Lynn, W. S., Jr., Staple, E., and Gurin, S. (1954), *J. Am. Chem. Soc. 76*, 4048.
Monod, J., Changeux, J., and Jacob, F. (1963), *J. Mol. Biol. 6*, 306.
Oertel, G. W., and Eik-Nes, K. B. (1959), *Anal. Chem. 31*, 98.
Péron, F. G., and Koritz, S. B. (1960), *J. Biol. Chem. 235*, 1625.
Roberts, K. D., Bandi, L., Drucker. W. D., and Lieberman, S. (1964), *J. Am. Chem. Soc. 86*, 958.
Rosenkrantz, H. (1953), *Arch. Biochem. Biophys. 44*, 1.
Samuels, L. T. (1960), *Metab. Pathways 2*, 431.
Shimizu, J., Dorfman, R. I., and Gut, M. (1960), *J. Biol. Chem. 235*, PC25.
Shimizu, J., Hayano, M., Gut, M., and Dorfman, R. I. (1961), *J. Biol. Chem. 236*, 695.
Solomon, S., Levitan, P., and Lieberman, S. (1956), *Rev. Can. Biol. 15*, 282.
Stone, D., and Hechter, O. (1954), *Arch. Biochem. Biophys. 51*, 457.

Fatty Acid Distribution in Bacterial Phospholipids. The Specificity of the Cyclopropane Synthetase Reaction*

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The distribution of fatty acids in purified phospholipids isolated from several different bacteria has been examined by use of the specific phospholipase A of snake venom. In general the distribution is in accord with that found elsewhere in nature; the unsaturated acids are found in the β position and the saturated acids in the γ position. The distribution of cyclopropane fatty acids follows closely that of the unsaturated fatty acids. A notable exception was encountered with the phosphatidylethanolamine of *Clostridium butyricum*, in which unsaturated and cyclopropane fatty acids were found in more abundance in the γ position. The specificity of the *C. butyricum* cyclopropane synthetase reaction has been examined by employing as substrate a phosphatidylethanolamine with a known distribution of unsaturated fatty acids and analyzing the distribution of cyclopropane fatty acids in the phospholipid produced by the enzyme reaction. These experiments indicate that the enzyme has a definite, but not absolute, specificity for an unsaturated fatty acid in the γ position.

Natural phosphoglycerides commonly show a nonrandom distribution of fatty acids such that the γ (or α') position is usually occupied by a saturated fatty acid, while unsaturated fatty acids are usually found in the β position. This fact finds its explanation, at least in part, in the specificity of certain enzymes for catalyzing acylation of lysophosphatides. Thus the studies of Lands (Lands and Merkl, 1963; Merkl and Lands, 1963) with liver enzymes show clearly that the acylating enzymes will discriminate between saturated and unsaturated fatty acid coenzyme A esters in determining which acid will occupy the β or γ position.

Nearly all studies of the distribution of fatty acids in phospholipids have employed the highly specific snake venom phospholipase A. The specificity of this enzyme has been in question during recent years, but because of the elegant work of Van Deenen and De Haas (1963), there is now no doubt that this enzyme cleaves a fatty acid esterified to a hydroxyl group on a carbon adjacent to a carbon bearing a phosphate ester. The steric relationship of this *vic*-glycol system is also important, so that in the natural phosphoglycerides only the β-ester in the L-α-glycerophosphate type would be subject to hydrolysis. The products from a natural phosphoglyceride are a free fatty acid arising from the β-ester and a lysophosphatide which contains the γ-ester. These products may be separated quite easily and examined.

The presence in several species of bacteria of fatty acids containing a cyclopropane ring (O'Leary, 1962), as well as the high proportion of phospholipid in these organisms (see, for example, Kaneshiro and Marr, 1962) lends particular interest to an examination of phospholipids from this source. Furthermore, recent studies of the synthesis of cyclopropane fatty acids in *in vitro* systems (Zalkin *et al.*, 1963; Chung and Law, 1964) have shown that this reaction involves olefinic fatty acids already esterified in intact phospholipids. On the basis of this finding, one could predict that if this reaction is an important one *in vivo* and if there is no subsequent redistribution of fatty acids, the distribution of cyclopropane fatty acids and olefinic fatty acids should be similar. Also, the techniques for examining the distribution of fatty acids enable one to investigate the question of whether the cyclopropane synthetase system *in vitro* has a specificity for an olefinic fatty acid in the β or γ position of the phospholipid substrate.

