Application of a Chemical Degradation of Coenzyme Q to Problems of Biosynthesis*

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ABSTRACT: The chemical degradation of certain protected derivatives of coenzyme Q with nine (CoQ_9) and ten (CoQ_{10}) isoprene residues on the side chain has been investigated. Ozonolysis in glacial acetic acid at room temperature gave low and variable yields of the desired 3′,4′,5′,6′-tetramethoxy-2′-methylphenylacetic acid or 3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid. In an improved procedure, the diacetate of CoQ hydroquinone in ethyl acetate was treated with damp ozone at low temperature. After separation of levulinaldehyde as the 2,4-dinitrophenylhydrazone, the aromatic aldehyde was oxidized with neutral permanganate to 3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid with a 25–40% yield. Appearance and disappearance of intermediates in the ozonolysis reaction and subsequent oxidation were followed by gas-phase chromatography.

The vitamin A–deficient rat was found to incorporate 0.005% of administered L-[U-^{14}C]phenylalanine into CoQ_9. Degradation of the [^{14}C]-CoQ_9 hydroquinone diacetate by the method outlined revealed approximately 80% of the radioactivity in the ring fragment and 20% in the side chain.

Potential precursors of both aromatic and isoprenoid portions of CoQ_9,[1] such as phenylalanine and acetate, respectively, are incorporated into this compound by the rat in significant amounts (Olson *et al.*, 1961). To determine the extent of incorporation of these precursors into the benzoquinone ring and isoprenoid side chain of CoQ, a chemical degradation of the molecule, adaptable to the small amounts available from biological studies, was necessary.

When the dimethyl ethers of CoQ hydroquinones were treated with aqueous alkaline $KMnO_4$ at 100°, Wolf *et al.* (1958) obtained tetramethoxyphthalic anhydride; with $KMnO_4$ in acetone, they isolated 3′,4′,5′,6′-tetramethoxy-2′-methylphenylacetic acid. In addition, levulinic and succinic acids were identified. Since we encountered great difficulty in repeating this work on a small scale, we investigated the ozonolysis of the diacetates of CoQ hydroquinones (Morton *et al.*, 1958). Again, the originally described conditions gave poor yields in our hands.

This paper is devoted to a report of the development of reliable semimicro methods for the ozonolysis of ^{14}C-labeled CoQ derivatives and their application to the study of distribution of radio carbon among ring and side chain of the molecule after administration of [U-^{14}C]phenylalanine to rats. Improved methods for the identification of degradation products and the synthesis of various intermediates and reference materials are also described. A preliminary report has appeared (Bentley *et al.*, 1961).

Experimental Procedure

Animals. Young male albino rats of the Sprague-Dawley strain (Charles River Breeding Laboratories, Brookline, Mass.) were used. Vitamin A deficiency was induced to increase the incorporation of precursor radioactivity into hepatic CoQ_9 (Gloor and Wiss, 1959). Weanling rats 50–60 g in weight were fed the diet of Mayer and Krehl (1948) for 2–4 weeks, at which time they weighed 140–180 g and had approximately doubled their hepatic CoQ content from 111 ± 11 μg/g to 276 ± 45 μg/g.

Chemicals. All solvents were redistilled except analytical grade diethyl ether (peroxide-free). L-[U-^{14}C]-Phenylalanine, 10.6 mc/mM, was obtained from Nuclear-Chicago, Desplaines, Ill. Its radiochemical purity was attested to by carrier dilution with authentic L-phenylalanine, by paper chromatography in 1-butanol–water–acetic acid, and by paper electrophoresis. Synthetic CoQ_9 was a generous gift from Dr. O. Wiss, Hoffmann-La Roche and Co. Ltd., Basle. Dr. Wiss also provided us with a sample of 3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid. We are grateful to Dr. K. Folkers, Merck, Sharp & Dohme Research Laboratories, Rahway, N.J., for gifts of CoQ_{10} and 3′,4′,5′,6′-tetramethoxy-2′-methylphenylacetic acid. A

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[1] The abbreviation CoQ is used for coenzyme Q (ubiquinone). CoQ_x indicates coenzyme Q with *x*-isoprene residues in the side chain.

sample of rubrogliocladin was kindly provided by Dr. P. W. Brian, Akers Research Laboratories, Imperial Chemical Industries Ltd., The Frythe, Welwyn, Herts. Ozonized oxygen was prepared with a Welsbach Ozonator, Model T 23, equipped with a Welsbach ozone meter, H 30 (The Welsbach Corp., Philadelphia, Pa.).

Isotope Experiments. Nonfasted, vitamin A–deficient rats were injected intraperitoneally with 100 μc of L-[U-^{14}C]phenylalanine and killed in 3 hours. Coenzyme Q was isolated from both liver and carcass (less head and intestinal contents) and purified separately to constant specific radioactivity and spectral purity on Brockman grade III alumina as previously described (Olson *et al.*, 1961). At this point, the two samples were combined and CoQ_9 (80–90% of the total CoQ) was isolated by preparative reverse-phase paper chromatography, using paraffin oil–impregnated paper and 1-propanol-water, 4:1. Paper chromatography was repeated (at least once) to obtain constant specific radioactivity of the CoQ_9. Rechromatography on alumina sufficed to remove the paraffin and the spectrally pure [^{14}C]-CoQ_9 was then crystallized to constant specific radioactivity with 4–8 times its weight of authentic cold CoQ_9. The diacetate of CoQ_9 hydroquinone was prepared by reductive acetylation (Lester *et al.*, 1959), and recrystallized to constant specific radioactivity prior to ozonolysis as subsequently described. Acetone from the terminal isopropylidene group was isolated as the 2,4-dinitrophenylhydrazone in preliminary experiments and found to agree in specific activity with that predicted from the levulinaldehyde moiety assuming consistent labeling of the side chain in agreement with established pathways for polyisoprenoid molecule biosynthesis (Bloch, 1957).

The radioactivity of CoQ_9 and its derivatives was determined in toluene by liquid scintillation counting using diphenyloxazole as a phosphor. Samples were routinely checked for quenching and corrected if necessary. The bis-2,4-dinitrophenylhydrazone of levulinaldehyde was counted as an infinitely thick sample against a calibrated standard.

Analytical Methods. IDENTIFICATION OF AROMATIC ACIDS BY GAS CHROMATOGRAPHY. A Barber-Colman Model 10 instrument (Barber-Colman Co., Rockford, Ill.) and a Chromalab instrument (Glowall Corp., Glenside, Pa.) were used. In each case, glass columns were pretreated with a 1% solution of dichlorodimethylsilane in chloroform, then rinsed with methanol and dried prior to packing (Sweeley and Chang, 1961). The standard liquid phases finally adopted were 4% silicone rubber gum, SE-30 (Silicone Products Div., General Electric Co., Waterford, N.Y.) or 4% nitrile silicone, XE-60. The inert support was acid-and-alkali-washed Chromosorb W (Johns-Manville), 80–100 mesh.

