3. Liver mitochondria isolated after suspension in 5 mM-sodium salicylate in 0·44 M-sucrose carried out oxidative phosphorylation nearly as efficiently as the controls.

4. Salicylate was taken up by mitochondria at 0°, but the binding was extremely labile. From 78 to 95 % of the salicylate taken up was removed by one rapid wash in sucrose medium.

5. The intracellular fluid of the livers of salicylate-poisoned rats contained concentrations of salicylate from 0·8 to 4·0 mM. These concentrations approximate to the concentrations of salicylate in the plasma of these animals.

6. The presence of salicylate in the intracellular fluid of the livers of rats poisoned with salicylate *in vivo* is compatible with a physiological uncoupling effect of salicylate on mitochondria *in vivo*. Uncoupling cannot be demonstrated in mitochondria isolated from intoxicated rats because the salicylate is washed out during the isolation procedure.

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Ubiquinone and Vitamin K in Bacteria

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The term 'ubiquinone' covers a family of 5,6-dimethoxytoluquinones with polyisoprenoid side chains; the member most commonly found in animals has a C_{50} side chain, i.e. with 10 isoprene units (Morton, 1961). The ubiquinones recall the naphthaquinones of the vitamin K series; vitamin K_1, found in plants, has a phytyl (C_{20}) side chain with only one double bond. The vitamins of the K_2 group are often found in micro-organisms and have side chains with different numbers of isoprene units but always have one double bond per C_5 unit. The length of the side chain may be indicated by a figure after the name, e.g. 'ubiquinone-50' and 'vitamin K_2-40' indicating C_{50} and C_{40} side chains, composed of 10 and 8 isoprene units respectively. The ubiquinones are also known as the 'coenzymes Q', the side chain being designated by the number of isoprene units incorporated; thus 'Q-10' is synonymous with 'ubiquinone-50'.

Ubiquinone is widely distributed in animal tissues, being found mainly, if not exclusively, in the mitochondria, where it occurs at about 1 μmole/g. dry wt. It is believed to play a part in electron transport, but its precise role is still unknown. Vitamin K occurs in animal tissues at a much lower concentration. It has not so far been isolated from animal tissues, though isotope experiments have recently shown that vitamin K_2-20 can be formed in the liver from other K vitamins present in the diet (Martius & Esser, 1958; Martius, 1961). The amount present is too small for chemical or spectroscopic determination but the sensitive bioassay (blood coagulation in chicks) indicates that the richest tissue, liver, contains about 0·01 μmole/g. (dry wt.); 0·04 μmole/g. is present in the mitochondria (Green, Sondergaard & Dam, 1956).

Like ubiquinone, vitamin K is also believed to be a catalyst in the energy metabolism of the cell. Its

function is not yet clarified but is believed to be in the phosphorylation rather than the oxidation mechanism (Martius & Nitz-Litzow, 1953, 1954; Brodie & Ballantine, 1960).

Different types of micro-organisms show more variety in their pathways of oxidative metabolism than do higher animals. One approach to the functions of ubiquinone and vitamin K is to study their distribution in bacterial species. This paper gives the results of such a survey. Some data are already available (cf. Lester & Crane, 1959; Jacobsen & Dam, 1960; Page *et al.* 1960).

METHODS

Materials. Samples of synthetically prepared ubiquinone-30, -35, -40, -45 and -50 and vitamins K_2-30, -35, -40 and -45 were provided by Hofmann–La Roche and Co. Ltd., Basle, Switzerland.

Organisms listed in Table 3 with National Collection of Type Cultures (N.C.T.C.) or National Collection of Industrial Bacteria (N.C.I.B.) numbers were obtained from these sources. Others were stock laboratory strains. *Corynebacterium diphtheriae* (Park-Williams 8 strain) was grown in bulk at the Wellcome Physiological Laboratories under the supervision of Dr D. C. Edwards, to whom we express our thanks. One batch, recorded as 'low-Fe', was grown on a medium of minimal iron content as used for commercial toxin production; the cells contained 13 μg. of Fe/g. dry wt. A second, 'high-Fe', batch was grown on the same medium with addition of iron salts to give optimum growth; these cells contained 350 μg. of Fe/g. dry wt. (analysis by Dr Edwards).

All organisms were maintained by periodic subculture on nutrient-agar slopes.

Media. Lipid analysis called for at least 25 g. dry wt. of organisms if constituents present in small amount were to be detected. This required the culture of one or more 15 l. batches for each species. The media used were mostly based on a compromise between the simplest media permitting growth and richer media giving better yields of organisms. Simple media had the advantage of reducing the risk of introducing extraneous lipid material. The following glucose medium was used in most cases: 0·1% of casein hydrolysate (Oxo Ltd., London), 2% (w/v) of NaH_2PO_4 and 0·1% of $(NH_4)_2SO_4$, in tap water adjusted to pH 7·4 (NaOH). Glucose (2%, w/v; autoclaved at pH 4) was added as a concentrated solution after sterilization of the other components.