EXPERIMENTAL

Materials

DEAE-cellulose for column chromatography was obtained from Carl Schleicher and Schuell Co., Keene, N.H., and was washed as suggested by Rouser *et al.* (1961). Silica gel for thin-layer chromatography was obtained from Brinkmann Instruments, Westbury, N.Y., or from Research Specialties Co., Richmond, Calif. These materials occasionally contained volatile impurities which later interfered with gas-chromatographic analysis of the fatty acid esters. This problem was eliminated by extraction of the silica gel with solvents before use, or by use of Adsorbosil-1 (Applied Science Laboratories, Inc., State College, Pa.), which was found to be relatively free of impurities.

Standard fatty acids were purchased from Applied

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Science Laboratories, State College, Pa. Standard cyclopropane fatty acids were prepared by the method of Simmons and Smith (1959).

S-[*methyl*-^{14}C]Adenosylmethionine with a specific activity of 13.78 mcuries/mmole was purchased from Tracerlab, Waltham, Mass. S-Adenosylmethionine was prepared from activated baker's yeast according to Schlenk *et al.* (1959), and purified by the method of Mudd (1959).

Dried venom of *Ancistrodon piscivorus piscivorus* was obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., and stored at 4°. Sodium lauryl sulfate was purchased from Sigma Chemical Co., St. Louis, Missouri. Radioactive lipid samples were assayed in a Packard Tri-Carb instrument using a scintillation fluid prepared by dissolving 15.18 g 2,5-diphenyloxazole and 189 mg 2,2-*p*-phenylenebis-(5-phenyloxazole) in 1 gallon of toluene. Crude and partially purified preparations of the *Clostridium butyricum* cyclopropane synthetase system were generously provided by Dr. A. E. Chung.

Methods

Growth of cells.—*Escherichia coli* B *and Serratia marcescens* (ATCC 8195) cells were grown in an inorganic salts medium supplemented with 2% glucose (Law *et al.*, 1963) at 37° and 30°, respectively. *Clostridium butyricum* (ATCC 6015) cells were grown anaerobically at 37° on either a synthetic medium (Goldfine, 1962) or the medium of Wolfe and O'Kane (1953). Cells were harvested during logarithmic or stationary phase of growth with a Sharples centrifuge and were stored at −20°.

Preparation of Bacterial Phospholipides.—Total lipids were extracted with chloroform-methanol mixtures, and the solutions were washed, by the method of Folch *et al.* (1957). The lipid solutions were evaporated to dryness under reduced pressure in a rotary evaporator at 30–40°. The residue was redissolved in chloroform-methanol, 7:1 (v/v) and subjected to chromatography on DEAE-cellulose according to Rouser *et al.* (1961). Phosphatidylethanolamine admixed with neutral lipids was eluted in the chloroform-methanol 7:1 (v/v) and 7:3 (v/v) fractions, which were evaporated to dryness under reduced pressure and redissolved in the minimal volume of chloroform. *E. coli* phosphatidylethanolamine was precipitated from this solution by the addition of 4–5 volumes of cold acetone. The precipitate was collected by centrifugation and dissolved in benzene, and this solution was lyophilized to give a white powder. The product was homogeneous when examined by thin-layer chromatography on silicic acid using the solvent system of Wagner *et al.* (1961). Phosphatidylethanolamines from *S. marcescens* and *C. butyricum* were purified by preparative thin-layer chromatography of the crude fractions from DEAE-cellulose chromatography. Silicic acid films were applied to 8 × 8-in. glass plates by the adhesive-tape method of Lees and DeMuria (1962), using two thicknesses of tape. The sample was dissolved in a minimal amount of chloroform and this solution was applied as a band across the plate. After development, the plate was dried and the lipid bands were stained by spraying the plate with a 0.2% solution of 2′,7′-dichlorofluorescein in 95% ethanol. The band containing phosphatidylethanolamine was identified by comparing R_F values with a pure phosphatidylethanolamine standard. The band was scraped from the plate and the phosphatidylethanolamine was recovered from the silicic acid by three successive extractions with methanol. The extracts were combined and evaporated to dryness under a stream of pure, dry nitrogen at 30–40°. The sample was stored at 4° until used. These preparations invariably contained some 2′,7′-dichlorofluorescein, but this did not interfere with any subsequent operations.