Solutions of the various acids or aldehydes were prepared in redistilled benzene. Samples of from 0.5 to 2.0 μl were injected into the instruments. For preparation of the methyl esters of the acids, the compound was dissolved in 2–3 ml of anhydrous ether containing 10% methanol and was treated with diazomethane as described by Schlenk and Gellerman (1960).

ASSAY OF TETRAMETHOXYPHTHALIC ANHYDRIDE. Appropriate aliquots of a stock solution of freshly sublimed tetramethoxyphthalic anhydride dissolved in water were diluted to 3 ml with 0.05 M HCl-KCl buffer, *p*H 2.4. The final solution used for assay had a *p*H of 2.6. The fluorescence spectrum obtained in the Aminco-Bowman spectrophotofluorometer, using an excitation wavelength of 300 mμ, showed one major peak at 420–400 mμ, a minor peak at 340 mμ, and a small peak at 300 mμ from the excitation wavelength. There was a linear relationship between concentration and the recorded peak height at 430–440 mμ over the range 5–30 μg.

Preparation of CoQ Derivatives. DIMETHYL ETHERS OF CoQ_9 AND CoQ_{10} HYDROQUINONES.[2] To a stirred suspension of 2.0 g of either CoQ_9 or CoQ_{10} in 20 ml of absolute ethanol was added 20 ml of freshly distilled dimethyl sulfate at room temperature (N_2 atmosphere), followed by ten 40-mg portions of KBH_4. After 20 minutes, a further 120 mg of KBH_4 was added to the now colorless reaction mixture followed by 30% NaOH added dropwise (over 2.5 hours) to slight excess. The mixture was refluxed under N_2 for 1.5 hours. After cooling and dilution with 50 ml H_2O, the mixture was extracted three times with 125 ml of ether. The combined ether extracts were washed three times with saturated NaCl solution, dried over $MgSO_4$, filtered, and evaporated to dryness to yield about 2.2 g of a viscous oil. The oil crystallized on standing overnight at 4°. The total solids were chromatographed on a 10-mm × 20-cm column of acid-washed alumina packed in Skellysolve B. Elution was carried out with 100 ml of each of the following: Skellysolve B, isooctane, 5% diethyl ether–95% isooctane (v/v), and ether.

The dimethyl ether of CoQ_9 hydroquinone isolated from the ether-isooctane fractions was recrystallized from ether-ethanol-isooctane (1:1:1), yielding 1.35 g of white plates, mp, 32–35°; $\lambda_{max}^{ethanol}$ 275 mμ; $E_{1\,cm}^{1\%}$ = 9.5.

Anal. Calcd for $C_{56}H_{88}O_4$: C, 81.49; H, 10.75; CH_3O, 15.07%. Found[3]: C, 80.76, 80.98; H, 10.58, 10.49; CH_3O, 14.47, 14.73%.

The dimethyl ether of CoQ_{10} hydroquinone, also isolated from the corresponding ether-isooctane fractions, crystallized spontaneously on evaporation, mp 36–37°. It was recrystallized from anhydrous ethanol to constant mp of 37–38.5°; $\lambda_{max}^{ethanol}$ 275 mμ; $E_{1\,cm}^{1\%}$ = 8.0.

Anal. Calcd for $C_{61}H_{96}O_4$: C, 82.01; H, 10.83; CH_3O, 13.90%. Found: C, 81.98; H, 10.64; CH_3O, 13.97%.

Preparation of Substituted Benzoic and Phenylacetic Acids. TETRAMETHOXYPHTHALIC ANHYDRIDE. Tetramethoxyphthalic acid (17.3 mg), prepared from rubrogliocladin (Vischer, 1953), was added to 2.0 g of

[2] We are indebted to Dr. Karl Folkers for sending us details of his preparation of the dimethyl ether of CoQ_{10} hydroquinone which was modified in regard to conditions for reduction and methylation.

[3] Microanalyses were by Dr. Carl Tiedcke, Teaneck, N.J., and the Microanalytical Laboratory, Mellon Institute of Industrial Research, Pittsburgh, Pa.

Celite impregnated with 8 drops of 27 N H_2SO_4. The mixture was added to a standard column of Celite (12 g) wet with 8 ml of 0.5 N H_2SO_4 (Phares *et al.*, 1952), which was eluted with chloroform. Fractions were collected every 30 minutes. Fractions 3–6 contained 15.4 mg of a tan solid, mp 138–140° (93% yield). The anhydride was sublimed at 95–105° (bath temperature) and 1 mm pressure to obtain 11.7 mg of pure tetramethoxyphthalic anhydride, mp 138–139°; literature (Vischer, 1953) mp, 136–137°.

3′,4′,5′,6′-TETRAMETHOXY-2′-METHYLPHENYLACETIC ACID. This compound was prepared by the general method of Shunk *et al.* (1960). 3,4-Dimethoxytoluquinone (500 mg) (Anslow *et al.*, 1938), in 5 ml ethanol and 5 ml dimethyl sulfate, was treated under stirring with 100 mg of KBH_4 in a N_2 atmosphere. The initially red solution became faintly yellow after 15 minutes. Six ml of 30% NaOH was then added dropwise (30 minutes) with cooling. After stirring for an additional hour at room temperature, the mixture was heated at 90° for 1 hour. After cooling and dilution with 25 ml H_2O, the mixture was extracted three times with 125 ml of ether. The combined ether extracts, after being washed several times with saturated NaCl solution, were dried over $MgSO_4$, then filtered and concentrated to dryness to obtain 1.15 g of 2,3,4,5-tetramethoxytoluene as a light-yellow oil. This compound was added to a mixture of 25 ml concentrated HCl and 1.0 ml of 37% formaldehyde and was stirred while HCl gas was added for 2.5 hours at 45°. The reaction mixture, diluted with water (25 ml), was extracted three times with 100 ml of ether. The combined ether extracts, after being washed with water, were dried over $MgSO_4$ and concentrated to dryness to yield 1.5 g of 2-methyl-3,4,5,6-tetramethoxybenzyl chloride.