Sarcina lutea and *Staphylococcus albus* (*Micrococcus pyogenes* var. *albus*) were grown on nutrient broth. Granules of Nutrient Broth no. 2 (Oxo Ltd., London) were dissolved in tap water in accordance with the manufacturer's instructions. The medium thus prepared contained meat extract (1%), peptone (1%) and NaCl (0·5%) at pH 7·4.

The *Bacillus* species are known to give good growth on glutamate medium (Shah & King, 1959) containing 0·1% of casein hydrolysate (Oxo Ltd., London), 2% (w/v) of NaH_2PO_4, 0·1% of $(NH_4)_2SO_4$ and 0·5% of L-glutamic acid in tap water adjusted (NaOH) to pH 7·4. *Clostridium sporogenes* was grown in thioglycollate broth, i.e. nutrient broth with addition of 0·1% of thioglycollic acid (Brewer, 1940). *Lactobacillus casei* was grown in whey broth (Mackie & McCartney, 1948). For *Azotobacter chroococcum* mannitol medium containing 0·02% of KH_2PO_4 and 2% (w/v) of mannitol in tap water was used (Wilson & Miles, 1957).

Samples of the constituents of nutrient broth and glutamate media were extracted with 2,2,4-trimethylpentane ('octane') and the extracts did not show appreciable absorption between 220 and 450 mμ. The whey-broth medium might have been regarded as a possible source of extraneous lipid, but no ubiquinone or vitamin K was obtained from the organism grown on this medium.

Culture conditions. Pyrex bottles (20 l.) were completely filled for anaerobic growth; for aerobic growth each bottle contained 15 l. and was fitted with tubes for forced aeration. Neoprene bungs and tubing were used to avoid the possible contamination with isoprenoid materials that might be encountered with rubber. Sterilization was by autoclaving at 25 lb./in.^{2} for 45 min. Aeration, at 5 l./min., was maintained with air sterilized by passage through a cotton-wool filter. The outlet from the vessel led through a trap containing dilute Lysol; the exit was guarded with cotton wool. Antifoaming agents were required only with the whey-broth medium; 10 drops of Silicone M.S. Antifoam Emulsion RD (Hopkin and Williams Ltd.) was added. This gave rise to difficulties as the silicone adhered to the bacteria and was difficult to separate from the bacterial lipids. However, only one organism (*L. casei*) was grown on the whey medium, and this contained neither vitamin K nor ubiquinone. This was confirmed in a run in which foaming was controlled by restricted aeration without recourse to antifoam agents.

For anaerobic culture four techniques (methods 1–4, below) were tried. Methods (1), (2) and (4) were used with *Escherichia coli* and yielded concordant results. *Proteus vulgaris* likewise gave close agreement between cultures obtained by methods (1) and (4). *Pseudomonas aeruginosa* and *Staphylococcus aureus* were grown under condition (1). Results recorded in Table 3 for these organisms apply to cultures grown by method (1).

Method (1). The surface of the medium was sealed with a $\frac{1}{2}$ in. layer of medicinal liquid paraffin, and sterile N_2 (O_2-free) was passed through for 30 min. before inoculation. However, the paraffin tended to adhere to the organisms and to contaminate the lipid extracts.

Method (2). A slow stream of sterile H_2 was passed through the medium, which was covered with a $\frac{1}{2}$ in. layer of medicinal liquid paraffin.

Method (3). *Clostridium sporogenes* grew satisfactorily on the thioglycollate medium without special precautions.

Method (4). No special precautions were taken beyond filling the vessel to the neck. This proved satisfactory for the growth of the facultative anaerobes. As soon as growth commenced anaerobiosis was maintained by the organisms themselves. This was demonstrated by addition of methylene blue to the medium. The dye was fully reduced, save for a thin layer at the small area of surface exposed at the neck of the vessel. The batches to which the dye was added were used only for demonstrating anaerobiosis and were discarded after incubation.

Culture vessels were inoculated by adding 300 ml. of an aerobic broth culture (for *Cl. sporogenes*, 300 ml. of thioglycollate–broth culture). The whole apparatus (including the air-compressor) was at 37° (except for *Azotobacter chroococcum* and *Sarcina lutea*, at 30°; *Pasteurella pseudo-*

tuberculosis and *Achromobacter hartlebii*, at 25°). In at least one run with each organism, samples were withdrawn aseptically at intervals and growth was estimated turbidimetrically; this enabled harvesting to be timed for the end of the logarithmic growth phase. In all cultures a sample was taken at harvesting and examined microscopically and by plate-culture for the presence of contaminating organisms. Batches containing contaminants were discarded. Aeration was not sufficiently vigorous to permit growth rates as rapid as those attained in thoroughly shaken cultures, though final yields were nearly as high. In test runs with *E. coli* and *Proteus vulgaris* methylene blue added to the cultures remained in the oxidized state as long as aeration was maintained but became decolorized in less than a minute when aeration was stopped.

The organisms were harvested in a Sharples centrifuge, sporing organisms being killed by autoclaving before centrifuging. The packed mass of the organism was scraped from the centrifuge bowl and washed by resuspending in 1–2 l. of water and centrifuging again, twice.

