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Isolation and Structure Proof of 3,4-Dimethyl-6-carboxy-α-pyrone as a Bacterial Degradation Product of Riboflavin

BY P. Z. SMYRNIOTIS, H. TODD MILES AND E. R. STADTMAN

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A new compound, $C_8H_8O_4$, has been isolated as a bacterial degradation product of riboflavin and shown to be 3,4-dimethyl-6-carboxy-α-pyrone.

In an effort to learn more about the intermediary metabolism of heterocyclic compounds, we have undertaken a study of the microbiological degradation of riboflavin. The hydrolysis of riboflavin to 6,7-dimethylalloxazine and ribitol and the further oxidation of the latter compound to carbon dioxide has been shown previously to be carried out by certain aerobic soil organisms.[1a,b] The accumulation of 6,7-dimethyl-9-(2'-hydroxyethyl)-isoalloxazine as a major product of riboflavin fermentation by anaerobic bacteria[1c] also has been observed. However, until recently[2] bacterial degradation of the isoalloxazine moiety of riboflavin has not been described. The present communication describes the isolation of 3,4-dimethyl-6-carboxy-α-pyrone (compound I) as an intermediate in the bacterial oxidation of riboflavin to carbon dioxide and ammonia. The organism used was isolated from the soil by the enrichment culture technique using aerobic conditions with riboflavin as the major carbon and nitrogen source.[2,3]

Isolation and Characterization.—A pure strain of the riboflavin decomposing bacterium (unidentified species) was used in the present experiments to inoculate a culture medium containing riboflavin (see Experimental). After an incubation period of a week the cells were removed, the supernatant passed over Florisil and the effluent acidified and extracted continuously with ether.

The brown residue obtained on evaporation of the ether was recrystallized several times from water to give the pure compound I. The colorless crystals melted at 183–184°, showed no optical rotation at 589 or at 436 mμ, had ultraviolet absorption maxima at 300 (ϵ_{max} 9,850) and 237 mμ (ϵ_{max} 2,650), and a neutral equivalent of 187. The elementary analysis indicated the composition $C_8H_{10}O_5$. The neutral equivalent calculated from this (assuming a monobasic acid) is 186, in agreement with the value obtained by rapid titration. A C-methyl determination on the free acid showed two such groups to be present.

When the compound was sublimed the analysis indicated that the composition had changed to $C_8H_8O_4$, indicating the loss of a molecule of water of crystallization. The melting point and absorption maxima were unchanged and the neutral equivalent was 169 (calculated 168).

(1) (a) J. W. Foster, *J. Bact.*, **47**, 27 (1944); (b) T. Yanagita and J. W. Foster, *J. Biol. Chem.*, **221**, 593 (1956); (c) H. T. Miles and E. R. Stadtman, THIS JOURNAL, **77**, 6747 (1955).

(2) P. Z. Smyrniotis and E. R. Stadtman, *Bact. Proc.*, 124 (1957).

(3) O. Hayaishi, "Methods in Enzymology," Vol. I, edited by S. P. Colowick and N. O. Kaplan, Academic Press, Inc., New York, N. Y., 1957, p. 126.

Determination of Structure.—When the neutralized compound was treated with an excess of 0.15 *N* alkali at room temperature, the absorption band at 300 mμ disappeared (60 min.) and a substance with an absorption band at 235 mμ was formed. This transformation was associated with the liberation of one equivalent of acid as determined by titration of the excess alkali.

Titration curves on the free dibasic acid, obtained by passing the reaction mixture over a Dowex-50 (H^+) column, showed the presence of two titratable groups (pK_1, 2.45; pK_2, 7.5) per mole of starting compound.

Continued incubation of the alkaline reaction mixture following the primary hydrolysis of I resulted in the slow development (24 hr.) of one equivalent of carbonyl per mole (Fig. 1). This secondary conversion is accompanied by a slight reduction and a shift of the absorption maximum to 242mμ. These observations suggest that the compound I contains a lactone ring that is easily hydrolyzed by alkali to form a dicarboxylic acid with an enolic hydroxyl group. Ketonization of the enolic hydroxyl could account for the slow development of the carbonyl function.

