Ubiquinone and Vitamin K in Bacteria

2. INTRACELLULAR DISTRIBUTION IN *ESCHERICHIA COLI* AND *MICROCOCOCCUS LYSODEIKTICUS**

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Ubiquinone and vitamin K show a characteristic distribution among bacterial species (Bishop, Pandya & King, 1962). Neither is present in cells lacking aerobic respiration. Otherwise, Gram-positive organisms contain vitamin K but not ubiquinone, and Gram-negative organisms contain ubiquinone but not vitamin K, except for a few types (e.g. *Escherichia coli*, *Proteus vulgaris*) which contain both. The interspecific distribution of these substances would thus suggest that they play a part in respiratory processes, as has been suggested for animal tissues (Wolstenholme & O'Connor, 1961). The sites of oxygen utilization and energy production are, in animal tissues, the mitochondria, but bacteria contain no analogous structures. Instead, the electron-transport enzymes appear to be located in the cell membrane (Marr & Cota-Robles, 1957; Mitchell & Moyle, 1956; Nakada, Nozu & Kondo, 1958; Weibull, Beckman & Bergstrom, 1959). This paper presents evidence that vitamin K and ubiquinone are similarly located.

Techniques for studying the intracellular distribution of cell constituents are less highly developed for bacterial than for mammalian cells. Three methods are employed here:

(a) Disruption of the cells by freezing and the sudden application of high pressures (Hughes, 1951). The soluble cell contents escape leaving the cell walls with the membranes attached.

(b) Ultrasonic disintegration, which not only breaks open the cells but also disrupts the 'hulls', i.e. the cell walls with the membranes attached. The hulls break down only on prolonged treatment to yield particles too small to be precipitated by centrifuging at moderate speed. This forms the basis of the method of 'differential release' described by Marr & Cota-Robles (1957). When a bacterial suspension is subjected to ultrasonic treatment the fall in cell count and fall in turbidity are both exponential but the time-constants are different. If a cell constituent is located entirely in the soluble contents of the cell its release from the

cell will follow the same time-relation as the drop in cell count. On the other hand, components of the hulls will pass into the supernatant fraction at a rate corresponding to the loss in turbidity.

(c) Neither of these methods distinguishes between cell-wall and plasma-membrane constituents. Preparation and analysis of the plasma membranes themselves is possible, however, in *Micrococcus lysodeikticus* by treatment with lysozyme, which hydrolyses the cell-wall material of this organism into products of relatively low molecular weight. The naked cells (or 'protoplasts') then rupture osmotically. The contents escape, and the only solid materials left are the ruptured cell membranes; these may be collected by centrifuging.

EXPERIMENTAL

Organisms

Micrococcus lysodeikticus (N.C.T.C. 2665) and *Escherichia coli* (laboratory stock strain no. 106) were maintained on nutrient agar. *E. coli* was grown on a simple glucose-salts medium at 37° and harvested at the end of the logarithmic phase (18 hr.). *M. lysodeikticus* was harvested after growth for 48 hr. at 30° in nutrient broth. Both organisms were grown in 15 l. batches with forced aeration as described by Bishop *et al.* (1962). The harvested cultures were washed three times with 2 l. of water and proved free of contamination by microscopic examination and by plating on nutrient agar.

Disintegration of bacteria

Hughes *press.* *E. coli*, 25 g. wet wt. (6.5 g. dry wt.), was suspended in 50 ml. of water and treated without abrasive at -20° in a Hughes (1951) press, modified for treatment of larger amounts of material (Sutton & King, 1962). After the suspension had been thawed, 20 mg. of deoxyribonuclease (L. Light and Co.) was added and the suspension incubated at 20° for 30 min. to degrade the viscous DNA. Batches (5 ml.) were then ultrasonically treated for 1 min. under standard conditions (see below) to free the bacterial hulls of their contents. The treated suspension was centrifuged at 75 000g for 100 min. The supernatant ('supernatant 1') was extracted with ether (3 × 100 ml.) and then refluxed with methanol (200 ml.) for 45 min. and re-extracted with ether (5 × 200 ml.). Both ether extracts were distilled down to 50 ml., and evaporated to dryness under nitrogen at 30°, to yield 12.0 and 13.7 mg. of lipid

* Part 1: Bishop, Pandya & King (1962).

respectively. The two fractions were combined for vitamin K and ubiquinone estimation.