The spores of *Bacillus subtilis* were obtained by growing the organism on the surface of nutrient agar (Oxo Ltd., London) for 12–15 days at 37°, and harvested by washing and detaching the spores with a rubber-tipped rod. The suspension was centrifuged and washed twice on the centrifuge. Microscopic examination revealed a pure spore preparation, and manometry indicated negligible respiration; the rate of glucose oxidation was less than 1% of that obtained with the vegetative cells of this organism. Fifteen Roux bottles yielded 40–50 g. (wet wt.) of spores.

Rate of respiration

Respiratory activity was determined by conventional manometry immediately after harvesting. A sample of the organism was suspended in 0·1 M-phosphate buffer, pH 7, and adjusted to give a reading of 60 in the EEL Portable Colorimeter without a filter; this corresponds to approx. 2 mg./ml. dry wt. for most organisms and this figure was used for calculating the Q_{O_2} values in Table 3. The main compartment of the manometers contained 1 ml. of the cell suspension and 1·3 ml. of buffer. The side arms contained 0·5 ml. of 0·05 M-glucose or of 0·05 M-DL-lactate. A portion (0·2 ml.) of NaOH (40%) was placed in the centre well. Determinations were performed in duplicate and blanks without substrate were run; Q_{O_2} values recorded in Table 3 are not corrected for endogenous respiration.

Extraction of lipids

Extraction of non-saponifiable materials from animal tissues is normally preceded by removal of the bulk of the glycerides and phosphatides by saponification. In most bacteria there is insufficient lipid material for preliminary saponification to be necessary. Elimination of this step is also advantageous in view of the susceptibility of certain non-saponifiable materials (including vitamin K and ubiquinone) to destruction by strong alkali. On the other hand, some lipids may be bound to the cell structure in a manner that prevents their extraction by solvents unless the tissue has been subjected to drastic hydrolytic procedures (Salton, 1953). We therefore employed a variety of extraction procedures involving both simple solvent extraction, and also extraction after hydrolysis with acid or alkali.

Octane extraction of dry material (*method* 1). The organisms were freeze-dried and 15 g. of the dry material was thrice extracted for 4–8 hr. in a Soxhlet apparatus with 200 ml. of 2,2,4-trimethylpentane ('octane').

Methanol extraction (*method* 2). A portion (100 g.) of the wet bacterial mass was refluxed with 300 ml. of methanol for 2 hr. The solid residue was filtered off and the methanol extract (250 ml.) diluted with 3 vol. of water. The lipid-soluble material was extracted with 3 × 400 ml. of ether. The cell residues were then refluxed with two further lots of 300 ml. of methanol, which were also diluted and extracted with ether. Considerable difficulty was encountered because of formation of emulsions at the ether-extraction stage: these were usually resolved on addition of NaCl.

The combined ether extracts were dried over anhydrous Na_2SO_4, filtered, reduced to small volume by distillation and the remaining solvent was evaporated off at 30° in a stream of N_2.

Saponification (*method* 3). A portion (100 g. wet wt.) of bacteria was refluxed for 45 min. with a mixture of 200 ml. of methanol, 50 ml. of KOH (60%, w/v) and 2 g. of pyrogallol (to prevent destruction of ubiquinone; Mervyn & Morton, 1959). The alkaline extract was diluted with 3 vol. of water and extracted with 3 × 400 ml. of ether. The ether extract was washed with water until the washings were no longer alkaline (phenolphthalein), dried over anhydrous Na_2SO_4 and the solvent was removed first by distillation and finally by evaporation at 30° in a stream of N_2.

Acid hydrolysis (*method* 4) (*Salton*, 1953). A portion (100 g. wet wt.) of bacteria was heated to 100° for 2 hr. with 200 ml. of 6 N-HCl. Water (1 vol.) was then added and the material extracted with 3 × 400 ml. of ether. The ether extracts were combined and washed with water until free of chloride, then dried over anhydrous Na_2SO_4 and the solvent was removed first by distillation and finally by evaporation at 30° in a stream of N_2.

Octane plus methanol (*method* 5). A portion (15 g.) of freeze-dried bacteria was extracted first with octane and then with methanol. The solvents were removed by distillation and evaporation as described above.

Fractionation of lipids

The quantity of lipid varied according to the organism and the mode of extraction (Tables 2 and 3). Normally, each 15 l. batch of culture provided at least 200 mg. of lipid for fractionation on alumina columns. The procedure adopted was similar to that described by Heaton, Lowe & Morton (1957) for examination of ubiquinone in animal tissues. Alumina (Type O; Peter Spence and Sons Ltd., Widnes, Lancs.) was allowed to stand overnight with N-HCl, then washed free of acid with water, dried at 120°, activated by heating to 500° for 45 min. and ground with sufficient water (6–8 ml./100 g. of alumina) to dilute to Brockmann grade 3. The amount of water required was determined for each batch of alumina by standardization with dyes (Brockmann & Schodder, 1941).