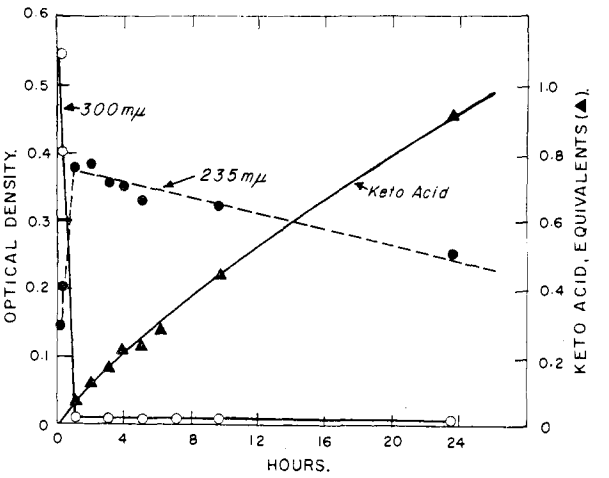


Fig. 1.—Influence of alkali on compound I: 10.3 mg. of compound I (61 micromoles) was dissolved in 1.5 ml. of 0.2 *M* potassium hydroxide. At times indicated aliquots were removed for spectral studies in water and for total carbonyl analysis.

Treatment of the acid with diazomethane in ether solution gave a crystalline ester, m.p. 136–137°, which analyzed for $C_9H_{10}O_4$. This reaction confirmed the presence of a single free acidic group in the original compound and indicated that the $C_8H_{10}O_5$ analysis of the unsublimed material in-

TABLE I

Starting compound	Micromoles per ml.	Ozonolysis products, micromoles/ml.[a] Keto acids Total	Keto acids Pyruvic	Bis-carbonyl Total	Bis-carbonyl Methyl-glyoxal	Oxalic acid Before hydrol.	Oxalic acid After hydrol.	Formic acid
Cmpd. I	26	13.2	14.8	7.0	6.1	5.5	5.4	8.6
Methyl ester	25	9.9	7.5	3.0	2.0	2.8	17.8	7.0
Dihydro cmpd.[b]	12	15.3	7.6	0.4		0.56	1.2	0.9

[a] For analytical methods see Experimental. [b] The dihydro compound used in this experiment still contained a small amount of starting material, which undoubtedly accounts for the oxalic and formic acids observed.

cluded a molecule of water of crystallization. The ultraviolet absorption maximum for the ester remained at 300 mμ. Analysis showed two C-methyl groups to be present.

When the free acid, compound I, was hydrogenated with palladium–charcoal catalyst in ethanol the reaction slowed appreciably after the uptake of one mole of hydrogen. Interruption of the reaction at this point gave a dihydro compound, $C_8H_{10}O_4$, m.p. 114–118°, which had an ultraviolet absorption maximum at 233 mμ. The dihydro compound did not react with either dinitrophenylhydrazine or periodic acid.

Hydrogenation with platinum catalyst in acetic acid resulted in the uptake of three moles of hydrogen. The hexahydro compound $C_8H_{14}O_4$, m.p. 76–80°, had no ultraviolet absorption, consumed no periodate and titrated as a dicarboxylic acid. The bis-*p*-nitrobenzyl ester, m.p. 50–53°, was prepared and the analysis, $C_{22}H_{24}N_2O_8$, agreed with that expected for the corresponding ester of a dicarboxylic acid.

On the basis of these experiments it is possible to conclude that the compound is a substituted α-pyrone. The absorption maximum agrees with the value of 300 mμ observed for scilliroside,[4a] bufotalidin and 5-methyl-α-pyrone,[4b] and the log *E* value of 4 is similar to that of 3.4 to 3.7 observed for the above compounds.

The formation of an additional carboxyl group by treatment with hydrogen indicates that hydrogenolysis of an enol lactone has occurred, the third mole of hydrogen being required to cleave the carbon–oxygen bond rather than to saturate a double bond. The hydrogenation of enol lactones to desoxy acids was studied by Jacobs and Scott[5] and was observed by Stoll[6] in those squill toxins which contained the α-pyrone nucleus. Of the different unsaturated lactones which could undergo such hydrogenolysis only an α-pyrone structure satisfied other criteria, such as the observed absorption spectrum or the ozonolysis products observed below.

