *in vitro* reactions should proceed rapidly. The mechanism accounts for the incorporation of the atoms of the oxygen molecule into the final product, and predicts the absence of any detectable quinone or hydrogen peroxide as intermediates.

In connection with the redox potential requirement, a recent report of an enzyme system of this class stated that both manganese and cobalt ions activated the enzyme, though to a lesser extent than the preferred ferrous ion (Cain, 1962). This is interesting in view of the activity of manganese and cobalt reported here, and may indicate that the redox potential requirement is less severe for this enzyme.

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Biosynthesis of Dipicolinic Acid and of Lysine in *Penicillium citreo-viride**

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The biosynthesis of 2,6-dipicolinic acid by *Penicillium citreo-viride* has been studied using a variety of radioactive substrates. Intermediates from terminal respiration and from the glyoxylate shunt appear in this end product. Radioactive CO_2 was fixed into the ring and into the carboxyl carbons of dipicolinate. The presence of unlabeled propionate enhanced the incorporation of labeled carbonate into dipicolinate and also shifted the ratio of carboxyl to ring activity to favor ring labeling. [1,7-^{14}C]Diaminopimelic acid was transformed by *P. citreo-viride* into dipicolinic acid labeled essentially in its carboxyl groups, but mycelial lysine was devoid of radioactivity. Diaminopimelate could not be detected however, in an acid hydrolysate of the fungal mycelium. Administration of α-[6-^{14}C]aminoadipic acid to *P. citreo-viride* also gave rise to dipicolinate which was predominantly carboxyl labeled. Here, mycelial lysine was revealed by degradation to have been formed directly from α-aminoadipate. D- and L-amino acid oxidases were demonstrated in crude cell-free extracts from *P. citreo-viride;* and it was shown that *meso-* and DL-diaminopimelic acids were oxidized to 2,6-diketopimelate. Chromatographic evidence was found for the presence of diketopimelate in minimal growth medium and in 80% ethanolic mycelial extracts obtained therefrom. The diketo acid was further identified by characterization as its bis-dinitrophenylhydrazone derivative. In replacement experiments which used glucosamine, glutamine, and glutamic acid, respectively, with diketopimelate, increased production of dipicolinic acid resulted. The amino group of L-[^{15}N]-glutamic acid was, however, not incorporated into dipicolinate. It is concluded that dipicolinic acid and lysine are ultimate products which arise from branchpoints in the metabolism of α-ketoadipic acid. This intermediate is postulated either to undergo transamination and reductive amination; or to add a C_2 fragment with subsequent oxidative decarboxylation, to give diketopimelic acid. The nonenzymatic conversion of diketopimelate and ammonia to dipicolinate was confirmed. The last steps of the biosynthesis of dipicolinic acid appear therefore to involve spontaneous reactions from the biologically formed 2,6-diketopimelic acid.

The isolation and characterization of dipicolinic acid (DPA)[1] from aerobic spore-forming bacteria was first reported by Udo (1936) and later by Powell (1953) and by Perry and Foster (1955). From a series of experiments employing radioactive substrates, it was concluded (Perry and Foster, 1955; Martin and Foster, 1958) that the biogenesis of this compound involves the condensation of pyruvate and aspartate (or of alanine and oxalacetate) to yield an unsaturated derivative of α-keto-ε-aminopimelic acid, which then undergoes cyclization and oxidation. It was also shown by these workers that uniformly labeled DAP was incorporated into DPA by *Bacillus megaterium*. Powell and Strange (1959) subsequently pointed out that a facile chemical

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[1] Abbreviations used in this work: DPA, pyridine-2,6-dicarboxylic acid; DKP, 2,6-diketopimelic acid; DAP, 2,6 diaminopimelic acid; α-AAA, α-aminoadipic acid; α-KAA, α-ketoadipic acid; DNPH, 2,4-dinitrophenylhydrazone.