A column (7·0 cm. × 1·3 cm.) containing about 10 g. of alumina was suitable for fractionating 100 mg. of lipid, which was dissolved in the minimum amount of light petroleum, b.p. 40–60° (dried over sodium and redistilled), and poured on to the top of the column. Elution was commenced with 100 ml. of the same solvent ('light petroleum fraction'). Elution was continued with successive 100 ml. volumes of light petroleum containing 2, 4, 6, 8 and 20% (v/v) of ether, and finally with pure ether. These eluates are referred to as the '2% ether', '4% ether fractions' etc. In

some cases a further elution with methylal was performed and minor variations of the standard procedure were adopted at times.

Each chromatographic fraction was reduced to small bulk by distillation and then to dryness by heating at 30° in a stream of N_2. Fractions were weighed, dissolved in cyclohexane and the ultraviolet-absorption spectrum was examined over the range 200–400 mμ in the 1 cm. cell of a SP. 500 or SP. 700 spectrophotometer (Unicam Ltd., Cambridge).

Infrared-absorption spectra (Infracord spectrophotometer, Perkin–Elmer Ltd., Slough, Bucks.) were determined for some fractions, usually by placing a drop of the oily material without solvent between a pair of rock-salt disks.

Determination of vitamin K and ubiquinone

Vitamin K. This was determined by measuring the absorption of the '2% ether fraction' at 249 mμ and accepting a molecular-extinction coefficient ϵ 19 000 (Isler & Wiss, 1959) at that wavelength. This applies to pure vitamins K_2 but allowance may be made for other material present and absorbing at this wavelength. In practice, however, we either found the characteristic four-banded spectrum well defined in the '2% ether fraction' (cf. Fig. 1) or else it was entirely absent and absorption was low in this fraction. The problem was thus simplified by the fact that either the bacteria examined contained substantial amounts of vitamin K or else the vitamin was absent within the limits of spectroscopic detection. The form of the absorption spectrum itself, with a peak at 270 mμ falling very abruptly to a low minimum at 285 mμ, also facilitates the decision whether irrelevant absorption will affect the results in any given case. Furthermore, Jacobsen & Dam (1960) have reported good agreement between vitamin K determinations by spectroscopy and bioassay in some bacteria.

Only one organism, *Sarcina lutea*, gave an anomalous absorption in the vitamin K region; this problem is discussed below.

Ubiquinone. This material has a single peak at 272 mμ in cyclohexane (Fig. 2) and can likewise be determined by measurement of the extinction at this wavelength, in the '6% ether fraction'. Like vitamin K, it was present either in substantial amount or in amount below the limit of detection. The direct spectroscopic determination of ubiquinone has been supplemented by a more specific assay based on the diminution of extinctions at 275 mμ when the fraction was reduced with sodium tetrahydroborate in ethanol (Lester & Crane, 1959). The estimations by direct spectroscopy and by tetrahydroborate reduction are recorded in Tables 2 and 3 as 'maximum' and 'minimum' ubiquinone values; in no case do these results diverge widely.

Paper chromatography. The molecular weight, and hence the side-chain length, of a vitamin K or a ubiquinone can be deduced from the spectroscopic data if a pure sample is available, since the ultraviolet molar-absorption coefficients are the same for each member of the series. However, the materials were not obtained at the requisite degree of purity and identification was more safely made by paper-chromatography comparisons with samples of known side-chain length (cf. Lester & Ramasarma, 1959). Whatman no. 1 paper was treated with white soft paraffin, B.P. (5%, w/v, in light petroleum, b.p. 60–80°). The solvent was allowed to evaporate and 10–20 μl. of a 0·1% solution of the standard or test material in light petroleum was applied. The chromatograms were developed as follows; ubiquinones in the ascending mobile phase of water–*NN*-dimethylformamide (2·5:97·5, v/v); vitamin K, in the descending mobile phase of butan-1-ol–butyl acetate–acetic acid–water (59:4·5:12:24·5, by vol.).

Chromatograms were run until the solvent front reached the end of the paper (30 cm. in about 18 hr.). Solvents were removed by heating in a current of air at 40–50°. The quinones were revealed as dark spots when the papers were examined under a mercury-vapour lamp emitting predominantly in the 254 mμ line (Chromatolite, Hanovia Ltd., Slough, Bucks.).

RESULTS

Fractionation of lipids

The results obtained for *Proteus vulgaris* are described in detail. This organism is chosen because it produces both ubiquinone and vitamin K.

Glucose medium, 15 l. with 0·01% of nicotinamide (a growth factor for this organism), was inoculated with 300 ml. of a 16 hr. broth culture and incubated at 37° with forced aeration. After 24 hr. the pH of the medium had fallen to pH 5·5 and was restored to the original value of pH 7·4 by aseptic addition of sodium hydroxide solution. Incubation was then continued for a total of 72 hr. The cells were harvested on the Sharples centrifuge and washed by suspending in 2 l. of water and centrifuging again. The packed mass of cells weighed 128·5 g. The organisms were freeze-dried and the 27·5 g. of dry material was extracted twice with octane (200 ml., 4 hr.). Evaporation of the solvent left 1·40 g. of lipid (5·1% of the dry wt. of the cells). The lipid was dissolved in 10 ml. of light petroleum and poured on to a 12 cm. × 3·8 cm. column containing 100 g. of alumina, acid-washed, diluted to Brockmann grade 3. Elution was performed with light petroleum, ether–light petroleum mixtures, and ether, and the eluates were evaporated to dryness (Table 1).