The material deposited in the centrifuge ('residue 1'; 3.41 g. dry wt., equivalent to 52% of cell material) was freeze-dried and extracted with methanol to yield 110 mg. of lipid.

Ultrasonic disintegration. The washed bacteria were suspended in water (200 mg. wet wt./ml.) and treated in 5 ml. batches in the MSE ultrasonic disintegrator, operating at 60 w and at 20 kcyc./sec. for the times stated. Before disintegration oxygen was removed by flushing the solution with nitrogen. A salt-ice bath kept the temperature below 5°. Turbidity measurements and total cell counts (also viable counts with *E. coli*) were made before and after disintegration. Portions (50 ml.) of the combined ultrasonically-treated bacteria were then centrifuged (*E. coli*, 75 000g for 100 min.; *M. lysodeikticus*, 20 000g for 10 min.). The supernatants were then assayed for phosphorus, nitrogen, total nucleic acid, cytochrome and (*E. coli*) glutamate decarboxylase or (*M. lysodeikticus*) carotenoids. The centrifuged deposits, consisting of hull residues and unbroken cells, were freeze-dried and extracted with methanol to obtain the lipid for vitamin K, ubiquinone and (for *M. lysodeikticus*) carotenoid.

Protoplast membranes

Batches (1.5 l.) of *M. lysodeikticus* were grown and harvested as above, then washed thrice in 2 l. of 0.05M-sodium phosphate buffer, pH 7.0, and suspended (6.9 g. dry wt.) in 690 ml. of this buffer. Lysozyme hydrochloride (from egg white; L. Light and Co.) (40 mg.) and sodium chloride (124 mg.) were added. After being left for 30 min. at room temperature (20°) the cells were lysed and a clear orange-yellow solution was obtained. The viscosity of this solution was decreased by treatment for 30 min. at room temperature with 10 mg. of deoxyribonuclease. The solution was then centrifuged at 20 000g for 30 min. Very little more material was spun down on continuing the centrifuging for a further 30 min. The deposited material was washed three times by centrifuging at 20 000g for 10 min. with 0.05M-sodium chloride (100 ml.). Electron microscopy showed an almost pure preparation of protoplast membranes. The membrane fraction ('residues 2'; 0.67 g. dry wt., equivalent to 9.8% of cell material) was extracted with methanol to obtain the lipids. The supernatant and first two washings were combined and the whole ('supernatant 2' fraction; 880 ml.) was extracted thrice with 400 ml. of ether. Methanol (2400 ml.) was then added and, after being refluxed for 60 min., the mixture was extracted with 3 × 1000 ml. of ether. The ether extracts were distilled to reduce bulk, dried with anhydrous sodium sulphate and then evaporated to dryness to yield 24 mg. of lipid.

Analytical methods

Chromatography of lipids and determination of vitamin K and ubiquinone. The methods used followed closely those described by Bishop *et al.* (1962). The lipids isolated from the various preparations were chromatographed on 10 g. of acid-washed alumina, Brockmann grade 3, and eluted successively with 100 ml. portions of pure light petroleum (b.p. 40–60°), ether-light petroleum (b.p. 40–60°) (2:98, v/v), ether-light petroleum (b.p. 40–60°) (4:96, v/v), and pure ether. In the *E. coli* materials, an ether-light petro-

leum (b.p. 40–60°) (6:94, v/v) fraction was taken instead of the ether-light petroleum (b.p. 40–60°) (4:96, v/v) fraction.