To 0.69 g of KCN dissolved in 8 ml of H_2O was added 1.5 g of 2-methyl-3,4,5,6-tetramethoxybenzyl chloride in 40 ml of ethanol. The mixture was stirred under reflux for 4.5 hours. After removal of ethanol *in vacuo* and addition of water (25 ml), the mixture was extracted three times with ether (50 ml). The combined ether extracts were washed with water, dried over $MgSO_4$, filtered, and concentrated to yield 1.2 g of 2-methyl-3,4,5,6-tetramethoxybenzyl cyanide. This latter material was refluxed with 200 ml of 50% methanol containing 30 g of KOH for 15 hours. Ammonia was evolved. After concentration *in vacuo*, the residue was extracted four times with chloroform (50 ml). The combined chloroform extracts were dried over $MgSO_4$, filtered, concentrated to dryness, and dried *in vacuo*. The residual yellowish oil (270 mg) was sublimed at 1 mm Hg to yield 213 mg of 3′,4′,5′,6′-tetramethoxy-2′-methylphenylacetic acid as a white crystalline solid, mp 70–72° (literature mp 75–76°). The acid (25 mg) was purified by chromatography on a standard Celite column, 11 mm × 32 cm (Phares *et al.*, 1952). Elution with chloroform saturated with 0.5 N H_2SO_4 yielded the pure white, crystalline acid, mp 74.5–76°.

PREPARATION OF 2,5-DIACETOXY-3,4-DIMETHOXY-*o*-TOLUIC ACID. Small portions of zinc dust were added to 1.1 g of resublimed 3,4-dimethoxytoluqinone in glacial acetic acid (4.0 ml) until the solution was colorless. Chloroform (50 ml) was added and the mixture was filtered through glass wool. The filtrate was washed with saturated NaCl and the chloroform solution then dried over Na_2SO_4. Vacuum evaporation of the solvent gave a yellow oil which was dissolved in pentane (50 ml), seeded, and allowed to stand overnight at 4°. There was obtained a 75% yield of white, crystalline hydroquinone (833 mg) with mp 73.5–74°. After purification by sublimation at 0.01 mm Hg (bath temperature, 45–60°) and two recrystallizations from ether-pentane (1:5), 3,4-dimethoxytoluhydroquinone had mp 74.5–75.5°; $\lambda_{max}^{ethanol}$ 289 mμ; $E_{1\,cm}^{1\%} = 177$.

Anal. Calcd for $C_9H_{12}O_4$: C, 58.68; H, 6.57%. Found: C, 58.70, 58.51; H, 6.01, 6.17%.

3,4-Dimethoxytoluhydroquinone (833 mg) and zinc cyanide (790 mg) (Vogel, 1956), both dried overnight over P_2O_5, were mixed with dry ether (15 ml). Dry HCl gas was *rapidly* passed into the mixture for 50 minutes, with stirring. A yellow oil separated. After the ether layer was decanted the oil was heated with 5 ml of H_2O at 90° for 3 minutes. Gas was evolved and a yellow solid precipitated. After standing overnight at 4°, the precipitate was filtered to yield 647 mg of material with mp 105°. By heating of the filtrate at 100° for 5 minutes, then cooling at 4° for 2.5 hours, an additional 35 mg of crude aldehyde was obtained.

The first crop of aldehyde was washed successively with one 2-ml, two 1-ml, and two 0.5-ml portions of H_2O. The residue, dried over P_2O_5 overnight, weighed 247 mg, mp 125–129°. The second crop was warmed at 60° for a few minutes with 1 ml of H_2O. After the solution had stood overnight at 4°, there was obtained 25 mg of aldehyde, mp 129–130°. The combined yield was 28% of the theoretical. Aldehyde (247 mg), dissolved in 5 ml of ether at 40°, was treated with 11 ml of pentane. After the solution had remained at 4° for 2 days, yellow needles of 3,4-dimethoxytoluhydroquinone-6-aldehyde (197 mg) were obtained by decanting the supernatant fluids and washing with pentane, mp 130–131°. A further 24 mg was obtained from the mother liquor.

Anal. Calcd for $C_{10}H_{12}O_5$: C, 56.59; H, 5.70%. Found: C, 56.46, 56.60; H, 5.15, 5.27%.

The infrared and ultraviolet absorption spectra of 3,4-dimethoxytoluhydroquinone-6-aldehyde were similar to those of the hydroquinone aldehydes studied by Quilico and Cardani (1953). In the infrared spectrum, determined by the KBr pellet technique, a carbonyl stretching absorption was observed at 1625 to 1620 cm^{-1}. A similar value was also determined for orsellinic aldehyde. This absorption appears at slightly lower frequency than those commonly reported for aromatic aldehydes. The following peaks were observed in the ultraviolet absorption spectrum of 3,4-dimethoxytoluhydroquinone-6-aldehyde: shoulder at 233 mμ, $E_{1\,cm}^{1\%} = 427$; $\lambda_{max}^{ethanol}$ 290 mμ, $E_{1\,cm}^{1\%} = 562$; $\lambda_{max}^{ethanol}$ 376 mμ, $E_{1\,cm}^{1\%} = 181$.

The foregoing aldehyde (172 mg), 1.5 ml freshly distilled acetic anhydride, 0.5 ml freshly distilled triethylamine, and 5 ml of sodium-dry ether were allowed to

stand at room temperature for 17 hours. After dilution with 25 ml ether, the mixture was extracted with small portions of saturated aqueous NaCl solution. The residue, obtained on evaporation of the ether in a stream of N_2 gas at 40°, was stirred with 12 g of crushed ice. A white solid (214 mg; 89% of theoretical) separated, mp 91–92°. Recrystallization from ether by addition of pentane gave 169 mg of colorless needles of 2,5-diacetoxy-3,4-dimethoxy-*o*-tolualdehyde, mp 92–94°. After sublimation at 0.01 mm Hg (bath temperature, 55–80°), the compound had mp 93–94°, $\lambda_{max}^{ethanol}$ 269 mμ; $E_{1\,cm}^{1\%} = 35.8$.

Anal. Calcd for $C_{14}H_{16}O_7$: C, 56.75; H, 5.44%. Found: C, 56.90; 56,98; H, 5.71, 5.73%.

Sublimed 2,5-diacetoxy-3,4-dimethoxy-*o*-tolualdehyde (88.5 mg dissolved in 80 ml of acetone) was oxidized with 400 mg each of $KMnO_4$ and $MgSO_4$ in water (8 ml) added dropwise with stirring, at room temperature. After 2.5 hours the mixture was cooled to 0° and treated with SO_2 gas until colorless. Following removal of acetone by vacuum evaporation at 45°, the aqueous solution was extracted with ether. The combined ether extracts were extracted with one 10-ml and four 5-ml portions of 5% aqueous sodium bicarbonate, and then with two 2-ml portions of water. The aqueous extracts were acidified to Congo red with concentrated HCl, saturated with NaCl, and extracted with one 20-ml and five 5-ml portions of chloroform. The combined chloroform extracts were washed with saturated NaCl, dried over sodium sulfate, then evaporated to an oily residue (93 mg, 99% yield) which crystallized slowly on standing.

