STUDIES ON THE ELECTRON TRANSPORT SYSTEM

XIX. THE ISOLATION OF COENZYME Q FROM *AZOTOBACTER VINELANDII* AND *TORULA UTILIS*

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SUMMARY

Procedures are described for the isolation of 3 crystalline compounds of the coenzyme Q family. One of these compounds was isolated from the nonsaponifiable fraction of cells of *Azotobacter vinelandii*. Two distinct compounds were isolated from *Torula utilis* by direct solvent extraction with or without saponification. Chromatographic procedures followed by crystallization techniques are used for the final purification of these three compounds.

INTRODUCTION

By a variety of methods a crystalline lipid of quinonoid structure has been isolated from beef heart mitochondria[1]. This substance, for which the name Coenzyme Q has been proposed, functions as an oxidation-reduction coenzyme in the respiratory system of beef heart particles[2–5]. Upon investigation of a number of organisms and tissues it became obvious that those cells with high aerobic respiratory activity contained relatively large amounts of substances related to the material first isolated from beef heart. It was also recognized that there occurs in nature a whole family of such compounds all related to one another by virtue of containing the same quinonoid group which is the key grouping involved in their respiratory function, *i.e.* undergoing alternate oxidation and reduction reactions.

This paper will describe the isolation in crystalline form of three different compounds of this family, one from cells of *Azotobacter vinelandii* and two from *Torula utilis*.

A substance called SA and later ubiquinone has been demonstrated in various animal tissues[6,7]. Its possible relation to this work has been discussed[1].

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EXPERIMENTAL

Methods

Chromatography on either decalso or silicic acid columns was carried out essentially as described previously[1]. In these procedures substances were adsorbed from iso-octane solutions onto the adsorbent which had been equilibrated with iso-octane. Elution of the decalso column was uniformly carried out with 5 % (v/v) ethyl ether in iso-octane and of the silicic acid column with 1/1 (v/v) chloroform–iso-octane.

The fractionations were followed by measuring the weight of a fraction after all solvent had been removed at water pump pressure in a dessicator over $CaCl_2$. The amount of coenzyme Q present was estimated after diluting the sample with ethanol by measuring the absorbance at 275 mμ before and after reduction with KBH_4. Among the components of the nonsaponifiable fraction of *Azotobacter* cells, coenzyme Q makes the predominant contribution in the spectral region examined, and its measurement consequently is fairly precise. However, there is a multitude of components in the crude nonsaponifiable fraction of *Torula* (particularly ergosterol) which makes the spectrophotometric estimation of quinone difficult at this stage of purification.

The reversed-phase paper chromatographic technique for qualitative identification will be described elsewhere[8].

Materials

The cells of *Azotobacter vinelandii* were generously furnished by Mr. N. NEUMANN of the Department of Biochemistry of this university. *Torula utilis* cells, both the dried powder and the fresh, pressed product, were generous gifts of the Lake States Yeast Corporation, Rhinelander, Wis. "Food grade" and "feed grade" products were equally effective.

RESULTS

Isolation of a coenzyme Q from Azotobacter vinelandii

The spectral characteristics of coenzyme Q were easily recognizable even in spectra of crude extracts. The concentration of this material in *Azotobacter* (approximately 2 mg/g dry wt. cells) was close to that found in beef heart mitochondria. The procedure used for the isolation of this compound was essentially similar to that described for the isolation of coenzyme Q from beef heart mitochondria. The substance is first extracted with heptane (or iso-octane) from the saponified cells. The extract is then chromatographed on a decalso column. The pertinent fractions are concentrated and purification is completed by a crystallization procedure. The following is a description of a typical isolation run:

1. *Saponification.* 100 g pyrogallol was dissolved in 2 l of 20 % (w/v) KOH in 95 % ethanol. To this solution was added approximately 2 kg (wet weight) of a cell paste of *Azotobacter vinelandii*. The mixture was stirred well and refluxed for 30 min and then cooled to room temperature.

2. *Extraction.* The saponified mixture was extracted twice with 1 l of *n*-heptane in a separatory funnel. (An emulsion which may form at this point can be broken by addition of solid KCl.) The heptane extracts were combined, dried over anhydrous Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was taken up in 20 ml of iso-octane and the solution was kept at —20°. After 2 days the white precipitate

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which formed was centrifuged off and discarded. The supernatant solution was then chromatographed.

