Studies on Coenzyme Q

THE ISOLATION, CHARACTERIZATION, AND GENERAL PROPERTIES OF A PARTLY REDUCED COENZYME Q₉ FROM PENICILLIUM STIPITATUM*

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The ubiquitous nature of members of the coenzyme Q group has been well established, the several forms of the coenzyme differing only in the number of isoprene groups in the side chain. Evidence has been obtained for the natural occurrence of all of the isoprenologues from coenzyme Q₆ to coenzyme Q₁₀. Since there have been relatively few reports on the coenzyme Q components of molds, it seemed worthwhile to examine the mycelial pads of Penicillium stipitatum which were available to us in connection with other work. It was soon observed that the coenzyme Q from this mold did not behave on paper chromatograms in the same way as any of the already known, naturally occurring coenzymes Q. A detailed study of this material was, therefore, undertaken; the present paper describes the isolation, characterization, and general properties of this new member of the coenzyme Q group. For convenience, our laboratory designation for this material, CoQ₉, will be used in the initial parts of this report.

EXPERIMENTAL PROCEDURE

The four strains of Penicillium stipitatum which were used were obtained through the kindness of Dr. C. W. Hesseltine, Northern Utilization Research and Development Laboratory, United States Agricultural Research Service; they were NRRL 1006, 2104, 2105, and 2166. Unless otherwise indicated, reference to *P. stipitatum* indicates use of culture NRRL 2104. The mold was routinely grown as stationary cultures in 2-liter Fernbach flasks containing 700 ml of the previously described growth medium (2). The flasks were sterilized at 15 pounds steam pressure for 20 minutes; after cooling, they were freely inoculated with spores from an agar slope. The usual growth period was 10 days at 28-30°. In a few experiments, mycelium from shaken cultures was used. For this purpose, the same Fernbach flasks containing 700 ml of medium were agitated on a Gyrorotatory shaker (New Brunswick Scientific Company, New Brunswick, New Jersey) with a 1-inch throw at about 250 r.p.m.

Assay of CoQ was carried out by the spectrophotometric method of Crane et al. (3); protein was estimated with the Folin-Ciocalteu reagent of Lowry et al. (4). Preparations of perhydro CoQ₉ and perhydro CoQ₁₀ were obtained by the method of Morton et al. (5); these quinones with fully reduced side chains were noncrystallizable oils.

Paper chromatography of CoQ preparations was routinely carried out on Whatman No. 1 paper, impregnated with 5% Vaseline petroleum jelly in toluene, and developed with the dimethylformamide-water, 97:3, system of Linn et al. (6). After development for 18 to 20 hours, the papers were examined under ultraviolet light; members of the CoQ group then appeared as brownish spots. Subsequently, this identification was confirmed by either spraying with, or dipping in, a solution of 0.5%, KMnO₄ for thin layer chromatography, plates were dipped into a mixture of 150 g of silica gel G (Brinkmann Instruments, Inc., Great Neck, Long Island, New York) in 300 ml of chloroform-methanol (2:1) containing 5% paraffin. The solvent system was acetone-water, 90:10, saturated with paraffin, as described by Wagner, Hörhammer, and Dengel (7). With a layer between 60 and 100 μ in thickness, the solvent moved approximately 9 cm in 45 minutes. The plates were subsequently exposed to iodine vapor for 10 seconds; as little as 0.1 μg of CoQ could be visualized in this way.

RESULTS

Extraction and Isolation of CoQ from Mycelial Pads—The culture fluid was decanted from the flasks after the appropriate growth period and the mycelial pads were then washed several times, by decantation, with distilled water. After blotting with filter paper to remove adhering water, 100- to 500-g portions of mycelium were macerated in a Waring Blender with methanol. For each 100 g of mycelium, 400-ml portions of methanol were used; the maceration time was usually 1 minute. After the addition of 50 ml of 100% aqueous KOH (weight per volume) and 50 ml of 20% pyrogallol in methanol (weight per volume), the slurry was saponified on a water bath (80°) for 30 minutes, with continuous stirring. Trial experiments established that 30 minutes was the optimum time for saponification. The saponified mixture was rapidly cooled under running tap water and in an ice bath prior to extraction with 3 equal volumes of petroleum ether (b.p. 40-60°). The combined ether extracts were washed with water (three portions of 1000 ml), and were then evaporated to dryness in a vacuum.