The crude solid, recrystallized from ether (2.5 ml) by careful addition of pentane (12 ml), yielded 74 mg of 2,5-diacetoxy-3,4-dimethoxy-*o*-toluic acid, mp 120–121.5°.

Anal. Calcd for $C_{14}H_{16}O_8$: C, 53.84; H, 5.17%. Found: C, 54.02, 54.20; H, 5.35, 5.37%.

The same toluic acid was also prepared from rubrogliocladin. Rubrogliocladin (200 mg), 200 mg of zinc dust, 5 ml of acetic anhydride, and 1 ml of freshly distilled triethylamine were heated for 4 minutes at 95°. After addition of 25 ml of cyclohexane and filtration of the solution through glass wool, the cyclohexane was washed with three 15-ml portions of 0.3 N HCl and water. The colorless oil obtained on evaporation was then crystallized from 80% ethanol to yield 112 mg of the diacetate, mp 91–92°. A 25-mg portion of this diacetate in 10 mg of acetone-water (1:1) at 70° was treated dropwise for 15 minutes with 5 ml of water containing 280 mg each of $KMnO_4$ and $MgSO_4$. After heating for an additional hour, all of the permanganate had been utilized. A further 1 ml of the oxidizing mixture was then added and heating was continued for 30 minutes. The cooled reaction mixture was treated with SO_2 gas until colorless. Most of the acetone was then removed by vacuum evaporation. The aqueous residue was extracted with ether and the combined ether extracts were back-extracted with water. After drying the extract over Na_2SO_4, the ether was evaporated. The oily residue was found on gas chromatography (see Analytical Methods) to contain 46% of the starting material and 54% of the acid. The oil, dissolved in ether, was separated into neutral and acid fractions in the usual way. From the acid extract there was obtained 12.5 mg of product. Following recrystallization from acetone–petroleum ether, 2,5-diacetoxy-3,4-dimethoxy-*o*-toluic acid had mp 117.5–118°, and showed no mp depression in admixture with material prepared from 3,4-dimethoxytoluquinone.

Anal. Calcd for $C_{14}H_{16}O_8$: C, 53.84; H, 5.17%. Found: C, 53.54; H, 5.16%.

Ozonolysis Techniques. OZONOLYSIS IN ETHYL ACETATE AT LOW TEMPERATURE. The "standard" conditions for low temperature ozonolysis as developed in many experiments are as follows. The diacetate of CoQ_9 hydroquinone (264 mg) was dissolved in ethyl acetate (7 ml) and 0.2 ml of water was added. After cooling in a dry ice–acetone bath, ozonized oxygen (which had been bubbled through water) was passed in at a rate of 0.01 standard cu ft/min. The voltage setting of the Welsbach ozonator was 58, and 18 mg O_3 per minute was obtained. The theoretical amount of O_3 would require 8 minutes of treatment. After 12 minutes a blue solution was obtained, showing the presence of excess ozone. The treatment with ozonized oxygen was continued for a total of 60 minutes.

The solvent was removed by vacuum distillation at 30°, and the residue was taken up in 70 ml of ether. The ether was extracted with five 20-ml and five 10-ml portions of water and each aqueous extract was in turn back-extracted with 5 ml of ether. The combined aqueous extracts are termed fraction I.

The combined ether extracts were dried over Na_2SO_4, filtered, and evaporated to dryness. The residual oil (125 mg), dissolved in acetone, 90 ml, was treated at room temperature with a solution of 250 mg each of $KMnO_4$ and $MgSO_4$ in 5 ml of water. This was added with stirring at the rate of 1 ml/minute and stirring was continued for an additional 40 minutes after all the oxidant had been added. The reaction mixture, cooled in ice, was treated with SO_2 gas until it was colorless. After vacuum evaporation of the acetone, the aqueous residue was extracted with three 50-ml portions of ether. The combined ether extracts were washed with six 5-ml portions of 10% $NaHCO_3$ and two 5-ml portions of H_2O. The washed ether layer was concentrated to obtain 18.9 of neutral material, fraction II (not further investigated).

The combined basic aqueous extracts, cooled in ice, were acidified to Congo red with concentrated HCl. The acid solution was extracted with six 10-ml portions of chloroform. The combined chloroform extracts were washed with water (10 ml), dried, filtered, and concentrated. The residue, 44 mg, was termed fraction III.

Fraction I. The combined aqueous extracts were treated with 200 mg of 2,4-dinitrophenylhydrazine in 160 ml of 6 N H_2SO_4. The precipitate, obtained on standing, was filtered, washed with water, and recrystallized, first from methanol, then from chloroform to yield 348 mg of levulinaldehyde bis-2,4-dinitrophenylhydrazone, mp 228–232° (32% yield).

Fraction III. This fraction was sublimed at 0.005 mm Hg, and the following fractions were collected: bath temperature 105–115°, 7.76 mg (yellowish oil); 115–130°, 19.10 mg (colorless oil); 130–140°, 15.16 mg (colorless oil). The fractions were separately crystallized from ether–petroleum ether. After recrystallization, there was obtained 24.0 mg of 3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid, mp 132–133° (24.5% of theory).

Higher yields of aromatic acid were frequently obtained; e.g., from 140 mg of the diacetate of CoQ_9 hydroquinone, the final yield of recrystallized aromatic acid, mp 134–136°, was 17.8 mg (34.8% of theory).

OZONOLYSIS WITH SUBSEQUENT OXIDATION OF BOTH AROMATIC ALDEHYDE AND LEVULINALDEHYDE. CoQ_9 hydroquinone diacetate (300 mg) was ozonized as previously described. The residue obtained on removal of solvent was immediately dissolved in acetone, 100 ml, and was oxidized with 400 mg each of $KMnO_4$ and $MgSO_4$ in 8 ml of H_2O under the usual conditions.

After the usual removal of ether-soluble neutral materials, the alkaline extracts were made acid to Congo red and extracted with one 10-ml and five 5-ml portions of chloroform. The combined chloroform extracts (containing aromatic acid) were washed with three 2-ml portions of saturated NaCl solution (washing discarded). The aqueous layer after chloroform extraction contained levulinic acid, which was isolated as the 2,4-dinitrophenylhydrazone (210 mg, mp 188–195°). On recrystallization from 7 ml of chloroform, the pure derivative had mp 211–213°.

The aromatic acid extract was dried over Na_2SO_4, filtered, and evaporated to yield 141 mg of a pale-yellow oil which was sublimed and recrystallized as previously described to give 53 mg of acid, mp 130–132° (48% of theoretical).