The occurence of the absorption maximum at a wave length as high as 233 mμ indicates that the double bond of the dihydro compound must be both conjugated and substituted.[7] When considered in conjunction with the information obtained from the ozonolysis of the original α-pyronecarboxylic acid, compound I, the absorption maximum indicates that the dihydro compound has a 2,3- rather than a 5,6-double bond. An analogous result was found in the case of 2-acetamino-6-acetoxymethyl-α-pyrone which was partially hydrogenated to the 5,6-dihydro compound.[8]

To this point it had been shown that the compound was an α-pyrone with two C-methyl groups and a carboxyl group as substituents, but their location was unknown. It was probable in view of the origin of the compound from riboflavin that the methyl groups were on adjacent carbon atoms. The presence of the carboxyl group on the 5-position is made unlikely by the observation of Fried and Elderfield[9] that methyl coumalate gave a red color when treated with diazomethane with eventual isolation of a product which had undergone C-methylation at position 6. In the present case there was no red color and no C-methylation.

For the definite assignment of positions to the substituents on the pyrone ring, ozonolysis of I, its methyl ester and its dihydro derivative was employed. The reactions were run in cold ethyl acetate and decompositions of the ozonides with hydrogen and platinum were carried out immediately afterward in the same solvent at 0°.

For the identification of the ozonolysis products specific and sensitive enzymatic analyses were employed. It is believed that this method has been employed here for the first time for the identification of ozonolysis products and that it may be generally useful for identifying on a micro scale the small fragments that are often produced by ozonolysis. As can be seen from the data in Table I, pyruvic acid, methyl glyoxal, oxalic acid and formic acid are all obtained as major products from the ozonolysis of compound I.

The production of pyruvic acid means that a methyl group must be present at position 3. While methyl glyoxal could arise from either a 3,4- or a 3,5-dimethyl-α-pyrone, the former is more likely in view of the origin from riboflavin. Oxalic acid could arise only from a 6-carboxyl substituent, but formic acid, if a primary product, would indicate that the 6-position is unsubstituted. The contradiction can be resolved, however, by the observation of Friedemann[10] that methyl glyoxal is converted almost instantaneously by peroxide to formic and acetic acids. It is probable that some of the methyl glyoxal was decomposed by peroxide present in the ozonolysis mixture before the reduction was complete. In this connection it is probably significant that the sum of the formic acid and methyl glyoxal is equal within experimental error to the pyruvic acid. The oxalic acid would then arise,

(4) (a) A. Stoll and J. Benz, *Helv. Chim. Acta*, **25**, 377 (1942); (b) J. Fried and R. C. Elderfield, *J. Org. Chem.*, **6**, 566 (1941).

(5) W. A. Jacobs and A. B. Scott, *J. Biol. Chem.*, **87**, 601 (1930); **93**, 139 (1931).

(6) A. Stoll, A. Hofmann and W. Kreis, *Helv. Chim. Acta*, **17**, 1334 (1934); A. Stoll and J. Benz, *ibid.*, **25**, 43 (1942).

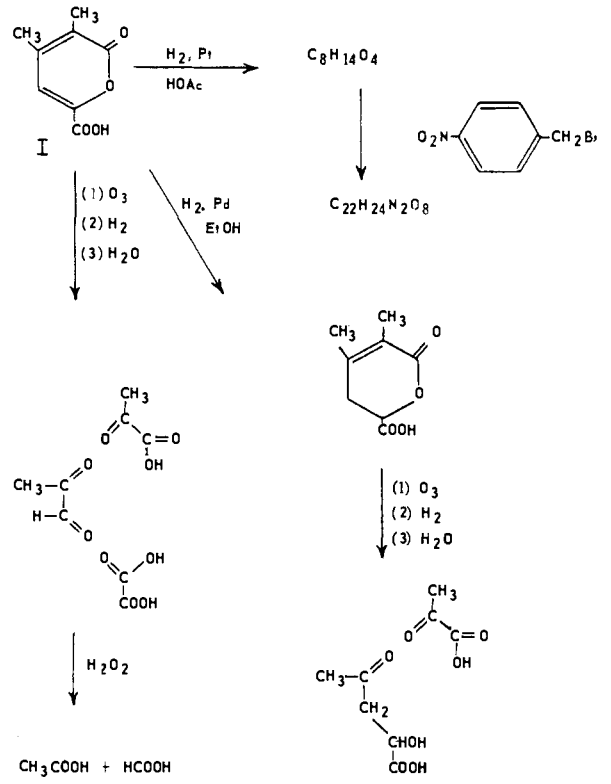
(7) A. T. Nielsen, Abstracts of Papers, 132nd Meeting of the American Chemical Society, p. 15, Sept. 8, 1957, and personal communication.