Of the total lipid 6·5% was either not adsorbed or was bound so lightly that it was washed through with light petroleum. This fraction showed no specific ultraviolet or visible absorption. Its low affinity for alumina suggested that it was relatively non-polar material and its infrared spectrum was consistent with the presence only of saturated hydrocarbons. In some organisms this fraction contained also triglycerides.

Fraction II showed the characteristic vitamin K spectrum (Fig. 1, continuous line); comparison with an authentic specimen (Fig. 1, broken line) suggested that it was virtually free of impurities absorbing selectively in this region. At $\lambda_{max.}$ 249 mμ, $E^{1\%}_{1cm.}$ was 177. Paper chromatography showed that only the vitamin K_2-40 was present, for which $E^{1\%}_{1cm.}$ was 264 (Isler & Wiss, 1959). This

Table 1. *Fractionation of octane extract of* Proteus vulgaris

Lipid (1·40 g.) was placed on the column. The total eluted was 131 mg.

Fraction no.	Eluent: Ether (%)	Eluent: Light petroleum (%)	Vol. of eluent (ml.)	Wt. of fraction (mg.)	Remarks
I	0	100	1000	92	Hydrocarbons?
II	2	98	500	19	Vitamin K (see Fig. 1)
III	4	96	500	0·1	—
IV	6	94	1000	18	Ubiquinone (see Fig. 2)
V	20	80	500	1·4	No defined u.v. spectrum
VI	100	0	500	10	Only slight u.v. absorption

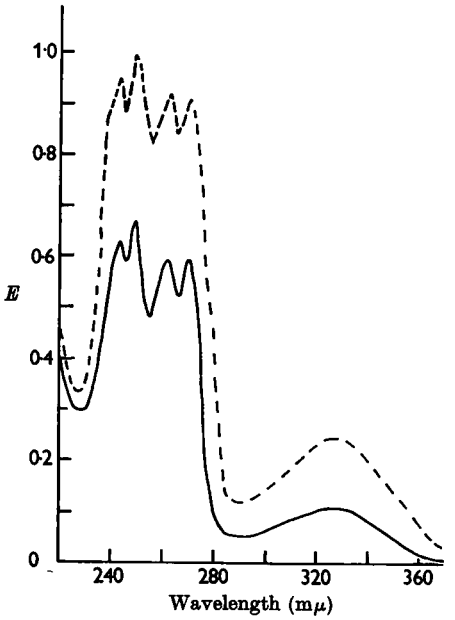

Fig. 1. Absorption spectra of fraction II (Table 1) from *Proteus vulgaris* (—) and authentic vitamin K_2-40 (- - -); both were at concentrations of 38 mg./l. in cyclohexane.

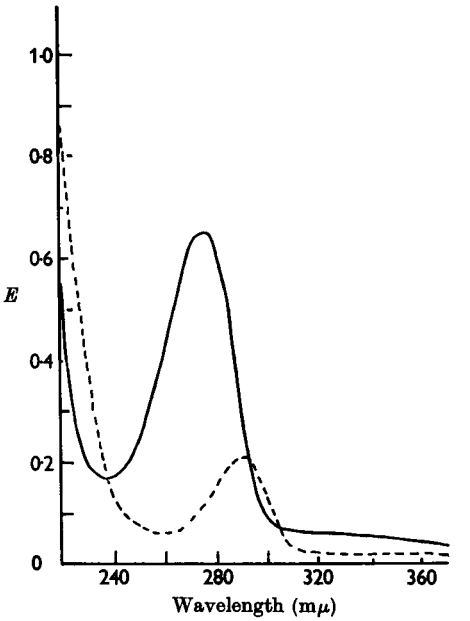

Fig. 2. Absorption spectrum of fraction IV (Table 1) from *Proteus vulgaris* before (—) and after (- - -) reduction with $NaBH_4$. Both substances were at concentrations of 36 mg./ml. in ethanol.

fraction must therefore contain 12·6 mg. of vitamin K_2.

Elution with 4% of ether in light petroleum removed no significant amount of material (fraction III), but the '6% ether fraction' (fraction IV) had the characteristic ultraviolet absorption of ubiquinone and showed the same changes on treatment with sodium tetrahydroborate (Fig. 2, continuous line) (Lester & Crane, 1959). Paper chromatography indicated ubiquinone-40. The fraction contained 18 mg. of material; estimation of ubiquinone gave a 'maximum' value of 13·1 mg. (from E at 272 mμ) or a 'minimum' of 12·6 mg. (by tetrahydroborate reduction) (Fig. 2, broken line).

Very little material was removed with 20% ether (fraction V) and the fraction eluted with 100% ether also contained no material with characteristic ultraviolet absorption.