Purified *Azotobacter agilis* phosphatidylethanolamine was kindly supplied by Dr. A. E. Chung. Phosphatidylethanolamine and phosphatidylcholine of *Agrobacterium tumefaciens* were purified from lipid extracts generously provided by Dr. T. Kaneshiro. The *A. tumefaciens* phosphatidylethanolamine contained some of the *N*-monomethyl derivative (Kaneshiro and Law, 1964).

In the case of phosphatidylethanolamine from *C. butyricum*, an organism which contains large amounts of plasmalogens and phosphatidyl-*N*-methylethanolamine, a special procedure was used. After preliminary chromatography on DEAE-cellulose, the crude phosphatidylethanolamine fraction was subjected to the acetic acid hydrolysis procedure of Gray (1958) for removal of plasmalogens. This method was modified in such a way that incubation of the reaction mixture for 24 hours at 37° was followed by lyophilization. The lyophilized product was dissolved in a small volume of chloroform and further purified by preparative thin-layer chromatography, using the solvent system of H. Goldfine (personal communication) which contains benzene-pyridine-water, 60:60:11. This resolves phosphatidylethanolamine and phosphatidyl-*N*-methylethanolamine. The phosphatidylethanolamine band was identified by the use of a standard of the pure reference compound, and was eluted as described previously. Aqueous dispersions of pure *A. agilis* phosphatidylethanolamine were prepared by the dialysis technique of Fleischer and Klouwen (1961).

Enzymatic Cyclopropane Fatty Acid Synthesis.—In the experiments with cyclopropane fatty acid synthetase, the lipid substrate used was an aqueous dispersion of purified *A. agilis* phosphatidylethanolamine. The *C. butyricum* enzyme preparation was incubated at 30° for 60 minutes with labeled *S*-adenosylmethionine and other additions as noted. The surfactant sodium lauryl sulfate, which is necessary for maximal enzyme rates (Chung and Law, 1964), was added to the reaction mixture. Reactions were terminated by addition of an equal volume of methanol. The phosphatidylethanolamine was extracted by a modification of the Folch procedure (Folch *et al.*, 1957), and was purified further by preparative thin-layer chromatography on silicic acid. The fatty acid content of each position of the phospholipid was then determined after enzymatic hydrolysis with snake venom phospholipase A and separation of the reaction products as described below.

Enzymatic Hydrolysis of Phospholipids.—The positions of the fatty acids in the phospholipids were determined with the aid of the phospholipase A of snake venom. Van Deenen and De Haas (1963) have demonstrated that the venom phospholipase A of *Crotalus adamanteus* specifically hydrolyzes the β-fatty ester linkage in phospholipids. Our choice of *Ancistrodon piscivorus piscivorus* venom was prompted by reasons of economy and convenience. While *A. piscivorus piscivorus* venom has frequently been used as a source of phospholipase A, the specificity of the venom enzyme from this source has not been rigorously tested. Dr. W. E. M. Lands has very kindly examined a sample of this venom for comparison with that of *C. adamanteus* in its action on phospholipids of known fatty acid content. Using methods previously dexcribed (Robertson and Lands, 1962), he has shown that the specificity for the β-fatty acid is identical in both venoms (personal communication).

In a typical experiment, 15–20 mg of purified phos-

TABLE I
DISTRIBUTION OF FATTY ACIDS IN BACTERIAL PHOSPHOLIPIDS[a]

	A. agilis PE[b]			*E. coli B* PE			*A. tumefaciens* PE			*A. tumefaciens* PC[c]			*C. butyricum* PE			*S. marcescens* PE		
Fatty Acid	Total	β	γ	Total	β	γ	Total	β	γ	Total	β	γ	Total	β	γ	Total	β	γ
14:0	4	5	3	4	4	4	1	1					8	6	2	6	7	2
16:0	29	10	51	41	9	75	8	2	12	8	3	12	44	66	32	39	5	89
16:1	48	85	20	3	4	3	1	1	2	<1	<1	1	17	8	20	5	7	<1
17:0Δ				39	54	5	6	2	10	4	<1	7	19	10	28	42	74	3
18:1	19		26	5	9	7	46	39	60	44	25	60	2	3	3	2	3	3
19:0Δ				8	20	6	38	50	16	44	70	20	7	7	15	2	4	3
Other[d]								5					3			4		
Saturated Fatty Acid	33	15	54	45	13	79	9	3	12	8	3	12	52	72	34	45	12	91
Unsaturated Fatty Acid[e]	67	85	46	55	87	21	91	97	88	92	97	88	48	28	66	55	88	9