(8) M. Bergman, L. Zervas and E. Silberkweit, *Ber.*, **64**, 2428 (1931).

(9) J. Fried and R. C. Elderfield, *J. Org. Chem.*, **6**, 577 (1941).

(10) T. E. Friedemann, *J. Biol. Chem.*, **73**, 331 (1927).

as indicated, from the 6-carboxyl substituent, and the compound must have the structure indicated by I.



A small amount of oxalic acid is also formed on ozonolysis of the methyl ester of I, in addition to the same products formed from the free acid, but after saponification the amount of oxalic acid increased almost to the theoretical value. This result confirms the original view that the carboxyl group present in the free acid is the one which gives rise to the oxalic acid.

It may be observed further that the absence of any 2,3-bis-carbonyl acid (if such a compound had been present it would have been detected as the blue bis-dinitrophenylhydrazone in the aqueous layer after extraction of the basic solution with ethyl acetate) in the reaction mixture from ozonolysis of the free acid shows that the carboxyl group could not be either on C 4 or C 5, regardless of where the methyl groups are. The dihydro compound on ozonolysis gave rise to pyruvic acid and to a keto acid which, on the basis of structure I, should be α-hydroxylevulinic acid. The second keto acid was formed in an amount equivalent to the pyruvic acid.

The hexahydro compound on the basis of structure I, should be α,β-dimethyladipic acid and is, undoubtedly, a mixture of diastereoisomeric forms. The only reference found to this compound was made in a patent[11] which gave no characterization of the material obtained.

(11) W. Schrauth, U. S. Patent 1,921,101, Aug. 8, 1933 (*C. A.*, **27**, 5084 (1933)).

Experimental[12,13]

Isolation of Metabolic Products.—Four 20-l. bottles, each containing 15 liters of sterile culture medium[14] and 10 g. of riboflavin (Nutritional Biochemical Co.), were inoculated with a pure strain of the riboflavin decomposing organism and were incubated for one week under aerobic conditions at room temperature in the dark. The decomposition of riboflavin was followed spectrophotometrically by periodic determination of the absorption spectrum. After 5–7 days most of the riboflavin was decomposed as was indicated by the decrease in optical density at the three major absorption maxima 445, 370 and 265 mμ. The decrease in riboflavin is associated with the simultaneous appearance of a compound having a strong absorption band at 300 mμ. This substance, compound I, was isolated from the growth medium as follows: The bacterial cells were removed by centrifugation in a Sharples centrifuge and the supernatant solution was passed through a Florisil column 35.0 × 8.5 cm. Any unchanged riboflavin and other as yet unidentified decomposition products are retained on the Florisil, whereas the compound I passes through with the effluent solution. The effluent was adjusted to *p*H 3.0 with addition of 18 *N* sulfuric acid (*ca.* 55 ml.) and concentrated under vacuum at 35° in a circulating evaporator to approximately 1.0 liter. The compound was quantitatively extracted from the concentrate by 12 hours continuous ether liquid–liquid extraction. The ether extract was then evaporated to dryness and the crude brown crystalline residue dissolved in a minimum amount of hot ethyl acetate (hot water was used in the earlier experiments which gave the hydrate, $C_8H_{10}O_5$). The solution was treated with charcoal and filtered hot, and compound I (2.6 g.) crystallized upon cooling. An additional 0.90 g. was obtained from the filtrate from the first crop of crystals on concentrating and cooling. The compound was recrystallized (3 ×) to constant absorption at 300 mμ and melting point (183–184°). Finally, the compound was sublimed by heating at 114° at 10^{-4} mm. in a high vacuum still.

Anal. Cmpd. recrystallized from water. Calcd. for $C_8H_{10}O_5$: C, 51.80; H, 5.40; C-CH_3, 16.0. Found: C, 51.68; H, 5.57; C-CH_3, 14.51. Sublimed compound. Calcd. for $C_8H_8O_4$: C, 57.20; H, 4.77. Found: C, 56.95; H, 4.77.