Purification of 3′,6′-Diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic Acid by Column Chromatography. Crude acid (37.8 mg) was dissolved in a little benzene and applied to a column (1.7 × 20 cm) of silicic acid (80–100 mesh). The column was prewashed with 100 ml benzene, 100 ml ether, and 100 ml benzene again. The eluting solvents were 400-ml portions of the following ether-benzene mixtures: 10:90, 15:85, 20:80. The combined 15:85 and 20:80 eluates were evaporated yielding 23 mg of residue which, after sublimation and recrystallization in the usual way, had mp 136–138° (corr). This was the highest mp recorded for the phenylacetic acid. On gas chromatography this material showed a single peak, both as free acid and methyl ester.

Results

Oxidation of Dimethyl Ethers of Coenzyme Q. The dimethyl ethers of the hydroquinones of coenzymes Q_9 and Q_{10} were prepared and attempts were carried out to degrade them oxidatively on a 0.5-mM scale. These oxidation studies were facilitated by the spectrophotofluorometric assay for the expected tetramethoxyphthalic anhydride. Permanganate oxidations of preparations of the dimethyl ethers of CoQ_9 and CoQ_{10} hydroquinones were carried out with either 1 M $KMnO_4$ and 1 M KOH, or with 5% $KMnO_4$ and 1 M KOH, and at temperatures in the range 45–100°. The resulting solutions were then analyzed; although a fluorescence peak at 420 mμ was usually detectable, only very small amounts of phthalate were present and it was impossible to isolate any pure aromatic acid. The tetramethoxyphthalic anhydride was found to be destroyed to a considerable extent on treatment with $KMnO_4$ under the oxidation conditions applied to the dimethyl ether derivatives. Attempts to obtain the required oxidation products by use of $KMnO_4$ in acetone, periodate-permanganate, OsO_4-$KClO_3$, or chromic acid were equally unsuccessful.

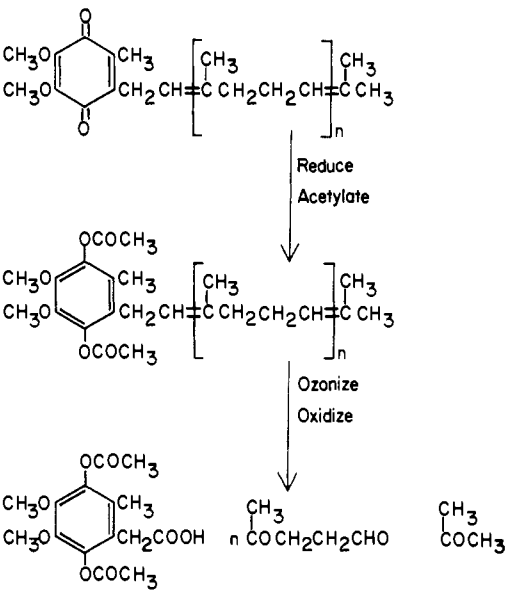

FIGURE 1: The ozonolysis reaction. The reactions shown are the reductive acetylation of the CoQ derivative, followed by cleavage of the double bonds with ozone. The oxidation of the aromatic aldehyde to acid is not shown as a separate step.

Oxidation of Diacetates of Coenzyme Q. A detailed study of the ozonolysis of the diacetates of either CoQ_9 or CoQ_{10} hydroquinone was then carried out, starting with the conditions of Morton *et al.* (1958) who used glacial acetic acid as solvent, at 15°. The reactions involved are shown in Figure 1. To avoid overoxidation, ozone in slightly less than the theoretical amount was used with the aid of an ozone meter. Table I gives the yields of two of the expected products (3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid and levulinaldehyde as the bis-dinitrophenylhydrazone); the yield of the third product, acetone, was not measured. The highest yield of the phenylacetic acid was approximately 13.5% in an experiment in which 78% of the theoretical amount of ozone was used. The use of more or less ozone apparently resulted in decreased yields of the phenylacetic acid. The acid obtained in this way usually had mp 119°. In these experiments,

TABLE I: Ozonolysis of Diacetate of CoQ_9 Hydroquinone Using Dry Ozone.[a]

Expt No.	Amount of Diacetate Used (g)	Amount of Ozone Used (% theory)	Aromatic Acid Yield (mg)	Aromatic Acid Mp (°)	Neutral Fraction Yield (mg)	Levulinaldehyde (as 2,4-DNP) Yield (mg)	Levulinaldehyde (as 2,4-DNP) Mp (°)
1	1.08	90	15.4[b]	[c]	7.65	770	225–228
2	1.01	98	17.9[b]		89.14	650	216–225
3	0.289	Excess[d]	46.2[b]		98.53	412	Crude, 205
4	0.290	78	14.4	116–118	16.76	600	216
5	0.500	93	22.0	117–119	43.35		
6	0.254	81	10.5	112–114	55.5	315	233–236
7a	0.250	90	5.93	112–115			
7b	0.250	80	6.36	110–114	116		
7c	0.250	70	3.54[e]	105–108	22		

[a] In these experiments, the diacetate was dissolved in glacial acetic acid and was treated with dry ozone at 15°. Less than theoretical amounts of ozone were used as indicated, with the exception of experiment 3. The amount of ozone in the gas stream was 39.7 mg/liter, or 0.67 g/hr. The ozone was metered into the solution on the basis of the time needed for the required amount. The reaction mixtures were fractionated following the general procedure of Morton *et al.* (1958); except as indicated, the aromatic acid was sublimed *in vacuo*, and further crystallized from ethanol-ether (1:4). [b] Determined as the crude oil, prior to sublimation. [c] Not determined. [d] Twice theory. [e] This includes a small amount, collected as a second fraction, having mp 134–136°.

we reduced the ozonide by zinc dust to obtain the corresponding phenylacetaldehyde, which was then oxidized to acid with permanganate. We investigated a number of other reagents without success for cleavage of the ozonide, such as trimethyl phosphite (Knowles and Thompson, 1960) and oxidation with H_2O_2, peracetic acid, and silver oxide.

Since it appeared that the highly O-substituted ring was particularly labile to ozonolysis at 15–25°, we investigated ozonolysis at lower temperatures. With ethyl acetate and damp ozone at −70°, it was possible to isolate some of the phenylacetic acid immediately following the treatment with ozone, and without a reductive cleavage and subsequent $KMnO_4$ treatment. It appeared that the ozonide cleavage was brought about by water and that the aldehyde was subsequently oxidized either by excess ozone or the hydrogen peroxide formed in the hydrolysis. Following this, additional amounts of acid could be obtained by oxidation of the residual aldehyde with $KMnO_4$. A series of experiments with this technique is shown in Table II. With the use of excess ozone, and in some cases of a further quantity of oxygen, the amount of acid present following ozonolysis increased, and, correspondingly, that added by subsequent $KMnO_4$ treatment declined.