[a] All data are expressed as per cent of total fatty acid esters detected in gas chromatography. For the β or γ position the values refer to per cent of fatty acids found in that position, whereas for the total fatty acids, the values refer to per cent of total fatty acids from the combined β and γ positions. [b] The abbreviation PE is used to indicate phosphatidylethanolamine. [c] The abbreviation PC refers to phosphatidylcholine. [d] These unidentified materials are probably impurities from silicic acid used for thin-layer chromatography. [e] Cyclopropane fatty acids are included with unsaturated fatty acids, as is the material designated "other."

phatidylethanolamine was dissolved in 2 ml of ethyl ether to which was added 30 μl of 1.5 M aqueous NH_4OH. After thorough mixing, 30–50 μl of venom solution, containing 3–4 mg of venom in 1 ml of 0.1 M Tris-chloride buffer, *p*H 7.2, and 0.01 M with respect to $CaCl_2$, was added with the aid of a disposable 10-μl microcapillary tube (Drummond Scientific Co., Broomall, Pa.). The mixture was again agitated, and then incubated for 4–10 hours at room temperature with occasional agitation. When the substrate was phosphatidylcholine, no NH_4OH was added; the ethereal solution of substrate was incubated with no additions other than venom With both substrates the reaction mixture was generally clear at the outset, but became turbid after 1–2 hours of incubation. When phosphatidylcholine was the substrate, a translucent precipitate often formed; this was the ether-insoluble lysophosphatidylcholine. The progress of venom hydrolysis was followed by thin-layer chromatography of 10-μl aliquots of the reaction mixture. At the end of the incubation period, the enzyme reaction was stopped by addition of an equal volume of ethanol, which rendered the mixture clear. The mixture was then evaporated to dryness under a stream of nitrogen at room temperature, and was stored at 4°. Controls, in which phospholipids were treated with ammonium hydroxide or ammonium hydroxide plus aqueous buffer, showed no evidence of nonenzymatic hydrolysis.

Chromatography of the Hydrolysis Products.—The residue from the venom hydrolysis was dissolved in a small volume or chloroform and this solution was spread as a band across a glass plate coated with a thin layer of silicic acid. This was developed and stained as described. Three bands were usually observed, corresponding to unreacted substrate, lysophosphatide, and free fatty acids. These bands were scraped off separately and extracted with methanol. The extracts were evaporated under nitrogen and stored at 4° until used.

Analysis of Fatty Acids from Hydrolysis Products.—The fatty acids from the lysophosphatides and unhydrolyzed phosphatides and the free fatty acids extracted from thin-layer plates were examined. The lipids were saponified with 15% KOH in 50% methanol. After extraction of the alkaline solution with petroleum ether to remove nonsaponifiable material, the aqueous phase was acidified with concentrated HCl and the fatty acids were extracted with ethyl ether. The extracted fatty acids were methylated with freshly distilled diazomethane, and the methyl esters were subjected to gas-liquid chromatographic analysis. Routinely, this was performed with an F & M instrument (Avondale, Pa.) equipped with a hydrogen-flame ionization detector and an 8-ft column packed with 10% polydiethyleneglycol succinate on Chromosorb Z. The column was operated at 190° with a nitrogen flow rate of 95–105 ml/min. For the examination and collection of isotopically labeled fatty acid esters, a Research Specialties Co. (Richmond, Calif.) gas chromatograph equipped with an ionization detector was employed. A 6-ft column packed with 10% polydiethyleneglycol succinate on Chromosorb W was operated at 175–185° with an argon flow rate of 40–70 ml/min. Carrier methyl esters of synthetic 17- and 19-carbon cyclopropane fatty acids were added to the mixture of labeled fatty acid esters to facilitate collection of minute quantities of labeled cyclopropane acid esters. An aliquot of the mixture was withdrawn for counting, and a larger aliquot was injected for chromatographic analysis. Glass U-tubes cooled in a dry ice–acetone bath were used to collect individual fatty acid esters as they passed from the chromatographic column. The contents of the U-tubes were flushed into scintillator vials with 15 ml of toluene scintillation fluid, and the samples were assayed for radioactivity. Collection efficiency of injected radioactive compounds ranged from 40 to 80%. It is assumed that collection efficiency for each ester is equal within a given run, and that radioactive compounds unaccounted for escaped collection. The amount of each ester present was estimated by peak area as determined by the method of Carroll (1961).

