Both intradiol and proximal extradiol dioxygenases are thought to produce the same product, α-hydroxy-
muconic acid, when pyrogallol (3-hydroxycatechol) is used as a substrate. However, when these enzymes were reacted with pyrogallol, they gave different products. A proximal extradiol dioxygenase, metapyrocate-
tachase (catechol:oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.2), gave a product having an absorption maximum at 290 nm, which was gradually converted to a more stable compound having an absorption maximum at 239 nm. On the other hand, an intradiol dioxy-
genase, protocatechuate 3,4-dioxygenase (protocate-
chuate:oxygen 3,4-oxidoreductase (decyclizing), EC 1.13.11.3), gave a product having an absorption maxi-
num at 300 nm. Based on the spectral data and direct comparison with authentic samples, the primary prod-
ucts obtained by the action of the former and the latter enzymes were identified as α-hydroxymuconic acid and
2-pyrone-6-carboxylic acid, respectively. While an-
other intradiol dioxygenase, pyrocatechase (catechol:
oxygen 1,2-oxidoreductase (decyclizing), EC 1.13.11.1),
gave a mixture of nearly equimolar amounts of the
two compounds.

Isotope labeling experiments indicated that 1 atom of
oxygen was incorporated in 2-pyrone-6-carboxylic acid
from the atmosphere. Based on these findings, the re-
action mechanism for the formation of 2-pyrone-6-car-
boxylic acid is discussed. This may be the first experi-
mental evidence indicating the presence of a seven-
membered lactone intermediate during the oxygena-
tive cleavage of catechols, proposed by Hamilton (Ham-
ilton, G. A. (1974) in Molecular Mechanisms of Oxygen
Activation (Hayashi, O., ed) pp. 405-451, Academic
Press, New York).

Dioxygenases are a group of enzymes which catalyze the
incorporation of 2 atoms of molecular oxygen into various
organic substrates. The cleavage of aromatic rings appears to
depend almost entirely upon this type of enzyme (1). When o-
dihydroxyphenyl compounds (catechols) are cleaved by
the action of individual microbial non-heme iron-containing dioxy-
genases, three modes of ring fission have so far been dem-
onstrated (2): (a) oxygenative cleavage of the bond between
the carbon atoms bearing the hydroxyl groups (intradiol cleav-
age); (b) cleavage of the bond between the carbon atoms at
positions 2 and 3 (proximal extradiol cleavage); and (c) that
between these at positions 1 and 6 (distal extradiol cleavage)
(Fig. 1).

Pyrocatechase (catechol:oxygen 1,2-oxidoreductase (decy-
lizing), EC 1.13.11.1) which catalyzes the conversion of cate-
chol to cis, cis-muconic acid (cis, cis-6-carboxy-2,4-pentadi-
enic acid) (Equation 1) is a typical enzyme catalyzing an
intradiol cleavage reaction (3). Metapyrocatechase (catechol:
oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.2) cata-
yzes the conversion of catechol into α-hydroxymuconic ε-
semi-aldehyde (2-oxo-6-oxo-2,4-hexadienoic acid) (4, 5) (Equa-
tion 2). When substituted catechol derivatives are subjected
to this enzyme as substrates, they are cleaved exclusively at
the proximal site (2). Thus, metapyrocatechase is a typical
enzyme which catalyzes a proximal extradiol cleavage reac-
tion. When protocatechueic acid is cleaved by the action of
protocatechuate 3,4-dioxygenase (protocatechuate:oxygen 3,4-
oxidoreductase (decyclizing), EC 1.13.11.3) (6) and protocate-
chuate 4,5-dioxygenase (protocatechuate:oxygen 4,5-oxidore-
ductase (decyclizing) EC 1.13.11.8) (7, 8), γ-carboxy cis, cis-
muconic acid (3,5-dicarboxy-2,4-pentadienoic acid) (Equation
3) and α-hydroxy-γ-carboxymuconic ε-semialdehyde (2-oxo-6-
oxo-4-carboxy-2,4-hexadienoic acid) (Equation 4) are formed,
respectively. The former is also a typical enzyme which carries
out an intradiol cleavage and the latter catalyzes an extradiol
 cleavage reaction at the distal position.