The fractions eluted totalled 131 mg. or 9·4% of the total octane extract. Most of the extracted lipids remained firmly attached to the alumina and were thus probably highly polar phospholipids. Indeed, the total lipid in certain micro-organisms has been reported to consist mostly of phospholipid (Macfarlane, 1961), and this would be expected to remain on the column under our conditions.

Five runs were carried through with *Proteus vulgaris* and results are summarized in Table 2. The three different extraction methods showed little divergence in respect of the values for total lipid, ubiquinone or vitamin K. The $E^{1\%}_{1cm.}$ given for

Table 2. *Ubiquinone and vitamin K in* Proteus vulgaris (*aerobic culture*)

Five batches of *P. vulgaris* were grown on glucose medium with addition of 0·01 % of nicotinamide (see text) and extracted by the method indicated in col. 2. The total lipid was fractionated by alumina chromatography. Col. 4 gives the yield of vitamin K (from *E* at 249 mμ) and col. 5 $E^{1\%}_{1\,cm.}$ for this fraction, the value for pure vitamin K_2-40 being 264. Col. 6 gives the ubiquinone content, estimated by *E* at 272 mμ, and col. 7 the value obtained from diminution of *E* at 275 mμ on tetrahydroborate reduction in ethanol.

Expt. no.	Extraction method	Total lipid (%, dry wt.)	Vitamin K (μmole/g.)	Vitamin K $E^{1\%}_{1\,cm.}$	Ubiquinone (μmole/g.) Max.	Ubiquinone (μmole/g.) Min.
1	Octane	5·1	0·72	177	0·66	0·63
2	Octane	4·3	0·65	191	0·69	0·60
3	Octane	5·3	Not determined			
4	Methanol	4·9	0·60	218	0·65	0·61
5	Octane + methanol	5·4	0·71	226	0·67	0·59

vitamin K indicate the degree of purity obtained in this fraction; they should be compared with the value of 264 for the pure compound.

Experience showed with *Proteus* and others that the yields of vitamin K and ubiquinone were relatively independent of the extraction procedure (except for the expected losses of vitamin K in extraction method 3, which involved saponification). The octane-extraction method (1) was adopted as best for general use, as it involved fewer technical difficulties. The spores of *B. subtilis* released very little lipid on octane extraction, however, and acid hydrolysis (method 4) was used. Some Gram-positive organisms also yielded more lipid, but not more vitamin K, after hydrolysis. They did not contain ubiquinone. *Micrococcus lysodeikticus* has also proved a difficult organism for lipid extraction by octane although the vitamin K of this organism is completely extracted by methanol.

Table 3 summarizes the results obtained with all organisms investigated. The first column lists the extraction procedures applied. The total lipid of Table 3 is the amount given by the extraction method giving the highest yield; the acid- and alkaline-hydrolysis methods (4 and 3) are excluded. The maximal and minimal values for ubiquinone have been explained above: they are the averages from the various extraction methods used. The vitamin K figures are also averages but results from the saponification method (3) have been excluded.

It is thought that vitamin K_2 rather than K_1 is always present because the former is the type hitherto reported in bacteria; moreover, the behaviour of our samples on paper chromatograms is consistent with the K_2 series and clearly eliminated ordinary vitamin K_1-20, the only K_1 homologue so far proved to occur in Nature. The K_1 and K_2 series may also be differentiated by differences in the fine structure of the infrared spectra; although we were unable to exploit this method critically, our infrared data for *E. coli*, *P. vulgaris* and *Corynebacterium diphtheriae* were consistent with the presence of vitamins belonging to the K_2 series.

Ubiquinone or vitamin K, when detected, were present in amounts much greater than the minimum required for tentative identification; there were thus no 'doubtful' results. *Sarcina lutea*, however, was anomalous in that the '2 % ether fraction', which normally contains vitamin K_2 when the latter is present, did not show the typical four-banded spectrum but gave a single main peak at 249 mμ (with a lower peak at 325 mμ), and inflexions at 243, 256 and 272 mμ; these are the approximate positions of the typical vitamin K peaks. Either vitamin K was present and its spectrum was being obscured by a chromogen absorbing in the same region or an 'abnormal' vitamin K was present. Further investigation will be needed to decide this point; but three separate runs with *Sarcina lutea* gave essentially similar results; the form of the spectrum did not change on rechromatography, and paper chromatograms showed only a single spot running in the position of vitamin K_2-40.

Examination for long-chain alcohols

Alcohols with long polyisoprenoid chains have been reported recently, e.g. solanesol, $C_{45}H_{73}OH$, from tobacco leaves (Rowland, Latimer & Giles, 1956) and dolichol, $C_{100}H_{161}OH$, from animal tissues (Pennock, Hemming & Morton, 1960). The role of these is not known but they may be related to the supply of the side chain of vitamin K or ubiquinone. Three organisms were examined for their presence; the saponification procedure (extraction method 3) was applied as it is necessary to remove triglycerides.

