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A Method for Determining the Concentration of Ubiquinone in Mitochondrial Preparations

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In the course of studies on the possible role of ubiquinone (coenzyme Q) in the respiratory chain it became necessary to devise a rapid and accurate method for the assay of the substance in small amounts of tissue preparations. The method consists essentially of: (*a*) the simultaneous termination of any enzymic reaction and denaturation of enzyme proteins without destroying the ubiquinone or altering the oxidation–reduction state of ubiquinone–ubiquinol mixtures; (*b*) extraction of the lipid with light petroleum; (*c*) removal of interfering lipids, such as phospholipids, by partitioning the lipid between light petroleum and methanol; (*d*) the spectrophotometric determination of the ubiquinone. The method can be used for the determination of the ubiquinone concentrations in mitochondria or other preparations, except in special cases where the concentration of other substances with u.v.-light absorption (e.g. vitamin A) is high enough to interfere with the determination. It can also be used for kinetic studies where a knowledge of the oxidation–reduction state of the quinone is required (Redfearn & Pumphrey, 1960).

MATERIALS AND METHODS

Mitochondria. These were prepared from animal tissues by the method of Schneider & Hogeboom (1950) and from plant tissues by the method of Simon (1957).

Heart-muscle preparations. These were prepared from pig heart by a modified Keilin & Hartree (1947) procedure. Fresh minced heart muscle (400 g.) was washed with cold water (2 l.) for about 15 min., collected by straining off the water through mutton cloth and squeezed to remove excess of liquid. The washing process was repeated six or seven times until the washings were colourless. The washed mince was homogenized for 1·25 min. in a Waring Blendor with 0·1 M-KH_2PO_4–Na_2HPO_4 buffer, pH 7·4 (400 ml.). The homogenate was centrifuged for 20 min. at 1800 *g* to remove nuclei, unbroken cells and muscle fibres from suspension. The dark-red turbid supernatant containing the enzyme particles (mitochondrial fragments) was decanted and centrifuged at 20 000 *g* for 30 min. The resulting precipitate was divided into two parts: one part was suspended in 0·1 M-phosphate buffer, the other in 0·1 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) –HCl buffer, pH 7·4, and both suspensions were centrifuged at 55 000 *g* for 15 min. The precipitates of washed enzyme particles were suspended in the same buffers to give a protein concentration of 10–30 mg./ml. The whole procedure was carried out at 0°.

Solvents. Ethanol was purified for spectrophotometric use by adding zinc dust (20 g.) and potassium hydroxide (40 g.) to ethanol (1 l.), and refluxing the mixture for at least 1 hr. before distilling. Light petroleum (A.R., b.p. 40–60°) was passed through a column (20 cm. × 1·5 cm.) of silica gel (200-mesh) to remove benzene and then distilled. Methanol (A.R.) was redistilled.

Protein determinations. These were made by the biuret method (Cleland & Slater, 1953), purified fat-free casein being used as the standard.

Assay of ubiquinone. A portion (1 ml.) of the tissue preparation (mitochondria or heart-muscle preparation) containing 10–30 mg. of protein was placed in a 15 ml. conical centrifuge tube. The preparation was denatured by the rapid addition from a hypodermic or spring-loaded pipetting syringe of cold (−20°) methanol (4 ml.)