Among these dioxygenases which contain non-heme iron as
a sole cofactor, the intradiol enzymes such as pyrocatechase
and protocatechuate 3,4-dioxygenase contain the ferric form of iron (9). It
was believed that the dioxygenation reaction was highly spe-
cific with respect to the cleavage site of the catechol ring.
Namely, the ferrous iron-containing dioxygenases catalyze the
extradiol cleavage, while the ferric iron-containing dioxy-
genases catalyze the intradiol cleavage (9).

Recently, however, we reported that pyrocatechase cata-

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Cleavage of Pyrogallol by Dioxygenases

FIG. 1. Three modes of ring fission of catechols.

FIG. 2. Oxygenative cleavage of pyrogallol by three different dioxygenases, metapyrocatechase, protocatechuate 3,4-dioxygenase, and pyrocatechase.

FIG. 3. Spectral changes during the cleavage reaction of pyrogallol by the action of metapyrocatechase (a), protocatechuate 3,4-dioxygenase (b), and pyrocatechase (c).

MATERIALS AND METHODS

Crystalline metapyrocatechase with a specific activity of about 300 and crystalline protocatechuate 3,4-dioxygenase with a specific activity of about 80 were prepared by the methods described previously from Pseudomonas arvilla (ATCC 23973) (11) and Pseudomonas aeruginosa (ATCC 23976) (12), respectively. Pyrocatechase was purified as previously described (10) from P. arvilla C-1 (ATCC 23974). Pyrogallol was obtained from Tokyo Kasei Co., Ltd. (Tokyo) and purified by sublimation under reduced pressure. 2-Pyrene-6-carboxylic acid, m.p. 228-230°C, was synthesized by cyclization of the condensation product obtained from diethyl oxalate and ethyl crotonate.

In this paper, we present some evidence indicating that the three dioxygenases gave different products when pyrogallol was used as a substrate. Metapyrocatechase converted pyrogallol into a-hydroxymuconic acid, whereas protocatechuate 3,4-dioxygenase yielded 2-pyrene-6-carboxylic acid. Pyrocatechase gave both products in nearly equimolar amounts (Fig. 2). The reaction mechanism for the formation of 2-pyrene-6-carboxylic acid is also discussed, based on the data obtained from isotope labeling experiments.
ate (13). α-Hydroxymuconic acid was synthesized from diethyl α-hydroxymuconic acid (14). 14O-enriched oxygen gas (60 atom % enriched) and water (20.3 atom % enriched) were obtained from Prochem, British Oxygen Company Ltd. (England). Precast plates of Silica Gel F254 for thin layer chromatography were products of Merck. Dizothemamine was prepared from N-nitrosomethyl urea by the method of Arndt (15). All other chemicals were of analytical grade and purchased from commercial sources.

**Instrument**—Spectrophotometric measurements were carried out with a Union Giken model SM 401 recording spectrophotometer. Infrared spectra were recorded on a Hitachi model EPS12 or model 225S spectrometer in a potassium bromide disk or in chloroform. The proton nuclear magnetic resonance spectra were recorded with a Varian T-60 spectrometer in deuterated chloroform with tetrakisylsilane as an internal standard. The mass spectra were obtained using a Hitachi model RMU 6D spectrometer operating at 75 eV. Samples were introduced into the ionization chamber by means of a direct inlet system. High pressure liquid chromatography was performed with a Hitachi HPLC model 635 equipped with a Union Giken spectrometer and a Yanagimoto micro-hot stage apparatus and were uncorrected.