Vitamin K was estimated from the extinction at $\lambda_{\max}$. 249 m μ (in cyclohexane) of the ether-light petroleum (b.p. 40–60°) (2:98, v/v) fraction. This takes no account of irrelevant absorption, but the extent of this was small in most of these fractions. In doubtful cases, the difference in extinction between $\lambda_{\max}$. 270 m μ and $\lambda_{\min}$. 290 m μ was used as an alternative measure (Bishop, 1962). Even at low concentrations of vitamin K, the error should not exceed 10%.

Ubiquinone was estimated by the decline in extinction at 275 m μ on adding sodium borohydride (tetrahydroborate) to an ethanolic solution of the ether-light petroleum (b.p. 40–60°) (6:94, v/v) fraction. No ubiquinone determinations were performed on the *M. lysodeikticus* fractions since this organism does not contain ubiquinone in detectable amounts.

Where the characteristic spectra of ubiquinone or vitamin K could not be seen, the amount present is reported as 'less than' the amount which would have been present if the whole of the absorption at the appropriate wavelengths were attributable to the substance in question. The actual amount present would certainly be far below the limiting figures quoted.

Cell counts. Total cell counts were made in a haemocytometer, viable counts by plating in nutrient agar.

Turbidity. This was measured in an EEL portable colorimeter (Evans Electroselenium Ltd.) without a filter.

Phosphorus and nitrogen. Total P was determined after perchloric acid digestion, by the method of King & Wootton (1956). Nitrogen was estimated by the micro-Kjeldahl method.

Total nucleic acids. In clear supernatants these were estimated by the extinction at 260 m μ ; in whole cells of *M. lysodeikticus* the same method was used after lysis with lysozyme and treatment with deoxyribonuclease.

Cytochrome. This was estimated from the difference in extinction between 434 and 395 m μ in clear supernatants (or lysed *M. lysodeikticus* preparations) after reduction with sodium dithionite. Neither cytochrome nor nucleic acid could be determined directly in intact cells of *E. coli* owing to excessive light-scattering. A value was obtained (for the purposes of Fig. 1) by extrapolation from the extracts from cells disintegrated for various times.

Carotenoids in Micrococcus lysodeikticus. These were estimated from the extinction at 470 m μ . The haematin have little absorption at this wavelength. The carotenoid in the pure ether fraction of the lipids from the hull residues was determined from the 440 m μ (in cyclohexane) peak.

The results of the spectroscopic assays for cytochrome, nucleic acid and carotenoids are expressed in terms of extinction units, since only comparative values were required.

Glutamate decarboxylase (α -glutamate 1-carboxy-lyase, EC 4.1.1.15) in Escherichia coli. This was determined by conventional Warburg manometry in phthalate buffer, pH 4.0, at 25° in nitrogen-filled manometers. The 'initial' value could not be obtained directly from the intact cells as a permeability barrier appeared to restrict activity; it was, however, obtained from cells which had been disintegrated but not centrifuged.

RESULTS

*Disintegration of Escherichia coli
in the Hughes press*

Table 1 shows that disintegration of over 99% of the cells in a preparation failed to release into solution any of the ubiquinone or vitamin K; neither compound was spectroscopically detectable in the extracts, and both were recovered almost quantitatively in the insoluble cell debris.

'Differential release' by ultrasonic disintegration

A suitable rate of disintegration was obtained when 5 ml. batches of a suspension containing 200 mg. wet wt. of cells/ml. were treated in a 20 ml. cup with the large probe of the MSE disintegrator.

