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STUDIES ON THE ELECTRON TRANSPORT SYSTEM

XVIII. ISOLATION OF COENZYME Q (Q_{275}) FROM BEEF HEART AND BEEF HEART MITOCHONDRIA

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SUMMARY

Two different methods for purification of coenzyme Q from beef heart mitochondria and from beef heart have been described. Selective extraction following saponification or direct extraction of total lipids have been used for initial extraction. The coenzyme Q in these extracts has then been purified by chromatography on Decalso or silicic acid followed by crystallization from ethanol. The coenzyme Q obtained by these two procedures have melting points which range from 49.3 to 50°, $E^{1\%}_{1\,cm}$ at 275 mμ ranges from 162 to 165. All purified preparations have the same R_F when chromatographed on silicone-treated paper and identical visible, ultraviolet and infrared spectra.

INTRODUCTION

The ease with which coenzyme Q** (Q_{275}) can be obtained in high purity from beef heart or from beef heart mitochondria has facilitated the recognition of this compound as an essential component in the electron transport system[1, 2, 3].

Coenzyme Q is a neutral lipid, insoluble in water, poorly soluble in polar organic solvents, but highly soluble in non-polar solvents, especially hydrocarbons. These properties can be made the basis of efficient purification procedures. The first step in

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** The nomenclature of the coenzyme Q series of compounds is discussed in paper XV of this series.

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the purification involves either selective extraction of coenzyme Q after alkaline treatment of the starting material (saponification) or, alternatively, removal of the phospholipid fraction from the total lipids by acetone precipitation. The extracts are then further purified by column chromatography on suitable adsorbants. Following chromatography, coenzyme Q can be easily crystallized from relatively polar solvents such as alcohols.

METHODS

The purification of coenzyme Q has been followed spectrophotometrically. The extinction coefficients of coenzyme Q in ethanol are as follows:

Oxidized form: Max 275 mμ $E^{1\%}_{1\,cm}$ = 165
Min 236 mμ $E^{1\%}_{1\,cm}$ = 28.4
Reduced form: Max 290 mμ $E^{1\%}_{1\,cm}$ = 46.4
275 mμ $E^{1\%}_{1\,cm}$ = 23

Therefore, $\Delta\ E^{1\%}_{1\,cm}$ (oxidized-reduced) 275 mμ = 142
This difference is taken as a measure of the amount of coenzyme Q.

The assay is carried out as follows. An amount of unknown corresponding to a concentration of quinone of about 0.05 mg/ml is dissolved in absolute alcohol, and the spectrum corresponding to the oxidized form is then measured. Reduction is carried out by adding an excess of a fresh aqueous solution of KBH_4 prepared by dissolving 40 to 50 mg of KBH_4 in about 0.5 ml water. About 0.01 ml of this solution is usually more than sufficient to reduce 1 ml of the diluted unknown. An excess of KBH_4 solution is to be avoided since it may lead to turbid solutions. After addition of the reducing agent and mixing of the solution, the spectrum is measured. Reduction can also be achieved by shaking the sample with a few grains of solid KBH_4 until the absorbance at 275 mμ decreases to a constant value. To confirm the concentration calculated from the change of absorbance (oxidized-reduced) at 275 mμ, the decreased absorbance at 405 mμ after addition of borohydride may be used. A more concentrated solution of coenzyme Q is required for this purpose since the absorption at 405 mμ is considerably lower than at 275 mμ [$\Delta\ E^{1\%}_{1\,cm}$ (oxidized-reduced) = 6.0 at 405 mμ].

When coenzyme Q is in the reduced form in extracts to be assayed, the solution is shaken with solid silver oxide and allowed to stand for 1 h. The silver oxide is removed by centrifugation, and then the solution is suitable for the reduction procedure.

Estimation of coenzyme Q in the total lipid extract of beef heart by reduction at 275 mμ is not reliable because of the presence of a material which shows increased absorbance at 275 mμ after addition of borohydride. This interfering material is removed during purification.

Initial extraction after saponification

Selective extraction of coenzyme Q by long-chain hydrocarbons such as heptane following saponification of tissue results in extensive purification. Several points concerning this method should be noted. The compound is irreversibly destroyed in

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alkaline solutions unless the saponification mixture contains pyrogallol which presumably acts as an antioxidant. There is also the possibility that this procedure could lead to isomerization of some of the double bonds in the molecule. It is preferable to extract the saponified mixture with heptane or isooctane rather than the low boiling hydrocarbons since the latter extracts undesirable material. The major identifiable impurities in the heptane extract are carotenoids and sterols which can be easily removed by chromatographic techniques or even by differential crystallization.