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Column Chromatography—The nonsaponifiable extract obtained from the petroleum ether extract was taken up in a small volume of redistilled petroleum ether (b.p. 40–60°) for chromatography on alumina. Neutral alumina (Alumina Woelm, supplied by Aluphar Chemicals, New Orleans, Louisiana) was treated with 6% water to be equivalent to Brockman grade III; 30 g were used as a column 21 mm in diameter and about 10 cm in length. After the petroleum ether extract had been run onto the column, washing was started, first with 100 ml of petroleum ether, next with 100 ml of 2% diethyl ether in petroleum ether. To elute a distinctly yellow band containing the CoQ components 5% diethyl ether in petroleum ether was then used. About 100 to 200 ml of this solvent were required; this fraction was used as described under “Purification of CoQ.” Elution was completed with diethyl ether (100 ml) followed by ethanol (50 ml); these eluates, containing sterols, were then combined and evaporated to dryness. The sterol residue was twice recrystallized from a minimum volume of absolute ethanol at -20°. The average yield of recrystallized sterol was 120 to 130 mg/100 g of mycelium, wet weight. The white flaky material, m.p. 155°, had an ultraviolet absorption spectrum showing peaks at 262, 271, 282, and 293 μν. The specific optical rotation, [α]D, was -126.4° (benzene, c = 1). These characteristics are those of the typical mold sterol, ergosterol. Further confirmation of this identity was provided by gas chromatography on a column of SE 30 at 225° (8). Examination of the crude sterol extracts in this way also revealed the presence of small amounts of other, unidentified sterols.

Purification of CoQ—The fraction containing CoQ was evaporated to dryness, then redissolved in a small amount of absolute ethanol. Prior to purification, aliquots of this solution were examined as follows. (a) The CoQ content was assayed by the spectrophotometric method; the average yield of crude CoQx was 6.0 mg/100 g of mycelium, wet weight. (b) A preliminary characterization by paper chromatography was undertaken; a typical chromatogram is shown in Fig. 1. The crude extract always contained a little CoQmix, amounting to about 3.5% of the total CoQ. In addition, traces of CoQx were also present.

Following these examinations, purification by preparative paper chromatography was carried out. The remainder of the ethanolic solution of crude CoQx was streaked on a large sheet of Whatman No. 3 paper impregnated with 5% paraffin in toluene. Development was carried out with the dimethylformamide-water system, usually for 10 to 12 hours at room temperature. The change from paper impregnated with Vaseline petroleum jelly to paper impregnated with paraffin for preparative purposes was made since the subsequent removal of paraffin by column chromatography was more efficient than that of Vaseline petroleum jelly. The CoQx band was marked out by visualization in ultraviolet light, and was then eluted with 50 to 100 ml of diethyl ether. The solvent was removed by evaporation and the residue was rechromatographed on an aluminum column to remove paraffin. The 5% ethyl ether-petroleum ether extract was evaporated yielding an oily residue. This material was crystallized three times from a minimum volume of absolute ethanol; the crystalline material separated on keeping the ethanol solution at -20°. The average recovery of purified CoQx was 3.0 mg/100 g of mycelium, wet weight.

Use of Shake Cultures—In shaken cultures, P. stipitatum mycelium is obtained as flocculent clumps, lacking most of the intense yellow pigmentation characterizing stationary cultures of this organism. Under these conditions of shaking, the growth period could be reduced to 6 to 7 days, and the yield of mycelium for a given volume of culture medium was higher than in stationary cultures. In a typical run, the material from five 7-day-old cultures was removed by filtration and was washed twice with water. The yield of wet mycelium was 205 g. The average yield of crude CoQx contained in the mycelium was 14.6 mg and of the purified CoQx was 14.6 mg and of the purified CoQx was based on the spectrophotometric assay.