Escherichia coli. In Fig. 1 (a) the logarithms of the total and viable cell counts are plotted against time, and the nearly-coincident straight-line relationships show both that there is an exponential rate of fall and that visible damage to the cell is a good measure of lethal action. If the fall in turbidity is taken to represent disintegration of the hulls, this too follows the same law but with a different time-constant. In Figs. 1 (a) and 1 (b), dry weight, nitrogen and phosphorus are expressed as the logarithm of the percentage of the initial amount retained in the solid particles. Nitrogen and phosphorus show (to different extents) the pattern of an initial release at a rate comparable with that of cell breakage, changing later to a slower rate which corresponds roughly to the decrease in turbidity. These are therefore cell constituents which are partly components of the soluble cell contents and partly of the insoluble cell hull. Total nucleic acids, on the other hand, follow closely the fall in cell count (Fig. 1b), suggesting that they are mostly in the soluble cell contents. Cytochrome, however, follows the pattern of hull

disintegration (Fig. 1b). The enzyme, glutamate decarboxylase, was included to test the behaviour of a metabolically active component of the cell, long regarded as a 'soluble' material. Its release follows closely the breaking and death of the cells themselves (Fig. 1c). The concepts of Marr & Cota-Robles (1957), worked out on *Azobacter vinelandii*, thus appear equally valid for *E. coli*.

The 'total lipid' (i.e. the methanol-soluble cell material) shows an intermediate pattern of release similar to that of total nitrogen, although ubiquinone and vitamin K follow a linear relationship at the same slope as the fall in turbidity (Fig. 1c). The points for vitamin K show some scatter, but this labile substance cannot be assayed as accurately as the other cell constituents considered here.

Micrococcus lysodeikticus. The cells of *M. lysodeikticus* were killed by ultrasonic treatment rather more slowly than those of *E. coli*, although the rate of breakage of the hulls was about the same in both organisms. The behaviour of nitrogen, phosphorus, nucleic acid and cytochrome follows similar patterns in the two organisms. *M. lysodeikticus* contains no ubiquinone; the behaviour of vitamin K, however, suggests that it is predominantly a component of the hulls. This organism also contains an appreciable amount of carotenoid pigment, and this appears to be distributed between the hulls and the cell contents.

Protoplast membranes in Micrococcus lysodeikticus

M. lysodeikticus was treated with lysozyme and the plasma membranes were centrifuged down and washed, as described in the Experimental section. The vitamin K content [shown to be the K₂-45 isoprenologue by paper chromatography (Bishop, 1962)] was estimated in the original cells, in the membrane fraction and in the supernatant, which contained both the cell contents and the lysozyme-degradation products of the cell wall. Table 2 shows that the vitamin K was quantitatively recovered in the membrane fraction. The appropriate chromatographic fraction [ether-light petroleum (b.p. 40–60°) (2:98, v/v)] of the lipids from the supernatant showed no trace of the vitamin K spectrum.

Table 1. *Ubiquinone and vitamin K contents of hull residues, cell contents and whole cells of Escherichia coli*

Extracts were prepared in the Hughes (1951) press. Ubiquinone-40 was estimated by ethanolic borohydride reduction, and vitamin K₂-40 from the $\lambda_{\max}$ 249 m μ value. Disintegration reduced the viable count to less than 1% of the initial value.

Cell fraction	Ubiquinone-40 (μ mole/g. dry wt. of whole cells)	Vitamin K ₂ -40 (μ mole/g. dry wt. of whole cells)
Whole cells*	0.32	0.32
'Residue 1'	0.32	0.29
'Supernatant 1'	<0.05	<0.08

* Data obtained from Bishop *et al.* (1962).

DISCUSSION

Analysis of the isolated cell membrane of *M. lysodeikticus* indicates that this is the sole site of vitamin K in this organism. This information provides a check on the validity of the differential-release technique. The latter also indicates that vitamin K is a hull component, though the difficulties of assaying the vitamin with the required precision would make it difficult to assert, on this evidence alone, that no appreciable amount was

Fig. 1. Time-curves for the release of cell components of *Escherichia coli* during ultrasonic disintegration. (a) O, Turbidity; ●, dry weight; □, total P; ▲, total cell count; ▽, viable cell count. (b) Uppermost line, turbidity; ▲, cytochrome; △, total N; ■, lipid; ●, nucleic acids; broken line, viable cell counts. (c) Uppermost line, turbidity; ■, ubiquinone; +, vitamin K₂-40; ▼, glutamate decarboxylase; broken line, viable cell count.