containing pyrogallol (1 mg./ml.). Light petroleum (5 ml.) was added immediately and the mixture shaken rapidly for 1 min. The tube was then given a short spin in a clinical centrifuge to separate the layers. The upper light petroleum layer was transferred to another 15 ml. centrifuge tube, and the denatured enzyme was extracted again with light petroleum (3 ml.). The combined light petroleum extracts were treated with 2 ml. of 95% (v/v) methanol and the mixture was shaken for 30 sec. After separation of the layers the light petroleum layer was transferred to a 5 ml. beaker and evaporated under reduced pressure in a vacuum desiccator. The residual lipid was redissolved in spectroscopically pure ethanol (3 ml.) and the spectrum of this solution was measured in the 230–320 mμ range. The presence of ubiquinone was indicated by selective absorption with a maximum at 275 mμ. The ubiquinone was reduced by the addition of a small crystal of sodium borohydride (approx. 0·5 mg.) followed by rapid stirring. The spectrum was determined over the same range; the absorption maximum had now shifted from 275 to 290 mμ, and had decreased in intensity. This spectrum is characteristic of ubiquinol. From the decrease in extinction at 275 mμ (ΔE_{275}) the concentration of ubiquinone in the lipid extract was calculated by using the molecular extinction coefficient for the difference in absorption of the oxidized and reduced forms of ubiquinone $(\epsilon_{ox.} - \epsilon_{red.})_{275} = 12\,250$ (Hemming, 1958). Ubiquinone concentrations are expressed as μmoles/g. of protein.

RESULTS AND DISCUSSION

Assay procedure

The aim of this method is to isolate ubiquinone from small amounts of tissue preparation in a state of purity sufficiently high for spectrophotometric estimation by the difference in absorption of oxidized and reduced forms. The spectrum of the whole lipid of mitochondrial preparations contained a great deal of end-absorption due to lipids, and this tends to obscure and distort the selective absorption due to ubiquinone.

A further difficulty is that this extraneous lipid may undergo absorption changes on treatment with sodium borohydride. In the present method, interfering lipid is largely removed by partitioning between light petroleum and methanol. Most of the phospholipid is preferentially soluble in 95% methanol, whereas ubiquinone is practically insoluble in this solvent. The spectra obtained in a typical assay of ubiquinone in a pig-heart-muscle preparation are shown in Fig. 1. Chromatography of the lipid extract of the pig-heart-muscle preparation on alumina (Heaton, Lowe & Morton, 1957) or silicic acid (Pumphrey & Redfearn, unpublished work), followed by spectrophotometric measurement of the fractions, showed that more than 97% of the ΔE_{275} could be attributed to ubiquinone. The ubiquinone was identified by its characteristic absorption spectrum and by observing the effects on the spectrum of sodium borohydride and of sodium ethoxide (Morton, Wilson, Lowe & Leat, 1957). The latter test is specific for ubiquinone. Ubiquinol is fairly easily autooxidizable and oxidation is particularly liable to occur in the denatured preparation before extraction into light petroleum. The purpose of the pyrogallol in the methanol used to denature the preparation is to protect any ubiquinol from oxidation during the extraction procedure. This is particularly important when the method is applied to kinetic measurements where a knowledge of the oxidation–reduction state of ubiquinone is required.

Ubiquinone concentrations in tissue preparations from various sources

The method has been applied to the measurement of ubiquinone concentration in heart-muscle preparations and in mitochondria from different sources (Table 1). The ubiquinone concentrations in pig-heart-muscle preparations are relatively high (4 μmoles/g. of protein). The cytochrome *c* concentration of Keilin & Hartree preparations has been given as 0·8 μmole/g. fat-free dry weight by

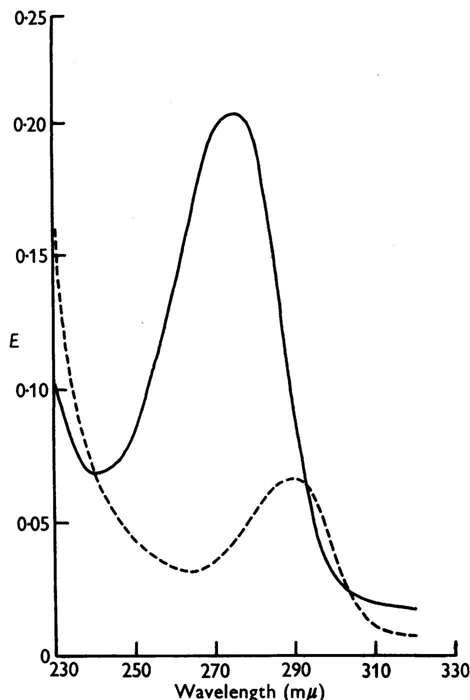

Fig. 1. Absorption spectrum of lipid extract of a pig-heart-muscle preparation in ethanol (——). This spectrum, with a maximum at 275 mμ, is typical of ubiquinone. On reduction with sodium borohydride a spectrum with a maximum at 290 mμ, typical of ubiquinol, is obtained (– – –).