Pasteurella pseudotuberculosis (107 g. dry wt.) yielded 461 mg. of non-saponifiable material, which was fractionated on alumina as described above. After elution of the ubiquinone in the '6 % ether fraction', elution with ether–light petroleum (10:90, v/v) yielded 37 mg. of material; this fraction would be expected to contain the long-chain alcohols, if present (Pennock *et al.* 1960). The ultraviolet spectrum showed no specific absorption save for that due to a trace of ubiquinone; the infrared

Table 3. *Ubiquinone and vitamin K in micro-organisms*

For details of extraction methods see Methods section.

Organism	Conditions of culture	Extraction methods	Total lipid (% dry wt.)	Q_{O_2}			Ubiquinone μmoles/g. dry wt.			Vitamin K_2	
				Glucose	Lactate	No substrate	Max.	Min.	Side chain	μmoles/g. dry wt.	Side chain
Gram-positive											
Bacillus subtilis	Aerobic	1, 2, 3, 5	1·1	75	105	45	<0·001	—	—	0·7	35
B. subtilis spores	Aerobic	2, 3, 4	0·2	0	0	0	<0·001	—	—	<0·001	—
B. megaterium (N.C.T.C. 9848)	Aerobic	1, 2, 3	1·9	98	83	28	<0·001	—	—	0·66	35
Lactobacillus casei	Aerobic	2, 3	1·5	—	—	—	<0·001	—	—	<0·001	—
Staphylococcus albus	Aerobic	2, 3, 4	3·8	37	111	6	<0·001	—	—	1·4	35
	Anaerobic	2, 4	3·0	5	8	4	<0·001	—	—	<0·01	—
Sarcina lutea	Aerobic	1, 2, 3	1·8	37	148	0	<0·001	—	—	1·8	40
Clostridium sporogenes	Anaerobic	2, 3	2·0	0	0	0	<0·001	—	—	<0·001	—
Corynebacterium diphtheriae (P.W. 8)	Aerobic 'low-Fe'*	2	—	12	35	—	<0·001	—	—	1·3	—
	Aerobic 'high-Fe'*	2	—	20	80	—	<0·001	—	—	3·9	45
Gram-negative											
Azotobacter chroococcum (N.C.I.B. 8003)	Aerobic	1, 2, 5	1·7	130	78	15	0·48	0·40	40	<0·05	—
Escherichia coli	Aerobic	1, 2, 3, 5	2·0	135	120	37	0·41	0·32	40	0·32	40
	Anaerobic	1, 2	2·3	20	7	2	0·40	0·33	40	0·28	40
Proteus vulgaris	Aerobic	1, 2, 5	5·4	87	46	3	0·67	0·62	40	0·62	40
	Anaerobic	1, 2	5·0	1	1	0	0·61	0·51	40	0·51	40
Pseudomonas (pyocyanea) aeruginosa	Aerobic	1, 2, 5	5·0	310	215	28	1·59	1·23	45	<0·03	—
	Anaerobic	1, 2	4·8	28	22	15	1·57	1·12	45	<0·03	—
Aerobacter aerogenes (N.C.T.C. 418)	Aerobic	1, 2	3·0	331	222	12	0·72	0·53	40	<0·05	—
Pasteurella pseudotuberculosis (N.C.T.C. 1101)	Aerobic	1, 2, 3, 5	1·2	124	80	7	0·12	0·12	40	<0·005	—
Chromobacter prodigiosum (N.C.T.C. 1377)	Aerobic	1, 2	5·2	200	112	11	0·35	0·30	40	<0·01	—
Neisseria catarrhalis	Aerobic	1, 2, 3	9·0	10	16	1	2·05	1·90	40	<0·05	—
Achromobacter hartlebii (N.C.I.B. 8129)	Aerobic	1, 2, 4	2·8	98	220	25	0·45	0·40	40	<0·08	—

* Data supplied by N. Lucas and P. B. Scholes.

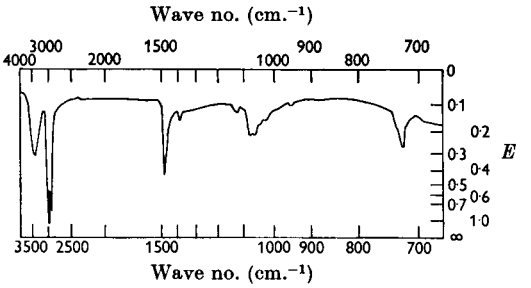

Fig. 3. Infrared-absorption spectrum of long-chain alcohol from *Pasteurella pseudotuberculosis*.

spectrum suggested a long-chain alcohol but the characteristic isoprenoid frequencies were not observed. The material was now purified by acetylation. The fraction was dissolved in 1 ml. of anhydrous pyridine and 15 ml. of acetic anhydride added at 0°. The mixture was left overnight at room temperature, then poured into hydrochloric acid (100 ml., 2 N) at 0°. The product was extracted with light petroleum, the extract washed with hydrochloric acid (2 N) to remove pyridine, then water (to remove hydrochloric acid), dried over anhydrous sodium sulphate and evaporated to dryness. The material was then dissolved in light petroleum and applied to a column containing 5 g. of alumina diluted with 3 % of water. Most of the material was eluted from the column with light petroleum; it was transparent in the ultraviolet region and the infrared absorption was consistent with the acetate of a long-chain saturated alcohol. The alcohol was recovered by saponification of the ester and its infrared-absorption spectrum is shown in Fig. 3. Well-marked peaks attributable to the OH group are seen at 3400 and 1065 cm.$^{-1}$. The lack of absorption around 1660 cm.$^{-1}$ suggests that no double bonds are present. The weak CH_3 group band at 1380 cm.$^{-1}$, in comparison with the strong CH_2 group band at 1490 cm.$^{-1}$, also suggests a long unbranched chain.