Table 2. Vitamin K₂-45 content of protoplast membrane and cell soluble fractions of *Micrococcus lysodeikticus*

The 'residue 2' fraction represents the protoplast membranes, and the 'supernatant 2' fraction the cell-soluble (and solubilized) materials after lysozyme digestion.

Cell fraction	Vitamin K ₂ -45 (μmoles/g. dry wt.)	
	Expt. 1	Expt. 2
Whole cells	1.30	1.25
'Supernatant 2'	<0.02	<0.05
'Residue 2'	1.28	1.27

present in the cell contents. Nor does the differential-release method permit distinction between cell walls and cell membranes.

The agreement of the differential-release method with the results of the direct analysis of the membranes themselves thus gives us confidence in the results of the method for not only *M. lysodeikticus* but also *E. coli*, where the preparation of cell membranes was not feasible. Here the differential-release method suggests that both ubiquinone and vitamin K are largely, if not exclusively, hull components; and this is supported by the evidence from cells disintegrated in the Hughes (1951) press.

In the animal cell, ubiquinone is present to the same extent (about 1 μmole/g. dry wt.) as in Gram-negative bacteria; it occurs mainly if not exclusively in the mitochondria. Vitamin K is present in animal cells at a much lower concentration; its distribution within the cell has not been firmly established. In bacteria the two quinones appear

to be located in the cell membrane, the region believed to be the site of many mitochondrial functions. It would be rash to ascribe to the quinones a function on electron-transport on these grounds alone, for in *M. lysodeikticus* over 90% of the total lipid of the organism is found in the membrane (Macfarlane, 1961). This is the region, therefore, in which lipid components would be expected to occur. The present findings are, however, quite consistent with a respiratory function for these quinones.

SUMMARY

1. The intracellular distribution of ubiquinone and vitamin K in certain bacteria has been investigated.

2. Suspensions of *Escherichia coli* were frozen and the cells broken by mechanical shock in the Hughes (1951) bacterial press. Both the vitamin K and the ubiquinone of this organism were found exclusively in the insoluble cell residues, representing the cell walls plus the plasma membranes.

3. Protoplast membranes were obtained from *Micrococcus lysodeikticus* by treating the cells with lysozyme followed by differential centrifuging. The entire vitamin K content of the organism was recovered from the membranes. This organism does not contain ubiquinone.

4. *E. coli* and *M. lysodeikticus* were subjected to progressive ultrasonic disintegration. The rate of liberation of ubiquinone and vitamin K in *E. coli*,

and of vitamin K in *M. lysodeikticus*, indicated that these quinones were components of the cell 'hulls', i.e. the cell walls plus attached cell membranes.

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The Onset of Lactose Synthesis after Injection of Prolactin

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The synthesis of lactose in the mammary gland was first demonstrated unequivocally by Grant (1935), who proved lactose synthesis in slices of guinea-pig mammary gland by fermentation with adapted yeast. Malpress & Morrison (1949, 1950) also demonstrated lactose synthesis in mammary glands, by preliminary fermentation with brewer's yeast followed by a non-specific trimethylamine method. Paper-partition chromatography of sugars (Partridge, 1948) was adapted for the quantitative estimation of lactose in the mammary gland by Heyworth & Bacon (1955), who confirmed the

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synthesis of lactose in surviving mammary-tissue slices from lactating guinea pigs. Other ways of estimating lactose, such as the chloramine-T method adopted by Folley & Greenbaum (1947) and the orcinol method described by Slater (1957), lack the specificity given by preliminary chromatographic separation of the sugars. For this reason the chromatographic method was adopted to study the initial stages of lactose synthesis in the mammary glands of the rabbit. In order to measure the concentration of lactose at the first possible moment after its appearance in the mammary gland, possibly before any milk appeared in the alveoli, it was necessary to concen-