3. *Chromatography.* An 18.5 × 3.3 cm column of 50/80 mesh decalso was washed with 500 ml iso-octane. The material soluble in iso-octane at —20° (see extraction step) was then adsorbed on this column. The column was then washed with 500 ml iso-octane; this fraction was discarded. The quinone was then eluted with about 2 l of 5 % w/v ethyl ether in iso-octane. Six equal fractions were collected, and their ultraviolet absorption spectra were plotted. The first and last fractions contained spectral impurities and were discarded. The middle four fractions were pooled and evaporated to dryness *in vacuo*. (It may be of interest to note that a material has been observed which emerges from the column prior to the quinone and which has four absorption bands in the ultra-violet. The location of these bands is the same as for vitamin K_1. The influence of solvents (ethanol, iso-octane) on the spectrum of the *Azotobacter* fraction was similar to that observed on the spectrum of authentic K_1 or other methylnapthoquinones.) The residue was treated with about 7 ml of hot ethanol and the small amount of insoluble material discarded. At this point the ethanolic solution contained 250 mg of coenzyme Q.

4. *Final purification.* After 1 day at 5°, a yellow precipitate formed in the ethanolic solution. The precipitate was separated by centrifugation and recrystallized 3 times at 5° from 3/1 (v/v) methanol–ethanol solutions, the volumes of which were, in order, 9, 15 and 7 ml. The yield of product was 92 mg. More pure material was recovered by reworking the mother liquors. The final product melts at 36–37°; λ max, 275 mμ; $E^{1\%}_{1\,cm}$ (ethanol) = 206. Neither the melting point nor the extinction coefficient has been increased by further recrystallization from glacial acetic acid, ethanol, or methanol.

Isolation of coenzyme Q from Torula utilis

Preliminary experiments indicated that in the crude state the coenzyme Q prepared from *Torula utilis* had distinctly higher solubility in ethanol than did the quinone isolated from beef heart. Although this could merely reflect the presence of impurities in these preparations, it seemed desirable to prepare very pure preparations. By a tedious fractional recrystallization procedure, two substances were isolated. Although these two materials had quite different melting points (each lower than the heart compound), they seemed to be very closely related to the material isolated from beef heart mitochondria on the basis of absorption spectra and ability to reactivate an iso-octane-extracted succinoxidase system[3]. In view of the many similarities among these products, it was felt that differences in melting point and insolubility properties were not alone conclusive in proving that these low melting lipid substances were different, unless the purity of each product could be rigorously established. That these products were indeed different was demonstrated unambiguously by means of reversed-phase paper chromatography. All of the products isolated—from beef heart, *Azotobacter*, and the two from *Torula*—migrated as single components at different R_F values when visualized with the various spray reagents[8]. A more rational isolation procedure could be devised once it was realized that there existed two forms of coenzyme Q in *Torula* and that both these differed in certain properties from each other and from the heart and *Azotobacter* compounds.

In *Torula* the low melting compound is present in roughly 5 to 10 times the

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amount of the high melting form (as judged by relative colors of paper chromatograms of crude extracts and by final yield). Both compounds have been obtained from the dried *Torula* powder (supplied commercially) either by a saponification procedure or by direct extraction with iso-octane. Both compounds have also been demonstrated in the nonsaponifiable fraction obtained from the fresh, pressed product. The combined amount of these compounds is about 0.31 mg/g of the dried powder.

Although these two compounds have been successfully separated from each other by a fractional crystallization procedure, the procedure will not be described in detail since it is too tedious, uneconomical of material and difficult to control precisely from run to run. In general, the procedure is based on the fact that in cold ethanolic solutions the low melting form is much more soluble than the high melting form.

The two closely related compounds can be separated from each other by reversed-phase paper chromatography carried out on a preparative scale. Two typical isolation runs will now be described.

Preparation by saponification

Step 1. Saponification. The following mixture was refluxed for 30 min: 1 l 15 % w/v KOH in 95 % ethanol, 37 g pyrogallol and 330 g of dried *Torula* powder (feed grade) slurried in 800 ml water. 8 batches were refluxed in this manner.

Step 2. Extraction. Each batch was extracted 3 times with 400 ml volumes of iso-octane in a separatory funnel. The last extract of one batch was used to extract a fresh batch. The pooled iso-octane extracts were dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to a volume of 500 ml. After standing overnight at —20°, a precipitate formed and was discarded after centrifugation. The supernatant solution contained 8.15 g of solids of which 20.1 % was coenzyme Q.

Step 3. Silicic acid chromatography. The material was adsorbed on a column containing 300 g of silicic acid and 150 g of super-cel equilibrated with iso-octane. The material was eluted with 1/1 (v/v) chloroform–iso-octane. The fractions containing the quinones, which emerged as a deep orange band, were pooled and evaporated to dryness *in vacuo*. The residue weighed 1.94 g of which 66 % was coenzyme Q.