Initial extraction of total lipids without saponification

Coenzyme Q can be extracted directly from mitochondria or beef heart by most organic solvents. The heptane or isooctane extracts are relatively rich in coenzyme Q, and these extracts lend themselves to easy purification of coenzyme Q. However, many extractions by these hydrocarbon solvents are required to remove all the coenzyme Q from the tissue. Ethanol–ether mixtures 3:1 v/v have been found to be most efficient for direct extraction of all coenzyme Q. A large part of the impurities in the ethanol–ether extract are phospholipids which can be removed by acetone precipitation without loss of coenzyme Q. The quinone can then be easily separated from the remaining acetone-soluble lipids by suitable chromatographic procedures. The advantage of this method is that coenzyme Q is not exposed to alkali or high temperatures during the isolation.

Chromatography

Good purification of the initial extracts can be achieved by column chromatography. The quinone can be adsorbed onto and eluted from alumina (Merck, acid and basic form), sodium aluminum silicate (Decalso 50/80 mesh), magnesium silicate (Florisil), fuller's earth and silicic acid (Mallincrodt 100 mesh) and silica gel (Davidson 20–65 mesh). We have found Decalso to be most efficient for rapid and extensive purification of saponified extracts, whereas silicic acid is preferable for purification of the acetone-soluble material obtained by direct ethanol–ether extraction. In general, all of these adsorbants are used in similar ways, *i.e.* the compound remains adsorbed when put on the column in heptane or issoctane and is eluted with slightly more polar solvent mixtures.

Crystallization

After preliminary purification coenzyme Q can be easily crystallized from ethanol or methanol. By making use of this property, it has even been possible to obtain coenzyme Q crystals directly from the first heptane extract after saponification of mitochondria. For this purpose the heptane solution is evaporated, and the ethanol extract of the residue is placed at —15° overnight. White crystals which form are removed, and the mother liquor is held at —15° for 48 h or until crystals of coenzyme Q form. These crystals can be purified then by further crystallizations at 5°. The yield of coenzyme Q is low since much of the coenzyme Q does not crystallize out of the initial mother liquor, but coenzyme Q thus isolated has not been exposed to the various adsorbents and can serve as a check on any structural modifications induced by the adsorbent.

Other solvents have also been found useful for crystallization of more highly purified samples of coenzyme Q. Crystals of coenzyme Q have been obtained from the

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following solvents at a concentration of 10 mg/ml and at the indicated temperatures: amyl alcohol (—15°), ethyl acetate (—15°), acetone (5°), acetic acid (5°), ethanol (25°), methanol (25°). No crystallization could be induced at —15° in the following solvents: carbon tetrachloride, pyridine, toluene, benzene, *n*-heptane, pentane, 2,2,4-trimethylpentane and ethyl ether.

Isolation of coenzyme Q after saponification

1.057 kg beef heart mitochondrial protein was worked up batchwise in the following manner. One volume of mitochondrial suspension in 0.25 *M* sucrose (60 to 70 mg protein/ml) was added to 2 vol. of 10% KOH in 95% ethanol which contained an amount of pyrogallol equal in weight to the weight of protein. This mixture was refluxed 30 min. The saponified mixture was extracted 3 times with 0.156 vol. of *n*-heptane. The heptane extract was then washed several times with distilled water until the pH of the aqueous extracts remained unchanged. The combined heptane extracts were dried over anhydrous Na_2SO_4, and the heptane distilled off *in vacuo*. The residue was dissolved in 250 ml of warm isooctane and placed at 5° overnight. The precipitate which formed was filtered with suction and washed with 50 ml cold isooctane. This precipitate (6.5 g) is mainly cholesterol. The filtrate and washings were combined. This solution was found by assay to contain 1.62 g of coenzyme Q. The isooctane solution was placed at —20° overnight and 0.31 g of precipitate was filtered off and discarded.

For chromatography a column of Decalso (50/80 mesh, 4.2 × 21 cm) was washed with 500 ml isooctane. The isooctane solution containing coenzyme Q was added to the column, and elution was carried out with the following solvent mixtures: isoctane, 5% ethyl ether in isooctane and 20% ethanol in isooctane. A description of the fractions obtained is presented in Table I.