Isolation of CoQx from different strains of Penicillium stipitatum

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age at harvest</th>
<th>Weight of mycelium used</th>
<th>Amount of crude CoQ</th>
<th>Amount of pure CoQ</th>
<th>Crystalline CoQ</th>
<th>Yield</th>
<th>M.p.</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL 2104</td>
<td>10</td>
<td>100</td>
<td>4.8</td>
<td>2.5</td>
<td>20°</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRRL 2105</td>
<td>10</td>
<td>80</td>
<td>3.0</td>
<td>2.0</td>
<td>9.9</td>
<td>0.9</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>NRRL 2106</td>
<td>13</td>
<td>80</td>
<td>5.0</td>
<td>4.5</td>
<td>0.5</td>
<td>0.38</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>NRRL 1006</td>
<td>11</td>
<td>90</td>
<td>4.5</td>
<td>4.0</td>
<td>0.8</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The wet weight of a single mycelial pad varied from 27 to 30 g. Pads from three flasks were available in all of the experiments, except that for NRRL 2104, four flasks were used.

* The amount of total crude CoQ and of the purified CoQx is based on the spectrophotometric assay.

Table I

Examination of Other Strains of P. stipitatum Although most of the work was carried out with P. stipitatum NRRL 2104, three other strains were examined. Of these, NRRL 1006 and 2105 closely resembled 2104 in general growth characteristics; NRRL 2106 formed a mycelial pad which was less strongly pigmented than the others, and tended to be more predominantly white. The results from these experiments, summarized in Table I, showed that CoQx was the major CoQ component in all of the strains.

General Properties of CoQx—The purified material was orange-
yellow with a melting point of 29°. The following optical properties characterized the material as a member of the CoQ group: \( \lambda_{\text{max}} \) ethanol 275 \( \mu \)m (\( E_{1\%}^\text{em} = 151 \)); \( \lambda_{\text{min}} \) ethanol 236 \( \mu \)m (\( E_{1\%}^\text{em} = 30 \)); \( \Delta E_{1\%}^\text{em} \) ox \( \rightarrow \) red = 125 (ethanol; \( \lambda = 275 \) \( \mu \)m). In addition, infrared spectra of the material (see Fig. 2) were almost identical with those of CoQ\(_8\) or CoQ\(_{10}\). A molecular weight determination was carried out by reduction to the hydroquinone and acetylation with \(^{13}\)C-labeled acetic anhydride (9); CoQ\(_{10}\) was used as the reference standard. The value for the molecular weight was 864, compared with that of 862 for CoQ\(_{10}\).

An indication of a very slightly lower \( R_F \) value for CoQ\(_8\) compared to CoQ\(_{10}\) had originally been obtained on reverse phase paper chromatography on Whatman No. 1 paper, impregnated with 5% Vaseline petroleum jelly in toluene (10) and developed with the 1-propanol-water system of Lester and Ramasarma (11). A clear-cut separation of CoQ\(_8\) from the other naturally occurring members of the series was only possible, however, with Vaseline petroleum jelly-impregnated paper and the dimethylformamide system (6). The average \( R_F \) value for CoQ\(_8\) was 0.28 and for CoQ\(_{10}\), 0.38 (see also Fig. 1). CoQ\(_8\) was also conveniently and rapidly separated from other coenzymes Q by thin layer chromatography (see "Experimental Procedure"). The following \( R_F \) values were determined: CoQ\(_8\), 0.12; CoQ\(_{10}\), 0.21; CoQ\(_{12}\), 0.30; CoQ\(_{10}\), 0.46.

