THE RELATIONSHIPS BETWEEN QUINONE AND CAROTENOID IN MICROCOCCUS LYSODEIKTICUS.

by

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The relationships between quinone and carotenoid in Micrococcus lysodeikticus.

There are differences between yellow wild-type and white carotenoidless-mutant strains of Micrococcus lysodeikticus, when they are grown in the dark. The white strain exhibits lower viability, quinone levels and respiratory activity of some membrane-bound enzymes.

Membrane and cell preparations, of both strains, incorporate [2-^{14}C]mevalonic acid into quinone and also carotenoid in the yellow strain, but labelled isopentenyl pyrophosphate is not incorporated. There is much higher incorporation into quinone in white cells and membranes than yellow. The incorporation in yellow is low, but can be increased, in membranes, by addition of supernatant from the lysis stage of membrane preparation. Three other carotenoidless mutants obtained, behaved similarly to the strain generally used (strain UVI). Treatment of yellow cells with diphenylamine, which inhibits the synthesis of coloured carotenoids, results in white cultures which exhibits similar properties to strain UVI.

The breakdown of quinone, in differing oxidation-reduction states, has been studied, but difficulties arise as conditions were not found when breakdown was completely independent from synthesis.

Control of synthesis has been studied. Menadione, a possible intermediate in quinone biosynthesis, increases the incorporation of [2-^{14}C]mevalonic acid into quinone, but menaquinone-7 reduces the incorporation.

The effect of inhibitors and substrates of the respiratory chain, on quinone levels and synthesis, was examined. Azide greatly decreases the incorporation of [2-^{14}C]mevalonic acid into quinone.

Malate appears to have only a small effect on quinone, lowering the levels. Under certain conditions, possibly a combination of malate and magnesium concentrations and the state of the membrane, oscillations of quinone levels, in white membranes, are observed.

ABBREVIATIONS.

TLC	thin layer chromatography
v/v	volume to volume
w/v	weight to volume
μCi	microCurie
HOQNO	2-n-heptyl-4-hydroxyquinoline-N-oxide
MK7 or 8	menaquinone -7 or -8
ppm	parts per million
Mg^{++}	magnesium ion
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
ATP	adenosine triphosphate
DPA	diphenylamine
8-MOP	8-methoxy psoralen
CPTA	2-(p-chlorophenylthio) trie thylammonium hydrochloride
MDA	malondialdehyde
TBA	thiobarbituric acid
PMS	phenazine methosulphate
DCPIP	dichlorophenolindophenol

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1. INTRODUCTION.

A. CAROTENOIDS.

(a) Structure and definition of carotenoids.

The basic building blocks of carotenoids are isoprenoid units. Most carotenoids are C40 structures, comprising eight units. The units are joined head to tail, except at the centre of the molecule, where the order is reversed. This means that the C40 skeleton is symmetrical. The conjugated double bond system in the molecule is the chromophore. Carotenoids are usually red, yellow or orange, although there are colourless ones. Xanthophylls are carotenoids,which have one or more oxygen functions.

C40 carotenoids are widespread in nature: they can be divided into groups on the basis of their structure:-

(i) Acyclic carotenes

(ii) Alicyclic carotenes

(iii) Acyclic xanthophylls

(iv) Alicyclic xanthophylls

(v) Aromatic carotenoids

(vi) Epoxy carotenoids

(vii) Cyclopentyl ketone carotenoids

(viii) Allenic carotenoids

(ix) Acetylenic carotenoids

Carotenes and xanthophylls of groups (i)-(iv) are the most widely found carotenoids. Those in groups (v) to (ix) tend to be found only in a limited range of species, for example, cyclopentyl ketone carotenoids are found in red peppers, and acetylenic and allenic carotenoids are

found in algae. Higher carotenoids (C45 and C50) have been found in some non-photosynthetic bacteria.

Apocarotenoids contain less than forty carbon atoms. They can be divided into two groups:

(i) those which are breakdown products of C40 carotenoids.

(ii) C30 carotenoids, which are believed to form a system homologous to the C40 Porter-Lincoln series.

In Staphylococcus aureus (Suzue et al 1968) and Halobacterium cutirubrum (Kushwaha et al 1972), the C30 homologue of phytoene has been found. However, in Streptococcus faecium (Taylor and Davies 1974, 1974 a), the C30 homologues of phytoene, phytofluene, zeta-carotene, 7, 8, 11, 12 - tetrahydrolycopene and neurosporene have been identified, as well as two C30 xanthophylls.

Using a widely accepted nomenclature, a degradation product which does not retain the C20 and C20' methyl groups of the original C40 structure, is no longer a carotenoid. The structures of lycopene and beta-carotene, and the nomenclature used, are shown in Fig. 1.

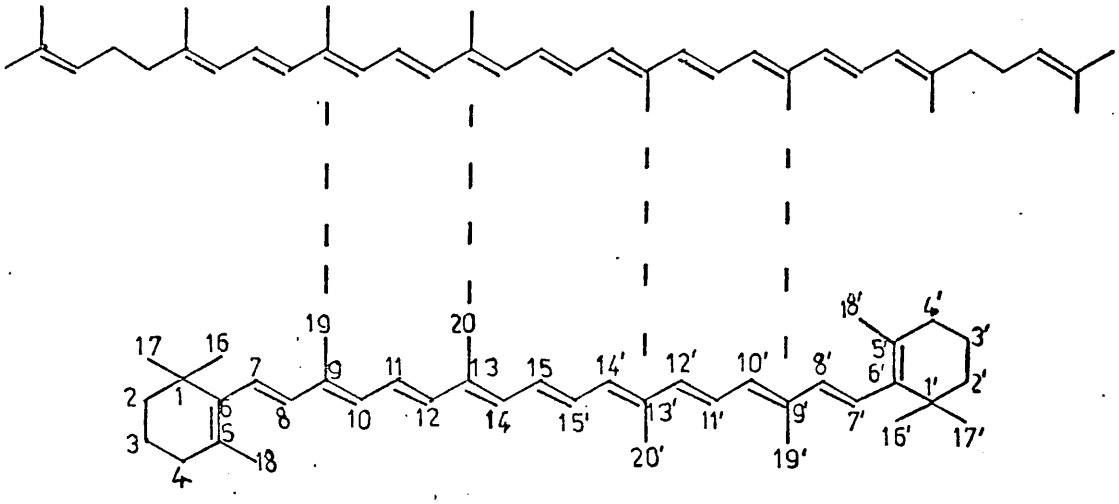
(b) Carotenoids of Micrococcus lysodeikticus.

The carotenoids of M. lysodeikticus were first examined by Rothblat et al in 1964. They found at least seven carotenoid pigments. From the absorbance spectra, they decided that the chromophoric system was the same, but that there were different polar groupings. They found no hydrocarbon carotenoids, nor any with acidic properties or epoxide groups. They concluded that the carotenoids were hydroxy carotenoids (xanthophylls).

Figure 1

The structures of lycopene and beta-carotene, demonstrating the nomenclature used with carotenoids.

LYCOPENE(ACYCLIC CAROTENE)



β CAROTENE

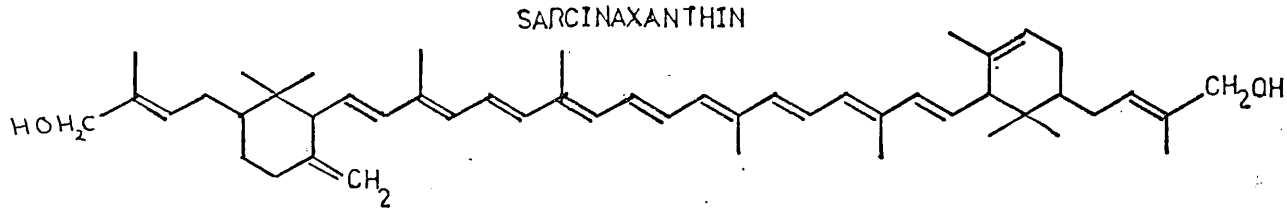
In 1969 Strang and Thirkell looked at the carotenoids of M. lysodeikticus. From their experiments, they decided that the carotenoids were practically the same as in Sarcina lutea. S. lutea is very closely related to M. lysodeikticus; Micrococcus luteus, M. lysodeikticus and S. lutea being members of the same taxonomic species, although M. lysodeikticus is probably more lysozyme sensitive than the other two. It therefore seems reasonable to assume that the carotenoids will be the same.

The carotenoids of S. lutea have been examined by a large number of workers. C50 carotenoids have been found in S. lutea. In 1970 Norgaard et al found a C50 carotenyl glycoside in this bacterium. In 1973 Jensen studied all the carotenoids in S. lutea. The major carotenoids found were sarcinaxanthin, a mono-D-glucoside of a C50 diol, probably sarcinaxanthin and decaprenoxanthin. 7, 8 or 7', 8' - dihydrosarcinaxanthin was the most abundant of the minor carotenoids, the others being three C45 mono-ols, and lycopene, the only carotene found (Hertzberg and Jensen 1977). The structures of the carotenoids of S. lutea are shown in Fig. 2. C50 carotenoids have also been found in another related bacterium, Sarcina flava (Thirkell et al 1967).

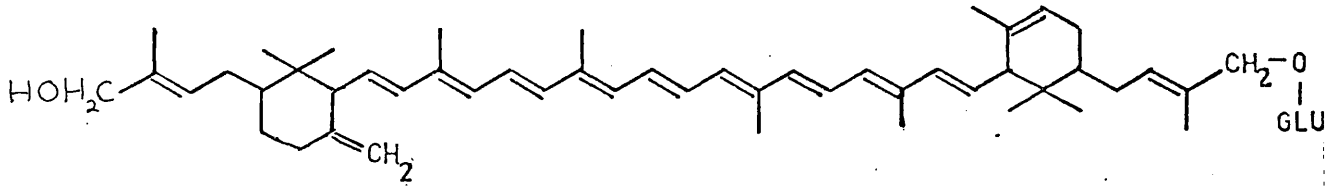
Figure 2

The carotenoids found in S. lutea and probably also in Micrococcus lysodeikticus.

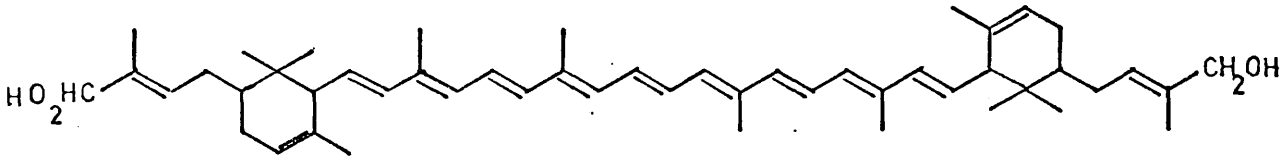
SARCINAXANTHIN



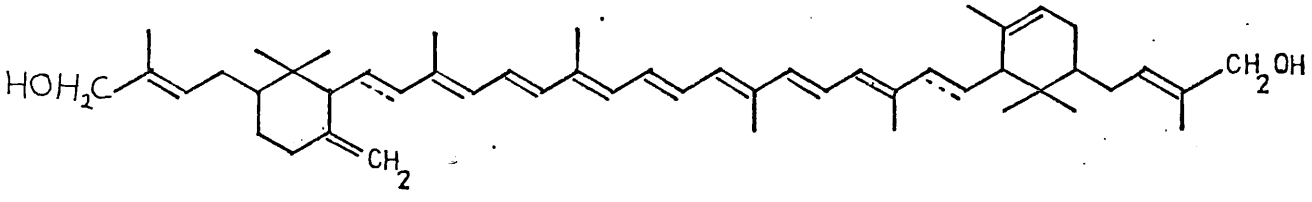
SARCINAXANTHIN D GLUCOSIDE



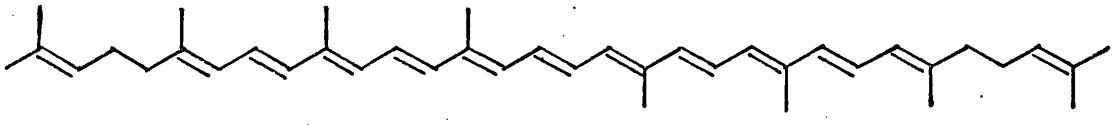
DECAPRENOXANTHIN



7,8 or 7,8' DIHYDROSARCINAXANTHIN



LYCOPENE



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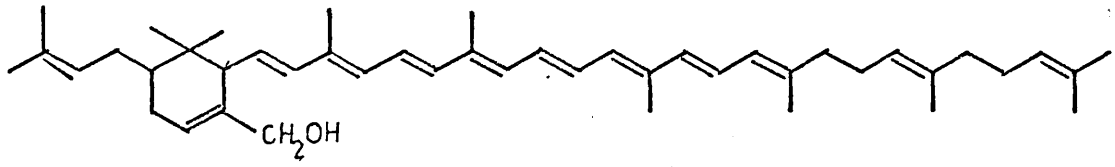
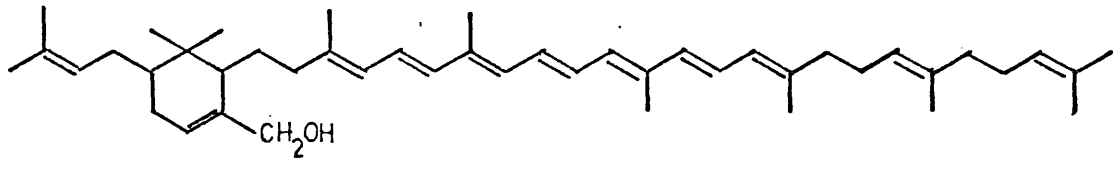
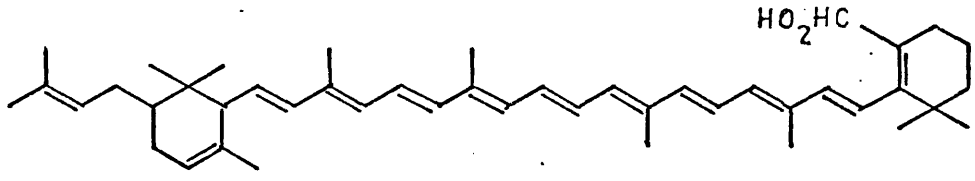
THE THREE C_{45} MONO-OLS







B. PHOTOFUNCTIONS OF CAROTENOIDS.

(a) In photosynthetic bacteria.

(i) Accessory pigments.

The first function of carotenoids was observed by Engelman in 1883, when using algal strands. He showed that carotenoids were accessory pigments in photosynthesis.

More recently Goedheer (1965) demonstrated that only certain carotenoids could function as accessory pigments in algae. He found that xanthophylls were very inefficient at transferring energy to chlorophyll, whereas carotenes were very efficient. This explained his earlier work (Goedheer 1959), using photosynthetic bacteria, when he observed two different categories of carotenoid, one efficient at energy transfer to bacteriochlorophyll, and the other efficient at reducing bacteriochlorophyll fluorescence.

Obviously certain carotenoids function as accessory photosynthetic pigments, using wavelengths of light not absorbed by bacteriochlorophyll, thus enabling the bacterium to make the best possible use of natural light.

(ii) Photoprotection.

Photoprotection is now believed to be a major function of carotenoids. This was first proposed by Sta nier and his colleagues (Griffiths et al 1955), who demonstrated carotenoid protection, using a mutant strain of Rhodopseudomonas spheroides, a purple non-sulphur bacterium, which lacked coloured carotenoids. This strain was photosensitive in the presence of air, as opposed to the wild-type,which was not. No difference was observed between the two strains in the dark, or in the absence of oxygen. They con-

cluded that this reaction was photodynamic, as defined by Blum (1941) as "the sensitivity of a biological system to light by a substance which serves as a light absorber for photochemical reactions, in which molecular oxygen takes part". Stanier and his co-workers (Griffiths et al 1955) also found that the light which killed the bacteria was that which was absorbed by bacteriochlorophyll.

Sistrom, also using R. spheroides, found that the photodynamic action was catalysed by bacteriochlorophyll (Sistrom et al 1956). She found an inhibition of pigment synthesis in aerobic conditions, which led to dilution of bacteriochlorophyll and consequent bleaching. In the wild-type strain, this inhibition was reversible, under anaerobic conditions, but not so in the carotenoidless mutant. She suggested that the carotenoids were acting as chemical buffers, possibly as preferred substrates for bacteriochlorophyll-catalysed photo-oxidations. This could occur via epoxide groups formed in the carotenoid molecule.

It is interesting to note that there is a high carotenoid content in bacteria which are subjected to light and oxygen in their natural environment, showing the importance of carotenoid protection.

Instead of using ultraviolet light induced carotenoidless mutants, diphenylamine (DPA) can be used to form colourless cultures, as it blocks carotenoid biosynthesis before the formation of coloured carotenoids.

Cohen-Bazire and Stanier (1958), using a DPA induced colourless culture of Rhodospirill um rubrum, found photodynamic killing with oxygen. Photokilling was also investigated by Dworkin (1958),

who found that the Q_{10} for the killing of R. spheroides, between 4 and 40°C, was 1. This shows that the killing is probably purely photochemical. However, the Q_{10} for the destruction of bacteriochlorophyll,between 20 and 30°C,is 3.7. Thus photokilling and bleaching are independent reactions, from which Dworkin suggested that bacteriochlorophyll transfers energy to an adjacent acceptor molecule, possibly carotenoid.

Another theory, suggested by Clayton (1960),was that the lethal effect of oxygen was mediated through hydrogen peroxide and blocked by catalase, or carotenoid.

It is obvious that without carotenoid, bacteriochlorophyll is the photosensitizer, in air, which results in the destruction of the bacteriochlorophyll and death of the bacterium. The exact mechanism of this destruction, and the mechanism of carotenoid protection, are not fully understood, although several possible theories will be discussed in a following section.

(b) In non-photosynthetic bacteria.

(i) Photoprotection.

In 1957, Stanier and Cohen-Bazire, who had previously worked on carotenoid protection of photosynthetic bacteria, proposed that carotenoids function similarly in non-photosynthetic bacteria.

Kunisawa and Stanier (1958), using Corynebacterium poinsettiae found no difference, in response to light and oxygen, between wild-type and carotenoidless-mutant strains, when no photosensitizer was added. However as soon as toluidine blue was added, the white mutant strain exhibited photosensitivity.

Mathews and Sistrom (1959) tried to explain this need for an

added photosensitizer, by suggesting that endogenous photosensitizers in non-photosynthetic bacteria, unlike photosynthetic bacteria, show little absorbance above 500 nm.

So, they used direct sunlight on a carotenoidless-mutant and wild-type strain of Sarcina lutea, in the absence of toluidine blue, and were able to show carotenoid protection against photokilling of the bacteria in natural light. They postulated that workers, who found that there were no differences between carotenoidless and wild-type strains, in light and oxygen, were not using a lamp emitting high enough intensities of the correct wavelength for the endogenous photosensitizer.

As they had already found that the carotenoid, in S. lutea, was located in the membrane, they looked at various membrane variables, to see if anything changed during photo-oxidation (Mathews and Sistrom 1959a). They observed an increase in permeability and also a decrease in the activity of two membrane-associated enzymes; succinate dehydrogenase and NADH oxidase.

Rottem et al (1968) showed that carotenoid can protect the membrane associated enzyme, adenosine triphosphatase, against toluidine blue sensitization in Acholeplasma laidlawii.

More recently Prebble and Huda (1973) showed that the respiratory chain of S. lutea, which is located in the membrane, can be protected by carotenoid. Using malate oxidase activity as a measure of the state of the respiratory chain, they found that wild-type bacteria were less susceptible to destruction by toluidine blue photosensitization, than carotenoidless bacteria. This occurred in cells and membranes, and it is believed that several sites are affected.

Histidine also gave protection.

These sites have been examined further, (Anwar and Prebble 1977). Isolated membranes of S. lutea were irradiated with blue light, in the absence of added photosensitizer. Three photosensitive sites were found,by comparing the effect of the blue light on membranes from the carotenoid-containing wild-type and a carotenoidless mutant. One of these sites was found to be the menaquinone, and only this site was found to be protected by carotenoid. Anwar et al (1977) have shown that quinone, in a white carotenoidless mutant of S. lutea, can be protected from photo-oxidation by carotenoid extracted from the wild-type with detergent.

It seems clear that in S. lutea, if not in other bacteria, the quinone is protected by carotenoid from destruction by light,in the presence of air.

In addition to protection of membrane enzymes and quinone in certain bacteria, other facets of carotenoid protection have been studied.

(ii) Carotenoid protection and chromophore length.

Having established that carotenoid protected non-photosynthetic bacteria from photosensitized oxidations, some workers then turned to the question of whether all carotenoids can protect.

Claes (1960, 1961, Claes and Nakayama 1959) looked at chlorophyll-sensitized light reactions (irreversible photo-oxidation and reversible photoreduction), using carotenoids of varying conjugated double bond length, in vitro. They found that nine double bonds were the minimum for protection against photo-oxidation and seven for photoreduction.

Foote et al (1970), using carotenoids with conjugated double bond lengths, five, seven, nine and eleven, found that a minimum of nine was required to quench singlet excited oxygen. Most naturally occurring carotenoid pigments contain an odd number of conjugated double bonds. Mathews-Roth and Krinsky (1970) isolated a mutant from S. lutea, which is believed to have a conjugated octaene carotenoid. Using toluidine blue as a photosensitizer, they found that this carotenoid could not protect the bacterium. From these observations, it seems that a minimum of nine conjugated double bonds is necessary for photoprotection in vitro and also in vivo.

(iii) Carotenoid levels, and photoprotection.

The question arises as to whether carotenoid protection is linear, with respect to concentration in the membrane, or if there is a threshold level for protection.

Mathews-Roth and Krinsky (1970a) examined this using wild-type and three mutant strains of S. lutea. One mutant strain contained no coloured carotenoids, whereas the other two did, but at a much lower concentration than in the wild-type strain. The mutants which contained coloured carotenoids were killed at a rate intermediate between that of the wild-type, and carotenoidless strains. From their results, Mathews-Roth and Krinsky concluded that the important factor in carotenoid protection may be the total amount of pigment per cell.

(iv) Effect of temperature on photoprotection.

Mathews and Sistrom (1960) have investigated the effect of temperature on carotenoid protection. They found that photokilling was independent of temperature between 6.5 and 34°C, using S. lutea

and toluidine blue as photosensitizer.

However Mathews (1964) showed that carotenoid protection was affected by temperature. Using S. lutea, she found that although the white carotenoidless mutant strain was killed at the same rate at 4° and 34°C, the yellow wild-type was less protected at 4°C than 34°C.

(v) Effects of various added photosensitizers.

In many of the studies reported, using non-photosynthetic bacteria, it has been found necessary to use photosensitizers,such as methylene blue or toluidine blue.

Mathews (1963) examined photosensitization with toluidine blue and 8-methoxy psoralen (8 MOP). Toluidine blue required light and oxygen, and seemed to affect the cytoplasmic membrane and associated enzymes. Carotenoid can protect against this photosensitization. 8MOP, on the other hand, did not require oxygen, and carotenoid offered no protection against its lethal effects. Its site of action seemed to be the DNA and not the membrane.

In 1967 Mathews followed up these experiments by investigating photosensitization using acridine orange. Acridine orange photosensitization required oxygen, but seemed to affect the cellular DNA. As with 8MOP, carotenoid gave no protection, as white and yellow strains of S. lutea were killed at the same rate.

Photosensitizers are added to amplify the effects of the natural endogenous photosensitizers. But from these studies by Mathews, it is clear that care must be taken when using exogenous photosensitizers, to ensure that the added photosensitizer is acting in a similar manner to the endogenous one.

(vi) Light induced carotenogenesis.

Certain bacteria only produce carotenoid when exposed to light. The light required to induce carotenogenesis however, is ten times less than that for photodynamic action (Mathews 1963a). One bacterium which operates this system is Myxococcus xanthus , which does not synthesise carotenoid when grown in the dark, and so when exposed to light is easily photolysed. This photolysis is temperature independent and oxygen dependent (Burchard and Dworkin 1966).

(vii) Natural endogenous photosensitizers.

One of the only bacteria where the endogenous photosensitizer is known, is Myxococcus xanthus. A porphyrin, similar in spectral and chemical properties to protoporphyrin IX, was isolated from photosensitized cells. Its absorbance spectrum was similar to the action spectrum for photolysis, (Burchard et al 1966). They concluded that protoporphyrin IX was the natural endogenous photosensitizer in these bacteria. A large number of naturally occurring compounds, such as porphyrins (both iron and magnesium derivatives), have been shown to be effective photosensitizers.

(viii) Inability of carotenoid to protect against ionising radiations.

Whatever the mechanism of protection against visible light (see section 1C), carotenoids cannot protect against ultraviolet light or X-irradiation and gamma irradiation (Kunisawa and Stanier 1958, Mathews and Krinsky 1965).

In the investigation of both photosynthetic and non-photosynthetic bacteria, using pigmented and carotenoidless strains, workers have looked at differences, observed only after illumination. The

possibility that lack of carotenoid has other effects on the bacterium, which can operate in the dark, has not been previously investigated.

This thesis is concerned with viability, respiratory enzyme activity and quinone levels, in wild-type and white carotenoidless strains of Micrococcus lysodeikticus, grown in the dark. Considerable differences between the two strains have been found, without illumination.

C CAROTENOID PROTECTION OF BACTERIA.

(a) Mechanisms of protection.

(i) Mechanisms of photodynamic action, and of carotenoid protection.

The exact mechanism of protection of bacteria from photosensitization in air, has still not been elucidated, mainly because the mechanism of photodynamic action is not understood. Various mechanisms, involving singlet excited oxygen, have been proposed, and *in vitro* experiments have shown that carotenoid can neutralize this potentially destructive molecule.

One of the earliest mechanisms for production of and protection against singlet excited oxygen, was put forward by Kautsky in the 1930's (Kautsky and de Bruijn 1931, Kautsky 1939). He postulated that the triplet sensitizer reacted with oxygen to give singlet excited oxygen ($^1O_2^*$), which then reacted with an acceptor:

$$^3SENS + O_2 \longrightarrow {}^1O_2^*$$

$$^1O_2^* + A \longrightarrow AO_2$$

Later Schenk (1954) proposed that the sensitizer itself, in the triplet form, is oxidised, and this oxygen is transferred to an acceptor molecule to yield ground state sensitizer:

$$SENS \xrightarrow{h\nu} {}^1SENS$$

$$^1SENS \longrightarrow {}^3SENS$$

$$^3SENS + O_2 \longrightarrow SENS - O_2$$

$$A + SENS - O_2 \longrightarrow AO_2 + SENS$$

Fujimori and Livingstone (1957) proposed that carotenoid pigments interacted with the triplet state of chlorophyll. In normal photosynthesis the singlet excited state of chlorophyll is involved.

Gaffron (1963) suggested that if there were excess quanta, then some of the excited chlorophyll could make the transition into the meta-stable triplet form, which could cause photosensitized oxidations, among other abnormal photoreactions.

In 1964,Foote and Wexler, and Corey and Taylor simultaneously announced that singlet oxygen could carry out reactions identical with photosensitized oxidations. They therefore proposed that singlet oxygen was an intermediate in photosensitized oxidations.

Singlet oxygen may be involved in various oxygenase reactions in biological systems, and as such seems a reasonable intermediate in photosensitized oxidations.

(ii) Singlet oxygen and carotenoid protection in vitro.

In 1968,Foote and Denny looked at the interaction between singlet oxygen and carotenoids. If the reaction between carotenoid and triplet sensitizers involved diffusion, then the quenching of the triplet sensitizer by oxygen would compete. An alternative, that carotenoids react directly with singlet oxygen, has been experimentally verified.

Beta-carotene (10^{-4}M) inhibited methylene blue sensitized oxidation of 2-methyl-2-pentene. The carotene went into the triplet state. It is not known how it was reversed, but this must have occurred as one beta-carotene molecule quenched about one hundred molecules of singlet oxygen. Foote (Foote el al 1970a) also found that for every thousand molecules of beta-carotene, which were quenching singlet oxygen, only one was oxidised.

The most efficient sensitizers are ones which have a long lived triplet state in high quantum yield (Foote 1968). Foote proposed

the mechanism below for carotenoid and singlet oxygen interaction:

$$^1O_2 + {}^1CAR \longrightarrow {}^3CAR + {}^3O_2$$

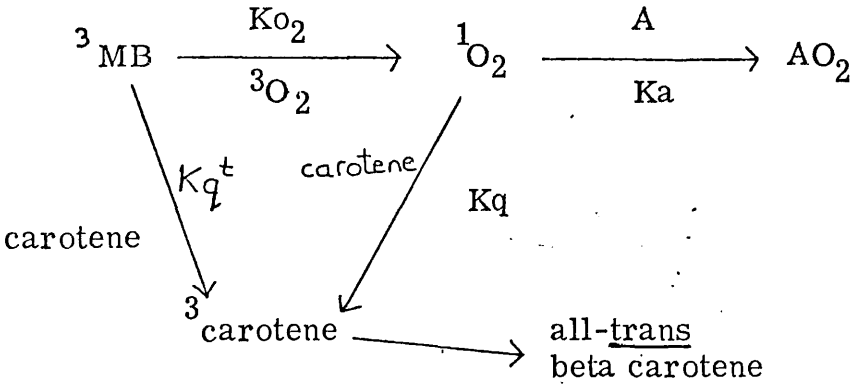
This reaction would only occur if the triplet energy of beta-carotene was near or below that of the $^1\Delta g$ of oxygen, which is 22.5 k. cal. Foote and his colleagues demonstrated the ability of various carotenoids to quench singlet oxygen, (Foote and Denny, 1968, Foote, Chang and Denny 1970, Foote et al 1970a). They were also able to show that carotenoids can protect chlorophyll a from photosensitized oxidations (Foote et al 1970). The protective ability of carotenoids is a function of the conjugated double bond length, the minimum operable being nine. To quench the triplet level of chlorophyll a, beta-carotene must have a triplet energy less than 29 k. cal (triplet energy of chlorophyll a). In order to quench singlet oxygen, the triplet energy must be less than 22.5 k. cal:- it is probable that the beta-carotene is near this value.

If,when the number of conjugated double bonds decreased, the triplet energy increased, this would explain why lower length conjugated double bond carotenoids will not protect. The triplet energy of a carotenoid with nine conjugated double bonds must be less than 22.5 k. cal and the triplet energy of one with eight must be more.

(iii) Cis-trans isomerization of carotenoid, as a possible mechanism for protection.

Cis-trans isomerization has been suggested as a mechanism for neutralizing singlet oxygen. Foote, Chang and Denny (1970), using 15-15' cis beta-carotene, found that under conditions of singlet oxygen production, cis beta-carotene was isomerised to the all-trans form. Under the same conditions, however, the trans beta-carotene would not revert to the cis-form. The conditions used for singlet oxygen production were irradiation of a solution of the cis (15,15') beta-carotene under oxygen,

and in the presence of methylene blue. A filter was used to ensure that light was absorbed only by the methylene blue. Each singlet oxygen molecule formed,caused isomerization of slightly less than one molecule of 15-15' cis-beta-carotene to all-trans beta-carotene. The scheme below was proposed by Foote, Chang and Denny (1970):-



Kq^t - rate of quenching of triplet methylene blue (MB)

Kq - rate of quenching of singlet oxygen

The presence or absence of oxygen does not affect the rate of isomerization,as the rate of quenching of singlet oxygen is very much greater than the rate of oxidation of an acceptor A. If the isomerization went only via methylene blue, oxygen should inhibit by competing, whereas if it went only via singlet oxygen, then the isomerization would not occur under nitrogen. In oxygen saturated solutions, the isomerization went entirely by way of singlet oxygen,as the rate of quenching of triplet methylene blue was very much lower than the rate of formation of singlet oxygen.