The 5% ether eluate (fractions 4, 5, 6) which contained most of the coenzyme Q was concentrated *in vacuo* and rechromatographed on Decalso essentially as described

TABLE I

DESCRIPTION OF FRACTIONS ELUTED FROM DECALSO COLUMN

Fraction number	*Solvent*	*Volume (ml)*	*Description of eluate*
1 to 3	isooctane	1250	1, 2 yellow 3 no color
4 to 8	5% ethyl ether– 95% isooctane	2750	4, 5, 6 very yellow* 7, 8 slight yellow
9 and 10	20% ethyl alcohol– 80% isooctane	1000	deep yellow band moves off column

* The 5% ether eluate contains 82% of the coenzyme Q as determined by Δ 275 mμ in alcohol. When this eluate was rechromatographed as described below, the recovery of coenzyme Q was 97%. When the isooctane and alcohol eluates are combined and rechromatographed as above, only 0.2% of the original coenzyme Q could be recovered in the ether eluates. Therefore, the "loss" of coenzyme Q may be due (1) to a compound other than coenzyme Q which reacts similarly with KBH_4, (2) to irreversible adsorption of a coenzyme Q-X complex or (3) to change in R_F due to complex formation. These possibilities are under investigation.

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above (50/80 mesh, 3.2 × 13 cm column washed with 500 ml isooctane). After addition of the isooctane solution containing coenzyme Q the column was eluted with 500 ml isooctane which gave a colorless eluate. The single yellow band was then eluted with 1500 ml of a mixture of 5 % ethyl ether and 95 % isooctane. These eluates contained 95 % of the added coenzyme Q.

The main portion of coenzyme Q from the second column was concentrated *in vacuo* to an orange oil. The oil was heated on a steam bath with 100 ml absolute ethanol. The hot ethanolic solution was separated by decantation from a small amount of insoluble brownish oil. Crystals of coenzyme Q formed in this solution after 24 h at 5°. The crystals were filtered off and recrystallized from 160 ml absolute alcohol at 0° after removing a small amount of alcohol insoluble oil. Yield 1.4 g m.p. 46.5 to 47°. $E^{1\%}_{1\,cm}$ = 160 (275 mμ). An additional 0.25 g of Q_{275} was recovered from mother liquors after crystallization. M.p. 48°. $E^{1\%}_{1\,cm}$ = 162.

Adaption of saponification procedure to whole heart

A procedure similar to that described for isolation of coenzyme Q from mitochondria can be applied to the isolation of large amounts of coenzyme Q from whole heart tissue. Since whole heart contains more unsaponifiable material than mitochondria, more treatment is required for complete purification.

Beef heart tissue is trimmed of fat and connective tissue and passed through a meat grinder. This chopped meat is then saponified with 600 ml of 10 % KOH in 95 % ethyl alcohol containing 30 g pyrogallol for each lb. (450 g) of heart tissue. The mixture is refluxed for ½ h. The saponified mixture is extracted with heptane, and the heptane extract is washed with water. A thick emulsion usually forms at the heptane–water interface, and this is separated from the other layers and treated separately with an equal volume of 95 % alcohol to break the emulsion. The heptane layer formed after breaking the emulsion is combined with the original heptane extract.

The heptane is removed *in vacuo*, and the residue taken up in 20 ml absolute ethanol. The ethanol solution is allowed to stand overnight at 5°, and the white residue that forms is removed by filtration. The ethanol is then removed *in vacuo*, and the residue taken up in isooctane. The isooctane solution is then cooled, filtered to remove cholesterol, and chromatographed on Decalso as described above for the mitochondrial extracts. For the extract from 1500 g of beef heart a column 1–½ inches by 8 inches will suffice. Coenzyme Q appears in the 5 % ethyl ether–95 % isooctane eluate. After chromatography twice on Decalso, the coenzyme Q is crystallized from absolute ethanol. The initial extract from 450 g of beef heart (saponified) contains 24 to 32 mg Q_{275}. At least 10 mg crystals will be obtained after chromatography. $E^{1\%}_{1\,cm}$ at 275 mμ is usually 160, and the m.p. varies from 35 to 45°. Further treatment with Decalso is not very effective in increasing the purity. The material may be further purified, however, by chromatography on silicic acid under the conditions described below for chromatography of coenzyme Q obtained by direct extraction of mitochondria.