**RESULTS**

**Spectra of Reaction Products**—Metapyrocatechase gave a product having an absorption maximum at 290 nm at pH 7.5 (Fig. 3a). This initial product was gradually converted to a more stable compound having an absorption peak at 238 nm (Fig. 3a). This spectral change indicated the conversion of a single species because of the presence of an isosbestic point at 265 nm. When the reaction medium was made acidic (pH 1.0) by the addition of 4 N HCl, the absorption maximum moved immediately to 303 nm and increased in absorbance. The addition of 4 N NaOH to make the reaction mixture basic (pH 12.0) caused a shift of the absorption peak to 350 nm. The peak at 350 nm returned to 303 nm on the addition of 4 N HCl (pH 1.0), whereas protocatechuate 3,4-dioxygenase gave a product having an absorption peak at 300 nm at pH 7.5 (Fig. 3b). Upon the addition of 4 N HCl (pH 1.0), the absorption peak moved slightly to 302 nm with a concomitant increase in the absorbance. The magnitude of the increase in absorbance was not to the same degree as noted in the case of metapyrocatechase. However, upon the addition of 4 N NaOH (pH 12.0), a new spectral species having a peak at 350 nm appeared which was found to be the same as noted in the case of metapyrocatechase, as shown in Fig. 3b. Further addition of 4 N NaOH (pH 1.0) resulted in the shift of the absorption maximum from 350 to 303 nm. Therefore, the primary reaction products of pyrogallol resulting from the action of metapyrocatechase and protocatechuate 3,4-dioxygenase were not identical. However, in a basic medium, both primary reaction products were converted to the same compound, having an absorption maximum at 350 nm.

When pyrogallol was incubated with pyrocatechase, an absorption peak at 295 nm appeared initially, which then gradually decreased with concomitant formation of an absorption peak at 238 nm. The product(s) finally formed at pH 7.5 showed absorption peaks at 238 and 298 nm as shown in Fig. 3c. This spectrum resembled that obtained on the addition of the two spectra of the reaction products of metapyrocatechase and protocatechuate 3,4-dioxygenase. The absorbance peak of the reaction product shifted to 303 nm under acidic conditions and to 350 nm under basic conditions as shown in the figure. Thus, it was suggested that the reaction product of pyrocatechase was a mixture of the reaction products obtained with metapyrocatechase and protocatechuate 3,4-dioxygenase.

**High Pressure Liquid Chromatography of the Reaction Products**—In order to determine whether the reaction products resulting from the action of pyrocatechase are a mixture of the compounds produced by the action of metapyrocatechase and protocatechuate 3,4-dioxygenase, high pressure liquid chromatography of the products was carried out using a column packed with Hitachi gel 3050 octadesyl-silanized silica particles and 30% methanol as a mobile phase at a flow rate of 1 ml/min. The products obtained by the action of metapyrocatechase and protocatechuate 3,4-dioxygenase gave single peaks with retention volumes of 3 and 1.75 ml, respectively, while the products of pyrocatechase showed two peaks, whose retention volumes were identical with those of the products obtained by the action of metapyrocatechase and protocatechuate 3,4-dioxygenase, respectively. Essentially the same results were obtained from thin layer chromatography using silica gel plates and chloroform-methyl acetate-methanol (1:2:2) as a developing solvent.

**Isolation and Identification of the Reaction Product Formed by Metapyrocatechase**—Incubation was carried out at 20°C in 100 nM potassium phosphate buffer, pH 7.5, containing 40 mg of metapyrocatechase and 30 mg of pyrogallol in a final volume of 50 ml. The reaction was performed by stepwise addition, 5 mg each time, of pyrogallol in order to prevent nonenzymic degradation. During the reaction, the pH was maintained at 7.5 by occasional addition of 2 N NaOH. After completion of the reaction, the reaction medium was made acidic (pH 2.9) by the addition of 4 N HCl. After the reaction mixture was saturated with NaCl, the reaction product was extracted three times with ethyl acetate, 30 ml each time. The ethyl acetate extracts were combined, washed with water saturated with NaCl, dried over anhydrous sodium sulfate, and evaporated to dryness. Thin layer chromatography...
Cleavage of Pyrogallol by Dioxygenases

Isolation and Identification of the Reaction Products Formed by Pyrocatechase—The cleavage reaction was carried out in 190 mM potassium phosphate buffer, pH 7.5, containing 50 mg of pyrocatechase and 80 mg of pyrogallol in a final volume of 100 ml. Incubation and isolation of the reaction products were carried out in the same ways as for the reaction product of protocatechuate 3,4-dioxygenase. Preparative thin layer chromatography gave dimethyl α-methoxymuconate (11 mg), m.p. 52-53°C, which was identified as dimethyl α-methoxymuconate by comparison with an authentic sample (10). 