There are however, alternative mechanisms. A reversible electron transfer,from beta-carotene to singlet oxygen,would fit in with both the isomerization results, and the conjugated double bond length dependence, as the ionization potential of carotenoids would increase with decreasing conjugation. Also the carotene

radical cation should isomerize relatively easily (although it might be expected to undergo other reactions as well). Foote concluded that the energy transfer mechanism seemed more satisfactory.

(iv) In vivo studies of singlet oxygen, and carotenoid protection.

So far, experiments showing interaction between singlet oxygen and carotenoids have been in vitro, and the possibility arose that the results may not be indicative of the situation in vivo.

Polymorphonuclear (PMN) leukocytes kill bacteria with the appearance of chemiluminescence, possibly due to the generation of singlet excited oxygen ($^1O_2^*$). Krinsky (1974) has studied these cells, to determine if the mechanism of destruction of bacteria involved singlet excited oxygen generation. The singlet excited oxygen could be formed from the superoxide radical ($^{\bullet}O_2^-$):-

$$^{\bullet}O_2^- + {}^{\bullet}O_2^- \xrightarrow{2H^+} H_2O_2 + {}^1O_2^*$$

As PMN leukocytes contain myeloperoxidase (MPO), another mechanism for production of singlet excited oxygen can be proposed:-

$$H_2O_2 + Cl^- \xrightarrow{MPO} H_2O_2 + OCl^-$$

$$OCl^- + H_2O_2 \longrightarrow H_2O + Cl^- + {}^1O_2^*$$

The mechanism, by which carotenoids protect bacteria from photosensitized reaction, is believed to involve their ability to quench singlet oxygen. Krinsky compared the rate of killing, by PMN leukocytes, of yellow wild-type and white carotenoidless strains of Sarcina lutea. He found that the yellow strain was protected in conditions where the white strain was rapidly killed. This protection by carotenoid, however, may be due to its ability to neutralize the superoxide radical, and not the singlet excited oxygen. Whichever

mechanism is involved, however, carotenoid does give protection to bacteria, against PMN leukocytes. From these results, Krinsky suggested that certain forms of virulence in bacteria, may be associated with strains which are particularly effective in dealing with singlet excited oxygen. Excited oxygen is generated in bacteria, in light and oxygen, which would be harmful, if not adequately dealt with by carotenoids. Carotenoids, in vitro , have the ability to quench singlet oxygen, the same conjugated double bond lengths being effective at quenching singlet oxygen, in vitro, and conferring protection on the bacteria, against photodynamic action, in vivo.

The fact that PMN leukocytes can produce singlet oxygen (or perhaps another highly reactive oxygen form) in the dark, shows that lack of carotenoid could have harmful effects on bacteria, without illumination.

D. MEMBRANES.

(a) The effect of carotenoid on the properties of the membrane.

(i) Composition and structure of membranes.

Since carotenoids are found in the bacterial membrane, it is possible that they have an effect on its physical properties. Before discussing the work on carotenoid stabilization of membranes, the composition and structure of bacterial membranes will be briefly considered, with specific reference to the membrane of Micrococcus lysodeikticus.

Bacteria have a plasma membrane and also invaginations of the membrane,known as the mesosome (Fitz-James 1960). The mesosome is more prolific in gram-positive bacteria. Data on mesosome structure and function are often contradictory, perhaps because of the difficulties in separating the two structures. However, Thomas and Ellar (1973) have reported differences in mesosomal and plasma membrane composition in M. lysodeikticus.

Gram-positive bacteria are often used for membrane studies, as the cell wall can easily be completely removed, by use of enzymes such as lysozyme. The M. lysodeikticus membrane contains 65-85% protein and 23-26% lipid. Phospholipids make up 75-80% of the total lipid:- diphosphatidyl glycerol (DPG) 67% , phosphatidyl glycerol (PG) 27%, and phosphatidyl inositol (PI) 6%. Menaquinone is 4-5% of the total lipid and carotenoids approximately 0.5% (Salton 1968). Macfarlane (1962) had earlier detected other components as well; glycolipids, lipoamino acids, fatty acids and glycerides.

The membrane also has an associated acylated mannan (Powell

et al 1974), which is non-phosphorylated (Owen & Salton 1975). The fatty acid composition of the M. lysodeikticus membrane is shown below (Salton 1968):-

Fatty acids	Percentage composition
$C_{12:0}$	0.4
$C_{13:0}$ branched	1.2
$C_{14:0}$	4.4
$C_{15:0}$ iso	0.4
$C_{15:0}$ anteiso	85.4
$C_{16:0}$	0.2
$C_{16:0}$ branched	5.0
$C_{17:0}$ iso	0.4
$C_{17:0}$ anteiso	2.6
$C_{18:0}$	trace

The above table, however, may not be accurate for all stages of growth, as De Siervo and Salton (1973) have shown that there are lipid changes during growth in the membrane of M. lysodeikticus. Using a peptone, water and yeast extract medium at 30°C, they found that the total lipid decreased from ten to two per cent of the dry weight of the cells. The total lipid phosphate increased and reached a plateau during the stationary phase. The two major lipids, phosphatidyl glycerol and diphosphatidyl glycerol, showed marked changes in their relative amounts during the growth period. A fall in phosphatidyl glycerol coincided with an increase in diphosphatidyl glycerol, and abrupt changes occurred as growth ceased, when the phosphatidyl glycerol reached a maximum and the diphosphatidyl glycerol a minimum.

There were only small changes in the phosphatidyl inositol.

Thus,the membrane lipid composition appears to be dependent on the growth phase, and medium.

(ii) Carotenoid stabilization of membranes.

Conflicting ideas have been put forward as to whether carotenoid stabilizes the membrane. However different bacteria have been used, so it is possible that the presence of carotenoid in the membrane does not necessarily always result in membrane stabilization.

An interesting observation was made by Prasad and Litwack (1960, 1961), using M. lysodeikticus. They found that by growing these bacteria in increasing concentrations of lysozyme, the bacteria eventually became lysozyme resistant. However by the time this had happened, the bacteria had stopped synthesising coloured carotenoids.

A conflicting observation was made by Salton and Freer (1965) and Salton and Ehlishan-ud-din (1965). They found that in occasional preparations of S. lutea and M. lysodeikticus, grown with diphenylamine so that they were colourless, the bacteria were less stable and had a tendency to lyse. Possibly the membrane was stabilised by carotenoid. Following this,Krinsky and Mathews-Roth (1970) looked at three different cultures of S. lutea: wild-type, a colourless mutant, and a colourless diphenylamine-induced. They used both whole cells and protoplasts, but could find no difference in osmotic fragility in the three strains. They concluded that the occasional lysis observed by Salton and his co-workers,was not related to carotenoid deficiency, which seems reasonable as it only occurred occasionally. Other workers have obtained similar results. Razin

et al (1966), using Acholeplasma laidlawii, found that a tenfold change in carotenoid levels produced no change in osmotic fragility.

But Leaf Huang and Haug (1974), also using A. laidlawii, found that increased carotenoid caused decreased membrane fluidity. They used a lipid extracted medium and added only arachidic acid ($C_{20:0}$). In that situation, the carotenoid levels depended on whether propionate (giving low carotenoid levels) or acetate (giving high carotenoid levels) was added to the medium. Bacteria containing higher levels of carotenoid were characterised by higher osmotic fragility, higher buoyant density, and lower glycerol permeability. Using spin labelling techniques, they found that bacteria with lower carotenoid levels had higher lipid fluidity.

Increasing the carotenoid to very high levels, however, did not result in a corresponding decrease in lipid fluidity. Faced with a situation, when its membrane contained so much carotenoid that it would be very rigid, the bacteria compensated by altering its fatty acyl residues to increase lipid fluidity. The average acyl chain length decreased, and there was an increase in unsaturated fatty acyl groups. Thus, bacteria are able to keep their lipid fluidity within narrow limits, despite large changes in carotenoid levels.

Carotenoids being fairly rigid molecules, might be expected to stabi lize the membrane, perhaps similarly to cholesterol.

Krinsky and Mathews-Roth (1970) found no difference in osmotic fragility between wild-type and carotenoidless mutant strains of S. lutea. However they did not appear to have examined the membrane lipid fluidity or the fatty acyl composition. Possibly the cells had

altered their fatty acyl composition to compensate for the drastic alteration in carotenoid content. If the S. lutea cells were able to maintain their lipid fluidity, then no significant change in osmotic fragility would be expected.

(iii) Carotenoid protection of membrane lipids.

Another function of carotenoid, in the membrane, could be to protect the lipids from peroxidative damage. Anderson and Krinsky (1973) demonstrated this using liposomes, as a model membrane system.

The liposomes were exposed to visible light and air, in the presence of toluidine blue. Light below 520 nm was removed by a filter, so that only the toluidine blue was absorbing light.

Without carotenoids, there was peroxidative damage to the membrane lipids, which can be measured by the coloured condensation product of thiobarbituric acid (TBA) and a product of lipid peroxidation, malonaldehyde (MDA). This peroxidation resulted in lysis. Addition of carotenoid to the membrane, or other singlet oxygen quenchers and free radical scavengers, protected the liposomes against peroxidative damage. However, it must be mentioned that Anderson and Krinsky used a highly unsaturated lipid, egg phosphatidyl choline, in the liposome, which would undergo peroxidation very readily.

Singlet oxygen would be expected to have a very disastrous effect on membrane lipids, causing peroxidative damage and eventual lysis of the bacterium. Therefore, if carotenoid can protect the respiratory chain against singlet oxygen, it is logical to assume it can also protect the membrane lipids.

E. CAROTENOID BIOSYNTHESIS.

(a) In bacteria.

Micrococcus lysodeikticus contains at least seven carotenoids, a large proportion of which are C_{45} and C_{50} carotenoids. The ability to synthesise carotenoids probably occurred early in evolution, as some bacteria, plants and algae retain this ability, but in animals, especially higher ones, it is lost.

Relatively little is known about the mechanisms of carotenoid biosynthesis, because of the nature of these pigments, which are sensitive to heat, light, oxygen and acids, and often also alkalis. They are, also, often unstable in dilute solution and on chromatography. However as they are tetraterpenes, their synthesis follows the general pathway for all terpenoids. In bacteria, their synthesis appears similar to that in plants and fungi.

Carotenoid biosynthesis can be divided into four stages:

(i) formation of first C_{40} compound

(ii) dehydrogenation

(iii) formation of alicyclic and aromatic compounds

(iv) oxygenation to xanthophylls and skeletal modifications

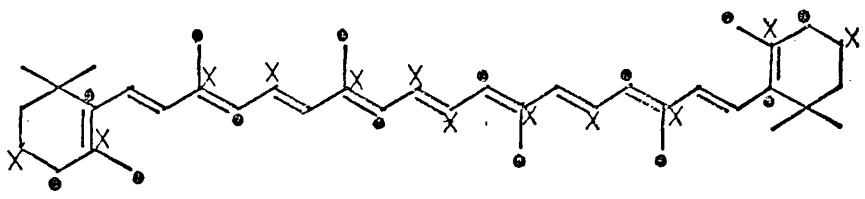
(i) Incorporation of radioactively labelled precursors.

Incorporation of radioactive label, into carotenoids, may be achieved using acetate or mevalonate, the former giving the labelling pattern, in beta-carotene, shown in Fig. 3a. [2-^{14}C]Mevalonic acid incorporation into carotenoids, in bacteria, has been demonstrated with mutants of Staphylococcus aureus (Suzue 1960) and in this study, using Micrococcus lysodeikticus. The labelling into beta-carotene,

Figure 3

The structure of phytoene and the labelling obtained in beta-carotene from [2-^{14}C]mevalonic acid and [^{14}C]-acetate; the acetate labelled either in the methyl or carboxyl group.

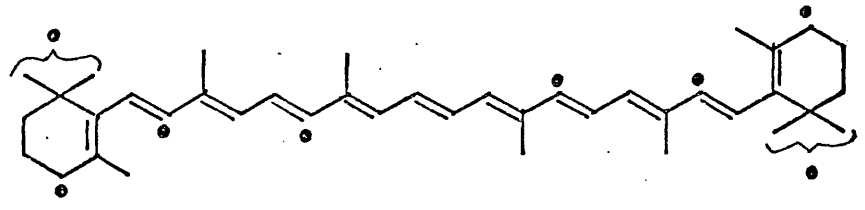
A LABELLING FROM ACETATE



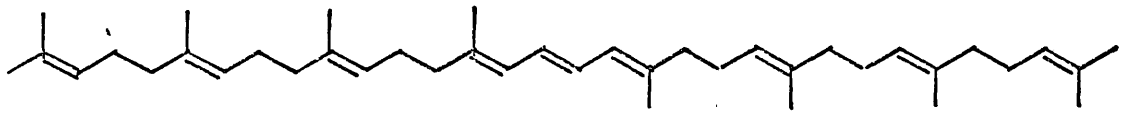
● FROM THE METHYL GROUP

X FROM THE CARBOXYL GROUP

B LABELLING FROM [2-C^{14}]MEVALONIC ACID



C PHYTOENE



from [2-^{14}C] mevalonic acid is shown in Fig. 3b. Sometimes difficulties have arisen in the incorporation of mevalonate into carotenoids, probably due to membrane impermeability.

(ii) Formation of the first C_{40} compound and dehydrogenation.

Enzymes of the general terpenoid pathway have been found in bacteria, indicating a similar route to that found in fungi and plants. Mevalonic acid kinase has been shown to be present in Staphylococcus aureus (Ohnoki et al 1962). More recently, an enzyme from M. lysodeikticus has been found, which catalyses the elongation of polyprenylpyrophosphate by isopentenyl pyrophosphate (Allen et al 1967).

The first C_{40} compound is believed to be phytoene (Fig. 3c). When diphenylamine was used to inhibit carotenogensis in Neurospora crassa, large amounts of phytoene accumulated, which contained the expected labelling from [2-^{14}C] mevalonic acid (Davies et al 1963). Also,a Mycobacterium sp. accumulated phytoene,under anaerobic conditions (Rilling 1962).

In phytoene, from tomatoes, the configuration is 13 trans, 15 cis, and 13' trans. In fungi and bacteria, isomerization of the polyenes from 15 cis to all trans must occur early in the dehydrogenation sequence, as zeta-carotene, 7, 8, 11, 12-tetrahydrolycopene, lycopene and neurosporene generally have the all trans configuration (Davis et al, 1966, Davies 1970). After dehydrogenation, several reactions can occur: (i) addition of water characteristics, hydrogen or a C_5 unit at the $C_{1,2}$ double bond, (ii) cyclisation.

(iii) Reactions at the $C_{1,2}$ double bond.

The introduction of a hydroxy group is an anaerobic process

(Liaaen-Jensen 1963, Britton 1971), and is inhibited by nicotine and CPTA (2- (p-chlorophenylthio) triethylammonium hydrochloride), McDermott et al (1973), Singh et al (1973).

Modification of the cyclisation reaction (see next section), could be used to form C_{45} and C_{50} carotenoids. It is believed that the proton, in cyclisation, is replaced by an electrophilic C_5 species, as the cyclisation-initiating species, in the formation of higher carotenoids.

(iv) Cyclisation.

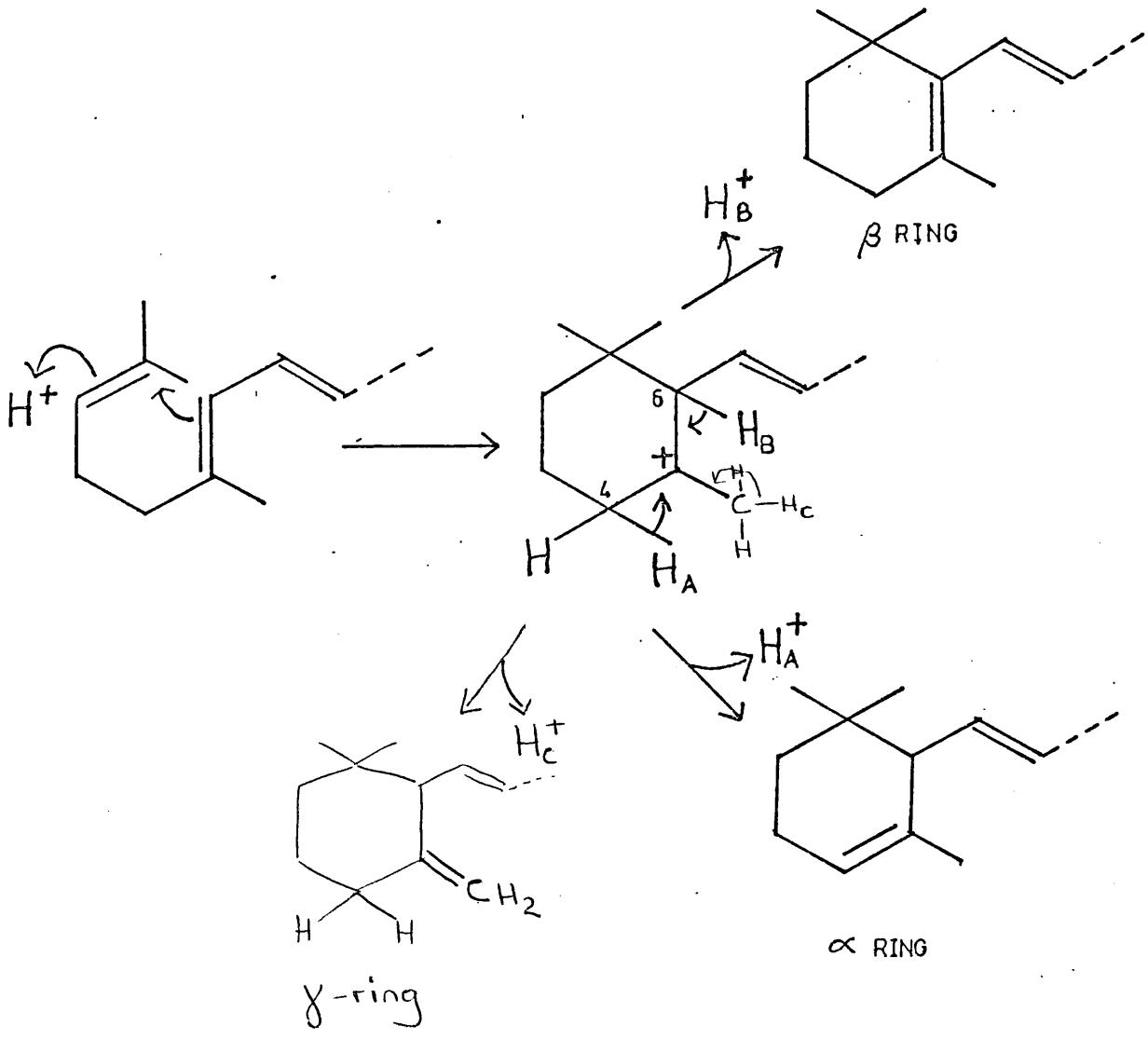
Many bacterial carotenoids contain one or two rings. These rings can be of the α or β or γ-ionone form, and a proposed mechanism is shown in Fig.4. In tomatoes, the α or β ring form is controlled by different genes.

The point, in the pathway, at which cyclisation occurs has not been proved. If normal desaturation went to completion before cyclisation, then lycopene would be the key intermediate in the formation of cyclic carotenes. However if cyclisation occurred before complete desaturation, then neurosporene and the zea-carotenes would be the key intermediates. Cyclisation is inhibited by nicotine and CPTA.

However, there is doubt as to the validity of the proposed cyclisation mechanism (Fig. 4), due to differences in the absolute configuration at C_2 and C_6 in various carotenoids. Therefore it has not been possible to define the stereochemistry. However, the mechanisms of cyclisation are probably similar, but the stereochemistry is different for different carotenoids. The stereochemistry

Figure 4

A proposed mechanism for cyclisation in carotenoid biosynthesis.


H+
6
4
HB
HA
HC
H
C
H
HB+
β RING
HA+
HC+
CH2
H
H
γ-ring
∝ RING

may depend on the cyclisation-initiating species. This general theory makes use of a carbonium ion, as the key intermediate. However, it has been suggested that, in Myxococcus fulvus , there is an assembly line of carotenogenic enzymes associated with the cytoplasmic membrane, (Kleinig 1975), which has given rise to the theory that cyclisation may involve an enzyme bound intermediate.

(v) C_{50} and C_{30} carotenoids.

Decaprenoxanthin was the first C_{50} carotenoid found. It was isolated from Flavobacterium dehydrogenans (Liaaen-Jensen and Weeks 1966) and could be formed from phytoene, as proposed by Weeks (1971), in Fig. 5. From decaprenoxanthin, other C_{50} carotenoids could be formed, by modifications.

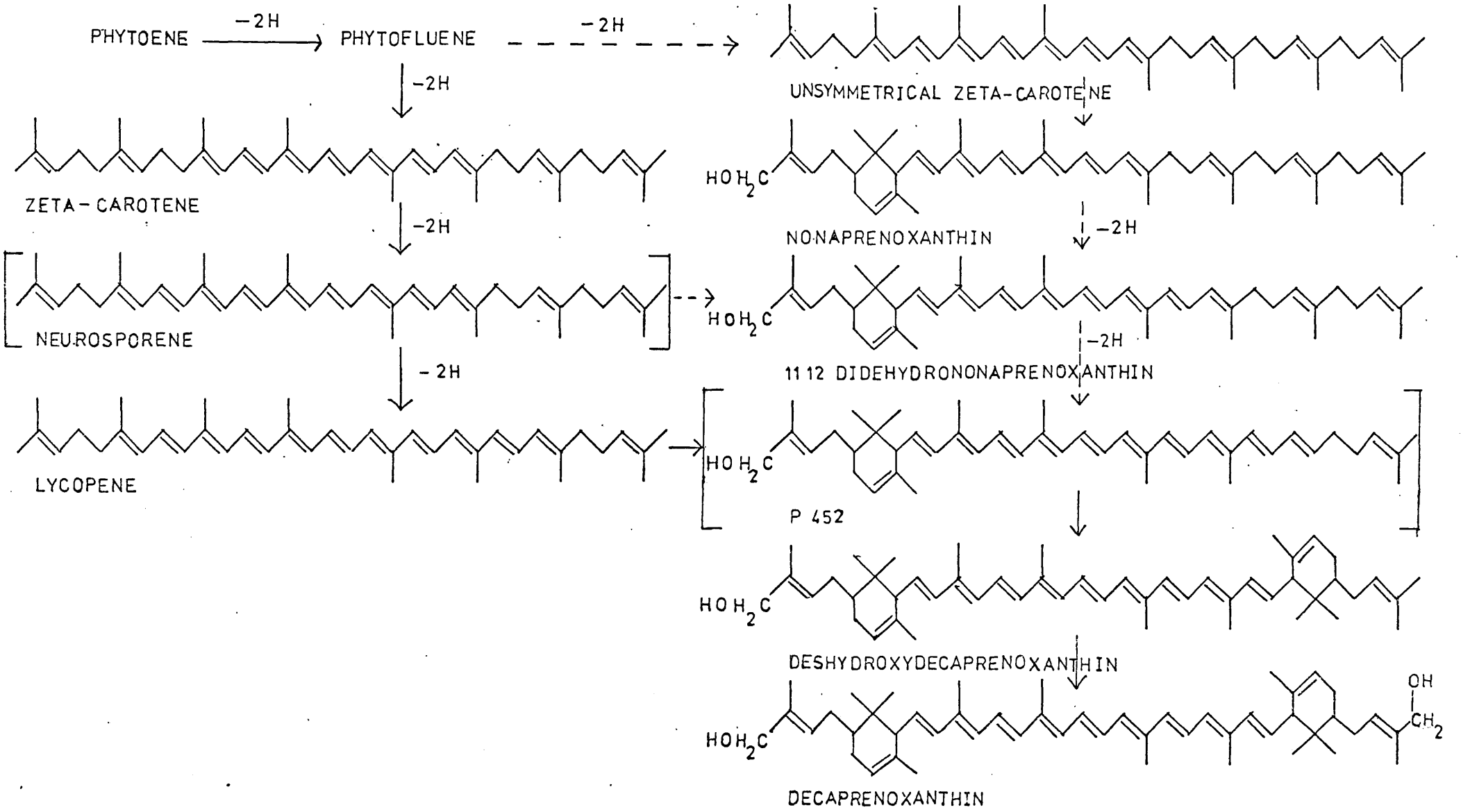
In Streptococcus faecium , the carotenes are all tri-terpenoid (C_{30}) analogues of the C_{40} series, and two major C_{30} xanthophylls have also been found. (Taylor and Davies, 1974, 1974a).

(vi) Theories of carotenoid biosynthesis.

Carotenoid biosynthesis can be simplified by use of the theory of half-molecule substrates. Then the substrate of each enzyme is a carotenoid half-molecule, rather than a specific compound. Using this idea, neurosporene and lycopene would appear identical to the cyclising enzyme, as each contains the required half-molecule substrate. Consideration of this, and all available data, has shown that desaturation at C $_{7,8}$ is essential, before cyclisation of the half-molecule can occur. The work on zeaxanthin biosynthesis in Flavobacterium , in the presence of inhibitors, has led to the

Figure 5

A proposed scheme for the formation of decaprenoxanthin from phytoene.


PHYTOENE
−2H
PHYTOFLUENE
−2H
UNSYMMETRICAL ZETA-CAROTENE
−2H
ZETA-CAROTENE
HO H2C
NONAPRENOXANTHIN
−2H
−2H
NEUROSPORENE
HO H2C
−2H
11 12 DIDEHYDRONONAPRENOXANTHIN
−2H
LYCOPENE
HO H2C
P 452
HO H2C
DESHYDROXYDECAPRENOXANTHIN
OH
CH2
HO H2C
DECAPRENOXANTHIN

suggestion that there may be two sites of enzyme activity, which would fit in with two half-molecule substrates (McDermott et al 1974)

However, Kleinig (1975) showed that lycopene, accumulated in Myxococcus fulvus , can be converted to the normal carotenoids of this organism, only if de novo synthesis from phytoene is prevented. He suggested that this indicates an arrangement of carotenogenic enzymes in an assembly line, with access at a late point (lycopene) not possible, unless the supply of earlier substrate (phytoene) has been removed.

(vii) Control of carotenoid biosynthesis.

Control mechanisms in bacteria are not fully understood. Certain bacteria only synthesise carotenoids after a short exposure to light and oxygen. These bacteria contain a light receptor, which triggers carotenoid synthesis. In Mycobacterium sp. it is thought to be a flavin (Rilling 1964), whereas in Mycobacterium marinum it is a porphyrin (Batra and Rilling 1964).

In the leaves of Phaseolus vulgaris, the concentration of phytol pyrophosphate exerts control on the formation of phytol, and so chlorophyll. It controls the incorporation of [2-^{14}C] mevalonic acid, so that if the phytol pyrophosphate is at a high level, the mevalonic kinase is depressed (negative feedback), Watts and Kekwick (1973).

Using extracts of the fungus, Phycomyces blakesleeanus, the incorporation of farnesyl pyrophosophate into carotenoids was stimulated by isopentenyl pyrophosphate or mevalonic acid.

In M. lysodeikticus, since carotenoid and quinone have a common pathway from mevalonic acid to isopentenyl pyrophosphate,

the possibility exists for a double control in the isoprenoid synthetic pathway, by both quinone and carotenoid, which could make the mechanism very complicated.

F. QUINONES.

(a) Structure and occurrence of quinones.

Quinones are widely distributed in nature. Ubiquinone structure was first elucidated by Morton et al and Wolf et al in 1958. Plants contain quinone, in their chloroplasts, of three types; phylloquinone, plastoquinone and quinones derived from ∝tocopherol and ∝tocotrienol.

Menaquinones (naphthoquinones) occur in some bacterial plasma membranes and higher animals, whereas demethyl menaquinones occur only in some bacteria.

Ubiquinones (benzoquinones) occur widely, in plant mitochondria (n=9, 10), bacteria (n = 7, 8, 9, 10) and animal mitochondria (n = 9, 10). In bacteria the ubiquinones are situated in the membrane, or cell envelope. Rhodospirill um rubrum , a photosynthetic bacterium, has evolved its own quinone, rhodoquinone, which is a benzoquinone, with an amine group attached to the ring.

Generally bacteria contain either naphthoquinones or benzoquinones, but some, such as E. Coli, may contain both types.

Micrococcus lysodeikticus contains a menaquinone, probably menaquinone - 8 (MK8) or MK8 (H), (Jeffries et al 1967).

Quinone structures, with their location, are shown in Fig. 6.

(b) Function of Bacterial quinones.

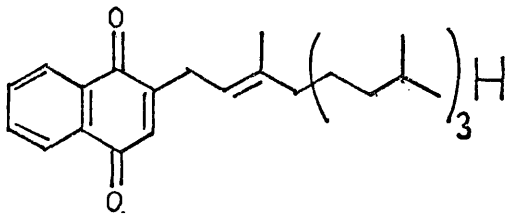
(i) Quinones in the electron transport system.

Kinetic studies, as well as studies with quinone-depleted systems, showed that certain quinones play a part in electron transport. Quinones can be dissociated from the respiratory chain, without disturbing the spatial orientation of the enzymes and co-enzymes

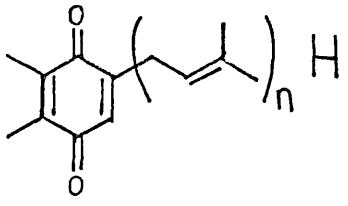
Figure 6

Structures of quinones from varying sources.

PLANT QUINONES

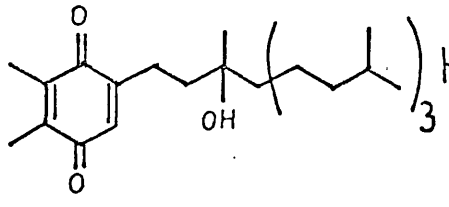


PHYLLOQUINONE

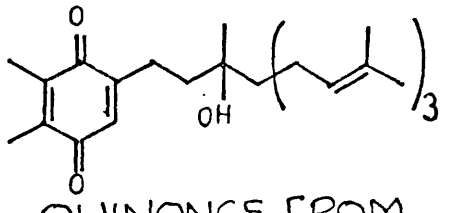


PLASTOQUINONES

n = 3,4,9



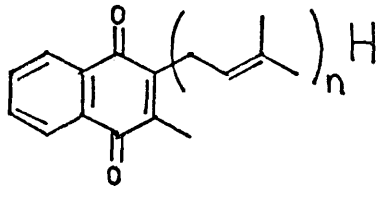
H from α tocopherol



H from α tocotrienol

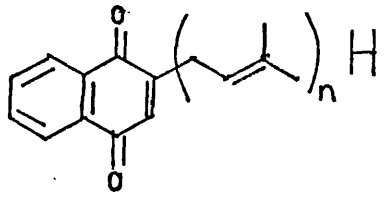
TOCOPHEROL QUINONES

QUINONES FROM OTHER SOURCES



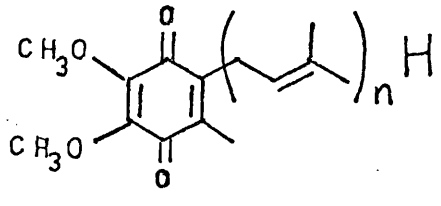
MENAQUINONES

n = 4,6,7,8,9



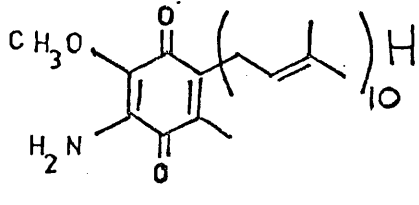
DEMETHYL MENAQUINONES

n = 5,6,7,8,9



UBIQUINONES

n = 7,8,9,10



RHODOQUINONE

associated with the chain. It seems likely that quinone is an integral part of the respiratory chain, as exposure to 360nm light results in a loss of oxidative phosphorylation, and loss of the ability to utilize oxygen. The chain can be reconstituted, but only with quinone of certain specific configurations. The rest of the chain can be shown to be active after treatment with 360 nm light, by use of dyes, which "bridge" the gap. In Mycobacterium phlei, if quinone was destroyed by 360 nm light, the loss of succinate oxidation, and NAD-linked substrate oxidation, could be restored by vitamin K_1. There was better restoration, if the natural quinone was used. However, restoration of activity is not enough to prove that quinone is an integral part of the respiratory chain. For example, with pyruvate as substrate,the addition of quinone to an irradiated system resulted in greater restoration of phosphorylation than oxidation, and the phosphorylation was found to be insensitive to cyanide. The phosphorylation also occurred equally well under anaerobic conditions, or when quinone was replaced by methylene blue (Phillips et al,1970). Therefore the restoration of activity must also be shown to be the same as with the natural quinone e.g. with respect to inhibition.