Nuclear Magnetic Resonance Spectroscopy—High resolution nuclear magnetic resonance spectroscopy of CoQ\(_8\), and related compounds was carried out at 60 mc in a Varian spectrometer. The CoQ\(_8\) sample that was available amounted to 22 mg; it was dissolved in 0.5 ml of CCl\(_4\). Representative spectra of CoQ\(_8\) and CoQ\(_{10}\) are shown in Fig. 3. Studies of the fully reduced CoQ\(_8\) and CoQ\(_{10}\) were also undertaken. Similar spectra were obtained for both perhydro CoQ\(_8\) and perhydro CoQ\(_{10}\); that of perhydro CoQ\(_{10}\) is shown in Fig. 4.

Ozonolysis of CoQ\(_8\)—To determine whether either of the terminal isoprene units was reduced, ozonolysis of the diacetate of CoQ\(_8\) hydroquinone was carried out. Recrystallized CoQ\(_8\), 5.3 mg, dissolved in a mixture of 0.25 ml of acetic anhydride and 0.1 ml of triethylamine, was subject to reductive acetylation (12). The oily diacetate of the CoQ\(_8\) hydroquinone was dissolved in 1 ml of ethanol, treated with 2 drops of water, then cooled to \(-20°\); a crystalline material which separated out was removed by centrifugation in the cold. This diacetate had a melting point close to room temperature and tended to become a semisolid unless kept cold. The ultraviolet absorption spectrum, determined on a small portion, showed a single maximum at \( \lambda = 270 \) \( \mu \)m (ethanol).

The diacetate, 0.9 mg, dissolved in ethyl acetate, 5 ml, containing 0.2 ml of water, was treated with ozone under the standard conditions described previously (13). Immediately following ozonolysis, the solution was distilled at room temperature in a closed vacuum system; the distillate was collected in a receiver cooled with Dry Ice and alcohol. This distillate was allowed to react with 2,4-dinitrophenylhydrazine in 6 N HCl and then extracted with carbon tetrachloride. Chromatography of the extract, first on a column of Hyflo Super-Cel treated with nitromethane (14), then on paper with heptane saturated with methanol (15), revealed at most only traces of acetone 2,4-dinitrophenylhydrazone. The crude aromatic acid fraction, obtained in the usual way, was a pale yellow solid, weighing 2.1 mg. On examination by gas chromatography (13) either as the free acid, or as the methyl ester, it was determined that the major component of this solid was 3',6'-diacetoxy-4',5'-dimethoxy-2'-methylphenylacetic acid. The presence of levulinic acid in
FIG. 3. Nuclear magnetic resonance spectra of CoQ.). These spectra were obtained as described in the text. In each case, the region from 150 to 350 c.p.s. is shown in an amplified form to give a better indication of the doublet peak at 6.92 r. The following are the r values for the peaks in the CoQ spectrum, reading from left to right: 5.60, 6.11, 6.92 (doublet, J = 7 c.p.s.), 8.07, 8.29, 8.45, 8.75, 9.13 (doublet, J = 6 c.p.s.). Tetramethylsilane was used as the internal reference standard.

the ozonolysis reaction products was confirmed by preparation of the bis-2,4-dinitrophenylhydrazone.

Catalytic Hydrogenation of CoQ).—To determine the number of reduced isoprene units in CoQ), a study of hydrogen uptake was carried out in the Warburg apparatus. Approximately 2.5-mg samples of CoQ, and a control of either CoQ, or CoQ,, were accurately weighed out on a Cahn electrobalance (Cahn Instrument Company, Downey, California) and were made to 5 ml with ethanol. The Warburg flasks contained 1 ml of CoQ solution, and 1.8 ml of ethanol in the main chamber; the side arm contained 3 mg of 10% palladium on charcoal and 0.2 ml of ethanol. The ground glass joints were greased with Lubrisel (A. H. Thomas Company, Philadelphia, Pennsylvania) rather than lanolin. The flasks were gassed with hydrogen (which had been bubbled through ethanol) for at least 15 minutes. After gassing was complete, the stopcocks were closed, and the flasks were shaken for at least 30 minutes prior to addition of catalyst. This was necessary since there were usually some erratic pressure changes in the first few minutes following closing of the stopcocks. Manometer readings were made without stopping the shaking. The results shown in Table II are in agreement with the uptake predicted for a dihydro CoQ.