Hydrolysis of the α-Pyrone and Isolation of the Dicarboxylic Acid Formed.—One hundred and thirty-one milligrams of compound I was dissolved in 7.5 ml. of 0.15 *N* potassium hydroxide. After 2 hours at 30° the hydrolysis was complete as measured by the complete disappearance of the absorption band at 300 mμ. Then the reaction mixture was passed over a Dowex-50 (H^+) resin column (9 cm. × 1.0 cm.) to remove excess base and to convert the hydrolysis product to the free acid. The effluent solution and the water washings were combined (15 ml. total). A 2.0-ml. aliquot was titrated with 0.1 *N* potassium hydroxide. Two acid equivalents per mole of compound I were found. The titration curve shows two inflections at *p*H 2.45 and 7.5. The remainder of the Dowex-treated material was lyophilized to obtain the dibasic acid as an amorphous material. When redissolved in water the compound had an absorption maximum at 235 mμ showing that little or no re-cyclization had occurred. An attempt to obtain the dimethyl ester of the hydrolysis product by reaction with diazomethane in ether solution led to the formation instead of the methyl ester of the original α-pyrone derivative with its absorption maximum at 300 mμ and m.p. 132–134°. This material showed no depression of m.p. when mixed with the methyl ester prepared originally from I.

Formation of Dihydro Compound.—A solution of 315 mg. of compound I (1.7 mmoles) in 15 ml. of ethanol was hydrogenated at atmospheric pressure with 200 mg. of 10% palladium-on-charcoal catalyst (Matheson, Coleman and Bell). The compound absorbed one mole of hydrogen in two hours, after which the rate decreased appreciably. The

(12) Elementary analyses by Dr. W. C. Alford, National Institute of Arthritis and Metabolic Diseases, and C-CH_3 determinations by W. Manser, Zurich, Switzerland.

(13) The ultraviolet spectra were measured in water.

(14) 15.0 liters of growth medium contained 15.0 g. of yeast extract, 15.0 g. of tryptone, 600.0 ml. of 1.0 *M* KH_2PO_4, 3.0 g. of $MgSO_4 \cdot 7H_2O$, 150 mg. of $CaSO_4 \cdot 2H_2O$, 45.0 mg. of $FeSO_4 \cdot 7H_2O$, 37.5 mg. of $MnSO_4 \cdot 4H_2O$, 37.5 mg. of $NaMoO_4 \cdot 2H_2O$ and 4.5 g. of $Na_2S \cdot 9H_2O$.

acid was allowed to take up an additional 10% of hydrogen before filtration of the catalyst and evaporation of solvent. The weight of the residue was 320 mg. When the material finally crystallized the m.p. was 87–110° and the absorption maximum was at 233 mμ (the presence of slight absorption at 300 mμ showed that a small amount of starting material remained). The material was crystallized from a mixture of ethyl acetate, ether, and hexane with poor recovery. After three crystallizations, the m.p. was 114–118°. There was no reaction with dinitrophenylhydrazine or periodic acid.

Anal. Calcd. for $C_8H_{10}O_4$: C, 56.46; H, 5.92. Found: C, 56.02; H, 5.79.

The difficulty of the purification probably accounts for the low carbon value, but there is no reason to doubt the composition of the material.

Formation of Hexahydro Compound.—Platinum oxide catalyst (300 mg., Bishop and Co. Platinum Works) was prereduced in 5 ml. of acetic acid, and then 308 mg. of I (1.65 mmoles) in 25 ml. of acetic acid was added to the flask. The compound took up three moles of hydrogen in an hour and no more during a further 30 minutes. The catalyst was filtered and the filtrate evaporated under vacuum to leave a largely crystalline residue. Chloroform was added and evaporated under vacuum several times. The material was extracted several times with ether and crystalline material obtained by allowing the combined ether extracts to evaporate at room temperature. A small residue, not soluble in ether, could not be induced to crystallize. The compound tended to be deposited as an oil rather than crystallize from mixed solvents, and for this reason it was dried without further purification at 60° and 0.1 mm. pressure. The white crystals then sintered at 73° and melted at 76–80°. Some sublimed material had the same melting point. The material reacted with neither dinitrophenylhydrazine nor periodic acid. On titration the compound consumed 2 moles of potassium hydroxide.

Anal. Calcd. for $C_8H_{14}O_4$: C, 55.16; H, 8.10. Found: C, 55.00; H, 8.37.

Preparation of Bis-*p*-nitrobenzyl Ester of I.—A solution of 106 mg. of the hexahydro compound was titrated to *p*H 8 with potassium hydroxide, evaporated to 0.5 ml. and refluxed in 2 ml. of ethanol with two equivalents of *p*-nitrobenzyl bromide for three hours. The reaction mixture was filtered hot twice to remove salt, the solvent evaporated, chloroform added, and the solution again filtered. The solution was extracted with bicarbonate to remove any mono ester and the chloroform solution filtered, dried with anhydrous magnesium sulfate, and evaporated. The resulting oil crystallized after several days and melted at 40–46°. The material was re-crystallized from ether–hexane (seeding each time) to a constant melting point of 50–53°.