(ii) Quinone in the electron transport system, of specific bacteria.

Work has been done on specific bacteria, to show that the quinone is part of their respiratory chains. Using Bacillus megaterium, Kroger and Dadak (1969) showed that the menaquinone was located in the membrane, along with the cytochromes. They also showed that the menaquinone was almost completely reduced in

anaerobic conditions, with substrate present. On addition of oxygen, it was reoxidised. The redox reactions also indicated that menaquinone participated in the oxidation of the substrates (∝glycerophosphate, malate and NADH) by oxygen and fumarate. Menaquinone also exhibited the steady state response expected for a respiratory component.

Lukoyanova and Kovoleva (1975), using M. lysodeikticus, found that ultraviolet irradiation of membranes resulted in a 90% inhibition of the respiratory enzymes, NADH, malate and lactate oxidase. NADH dehydrogenase was not affected, but the malate and lactate dehydrogenases were slightly deactivated, due to partial photodegradation of flavine coenzymes.

(iii) Function of quinone in bacteria containing both ubiquinone and naphthoquinone.

E. Coli contains both menaquinone and ubiquinone. Ubiquinone appeared to be the major quinone during aerobic growth. However during anaerobic or oxygen-limited conditions, the menaquinone was the major quinone (Whistance and Threlfall 1968). The menaquinone also seemed to be the important quinone, under aerobic conditions, in the presence of low concentrations of cyanide (Ashcroft and Haddock 1975).

(iv) Quinone oxidation.

The quinone is an integral part of the respiratory chain in M. lysodeikticus, and will be destroyed by photooxidation, if not protected. Morimoto and Imado have investigated the photooxidative products of quinone, in solvent systems (Morimoto and Imado 1964,

Imado and Morimoto 1964). They found that the first products involved changes in the β-γ position in the side chain. It was found that an unsaturated β-γ position was necessary for the restoration of phosphorylation.

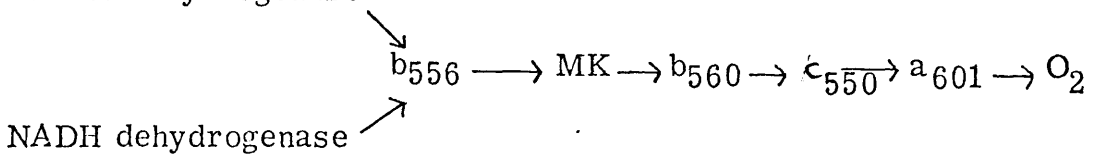
Further proposals for the mechanism of photooxidation of quinone have come from Mee et al (1975). They used menadione, which is a quinone ring structure, and examined both photooxidation and chemical oxidation. They suggested that a menadione epoxide is formed, in both types of oxidation. However this has not been proved, due to difficulties involved in unambiguous identification of one of the gas chromatography peaks, by mass spectroscopy. However, photooxidation of menadione only appears to occur in the presence of oxygen.

(v) Respiratory chain of M. lysodeikticus.

In bacteria, there is usually a major quinone, but there will also be small quantities of other quinones, e.g. Mycobacterium phlei contains mainly menaquinone 9 (II H), but also contains small quantities of menaquinone 8 (II H) (Campbell and Bentley 1968, Dunphy and Brodie 1971).

Gel'man et al (1970) disrupted the respiratory chain of M. lysodeikticus membranes, with detergents, into two complexes. One contained the malate and NADH dehydrogenases, with cytochrome b_{556}, and the other, cytochromes b_{560}, c_{550} and a_{601}. These blocks maintained their functional activity, exhibiting various degrees of bonding with the membrane. Lukoyanova and Kovoleva (1975) found that with loss of menaquinone, and addition of substrate, the cytochrome b_{556} was reduced, but the b_{560} was not, therefore they suggested

that menaquinone must lie between these cytochromes. The addition of menaquinones O to 9 restored the activity. From the above results, the scheme below was proposed, for the respiratory chain of M. lysodeikticus:



Quinone has been shown to be an integral part of the respiratory chain in bacteria. Generally either naphthoquinone or ubiquinone is present. However in certain bacteria, which possess both, they function under different conditions. The respiratory chain, in bacteria, can be very diverse, with different cytochromes and quinones, and utilizing different substrates. Because of its important biological role, it is protected from photo-oxidative damage, by carotenoid, as a loss of quinone would have disastrous consequences for the respiration, and hence viability, of obligately aerobic cells.

(c) Naphthoquinone biosynthesis.

In 1971 Thomson estimated that there were 380 members of the three main structural classes of quinone (anthraquinones, benzoquinones and naphthoquinones). There are also at least five biosynthetic pathways to both naphthoquinones and benzoquinones (Bentley 1975). Benzoquinones and naphthoquinones have important and well studied biological roles, but their synthesis is not so well elucidated.

(i) The role of shikimic acid.

Shikimic acid is a precursor of the aromatic amino acids, phenylalanine, tyrosine and tryptophan, but from it also come benzoate derivatives which are precursors of quinones.

In 1964, Cox and Gibson showed that shikimic acid was incorporated into vitamin K_2 and ubiquinone in E. Coli. Unlabelled 4-hydroxybenzoic acid stimulated incorporation of [^{14}C]- shikimic acid into ubiquinone, whereas addition of 3,4-dihydroxybenzaldehyde suppressed the incorporation into vitam$_{\lambda}$ K_2. From this, they concluded that 4-hydroxybenzoic acid was on the ubiquinone pathway, and 3,4-dihydroxybenzaldehyde on the naphthoquinone route. In 1966 they showed shikimic acid to be the precursor of ubiquinone and menaquinone-8, in E. Coli. They also showed that quinone synthesis was related to the general pathway of aromatic biosynthesis. Their proposed scheme is shown in Fig. 7.

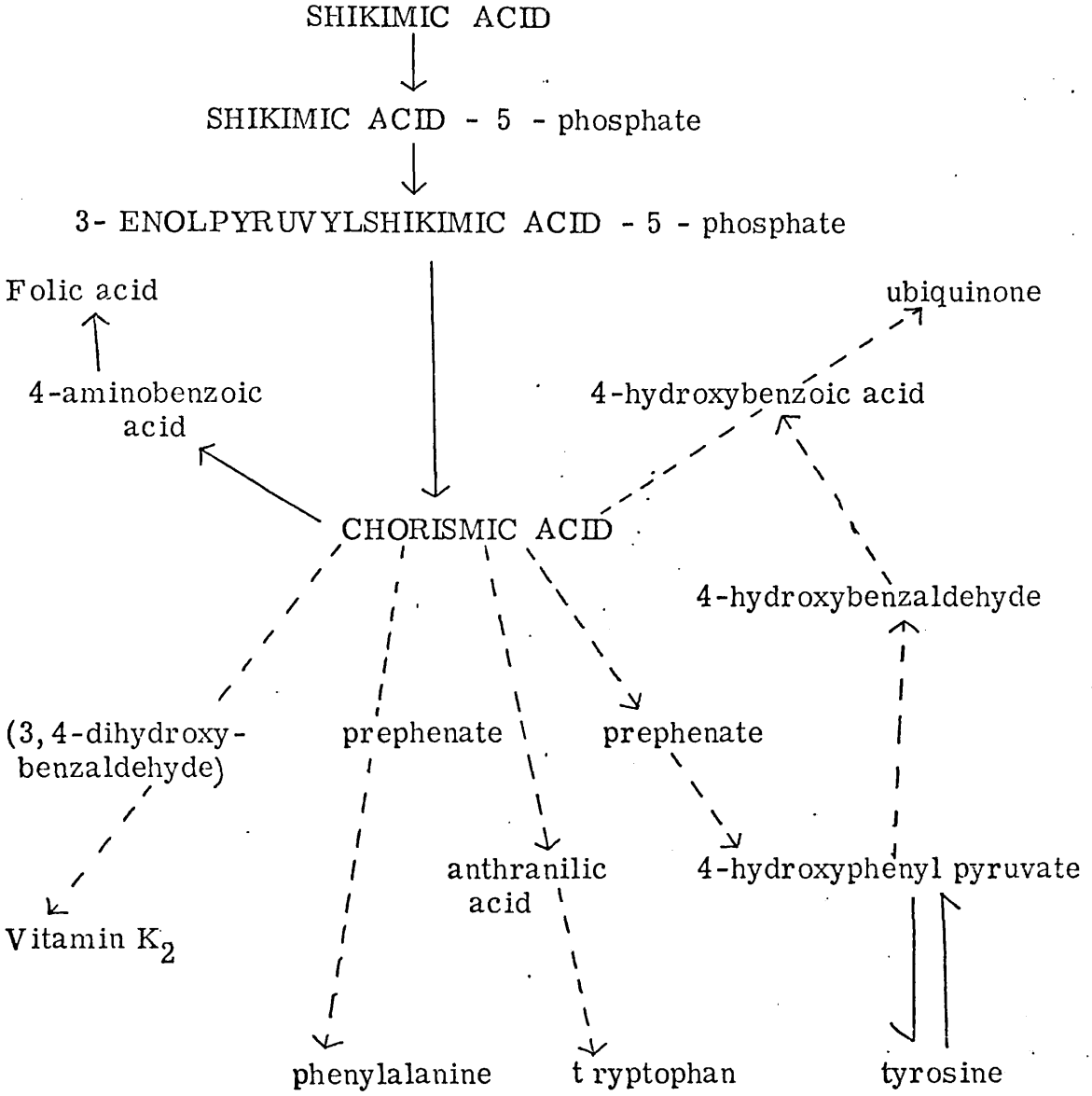
(U-^{14}C) Shikimate was also incorporated into menaquinone in Mycobacterium phlei and Streptomyces albus, but less efficiently than in E. Coli. From their results, they suggested that the intact carbon skeleton of shikimate was used in menaquinone biosynthesis. Leistner et al (1967) obtained a similar result using Bacillus megaterium.

(ii) Origin of the methyl group.

Jackman et al (1967) investigated the origin of the methyl group, and found that it was supplied by methionine, in both naphthoquinone and ubiquinone, in E. Coli. They used tritiated methionine, which was incorporated into the ring methyl group of

Figure 7

Scheme linking quinone synthesis to aromatic synthesis, through chorismic acid.


SHIKIMIC ACID
SHIKIMIC ACID - 5 - phosphate
3- ENOLPYRUVYLSHIKIMIC ACID - 5 - phosphate
Folic acid
4-aminobenzoic acid
ubiquinone
4-hydroxybenzoic acid
CHORISMIC ACID
4-hydroxybenzaldehyde
(3,4-dihydroxy-benzaldehyde)
prephenate
prephenate
anthranilic acid
4-hydroxyphenyl pyruvate
Vitamin K_2
phenylalanine
t ryptophan
tyrosine

vitamin K_2 and the C-methyl and O-methyl groups of ubiquinone, but nowhere else in the molecule.

Methionine appears to be the source of the ring methyl groups, of vitamin K_2, in a wide range of living things; Mycobacterium phlei, Fusiformis nigrescens, rats and Rhodospirillum rubrum.

(iii) o-Succinyl benzoic acid.

[^{14}C]-o-Succinylbenzoate (OSB) was efficiently incorporated into bacterial menaquinone in Mycobacterium phlei and Aerobacter aerogenes, without randomization (Dansette and Azerad, 1970).

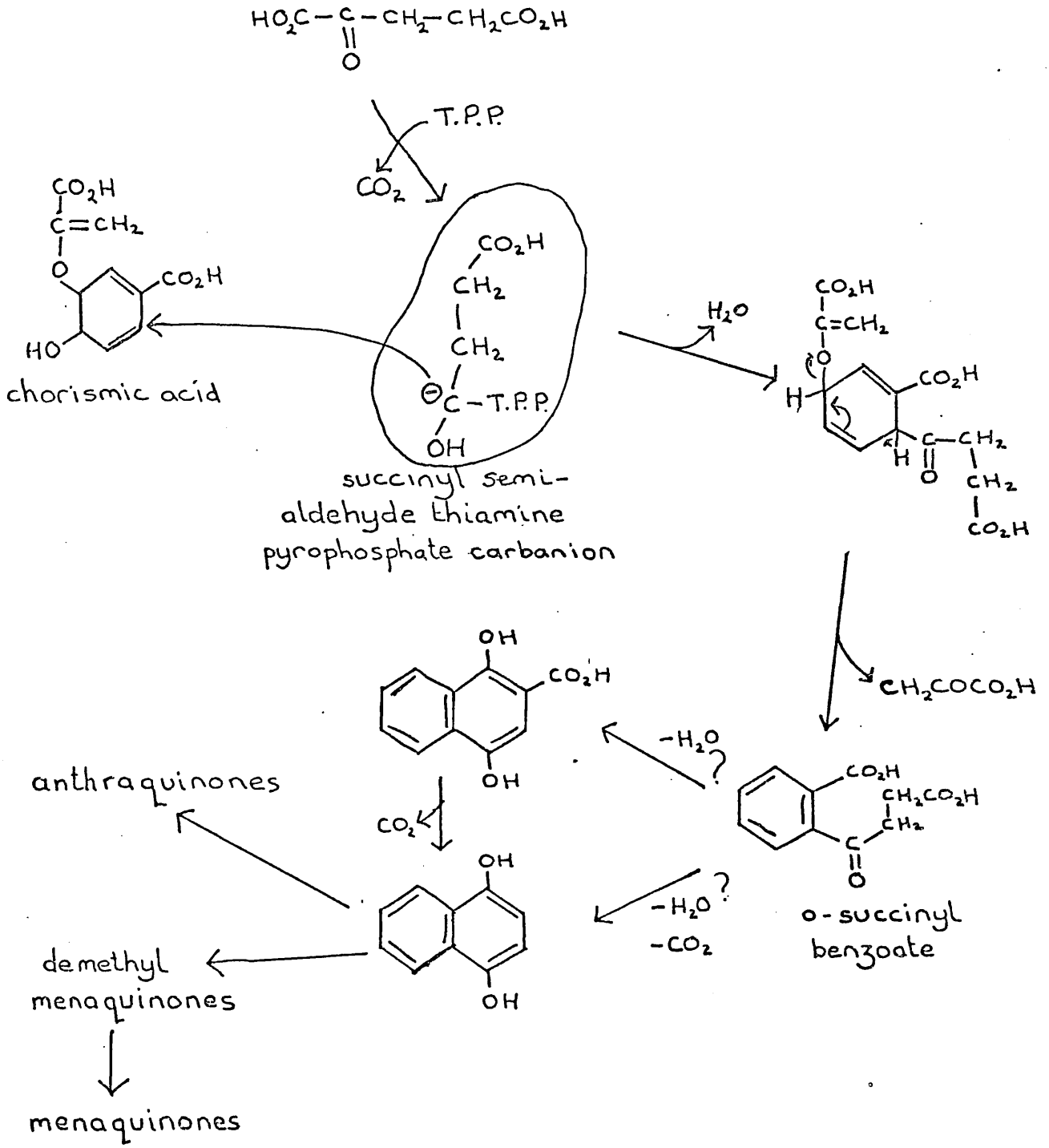
Previously the origin of all the carbon atoms in menaquinone had been determined, apart from three. These three were derived from a five-carbon dicarboxylic acid, glutamate or 2-ketoglutarate, which lost carboxyl groups at both ends. Then there was probably an initial Michael-type addition of the succinic semialdehyde-TPP complex to the double bond of chorismic acid, followed by a Claisen condensation to form the second ring (Fig. 8). Young (1975) proposed a similar scheme, from results using biosynthetic mutants of E. Coli. However he was not sure of the identity of the succinyl donor.

(iv) Other intermediates in naphthoquinone biosynthesis.

In some bacteria 1-naphthol is an intermediate, and (1-^{14}C)-naphthol was incorporated into menaquinone 7, in Bacillus megaterium, into carbon atoms C1 and/or C4 (Leistner et al 1967). 1-Naphthol was at first thought to be a general intermediate, as Staphylococcus aureus and Aerobacter aerogenes also convert it into menaquinone (Guerin et al, 1970, Hammond and White 1969), but now it has

Figure 8

Proposed pathway for quinone synthesis from chorismic acid.


T.P.P.
CO_2
chorismic acid
succinyl semi-aldehyde thiamine pyrophosphate carbanion
H_2O
CH_2COCO_2H
o-succinyl benzoate
$-H_2O$?
$-H_2O$?
$-CO_2$
CO_2
anthraquinones
demethyl menaquinones
menaquinones

been found that most bacteria do not utilize it to form menaquinone.

There is conflicting evidence as to whether menadione is an intermediate. Guerin et al (1970), after comparing radioactivity dilutions using the precursors (^{14}C)-2-methyl-1, 4-naphthoquinone and (^{14}C)-1, 4-naphthoquinone,came to the conclusion that menadione is probably not a true intermediate in menaquinone biosynthesis. However Goldenbaum et al (1975), using a Staphylococcus aureus mutant, found that menadione deprivation resulted in vitamin K_2 synthesis being stopped, as well as the synthesis of other electron transport components. He concluded that it is a true intermediate in quinone biosynthesis.

(v) Investigation of the branch point of naphthoquinone biosynthesis, and aromatic amino acid synthesis.

Dansette and Azerad (1970) considered this question, and came to the conclusion that it was at chorismic acid, as did Cox and Gibson (1966). Their reasons were that E. Coli mutants, blocked in the early reactions of aromatic biosynthesis,could not form menaquinone, and that when an aromatic supplement and 10^{-4}M succinyl benzoate was added, the same amount of menaquinone was obtained as when 0.2×10^{-4} shikimate was added. However it is still not clear if chorismate is a mandatory precursor to succinyl benzoate in all cases.

(vi) Incorporation of [2-^{14}C]mevalonic acid into menaquinone.

The origin of the side-chain is believed to be mevalonic acid, thus linking quinone and carotenoid synthesis, as previously discussed. However, in some cases, [2-^{14}C]mevalonic acid is not incorporated into quinones. Using M. tuberculosis, Ramasarma

and Ramakrishnan (1961) found that [2-^{14}C]mevalonic acid, the recognised isoprenoid precursor in nature, was incorporated not into quinone, but into neutral and esterified lipids. Following this, in 1965, Ramasarma et al looked at four more bacteria, with respect to [2-^{14}C]mevalonic acid incorporation into quinones, using E. Coli , a Pseudomonas sp., Azobacter vinelandii, and Agrabacterium tumefaciens . They again found that there were significant counts in the lipids, but not in the quinone. However both [^{14}C]-acetate and [^{14}C]-mevalonate were incorporated into quinone and sterols in moulds and yeast. The [^{14}C]-mevalonate only incorporated into the side-chain, whereas the [^{14}C]-acetate was incorporated into both the ring and the side chain.

They suggested that mevalonate is only a precursor for the quinone side-chain in organisms which also contain other isoprene compounds,such as sterol and carotene.

(vii) Conclusion.

As previously stated, there are at least five different routes for quinone ring biosynthesis. A lot of research has been done, using E. Coli, but when Guerin et al (1970) tried to incorporate various carbon and tritium -labelled compounds into quinone, in various bacteria, including Mycobacterium phlei, Mycobacterium avium, Bacillus megaterium, Micrococcus lysodeikticus, Aerobacter aerogenes and Proteus vulgaris , they found that 3,4-dihydroxybenz-aldehyde, thought to be on the menaquinone biosynthetic route, was not incorporated. Therefore bacterial synthetic routes for the quinone ring, worked out for E. Coli, are probably not always operable in other bacteria.

The inability of a wide range of bacteria to incorporate [2-^{14}C] mevalonic acid, the natural isoprenoid precursor, into quinone, as reported by Ramasarma and Ramakrishnan (1961), and Ramasarma et al (1965), is surprising. In the present study, significant incorporation of [2-^{14}C] mevalonic acid into quinone, in wild-type and carotenoidless-mutant strains of M. lysodeikticus, was observed. This would fit in with the proposal by Ramasarma et al (1965), as the mutant is synthesising colourless carotenoids.

2. MATERIALS AND METHODS

A. MATERIALS

All reagents used were of analytical grade, unless otherwise stated.

All the reagents were obtained from BDH Chemicals Ltd., with the following exceptions:-

Kieselguhr G and Kieselgel 60 from Merck Chemicals, alumina and antifoam agent from Hopkins and Williams, PPO (2,5-diphenyloxazole) and POPOP (1,4-di (2-(5-phenyloxazolyl))benzene) from Koch-Light Laboratories, 2-n-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), mevalonic acid lactone, vitamin K_1, and menadione from Sigma Chemicals, [2-^{14}C] and [1-^{14}C] isopentenyl pyrophosphate mevalonic acid lactone from the Radiochemical Centre, Amersham, light petroleum (b.p. 40-60^{o}C), aromatic hydrocarbon free, from May and Baker, and nutrient agar and nutrient broth from Oxoid Ltd.

B. METHODS.

(a) Cultures and culturing techniques.

(i) Bacterial strains.

Micrococcus lysodeikticus, a gram-positive coccus, strain number NCTC 2665, was used in this study, with four white-mutant strains, obtained by ultraviolet irradiation of the wild-type strain (carried out in this laboratory). The white mutants were numbered strains UV1, 2, 3 and 4, and were examined microscopically and by means of biochemical tests, to show their relationship with the parent strain.

(ii) Culture preparation.

Cultures were maintained on agar slopes at 26°C, a reserve supply being stored at 4°C. Both were subcultured regularly.

For experimental purposes, cultures were grown in nutrient broth. Pilot cultures, from agar slopes, were grown for 2-3 days at 30°C in a shaking incubator, and then a few ml added aseptically to 1-litre flat-bottomed culture flasks, containing 500ml medium. For most experiments, an overnight culture was used, grown at 30°C in a shaking incubator, (60-70 oscillations per minute).

(iii) Large scale batch culture.

A New Brunswick fermentor (New Brunswick Scientific Co., New Jersey) was used. Twelve litres of nutrient broth was prepared in the fermentor, and autoclaved thoroughly. The temperature was controlled, at 30°C. 200ml of pilot culture was added, and the culture aerated and stirred. An antifoam agent (Dows Corning Silicone MS Antifoam RD emulsion) diluted 1:1 (v/v) with water, was added at a slow rate, continuously (less than 100ml total addition).

300ml batches of bacterial culture were removed, aseptically, at suitable intervals.

During the growth period (40 hours), the volume of bacteria dropped considerably, as the 300ml batches removed were not replaced with fresh medium. The fall in volume was unavoidable in the batch culture method, as large samples were necessary for the assays.

(b) Cell count and viability measurements.

(i) Cell count.

A bacterial suspension, with an optical density of 0.1 at 600nm, was diluted with distilled water, until the cells could be easily counted, when put on a modified haemocytometer slide and viewed under a microscope. The cells were counted, and the volume of bacterial suspension on the slide was calculated. This was repeated several times, and the number of cells per ml of the suspension (0.1 absorbance) determined. The cell count was determined for both the yellow wild-type and white mutant strains of bacteria.

(ii) Viability.

Bacteria were harvested by centrifugation and suspended in sterile distilled water, to give an optical density of 0.1 at 600nm. This suspension was further diluted $1 \rightarrow 5 \times 10^4$ (in three steps), with sterile distilled water. 0.1ml samples were plated out, in triplicate, on nutrient agar, and incubated at $26^{\circ}C$ for 3 days, before the colonies were counted.

(c) Vesicle and membrane preparation.

(i) Vesicle preparation (Ishikawa 1967).

The cells were spun down at room temperature (13,000g x 10mins), and washed twice with 50ml of a medium containing 0.25M sucrose, 0.02M Tris-HCl pH7.4, and 5mM magnesium chloride, for each 200ml of original culture. The pellet was then suspended in a solution containing 1M sucrose, 0.02M Tris-HCl, pH 7.4, and 5mM magnesium chloride, and incubated at 37°C for 15 minutes with 3.5mg crystalline lysozyme, for each 10ml of resuspended bacteria. The resulting protoplasts were centrifuged down (30,000g x 40 mins) and suspended in 30ml of a medium containing 0.25M sucrose, 0.02M Tris-HCl, pH7.4, and 5mM magnesium chloride, for each 10ml of lysozyme-incubation suspension, and stirred in an ice bath. After 20 minutes, the suspension was sonicated for 3x2 minutes (a minutes gap inbetween) in an ice bath, using an MSE 150 watt ultra disintegrator (peak to peak amplitude 12 microns) and a $\frac{3}{4}$inch end diameter titanium probe.

The suspension was then centrifuged at 9000g x 20 mins to remove any debris, and the supernatant removed and then centrifuged at 68,000g x 70 mins to precipitate the vesicles, which were then suspended in a solution containing 0.25M sucrose, 0.02M Tris-HCl, pH7.4 and 5mM magnesium chloride (approximately 10ml). The expected P/O ratio of 0.3 was obtained with vesicles prepared by this method.

(ii) Membrane preparation.

1. The cells were spun down at room temperature (13,000g x 10 mins) and resuspended in a medium containing IM sucrose, 0.02M Tris - HCl, pH 7.4, and 5mM magnesium chloride (20ml per 500ml

culture). Crystalline lysozyme was added, 3.5mg per 20ml of resuspended bacteria, and the suspension incubated at 37°C for 15 minutes. The protoplasts were spun down (36,000g x 30 mins), where they lysed, and the membranes resuspended in 0.05M phosphate buffer pH7.0, washed once.

2. The cells were spun down at room temperature (13,000g x 10 mins) and resuspended in 0.05M phosphate buffer pH7.0 (20ml per 500ml culture). 3.5mg crystalline lysozyme were added, for each 20ml of resuspended bacteria, and the suspension was incubated at 37°C for 15 minutes. The membranes were spun down (36,000g x 30 mins) and resuspended in 0.05M phosphate buffer pH7.0. Membranes were shown to be prepared by the above methods, by their "ghost"-like appearance under a phase contrast microscope.

(d) Determination of membrane enzyme activities.

(i) Oxygen electrode.

Malate, succinate, lactate and NADH oxidase and malate dehydrogenase activities of the membrane were measured with an oxygen electrode (Beckman 39550). The oxygen electrode, together with a Beckman 100801 Fieldlab TM analyser, measures dissolved oxygen. The meter was connected to a chart recorder via a zero suppressor and scale expander.

The electrode was calibrated with water saturated with air, at 30°C (the temperature at which all enzyme activities were measured). At 30°C the solubility of oxygen in distilled water is 7.78 ppm. The chart recorder was set to read 0 to 10ppm of oxygen, and the chart speed was 1cm per minute.

(ii) Calculation of oxygen uptake.

A rate of 0.5 ppm per minute is equivalent to

$0.5 \times 10^{-6} \times X \times \frac{10^6}{16}$ microgram atoms of oxygen uptake per minute

X = volume of cuvette

(iii) Oxidase assays (using oxygen electrode).

0.5ml of 0.1M substrate (lactate, malate or succinate at pH 7.0), 0.5ml of membrane preparation and 1.0ml of 0.05M phosphate buffer, pH 7.0,were incubated at 30°C for 2 minutes, and then aerated, using a whirl mixer. The mixture was then transferred to the electrode cuvette, and the reaction rate measured. Reaction rates are expressed as microgram atoms of oxygen uptake per minute per mg protein.

(iv) NADH oxidase assay (oxygen electrode).

The NADH oxidase activity of the membrane was measured using the following reaction mixture: 0.2ml NADH (0.1%), 0.2ml membrane preparation and 1.6ml 0.05M phosphate buffer pH7.0. The membrane and substrate were incubated separately, at 30°C for 2 minutes. They were mixed just before aeration, and then transferred to the electrode cuvette, where the reaction rate was measured. The rate was expressed as microgram atoms of oxygen uptake per minute per mg protein.

(v) Malate dehydrogenase assay (oxygen electrode), (Benziman and Galanter, 1964).

The cytochrome oxidase activity of the membrane was inhibited with azide, and the malate dehydrogenase activity measured by the reduction and auto-oxidation of phenazine methosulphate (PMS). The reaction mixture contained 0.1ml PMS (0.009M), 0.1ml sodium azide (IM), 0.5ml malate (0.1M, pH7.0), 0.5ml membrane preparation, and 0.8 ml 0.05M phosphate buffer pH7.0. The sodium azide and membrane preparation were incubated separately from the other components, at 30°C for 2 minutes, and then mixed prior

to aeration. The reaction mixture was transferred to the electrode cuvette, and the rate measured. It was expressed as microgram atoms of oxygen uptake per minute per mg protein.

(vi) Malate - DCPIP reduction (Benziman and Galanter 1964).

Reduction of DCPIP (dichlorophenolindophenol) was measured spectrophotometrically by loss of absorbance at 600nm. The reaction mixture,total volume,was 3ml, and contained 0.5ml membrane preparation, 0.5ml malate (0.1M, pH 7.0), 0.1ml azide (1M), 0.3ml DCPIP (0.53mM) and 1.6ml 0.05M phosphate buffer pH 7.0. The blank contained everything but the substrate. Activity was expressed as (micromoles DCPIP reduced per minute) per mg protein:-

$$\frac{A_{600}}{W \times 7.0}$$

A_{600} = change in absorbance at 600nm per minute

W = the protein, in mg, in the 3ml assay mixture

(vii) Protein estimation (Jacobs 1959).

The protein was estimated by the microkjeldahl method. This involved digestion of the protein with concentrated sulphuric acid, in the presence of a selenium catalyst, and determination of the ammonia quantitatively by means of indanetrione hydrate (ninhydrin).

(e) Quinone extraction, purification and estimation.

(i) Quinone extraction from Micrococcus lysodeikticus.

Membranes were prepared, and suspended in a small volume, approximately 5ml, of 0.05M phosphate buffer, pH 7.0. 16ml of an acetone: methanol (1:1 v/v) mixture was added, and the suspension

shaken vigorously before being left in the dark for 30 minutes. 10ml of redistilled light petroleum (bp 40-60°C) was added, the mixture shaken, and then allowed to stand in the dark for 7-10 minutes, until the two phases had separated. If there was no separation, a small volume of 10% aqueous sodium chloride was added. The upper, light petroleum, phase was removed, and the aqueous phase re-extracted twice with light petroleum. The combined petroleum phases were evaporated to dryness, under nitrogen, and the residues dissolved in absolute alcohol. Under these conditions the menaquinone is recovered in the fully oxidised state, (modified method of Kroger and Dadak 1969).

Redistilled, aromatic hydrocarbon free, light petroleum (bp 40-60°C) was used to ensure the absence of ultraviolet absorbing material in the extract, which might interfere with the quinone estimation.

(ii) Quinone purification.

Quinone, extracted from the bacterial membrane, was purified by absorption - desorption chromatography (Brodie 1963, Dunphy and Brodie 1971a).

A pyrex column ($\frac{1}{2}$ inch diameter), containing 10 inches of silica gel (Kiesel gel 60 Art 7734, mesh size 70-230 ASTM) was used.