Further investigations of this general system may be summarized as follows: The addition of water to the ethyl acetate solution was helpful in increasing the yield of aromatic acid, while the addition of catalysts such as acetic acid or triethylamine had little effect. An excess of ozone was desirable and further treatment with oxygen could then be omitted. Despite the fact that some phenylacetic acid could be obtained immediately after ozonolysis, we concluded that extraction of the aromatic aldehyde followed by $KMnO_4$ oxidation was the most useful routine procedure. This has been described above as our "standard" procedure. Reproducible yields up to 40% of theoretical have been obtained under these conditions, as well as adequate amounts of levulinaldehyde as the bis-2,4-dinitrophenylhydrazone.

It was also possible to oxidize the crude ozonolysis mixture directly with permanganate, with isolation of levulinic acid and the aromatic acid. Good yields of aromatic acid, ranging from 47.7 to 56.7% of theory, were obtained at the 0.3 mM level. The yields of levulinic acid 2,4-dinitrophenylhydrazone were about 30% of theoretical.

The low temperature ozonolysis was applied to the dimethyl ether of CoQ_9 hydroquinone. The expected aromatic product, 3′,4′,5′,6′-tetramethoxy-2′-methylphenylacetic acid, was obtained with mp 71–72° after purification by column chromatography (literature mp 75–76°). In these experiments, however, yields of only 5–10% of the aromatic acid were obtained and the procedure was not pursued further.

Identification of Products of Oxidation. Gas-liquid chromatography provided a rapid method for the identification of the aromatic fragments (both aldehydes and acids) obtained in the ozonolysis. From studies using 3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid and 3′,4′,5′,6′-tetramethoxy-2′-methylphenylacetic acid as reference compounds, it was found that the silicone rubber gum, SE-30, at 170–180° was the best liquid phase. Further results with various model compounds and expected products from the ozonolysis experiments are shown in Table

TABLE II: Ozonolysis of Diacetate of CoQ_9 Hydroquinone at Low Temperature and with Various Solvents.[a]

Expt No.	Amount Diacetate (mg)	Solvent	O_3 Treatment (min)	O_2 Treatment (min)	Aromatic Acid after Ozonolysis (mg)	(mp, °)	Levulinaldehyde as 2,4-DNP (mg)	(mp, °)	Aromatic Aldehyde (mg)	$KMnO_4$ Oxidation Products: Acidic (mg)	(mp, °)	Neutral (mg)
1	131.1	Pyridine-CH_2Cl_2[b]	6[c]	0	0		277.5	228–232	28.0	12.2	126–131	5.9
2	50.1	Ethyl acetate[d]	4.5[e]	0	3.0	[f]			12.7	3.4		5.0
3	51.3	Ethyl acetate	20	60	9.4	124–127			11.2	2.8		4.6
4	63.8	Ethyl acetate	60		10.5	129	77.6	205–210	9.5	1.0		2.1
5a	144.8[g]	Ethyl acetate	60	0						16.1	133–134	5.0
5b					21.6		134.1	195–205		15.0	128–132	4.7
6	52.8	Ethyl acetate	60	60	20.6		25	205–215	8.5	1.6		7.6

[a] In each case, the ozonized oxygen at a flow rate of 0.01 standard cu ft/min (approximately 18.0 mg O_3 per minute) was first bubbled through water. Following treatment with ozone, and, in some cases subsequently with oxygen, any aromatic acid present was isolated as follows: Immediately after ozonolysis was complete, the reaction mixture was concentrated to dryness under N_2, and the residue was dissolved in ether. The ether solution was washed several times with water to remove levulinaldehyde. Acidic material was then extracted by shaking with 5% $NaHCO_3$ solution; the aqueous extract was carefully acidified, with cooling, and the aromatic acid was reextracted into chloroform. After removal of solvent, the acid was sublimed and recrystallized in the usual way. The ether solution from which acid had been removed was dried, filtered, and evaporated to obtain the weight of the "aromatic aldehyde." This residue was then oxidized with neutral permanganate; the yields of phenylacetic acid and of neutral material from this oxidation were determined in the usual way and are given as "$KMnO_4$ Oxidation Products" in the final columns of this table. [b] Methylene chloride containing 1% of pyridine was used. [c] 140% of theoretical. [d] Ethyl acetate indicates 7 ml of redistilled ethyl acetate containing 0.2 ml of water. [e] 160% of theoretical. [f] Not determined. [g] In this experiment, the solution following ozonolysis was divided into two parts; one-third (5a) was oxidized directly, as described under Experimental Procedure, and the other two-thirds (5b) was used as described above.

TABLE III: Gas-Liquid Chromatography of Derivatives of Benzoic and Phenylacetic Acids.[a]

Structure of Parent Acid	Retention Times			Mp of Acid (°)
	Aldehyde (min)	Acid (min)	Methyl Ester (min)	
m-Methoxybenzoic		1.0	0.8	110
m-Methoxyphenylacetic		1.4	1.2	67
p-Methoxybenzoic		1.3	0.94	184
p-Methoxyphenylacetic		1.5[b]	1.2	85–87
3,4-Dihydroxybenzoic			1.5, 1.9, 2.8	199
3,4-Dihydroxyphenylacetic			1.9, 2.1	127
3,4-Dimethoxybenzoic		2.5	1.9	181
3,4-Dimethoxyphenylacetic		2.8	2.1	82
3,4,5-Trimethoxybenzoic	2.0	3.4	3.2[b]	170
3,4,5-Trimethoxyphenylacetic		3.8[b]	4.0[b]	120
3′,6′-Diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid[c]	8.4	7.7	11.4	120–125
3′,4′,5′,6′-Tetramethoxy-2′-methylphenylacetic acid[d]	3.1	6.2	4.4	Oil
3′,4′,5′,6′-Tetramethoxy-2′-methylphenylacetic acid[e]		6.1	4.5	74.5–76

[a] The samples were chromatographed on a 4% column of SE-30, operated at 180°, as described in the text. [b] In these cases, column temperature = 176°. [c] Obtained by ozonolysis of diacetate of CoQ_9 hydroquinone. [d] Obtained by ozonolysis of dimethyl ether of CoQ_9 hydroquinone. [e] Synthetic.

III. When similarly substituted phenylacetate derivatives were compared with benzoate derivatives on SE-30, the phenylacetic acid derivatives had the longer retention times. Generally, the esters moved more rapidly than the acids themselves, but there were some exceptions. For example, the methyl ester of 3′,6′-diacetoxy-4′,5′-dimethoxy 2′-methylphenylacetic acid had a longer retention time than the acid on SE-30 columns. When hydroxyaromatic acids were treated with diazomethane, two or three peaks were usually obtained indicating varying degrees of methylation. The phenylacetic acid derivative, obtained from ozonolysis experiments, which had a mp higher than 132°, showed a single peak on the gas chromatograms, both as free acid and as methyl ester.