Isolation and Identification of the Reaction Product Formed by Protocatechuate 3,4-Dioxygenase—Incubation was carried out in essentially the same manner as above using 126 mg of pyrogallol and 30 mg of protocatechuate 3,4-dioxygenase in a total volume of 150 ml. After completion of the reaction, the reaction mixture was subjected to ultrafiltration using a Diaflo apparatus with a UM-30 membrane to separate protein. To the filtrate, 6 N HCl was added to decrease the pH of the filtrate to 2.0. After the filtrate was saturated with NaCl, the reaction product was extracted three times with ethyl acetate, 50 ml each time. The ethyl acetate extracts were combined, dried over anhydrous Na2SO4, and evaporated to give an oil (105 mg), which showed a major spot (Rf 0.38) that co-migrated with authentic 2-pyrone-6-carboxylic acid on a silica gel thin layer chromatogram using chloroform:ethyl acetate:methanol (1:2:2) as a solvent system. The oil was dissolved in a minimum volume of methanol and methylated with diazomethane. Preparative thin layer chromatography on silica gel using chloroform:methanol (99:1) as a solvent system. The oil was dissolved in a minimum volume of methanol and methylated with diazomethane. Preparative thin layer chromatography on silica gel using chloroform:methanol (99:1) as a solvent system. The oil was dissolved in a minimum volume of methanol and methylated with diazomethane. Preparative thin layer chromatography on silica gel using chloroform:methanol (99:1) as a solvent system. The oil was dissolved in a minimum volume of methanol and methylated with diazomethane. Preparative thin layer chromatography on silica gel using chloroform:methanol (99:1) as a solvent system. The oil was dissolved in a minimum volume of methanol and methylated with diazomethane.

Tracer Experiments—To elucidate the reaction mechanism for the formation of 2-pyrone-6-carboxylic acid from pyrogallol by the action of protocatechuate 3,4-dioxygenase, experiments were carried out with 15O2 and H18O as tracers. The reaction with 15O2 was carried out in a 100-ml two-necked round-bottomed flask. By use of a three-way stopcock, the flask containing the reaction mixture containing 50 mM potassium phosphate buffer, pH 7.5, and 10 mg of protocatechuate 3,4-dioxygenase in a total volume of 20 ml was evacuated and then filled with oxygen-free dry nitrogen. After repeating this

Fig. 6 Mass spectra of methyl esters of reaction products from pyrogallol with protocatechuate 3,4-dioxygenase. a, methyl ester obtained from the unlabeled reaction product; b, methyl ester obtained from the reaction product formed in the presence of 13C15O2; and c, methyl ester obtained from the reaction product formed in the presence of H18O.

Fig. 7 Fragmentation pattern of methyl 2-pyrone-6-carboxylate.
process five times, a mixture of 60 atom % enriched $^{18}\text{O}_2$ and oxygen-free nitrogen (1:4 by volume) was introduced. After the reaction mixture was stirred for 30 min to equilibrate with $^{18}\text{O}_2$, the reaction was started by the addition of an oxygen-free solution of pyrogallol (100 mg) in potassium phosphate buffer through a rubber stopper using a syringe. The reaction was continued for 2.5 h at 20°C with stirring. The reaction product was isolated as its methyl ester, $\text{z.e.}$ methyl 2-pyrone-6-carboxylate (21 mg), m.p. 123-124.5°C, by preparative thin layer chromatography as described above.

The mass spectrum of the unlabeled ester is shown in Fig. 6b.