A value for the molecular weight was obtained by forming the azobenzoate, and determining the intensity of the chromophore in the recrystallized compound (Pennock *et al.* 1960). The value of 290–320 suggested a chain length of C_{20}–C_{22}. No further characterization was attempted.

Proteus vulgaris and *E. coli* were similarly examined for long-chain isoprenoid alcohols, with negative results. Such compounds, if present, must have been less than 5 mg./100 g. dry wt. of organism.

DISCUSSION

The results incorporated in Table 3 show that organisms with a well-developed oxidative metabolism contain either ubiquinone or vitamin K (or, in two cases, both) in substantial amount, i.e. at concentrations comparable with those of ubiquinone found in the mitochondria of animal tissues and also in the spadix of *Arum* (Pumphrey & Redfearn, 1960). These values, of the order of 1 μmole/g. dry wt., represent roughly the amount of an oxidation–reduction substance required for participation in the main electron-transport chain (Redfearn, 1961). In the three organisms studied that are known to be lacking in a cytochrome system, neither quinone could be detected; and it seems certain that, if present, their concentration must have been much lower. There is a hint here that ubiquinone and vitamin K may be alternatives for some function connected with oxidative processes. Clearly, however, the situation is not as simple as this. Vitamin K, by nutritional criteria, plays a vital part in animal metabolism, but at tissue concentrations too low for easy detection. The bacteria that we have reported as lacking vitamin K might still contain it in trace amounts. Among the aerobic organisms recorded in Table 3, there is no quantitative relation between the amounts of the quinones and the Q_{O_2} values. The latter, however, were determined on intact cells and factors other than the electron-transport mechanism may have been governing oxygen uptake.

The behaviour of the facultative anaerobes is interesting. Four organisms were grown, both with forced aeration and also under conditions proved to be anaerobic by the criteria of methylene-blue reduction and also the production of cells with drastically reduced oxidative capacity. Yet in three out of the four organisms no appreciable fall in amounts of quinone was found. Lester & Crane (1959), however, reported a cessation of ubiquinone formation when *E. coli* was grown anaerobically, and Kashket & Brodie (1960) record a reduction in the formation of both quinones in this organism. Although we examined a strain of this organism different from that employed by either of these groups, we consider that there is an unexplained conflict in these findings.

A noteworthy feature of these results has been the finding that, in the aerobic organisms, ubiquinone is found in the Gram-negative types, vitamin K in the Gram-positive types, with both quinones present in *E. coli* and *Proteus vulgaris*. This adds another item to the list of characteristics that separate these organisms, but its significance is not known.

SUMMARY

1. Sixteen species of bacteria have been examined for their content of vitamin K and ubiquinone.

2. *Escherichia coli* and *Proteus vulgaris* contain both ubiquinone (-40) and vitamin K_2 (-40).

3. Seven other Gram-negative bacteria contain ubiquinone but not vitamin K.

4. None of the Gram-positive organisms examined contains ubiquinone. Vitamin K_2 was found in five types, but was not in *Lactobacillus casei*, *Clostridium sporogenes*, anaerobically cultured *Staphylococcus albus* or in spores of *Bacillus subtilis*.

5. No long-chain polyisoprenoid alcohols were detected in *Escherichia coli*, *Proteus vulgaris* or *Pasteurella pseudotuberculosis*. The last-named, however, yielded a compound tentatively identified as a long-chain saturated alcohol.

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The Localization of Cerebral Phosphoprotein Phosphatase

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The partial purification of a phosphoprotein phosphatase from ox brain has been reported by Rose & Heald (1960, 1961). The enzyme was labile, magnesium-activated, and extremely specific, acting only on certain phosphoproteins, e.g. casein, phosvitin and brain phosphoprotein, and partially hydrolysed phosphopeptides prepared from α-casein. The enzyme differed from other mammalian phosphoprotein phosphatases (Hofman, 1958; Glomset & Porath, 1960; Revel & Racker, 1960) in that it liberated only a small portion of the phosphoprotein phosphorus before ceasing to act. This released phosphorus included all the radioactivity incorporated into phosvitin during phosphorylation with radioactive phosphate in a mitochondrial preparation (Burnett & Kennedy, 1954; Rabinowitz & Lipmann, 1960).

Such distinctive properties made desirable a knowledge of the distribution and intracellular localization of the enzyme. In this paper some data

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