Anal. Calcd. for $C_{22}H_{24}N_2O_8$: C, 59.45; H, 5.40; N, 6.30. Found: C, 59.66; H, 5.49; N, 6.72.

Preparation of Methyl Ester with Diazomethane.—To a chilled solution of 300 mg. of I in 30 ml. of ether, diazomethane (prepared from N-methyl-N-nitroso-N¹-nitroguanidine) was added until a faint yellow color persisted and nitrogen evolution ceased. The product crystallized almost at once from the ether and was filtered to give 260 mg., m.p. 127–130°. The compound was recrystallized from ethyl acetate to a m.p. of 136–137°. The absorption maximum was 300 mμ.

Anal. Calcd. for $C_9H_{10}O_4$: C, 59.33; H, 5.53; C-CH_3, 16.5. Found: C, 59.31; H, 5.77; C-CH_3, 14.99.

Ozonolysis of I.—A Welsbach ozonator (style T-19) was the source of ozone used and delivered about 0.14 mmole of ozone/min. at the flow rate, pressure and voltage employed.

The stream of ozone and oxygen was passed for three minutes through a solution of 113 mg. of I in 15 ml. of ethyl acetate, cooled in a Dry Ice-bath. The absorption of ozone ceased in about three minutes, though the calculated duration of the reaction would have been about eight or nine minutes. A solid had precipitated during the reaction, probably a mixture of solid ozonide and unchanged starting material.

The ethyl acetate solution was transferred to a hydrogenation flask containing 50 mg. of pre-reduced platinum oxide catalyst and reduced at 0° until a starch-iodide test was negative. It may be noted that even a rapid transfer of the cold ozonide solution may permit some water to condense into the solution and that water reacts with an ozonide to produce hydrogen peroxide, which can in turn react with α-dicarbonyl compounds to produce secondary products. The solution was filtered to remove catalyst and stored in the deep freeze for the analyses described below.

Ozonolysis of Methyl Ester and of Dihydro Derivative of I.—These reactions were carried out as those described above on 101 mg. and 32 mg. of material, respectively. In the latter case, an earlier preparation of dihydro compound still containing a little compound I was used.

Materials and Methods for Enzymatic Assays.—Crystalline lactic acid dehydrogenase was obtained commercially from Sigma Chemical Co. Highly purified oxalic acid decarboxylase was a gift from Dr. O. Hayaishi. Purified glyoxalase I was obtained from Dr. W. W. Kielley and crystalline tetrahydrofolic acid formylase was generously provided by Dr. J. C. Rabinowitz.

Estimation of Total Keto Acid.—Total keto acid and bis-carbonyl compounds were determined by a modification of the method developed by Friedemann and Haugen.[15] To 2.0 ml. of sample containing 0.1–0.5 micromole of keto acid or 0.01–0.05 micromole of bis-carbonyl compound was added 0.5 ml. of 0.1% 2,4-dinitrophenylhydrazine solution (in 2 *N* HCl). After 10 minutes at room temperature 3.0 ml. of 2.5 *N* sodium hydroxide solution was added. The resulting solution was extracted with 3.0 ml. of *t*-amyl alcohol and the optical density of each phase was read in a Klett colorimeter using a green filter #54. In all of the instances examined, the aqueous phase which contains the keto acids was red in color indicating the absence of any significant amount of bis-carbonyl acids. The amyl alcohol extracts were intense blue in color indicating the presence mainly of neutral bis-hydrazones.[16] Pyruvate and methyl glyoxal of known concentration were used to standardize the method.

Pyruvic Acid.—Pyruvic acid was determined quantitatively by its stoichiometric reduction to lactic acid by DPNH in the presence of lactic acid dehydrogenase.[17] The reaction is followed spectrophotometrically by measuring the decrease in optical density at 340 mμ associated with the oxidation of DPNH. Since other α-keto acids such as glyoxylic acid are also reduced in the assay system at slower rates[17] pyruvic acid was further characterized as the ozonolysis product by showing that the kinetics of the enzymatic reaction are identical with those obtained by pyruvic acid (Fig. 2). Pyruvic acid was also identified by showing that it and its 2,4-dinitrophenylhydrazone derivative are indistinguishable from the known compounds when chromatographed on paper in a variety of solvent systems.[18]

Methyl glyoxal was estimated by its enzymatic conversion to S-lactylglutathione in the presence of glutathione and the specific enzyme catalyst, glyoxalase I.[19] The S-lactylgluta-

(15) T. E. Friedemann and G. E. Haugen, *J. Biol. Chem.*, **147**, 415 (1943).