**Table I**

| Experiment | Peak height ratio |
|------------|------------------|
| 1          | 154:156          |
| 2          | 136:128          |
| 3          | 123:125          |
| 4          | 95:97            |
| 5          | 67:69            |
| 6          | 59:61            |

The numbers in parentheses are atom % of excess $^{18}\text{O}_2$ incorporated into the product.

**Fig. 9.** Spectral changes of 2-pyrone-6-carboxylic acid during pH-dependent hydrolysis and isomerization. The sample cuvette contained 63 nmol of 2-pyrone-6-carboxylic acid in 50 mM potassium phosphate buffer, pH 7.5, in a total volume of 3 ml. The pH of the mixture was changed stepwise with the dropwise addition of either 4 N HCl or 4 N NaOH in the following order: 7.5(I) → 1.0(II) → 12(III) → 7.5(IV and V) → 1.0(VI). Curves I to VI represent spectra taken at each pH, respectively. Curve III was recorded with an absorbance range of 0 to 2. See the text for details.

**Fig. 10.** Hydrolysis and isomerization process of 2-pyrone-6-carboxylic acid. Spectra of these compounds are shown in Fig. 9. Compound number in this figure corresponds to the spectrum number in Fig. 9. See the text for details. Names of compounds are as follows: I, 2-pyrone-6-carboxylic acid anion; II, 2-pyrone-6-carboxylic acid; III, $\text{a-hydroxymuconic acid trianion}$; IV, $\text{a-hydroxymuconic acid enol dianion}$; V, $\text{a-hydroxymuconic acid enol dianion keto isomer}$; and VI, $\text{a-hydroxymuconic acid}$.

6a. Besides the parent ion peak at $m/e$ 154, the presence of four prominent fragment ion peaks at $m/e$ 126, 123, 55, and 67 can readily be interpreted (16, 17) in terms of the process shown in Fig. 7. The measurement of the relative peak heights at $m/e$ 154, 156, and 158 showed the total incorporation of $^{18}\text{O}$ into the molecule. The presence of the isotope peaks of the following fragment ions, $m/e$ 126, 123, 95, and 67, and the peak height ratios of these ions allowed the position of any isotopic oxygen incorporated into the ester to be determined. The mass spectrum of the ester obtained by the reaction under $^{18}\text{O}_2$ (Fig. 6b) showed a substantial peak at $m/e$ 156. Thus, the ester contained 1 atom of $^{18}\text{O}$. The presence of an isotope peak at $m/e$ 128 and the absence of a peak at $m/e$ 97 indicated that the isotope oxygen was incorporated into ester oxygens but not into lactone oxygens. As shown in Table I, isotope oxygen was distributed in both ester oxygens in preference to methoxy oxygen, that is, ester carbonyl oxygen was enriched by a 27 atom % excess. A control experiment using a 5 atom % excess of H$_2^{18}$O indicated no incorporation of isotope oxygen into ester oxygen.
spectroscopy. As shown in Fig. 8, lactone carbonyl and ester carbonyl bands appeared at 1748 and 1730 cm⁻¹, respectively, and the intensity of the lactone carbonyl band is lower than that of the ester carbonyl band (upper trace). However, the relative intensity of these two carbonyl bands was inverted in isotope labeled ester (lower trace) and a new absorption band appeared at 1705 cm⁻¹, which was assignable to the C=¹⁸O ester carbonyl stretching mode.

Isomerization of 2-Pyrene-6-carboxylic Acid—An aqueous solution of 2-pyrene-6-carboxylic acid showed an absorption maximum at 303 nm at pH 7.5 (I in Fig. 9), the major absorption coefficient of which was calculated to be ϵ = 7,500. In acidic medium (pH 1.0), this peak shifted to 301 nm (ε = 7,600 (II in Fig. 9). Upon addition of alkali (pH 12.0), an intense absorption peak appeared at 350 nm (ε = 21,000) (III in Fig. 9). When the pH of the solution was decreased to 7.5 by the addition of 4 N HCl, this intense peak disappeared and a new absorption spectrum having a maximum at 295 nm was obtained (IV in Fig. 9). Under the neutral conditions (pH 7.5), the peak at 295 nm gradually disappeared with a concomitant increase in absorbance at 237 nm (V in Fig. 9). Upon further addition of 4 N HCl to make the solution acidic (pH 1.0), an absorption maximum appeared at 302 nm (VI in Fig. 9). On addition of 4 N NaOH to this solution (pH 12.0), the peak at 350 nm reappeared.