Solutions of reduced CoQ, obtained either from the Warburg experiments, or by reduction on a larger scale according to Morton et al. (5), were diluted with water and extracted with petroleum ether. The petroleum ether extracts, after removal of solvent, were dissolved in ethanol containing 0.2% FeCl₃ to oxidize hydroquinone to quinone. The quinone form was recovered by dilution with water followed by petroleum ether extraction. Since the fully reduced quinones were found to have zero Rₚ value in the usual dimethylformamide solvent system, a new method was necessary to characterize these compounds. Whatman No. 1 paper was impregnated with 2% Vaseline petroleum jelly in toluene; 1-propanol-water, 9:1, was used as a sol-
**Fig. 4.** Nuclear magnetic resonance spectra of perhydro CoQ₁₀. In this case, the sweep width was 250 c.p.s. so that the values recorded on the instrument scale must be halved. The region from 120 to 165 c.p.s. is shown in an amplified form to give a better indication of the triplet at 7.60 ppm. The spectrum was obtained with 75 mg of perhydro CoQ₁₀ in 0.5 ml of carbon tetrachloride. The following are the values of the peaks, reading from left to right: 6.03, 7.60 (triplet, \(J = 8\) c.p.s.), 8.02, 8.78, 9.07 (doublet, \(J = 4\) c.p.s.), 9.13 (doublet, \(J = 4\) c.p.s.). Tetramethylsilane was used as the internal reference standard.

**TABLE II**

**Catalytic hydrogenations of CoQₑ**

These hydrogenations were carried out with the precautions described in the text. The bath temperature was 30°C. The experiments were carried out until there was no further uptake of hydrogen; in no case was the run discontinued before 2 hours. The results shown here were obtained with three separate weighings of CoQₑ which are indicated by 1, 2, or 3 in parentheses, following the actual hydrogen uptake value.

<table>
<thead>
<tr>
<th>Run</th>
<th>CoQₑ</th>
<th>CoQₑ</th>
<th>CoQₑ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.47</td>
<td>12.43</td>
<td>11.35 (1)</td>
</tr>
<tr>
<td>2</td>
<td>12.51</td>
<td>11.42 (1)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>12.71</td>
<td>11.51 (1)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>13.11</td>
<td>11.58 (1)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>12.22</td>
<td>11.61 (2)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>12.94</td>
<td>11.26 (2)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>12.52</td>
<td>11.22 (2)</td>
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</tr>
<tr>
<td>8</td>
<td>12.65</td>
<td>11.19 (2)</td>
<td></td>
</tr>
</tbody>
</table>

Mean value | 12.40 | 12.45 | 11.30 |
Standard deviation | 0.33 | 0.46 | 0.26 |
Calculated for CoQₑ | 12.57 | 12.75 | 11.55 |
Calculated for CoQₑH₂ | 11.70 | 10.71 (3) |
Calculated for CoQₑH₄ | 10.38 |

Although this value almost certainly represents an experimental error, it has been included in the calculation of the mean and standard deviation.