Slater (1949). Thus the ubiquinone concentration, on a molar basis, is approximately five times that of the cytochrome *c*.

The spadix of *Arum maculatum*, an active non-photosynthetic tissue, contains 1·4 μmole of ubiquinone/g. of mitochondrial protein. A mean figure for cytochrome *c* was calculated from Bendall's (1958) data for young spadices at approximately the same period of development. This was 0·23 μmole/g. of protein, or about one-sixth of the concentration of ubiquinone.

A limitation of the method is that it cannot be used on mitochondria which contain large amounts of material absorbing in the same spectral region as ubiquinone. Thus it is impossible to determine directly the ubiquinone concentration of normal liver mitochondria because of the intense absorption of vitamin A. However, the method could probably be successfully used in conjunction with a suitable paper-chromatographic technique.

Effect of phosphate buffer on extractability of ubiquinone

Table 2 shows the ubiquinone concentrations obtained for a series of pig-heart-muscle preparations suspended in tris and in phosphate buffers. It will be seen that the apparent concentration of ubiquinone in the phosphate-buffered enzymes is only 70–80% of that in the tris-buffered enzymes.

Several tests were carried out to determine whether this lower yield of ubiquinone was due to a partial reduction to ubiquinol in the phosphate medium. Endogenous ubiquinol, formed by incubating the enzyme preparation anaerobically with substrates such as succinate, is readily oxidized to the quinone by passage of oxygen through the enzyme suspension, or by omission of pyrogallol from the methanol used for denaturation. Neither passage of oxygen through the enzyme nor omission of pyrogallol had any effect on the ubiquinone yield from phosphate-buffered enzymes. Ubiquinol is also easily oxidized to the quinone by treatment with ferric chloride. Accordingly, a small amount of ferric chloride (1–2 mg.) in ethanol (3 ml.) was added to the light-petroleum extracts from a denatured sample of phosphate-buffered enzyme (1 ml.). After 5 min. at room temperature the ferric chloride was removed from the petroleum by two washes with a few millilitres of water, and the amount of ubiquinone present in the extract was measured in the usual way. This treatment also had no effect on the ubiquinone yield. These results show that the lower values obtained for ubiquinone in phosphate-buffered preparations are not due to a partial reduction to ubiquinol.

It seemed possible therefore that, in the phosphate medium, some of the endogenous ubiquinone might exist in a 'bound' form which was still firmly bound even after denaturation of the protein. To test whether the 'bound' ubiquinone was readily dissociable from the binding agent, enzyme preparations were treated with acid and alkali. Either 2N-hydrochloric acid or 2N-sodium hydroxide (0·2 ml.) was added to a sample (1 ml.) of the pig-heart-muscle preparation. After 1 or 2 min. at room temperature the enzyme was denatured and the extractable ubiquinone measured in the usual way. Acid treatment of the enzyme in both buffers lowered the yield of ubiquinone by 30–50%. The reason for this is not apparent since pure ubiquinone is relatively stable to acid. Alkali treatment of the phosphate-buffered enzyme, on the other hand, increased the yield of ubiquinone to a level approximating to that obtainable from the untreated tris-buffered enzyme (Table 2). Alkali-treatment of the extracted protein residue from a standard ubiquinone assay of the phosphate-buffered enzyme, followed by a further extraction, was found to release more ubiquinone. The combined yields approximately equalled the yield from the corresponding tris-buffered enzyme.