The above mentioned spectral changes of 2-pyrene-6-carboxylic acid in acidic and basic conditions could be explained in terms of the hydrolysis and isomerization process shown in Fig. 10. 2-Pyrene-6-carboxylic acid exists as its anion (I) having an absorption maximum at 303 nm at pH 7.5, whereas its protonated form (II) showed an absorption peak at 301 nm at pH 1.0. In the basic medium (pH 12.0), hydrolysis of the δ-lactone moiety occurred to form α-hydroxymuconic acid as its trianion form (III) which showed an absorption peak at 350 nm. Protonation of the enolate anion moiety to form the enol dianion (IV) occurred at pH 7.5. Under the neutral conditions, this enol was isomerized to a more stable keto form (V) which had an absorption maximum at 237 nm. The keto dianion (V) was protonated to form VI having an absorption maximum at 302 nm in acidic medium (pH 1.0). Structural interconversions among forms III, IV, V, and VI are reversible processes depending on the pH of the medium.

DISCUSSION

It was supposed that both intradiol and proximal extradiol cleavage reactions on pyrogallol produced the same product, α-hydroxymuconic acid. Actually, however, as indicated in Fig. 2, pyrogallol was converted to different products by the action of intradiol and proximal extradiol types of non-heme iron-containing dioxygenases. That is, metapyrocatechase which catalyzes the proximal extradiol cleavage reaction afforded α-hydroxymuconic acid as a sole product. On the other hand, protocatechuic 3,4-dioxygenase catalyzing the intradiol cleavage reaction yielded 2-pyrene-6-carboxylic acid; while a mixture of nearly equimolar amounts of these two compounds was obtained from pyrogallol by the action of another intradiol enzyme, pyrocatechase. Therefore, with pyrogallol, pyrocatechase catalyzed both intradiol and proximal extradiol cleavage simultaneously. We reported previously that pyrocatechase catalyzed simultaneously both intradiol and proximal extradiol cleavage reactions with 3-methoxycatechol and 3-methoxyacetophenone. These findings were recently confirmed by other investigators (18). The ratios of intradiol to extradiol cleavage for 3-methoxycatechol and 3-methoxyacetophenone were reported to be 17 and 5, respectively (10), and that for pyrogallol (3-hydroxycatechol) was found to be about 1. The difference in the ratio of the two cleavage reactions is probably due to the electron distribution of substrates, but further studies are needed to confirm this.

As to the cleavage of the catechol ring to form muconic acid derivatives, two pathways have so far been proposed (Fig. 11). 1) A cyclic four-membered ring dioxetane (ii), possibly formed from the peroxide (i), is involved as an intermediate (3); and 2) a seven-membered lactone (iii) is involved as an intermediate. The latter pathway was proposed by Hamilton (19) based on the calculation of bond energy and the absence of chemiluminescence during the dioxygenation reaction. Provided that the cis,cis-muconic acid derivative is involved as an intermediate for the formation of 2-pyrene-6-carboxylic acid from pyrogallol, there are two ways to form the lactone from α-hydroxymuconic acid as shown in Fig. 11. By pathway 1, 2-pyrene-6-carboxylic acid is formed by proton-promoted nucleophilic addition of a carboxylic anion to the double bond and subsequent dehydration. In this case, the 2-pyrene-6-carboxylic acid formed must contain 2 atoms of oxygen from the atmosphere. Through pathway 2, dehydration between C₂=O—OH and C₆=COOH forms 2-pyrene-6-carboxylic acid carrying 1 atom of oxygen from molecular oxygen. With this mechanism, the isomerization of the double bond between C₂ and C₆ must occur provided that the geometry of the double bond was conserved during the oxygenative fission as proposed previously (3). To ascertain which pathway is used during the formation of 2-pyrene-6-carboxylic acid, it is necessary to know whether or not the isotope oxygen from ¹⁸O is incorporated into lactone ether oxygen by an isotope labeling experiment. The tracer experiment indicated that 1 atom of ¹⁸O was incorporated into 2-pyrene-6-carboxylic acid which was isolated as its methyl ester. Mass and infrared spectroscopy clearly indicated that the isotopic oxygen was distributed in both oxygens of the ester moiety. However, neither of the oxygens of the lactone moiety contained an isotopic oxygen. Distribution of isotopic oxygen in both oxygens of the ester group is explained by the well known equilibration of the carboxylate group in the reaction mixture (20). However, the fact that no isotopic oxygen is incorporated into the lactone moiety suggests that the free dicarboxylic acid is not involved in the formation of the lactone.