<table>
<thead>
<tr>
<th>Run</th>
<th>CoQₑ</th>
<th>CoQₑ</th>
<th>CoQₑ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.57</td>
<td>12.57</td>
<td>11.55</td>
</tr>
<tr>
<td>2</td>
<td>12.57</td>
<td>12.57</td>
<td>11.55</td>
</tr>
<tr>
<td>3</td>
<td>12.57</td>
<td>12.57</td>
<td>11.55</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The nuclear magnetic resonance spectrum of CoQₑ at 60 mc revealed all of the bands shown by CoQ₉ and CoQₑ (16, 17). The major differences were a new singlet in CoQₑ observed at 8.75 ppm, and a new doublet at 9.13 ppm (\(J = 6\) c.p.s). The peak at 8.75 ppm was assigned to the protons of a methylene group attached to 2 saturated carbons (–CH₂–CH₃); \(\tau\) values for these paraffinic hydrogen atoms normally range from 8.4 to 8.8 (18). The new doublet is assigned to methyl protons attached to a saturated carbon atom; typical values for these protons range from 9.05 to 9.15 ppm (18). These new peaks provided the first evidence that CoQₑ contained a partly reduced side chain. A doublet at 9.12 ppm (\(J = 5\) c.p.s) had been observed previously by Shunk et al. (17) in the 40 mc nuclear magnetic resonance spectrum obtained from a synthetic sample of 2',3',4'-dimethoxy-5'-methyl-6'-phytylbenzoquinone, but no mention had been made of the peak at 8.75 ppm corresponding to the saturated methylene protons. A minor difference between CoQₑ and CoQ₉ or CoQₑH₂ was that the peak at 8.29 ppm was more obvious in CoQₑ (see Fig. 3). This resulted from a decreased and sharper peak.

* Reported in the original paper as -154 and -159 c.p.s.
TABLE III

Assignment of absorptions in nuclear magnetic resonance spectra of CoQ₁₀ and perhydro CoQ₁₀

The observed numbers of protons are based on a calibration value of 6 protons in the 6.03 to 6.11 ppm peak due to the two methoxyl groups. These values are shown in parentheses in the columns headed "No. protons, observed."

<table>
<thead>
<tr>
<th>Proton</th>
<th>CoQ₁₀</th>
<th>Dihydro CoQ₁₀</th>
<th>Perhydro CoQ₁₀</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ϱ</td>
<td>No. protons, observed</td>
<td>No. protons, theory</td>
</tr>
<tr>
<td>CH=C-</td>
<td>5.00</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>CH₃O</td>
<td>6.11</td>
<td>(6)</td>
<td>6</td>
</tr>
<tr>
<td>CH₃O</td>
<td>6.92</td>
<td>2.6</td>
<td>2</td>
</tr>
<tr>
<td>CH₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH₃</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH₁  (side chain, other than ArCH₃)</td>
<td>8.07</td>
<td>38</td>
<td>35</td>
</tr>
<tr>
<td>CH₂ (unit 9)</td>
<td>8.29</td>
<td></td>
<td>3</td>
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<tr>
<td>C-CH₂</td>
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<td>29.5</td>
</tr>
<tr>
<td>CH₃ (units 1 to 8)</td>
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<td></td>
<td>24</td>
</tr>
<tr>
<td>C-CH₂</td>
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<tr>
<td>CH₃</td>
<td>8.75</td>
<td>6.5</td>
<td>5</td>
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<tr>
<td>CH₁-CH₂-CH</td>
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</tr>
<tr>
<td>CH₃</td>
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<tr>
<td>CH</td>
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<tr>
<td>CH₃</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH</td>
<td>9.13*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The protons under consideration are shown by italic capitals.
* Doublet, J = 7 c.p.s.
* Triplet, J = 8 c.p.s.
* For CoQ₁₀, this absorption includes also the ring CH₁, and excludes the CH₃ protons in unit 10. The 5 carbon units are numbered starting at the aromatic ring.
* Doublet, J = 6 c.p.s.
* Doublet, J = 4 c.p.s.
at 8.45 τ, which is itself the result of a decrease in the number of CH₃ – CH – units.