(16) In the method used, the optical density of bis-hydrazone is almost 6 times greater than that of monohydrazones; therefore assuming the presence of no bis-hydrazones at all (an assumption that is far from correct since the color is blue) the amount of neutral monohydrazones could not possibly be more than one-sixth the amount reported as bis-hydrazones. This would represent such a small amount of material that neutral monocarbonyl compounds can be excluded from consideration as a major product of ozonolysis. This is further evidenced by the fact that in the experiment cited (Table I) the amounts of bis-hydrazones, as determined by the chemical method, are in good agreement with the amounts of methyl glyoxal as determined by the enzymatic procedure. This proves also that methyl glyoxal is the only major neutral carbonyl compound formed during ozonolysis.

(17) A. Meister, *J. Biol. Chem.*, **184**, 117 (1950); **197**, 309 (1952).

(18) The keto acids and their 2,4-dinitrophenylhydrazones were chromatographed in the solvents indicated below. The R_f's given are for pyruvic acid and its 2,4-dinitrophenylhydrazone, respectively. (A) propionic acid:water:1-butanol (5:7:1), R_f 0.89, dinitrophenylhydrazone not measured; (B) 1-butanol:formic acid (95:1) water saturated, R_f's 0.68 and 0.90; (C) 1-propanol:concentrated ammonium hydroxide (90:10), R_f 0.27, dinitrophenylhydrazone not measured; (D) ethanol:NH_4OH:H_2O (80:4:16), R_f's 0.77 and 0.67; (E) propionic acid:H_2O:1-butanol (5:7:10), R_f's 0.49 and 0.88; (F) ethyl ether:acetic acid:H_2O (13:3:1), R_f's 0.65 and 0.36.

(19) E. Racker, "Methods in Enzymology," Vol. III, edited by S. P. Colowick and N. O. Kaplan, Academic Press, Inc., New York, N. Y., 1957, p. 296.

thione formed is determined by spectrophotometric measurements of its thiol ester absorption at 233 mμ (ϵ_{max} 4,500). Glyoxal also reacts in the glyoxalase system to form S-glycolylglutathione. Therefore, to identify positively the ozonolysis product, the thiol ester produced in the enzymatic assay was allowed to react with hydroxylamine,[20] and the hydroxamic acid derivative was identified as lactylhydroxamic acid by paper chromatography.[21] No glycolyl hydroxamate was present; thus glyoxal is excluded as a product.

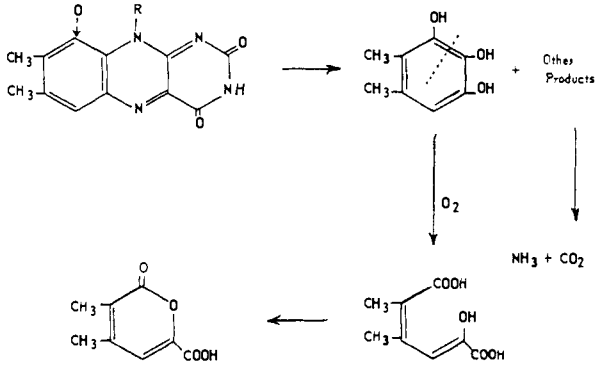
Oxalic acid was determined manometrically in a Warburg apparatus by measuring the CO_2 liberated when oxalic acid is decarboxylated in the presence of the highly specific oxalic acid decarboxylase from *Collyvia veltipes*.[22] The formation of a stoichiometric amount of formic acid during decarboxylation is additional proof that the CO_2 comes from oxalic acid.