The silica gel was suspended in redistilled light petroleum (bp 40-60°C), allowed to sediment and the fine particles were removed, twice. Then it was added, as a slurry, into the pyrex column, to a height of 10 inches. Glass wool plugged the bottom of the column.

the silica
Petroleum was run through the column until ⅄ had settled, and then a small piece of glass wool was placed on top of the column.

The sample was added in 2ml of light petroleum, and washed in with three 2ml portions of petroleum. Petroleum, in 50ml portions, containing increasing amounts of peroxide free diethyl ether (0, 0.5, 1.0, 1.5 and 2.0% v/v) was run through the column. The quinone eluted in the 2·0% fraction, which was collected. The ultraviolet absorbance of the effluent was measured, with an LKB Uvicord optical unit (Type 4701A, fixed wavelength 254nm) with an LKB control unit (Type 4701A). The optical unit was connected to a TOA electronic polyrecorder, model EPR 2TB.

The quinone fraction was evaporated down under nitrogen, and Quinone is destroyed by light, so the column and collecting dissolved in ethanol before estimation vessel were screened from light, by covering with aluminium foil.

(iii) Estimation of menaquinone (based on method of Kroger and Dadak 1969).

Menaquinone was determined spectrophotometrically, by means of a Hilger and Watts Uvichem H 1620 single-beam spectrophotometer. The absorbances at 265 and 289 nm, of the oxidised and reduced quinone, were measured, and the differences used to find the amount of quinone, as shown below. As extracted, the quinone was oxidised, and it was reduced by the addition of sodium borohydride, in the presence of acetate buffer, to avoid auto-oxidation under alkaline conditions. 0.04ml of 0.4M acetate buffer, pH 5.4, was added to the (i.e. the petroleum:ether 200:4 fraction, evaporated down under nitrogen, and dissolved in ethanol), ethanolic extract, followed by approximately 0.5mg of sodium borohydride. The quinone was in 3ml absolute alcohol. These values are higher than those recommended by Kroger and Dadak (1969), as their values, for acetate buffer and sodium borohydride, did not give complete reduction. Kroger and Dadak (1969) showed

that the molar difference absorbance coefficient of menaquinone at 265-289 nm is:

$$(\varepsilon \text{ red} - \varepsilon \text{ox})_{265} - (\varepsilon \text{ red} - \varepsilon \text{ox})_{289} = -14.7 \text{mM}^{-1}.\text{cm}^{-1}$$

(iv) Reversed phase TLC for menaquinone.

Thin layer plates were prepared from Kieselguhr G, (30g of Kieselguhr G and 60ml of distilled water). The gel layer was 0.5mm thick. The plates were left for 40 minutes, and then activated by heating for 1 hour at 110°C. After cooling, the plates were dipped in 5% (w/v) liquid paraffin in redistilled light petroleum (bp 40-60°C), and the petroleum was evaporated.

The samples were added to the plate, and the plate was run in acetone-water (95:5 or 90:10 v/v) saturated with 5% (w/v) liquid paraffin. After the run, which was 15cm, the plates were left for the solvent to evaporate, and then examined under an ultraviolet lamp. The quinone appears as a dark spot, and was eluted from the plate, by scraping the gel from the plate and eluting the quinone with light petroleum, which could then be taken for radioactive estimation.

(f) Carotenoid extraction and determination.

(i) Carotenoid extraction.

Carotenoid was extracted in the acetone-methanol used to extract the quinone. When the quinone was further extracted with light petroleum, the less polar carotenoids were also extracted. They were separated from the quinone on the purification column (section 2f(ii)). The quinone eluted from the column in 2% ether in light petroleum (v/v), and the carotenoid was eluted in methanol, along with other polar lipid material, such as polyprenols, menaquinone precursors and possibly mevalonolactone.

(ii) Carotenoid determination.

Carotenoid was determined spectrophotometrically. The absorbance at 440nm was measured. Using a 1% extinction co-

efficient of 2500 (that of a carotenoid of average molecular weight), the concentration of carotenoid can be determined as follows:

$$A_{440} = \mathcal{E}_{1\%} \times c \times d$$

$\mathcal{E}_{1\%} = 2500$ d = path length = 1cm

c = concn of carotenoid (as fraction of 1% solution)

$$c = \frac{A_{440}}{2500}$$

$$\text{Carotenoid in mg} = \frac{A_{440}}{2500} \times \frac{v}{100} \times 1000$$

$$= \frac{A_{440}}{2500} \times v \times 10$$

v = volume of carotenoid in methanol

(g) Determination of radioactivity.

(i) Scintillant.

(a) For use with radioactive material in an organic solvent, 0.4g PPO (2, 5-diphenyloxazole) and 0.01g POPOP (1,4-di/2-(5-phenyloxazolyl)/ benzene) were dissolved in 100ml of toluene. The solution was left at 4°C overnight for the POPOP to dissolve completely. 10ml of this solution, and 1.0ml of the radioactive solution were pipetted into each vial, and mixed, before counting.

(b) For use with radioactive material in an aqueous solution.

The above mixture of PPO and POPOP in toluene was mixed, in a 2:1 (v/v) ratio, with the detergent Triton X-100. The triton had previously been purified by shaking with silica gel for 30 minutes, and then straining through glass wool. 10ml of the triton scintillant mixture and 0.5ml of radioactive solution were added to each vial and mixed well, before counting.

(ii) Counting

The activity in the vials was assayed in a Packard Tri-carb liquid scintillation counter, model 3375, fitted to a teletype. Previously the vials and scintillation fluid alone had been counted, to give a background reading. Blanks, of the solvent alone, with no radioactive material were also counted.

The background counts were usually counted for a period of 50 minutes, which gave a standard deviation of 7.5%. Background counts were usually of the order of 20 counts per minute, $\equiv 20 \pm 1.5$ counts per minute. The samples were counted for fifty minutes also. Vials with high activity had a 0.1% standard deviation, and those with lower activity, 2.5%. Although theoretically, 2 counts above background would be outside the counting error, other errors may occur, such as measurement of sample, so counts less than 5 above background were regarded as not significant.

Problems can arise when the radioactive substance e.g. carotenoid, can quench. Quenching reduces the efficiency with which the scintillations are detected by the photomultiplier. So a quench curve was constructed. A known amount of carbon-14 was added to each vial, and varying amounts of beta-carotene added, to give a range of quenching. The vials were compared with an external ^{14}C standard and a machine standard, consisting of americium-241 and radium-226. The counting efficiency at different levels of quenching can be plotted, and from the curve, the correction for quenched samples can be determined, to give comparable results.

In all experiments one 500ml flask of culture was used for each set of conditions. Of this one third of the quinone extracted was used for radioactive estimation.

(h) Phytoene.

(i) Preparation from tomatoes.

$1\frac{1}{2}$lb of semi-ripened tomatoes were cut into pieces and ground using a pestle and mortar. The juice was separated, by straining through muslin, and the remaining pieces were ground with acetone and methanol (approximately 50ml; 1:1 v/v), to extract the carotenoids. The coloured solution was separated from the pieces of tomato by straining through muslin. Eight or nine extractions were carried out, until the remaining tomato pieces were nearly colourless.

The acetone-methanol solution was repeatedly extracted with redistilled light petroleum (bp 40-60°C), until the acetone-methanol extract was colourless. The combined light petroleum extracts were dried over anhydrous sodium sulphate, the solution decanted off, and evaporated to dryness, under nitrogen. The phytoene was separated from the other carotenoids by column chromatography. (Brockmann Activity = 1)

Alumina (100-240 mesh) was suspended in redistilled petroleum (bp 40-60°C), allowed to settle, and the fine particles were removed, twice. The bottom of a glass column (diameter 5cm) was plugged with glass wool, and the prepared alumina poured into it, as a slurry, to give a column 15cm long. The extracted carotenoids were added to the column in 3ml of petroleum and washed in with two 3ml portions of light petroleum. The column was eluted with successive additions of 50ml portions of light petroleum (bp 40-60°C), 0.75%, 1.5% diethyl ether in light petroleum, and then 80ml of 4% diethyl ether in petroleum. 20ml fractions were collected and

examined spectrophotometrically.

The phytoene runs just behind phytofluene, which is fluorescent, and both were eluted in the 4% diethyl ether in light petroleum fractions.

3. RESULTS.

A. DIFFERENCES BETWEEN WHITE AND YELLOW STRAINS OF MICROCOCCUS LYSODEIKTICUS.

Many workers have found differences between wild-type and carotenoidless-mutant strains of various bacteria; for example Corynebacterium poinsettiae (Kunisawa and Stanier, 1958), Sarcina lutea (Mathews and Sistrom 1959, Prebble and Huda 1973) and Acholeplasma laidlawii (Rottem et al 1968). The differences they observed were in photosensitivity of cell suspensions and membrane enzyme activity, but only in the presence of visible light and oxygen. In many cases an exogenous photosensitizer was used. From these and other results, the major role of carotenoid, in non-photosynthetic bacteria, has been suggested as a protector against photo-oxidative damage to the bacterium.

(a) Respiratory activity.

In this laboratory, using M. lysodeikticus, it was observed that carotenoidless-mutant cultures, kept in reserve in the cold room, lost viability much more rapidly than the wild-type cultures. These cultures were kept in the dark. This suggested that lack of carotenoid had a detrimental effect on bacteria, without irradiation.

An examination of several properties of the yellow wild-type and white carotenoidless-mutant strains has therefore been made, in order to determine the reasons for this difference. The first aspect to be studied was respiratory activity.

(i) Over a four day growth period.

Cells of both white and yellow strains were grown, over a period of days, in the dark, and their malate and NADH oxidase

activities measured (Table 1). In the white mutant the activities of these enzymes were lower, after a one day incubation, and they also decreased much more rapidly over the four day period, than did the enzyme activities of the wild-type cells.

NADH oxidation by whole cells is a little unexpected, as the NADH oxidase is believed to be on the inside of the membrane, impermeable to NADH. Either the M. lysodeikticus membrane is permeable to NADH or the catalytic site on the dehydrogenase is on the outside of the membrane.

(ii) After an overnight incubation.

A fuller examination of respiratory activity, using malate, lactate, succinate and NADH,on membranes from white and yellow cells, showed that there were significant differences in respiratory ability between the two strains (Table 2). It can be seen that, except for the NADH oxidase, all the other enzyme activities were significantly different. The lactate and succinate oxidase activities were higher in the white membranes. Malate oxidase, malate dehydrogenase, and NADH oxidase activities were lower in white membranes, but the high variability in NADH oxidase activity measurements prevented this difference in activity from being significant.

It has been found, using S. lutea (Anwar and Prebble 1977) and Mycobacterium phlei (Asano and Brodie 1964) that while malate oxidation requires quinone, the oxidation of succinate does not. Malate dehydrogenase activity would be expected to be the same,in both white and yellow membranes, and as it is not, this suggests that malate oxidation is depressed in white membranes. The lower activity of lactate and succinate oxidase, in yellow membranes, could

Table 1

Respiratory activity of white (strain UVI) and yellow cells, using malate and NADH as added substrates, over a period of 4 days.

	MALATE OXIDASE ACTIVITY		NADH OXIDASE ACTIVITY	
Time (days)	wild-type	UVI strain	wild-type	UVI strain
1	0.068	0.032	0.31	0.041
2	0.072	0.017	0.20	0.0011
3	0.028	0.0034	0.064	0.0014
4	0.022	0.0024	0.052	0.00078

Cells were grown in a shaking incubator at 30°C overnight. Then the cells were spun down, and resuspended in 0.05M phosphate buffer pH 7.4, containing 5mM magnesium chloride. Activities were measured with an oxygen electrode (see methods) and expressed as microg. atoms of oxygen uptake per minute per 10^{10} cells.

Each result is the mean of two estimations. The results were corrected to 2 significant figures.

Table 2

Respiratory activity of yellow and white membranes, from overnight cultures, using malate, lactate, succinate and NADH as added substrates

Enzyme Assayed	Yellow: mean value of activity	Yellow: no. of exp's from different preparations	UVI strain: mean value of activity	UVI strain: no. of exp's from different preparations	Probability of significant difference between the two strains
MALATE OXIDASE	0.034	16	0.016	21	.002 (0.2%)
LACTATE "	0.0040	12	0.075	11	> .001
SUCCINATE "	0.0022	12	0.029	11	> .001
NADH "	0.15	13	0.040	13	0.2
MALATE DEHYDROGENASE	0.052	6	0.012	12	> .001

Cells were grown overnight at 30°C. Membranes were prepared by method 1. Activities, measured at 30°C and pH 7.0, with an oxygen electrode (see methods), are expressed as (microg. atoms of oxygen uptake per minute) per mg. protein.

Results are the mean value of several experiments on different preparations, and are corrected to 2 significant figures.

be due to quinone having a regulatory role on dehydrogenase activity, higher amounts depressing oxidation, although the quinone is probably not directly involved in the oxidation of these substrates. From the results in Table 2, quinone does not appear to be involved in lactate oxidation, although Lukoyanova and Kovoleva (1975) found that irradiation of membranes, with ultra-violet light, resulted in a 90% inhibition of lactate oxidase in M. lysodeikticus, indicating that the quinone is involved.

(b) Viability.

In an obligately aerobic organism, such as M. lysodeikticus, loss of respiratory activity would lead to a loss of viability, as observed with cells stored in the cold room. The viability of yellow and white cells was examined over a period of four days growth, at both 26° and 37° C (Figs. 9, 10).

The media were inoculated at zero time, and the viability of the inoculum was measured. Cells were grown at 26°C and 37°C, and samples taken after 1, 2,3 and 4 days growth, to determine viability. The viability of the yellow inoculum was higher (65%) than that of the white (48%). If all the cells had been viable, one hundred should have grown per plate.

At 26°C the viability of the yellow cells fell linearly, to two thirds of the value at zero time, after four days. The loss of viability of the white cells, at 26°C, was more rapid. After three days only 10% of the original viable cells were still viable.

At 37°C, both yellow and white cells were less viable, probably because this temperature is above the optimum growth temperature

Figure 9

Viability of the white and yellow strains of Micrococcus lysodeikticus at 26°C, over a four day growth period.

The cells were grown in the dark at 26°C. Batches were removed after 1, 2, 3 and 4 days. These cells were spun down and resuspended in sterile distilled water, to give an optical density of 0.1 at 600nm. From cell count calculations, the number of cells per ml can be determined and an appropriate dilution made, to give 100 cells per 0.1ml. 0.1ml samples were plated out, in triplicate, and after a few days incubation at 26°C, the colonies were counted.

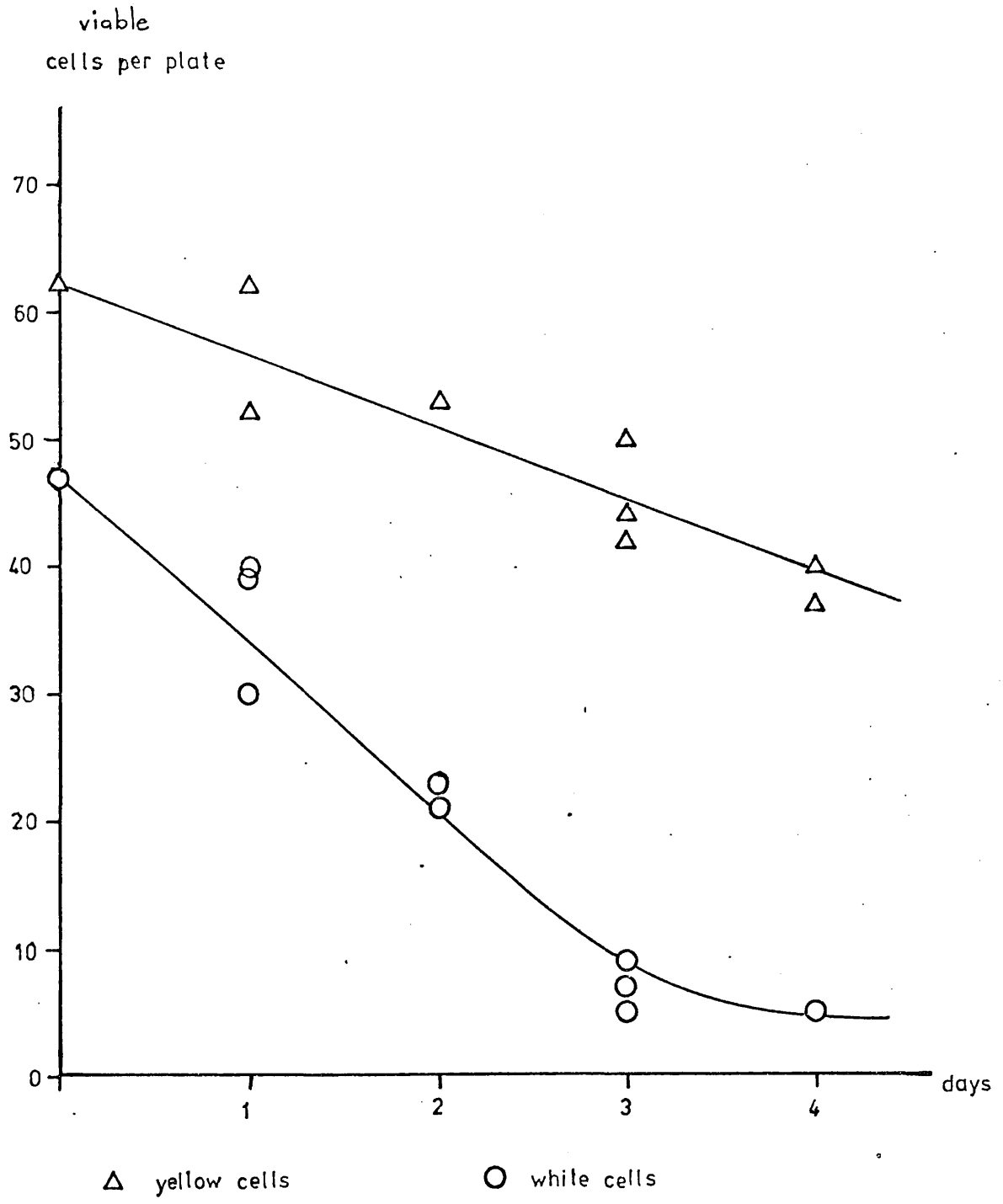
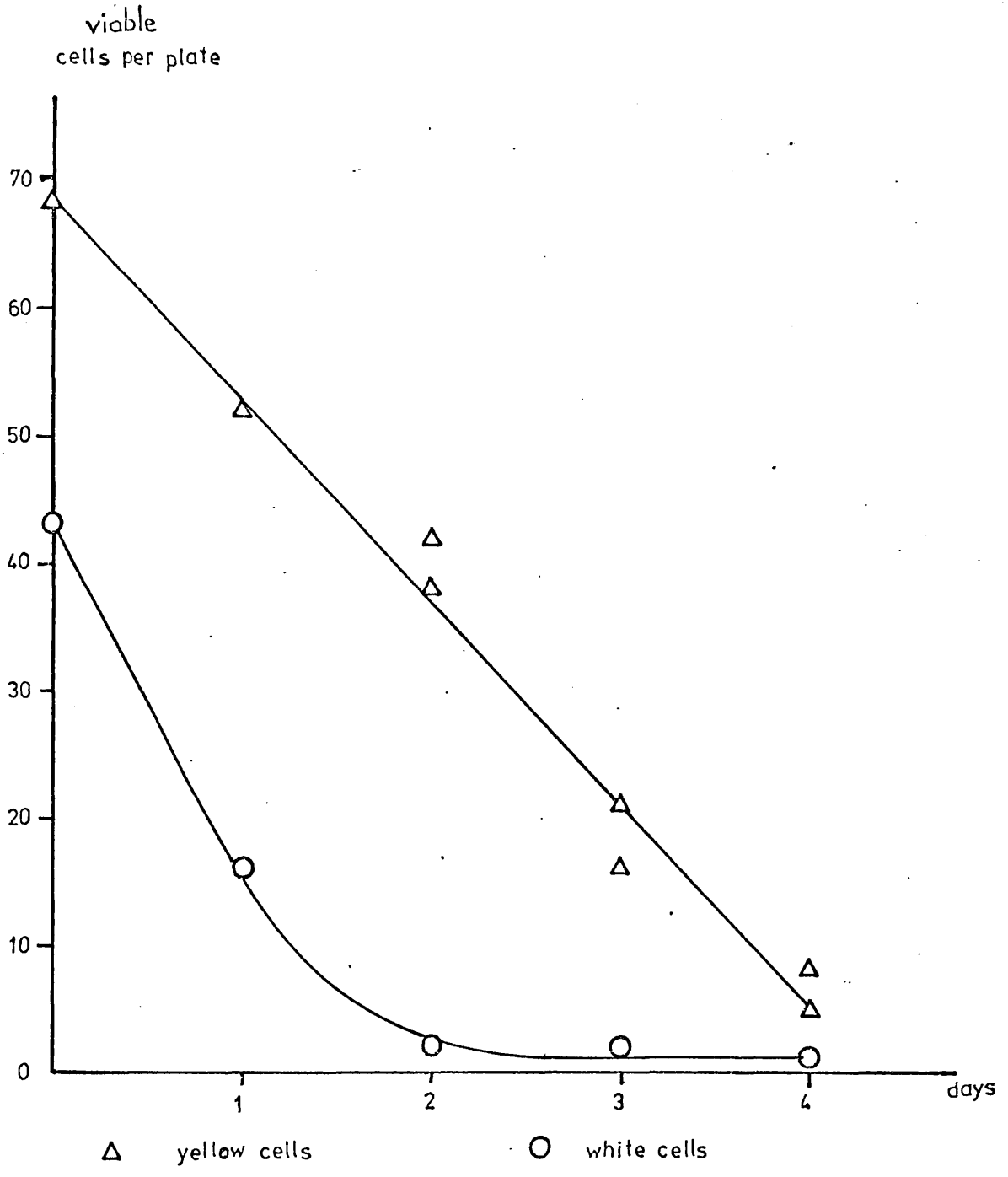

viable
cells per plate
70
60
50
40
30
20
10
0
1
2
3
4
days
yellow cells
white cells

Figure 10

Viability of wild-type and carotenoidless-mutant strains of M. lysodeikticus at 37°C, over a four day growth period.

Cells were grown in the dark at 37°C. Batches were removed after 1, 2, 3 and 4 days. These cells were spun down and resuspended in sterile distilled water, to give an optical density of 0.1 at 600nm. From cell count calculations, the number of cells per ml can be determined and an appropriate dilution made, to give 100 cells per 0.1ml. 0.1ml samples were plated out, in triplicate,and after a few days incubation at 37°C, the colonies were counted.


viable
cells per plate
70
60
50
40
30
20
10
0
1
2
3
4
days
yellow cells
white cells

of 27°C (Bergey 1957). With yellow cells, the viability dropped linearly to only 7% of the original, after four days. The viability of the white cells fell considerably, and after two days there were practically no viable cells left.

These results are consistent with the low respiratory activity of the white cells, after three and four days.

(c) Quinone.

(i) Levels in membranes from cells cultured overnight.

From the respiratory activity studies, it was indicated that the quinone level might be low in the carotenoidless-mutant cells. Preliminary studies measured the amount of quinone in white and yellow membranes, from cells grown in overnight cultures (Table 3). In four different preparations, the quinone level in white membranes was only one third that in yellow membranes.

The low level of quinone in the white-carotenoidless mutant (strain UVI) is a significant result, but it must be shown to be a characteristic of all the carotenoidless mutants (Table 4). All four carotenoidless-mutant strains, (including strain UVI), showed lower quinone levels than the yellow wild-type cells. The levels vary, possibly due to the difference in mutations, indicated by the fact that two accumulate phytoene and the other two do not (see section 3B(a)). Strains UVI and UV3, which do not accumulate phytoene, have similar quinone levels. As all the carotenoidless mutants show low quinone levels; this is presumed to be a specific consequence of lack of carotenoid.

Table 3

Quinone levels in yellow wild-type and white UVI strain membranes, prepared from cultures grown overnight.

	Yellow wild-type	White UVI strain
Mean value of quinone, millimoles per mg. protein ($\times 10^{-6}$)	1.52	0.49
Standard deviation ($\times 10^{-6}$)	0.07	0.05

Cultures were grown overnight, and then the cells were spun down. Membranes were prepared by method 1. The quinone was extracted and estimated as in Methods.

Quinone values are the mean values of four experiments on different preparations.

Table 4

Quinone levels in cells of wild-type and the four ultraviolet carotenoidless mutants.

		Quinone, micromoles per 500ml of culture	Number of cells x 10^{10} per 500ml of culture	Quinone per cell, micromoles x 10^{-11}
Yellow	Wild-type	22.9	24.6	9.31
w/strain	UV 1	7.96	21.0	3.79
w/strain	UV 2	6.12	30.1	2.03
w/strain	UV 3	7.35	19.2	3.77
w/strain	UV 4	5.51	13.8	4.00

Cells were grown overnight, and then spun down. A small sample was taken for cell estimation (methods). The quinone was extracted and estimated (as methods).

(ii) Quinone levels over a 40 hour growth period, in a large scale batch culture.

The previous results led to the examination of quinone levels over a 40 hour growth period, in conjunction with estimations of the number of cells, both total and viable (Figs 11, 12). Both white (strain UVI) and yellow cells were used, and the experiment was carried out with a 12 litre culture (see methods).

With the yellow cells, the expected growth curve, with increasing quinone per cell in the logarithmic phase, was observed. However with white cells, during growth, the amount of quinone per cell decreased. Clearly the white mutant was unable to meet quinone requirements, which would contribute to the low number of viable cells found. Problems were encountered when measuring the number of viable cells. The shape of the plots was as expected, an increase in the logarithmic phase, followed by a decrease in the stationary phase, as was the fact that the white cells were less viable. But if the results were calculated per 1ml of original suspension, then there were more viable cells than total cells, the latter being based on turbidity measurements calibrated with a mature culture. It is believed that this was due to clumping of the cells, although suspensions were lightly homogenised. In shake cultures, clumping varies, depending on the growth phase, with maximum clumping in the logarithmic phase, when the cells are dividing. The results for viable cells, both white and yellow, are plotted as a percentage of the maximum number obtained in yellow cells.

The results clearly show differences in quinone levels between

Figure 11

Quinone levels, viable cells and total cells, over a period of 40 hours, in the yellow wild-type strain.

Twelve litres of broth was inoculated and the cells were grown at 30°C in a New Brunswick fermentor (see methods). The culture was aerated and agitated constantly. An anti-foam agent was added continuously, at a slow rate (less than 100ml total addition). 300ml batches of culture were removed aseptically at suitable periods. A small volume was used to determine the total cells per ml (based on turbidity measurements). The viable cells per ml were determined by dilution to give 100 cells per 0.1ml (using turbidity measurements) and plating out 0.1ml samples, in triplicate. The plates were incubated for a few days at 26°C and the colonies counted.

The remainder of the cells were spun down and resuspended in 5-6 ml 0.05M phosphate buffer pH7.0. The quinone was extracted and purified on a silica gel column (as methods). Samples of quinone were then estimated (as methods).

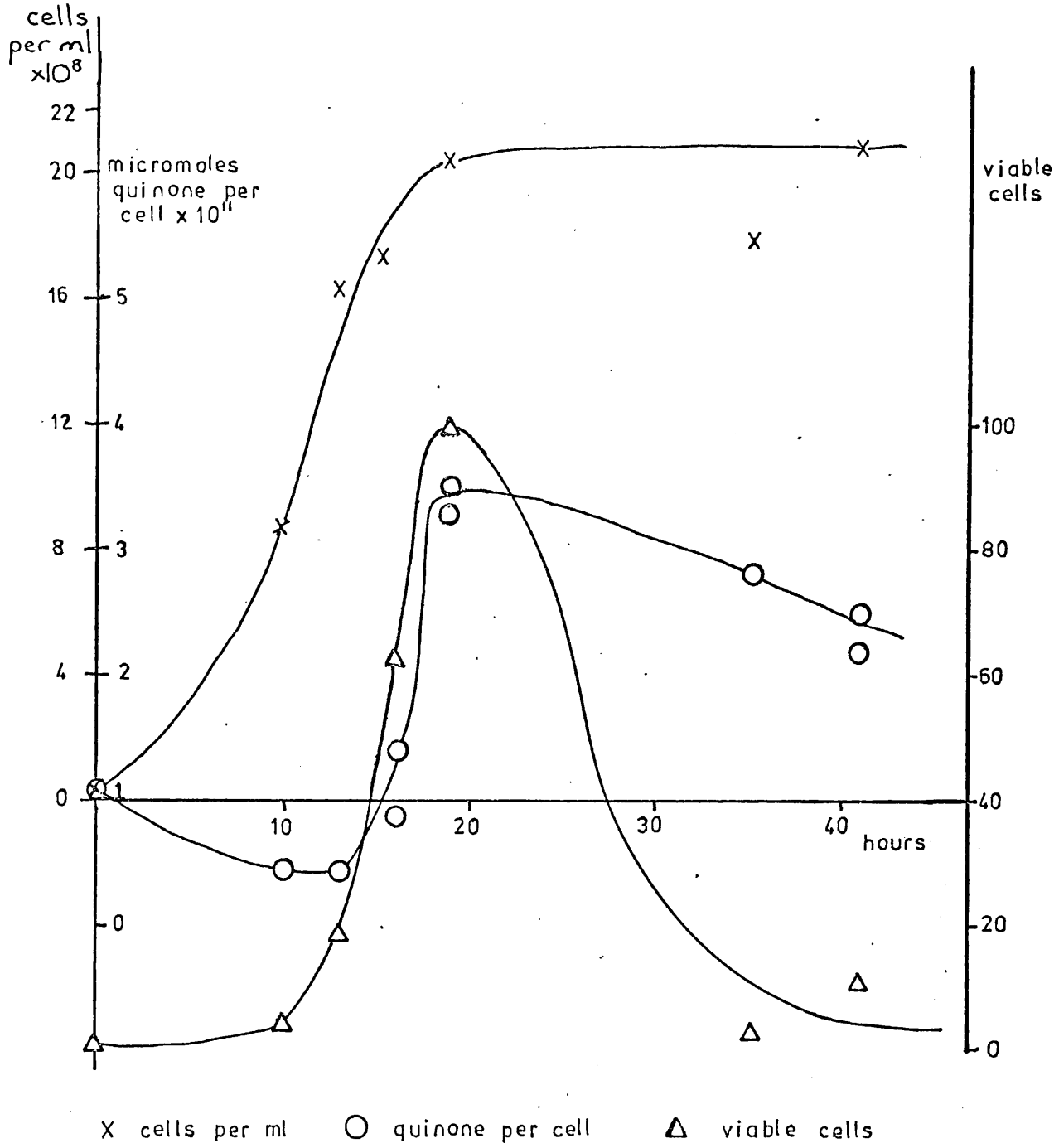
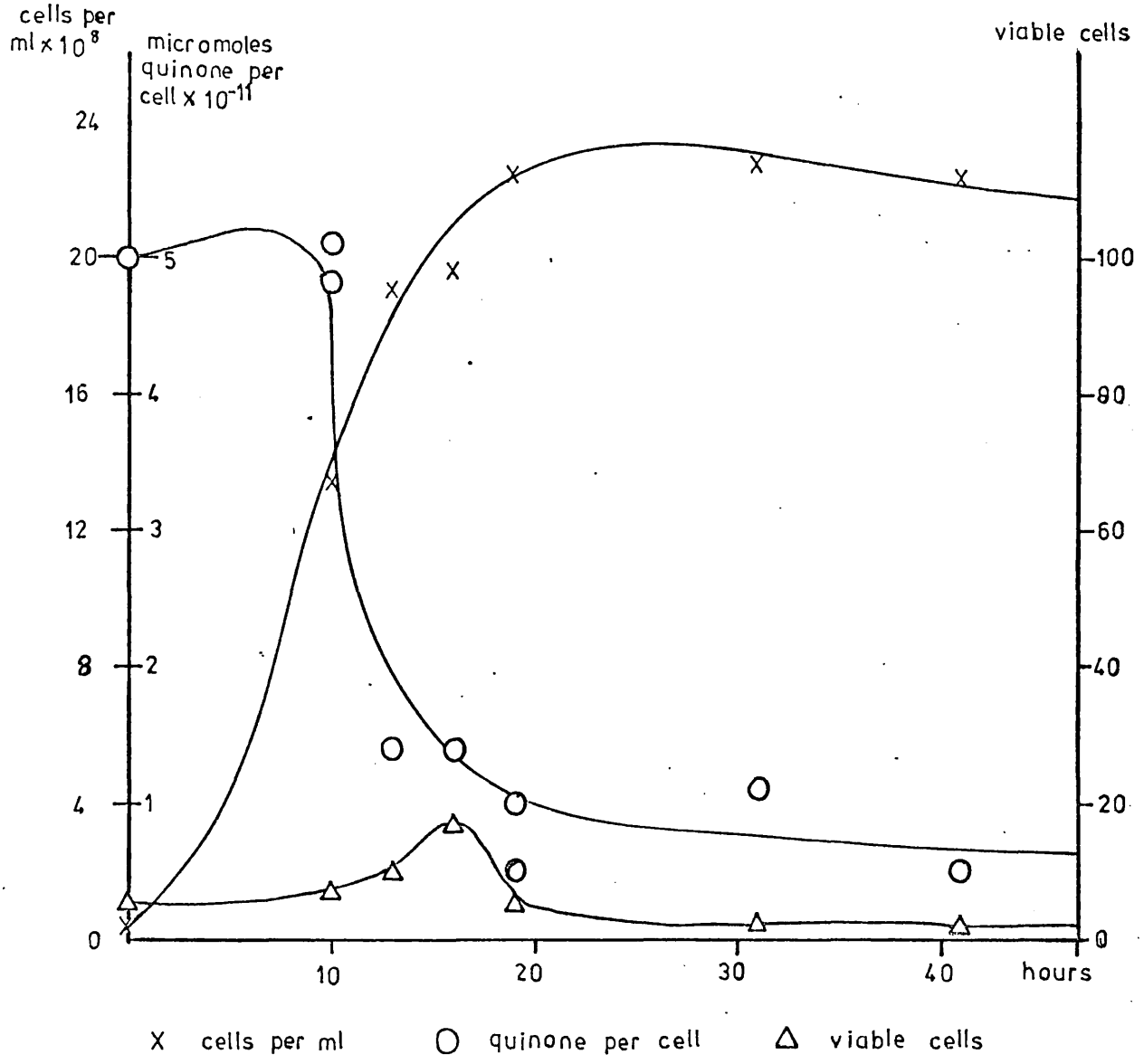

cells per ml x10⁸
22
20
16
12
8
4
0
micromoles quinone per cell x 10¹¹
5
4
3
2
1
0
viable cells
100
80
60
40
20
0
10
20
30
40
hours
X cells per ml
quinone per cell
viable cells

Figure 12

Quinone levels, viable cells and total cells, over a period of 40 hours, in the white-mutant strain.