These results indicate that, in the presence of phosphate buffer, endogenous ubiquinone in the pig-heart-muscle preparation exists in two forms. One form, representing about 75%, is easily extractable by light petroleum after denaturation of the enzyme with methanol, whereas the remaining 25% is extractable only after treatment of the protein with alkali. It is not yet known whether the two forms of ubiquinone differ chemically or merely in their location within the mitochondrial fragments. In contrast with the results with phosphate-buffered preparations, alkali-treatment of

Table 1. *Concentrations of ubiquinone in tissue preparations*

Preparation	Ubiquinone (μmole/g. of protein)
Pig-heart-muscle preparation	4·0
Guinea-pig-kidney mitochondria	1·6
Pig-kidney mitochondria	1·1
Arum-spadix mitochondria	1·4

Table 2. *Apparent ubiquinone concentrations of phosphate- and tris-buffered pig-heart-muscle preparations and the effect of treatment with alkali*

For details of alkali-treatment see text. Numbers in parentheses indicate the number of determinations.

Buffer used to suspend preparation	Treatment	Extractable ubiquinone (μmoles ± S.D./g. of protein)
Phosphate	None	2·80 ± 0·41 (12)
Phosphate	NaOH	3·94 ± 0·38 (5)
Tris	None	4·0 ± 0·21 (12)

the tris-buffered preparation resulted in a lower than normal yield of ubiquinone. This is what might be expected since ubiquinone is rather labile in alkaline solution. For the same reason the large increase in ubiquinone yield from the phosphate-buffered enzyme after treatment with sodium hydroxide was surprising. Presumably there exists in the phosphate-buffered enzyme suspension some agent which protects ubiquinone very effectively from destruction by alkali. Nothing is known about the nature of the bound form of ubiquinone.

SUMMARY

1. A method for determination of the ubiquinone concentration in small amounts of mitochondrial preparations is described.

2. Ubiquinone occurs in animal and plant mitochondria in relatively high concentrations. In heart-muscle preparations, for example, the concentration, on a molar basis, is four to five times those of the individual cytochromes.

3. A 'bound' form of ubiquinone appears to exist in phosphate-buffered heart-muscle preparations. This bound form, which amounts to about 25% of the total ubiquinone, can be extracted only after treatment of the preparation with alkali.

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The Kinetics of Ubiquinone Reactions in Heart-Muscle Preparations

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A great deal of evidence has been presented (Crane, Hatefi, Lester & Widmer, 1957; Lester & Fleischer, 1959; Pumphrey, Redfearn & Morton, 1958; Pumphrey & Redfearn, 1959; Redfearn, 1959) which suggests that ubiquinone (coenzyme Q) is a functional component of the respiratory chain in living organisms. It is widely distributed in animal and plant tissues, where it is located principally in the mitochondria in a concentration four to five times as great as those of the individual cytochromes. The endogenous ubiquinone is reduced to the quinol when the mitochondrial preparation is incubated with dihydrodiphosphopyridine nucleotide or succinate under anaerobic conditions, and is reoxidized when the preparation is aerated; the addition of ubiquinone to preparations in which the endogenous ubiquinone has been removed by acetone-extraction restores enzymic activity. Thus ubiquinone fulfils some of the criteria necessary for a substance to be considered as a functional component of the respiratory chain (Slater, 1958). However, before ubiquinone can be assigned a position in the chain the kinetics of its oxidation and reduction must be known. The current concept of the electron-transport system for the oxidation of succinate and dihydrodiphosphopyridine nucleotide is a chain of cytochromes with branches at the 'substrate end' to link with dihydrodiphosphopyridine nucleotide and succinate through their specific flavoproteins (Chance, 1957; Slater, 1958). If ubiquinone is a member of such a system then its rates of oxidation and reduction must be at least equivalent to the overall rate of substrate oxidation.

In this paper the results of a study of the kinetics of the reactions of endogenous ubiquinone in mitochondrial preparations are described and they are discussed in relation to a possible position for ubiquinone in the respiratory chain. Part of this work has already been presented as a preliminary communication (Pumphrey & Redfearn, 1959).