In a preliminary report we identified CoQ₉ as tetrahydro CoQ₁₀ (1). Following this report, Dr. Folkers informed us that he and his colleagues had isolated a compound, identified as dihydro CoQ₁₀, from another microorganism. A sample of our CoQ₁₀ was provided to Dr. Folkers and appeared to be identical with his material on the basis of paper chromatography. A major factor in our assignment of a tetrahydro structure to CoQ₁₀ was the results from hydrogenation studies. In six hydrogenations, which had involved two separate weighings, the mean hydrogen uptake was 10.70 μmoles per mg (standard deviation, 0.24); this value was, therefore, only in agreement with that of 10.38 calculated for tetrahydro CoQ₁₀. The mean value for CoQ₁₀ itself, used as a control in each of these six hydrogenations, was 12.80 μmoles per mg (standard deviation, 0.42); the theoretical value is 12.75. When further hydrogenations of CoQ₁₀ were carried out, the mean value in 10 runs (see Table II) was 11.30 (standard deviation, 0.26). The only significant operational difference between the two series relates to the preparation of the solutions. In the first series, the CoQ₁₀ was weighed on the pan of the electrobalance and then washed into a 5-ml volumetric flask with ethanol. In the second series, the pan plus CoQ₁₀ was dropped into a vial, and 5 ml of ethanol were then added from a volumetric pipette. It is possible that with the technique used in the first series, some of the waxy CoQ₁₀, which is not too readily soluble in ethanol, remained behind on the weighing pan; such an error would lead to a low value for the hydrogen uptake.

It must, therefore, be acknowledged that CoQ₁₀ is a dihydro CoQ₁₀, and that the material isolated by Gale et al. (19) from Gibberella fujikuroi is identical with that from P. stipitatum. Since the fully reduced CoQ₁₀ could not be distinguished chromatographically from perhydro CoQ₁₀, there is no doubt that the material contains a total of 10 C₅ units.

With a clarification of the number of reduced C₅ units, our results on the nuclear magnetic resonance spectroscopy of CoQ₁₀ lead to the further conclusion, in agreement with Gale et al. (19), that the reduced C₅ unit is at the chain terminal remote from the aromatic ring. The relative numbers of protons assigned to the various absorptions are shown in Table III. The assignments are in good agreement with the peaks observed in a sample of perhydro CoQ₁₀, also recorded in Table III. The absorption at τ = 8.29 is to be assigned to either the first (next to the aromatic ring) or the ninth CH₃ – C =, since these are the two that are different. The definite isolation, in good yield, of 3',6'-di-acetoxy-4',5'-dimethoxy-2'-methylphenylacetic acid on ozonolysis of the diacetate of CoQ₁₀ hydroquinone confirms that the C₅ residue next to the aromatic ring must be unsaturated.

Our results provided the first isolation of a reduced form of the CoQ structure in nature; Diplock et al. (21) claimed to have identified tentatively a dihydro CoQ₁₀ in a rat liver extract, but this material has apparently never been characterized. The analogy between the normal and reduced CoQ₁₀ on the one hand, and between vitamin K₁ and K₃ on the other, is of interest; furthermore, Gale et al. (22) have reported recently on a vitamin K₃, isolated from Microbacterium phlei, that contains nine C₅ units, one of which is reduced.

† K. Folkers, personal communication.

‡ We had independently observed that CoQ₁₀ was the major CoQ component in Gibberella fujikuroi, ATCC 12016 (20).