Chromatography on decalso columns has been used successfully for this step, essentially as described in Step 3 of the purification procedure for the *Azotobacter* compound.

Step 4. Separation of the quinones by reversed phase paper chromatography. The residue from the previous step was dissolved in 50 ml ethanol. This ethanolic solution of coenzyme Q was applied (1 mg coenzyme Q/cm) to Whatman No. 17 filter paper 4 cm from the bottom. The paper had been previously treated with Dow Corning No. 550 silicone. The paper was then hung in the chromatography chamber and allowed to equilibrate with 7/3 (v/v) *n*-propanol/H_2O for about 24 h. After the equilibration period the material was chromatographed in the ascending manner with the above mentioned solvent system. When the solvent front had progressed 20 cm or more, two distinct yellow bands could be observed. The papers were then air dried and the appropriate bands were cut out. The upper and lower bands contained the low melting and high melting compounds respectively. The quinones were eluted from the paper with warm ethanol until no color remained. The alcohol eluates were filtered to remove paper particles and then evaporated to dryness. The small amount of silicone which was now present was removed by silicic acid chromatography essentially

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as described in step 3 except that smaller columns were now used. From these columns were obtained the following amounts of quinone: low melting compound, 465 mg; high melting compound, 80 mg.

Step 5. Final purification.

(a) High melting compound. The eluate from the silicic acid column was taken to dryness, and the residue was dissolved in 10 ml absolute ethanol. After a day at 5°, a small amount of whitish precipitate had formed and was discarded after centrifugation. The supernatant was placed at —20° overnight. The yellow precipitate which had formed was centrifuged off and redissolved in 8 ml of warm 1/1 (v/v) methanol–ethanol and placed at 5°. The yellow crystals which formed were recrystallized from 4 ml ethanol–methanol solution at room temperature. Yield: 44 mg, M.P. 43 to 45°. More compound can be obtained from the mother liquors.

(b) Low melting form. The eluate from the silicic acid column was taken to dryness, the residue taken up in 36 ml 4/1 (v/v) ethanol–methanol and placed at 5° overnight. A small amount of colorless oil separated and was discarded after centrifugation. The supernatant was placed at —20° overnight. The yellow precipitate which formed was centrifuged and redissolved in 12 ml 4/1 ethanol–methanol and placed at 5° for two days. The crystals were filtered off in the cold and recrystallized from 7 ml solvent yielding 233 mg crystals, M.P. 30°. More material can be recovered from the various mother liquors.

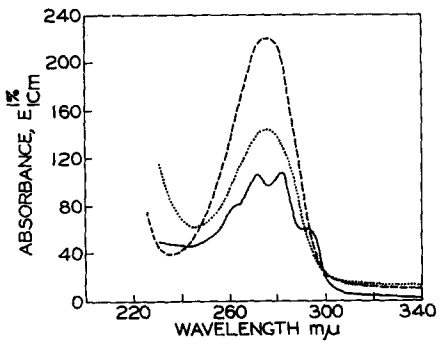

Fig. 1. The curves represent spectra of material obtained at various stages of purification of coenzyme Q from *Torula utilis via* the saponification procedure. The ordinate represents the absorbance of 1 % (w/v) ethanolic solutions of these materials: initial crude extract, step 2, solid line; after silicic acid chromatography, step 3, dotted line; crystalline low melting compound, step 5, dashed line.

It has been very useful to monitor various isolation procedures by examination of the absorption spectrum of various fractions. Fig. 1 represents a plot of the ultraviolet absorption spectra of material obtained at various stages in the above mentioned procedure. Several criteria have been applied which should be of general use in the isolation of these and related compounds from various sources. Where other spectral impurities predominate, as in the crude *Torula* fractions, the assay of coenzyme Q can be followed by measuring the absorbance of both the oxidized form and its KBH_4 reduction product in the ultraviolet range. The largest change in absorbance produced by this treatment is near 275 mμ. For all of the pure compounds of this family studied, the % change in absorbance produced by KBH_4 treatment at 275 mμ has been between 85 and 86, which affords a qualitative criterion of purity. The absolute change on a weight basis, $\Delta E^{1\%}_{1\,cm}$, (275 mμ) which is dependent on the compound in question is used as a quantitative measure of the purification step in question. Reduction of fractions with KBH_4 decreases the absorbance of the compound in the ultraviolet and

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visible region, thus bringing into sharper focus the nature of the absorption spectrum of the impurities.