When the mother liquors, from which pure phenylacetic acid had been crystallized, or crude preparations of this acid were examined on 4% nitrile silicone columns (210–215°), a number of minor peaks were usually observed (see Figure 2). Suspecting that one of these peaks might be the corresponding benzoate derivative (2,5-diacetoxy-3,4-dimethoxy-*o*-toluic acid), the latter was synthesized by the two procedures described earlier. The authentic *o*-toluic acid, both as the free acid and as the methyl ester, corresponded with one of the peaks observed after ozonolysis. It appears likely that the phenylacetic acid is oxidized to some extent to the *o*-toluic acid during the $KMnO_4$ oxidation. In some degradations, another minor peak has been tentatively identified as the diacetate of CoQ_0 hydroquinone (2,5-diacetoxy-3,4-dimethoxytoluene) by the same gas

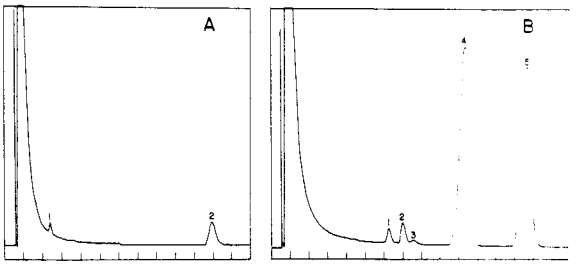

FIGURE 2: Gas-liquid chromatography of aromatic degradation products. The liquid phase was 3% nitrile silicone; a coiled column 183 cm long (i.d., 6.35 mm) was used in the Chromolab instrument at 214°. In each case, the marked time divisions are at intervals of 1.5 minutes. (A) Free acids. The chromatogram was obtained with the mother liquor residues after crystallization of the crude phenylacetic acid from ozonolysis of the diacetate of CoQ_9 hydroquinone. The numbered peaks are identified as follows on the basis of comparison with standard compounds; the figures are the retention times in minutes: 1 = 2,5-diacetoxy-3,4-dimethoxy-*o*-toluic acid, 2.6; 2 = 3′,6-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid, 15.3. (B) Methyl esters. The mixture analyzed in (A) was reacted with diazomethane, and a larger aliquot was injected to reveal other trace components. 1, 2, and 3, are unidentified peaks; 4 = methyl ester of 3′,6′-diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid, 14.7; 5 = methyl ester of 2,5-diacetoxy-3,4-dimethoxy-o-toluic acid, 19.7.

chromatographic procedure. This apparently represents a further degradation product from the *o*-toluic acid derivative. Preparations of the latter acid, on standing in benzene solution for long periods, have been observed to undergo decomposition with the formation of 2,5-diacetoxy-3,4-dimethoxytoluene.

Isotope Experiments. L-[U-^{14}C]Phenylalanine was incorporated into total rat body CoQ_9 with an over-all yield of only 0.005%. Although low, this is of the same order as that observed for [1-^{14}C]acetate (Olson *et al.*, 1964).

Table IV presents the results of degradation of the radioactive CoQ_9 hydroquinone diacetate isolated in two experiments. The radioactivity reported for the CoQ_9 hydroquinone diacetate is the radioactivity of the sample which was degraded. The results indicate that between 72 and 88% of the radioactivity from uniformly labeled phenylalanine appears in the ring fragment and from 0 to 33.5% appears in the side chain. This is consistent with two pathways of incorporation of aromatic amino acid carbon into CoQ. One proceeds by way of homogentisate to acetoacetate and then reincorporation via mevalonate into the side chain; the other proceeds by direct incorporation of a nuclear fragment derived from the ring of phenylalanine into the ring of CoQ_9.

Discussion

The report by Wolf *et al.* (1958) that the permanganate oxidation of the dimethyl ether of coenzyme Q_{10} hydroquinone led to the isolation of tetramethoxyphthalic anhydride and 3′,4′,5′,6′-tetramethoxy-2′-methylphenylacetic acid in unspecified yields stimulated us to attempt the same procedure on a "semimicro" scale. Our failure to duplicate their results seems less surprising in retrospect. We have subsequently learned that the permanganate oxidations of the dimethyl ethers of CoQ_{10} hydroquinone reported by Wolf *et al.* (1958) gave very poor yields of aromatic fragments.[4] It may also be noted that Lester *et al.* (1959) were unable to obtain any phthalate derivatives from the oxidation of 236 mg of CoQ_{10} with aqueous alkaline permanganate. The original conditions described for the ozonolysis of the diacetates of CoQ hydroquinones (Morton *et al.*, 1958) have given variable results in other laboratories. For example, Lawson *et al.* (1961) obtained only a poor yield of the phenylacetic acid even after conversion to the *S*-benzylisothiouronium salt (which was "not easily crystallized").

In glacial acetic acid at 15°, we obtained low yields of the substituted phenylacetic acid from CoQ_9 hydroquinone diacetate. The mp of the product was usually 120°, which agreed well with that of 121–124° reported originally by Morton *et al.* (1958). However, with the low temperature ozonolysis, the mp of this acid was observed to be considerably higher, averaging about 132° in many preparations. For one sample, purified

TABLE IV: Specific Radioactivity of Rat Body CoQ_9 Hydroquinone Diacetate and Its Degradation Products after Administration of L-[U-^{14}C]Phenylalanine.

Expt No.	Substrate	No. Animals	Total Dose Substrate (mc)	CoQ_9 Hydroquinone Diacetate[a] (μc/mM × 10^2)	Degradation Products: Phenylacetic Acid[b] (μc/mM × 10^2)	Levulinaldehyde[c] (μc/mM × 10^2)	Acetone[d] (μc/mM × 10^2)	Distribution of Radioactivity: Ring Fragment (%)	Side Chain[e] (%)	Total (%)
1	L-[U-^{14}C]Phenylalanine	10	0.5	2.99	2.64	0	0	88.0	0	88
2	L-[U-^{14}C]Phenylalanine	10	1.0	8.30	5.92	0.32	0.19	71.5	33.5	105

[a] Dilution factor from body CoQ_9 due to addition of carrier ranged from 5 to 18. [b] 3′,6′-Diacetoxy-4′,5′-dimethoxy-2′-methylphenylacetic acid. [c] Measured as the bis-dinitrophenylhydrazone. [d] Calculated from the activity of the levulinaldehyde. [e] Side-chain activity calculated by multiplying molar levulinaldehyde specific activity by 8 plus distribution of acetone.