Formic acid was determined spectrophotometrically by measuring the stoichiometric formylation of tetrahydrofolic acid in the presence of crystalline tetrahydrofolic acid formylase derived from *C. cylindrosporum*.[23]

Discussion

The α-pyrone ring system, aside from benzopyrones, has been found in nature in only a few instances, the most interesting being the cardiac glycosides scilliroside and scillaren A investigated by Stoll[6] and bufotalin investigated by Wieland.[24] In these compounds the α-pyrone nucleus exists as a substituent on the 17-position of a perhydrocyclopentanophenanthrene nucleus. In addition two compounds, 6-piperonyl-α-pyrone and 6-phenyl-α-pyrone, have been isolated from coto bark.[25]

The present discovery of 3,4-dimethyl-α-pyrone-6-carboxylic acid as a metabolite in the bacterial degradation of riboflavin is believed to be the first instance in which a monocyclic α-pyrone derivative has been obtained from a natural source. Although the biological mechanism of its formation remains to be determined, by analogy to established mechanisms of aromatic ring metabolism[26] it is likely that the pyrone is derived from riboflavin with the intermediary formation of dimethylpyrogallol. The latter can be visualized to arise from riboflavin by hydrolytic cleavage of the nitrogen substituents on the xylene ring and hydroxylation of the number 8 carbon. The further conversion of dimethylpyrogallol would then be completely analogous to the oxidation of catechol[24] to *cis–cis*-muconic acid and the subsequent lactonization of the latter to α-carboxymethyl-Δα-buteneolide.[27]



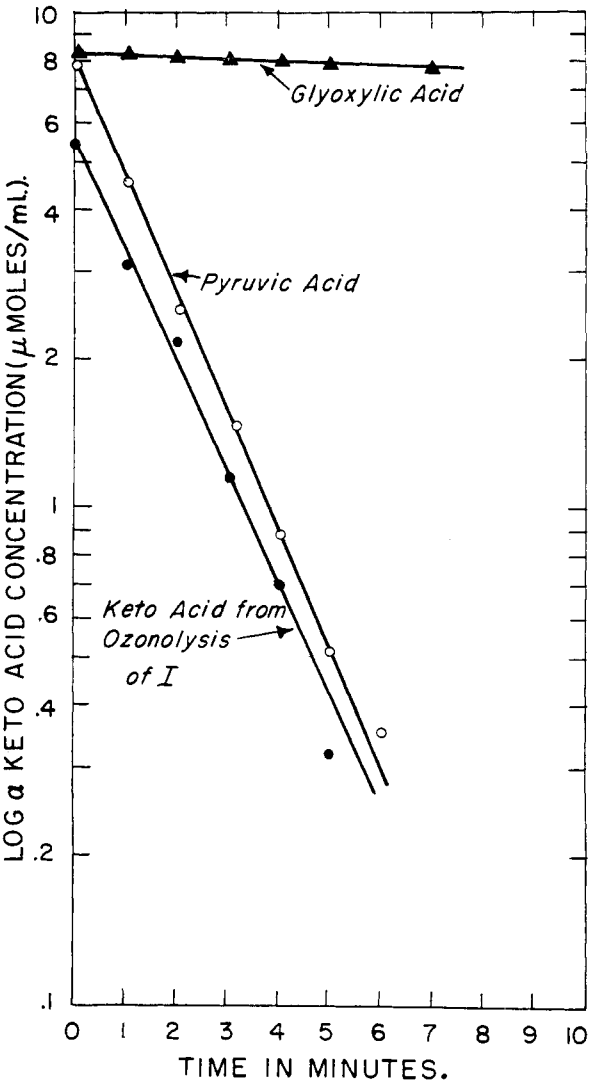


Fig. 2.—Enzymatic assay of pyruvic acid: each quartz cuvette (1.5 ml., 1.0-cm. light path) contained initially potassium phosphate buffer (pH 7.5), 100 micromoles; reduced diphosphopyridine nucleotide, 0.15 micromole; α-keto-acid as indicated, approximately 0.08 micromole; water to 1.0 ml. The reference cuvette contained all components except α-keto-acid and reduced diphosphopyridine nucleotide. After initial optical density measurements at 340 mμ (zero time), 0.01 ml. of lactic acid dehydrogenase (1–100 dilution) was added, and the decrease in optical density was recorded as a function of time. The amount of α-keto acid is computed from the total decrease in optical density.

In addition to the α-pyrone derivative, several other compounds accumulate during the bacterial degradation of riboflavin.[2] Some of these have been isolated as pure compounds and are under investigation. It should be pointed out also that the α-pyrone derivative is not the ultimate end product of metabolism, but it is slowly oxidized further by the organism to CO_2.

BETHESDA 14, MD.

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