RESULTS

The distribution of fatty acids in phosphoglycerides of several bacterial species is summarized in Table I.

We have found considerable variation in the fatty acid composition of lipids from the same species grown at different times and under slightly different conditions, especially with regard to the amounts of unsaturated and cyclopropane fatty acids (Law *et al.*, 1963). However, the sum of unsaturated and cyclopropane fatty acids is a much more constant figure.

Table II summarizes the results of experiments

TABLE II
METHYLATION SPECIFICITY OF *C. Butyricum* CYCLOPROPANE SYNTHETASE[a]

Fatty Acid	β Position			γ Position		
	Experiment 1[b] Crude Enzyme	Experiment 2[b] Partially Pure Enzyme	Substrate[c]	Experiment 1[b] Crude Enzyme	Experiment 2[b] Partially Pure Enzyme	Substrate[c]
14:0	0	9	5	0	0	3
16:0	1	1	10	1	1	51
16:1	1	1	85	1	1	20
17:0Δ	92	82	0	57	54	0
18:1	2	4	0	1	2	26
19:0Δ	4	3	0	40	42	0
Total radioactive acids recovered from venom reaction[d]	25,800	5,500		28,700	11,000	
Input to gas chromatograph[d]	4,550	1,980		2,340	7,515	

[a] The incubation mixtures contained the following: *Experiment 1:* Tris-acetate, pH 7.0, 500 μmoles; phosphatidylethanolamine, 14 μmoles; sodium lauryl sulfate, 2.5 μmoles; *S*-[^{14}C]adenosylmethionine, 0.475 μmole (7×10^5 dpm/μmole); enzyme, 30–40 mg protein; total volume, 5.55 ml. *Experiment 2:* Tris-acetate, pH 7.0, 500 μmoles; phosphatidylethanolamine, 14 μmoles; sodium lauryl sulfate, 2.5 μmoles; *S*-[^{14}C]adenosylmethionine, 0.410 μmole (1.6×10^6 dpm/μmole); enzyme (purified *ca.* 10-fold); total volume 4.50 ml. Incubation was at 30° for 60 minutes. Reactions were terminated by the addition of methanol and the products were purified as described in the text. [b] The data are expressed as per cent of total radioactive esters recovered in gas chromatography, except as noted below. [c] The units for the substrate data are per cent of total fatty acids esterified to the β or γ position. [d] The data here are expressed in cpm, corrected for counter and gas chromatograph background.

designed to determine the methylation specificity of the cyclopropane-synthetase system of *C. butyricum*. Two enzymatic experiments were performed, one with crude bacterial extracts and another with enzyme purified approximately 10-fold (Chung and Law, 1964). The composition of the *A. agilis* phosphatidylethanolamine used as a substrate is also included for a comparison of the potential substrate composition of each glyceride position with the product formed by the enzyme.

DISCUSSION

All the bacterial phosphoglycerides examined reacted readily with crude snake venom phospholipase A. In several cases where the hydrolysis was allowed to proceed for longer periods of time the reaction went very nearly to completion. This demonstrates that bacterial phosphoglycerides are of the normal L-α-glycerophosphate type, if we can assume that the rigorous stereospecificity observed by Van Deenen and De Haas (1963) for *Crotalus adamanteus* venom also applies to venoms taken from snakes of the genus *Ancistrodon*.

In general, the positional distribution of fatty acids in the bacterial phospholipids is in accord with that found elsewhere in nature. An exception is the distribution found in *C. butyricum*, which is very nearly the reverse of the general case observed elsewhere. In all examples studied, however, the distribution of unsaturated fatty acids and cyclopropane fatty acids is in harmony with the *in vitro* experiments (Zalkin *et al.*, 1963; Chung and Law, 1964) which showed a conversion of the double bond to the cyclopropane ring at the phospholipid level.

The positional distribution of fatty acids in bacterial phospholipids can result from at least two different enzymatic specificities. During the biosynthesis of the phosphoglyceride molecule an enzymatic process may be subject to a specificity which dictates that different classes of fatty acids will occupy the β and γ positions. This may be termed an *acylation specificity*. In the special case of organisms which produce cyclopropane fatty acids, a subsequent enzymatic process, which has an intact phospholipid as a substrate, can exhibit a second positional specificity. That is, if the cyclopropane–fatty acid synthetase acts upon a phosphatidylethanolamine which has unsaturated fatty acids in both positions, the enzyme may have a partial or complete specificity for one or the other position. This may be called a *methylation specificity*.