[4] Private communication from Dr. Karl Folkers.

first by column chromatography on silicic acid, then sublimed and crystallized, a mp of 136–138° was obtained. Subsequent to our preliminary communication (Bentley *et al.*, 1961), Dr. Wiss informed us that an mp of 136° had been obtained for the same phenylacetic acid derivative in his laboratory.[5] A sample of his material showed no mp depression in admixture with our preparations, and behaved identically on gas chromatography.

Our "standard" low temperature method employing moist ozone described here has consistently given yields of pure phenylacetic acid in the range from 25 to 40% of theory. Somewhat higher yields of the phenylacetic acid, from 48 to 49% of theoretical, were obtained by treatment of the entire ozonolysis mixture, directly with $KMnO_4$. A disadvantage of this latter technique, however, is that the C_5 units are obtained as the 2,4-dinitrophenylhydrazone of levulinic acid. This derivative is more soluble in the usual solvents than levulinaldehyde bis-2,4-dinitrophenylhydrazone, and hence losses in crystallization of the levulinic acid derivative are larger than in the case of the levulinaldehyde hydrazone. These losses would be of concern, however, only if very small quantities of material were being degraded.

With our standard conditions, some aromatic aldehyde remained unoxidized, so that we have routinely preferred to include an oxidation step with neutral permanganate. Our reason for the use of moist ozone, and the actual addition of water to the ozonolysis solvent, was that Dean *et al.* (1959) had shown that 3,4,6-trimethoxy-2-propenylacetophenone formed an isoozonide so stable that the corresponding 2-acetyl-3,5,6-trimethoxybenzaldehyde could not be prepared. By addition of water to the reaction mixture, it was found that the aldehyde could be obtained in good yield. The water apparently competed for the carbonium ion center in an intermediate, and thus promoted fission in the desired direction to aldehyde.

The incorporation of radioactivity from labeled phenylalanine into CoQ_9 is unequivocally established by these experiments. Wiss *et al.* (1961a) reported that uniformly labeled phenylalanine orally administered to rats yielded only traces (0.001%) of radioactivity in the nonsaponifiable fraction from liver after 2 hours. In a later report (1961b) these same workers observed that when tyrosine of higher radioactivity was administered *per os* to rats, 0.2% of the administered dose was found in the nonsaponifiable lipids of the liver and 0.0008% of the dose was found in hepatic CoQ. No degradations of this biosynthesized CoQ were reported. Our chemical degradations of the radioactive CoQ_9 biosynthesized in the rat from L-[U-^{14}C]phenylalanine show that phenylalanine contributes carbon largely to the nucleus and, to a lesser extent, to the side chain of CoQ_9.

Additional preliminary work in our laboratory (Olson *et al.*, 1963) has suggested that, whereas the carbon from phenylalanine entering the isoprenoid side chain follows the classical homogentisate pathway to acetoacetate, the ring carbon appears to enter the coenzyme Q molecule via a novel degradation to *p*-hydroxybenzoate with subsequent loss of the carboxyl group. The details of the biosynthesis of the aromatic ring of coenzyme Q are under continuing study.

[5] Private communication from Dr. O. Wiss.

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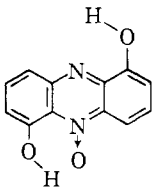
1,6-Phenazinediol-5-oxide from Microorganisms*

Nancy N. Gerber and Mary P. Lechevalier

ABSTRACT: The isolation and identification of 1,6-phenazinediol-5-oxide from *Microbispora aerata*, *Pseudomonas iodina*, and *Streptomyces thioluteus* is reported. The title compound was also synthesized by the partial reduction of 1,6-phenazinediol-5,10-dioxide (iodinin) with sodium hydrosulfite. The antimicrobial activity of 1,6-phenazinediol-5-oxide is intermediate between that of iodinin and phenazinediol. Experiments with cell suspensions indicate that 1,6-phenazinediol-5-oxide is an intermediate both in the biosynthesis of iodinin from 1,6-phenazinediol and in the microbial reduction of iodinin.

Recently we reported the isolation of iodinin (1,6-phenazinediol-5,10-dioxide), 1,6-phenazinediol, 2-aminophenoxazin-3-one, and 2-acetamidophenoxazin-3-one from *Microbispora*[1] *aerata* and *Pseudomonas iodina* (Gerber and Lechevalier, 1964). In this paper we noted the presence of an unidentified orange spot on certain paper chromatograms. The orange material could be obtained from either organism. Since in its color, ultraviolet spectrum, solubility, and chromatographic behavior the orange material was invariably intermediate between iodinin and 1,6-phenazinediol we suspected it had the intermediate, hitherto unreported structure, 1,6-phenazinediol-5-oxide.



The structure of the orange material was proved by analysis and by reduction to 1,6-phenazinediol with sodium hydrosulfite. Furthermore, when iodinin was incompletely reduced with sodium hydrosulfite there was obtained a mixture of iodinin, 1,6-phenazinediol-5-oxide, and phenazinediol which could be resolved (as was the naturally occurring mixture) by paper chromatography, countercurrent distribution, or partition chromatography on silica gel. 1,6-Phenazinediol-5-oxide could not be prepared free of iodinin by recrystallization or adsorption chromatography. The natural and synthetic products were identical in every respect. In a typical fermentation (Gerber and Lechevalier, 1964) from *Ps. iodina* 26, 12 and 3.8 mg/liter, respectively, of 1,6-phenazinediol-5-oxide and phenazinediol were obtained; from *M. aerata* the yields were much lower.

These three phenazines were also detected as minor components in chloroform extracts of *Streptomyces thioluteus* 12310[2] which had been grown on Pablum medium. A strain of this organism has been reported to produce aureothin (Hirata, 1961), 1,6-phenazinediol, aureothricin, and thiolutin (Akabori and Nakamura, 1959). The phenazines were separated from other metabolic products and partially from each other by column chromatography on silica gel, eluting with chloroform. Paper chromatography resolved the three phenazines completely and they were found to be identical with authentic specimens by paper chromatographic and spectral comparisons. In two fermentations the yields were in the range of 0.3 to 1 mg/liter for all three phenazines.

Antimicrobial assays of 1,6-phenazinediol-5-oxide were carried out as previously reported (Gerber and Lechevalier, 1964). The results are given in Table I. In most cases the activity was intermediate between that of iodinin and the phenazinediol. The static activity of 1,6-phenazinediol-5-oxide against *Hansenula anomala*, *Saccharomyces cerevisiae*, and *Nocardia coeliaca*

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[1] *Microbispora* = *Waksmania*. The former name has recently been found to have priority.

[2] Strain designations are those of the Institute of Microbiology, Rutgers Uuiversity.