Twelve litres of broth was inoculated and the cells were grown at 30°C in a New Brunswick fermentor (see methods). The culture was aerated and agitated constantly, so an anti-foam agent was added continuously, at a slow rate (less than 100ml total addition). 300ml batches of culture were removed, aseptically, at suitable periods. A small volume was used to determine the total cells per ml (based on turbidity measurements). The viable cells per ml were determined by dilution to give 100 cells per 0.1ml. Samples (0.1ml) were plated out, in triplicate. The plates were incubated for a few days at 26°C and the colonies counted.

The remainder of the cells were spun down and resuspended in 5-6ml 0.05M phosphate buffer pH7.0. The quinone was extracted and purified on a silica gel column (as methods). Samples of quinone were then estimated (as methods).


cells per ml x 10^8
micromoles quinone per cell x 10^-11
viable cells
24
20
16
12
8
4
0
5
4
3
2
1
0
100
80
60
40
20
0
10
20
30
40
hours
X cells per ml
O quinone per cell
Δ viable cells

the two strains, and also the marked loss of viability of the white cells. This correlates with the low respiratory activity in white cells. Thus,there are differences between the wild-type and carotenoidless-mutant strains, when grown in the dark.

(d) Membrane.

Other differences between the two strains were found, which are of interest, although some of these were not reliably reproducible.

If carotenoid, which is in the membrane (Mathews and Sistrom 1959a),is absent, then it seems likely that there will be differences in the membrane properties, and possibly also the structure.

(i) Vesicle preparation.

A reproducible difference between yellow and white cells was observed during vesicle preparation. The yellow strain formed vesicles, which gave the expected P/O ratio of 0.3 (Ishikawa 1967). However, by the same method, vesicles could not be formed from white cells. The material, from white cells,was precipitated in the low speed spin, prior to the high speed spin, which should precipitate the vesicles.

The method involved the bursting of protoplasts in 0.25M sucrose. The protoplasts were prepared by the action of lysozyme in isotonic sucrose (IM). Perhaps, due to membrane differences, the protoplasts were not stable in molar sucrose, and disintegrated into pieces, which could not form vesicles. Or, more likely, there was an increase in permeability in white cells. This could result in the protoplasts splitting to form large membrane pieces, which could not form vesicles. These large membrane pieces would preci-

pitate during the low speed spin.

Leaf Huang and Haug (1974), using Acholeplasma laidlawii, found that increasing the carotenoid levels in the membrane caused increased rigidity, up to a certain level, above which the membrane lipids were altered to increase lipid fluidity. They also found that partial carotenoid loss resulted in an increased membrane fluidity and higher glycerol permeability.

(ii) Peroxidation of lipids.

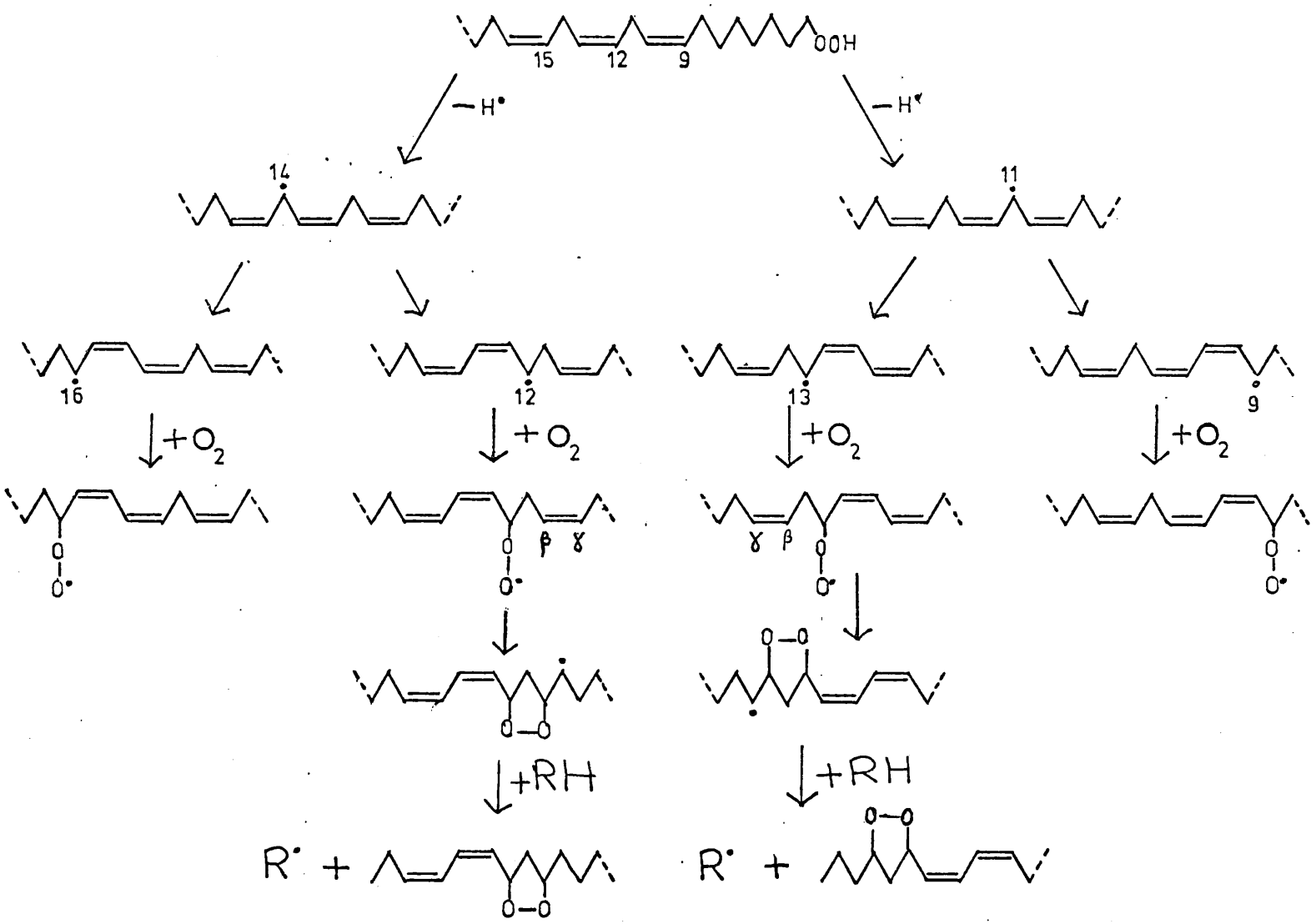
Another proposed function for carotenoid is as a protector of membrane lipids against peroxidative damage. Anderson and Krinsky (1973) using liposomes as a model membrane system, showed that carotenoid can protect the membrane lipids. They estimated lipid peroxidation by measuring the malonaldehyde (MDA) formed. MDA is estimated by condensation with thiobarbituric acid (TBA), to give a coloured product, with an absorbance maximum at 548nm (Placer et al 1966). Dahle showed that for a positive TBA reaction, the lipids must contain at least three double bonds, (Dahle et al 1962). The mechanism of MDA production, proposed by Dahle, is shown in Fig. 13a, b. For MDA to be formed, a 5-membered ring must be formed first. This will only occur if there is a double bond in the β-γ position to the oxygen (Fig. 13a). The mechanism explains observations, that there are two types of peroxides formed from polyunsaturated fatty acids.

The fatty acids of M. lysodeikticus would not be expected to give a positive TBA test. However, Leaf Huang and Haug (1974) have shown that lipids can be modified, by bacteria, to regulate lipid fluidity, and De Siervo and Salton (1973) have found that the lipids of

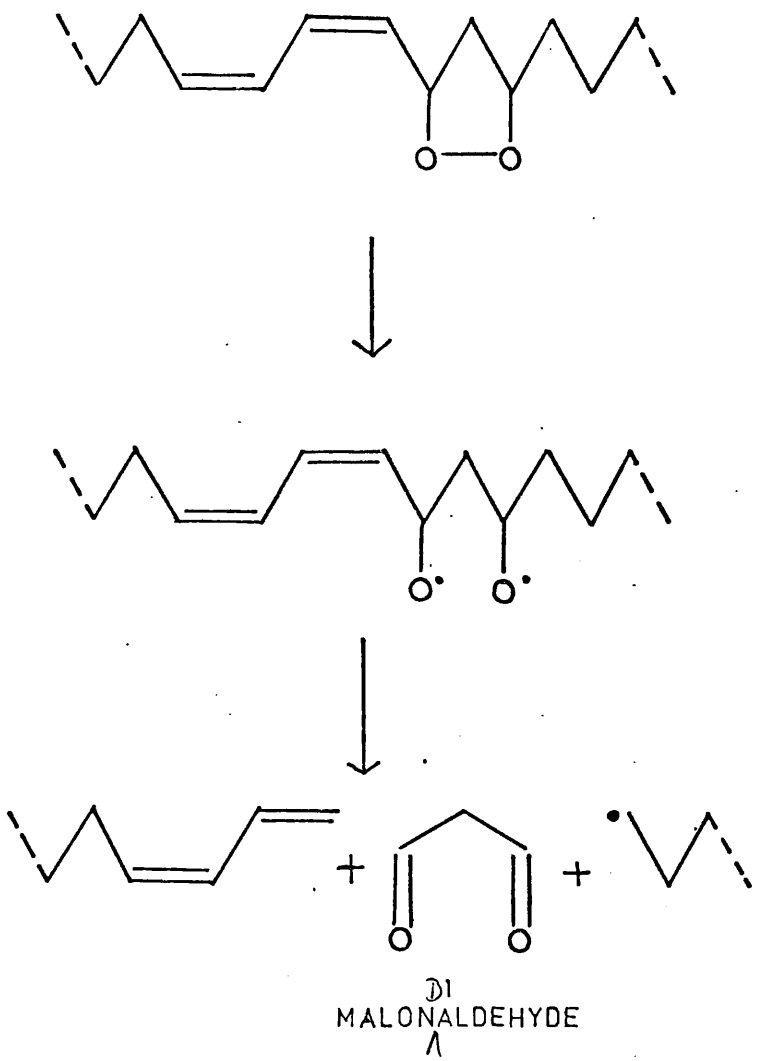
Figure 13 a, b
di

Proposed mechanism for malonaldehyde (MDA) production, after peroxidative damage to membrane lipids.

a.



b.



MALONALDEHYDE

M. lysodeikticus alter in their proportions,during growth. Therefore, the TBA test (Placer et al 1966) was tried,using white and yellow membranes. With yellow membranes, there was always a negative result. However, on two occasions, with white membranes, a pink colour, with an absorbance maximum at 548 nm, was obtained, although on other occasions the result was negative. The white membranes gave the following results: 10.1 and 8.4 x 10^{-2} nanomoles MDA per mg protein.

(iii) The salt effect.

The last effect, probably also attributable to membrane differences, was found by chance, when determining whether an addition of sodium chloride increased lysis in lysozyme treated cells. In the yellow strain, sodium chloride, and other salts, solutions induced a steady decrease in absorbance (at 340 nm) of cell suspensions, either in the presence or absence of lysozyme (Table 5). This system was reproducible for several weeks, using overnight or 2-day-old shake cultures, or suspensions made from agar-slope cultures. Later attempts to reproduce these results failed. However the effects of salt solutions were never observed in the carotenoidless strain. The effect is believed to be due to permeability differences between the two strains.

(iv) Summary.

Thus,there are differences between the membranes of the two strains, due to lack of carotenoid. The lack of ability to form vesicles, in the white cells,was completely reproducible, and is probably due to increased permeability. Leaf Huang and Haug (1974) have shown that loss of carotenoid increases membrane fluidity and permeability.

Table 5

Rate of decrease of absorbance, at 340nm, of yellow cells suspended in varying salt solutions.

Salt solution used	Rate of decrease (absorbance units per five minutes) of a suspension of 0.5 absorbance at 340nm
sodium chloride	0.20
potassium chloride	0.12
caesium chloride	0.04
lithium chloride	0.12
barium chloride	0.03
calcium chloride	0.03
magnesium chloride	0.04
sodium nitrate	0.12
potassium nitrate	0.12
calcium nitrate	0

A bacterial suspension in 2ml of distilled water was prepared. Its absorbance at 340nm was recorded. Then 0.9ml distilled water and 0.1ml 10% salt solution was added, with stirring, and the rate of change of absorbance recorded.

Results are corrected to 2 significant figures.

The effect of salt solutions could be due to the permeability differences. MDA production was observed twice in white membranes, but was not reproducible. Anderson and Krinsky (1973) have shown that carotenoids can protect lipids from peroxidative damage, although they used very unsaturated lipids, which are very susceptible to peroxidative damage. The differences observed, due to lack of carotenoid, could be a consequence of membrane changes, as the quinone and enzymes studied are located in the membrane.

B. INVESTIGATION OF QUINONE SYNTHESIS AND BREAKDOWN IN CELLS.

The low levels of quinone in white cells may be due to a low rate of synthesis, a high rate of breakdown, or both. To study quinone synthesis and breakdown, a satisfactory method of purification was needed, especially when incorporation of radioactive precursors was investigated.

(a) Quinone content and purification.

Jeffries et al (1967) studied the menaquinones of aerobic Micrococcaceae. According to the Baird-Parker classification (Baird-Parker 1965), M. lysodeikticus is classified in Micrococcus Group 2, subgroup 7. Jeffries et al (1967) found that the Micrococci in this group contained a quinone with a spectrum identical to that of menaquinone-8. They used thin layer silica gel chromatography (solvent; light petroleum: benzene, 1:1 v/v), to separate the quinone from lipids. Then, using descending paper chromatography (reversed phase), with aqueous 85% (v/v) ethanol as solvent, they found that the extracted quinone did not quite co-chromatograph with pure menaquinone-8. They suggested that it may have an extra pair of hydrogen atoms, possibly making the second isoprenoid unit away from the ring fully saturated, menaquinone-8(H).

In this study, the quinone was extracted and purified on a silica gel column (methods) . The quinone, extracted from both white and yellow cells, was eluted in the 200:4 (v/v) light petroleum: ether fraction. This fraction, when evaporated down under nitrogen, and run on a reversed phase TLC plate, appeared as one spot under ultraviolet light. The solvent used was acetone: water (93:7, v/v), and

in this system the extracted menaquinone and pure menaquinone-8 co-chromatographed, with an R_f of 0.35, very similar to that found by Dunphy and Brodie (1971b), using the same solvent system (0.33).

However, a full examination of quinone was not undertaken, and it is possible that under these conditions, menaquinone-8 and menaquinone-8(H) would not have been separable.

If purified radioactively labelled quinone was chromatographed on a reversed phase TLC plate, 95% of the original activity could be eluted from the spot which co-chromatographed with pure menaquinone-8.

During the course of experimentation, it was found that if quinone, extracted from membranes, was measured before and after purification on the column, a loss was occurring. If pure vitamin K_1, menaquinone-7, or menaquinone-8 was run on the column, 85-90% was recovered in the quinone fraction. The lost quinone was found to elute in a 200:8 light petroleum:ether (v/v) fraction. The quinone-like compound, eluting in the 200:8 fraction, could be estimated by borohydride reduction. It could be a second quinone, of shorter chain length, or a breakdown product. However as there was a consistent, small percentage of the total quinone (10-15%), in both white and yellow cells, it is likely to be a second quinone. Because it was a small, but consistent component, it was not taken into account in the results.

The four ultraviolet mutants obtained were examined for phytoene accumulation. Two of the mutants (strains UV2 and UV3)

accumulated phytoene, which was determined spectrophotometrically, (absorbance maxima in light petroleum 275, 285 and 296nm, Liaaen-Jensen and Jensen 1971), and from its elution from the quinone purification column, as compared with phytoene extracted from tomatoes. However with strains UV1 and UV4, phytoene could not be detected spectrophotometrically. Phytoene was eluted from the silica gel column in the 200:2 (light petroleum: ether v/v) fraction. This fraction was found to contain radioactively-labelled material, using white cells (strain UV1), when quinone was labelled. If this fraction was evaporated down and run on a reversed phase TLC plate, several spots of radioactivity were found. Sixteen per cent of the radioactivity was found in a spot which co-chromatographed with phytoene (isolated from tomatoes). The position on the plate of the radioactivity is shown in Fig. 14.

The white mutant (strain UV1) might not accumulate large amounts of phytoene, if the block in carotenoid synthesis was before phytoene. However if it were a "leaky" mutant, this would mean that the enzyme which is blocked, is not completely inactive. So, phytoene and all the other carotenoids along the pathway would be formed, slowly. This could account for the phytoene and the other spots of radioactivity on the plate, as small amounts of phytoene and the other carotenoids would be present.

(b) Use of radioactively labelled precursors.

(i) Incorporation of [2-^{14}C]mevalonic acid into quinone and polar material including carotenoid in cells.

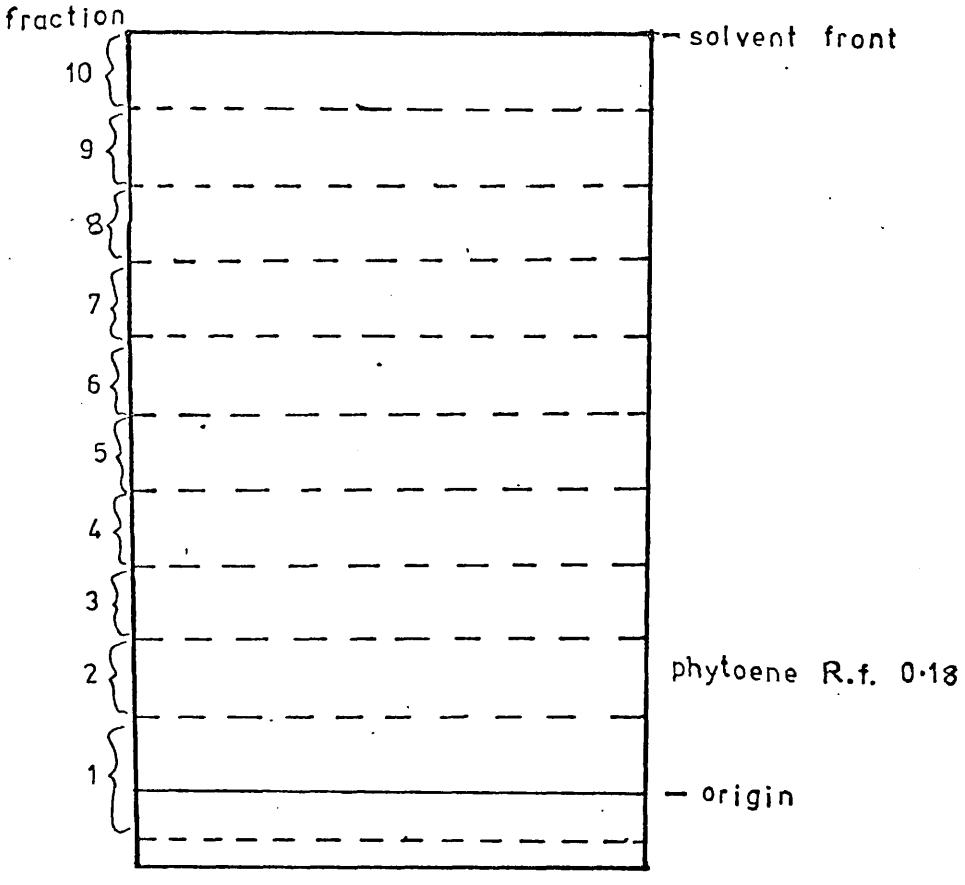
Mevalonic acid is the natural precursor of isoprenoid units, which form the quinone side-chain. Ramasarma and Ramakrishnan

Figure 14

Separation of radioactive material in the "phytoene" fraction (from white strain UVI) from the quinone purification column, by reversed phase TLC.

The "phytoene" fraction (200:2 light petroleum: ether v/v) was collected from the quinone purification column. A sample was run on a reversed phase TLC plate (see methods). The solvent was an acetone: water mixture (93:7 v/v) saturated with 5% (w/v) liquid paraffin. A purified phytoene sample (from tomatoes) was also run. After the run, the plate was divided into fractions as shown, the Kieselguhr removed and eluted with light petroleum (b.p.40-60°C). Samples from each fraction on the plate and the original fraction from the column were taken for radioactive counting (methods).

(a)


fraction
10
9
8
7
6
5
4
3
2
1
solvent front
phytoene R.f. 0·18
origin

(b)

Fraction Number	Counts per min	Percentage of total
1	0	0
2 (phytoene)	660	16
3	1195	30
4	870	22
5	240	6
6	53	1
7	28	0.7
8	0	0
9	0	0
10	70	2
		77.7

Total counts = 4010 per min (before running)

(1961) and Ramasarma et al (1965), using a wide variety of bacteria, were unable to incorporate labelled mevalonic acid into quinone. Instead they found the label in the neutral and esterified lipids.

However in this laboratory, with white and yellow cells suspended in phosphate buffer (0.05M, pH 7.0) and incubated with 5 μCi [2-^{14}C] mevalonic acid, incorporation of labelled mevalonic acid was achieved. The [2-^{14}C] mevalonic acid was always added as the lactone. In the yellow cells, label was also found in polar material, including carotenoids. There was much more incorporation into quinone in carotenoidless cells than in yellow, where the labelling was fairly low. Despite the high rate of synthesis of quinone, in white cells, from labelled mevalonic acid, the quinone levels remained low, showing that there is also a high rate of breakdown in white cells.

In Figs. 15, 16, the relationship between radioactivity, quinone and carotenoid, in fractions eluted from the purification column, can be seen. Most of the radioactivity was eluted in the quinone and polar fractions.

Despite the fact that Ramasarma and his co-workers have found difficulties in incorporating labelled mevalonic acid into quinone, the system described above, using white and yellow cells of M. lysodeikticus, effectively incorporates mevalonic acid into quinone. A difference between white and yellow cells is also apparent. The white cells incorporate labelled mevalonic acid very rapidly, whereas in yellow cells, the rate is very much slower.

(ii) Fate of labelled quinone in cells.

Having established that in M. lysodeikticus, [2-^{14}C] mevalonic acid was converted into quinone and other products and that there was

Figure 15

Position of quinone and radioactive counts eluting from the quinone purification column; the quinone having been extracted from white membranes.

The quinone was extracted from white membranes (as methods), in light petroleum, which was then evaporated down under nitrogen and added in 2ml petroleum to the purification column (see methods). It was washed in with three 2ml portions of petroleum. Approximately 50ml portions of light petroleum 200:1, 200:2, 200:3 and 200:4 (light petroleum : ether v/v) were run through the column. Fractions were collected (20-25ml each) and the radioactive counts and amount of quinone determined in each fraction. The positions of the solvent changes are indicated.

Labelled quinone prepared by: the incubation of cells (from 500ml culture) in phosphate buffer (0·05M pH7·0) and 2μCi [2-^{14}C] mevalonic acid, for 1 hour at 30°C with shaking (total volume 50ml). The cells were spun down, and quinone was extracted (as methods).

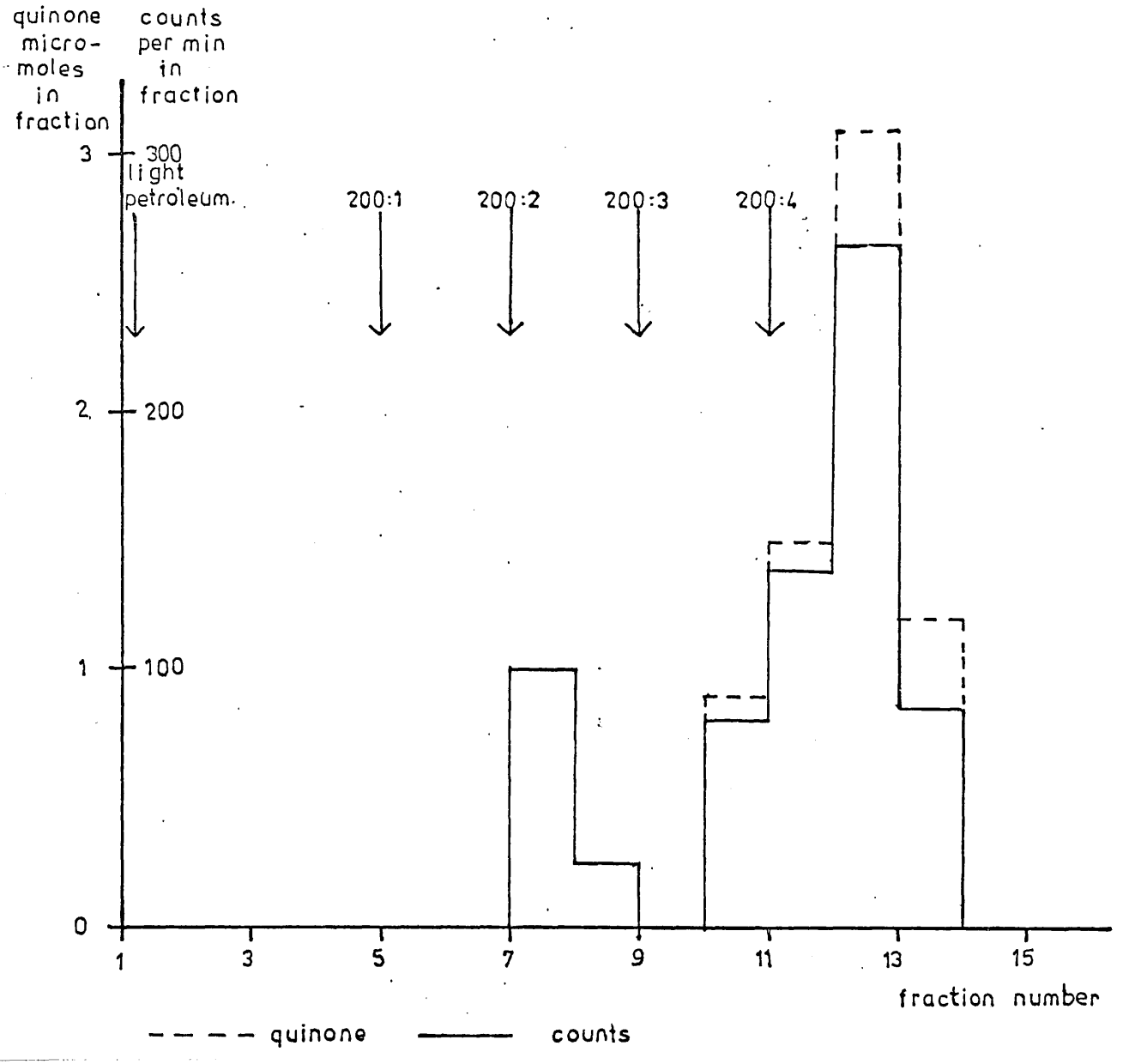

quinone micro-moles in fraction
counts per min in fraction
3
300
light petroleum
200:1
200:2
200:3
200:4
2
200
1
100
0
1
3
5
7
9
11
13
15
fraction number
quinone
counts

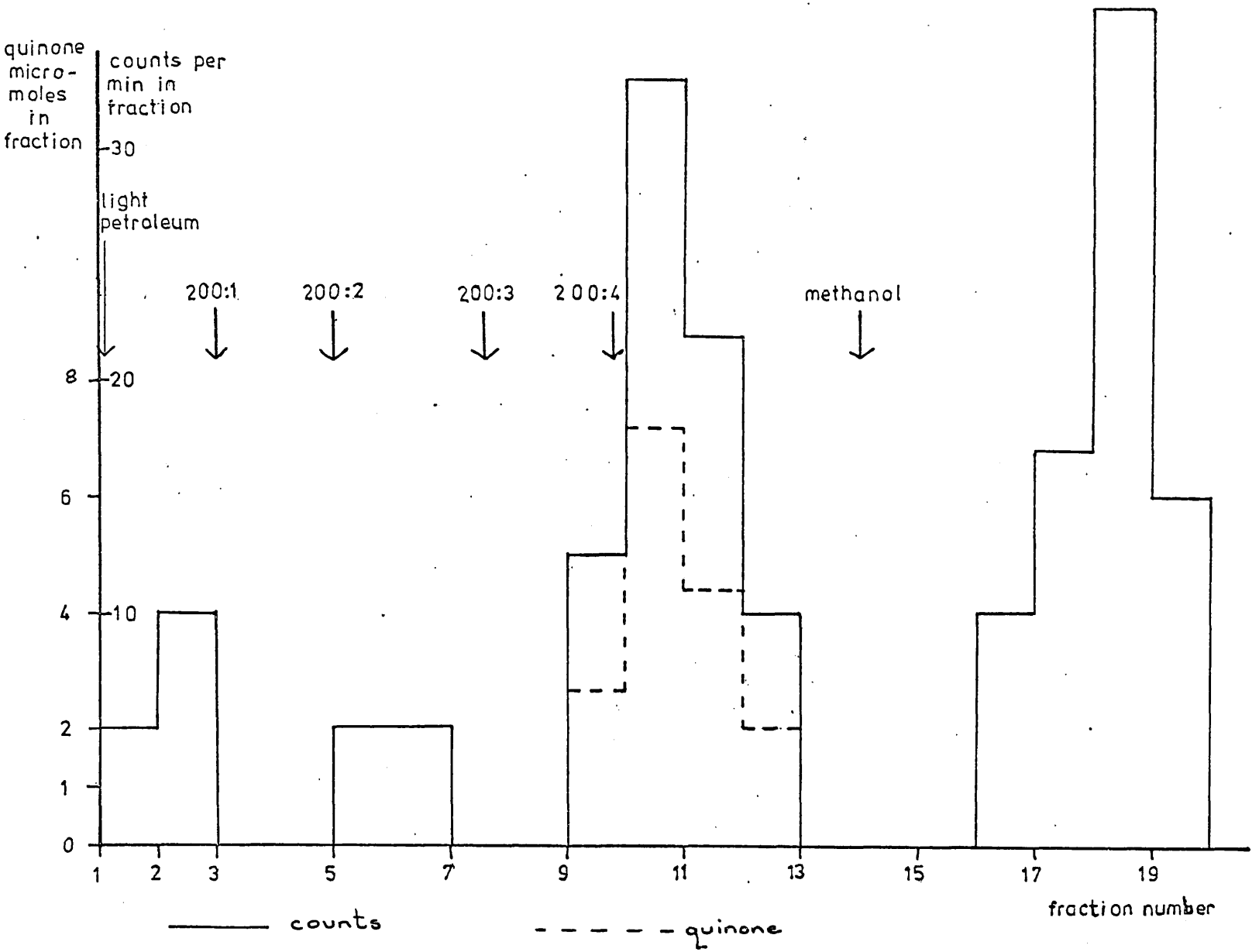
Figure 16

Position of quinone, carotenoid and radioactive counts eluting from the quinone purification column, the quinone and carotenoid having been extracted from yellow membranes.