Isolation by direct extraction

Step 1. Extraction. 6.4 kg of *Torula* powder was extracted with 10 l of iso-octane. The suspension was shaken vigorously at intervals and then allowed to settle overnight. The supernatant solution (3 to 4 l) was siphoned off, and fresh iso-octane was added. The procedure was repeated for several days until about 20 l of extract was obtained. This extract was clarified by filtering through super-cel under suction, and it contained 54 g of solids of which about 1.8 % was coenzyme Q.

Step 2. Chromatography. The extract was first reduced in volume by passing it through a 2/1 (w/w) silicic acid–super-cel column, 6 × 42 cm. The quinones were concentrated in a dark orange band near the top of the column. The adsorbant was extruded, the orange band cut out, and the quinones eluted with ethanol. The ethanol was evaporated, and the residue taken up in 95 ml iso-octane. Some material was insoluble in the iso-octane and was discarded. The extract was chromatographed on a silicic acid column (40 × 2.8 cm) with 1/1 $CHCl_3$–iso-octane as eluting agent. Several colored bands were resolved in this procedure. The main orange band containing the quinones was eluted with the second 759 ml of solvent. This fraction was evaporated to dryness *in vacuo*, and the residue was redissolved in 40 ml ethanol for paper chromatography. At this stage the fraction contained 3.07 g solids of which 40 % was coenzyme Q.

Decalso chromatography is less successful for this step since it yields a product of lower purity (15 to 20 % of the solids).

Step 3. Final purification. The quinones were then separated by paper chromatography as described above and their respective solutions then adsorbed on a silicic acid column to remove the contaminating silicone. The following yields were obtained: low melting compound, 405 mg; high melting compound, 61 mg. The final purification *via* recrystallization was carried out as described above yielding 205 mg of low melting compound and 36 mg of high melting compound.

The low melting compound obtained by both procedures melted at 30° [$E^{1\%}_{1\,cm}$ (275) = 221], and the high melting compound at 45° [$E^{1\%}_{1\,cm}$ (275) = 185]. No depression of melting point was observed by mixing the products obtained from the two different isolation procedures.

Further characterization of these products will be the subject of a future communication[9].

DISCUSSION

The isolation of the two distinct compounds from *Torula* raises certain questions which cannot at this time be answered unequivocally. The starting material was grown commercially on sulfite waste liquor, and it is difficult to tell whether the presence of these two compounds reflects the existence of two strains of organism in the starting material. If these compounds actually do coexist in one strain, it would not be unlikely that these compounds have a precursor–product relationship.

By the methods outlined above, these compounds can be routinely obtained in pure form with an overall yield of from 20 to 50 % depending on the source and method of isolation. For good sources, such as beef heart mitochondria and *A. vinelandii*,

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the isolation does not involve too many steps and very high yields can be obtained. In the case of the *Torula* compounds the largest losses have occurred in the separation of the two forms by reversed-phase paper chromatography. There is a marked tendency for the two compounds to be resolved on silicic acid columns when 1/1 (v/v) chloroform–iso-octane is used as the eluting agent. The first fractions to emerge contain the high melting compound, and the last fractions contain the lower melting, more polar compound. A similar elution pattern is observed on decalso columns. Unfortunately the bulk of the material is still a mixture of both forms. It is felt that a gradient elution technique could be devised that would give a quantitative resolution.

For sources of lower coenzyme Q content such as *Torula*, the saponification method is the one of choice for the preparation of small amounts of product. For the preparation of gram quantities or more, a direct extraction technique should be used. For large-scale isolations from *Torula* several modifications from the procedures described have been found to be useful. The initial extraction of the dry powder with hydrocarbon solvents is allowed to continue for about a week. During this time extracts are drawn off and are passed through 50/80 mesh decalso columns. The desired compounds are adsorbed and the eluates reused in the extraction of the powder. The product is then eluted from the column with ether–iso-octane mixtures and rechromatographed in a similar manner on decalso. In this way 100 lb quantities of powder have been handled to produce a concentrate which contains 20 % coenzyme Q by weight.

At this stage many colorless impurities can be removed by the following technique. The concentrate is dissolved in a solution of 7/3 (v/v) iso-octane–ethanol and placed at −20° for a day or more. The desired material is found in the supernatant after centrifugation. It is preferable to use silicic acid columns on rich concentrates because of the long time required in running these columns on a very large scale. The fast running decalso columns which have a somewhat lower resolving power are preferred in the early steps of a purification.

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