Purification following direct solvent extraction

10 l of mitochondria suspended in 0.25 *M* sucrose (approximately 650 g of

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protein) were extracted batchwise by mixing with 5 vol. ethanol–ether (3:1 v/v) and stirring for 2 h. Solids were filtered off on a Büchner funnel and extracted a second and third time with ethanol–ether by the same procedure. The combined ethanol–ether extracts were evaporated under reduced pressure over a warm bath. When the volume of extract was reduced to one-fifth the original volume, the aqueous ethanol suspension was extracted with petroleum ether (30° to 60°). The petroleum ether extract was reduced to a volume of 200 ml under vacuum. The petroleum ether suspension of lipids was then poured with stirring into 3 l of acetone. Another 50 ml of petroleum ether which was used to rinse the flask was also added to the acetone. The acetone suspension was set at —15° overnight. The curdy phospholipid precipitate was then removed, while the suspension was still cold, by filtration through a Büchner funnel, and the precipitate rinsed with cold acetone. The acetone was removed under reduced pressure, and the acetone soluble lipids taken up in 50 ml *n*-heptane.

The heptane solution of neutral lipids was placed on a silicic acid column (30 g silicic acid 15 g Hiflo Super Cel slurried in heptane and allowed to settle overnight followed by removal of excess heptane).

The column was run under 4 lb./sq. inch of nitrogen gas. Elution was made in 25 ml fractions with the following solvents or solvent combinations: 250 ml heptane (fractions 1 to 10), 800 ml 20% chloroform in heptane (fractions 11 to 43), 400 ml chloroform (fractions 44 to 60), 100 ml methanol (fractions 61 to 64). Yellow material was eluted by each of the solvent combinations. Thus the Q_{275} content of each fraction had to be determined spectrophotometrically. In this run 261 mg Q_{275} were in fraction 46 and 23 mg in fraction 47 (eluted with chloroform). The $\Delta E^{1\%}$ at 275 mμ for fraction 46 was 142. When fraction 46 was transferred to warm ethanol after removal of chloroform, coenzyme Q crystallized out after a few hours at room temperature.

The material in fraction 46 was further purified by chromatography on Decalso as described for saponified extract. The final product consisted of 185 mg coenzyme Q with m.p. 49.2 to 49.3°, $E^{1\%}_{1\,cm}$ at 275 mμ 164.

Application of direct extraction procedure to preparation from whole heart

Extraction and acetone treatment of lipids from whole heart tissue is carried out as described except that the heart tissue is first passed through a meat grinder and then homogenized in alcohol by means of a Waring blendor. The use of the silicic acid column for separation of coenzyme Q from the lipids of the heart is not as successful as for the separation from the lipids of mitochondria. Preliminary purification by chromatography on Decalso is desirable. The acetone-soluble fraction is passed through Decalso as described for the saponified extract except that the coenzyme Q adheres more strongly to the column and must be eluted with 20% ether in isooctane. The coenzyme Q in the 20% ether fraction from this first Decalso treatment is then rechromatographed on Decalso. In this second run the coenzyme Q will be found in the fraction eluted with 5% ether. The fraction containing coenzyme Q is then chromatographed on silicic acid–Super Cel (2:1 w/w) and the elution schedule described above for mitochondrial lipid is followed. The chloroform is removed, and the coenzyme Q is taken up in hot ethanol. Crystals form on cooling to room temperature. M.p. 50°, $E^{1\%}_{1\,cm}$ at 275 mμ 165.

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DISCUSSION

The chemical properties of coenzyme Q obtained by the procedures described here will be discussed in another publication of this series[4], in which it will be shown that samples of coenzyme Q prepared by the procedures described here have identical R_F when chromatographed on silicone-treated paper, and identical visible, ultraviolet and infrared spectra. All preparations have the same coenzyme Q activity as measured in the succinoxidase assay described in the preceding paper[3].

MORTON and coworkers have described a compound which they first referred to as SA and which they originally thought was a steroid. Subsequent to our publication of a note[1] in which we showed that coenzyme Q is a quinone, that it is concentrated in the succinoxidase system of mitochondria and that it functions in electron transport, MORTON *et al.*[6,9] have published preliminary notes which indicate that SA is widely distributed in animal tissues, that it is associated with mitochondria and that it is a quinone. MORTON *et al.* now recommend the name ubiquinone for the SA compound isolated from rat liver and horse intestine. Ubiquinone greatly resembles coenzyme Q in its ultraviolet and visible spectral characteristics. It also follows the same pattern as coenzyme Q when chromatographed on alumina. However, the material they report does not show the same infrared spectrum or the same melting point as beef heart coenzyme Q. No evidence has been presented that the material described as ubiquinone has coenzyme Q activity. The reasons for the differences in properties between coenzyme Q and ubiquinone are unknown. The material which they report may be different than the beef heart coenzyme Q since we have already presented evidence that different varieties of coenzyme Q do exist[7,8].

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