The quinone and carotenoid were extracted from yellow membranes (as methods) in petroleum, which was then evaporated down under nitrogen and added in 2ml petroleum to the purification column (see methods). It was then washed in with three 2ml portions of petroleum. Approximately 50ml portions of light petroleum, 200:1, 200:2, 200:3 and 200:4 (light petroleum: ether v/v) were run through the column. Then 60-70ml of methanol were run through. Fractions were collected (20-25ml each) and the radioactive counts and amount of quinone determined in each fraction.

The positions of the solvent changes are indicated. Carotenoid elutes in the methanol fraction.

Labelled quinone, prepared by the incubation of cells (from 500ml culture) in phosphate buffer (0·05M pH 7·0) and 2μCi [2-^{14}C] mevalonic acid for 1 hour at 30°C, with shaking, (total volume, 50ml). The cells were spun down, and quinone was extracted (as methods).


quinone micro-moles in fraction
counts per min in fraction
30
light petroleum
200:1
200:2
200:3
200:4
methanol
8
20
6
4
10
2
1
0
1
2
3
5
7
9
11
13
15
17
19
fraction number
counts
quinone

more synthesis in the white cells, the fate of labelled quinone, in white cells, was examined. This was achieved by labelling the quinone, as above, removing the remaining label from the external medium by washing, and then comparing the effect of addition of a substrate, or a substrate and an inhibitor, with no addition (Table 6).

Incubation of labelled cells for one hour greatly increased the total counts in quinone, but decreased the quinone content. Addition of malate decreased quinone and total counts, as did the addition of malate and HOQNO (an inhibitor of respiration in this organism (Erickson and Parker 1969)). When the cells were not shaken for one hour, there was a loss of quinone, but the radioactivity was reduced to a very low value. Several experiments of this type were carried out, but the results were not always reproducible. However, incubation of the cells alone resulted in a loss of quinone, and addition of malate or malate and HOQNO also resulted in a loss of quinone. The counts in an unshaken sample were always very low.

If succinate was added to the incubation with [2-^{14}C] mevalonic acid, increased incorporation of label into quinone was observed (compare zero time incorporation of label in Tables 6 and 7).

A similar experiment to the previous one was performed, utilizing succinate, and also increasing the incubation time after the removal of the [2-^{14}C] mevalonic acid (Table 7).

Incubation of the labelled cells alone, for up to 2 hours, resulted in a decrease and then an increase in quinone levels, with a steady rise in counts in the quinone fraction. The addition of malate, or malate and HOQNO resulted in a rise, then fall in quinone levels, with

Table 6

Fate of labelled quinone, in various respiratory conditions, using white cells.

Addition	Time (hr)	Quinone. micromoles per 500ml of culture	Counts per min in quinone from 500ml of culture	Counts per min per 0.01 m.moles quinone
	0	7.35	212	287
none	1	5.17	501	969
malate	1	3.40	118	347
malate	2	5.71	13	23
none, but non-shaken	1	3.27	8	24
malate + HOQNO	1	4.08	150	368

(6 x 500ml Flasks)

Cells were grown overnight, spun down and suspended in 0.05M phosphate buffer pH7.0 (120ml). Cells were incubated with 10 µCi [2^{14}-C] mevalonic acid for 1 hour, with shaking, at 30°C. Cells were spun down and resuspended in phosphate buffer as above. They were divided into six equal parts (10ml each). Malate (0.05M final concn) and HOQNO (1.2×10^{-5}M final concn) were added to the appropriate flasks (total volume 20ml per flask). After the allocated time, the cells were spun down, and quinone was extracted and purified, (as methods). Samples were taken for estimation and counting.

Table 7

Fate of quinone, labelled with carbon-14 in the presence of succinate, in white cells.

Addition	Time (hr)	Quinone, micromoles per 500ml of culture	Counts in extract before chromatography (per min) per 500ml of culture	Counts in purified quinone fraction (per min) per 500ml culture	Counts per 0.01 m.moles quinone (per min)
	0	9.80	20250	4053	4136
none	1	8.57	13740	6570	7666
none	2	10.7	33180	8520	7960
malate	1	13.1	24240	5720	4366
malate	2	10.5	22000	3140	2990
malate+HOQNO	1	12.3	27900	24200	19670
malate+HOQNO	2	10.6	24480	14970	14120

(7 x 500ml Flasks)

Cells were grown overnight, spun down and resuspended in 0.05M phosphate buffer pH7.0 (140ml). 10 μCi [2-^{14}C] mevalonic acid, and succinate (0.05M final concn) were added and the cells incubated (total volume 200ml) at 30°C for 1 hour, with shaking. The cells were spun down and (70ml) resuspended in phosphate buffer, as above. The cells were divided (10ml each) into 7 equal parts. Malate (0.05M final concn) and HOQNO (1.2 x 10^{-5}M Final concn) were added to the appropriate flasks (final volume per flask = 20ml). After the allocated time period, the cells were spun down, and quinone was extracted and purified (as methods). Samples were taken for estimation and counting. Malate was added after 1 hour to the 2 hour incubation with malate.

a corresponding increase and then decrease in counts. However, the incorporation with malate and HOQNO was 4 to 5 times greater than with malate alone.

The difference in the total counts in the sample put on the column, and those found in the purified quinone, can be explained by the results obtained by Ramasarma and Ramakrishnan (1961) and Ramasarma et al (1965). They found a large amount of label in the lipids, and the extra counts in the impure extraction are probably due to those in lipid.

The results with white cells were inconclusive, as breakdown and synthesis were occurring simultaneously. Theoretically, the zero time value, for total counts in quinone, should be higher than that obtained after reincubation in the absence of labelled substrate, but usually this value was one of the lowest. This is probably due to the cells storing labelled mevalonic acid and/or the accumulation of labelled intermediates of the quinone biosynthetic pathway, which are subsequently converted into quinone.

(iii) Incorporation of [1-^{14}C]-isopentenyl pyrophosphate into quinone and polar fractions, in white and yellow cells.

In an attempt to overcome some of these problems, a precursor further along the biosynthetic pathway, isopentenyl pyrophosphate, was tested as a substrate for quinone synthesis in whole cells.

It was hoped that due to its position in the pathway, there would be less likelihood of a large number of intermediates being stored by the bacteria. Cells of both white and yellow strains were incubated with labelled isopentenyl pyrophosphate. Controls, incorporating

[2-^{14}C]mevalonic acid, were used to compare incorporation (Table 8).

There was very little incorporation of [1-^{14}C]isopentenyl pyrophosphate into quinone, in white or yellow strains, or into more polar lipids. With [2-^{14}C]mevalonic acid, there was rapid incorporation into quinone in the white strain.

The presence of unlabelled mevalonic acid did not increase the incorporation of labelled isopentenyl pyrophosphate. This might have occurred if quinone synthesis was regulated, in part, by the presence of its natural precursor, mevalonic acid.

The inability of both strains of bacteria to utilize isopentenyl pyrophosphate could be due to either cell impermeability or to dephosphorylation of the isopentenyl pyrophosphate in the cell.

Table 8

Comparison of incorporation of labelled isopentenyl pyrophosphate and mevalonic acid, in both white and yellow cells.

ISOPENTENYL PYROPHOSPHATE
INCORPORATION INTO QUINONE

	Quinone. micromoles per 500ml culture	Counts per min in quinone from 500ml of culture	Counts per min per 0.01 m.moles quinone	Counts per min in polar fraction per 500ml culture
Yellow	31.3	17.0	5.0	198
White	13.0	21.0	16.0	

MEVALONIC ACID
INCORPORATION INTO QUINONE

	Quinone. micromoles per 500ml of culture	Counts per min in quinone from 500ml of culture	Counts per min per .01 m.moles quinone	Counts per min in polar fraction per 500ml culture
Yellow	38.2	206	54	192
White	10.9	24060	22070	

(2 x 500ml flasks of both yellow and white cells)

Cells were spun down and resuspended in 0.05M phosphate buffer, pH 7.0, and both white and yellow cells divided into two 100ml portions. The cells were incubated with succinate (0.05M final concn) and either 2 µC [2-^{14}C] mevalonic acid, or 2 µC [1-^{14}C]-isopentenyl pyrophosphate, for 1 hour at 30°C, with shaking (total volume 200ml). Cells were spun down, and quinone was extracted and purified as methods. Samples were taken for quinone estimation and radioactive counting. A polar fraction, containing Carotenoid, was eluted from the column in methanol, and counted.

C. PRELIMINARY EXPERIMENTS USING WHITE AND YELLOW MEMBRANE PREPARATIONS.

The ability of whole cells to retain and use labelled precursors of quinone, meant that breakdown alone could not be studied, in the absence of synthesis. To try to overcome this difficulty, the incorporation of labelled precursors into quinone in membrane preparations was attempted, and preliminary experiments similar to those with cells were carried out to determine if results were similar.

(a) Investigation of quinone synthesis in membranes.

(i) Incorporation of [2-^{14}C] mevalonic acid and [^{14}C] isopentenyl pyrophosphate into quinone and polar fractions, in white and yellow membranes.

Soluble preparations from M. lysodeikticus are able to form polyprenyl pyrophosphates (C35 - C50) from isopentenyl pyrophosphate. These extracts can be used to enhance the production of 2-polyprenylphenols in cell-free extracts of Rhodospirillum rubrum (Raman et al 1969). This enzyme, or enzymes, would therefore appear to be in the soluble fraction, and so membrane preparations alone would not be expected to synthesise quinone.

However, when the membrane preparation was incubated with [2-^{14}C] mevalonic acid and various cofactors: ATP and magnesium chloride (both 0.01M final concn Tchen 1963) and NADPH (approximately 0.03mM final concn), labelled quinone was synthesised from the [2-^{14}C] mevalonic acid, but the incorporation of labelled isopentenyl pyrophosphate was negligible (Table 9). There was much more incorporation in white than yellow membranes, as was found earlier when using whole cells. The addition of supernatant from the lysis

Table 9

Incorporation of [2-^{14}C] mevalonic acid and [1-^{14}C] isopentenyl pyrophosphate into quinone and polar fraction, in membranes of pigmented and white mutant, UVI, strains of M. lysodeikticus, and the effect of added supernatant on incorporation in pigmented membranes.

INCORPORATION INTO QUINONE

	a) with MEVALONIC ACID			b) with ISOPENTENYL PYROPHOSPHATE		
	Quinone. micromoles per 500ml culture	Counts per min in quinone per 500ml culture	Counts per min per 0.01m. moles quinone	Quinone. micromoles per 500ml culture	Counts per min in quinone per 500ml culture	Counts per min per 0.01 m.moles quinone
Yellow	7.59	129	170	12.7	7.0	6.0
Yellow + supernatant	10.8	365	338	10.3	23	22
White Strain UVI	4.78	4687	9808	4.15	204	492

INCORPORATION INTO POLAR FRACTION.

	a) with MEVALONIC ACID		b) with ISOPENTENYL PYROPHOSPHATE	
	Counts per min in 0.5ml of polar Fraction solution	Counts per min in total polar fraction per 500ml culture	Counts per min in 0.5ml of polar Fraction solution	Counts per min in total polar fraction per 500ml culture
Yellow	4*	21*	0	0
Yellow + supernatant	6*	35*	14*	120*

* probably not significantly above background

Table 9 (contd)

(4×500ml flasks yellow, 2×500ml flasks white cells)

Cells, from overnight culture, were spun down and membranes prepared by method 1. The First supernatant after splitting of yellow cells, was kept. The membranes were suspended in 0.05M phosphate buffer pH7.0, and divided:- white membranes into two, and yellow membranes into four equal parts (20 ml each). [2-^{14}C]Mevalonic acid (2μCi) or [1-^{14}C]-isopentenyl pyrophosphate (2μCi) were added to the appropriate flasks. 2ml supernatant was added to one yellow + [2-^{14}C]mevalonic acid flask, and one yellow + [1-^{14}C]isopentenyl pyrophosphate flask. ATP (0.01M final concn), NADPH (0.03mM final concn) and magnesium chloride (0.01M final concn) were added to all the flasks (total volume 50ml). The flasks were incubated for 1 hour, with shaking, at 30°C. The membranes were spun down, and quinone and carotenoid polar fractions eluted and purified (as methods). Quinone samples were taken for estimation and radioactive counting. The polar fraction in methanol, from the chromatographic column, was evaporated down and counted.

of the yellow cells, increased the incorporation of both [2-^{14}C] mevalonic acid and [1-^{14}C] isopentenyl pyrophosphate, in yellow membranes. This could be because certain enzymes, such as those in the cell free extracts used by Raman (Raman et al 1969), had been washed off or partially removed from the membrane preparation, when the cells lysed.

However, incorporation was still much lower in supplemented yellow membranes than in white membranes. The low incorporation of isopentenyl pyrophosphate could be due to dephosphorylation, although with yellow membranes and supernatant, some radioactivity was recovered in the polar lipid fraction, but not in quinone. The incorporation of [2-^{14}C] mevalonic acid and [1-^{14}C] isopentenyl pyrophosphate into quinone is similar in both cells and membranes. The use of isolated membranes avoids possible complicating effects associated with permeability and accumulation of [2-^{14}C] mevalonic acid inside the cell.

(ii) Incorporation of [2-^{14}C] mevalonic acid into quinone, by membranes from the yellow wild-type and four white carotenoidless strains.

Most of the experiments have involved using white strain UV1 as the carotenoidless strain, but it is necessary to check significant results using the other three carotenoidless mutants (strains UV2, UV3 and UV4). The ability of these three mutants to incorporate [2-^{14}C] mevalonic acid into quinone in the membrane, was studied (Table 10). All the mutants incorporated [2-^{14}C] mevalonic acid into quinone, much more rapidly than did the yellow wild-type strain. This shows that the high incorporation is a consequence of lack of

Table 10

Incorporation of [2-^{14}C]mevalonic acid into quinone in membranes, in yellow and white strain UV1, and the three other carotenoidless mutants, strains UV2, UV3 and UV4.

	Quinone. micromoles per 500ml culture	Counts per min in quinone per 500ml culture	Counts per min per 0.01 m.moles quinone	Total counts per min in polar lipids per 500ml culture
Yellow	11.6	31	27	175
w/strain UV1	3.53	560	1586	
w/strain UV2	2.18	117	357	
w/strain UV3	3.54	4950	13990	
w/strain UV4	3.13	1261	4029	

(1x500ml flask of each strain)

Cells, from overnight cultures, were spun down and membranes prepared by method 1. Each membrane preparation (10ml) was incubated with ATP (0.01M final concn), magnesium chloride (0.01M final concn) 3mM (final concn) mevalonic acid, NADPH (0.03mM final concn) and 5μCi [2-^{14}C] mevalonic acid for 1 hour, with shaking, at 30°C. [Total volume = 20ml]

The membranes were spun down, and quinone and carotenoid extracted (as methods). The quinone was purified, (as methods) and samples were taken for estimation and radioactive counting. Polar lipid eluted from the column in methanol, was counted.

carotenoid,rather than of a second unrelated mutation, in addition to the one which blocks the synthesis of coloured carotenoid.

(b) Investigation of quinone breakdown in membranes.

The method used was similar to that used for cells. The quinone was labelled, by [2-^{14}C]mevalonic acid, as above, the superfluous label removed, and the membranes were reincubated. Various treatments were applied to this reincubation medium.

(i) Effect of the oxidation-reduction state of quinone on its breakdown,in white membranes.

The process of repeated oxidation and reduction of the quinone could influence its susceptibility to breakdown. Alternatively it could be either the quinone state, the quinol state or the semi-quinone state which is destroyed. Quinone was labelled in white membranes, and excess label removed. If membrane suspensions were shaken in air,without oxidisable substrate, the quinone would be mainly in the oxidised state. Malate was added as a good substrate for the respiratory chain, the malate oxidase activity being strong in these membranes. This should reveal the breakdown of quinone were it to occur primarily in the reduced state or when undergoing oxidation-reduction.

If malate and an inhibitor, HOQNO,which blocks near the quinone in the respiratory chain, probably before cytochromes b and c (Kogut and Lightbrown 1962),were added, the quinone should be mostly reduced (Table 11). However HOQNO can also react with flavins to alter their oxidation-reduction properties (Kogut and Lightbrown 1962). This would inhibit flavin-requiring enzymes. So with malate and

Table 11

Investigation of the breakdown of labelled quinone, in the presence of malate, malate and HOQNO, or alone, in white strain UV1 membranes.

Addition	Time (hr)	Quinone, micromoles per 500ml culture	Counts per min in quinone per 500ml culture	Counts per min per 0.01 m.moles quinone
	0	5.7	90	158
none	1	5.0	586	1172
none	2	4.53	993	2192
malate	1	6.3	175	278
malate	2	6.0	283	472
malate+HOQNO	1	4.53	557	1230
malate+HOQNO	2	3.68	416	1130

(7 x 500ml flasks)

Cells, from overnight cultures, were spun down and membranes prepared by method 1 (in 100ml). The membranes were incubated for 1 hour at 30°C, with shaking, with 5 μCi [2-^{14}C] mevalonic acid, ATP (0.01M final concn), magnesium chloride (0.01M final concn) and 0·03mM (final concn) NADPH. The membranes were spun down, and resuspended in 0.05M phosphate buffer, pH 7.0. The membrane suspension was divided into 7 equal parts (10ml each). Malate (0.05M final concn) and HOQNO (1.2×10^{-5}M final concn) were added to appropriate flasks (total volume 20ml). After the allocated time period, the membranes were spun down, and quinone was extracted and purified (as methods). Samples were taken for estimation and radioactive counting. Malate was added after 1 hour to the 2 hour incubation with malate.

HOQNO, there would be inhibition of the chain near quinone and probably also inhibition of the flavin-requiring malate oxidation. Under these conditions the quinone would be mainly oxidised, although if the malate oxidase were not completely inhibited, the quinone might be in the semi-quinone state.

When the membranes were incubated alone, the quinone levels fell, but there was a large incorporation of [2-^{14}C]mevalonic acid. The addition of malate decreased quinone levels less, and substantially reduced the counts in the quinone fraction, although they still increased during the 2 hour period. With malate and HOQNO, the quinone levels fell, more than with no substrate. The counts in quinone increased, similarly to those with no substrate, during the first hour, but then decreased.

A second similar experiment was carried out, except that menadione was added to the overnight incubation, as this increases the incorporation of [2-^{14}C]mevalonic acid (see section 3D(a)). The effect of malate, with and without HOQNO, was compared with incubation in the absence of substrate, with respect to the level of labelled quinone and the amount of label, in white membranes (Table 12). Without substrate the quinone level fell steadily, whilst the radioactivity first increased and then decreased. The specific activity increased. The addition of malate, or malate and HOQNO, resulted in the quinone level falling and then rising. The radioactivity in the quinone fraction increased over the two hour incubation period, although the increase was lower in the presence of HOQNO.

In the experiment utilizing menadione, the quinone was labelled in the presence of carrier unlabelled mevalonic acid (3mM) and as

Table 12

Effect of malate, and malate and HOQNO on the breakdown of labelled quinone in white, strain UVI, membranes, prepared from cells grown overnight with menadione.

Addition	Time (hr)	Quinone. micromoles per 500ml culture	Counts per min in quinone per 500ml culture	Counts per min per 0.01 m.moles quinone
	0	5.92	34	57
none	1	4.90	104	212
none	2	1.22	76	623
malate	1	1.02	72	706
malate	2	2.04	106	520
malate+HOQNO	1	1.02	50	490
malate+HOQNO	2	3.06	68	222

Cells were grown overnight with menadione (5×10^{-7}M final concn) (7 x 500ml flasks) and then spun down and membranes prepared by method 1. Membranes were incubated with ATP (0.01M final concn) magnesium chloride (both final concn) (0.01M final concn), NADPH 0·03mM, 3mM mevalonic acid, and 5 µCi [2-^{14}C] mevalonic acid, for 1 hour with shaking (100ml volume). The membranes were spun down, washed twice, and then resuspended in 70ml 0.05M phosphate buffer pH7.0 and divided into 7 equal parts (10ml each). Malate (0.05 M final concn) and HOQNO (1.2×10^{-5}M final concn) were added to the appropriate flasks (total volume per flask 20ml). Malate was added after one hour to the two hour incubation with malate. After the appropriate time, the membranes were spun down, and quinone was extracted and purified (as methods). Samples were taken for estimation and radioactive counting.

the membrane is obviously storing intermediates for future use, it would be expected that quinone levels in this second experiment would be higher.

Certain results obtained after these results are relevant to their interpretation. Firstly, in both these experiments, the quinone was labelled in the presence of 10mM magnesium chloride, and in section 3E it can be seen that this level decreases incorporation and quinone levels. Also, in experiments investigating the effect of malate and magnesium (Sections 3E and 3F), under certain conditions, in white membranes, oscillations of the quinone level and radioactivity in quinone appear to occur. These oscillations occur with and without malate, and may also depend on magnesium concentration during labelling, and time between further additions of malate. This may explain why these results do not seem to be reproducible, and possibly also the ones in cells, if oscillations were occurring. These experiments show that the membranes are able to retain labelled mevalonic acid, or labelled intermediates in quinone biosynthesis, which were later used to synthesise labelled quinone, after the removal of excess labelled mevalonic acid. This meant that synthesis and breakdown were occurring simultaneously.

The fact that membranes can retain radioactively labelled intermediates of quinone biosynthesis, is surprising. Although if the total synthesis from mevalonic acid to quinone can be performed by a membrane preparation, presumably the enzymes are membrane bound, and the products of each individual reaction remain bound to the membrane. However, in several experiments, only labelled mevalonic acid was added, with no carrier, giving very low concen-

trations (5 micro Curie = 0.23 micromoles). Possible explanations include a reserve pool of intermediates part way along the pathway or a pool of intermediates between enzyme complexes.

From this section, it may be concluded that:

1. Membrane preparations can incorporate [2-^{14}C] mevalonic acid into quinone.
2. The incorporation is lower in yellow than white membranes.
3. Incorporation of [1-^{14}C] isopentenyl pyrophosphate into quinone is low, in both yellow and white membranes.
4. Addition of supernatant to yellow membranes increased the incorporation of both [2-^{14}C] mevalonic acid and [1-^{14}C] isopentenyl pyrophosphate into quinone.
5. There was significant incorporation of [1-^{14}C] isopentenyl pyrophosphate into polar material in yellow membranes, with supernatant.
6. All the carotenoidless-mutant strains behaved similarly to strain UVI.
7. Incubation of membranes, containing labelled quinone, alone results in a decrease in quinone levels.
8. Membranes can store [2-^{14}C] mevalonic acid, or labelled intermediates in quinone biosynthesis, and utilize them after excess label has been removed from the incubation medium.
9. The effect of malate, and malate and HOQNO varies from experiment to experiment, possibly because under certain conditions involving malate and magnesium, oscillations in quinone levels can be observed, and the probability of "catching"

the oscillation at exactly the same point, in two different experiments, would be fairly small. These oscillations are examined in Sections 3E and 3F.

D. STUDIES ON THE EFFECTS OF MENADIONE, MENAQUINONE-7 AND DIPHENYLAMINE ON QUINONE SYNTHESIS IN MEMBRANES.

(a) Effect of menadione and menaquinone-7 on quinone synthesis.

Menadione has a quinone ring structure without the isoprenoid sidechain. Guerin et al (1970) concluded that it was not a true intermediate in quinone biosynthesis, after comparing radioactivity dilutions using (^{14}C)-2-methyl-1, 4-naphthoquinone and (^{14}C)1, 4-naphthoquinone as precursors. However Goldenbaum et al (1975), using a mutant of Staphylococcus aureus, found that menadione deprivation resulted in no vitamin K_2 synthesis, and concluded that it was a true intermediate. Even if it were not a true intermediate, it could be modified by the bacterium to use as a source of the ring in naphthoquinone biosynthesis.

(i) Menadione addition to overnight cultures, and menadione and menaquinone-7 addition to white membranes incorporating [2-^{14}C] mevalonic acid into quinone.

Menadione was added either to the incubation mixture with [2-^{14}C] mevalonic acid or to an overnight culture, of the white carotenoidless strain. The effect of adding menaquinone-7 to the [2-^{14}C] mevalonic acid incubation mixture was also studied (Table 13). The "phytoene" fraction from the quinone purification column was counted, to determine whether label was accumulating in the blocked carotenoid pathway.

Menadione increased incorporation of [2-^{14}C] mevalonic acid into quinone, especially if added to the overnight growing culture. Addition of menadione to the [2-^{14}C] mevalonic acid incubation mixture also increased incorporation of label into quinone, but only to 50%

Table 13

Effect of menadione and menaquinone-7 (MK7) on [2-^{14}C] mevalonic acid incorporation into quinone, in M. lysodeikticus, strain UVI, membranes.

	Quinone. micromoles per 500ml culture	Counts per min in quinone per 500ml culture	Counts per min per 0.01 m.moles quinone	Counts per min in "phytoene" fraction per 500ml culture
Menadione in overnight culture	2.04	2340	11440	236
No menadione	1.63	104	638	428
Menadione in [2-^{14}C] mevalonic acid incubation	1.77	1096	6192	858
MK7 in [2-^{14}C] mevalonic acid incubation	1.63	80	490	112

Final concentration of menadione in overnight culture; 5×10^{-7}M. Final concentration of menadione and MK7 in [2-^{14}C] mevalonic acid incubation; 10^{-5} M.

(4x500ml flasks)

Cells, from overnight culture, were spun down and membranes (40ml) The membranes were divided into 4 equal parts. prepared by method 1. The membranes were incubated with ATP (0.01M final concn), magnesium chloride (0.01M final concn), NADPH 0·03 mM (final concn), mevalonic acid (3mM final concn) and 2 μC [2-^{14}C] mevalonic acid. Menadione and MK7 were added to the appropriate flasks. The membranes were incubated for 1 hour at 30°C, with shaking (20ml final volume). Quinone was extracted from the membranes, and purified, estimated and counted, (as methods). The "phytoene" fraction was evaporated down, under nitrogen, dissolved in ethanol and counted.

The "phytoene" fraction is the 200:2 (light petroleum : ether) fraction from the quinone purification column.

of the activity obtained when menadione was included in the growth medium. Menadione, in the overnight culture, also increased the level of quinone. Perhaps a significant time period is needed for the menadione to be modified, for use in quinone biosynthesis, or alternatively, the biosynthetic enzyme activities are higher in the presence of menadione. Addition of menadione to the [2-^{14}C] mevalonic acid incubation mixture also increased counts in the "phytoene" fraction.

The presence of menaquinone-7 in the [2-^{14}C] mevalonic acid incubation mixture lowered the counts by 23% (0.6 micromoles added, and 1.63 micromoles of endogenous quinone in membrane sample). This indicates that the level of quinone may play a part in regulating its own synthesis, through a negative feed-back system.

(ii) Effect of menadione in overnight cultures, on the incorporation of [2-^{14}C] mevalonic acid into quinone and polar fraction in white and yellow membranes.

The effect of menadione, in overnight cultures, on quinone synthesis was compared in white and yellow membranes (Table 14). In both white and yellow membranes, the menadione increased incorporation of [2-^{14}C] mevalonic acid into quinone. However in yellow membranes, the radioactivity in the polar fraction was decreased with menadione, whereas that in the "phytoene" fraction, from white membranes, were increased with menadione.

Menadione is acting either as an intermediate in quinone synthesis or as a regulator of quinone synthesis. The difficulty in obtaining labelled menadione prevented this matter being pursued further.

Table 14

Effect of menadione addition to overnight cultures on the incorporation of [2-^{14}C] mevalonic acid into quinone, polar material and "phytoene" fraction, in membranes of yellow pigmented and white UVI strains of M. lysodeikticus .

	Quinone. micromoles per 500ml culture	Counts per min in quinone per 500ml culture	Counts per min per 0.01 m.moles quinone	Counts per min in 'phytoene' fraction per 500ml culture	Counts per min in polar material per 500ml culture
Yellow	9.4	21	22	0	687
yellow + menadione	5.6	31	55		166
white (strain UVI)	3.3	420	1273	21	
white (strain UVI)+ menadione	1.0	492	4920	124	

Results are the mean of 2 experiments on different preparations.

Menadione in overnight culture, 5×10^{-7}M (final concn).

(2 x 500ml flasks of yellow and white cells were) Cells from overnight culture, spun down and membranes were prepared by method 1. (of membranes (20ml volume per experiment)) Incubation with 0.01M ATP (final concn), (0.03mM (final concn)) 0.01M magnesium chloride (final concn), NADPH, 2 μCi [2-^{14}C] mevalonic acid and (3mM (final concn)) mevalonic acid for 1 hour at 30°C, (total volume 40ml) with shaking. The membranes were spun down, and quinone, polar and "phytoene" fractions (were) extracted and purified, as methods. Samples were taken for quinone estimation and radioactive counting. "Phytoene" and polar fractions from the column were evaporated down and counted.

(b) Effect of diphenylamine on quinone synthesis in yellow membranes.

Diphenylamine is often used to produce carotenoidless cultures as it blocks the synthesis of coloured carotenoids (Salton and Freer, 1965, Salton and Ehtisham-ud-din, 1965). Diphenylamine-treated cultures behave similarly to carotenoidless mutant strains, with respect to aerobic photosensitivity (Cohen-Bazire and Stanier 1958), which is due to lack of carotenoid.

(i) Comparison of quinone levels, and synthesis from [2-^{14}C] mevalonic acid, in membranes from wild-type, diphenylamine induced and ultraviolet mutant cultures.

Preliminary experiments showed that the best final concentration of diphenylamine to use was 6×10^{-5} M. Higher concentrations caused severe inhibition of growth.

White mutant cells, diphenylamine induced white cells and yellow wild-type cells were compared, for their ability to incorporate [2-^{14}C] mevalonic acid into quinone, polar material and the "phytoene" fraction, in membrane preparations (Table 15).