In the simplest case we have examined, a phosphatidylethanolamine from *A. agilis*, only acylation specificity is involved, for this organism lacks cyclopropane–fatty acid synthetase. The acylation specificity clearly determines that most of the saturated fatty acids occupy the γ position, while the β position is almost exclusively occupied by hexadecenoic acid. It is interesting to note that only 16-carbon olefinic acid is found in the β position, while both 16- and 18-carbon olefinic acids are found in the γ position.

In the case of phosphatidylethanolamine from *E. coli* and *S. marcescens*, acylation specificity also controls the distribution, because the phospholipid seems to be built up with the unsaturated fatty acids nearly entirely in the β position, the γ position being occupied by saturated fatty acids. The subsequent methylation reaction can then proceed only at the β position. *A. tumefaciens* produces phosphoglycerides which have a large amount of olefinic acids, distributed by the acylation process into the γ position as well as the β position. The small amount of saturated acid is placed nearly exclusively in the γ position. However, the subsequent methylation reaction shows a definite preferential specificity for the olefinic acids in the β position. If cells are maintained for a long time in the stationary phase of growth, the methylation reaction will also convert olfinic acids in the γ position to cyclopropane acids (H. Zalkin and J. H. Law, unpublished data). The very strong similarity between the positional distribution of fatty acids in the phosphatidylethanolamine and that in the phosphatidylcholine of *A. tumefaciens* is readily explained by the demonstration that this organism converts, phosphatidylethanolamine to phosphatidylcholine by a series of *N*-methylation reactions (Kaneshiro and Law, 1964).

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.

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REFERENCES

Carroll, K. K. (1961), *Nature 191*, 377.
Chung, A. E., and Law, J. H. (1964), *Biochemistry 3*, 967.
Fleischer, S., and Klouwen, H. (1961), *Biochem. Biophys. Res. Commun. 5*, 378.
Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957), *J. Biol. Chem. 226*, 497.
Goldfine, H. (1962), *Biochim. Biophys. Acta 59*, 504.
Gray, G. M. (1958), *Biochem. J. 70*, 425.
Kaneshiro, T., and Law, J. H. (1964), *J. Biol. Chem. 239*, 1705.
Kaneshiro, T., and Marr, A. G. (1962), *J. Lipid Res. 3*, 184.
Lands, W. E. M., and Merkl, I. (1963), *J. Biol. Chem. 238*, 898.
Law, J. H., Zalkin, H., and Kaneshiro, T. (1963), *Biochim. Biophys. Acta 70*, 143.
Lees, T., and DeMuria, P. (1962), *J. Chromatog. 8*, 108.
Merkl, I., and Lands, W. E. M. (1963), *J. Biol. Chem. 238*, 905.
Mudd, S. H. (1959), *J. Biol. Chem. 234*, 87.
O'Leary, W. M. (1962), *Bacteriol. Rev. 26*, 421.
Robertson, A. F., and Lands, W. E. M. (1962), *Biochemistry 1*, 804.
Rouser, G., Bauman, A. J., Kritchevsky, D. H., and O'Brien, J. S. (1961), *J. Am. Oil Chemists' Soc. 38*, 544.
Schlenk, F., Dainko, J. L., and Stanford, S. M. (1959), *Arch. Biochem. Biophys. 83*, 28.
Simmons, H. E., and Smith, R. D. (1959), *J. Am. Oil Chemists' Soc. 81*, 4256.
Van Deenen, L. L. M., and De Haas, G. H. (1963), *Biochim. Biophys. Acta 70*, 538.
Wagner, H., Horhammer, L., and Wolfe, P. (1961), *Biochem. Z. 334*, 175.
Wolfe, R. S., and O'Kane, D. J. (1953), *J. Biol. Chem. 205*, 755.
Zalkin, H., Law, J. H., and Goldfine, H. (1963), *J. Biol. Chem. 238*, 1242.

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

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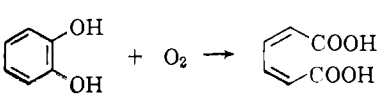
Oxidation of 3,5-di-*t*-butyl pyrocatechol by O_2 was carried out in the presence of metal salts in slightly alkaline aqueous methanol. The main product was 3,5-di-*t*-butyl *o*-benzoquinone (II). The yields, which reached as high as 96%, based on starting material, could be correlated roughly with oxidation 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):



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