In P. stipitatum, dihydro CoQ₁₀ is the major CoQ component; CoQ₁₀ is present to the extent of about 3.5% of the total CoQ, and very small traces of CoQ₁₀ may also be detected. The highest yields of dihydro CoQ₁₀ from P. stipitatum were 6.0 mg/100 g of mycelium, wet weight, equivalent to a dry weight of 20 g. The yield is, therefore, 300 μg per g, dry weight, or 0.35 μmole per g, dry weight. Comparable figures for other micro fungi are as follows: Penicillium chrysogenum, 0.47 μmole of CoQ₁₀ per g (23); Aspergillus fumigatus, 0.8 μmole of CoQ₁₀ per g (24); Aspergillus niger, 0.2 μmole of CoQ₁₀ per g; Ustilago zeae, 0.2 μmole of CoQ₁₀ per g (26). The level of the reduced CoQ₁₀ of P. stipitatum is, therefore, quite comparable with that of the "normal" coenzymes Q in other molds. In shaken cultures, an average wet weight of 41 g of mycelium was obtained as compared to 30 g in surface cultures with the same volume of medium. The dry weight of the mycelium from shaken flasks was 13.5 g, compared to 6 g for the surface pads. The same dihydro CoQ₁₀ was also obtained under conditions of shaking, the yield being 216 μg per g, dry weight. Despite the more strongly aerobic conditions prevailing in the shaken flasks, and the more rapid rate of synthesis of cell components, the CoQ₁₀ yield was slightly lower than from surface cultures. An increased yield of dihydro CoQ₁₀ in the shaken cultures might have been anticipated since it is known that the CoQ content of yeast is much increased when growth takes place under aerobic conditions as opposed to anaerobic conditions (27, 28). In Saccharomyces cerevisiae, the CoQ₁₀ level in anaerobically grown cells is <0.001 μmole per g, while in aerobically grown cells the level is 0.35 μmole per g (27). It is apparent that dihydro CoQ₁₀ synthesis is influenced only slightly by the more vigorous aeration provided by shaking.

The melting point of dihydro CoQ₁₀, 29⁰, is considerably lower than that of 50⁰ observed with CoQ₁₀ itself. It may be noted in this connection that neither perhydro CoQ₁₀ nor perhydro CoQ₁₀ has been obtained crystalline; this may result from the potential formation of multiple asymmetrical centers on reduction. The effect of partial and complete side chain reduction on the mobility of CoQ derivatives on paper chromatography was striking. In the dimethylformamide-water system, dihydro CoQ₁₀ has a lower RF than CoQ₁₀, while the perhydro compounds remain at the origin. It seems likely that a compound such as dihydro CoQ₁₀ might have an RF value similar to that of CoQ₁₀; it is apparent that in the characterization of this series of compounds, paper chromatography may be quite misleading.

In some preliminary experiments, the ability of dihydro CoQ₁₀ to restore the activity of the succinic oxidase system in acetone-treated rat liver mitochondria was found comparable to that of CoQ₁₀. Similar activity was observed with the perhydro derivatives of CoQ₁₀ and CoQ₁₀. This again emphasizes that the biological activity of this group is not markedly dependent on the nature of the polyisoprenoid side chain. It has also been observed by Moret, Pinamonti, and Fornasari (28) in studies with CoQ₁₀, CoQ₁₀, and CoQ₁₀ that the isoprenoid side chain did not influence the standard oxidation-reduction potential.

SUMMARY

A new coenzyme Q component, with a melting point of 29⁰, is isolated from four strains of the mold, Penicillium stipitatum.

J. Jayaraman, unpublished results cited by Ramasarma (25). It should also be noted that under these conditions, no accumulation of tropolones takes place in the culture medium. This biosynthesis of tropolone compounds is very characteristic of surface cultures.
On the basis of the following evidence, the material is characterized as dihydro coenzyme Q₁₀ in which the Cs unit farthest from the benzoquinone ring is reduced: (a) interpretation of the nuclear magnetic resonance spectrum at 60 mc; (b) the formation of 3',6'-diacetoxy-4',5'-dimethoxy-2'-methylphenylacetic acid on ozonolysis of the diacetate of dihydro coenzyme Q₁₀ hydroquinone; (c) the observed hydrogen uptake of 11.30 μmoles per mg; and (d) the fact that the quinone with a fully reduced side chain, derived from dihydro coenzyme Q₁₀, is indistinguishable from perhydro coenzyme Q₁₀. The dihydro coenzyme Q₁₀ is readily separated from other naturally occurring coenzymes Q by paper chromatography on paper which has been impregnated with 5% Vaseline petroleum jelly in toluene and developed with dimethylformamide-water, 97:3; another convenient separation is possible by thin layer chromatography on silica gel G containing paraffin which is developed with acetone-water, 90:10.

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