The loss of coloured carotenoid brought about by the use of diphenylamine, caused these membranes to behave similarly to those of the carotenoidless mutant strain. Thus, the total amount of quinone was reduced, and the activity in quinone increased. There were fewer counts in the "phytoene" fraction from diphenylamine induced white membranes.

The results obtained with diphenylamine treated cells were not exactly the same as with the carotenoidless mutant cells, but lie between the yellow and white mutant strains results. This is to be expected, since a small amount of coloured carotenoid is still being

Table 15

Comparison of incorporation of [2-^{14}C] mevalonic acid into quinone, polar and "phytoene" fractions in membranes of yellow, white strain UVI and diphenylamine-induced white cells.

	Quinone. micromoles per 500ml culture	Counts per min in quinone per 500ml culture	Counts per min per 0.01 m.moles quinone	Counts per min in 'phytoene' fraction per 500ml culture	Counts per min in polar fraction per 500 ml culture
yellow	8.89	38	43	0	504
diphenylamine induced white membranes	2.29	60	262	117	35
white strain UVI	1.12	88	786	-	-

The results are the mean of four experiments on different preparations.

Concentration of diphenylamine used in overnight culture = 6×10^{-5}M (Final concn)

(1x500ml Flask of yellow, white and yellow-DPA cells)

Cells, from overnight culture, were spun down and membranes were prepared by method 1. The membranes were incubated with NADPH 0.03 mM (final concn) magnesium chloride (0.01M final concn), ATP (0.01M final concn), 10mM (final concn) mevalonic acid and 5 μC [2-^{14}C] mevalonic acid for 1hr at 30°C, with shaking. Quinone and polar material were extracted and purified (as methods). Samples were taken for quinone estimation and radioactive counting. "Phytoene" and polar fractions, from the column, were evaporated down, and counted.

Total volume of incubation mixture per flask ≡ 20ml.

formed in the diphenylamine treated cells.

From this section it may be concluded that:

1. Menadione increases [2-^{14}C] mevalonic acid incorporation into quinone, especially if added to the growth medium.
2. Addition of menaquinone-7 to the [2-^{14}C] mevalonic acid incubation decreases quinone synthesis, from [2-^{14}C] mevalonic acid.
3. Menadione increases quinone synthesis, in both white and yellow membranes, from [2-^{14}C] mevalonic acid.
4. Diphenylamine treatment of yellow cells, results in white cells, which behave similarly to white ultraviolet-induced mutant cells. The diphenylamine-treated white cells have lower quinone levels, and higher incorporation of [2-^{14}C] mevalonic acid into quinone, than the yellow wild-type.

E. EFFECT OF MAGNESIUM ON QUINONE LEVELS, QUINONE SYNTHESIS AND ON THE FATE OF LABELLED QUINONE.

(a) Effect of factors, including magnesium, on quinone synthesis, from [2-^{14}C]mevalonic acid.

(i) Deletion of [2-^{14}C]mevalonic acid incubation components, using white membranes.

Incorporation of [2-^{14}C]mevalonic acid into quinone in white membranes is high, so the effect of deleting some of the factors previously added; ATP, NADPH and magnesium chloride, was studied (Table 16). The effect of addition of malate and succinate, on incorporation of [2-^{14}C]mevalonic acid into quinone, was also investigated.

Lack of ATP or NADPH resulted in no incorporation of label. ATP is required by some of the enzymes at the beginning of the pathway, for example mevalonate kinase (Popjak 1969). The increase in quinone levels, without ATP, is surprising. It suggests that ATP increases breakdown, or inhibits the synthesis of quinone from a pool of intermediates.

Malate and succinate addition both significantly increased incorporation of [2-^{14}C]mevalonic acid into quinone, but quinone levels were decreased with malate, and increased with succinate. Levels with succinate were approximately 8 times higher than with malate.

The surprising, and also interesting, result was the apparent inhibition of incorporation of [2-^{14}C]mevalonic acid into quinone by 10mM magnesium chloride, as this concentration was used by Tchen (1969). Magnesium ions, at high concentrations, appear to inhibit incorporation, but malate and succinate prevent this, possibly by binding magnesium ions. Magnesium ions could inhibit incorporation both by chelating the [2-^{14}C]mevalonic acid or affecting its utilization as a precursor for quinone.

Table 16

Effect of deletion of factors from the incubation mixture, on quinone levels and radioactive labelling, and the effect of addition of malate and succinate, in white strain UVI membranes.

	Quinone. micromoles per 500ml culture	Counts per min in quinone per 500ml culture	Counts per min per 0.01 m.moles quinone
normal	1.22	40	328
-NADPH	1.22	5*	0
-ATP	2.44	4*	0
-magnesium chloride	6.54	240	367
+ malate	0.82	202	2463
+ succinate	6.94	192	277

* not significantly above background counts.

normal = + ATP, NADPH and magnesium chloride.

Cells, from overnight culture (6x500ml flasks), were spun down and membranes prepared by method 1. The membranes were divided into 6 equal parts (10ml each). Normal incubation was with 0.03mM (final concn) NADPH, 2 μC; [2-^{14}C] mevalonic acid, ATP (0.01M final concn) magnesium chloride (0.01M final concn) and 10mM (final concn) mevalonic acid. Deletions were made to the appropriate flasks (Total volume per flask = 20ml). Malate and succinate (0.05M final concn) were added to normal incubation flasks. After incubation at 30°C for 1 hour, with shaking, the membranes were spun down, and the quinone was extracted and purified (as methods). Samples were taken for quinone estimation and radioactive counting.

Malate and succinate can both bind magnesium, but malate binds twice as strongly as succinate (Cannan and Kibrick 1938). Deletion of magnesium, or addition of succinate, which can remove magnesium by complexing it, therefore increases quinone levels by increasing synthesis. Malate can also bind magnesium, more effectively than succinate, but although there is increased incorporation, there is a decrease in quinone levels. Therefore malate is probably having another effect; increasing the breakdown of quinone.

(ii) Effect of magnesium concentration, in the presence and absence of succinate, on the incorporation of [2-^{14}C]mevalonic acid into quinone and polar fraction, in white and yellow membranes.

On the basis of the previous results, which showed that 10mM magnesium inhibited the incorporation of [2-^{14}C]mevalonic acid into quinone, the effects of 10 and 1 mM magnesium chloride on incorporation were compared, in the presence and absence of succinate (Table 17).

Results from yellow membranes.

1. Quinone levels were lower with 1 than 10 mM magnesium, in the presence or absence of succinate.
2. The radioactivity in quinone was similar with 1 and 10mM magnesium, but the specific activity was higher with 1mM magnesium, with or without succinate.
3. The higher specific activities with 1mM magnesium suggest increased synthesis, assuming that labelled and unlabelled quinone have an equal chance of being broken down. However the lower quinone levels with 1mM magnesium, indicate that there is probably an increased rate of breakdown as well.

Table 17

Effect of magnesium levels, in the presence and absence of succinate, on [2-^{14}C] mevalonic acid incorporation into quinone and polar fractions, in white strain UVI and yellow wild-type membranes.

		Quinone. micromoles per mg protein ($x10^{-2}$)	Counts per min in quinone per mg protein ($x10^{-2}$)	Counts per min per 0.01 m.moles quinone	Counts per min in polar fraction per mg protein ($x10^{-2}$)
(i)	yellow, high Mg^{++}	5.8	23	40	109
(ii)	yellow, low Mg^{++}	2.2	24	109	77
(iii)	yellow, high Mg^{++} + succinate	2.25	31	138	91
(iv)	yellow, low Mg^{++} + succinate	0.82	29	354	53
(v)	white, high Mg^{++}	0.17	60	3529	
(vi)	white, low Mg^{++}	0.52	59	1146	
(vii)	white, high Mg^{++} + succinate	1.3	122	938	
(viii)	white, low Mg^{++} + succinate	0.35	37	1057	

Cells, from overnight culture (4x500ml flasks of both white and yellow cells) were spun down and membranes prepared by method 1. The membrane suspensions were divided into 4 equal parts (for both white and yellow preparations; 10ml per estimation). ATP (0.01M final concn), 10mM (final concn) mevalonic acid, NADPH 0.03mM (final concn) and 2 μC [2-^{14}C] mevalonic acid were added to each flask. Succinate (0.05M final

Table 17 (contd)

conc) was added to the appropriate flasks. High Mg^{++} (= 10mM magnesium chloride, final concn) or low Mg^{++} (= 1mM magnesium chloride, final concn) was added to the flasks (total volume = 30ml). The membranes were incubated at 30°C for 1 hour, with shaking, and then spun down. Quinone was extracted, purified by chromatography, and samples were taken for estimation, and radioactive counting. The polar fraction, from the chromatographic column, was evaporated down, and counted.

4. Counts in the polar fraction were higher with 10 than 1mM magnesium, irrespective of succinate.

5. Quinone levels were lower when succinate was present, irrespective of magnesium concentration.

Results from white membranes.

1. Without succinate, 1mM magnesium addition resulted in an increase in quinone levels, compared with 10mM. The radioactivity did not increase, and therefore the specific activity was lower with 1 than 10mM magnesium.

2. With succinate, the quinone levels were higher with 10mM magnesium. The radioactivity was also higher, when compared with that obtained with 1mM magnesium. The specific activities were similar with 1 and 10mM magnesium.

Low (1mM) magnesium increased quinone levels in white membranes, when compared with 10mM magnesium, but with no increase in radioactivity. This suggests that quinone is being synthesised from unlabelled intermediates. Succinate addition, in white membranes, resulted in increased quinone levels, with high magnesium, probably because it was complexing the magnesium ions, but there was a decrease in quinone levels with low magnesium. With low magnesium, succinate appears to inhibit synthesis. The results suggest that in the absence of carotenoid, high magnesium levels promote breakdown.

(b) Effect of magnesium concentration during labelling, on the fate of labelled quinone.

The fate of labelled quinone was investigated, in the presence and absence of malate, as malate is a good substrate for the respiratory chain. It has been proposed that certain reactions, like those occurring in the respiratory chain, can generate the superoxide radical (Tyler 1975),

which could dismutate to form singlet oxygen (Pederson and Aust, 1973, Khan, 1970). Carotenoids can quench singlet oxygen (Foote and Denny, 1968) but in this event, the white mutant strain would have no protection.

(i) The effect of 10mM magnesium, during labelling, on the fate of labelled quinone, in the presence and absence of malate.

White membranes were used. Without malate, the quinone levels were maintained for 80 minutes and then fell significantly. The radioactivity in the quinone fraction fell steadily, as did the specific activity. Malate addition resulted in an even decrease in quinone levels. The specific activity increased and then decreased, as did the total radioactivity in the quinone fraction. In this experiment, no evidence of oscillations, to be described below, was observed, (Table 18). Without malate, the initial fall in specific activity, with no decrease in quinone levels, indicates that quinone is being synthesised from unlabelled intermediates, possibly in a reserve pool. With malate, the levels of quinone were initially lower and decreased evenly. Malate appears to inhibit the synthesis of quinone from unlabelled intermediates, for the first 80 minutes, however then the pool of intermediates is utilized to make quinone.

(iii) The effect of 1mM magnesium, during labelling, on the fate of labelled quinone, in the presence and absence of malate

The experiment was carried out with white and yellow membrane preparations.

Results from white membranes, (Table 19a).

1. Quinone levels were much higher with low (1mM) than high (10mM) magnesium.

Table 18

Effect of malate on the fate of labelled quinone levels, using white, strain UVI, membranes; the quinone labelled in the presence of high (10mM) magnesium chloride.

Addition	Time (mins)	Quinone. micro moles per 500ml culture	Counts per min in quinone per 500ml culture	Counts per min per 0.01 m.moles quinone
none	40	1.63	228	1399
none	80	1.63	143	877
none	120	0.61	16	261
malate	40	1.22	96	787
malate	80	1.02	556	5451
malate	120	0.82	40	490

Cells, from overnight culture (6x500ml Flasks), were spun down and membranes prepared by method 1. The membranes were incubated with NADPH, 0·03 mM (final concn), ATP (0.01M final concn), magnesium chloride (0.01M final concn) and 5 μ Ci [2-^{14}C] mevalonic acid, for 1 hour, with shaking (100ml, total volume).

The membranes were spun down, washed twice and then divided into 6 equal parts (10ml each). Malate (0.05M final concn) was added to the appropriate flasks. More malate was added at 40 minute intervals, to maintain the level. After the appropriate time, the membranes were spun down, and the quinone was extracted, purified and estimated, (as methods). Samples of quinone were also taken for radioactive counting.

Table 19a

Effect of low magnesium levels, during labelling, on the breakdown of labelled quinone, in the presence and absence of malate, in white, strain UVI, membranes.

Magnesium level	Time (mins)	Quinone. micromoles per mg protein ($x10^{-2}$)	Counts per min in quinone per mg protein ($x10^{-2}$)	Counts per min per 0.01 m.moles quinone
+ Malate				
high	0	0.35	6.4	183
high	80	0.35	22	629
low	0	2.3	15	65
low	40	1.8	18	100
low	80	3.0	29	97
low	120	3.4	38	158
- Malate				
low	0	2.3	15	65
low	40	0.43	36	837
low	80	3.3	74	224
low	120	0.43	17	395

Table 19b

Effect of low magnesium levels, during labelling, on the breakdown of labelled quinone, in the presence and absence of malate, in yellow wild-type membranes.

Magnesium Level	Time (mins)	Quinone. micromoles per mg protein ($x10^{-2}$)	Counts per min in quinone per mg protein ($x10^{-2}$)	Counts per min per 0.01 m.moles quinone	Carotenoid microg. per mg. protein (10^{-2})	Counts per min in polar fraction per mg. protein ($x10^{-2}$)
+ Malate						
high	0	6.9	74	107	0.84	174
high	80	19.0	44	23	2.1	-
low	0	18.0	16	9	2.4	413
low	40	24.0	11	5	2.2	439
low	80	27.0	52	19	2.9	232
low	120	46.0	29	6	3.0	156
- Malate						
high	0	6.9	74	107	0.84	174
high	80	24.0	69	29	2.0	55
low	0	18.0	16	9	2.4	413
low	40	15.0	0	0	1.6	335
low	80	24.0	47	4	1.6	61
low	120	53.0	0	0	3.1	-

Table 19 (contd)

(10 x 500 ml flasks of both yellow and white cells)
Cells, from overnight culture, were spun down, and membranes were prepared by method 1.

Membranes were incubated with ATP (0.01M Final concn), high magnesium ≡ 10mM magnesium chloride (final concn), or low magnesium ≡ 1mM magnesium chloride (final concn), NADPH 0·03mM (final concn) 5μ Ci [2-^{14}C] mevalonic acid and 15mM mevalonic acid (final concn) for 1 hour at 30°C, with shaking (total volume, 100ml).

The membranes were spun down and washed twice. They were then divided (10ml per estimation). Malate (0.05M final concn) was added to the appropriate flasks (total volume 20ml). After the time interval, the membranes were spun down and quinone was extracted and purified as methods. Samples were taken for quinone estimation and radioactive counting. The polar fraction from the quinone purification column was evaporated down, and samples were taken for estimation and radioactive counting.

Malate was added at 40 minute intervals.

2. Without malate the quinone levels appeared to oscillate, the specific activity oscillated, and the radioactive counts in quinone increased for 80 minutes and then fell.

3. Malate addition resulted in a dampening of the oscillations in quinone levels. The total radioactive counts in quinone rose, but the specific activity increased, plateaued and then increased again.

Results from yellow membranes,(Table 19b).

1. Quinone levels were higher with 1 than 10mM magnesium.

2. Quinone levels increased steadily, with or without malate, but reached a higher level without malate.

3. Total radioactivity in quinone and specific activities were low.

4. Radioactivity in polar material was greater than that in quinone.

5. Malate addition resulted in increased radioactivity in polar material and slightly increased carotenoid levels.

6. The increase in quinone levels, in yellow membranes, could not be accounted for by the change in the incorporation of radioactivity in quinone, indicating that quinone is being synthesised from unlabelled precursors.

Oscillations in quinone levels were not observed in yellow membranes, suggesting that they may be a consequence of lack of carotenoid. It must be pointed out, however, that these oscillations depend on a single point.

Several conclusions can be drawn from the study of the effect of magnesium:

(i) ATP and NADPH are essential for incorporation of [2-^{14}C] mevalonic acid into quinone.

(ii) High magnesium (10mM) inhibits the incorporation of [2-^{14}C] mevalonic acid into quinone.

(iii) Malate and succinate increase incorporation, in the presence of 10mM magnesium, probably by binding the magnesium.

(iv) Although malate can bind magnesium more strongly than succinate does, its addition results in much lower quinone levels than addition of succinate, suggesting that it is having a second effect, probably increasing breakdown.

(v) In yellow membranes, with or without succinate, lowering the magnesium concentration from 10 to 1mM decreased the level of quinone. The radioactive counts in quinone were similar, so perhaps synthesis and breakdown are affected.

(vi) In yellow membranes, succinate addition lowers quinone levels by approximately 38% in the presence of 1 or 10mM magnesium.

(vii) In the absence of carotenoid, high magnesium (10mM) increases quinone breakdown.

(viii) In white membranes, in the presence of succinate, low magnesium (1mM) appears to increase quinone breakdown.

(ix) Succinate has an opposite effect on quinone levels with high and low magnesium; increasing quinone levels with high magnesium and decreasing quinone levels with low magnesium, in white membranes.

(x) If quinone is labelled in the presence of high (10mM) magnesium, in white membranes, malate initially appears to increase breakdown.

(xi) If quinone is labelled in the presence of low (1mM) magnesium, in white membranes, then without malate the quinone levels

oscillate. Malate dampens these oscillations.

(xii) In white membranes, lowering the magnesium concentration, from 10 to 1mM, renders the system liable to oscillations, which malate partially inhibits.

(xiii) In yellow membranes, with low (1mM) magnesium, there are no oscillations. The quinone levels rise steadily, but the quinone is probably synthesised from unlabelled intermediates.

F. EFFECT OF MALATE ON QUINONE, IN MEMBRANES.

In this section the effect of malate was studied, on quinone levels and on the fate of labelled quinone. To ensure that the effect of malate alone was being studied, no magnesium could be added to the system, as it interferes with the utilization of the [2-^{14}C]mevalonic acid. Malate can complex the magnesium, but this complex may not give the same results as malate alone. To avoid using magnesium, the quinone was labelled in the cell, in the presence of succinate, and then the membranes were prepared.

(a) Effect of malate on quinone levels.

(i) In white and yellow membrane preparations.

The effect of malate on the level of extractable quinone was examined in membranes, prepared from cells grown overnight. With white membranes, the quinone levels oscillated in two experiments (Fig. 17a, 17b), but not in the third (Table 20). However, quinone levels in membranes which did not show oscillations were higher. Again, however, it must be mentioned that the oscillations depend on single points, but since the differences are so great, it would seem that the oscillations are genuine. The oscillations appear earlier without malate. Malate appears to keep the quinone levels lower for a longer period, before oscillations occur. When there were no oscillations (Table 20), malate lowered quinone levels.

With yellow membranes, there was no evidence of oscillations in quinone levels (Table 21). The quinone levels increased steadily and then plateaued, in the presence and absence of malate, although malate slightly lowered the levels. There was also little difference

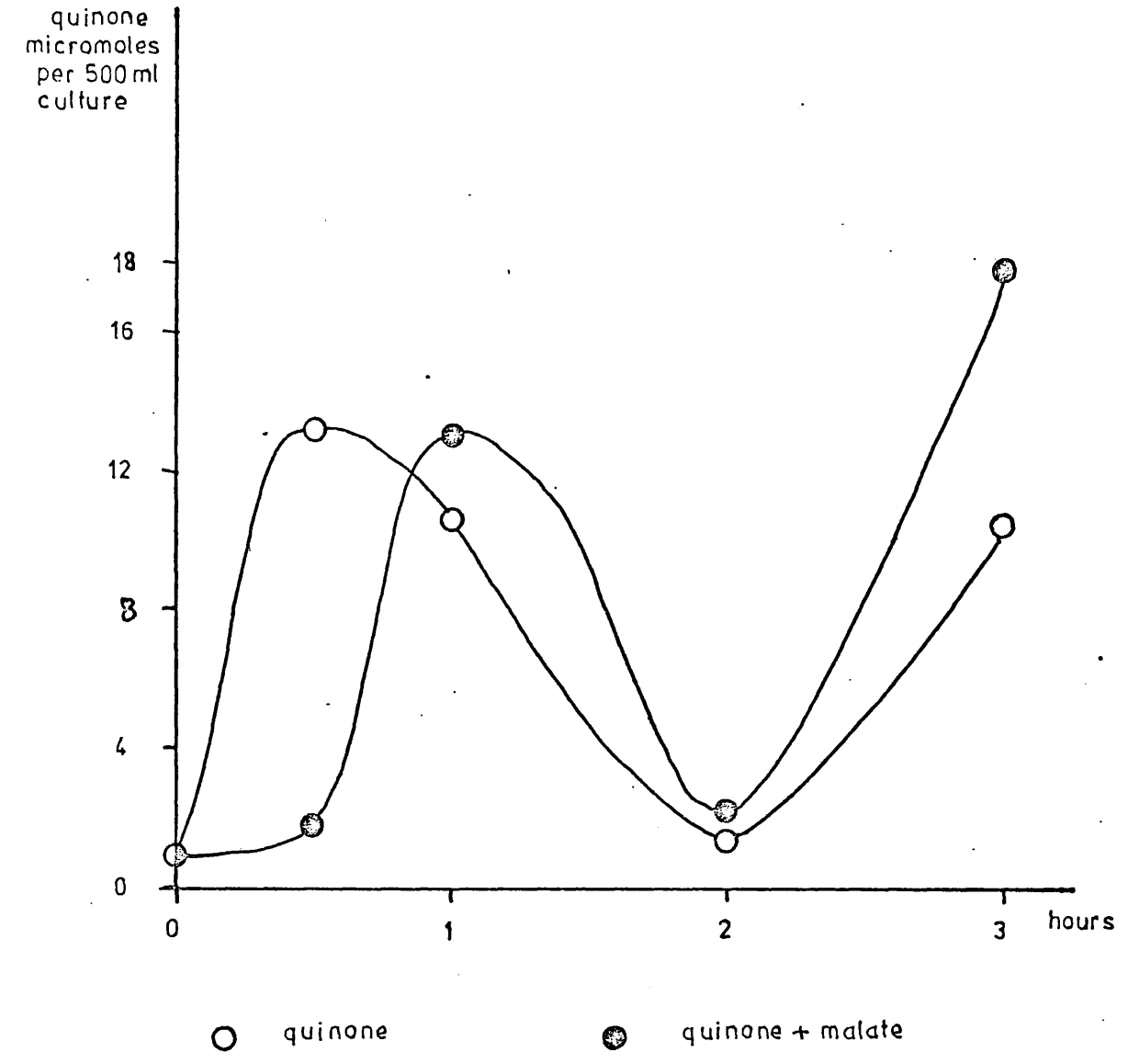
Figure 17 a, b

Oscillations in quinone levels, in white membranes, with and without malate.

The cells were spun down, after overnight culture, and membranes prepared by method 1. The membranes were divided into 9 equal parts (10ml each). Malate (0.05M final concn) was added to the appropriate flasks. More malate was added at hourly intervals. After the allocated time period, the membranes were spun down. Quinone was extracted and purified (as methods). Samples were then taken for quinone estimation.

Experiments 17a and 17b were carried out under the same conditions; those shown above.

(a)


quinone micromoles per 500 ml culture
18
16
12
8
4
0
0
1
2
3
hours
quinone
quinone + malate

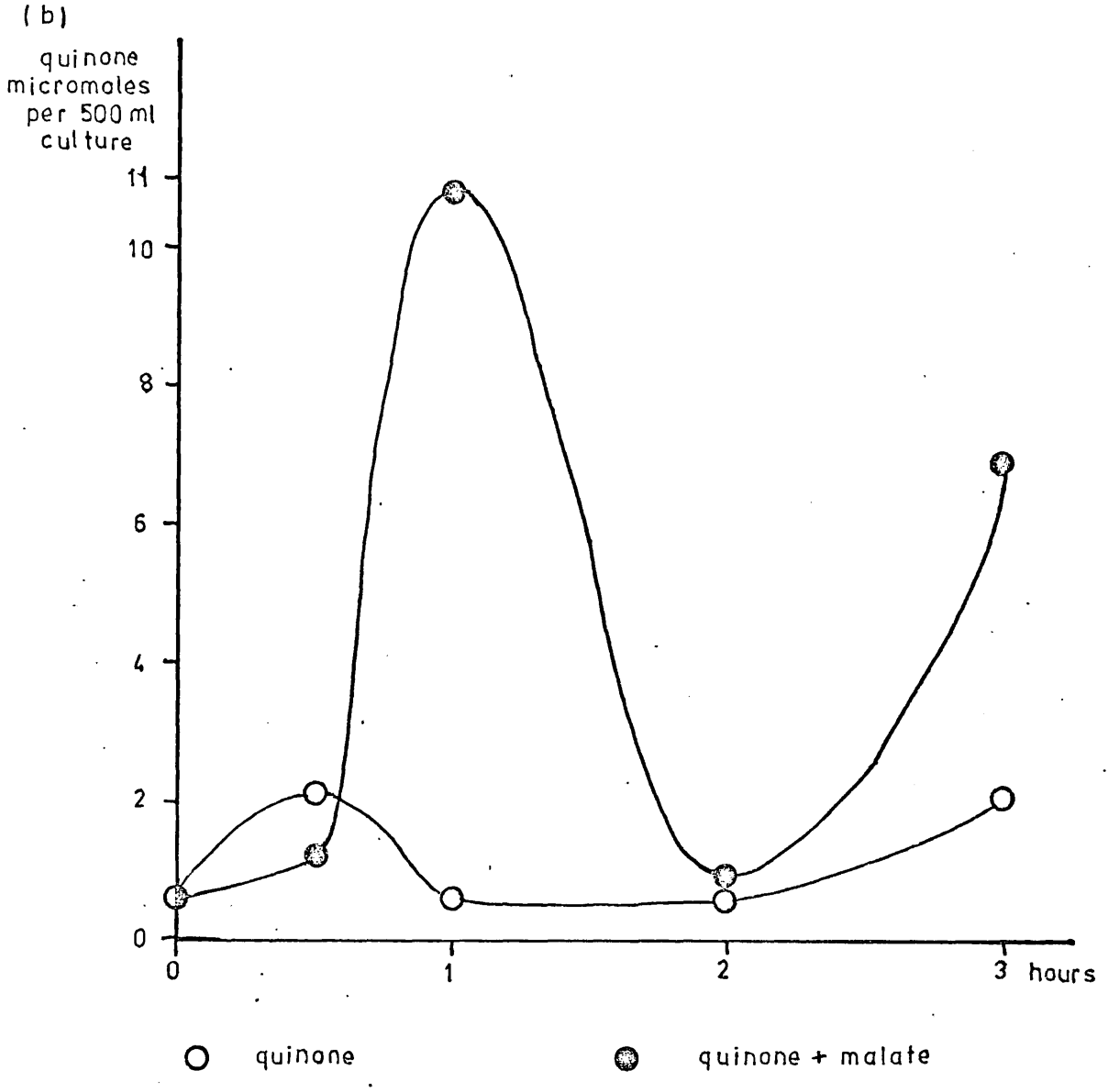

(b)
quinone micromoles per 500 ml culture
11
10
8
6
4
2
0
0
1
2
3
hours
quinone
quinone + malate

Table 20

Effect of malate on quinone levels in white, strain UVI, membranes.

Addition	Time (hr)	Quinone. micromoles per 500ml culture
none	1	22.4
none	2	5.1
none	3	3.3
malate	1	8.1
malate	2	8.4
malate	3	4.7

(6×500ml Flasks)

Cells, from overnight culture, were spun down, and membranes prepared by method 1. The membranes were divided into 6 equal parts (10ml each). Malate (0.05M final concn) was added to the appropriate flasks (Total volume per flask = 20ml). More malate was added hourly. After the appropriate time, the membranes were spun down and the quinone was extracted and purified, (as methods). Samples were taken for quinone estimation.

Table 21

Effect of malate on quinone and carotenoid levels, in yellow wild-type membranes.

Addition	Time (mins)	Quinone. micromoles per mg protein ($x10^{-2}$)	Carotenoid microg. per mg. protein ($x10^{-2}$)
	0	6.55	0.61
none	30	11.7	0.59
none	60	12.2	0.64
none	120	13.8	0.68
none	180	13.5	0.54
malate	30	10.9	0.47
malate	60	10.8	0.54
malate	120	13.2	0.52
malate	180	12.5	0.55

Cells, from overnight culture (7x500ml flasks), were spun down and membranes prepared by method 2. The membranes were suspended in 0.05M phosphate buffer pH7.0, and divided into 9 equal parts (10ml each). Malate (0.05M final concn) was added to the appropriate flasks (total volume per flask = 20ml). After the allocated time period, the membranes were spun down and quinone was extracted and purified, as methods, and then estimated. Carotenoid, eluted from the purification column in methanol, was evaporated down, under nitrogen, and estimated spectrophotometrically.

in carotenoid levels during the incubation period.

(ii) Comparison of the effects of malate and succinate on quinone levels, in white membranes.

In an earlier experiment (Section 3Ea(i)), malate and succinate seemed to have opposite effects on quinone levels, when the quinone was labelled in the presence of 10mM magnesium chloride. However in this experiment, with no added magnesium, the effects of malate and succinate are practically the same (Table 22). After an initial drop in quinone levels during the first hour, the levels fluctuated slightly during the next two hours.

(b) Effect of malate on quinone levels and on the fate of labelled quinone.

As stated previously, the quinone was labelled in the cell, in the presence of succinate, from [2-^{14}C] mevalonic acid. To try to inhibit synthesis from labelled intermediates, after the excess [2-^{14}C] mevalonic acid had been removed, the membranes were washed several times.

(i) Over a two hour incubation period.

With white membranes, the quinone levels and counts in quinone oscillated, in the presence and absence of malate (Table 23) The oscillations can be seen in Fig. 18, plotted from the results. Malate slightly increased quinone levels and counts in the quinone fraction. In both cases, the specific activities increased for 80 minutes and then fell. This indicates that towards the end of the incubation period, the quinone was being synthesised from unlabelled intermediates.

Table 22

Comparison of the effect of malate and succinate on quinone levels, using white, strain UVI, membranes.

Addition	Time (mins)	Quinone. micromoles per mg protein ($x10^{-2}$)
	0	9.7
malate	30	5.6
malate	60	6.4
malate	120	6.3
malate	180	5.3
succinate	30	6.0
succinate	60	6.3
succinate	120	5.5
succinate	180	6.0

Cells, from overnight culture (9x500ml flasks), were spun down and membranes prepared by method 2. The membranes were divided into 9 equal parts (10ml each).

Malate and succinate (0.05M final concn) were added to the appropriate flasks (total volume = 20ml). After the allocated time period, the membranes were spun down and quinone was extracted, purified and estimated, (as methods).

Malate and succinate were added hourly to maintain their level.

Table 23

Effect of malate on quinone levels, in white, strain UVI, membranes: the quinone labelled by incubation of the cells with [2-^{14}C]mevalonic acid, in the presence of succinate.