Metapyrocatechase showed no esterase activity on 2-pyrene-6-carboxylic acid since the formation of α-hydroxymuconic acid was not observed when the lactone was incubated
with the enzyme. Likewise, protocatechuic 3,4-dioxygenase showed no lactonizing enzyme activity since 2-pyrene-6-carboxylic acid was not formed when α-hydroxymuconic acid was incubated with the enzyme.

This experimental evidence indicates that 2-pyrene-6-carboxylic acid is a primary product formed directly from pyrogallol by the action of protocatechuic 3,4-dioxygenase and eliminates the possibility of the existence of free dicarboxylic acid as a dissociable intermediate. If the free dicarboxylic acid exists as an intermediate, at least one of the two oxygens of the lactone moiety must be derived from molecular oxygen. Therefore, the formation of 2-pyrene-6-carboxylic acid from pyrogallol could not be explained by the pathway shown in Fig. 11, in which free carboxylic acid is involved via either a four-membered dioxetane intermediate (ii) or a seven-membered lactone (iii). Hitherto, none of the evidence reported concluded whether the dioxygenase reaction proceeds through an intermediate such as a cyclic four-membered dioxetane or a seven-membered lactone. The present experimental results may be explained by the reaction mechanism shown in Fig. 12. In this scheme, the presence of an enol group is important and tautomerization of the enol to the keto form in the fifth step may make it possible to form the lactone, as shown in Fig. 12. When the tautomerization of the enol group is hampered in some way by the specificity of the enzyme, the seven-membered lactone would be cleaved to form the muconic acid derivative. The question whether or not this reaction mechanism is applicable to other dioxygenase reactions needs further experiments to be answered. Nevertheless, this is the first experimental evidence indicating the participation of the seven-membered lactone proposed by Hamilton (19) in the dioxygenase reaction.

Oxygenases are classified into two groups, monooxygenases and dioxygenases (21). They are defined as groups of enzymes that catalyze the incorporation of 1 and 2 atoms of molecular oxygen into their substrate, respectively. In this sense, the formation of 2-pyrene-6-carboxylic acid from pyrogallol catalyzed by protocatechuic 3,4-dioxygenase may belong to the category of monooxygenase reactions, judging from the stoichiometry of the overall reaction. However, in a monooxygenation reaction, 1 atom of oxygen is incorporated into the substrate and the other atom of oxygen is reduced by either an external or internal reductant to form water. In the formation of 2-pyrene-6-carboxylic acid from pyrogallol, however, a reductant is not required and 1 of the 2 oxygen atoms incorporated into the substrate is eliminated as water. Thus, mechanistically the reaction would be essentially the same as a dioxygenase reaction. In any case, it is of interest that individual dioxygenases act on pyrogallol to form either 2-pyrene-6-carboxylic acid or α-hydroxymuconic acid, both of which are converted to the same product, compound II shown in Fig. 10, in an alkaline solution. These facts may provide a clue for the understanding of the reaction mechanism of dioxygenases.

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