Addition	Time (mins)	Quinone. micromoles per mg protein ($x10^{-2}$)	Counts per min in quinone per mg protein ($x10^{1}$)	Counts per min per 0.01 m.moles quinone ($x10^{3}$)
	0	10.3	124	120
none	40	6.9	160	232
none	80	4.3	175	407
none	120	6.1	162	266
malate	40	9.3	181	195
malate	80	5.1	189	371
malate	120	6.6	169	256

Cells, from overnight culture (7×500ml flasks), were spun down and resuspended in 0.05M phosphate buffer pH7.0. They were then incubated with succinate (0.05M final concn) and 10 μCi [2-^{14}C]mevalonic acid (total volume 100ml), for 1.5 hours at 30°C, with shaking. The cells were spun down, and membranes prepared by method 2, followed by 2 washings. The membranes were divided into 7 equal parts (10ml each). Malate (0.05M final concn) was added to the appropriate flasks (total volume per flask = 20ml). After the allocated time period, the membranes were spun down, and the quinone was extracted and purified, (as methods). Samples were taken for counting and quinone estimation.

Malate was added at 40 minute intervals.

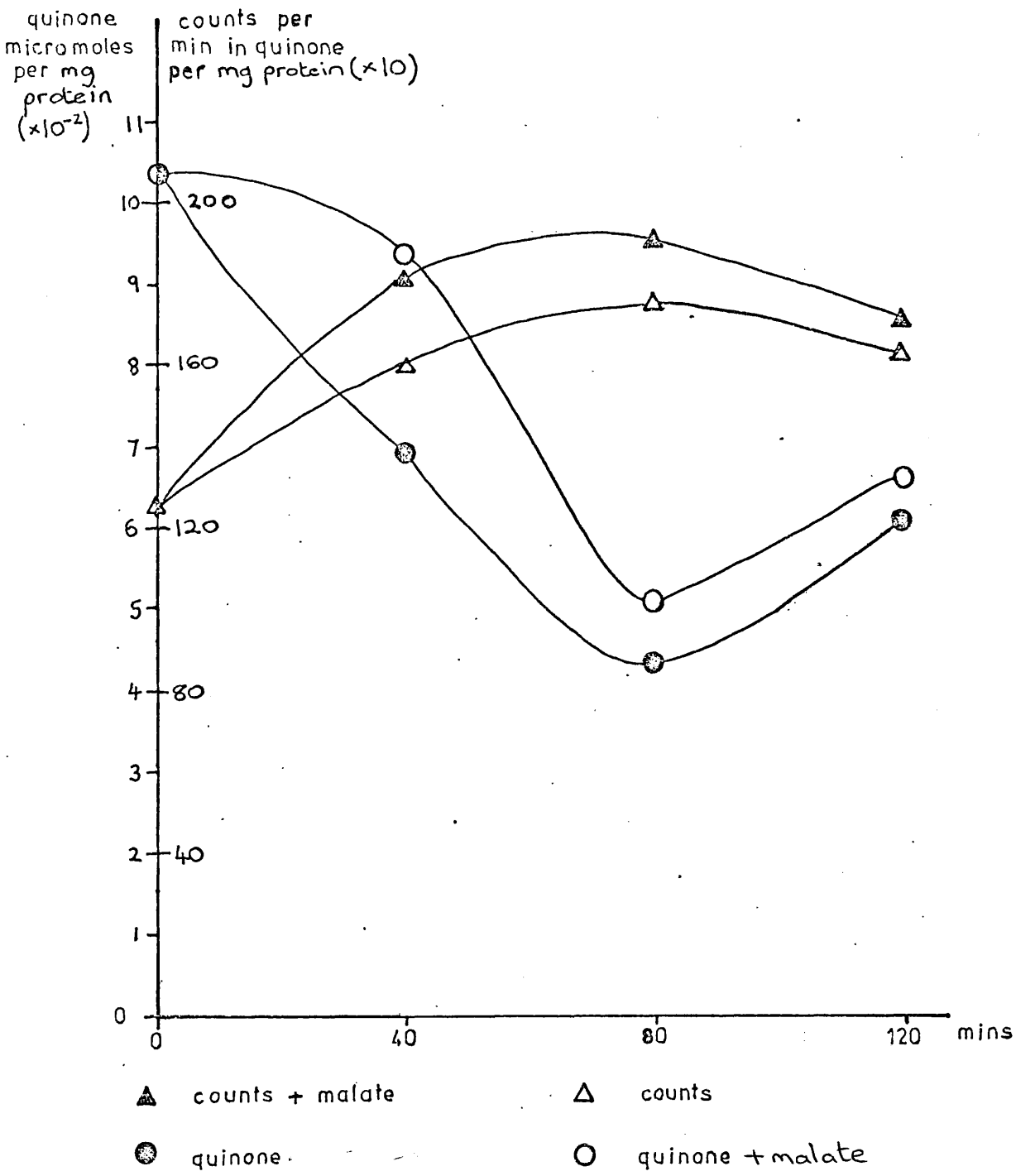
Figure 18

Effect of malate on quinone levels and radioactivity in quinone, in white membranes.

Cells were spun down after overnight culture and resuspended in 0.05M phosphate buffer pH 7.0. They were then incubated with succinate (0.05M final concn) and 10 micro Ci [2-14 C] mevalonic acid for 1.5 hours, with shaking (total volume 100ml). The cells were spun down and membranes prepared by method 2, followed by 2 washings. The membranes were divided into 7 equal parts (10ml each). Malate (0.05M final concn) was added to the appropriate flasks. After the allocated time period, the membranes were spun down and the quinone was extracted and purified (as methods). Samples were taken for radioactive counting and quinone estimation.

Malate was added at 40 minute intervals.

Total volume of incubation + malate = 20ml.


quinone micromoles per mg protein (×10⁻²)
counts per min in quinone per mg protein (×10)
11
10
9
8
7
6
5
4
3
2
1
0
200
160
120
80
40
0
40
80
120
mins
counts + malate
counts
quinone
quinone + malate

(ii) Over a four hour incubation period.

In this experiment, using white membranes, the malate was added hourly, whereas in the previous experiment it was added every 40minutes. In this experiment there were no oscillations, although the quinone levels did fluctuate slightly. Without malate, there was an initial increase in quinone levels, and the specific activity fell, indicating that quinone was being synthesised from unlabelled intermediates. However with malate, the quinone level decreased initially and the specific activity remained the same (Table 24a, b). Malate appears to inhibit the synthesis of quinone from unlabelled intermediates, as it also did when quinone had been labelled in the presence of 10mM magnesium chloride (see Section 3E b (i))

(iii) Summary of Section F.

1. In white membranes, when oscillations occurred in quinone levels, with and without malate and with no labelled quinone, malate retarded the onset of oscillation.
2. When oscillations occurred, in white membranes, in labelled quinone levels, with and without malate, malate slightly increased quinone levels and λ = (radioactivity) in the quinone fraction.
3. No oscillations were observed in quinone levels in yellow membranes. The levels increased and then plateaued in the presence and absence of malate, although malate slightly lowered quinone and carotenoid levels.
4. There was no significant difference in the effect of malate and succinate on quinone levels in white membranes.
5. Malate lowers quinone levels slightly in conditions when oscillations do not occur.

Table 24a, b

Effect of malate, over an extended time period, on quinone in white, strain UVI, membranes; the quinone labelled by incubation of the cells with [2-^{14}C] mevalonic acid, in the presence of succinate.

Addition	Time (mins)	Quinone. micromoles per mg protein x10^{-2}	Counts per min (x10^3) in quinone (per mg protein x10^{-2})	Counts per min per 0.01 m.moles quinone x10^3
(a)				
	0	2.2	118	536
none	60	4.6	104	226
none	120	4.2	92	219
none	180	4.3	92	214
none	220	3.8	91	239
none	270	5.9	84	142
(b)				
	0	5.7	435	760
malate	60	4.1	317	773
malate	120	2.7	251	930
malate	180	3.8	264	695
malate	240	3.0	272	907
none	240	4.1	315	768

(6x500ml flasks)

Cells, from overnight culture, were spun down and resuspended in 0.05M phosphate buffer pH7.0. They were then incubated with succinate (0.05M final concn) and 10 μCi [2-^{14}C] mevalonic acid for 1.5 hours, at 30°C, (total volume 100ml), with shaking. The cells were spun down and membranes prepared by method 2, followed by 4 washings. The membranes were divided, into 6 equal parts, (10ml each). Malate (0.05M final concn) was added to the appropriate flasks (total volume = 20ml per flask). After the allocated time period, the membranes were spun down, and quinone was extracted, purified and estimated, (as methods). Samples were taken for radioactive counting. Malate was added at hourly intervals.

6. Malate appears, under certain conditions,to inhibit the synthesis of quinone from an unlabelled pool of intermediates.

(iv) Summary of the conditions when oscillations in quinone levels were observed in membrane preparations.

1. In white membranes only.

2. With and without malate, if the quinone levels were low and there was no added magnesium,(malate added at hourly intervals).

3. With and without malate, if the quinone was labelled in the cell, so there was no added magnesium,(malate added every 40 minutes).

4. Without malate, if the quinone was labelled in the presence of 1mM magnesium. Malate dampened the oscillations, when added every 40 minutes.

(v) Conclusions.

Under conditions when oscillations occurred,malate retarded the onset of oscillations, possibly by increasing breakdown, or inhibiting the synthesis of quinone from unlabelled intermediates. Both of these situations would decrease quinone levels and retard the rapid increase in quinone, which signals the onset of oscillations. In white membranes, when oscillations did not occur, malate generally lowered quinone levels. In yellow membranes too, malate lowered quinone levels. However in one experiment, in white membranes, malate slightly increased quinone levels. The effect of malate is not always consistent. The level of malate may not be the same in all experiments, as its concentration would be affected by the rate at which it was utilized, and the time interval between further additions. Oscillations,although affected by malate, are probably not a conse-

quence of the absence of malate, but may be due to the oxidation-reduction state of the membrane.

G. EFFECT OF RESPIRATORY INHIBITORS AND SUBSTRATES ON QUINONE AND CAROTENOID, IN CELLS AND MEMBRANES.

In this section no magnesium was added, so that the effects observed would not be complicated by magnesium.

(a) Effect of acetate and glycine on quinone synthesis and quinone levels, in cells and membranes.

(i) Effect of glycine and acetate on quinone levels, in white and yellow cells.

As malate had not proved as useful as had been hoped in elucidating the states of quinone which increase breakdown and synthesis, the effect of other substances, on quinone levels and quinone synthesis, was examined. Glycine and acetate were examined initially. Acetate could be used by the bacterium to synthesise quinone, as it is a precursor of mevalonic acid. However acetate had no effect on quinone levels in white or yellow cells, possibly due to impermeability (Table 25). Shaking suspensions of white and yellow cells alone for 5.75 hours resulted in a sixty per cent decrease in quinone levels in yellow cells, but only a small decrease in white cells. This was surprising as yellow cells would be expected to have the ability to maintain their quinone levels, whereas in white cells it should have been more difficult. But the quinone level in white cells is much lower than in yellow, and therefore the white cells may be able to maintain this lower level more easily, if the rate of breakdown is not too rapid. The addition of glycine decreased quinone levels in yellow cells, but in white cells the quinone levels were depressed further. These results were obtained in several experiments.

Table 25

Effect of glycine and acetate on quinone levels, in white and yellow cells.

Yellow wild-type cells

Addition	Time (hr)	Quinone. micromoles per 500ml culture	Cells per 500ml culture $x10^{10}$	Quinone per cell micromoles $x10^{-10}$
	0	75.1	1.67	43.8
none	5.75	20.0	1.31	15.3
glycine	5.75	39.6	1.48	26.8
acetate	5.75	19.2	1.14	16.8
White carotenoidless cells (strain UVI)				
	0	45.5	3.49	13.0
none	5.75	45.1	2.66	17.0
glycine	5.75	25.5	2.78	9.17
acetate	5.75	47.6	2.96	16.1

(4 x 500ml Flasks of both yellow and white cells)

The cells, from an overnight culture, were spun down and re-suspended in 0.05M phosphate buffer, pH 7.0. The cells, both white and yellow, were divided into four equal parts (10ml each). Glycine and acetate (both 1% final concn) were added to the appropriate flasks (total volume per Flask 20ml). After the allocated time period, a sample was taken for cell estimation and then the quinone was extracted and estimated, (as methods). Estimation of cell numbers was based on turbidity measurements.

(ii) Effect of glycine and acetate on quinone levels, in white and yellow membranes.

The effect of glycine and acetate on membrane quinone levels was compared with that in cells. Shaking membranes for 5.75 hours resulted in smaller differences in quinone levels. The quinone level in yellow membranes decreased by only one quarter, but in white the quinone level was increased by half again. The addition of glycine again decreased quinone levels in yellow, but decreased the level of quinone further in white membranes. Acetate decreased the quinone levels in both white and yellow membranes (Table 26).

The effects of glycine and acetate are similar in cells and membranes of the white and yellow strains. Presumably, in membranes the enzymes for full metabolism of glycine and acetate are missing. Therefore the effects observed in whole cells probably only partly reflect acetate and glycine metabolism.

(iii) Synthesis of labelled quinone in the presence of glycine, by yellow wild-type cells.

As previously, the incubation of yellow cells alone for 5.75 hours resulted in a large decrease in quinone levels. The addition of glycine decreased the level of quinone less than no addition and slightly decreased the counts in the quinone fraction, indicating that it is probably affecting breakdown rather than synthesis of quinone (Table 27).

Several conclusions can be drawn from the experiments with glycine and acetate:

1. Incubation of yellow cells and membranes alone results in a decrease in quinone levels, the decrease being greater in cells.

Table 26

Effect of glycine and acetate on quinone levels in membranes, from white and yellow cells.

Yellow membranes

Addition	Time (hr)	Quinone. micromoles per 500ml culture
	0	32.4
none	5.75	25.5
glycine	5.75	31.2
acetate	5.75	19.4

White membranes

	0	14.5
none	5.75	23.2
glycine	5.75	19.1
acetate	5.75	16.9

(4x500ml flasks of both white and yellow cells)

Cells, from an overnight culture, were spun down and membranes prepared by method 2.

The membranes, of both white and yellow strains, were divided into four equal amounts (10ml each). Glycine (1% final concn) and acetate (1% final concn) were added to the appropriate flasks (total volume per flask 20ml). After the allocated time period, the membranes were spun down, and the quinone was extracted and estimated as methods.

Table 27

Synthesis of quinone from [2-^{14}C]mevalonic acid, in the presence and absence of glycine, in wild-type cells.

Addition	Time (hr)	Quinone. micromoles per 500ml culture	Counts per min in quinone from 500ml culture	Counts per min per 0.01 m.moles quinone
	0	58.6	-	-
none	5.75	10.4	585	563
glycine	5.75	12.2	523	429

(3×500ml flasks)

Cells, from overnight culture, were spun down and resuspended The cells were divided into 3 equal amounts (10ml each) in 0.05M phosphate buffer, pH7.0. Glycine (0.1M final concn) and 2 μCi [2-^{14}C]mevalonic acid were added to the appropriate flasks (total volume per flask 20ml) After the allocated time period, the cells were spun down, and the quinone was extracted (as methods). Samples were taken for quinone estimation and radioactive counting.

2. Incubation of white cells alone results in a small decrease in quinone levels, but when membranes from white cells are incubated, there is an increase in quinone levels.

3. Acetate has no effect on quinone levels, in white or yellow cells, but there is a decrease in quinone levels with membranes from white and yellow cells.

4. Incubation of yellow cells and membranes with glycine results in decreases smaller than with no addition in quinone levels and a decrease in total radioactivity.

5. Incubation of white cells and membranes with glycine resulted in a decrease in quinone levels.

(b) Effect of inhibitors of the respiratory chain, on quinone levels and quinone synthesis from [2-^{14}C]mevalonic acid, in white and yellow cells.

(i) Effect of HOQNO, azide and low oxygen levels on quinone levels, in yellow cells.

Azide and HOQNO were used, as inhibitors of the respiratory chain, in order to decrease respiratory activity in the cells. The low oxygen levels, which were a consequence of not shaking the cells, and which appeared to stabilize quinone in earlier experiments, would also decrease the respiratory activity.

Incubation of the suspended cells alone resulted in a decrease in quinone levels and a decrease in the number of cells, estimated from turbidity measurements. HOQNO and azide both decreased quinone levels, less compared to no addition, azide to a greater extent (Table 28). The number of cells fell slightly. With azide and HOQNO, the quinone per cell was increased compared with no addition, azide nearly maintaining the level obtained at zero time. Not shaking the cells resulted in the smallest decrease in quinone levels, and as the number of cells fell only slightly, the quinone per cell was higher than

Table 28

Effect of inhibitors of the respiratory chain, on quinone levels, in yellow wild type cells.

Addition	Time (hr)	Quinone. micromoles per 500ml culture	Cells per 500ml culture $x10^{10}$	Quinone per cell. m.moles $x10^{-10}$
	0	49.8	14.3	3.48
none	5.75	20.4	11.4	1.79
HOQNO	5.75	24.4	11.0	2.22
azide	5.75	36.8	10.9	3.38
none, but unshaken	5.75	41.2	10.6	3.89

(5x500ml flasks)

Cells, from overnight culture, were spun down and resuspended (the cells were divided into 5 equal parts (10ml each)) in 0.05M phosphate buffer pH7.0. Azide (0.2M final concn) and (total volume 10.5ml) HOQNO ($1.2x10^{-5}$M final concn) were added to the appropriate flasks. After the allocated time period, the cells were spun down, a small sample having been taken for cell estimation. The quinone was extracted from the cells and estimated, (as methods). The cell estimation was based on turbidity measurements.

at zero time. Inhibitors of the respiratory chain result in lower decreases in quinone. Thus, respiratory activity may be responsible for quinone breakdown. Decrease in oxygen levels resulted in the greatest protection of quinone, indicating that oxygen is involved in quinone breakdown.

(ii) Synthesis of quinone, from [2-^{14}C]mevalonic acid, in the presence of inhibitors of the respiratory chain, in yellow cells.

Incubation of the yellow cells alone resulted in a large decrease in quinone levels. The addition of HOQNO resulted in a slight lowering of the quinone levels, but a large increase in incorporation of radio activity into quinone. Azide and low oxygen (unshaken) both resulted in a lower decrease in quinone levels, with a slight lowering of total counts in quinone, however the specific activity was halved (Table 29). Addition of azide and low oxygen seem to result in synthesis of quinone from unlabelled intermediates, whereas HOQNO resulted in a high incorporation, but quinone levels remained low.

(iii) Effect of azide on quinone and carotenoid levels, in white and yellow cells.

The effect of azide, over a three hour period, was studied (Table 30). Shaking the cells alone, in a suspension containing sucrose, resulted in an increase in quinone levels in both white and yellow cells. Azide decreased quinone levels compared with no addition in both white and yellow cells, in yellow by approximately half, but in white by nearly ninety per cent. The level of carotenoid decreased when the yellow cells were shaken alone and the addition of azide decreased the carotenoid level further.

When yellow cells were shaken alone, the quinone could have been synthesised at the expense of carotenoid biosynthesis. Perhaps, in previous experiments when 0.25M sucrose was not added to the

Table 29

Synthesis of quinone from [2-^{14}C] mevalonic acid in the presence of respiratory chain inhibitors, in yellow cells.

Addition	Time (hr)	Quinone. micro moles per 500ml culture	Counts per min in quinone per 500ml culture	Counts per min per 0.01 m.moles quinone
	0	58.6	-	-
none	5.75	10.4	585	563
azide	5.75	22.0	498	226
HOQNO	5.75	8.0	1220	1525
none, but unshaken	5.75	20.8	523	251

(5x500ml flasks)

Cells, from overnight cultures, were spun down and resuspended (The cells were divided into 5 equal parts (10 ml each)) in 0.05M phosphate buffer pH7.0. Azide (0.2M final concn), HOQNO (1.2×10^{-5}M final concn) and 2 μ Ci [2-^{14}C] mevalonic acid (total volume 20ml) were added to the appropriate flasks. After the allocated time period, the cells were spun down, and quinone was extracted (as methods). Quinone samples were taken for estimation and radioactive counting.

Table 30

Effect of azide on quinone and carotenoid levels, using white and yellow cells.

Yellow wild-type cells

Addition	Time (hr)	Quinone. micromoles per 500ml culture	Cells per 500ml culture x10^{10}	Quinone per cell. micromoles x10^{-10}	Carotenoid. microg. per 500ml culture	Carotenoid per cell. microg. x10^{-10}
	0	14.8	1.8	8.2	5.44	3.02
none	3.0	53.9	-	-	3.73	-
azide	3.0	29.4	-	-	1.94	-

White carotenoidless (strain UVI) cells

Addition	Time (hr)	Quinone. micromoles per 500ml culture	Cells per 500ml culture x10^{10}	Quinone per cell. micromoles x10^{-10}
	0	7.55	0.9	8.4
none	3.0	11.2	-	-
azide	3.0	1.43	-	-

(3×500ml flasks of both yellow and white cells)

Cells, from overnight culture, were spun down and resuspended in 0.05M phosphate buffer pH7.0, containing 0.25M sucrose. 0.01M azide (final concn) was added to the appropriate flasks (total volume = 20ml per estimation). After the allocated time period, the cells were spun down. A small sample was taken from the zero time, for a cell count. Quinone and carotenoid were extracted, (as methods). Samples were taken for quinone estimation. Carotenoid was determined spectrophotometrically.

incubation medium, the cells were liable to autolyse and consequently in a poor state. So quinone levels fell over the incubation period, when there were no added effectors.

(c) Effect of substrates and inhibitors of the respiratory chain on quinone levels and synthesis from [2-^{14}C] mevalonic acid in white and yellow membranes.

From previous experiments, azide and HOQNO seem to have different effects on quinone synthesis from [2-^{14}C] mevalonic acid, in yellow cells. In the presence of 0.25M sucrose, azide resulted in a decrease in quinone levels in white as well as yellow cells. However, without sucrose, in yellow cells, the quinone levels increased with azide compared to no addition. HOQNO addition resulted in a rapid incorporation of [2-^{14}C] mevalonic acid into quinone, but quinone levels remained low. To try to clarify the results, which could be complicated by lysis of the cells in the absence of sucrose, the effects of azide and HOQNO on quinone levels in membranes were investigated. This was carried out in the presence and absence of a substrate, malate, as the membranes, unlike whole cells, would have little endogenous substrate.

(i) Effect of malate and azide on quinone levels and synthesis of quinone from [2-^{14}C] mevalonic acid, in yellow membranes.

Incubation of the membranes alone resulted in a decrease in quinone levels. Addition of malate decreased quinone levels and had only a small effect on incorporation of radioactivity into quinone, lowering it slightly. The radioactivity in the polar fraction (including carotenoid) increased, although the level of carotenoid decreased. Addition of azide also lowered quinone levels but the incorporation of label was greatly reduced (approximately thirty times). Carotenoid levels and radioactivity in the carotenoid-containing polar fraction were also reduced. Malate and azide addition also lowered the level

of quinone but not as much as malate or azide separately (Table 31). The total radioactive counts in quinone, with malate and azide, were also greatly decreased, but there were twice as many as with azide alone. The radioactivity in carotenoid and carotenoid levels were also decreased when malate and azide were added.

Azide is having a strong inhibitory effect on incorporation of [2-^{14}C]mevalonic acid into quinone and also to a lesser extent into polar isoprenoids in yellow membranes.

(ii) Effect of malate and HOQNO on quinone synthesis from [2-^{14}C]mevalonic acid, and quinone levels, in yellow membranes.

To see if azide was behaving similarly to other inhibitors of the respiratory chain, the effect of HOQNO, in yellow membranes, was studied (Table 32).

Incubation of the membranes alone, resulted in a loss of quinone. HOQNO addition increased quinone levels compared to no addition and lowered the total radioactivity by forty per cent. Malate and HOQNO addition also increased quinone levels compared to no addition but not as much as HOQNO alone. The radioactive counts with malate and HOQNO were also decreased, but again, not as much as with HOQNO alone. HOQNO increases the level of quinone compared to no addition, possibly by synthesis from unlabelled intermediates. Malate and HOQNO addition decreases the quinone level, compared to HOQNO alone, but there is more incorporation of radioactivity and the utilization of unlabelled intermediates is inhibited.

A low oxygen situation, produced by not shaking, increased quinone levels, compared to no addition, to the greatest extent and the radioactivity was also increased, although the increase in the counts could not account for the increase in quinone levels. From the specific activity results, there is probably some synthesis from unlabelled intermediates, but the breakdown rate is also decreased.

Table 31

Effect of malate and azide on quinone levels and [2-^{14}C] mevalonic acid incorporation into quinone and the carotenoid fraction in yellow membranes.

QUINONE

Addition	Time (hr)	Content: micromoles per mg protein ($\times 10^{-1}$)	Counts per min per mg protein (in quinone)	Counts per min per 0.01m.moles
	0	5.7	-	-
none	3.0	4.8	360	7500
malate	3.0	3.0	385	12833
azide	3.0	2.4	10	417
malate+ azide	3.0	3.6	20	556

CAROTENOID

Addition	Time (hr)	Content: microg. per mg protein ($\times 10^{-2}$)	Counts per min per mg protein (in polar fraction)
	0	6.1	-
none	3.0	8.1	14
malate	3.0	4.4	35
azide	3.0	3.1	10
malate+ azide	3.0	2.1	9

Cells, from overnight culture (5×500ml flasks), were spun down and membranes were prepared by method 2. The membranes were divided into 5 equal parts (10ml each). Malate (0.05M final concn), azide (0.2M final concn) and 2 μCi [2-^{14}C] mevalonic acid were added to the appropriate flasks (total volume per flask = 20ml). After the allocated time period the membranes were spun down.

Table 31 (contd)

Quinone was extracted and purified (as methods), and samples were taken for quinone estimation and radioactive counting. Polar material including carotenoid was eluted from the quinone purification column, in methanol, evaporated down, and samples were taken for carotenoid estimation and radioactive counting.

Table 32

Effect of HOQNO , malate and low oxygen levels on incorporation of [2-^{14}C] mevalonic acid into quinone and quinone levels, in yellow membranes.

Addition	Time (hr)	Quinone. micromoles per mg protein ($x10^{-1}$)	Counts per min per mg protein	Counts per min per 0.01 m.moles quinone
	0	7.1	-	-
none	3.0	3.0	49	1633
malate + HOQNO	3.0	3.3	42	1273
HOQNO	3.0	3.8	30	789
none, but unshaken	3.0	4.3	54	1256

Cells, from an overnight culture (5x500ml flasks), were spun down and membranes prepared by method 2. The membranes were divided into 5 equal parts (10ml each). Malate (0.05M final concn) and HOQNO ($1.2x10^{-5}$M final concn) were added to the appropriate flasks, as was 2 µCi [2-^{14}C] mevalonic acid (total volume per flask = 20ml). After the allocated time period, the membranes were spun down and the quinone was extracted and purified, as methods. Quinone samples were taken for estimation and radioactive counting.

(iii) Effect of HOQNO, malate, azide and low oxygen levels on quinone synthesis from [2-^{14}C] mevalonic acid, and quinone levels in white membranes.

Comparable experiments to the above, but with white membranes, are shown in Table 33. Shaking the membranes alone resulted in a decrease in quinone levels. The effect of azide was similar to that found in yellow membranes; the quinone levels were decreased and the total counts reduced substantially. Malate maintained quinone levels similar to no addition, with a slight decrease in radioactivity. Malate and azide together increased quinone levels compared with no addition but there was a large reduction in quinone synthesis from [2-^{14}C] mevalonic acid, as with azide alone.

HOQNO increased quinone levels compared to no addition and reduced incorporation of [2-^{14}C] mevalonic acid into quinone, but not to the same extent as azide. Malate and HOQNO together, increased quinone levels compared with no addition, but not as much as did HOQNO alone. The incorporation of [2-^{14}C] mevalonic acid into quinone with malate and HOQNO was decreased and was lower than with HOQNO alone.

Low oxygen levels, produced by not shaking the incubated suspension, increased quinone levels and also decreased radioactivity in the quinone, indicating that oxygen is involved in quinone breakdown.

(iv) Summary of effects of respiratory inhibitors and substrates on quinone and carotenoid levels, and synthesis, in white and yellow cells and membranes.

1. In most experiments, shaking alone decreased the level of quinone, probably due to lysis. With 0.25M sucrose in the incubation medium, the quinone levels increased.

2. With low oxygen levels, produced by not shaking during the incubation period, there was always an increase in quinone

Table 33

Effect of HOQNO, malate, azide and low oxygen levels on the synthesis of quinone from [2-^{14}C] mevalonic acid and quinone levels, in white membranes.

Addition	Time (hr)	Quinone. micromoles per mg protein ($x10^{-2}$)	Counts per min in quinone per mg protein	Counts per min per 0.01 m.moles quinone ($x10^{3}$)
(a)				
	0	9.5	-	-
none	3.0	5.3	1025	193
azide	3.0	3.8	9.0	2.4
malate	3.0	5.3	763	144
malate+ azide	3.0	5.8	11.0	1.9
(b)				
	0	9.7	-	-
none	3.0	5.7	3890	682
malate	3.0	6.0	3360	560
HOQNO	3.0	7.8	2590	332
malate+ HOQNO	3.0	6.8	1730	254
none, but un-shaken	3.0	6.9	3140	455

(5 x 500 (exp a) or 6 x 500 (exp b) ml flasks)

Cells, from overnight culture, were spun down and membranes were prepared by method 2. Membranes were divided into 5 (in experiment (a)) or 6 (in experiment (b)) equal parts (10ml each). Malate (0.05M final concn), HOQNO ($1.2x10^{-5}$M final concn), azide (0.2M final concn) and 2 μC [2-^{14}C] mevalonic acid were added to the appropriate flasks (total volume per flask = 20ml). After the allocated time period, the membranes were spun down and the quinone was extracted and purified, (as methods). Quinone samples were taken for estimation and radioactive counting. Results from two experiments on different preparations are shown.

levels (compared with no addition) in both white and yellow cells and also membranes. This was accompanied by only a slight increase or decrease in counts in quinone. The lack of oxygen resulted in little change in synthesis from labelled mevalonic acid and so presumably, the higher quinone levels are due to a decrease in the rate of breakdown, in the presence of only low levels of oxygen.

3. Addition of malate, in yellow membranes, decreased quinone levels but in white membranes quinone levels were maintained or increased (compared with no addition). In both cases there was little change in the total counts.

4. Without 0.25M sucrose in the incubation medium, azide increased quinone levels (compared with no addition) in yellow cells and decreased the level (further) in white cells. The (radioactive) counts in quinone, in yellow cells, were decreased slightly. With 0.25M sucrose the quinone levels were reduced in both white and yellow cells, but to a greater extent in the white cells.

5. Carotenoid levels in yellow cells and membranes were always decreased in the presence of azide.

6. In white membranes, azide decreased the quinone with a substantial reduction in (incorporation of radioactivity) (approximately 110 times). In yellow membranes there was also low (incorporation) but the level was only 30 times less than that of membranes alone. The level of quinone was also reduced.

7. With malate and azide, in white and yellow membranes, the quinone levels were higher than with either malate or azide alone.

The counts in quinone were reduced, but were not as low as with azide alone. Malate appears to reduce partially the effect of azide as when added with azide it increased quinone levels and counts in quinone, above those for azide alone.

8. In white and yellow membranes, HOQNO increased quinone levels compared with no addition and lowered incorporation of label into quinone, possibly by decreasing synthesis and breakdown.

9. In yellow membranes, the addition of malate and HOQNO lowered the level of quinone but increased the counts in quinone, when compared with HOQNO alone.

10. In white membranes, malate and HOQNO lowered quinone levels and lowered the incorporation of radioactivity into quinone, when compared with HOQNO alone.

11. In yellow cells HOQNO addition resulted in a slight decrease in quinone levels, but a large increase in incorporation of [2-^{14}C] mevalonic acid into quinone, indicating very high rates of synthesis and breakdown.

(v) Conclusions.

From the non-shaking experiments, it seems likely that some form of oxygen is involved in the breakdown of quinone.

Azide directly inhibits the synthesis of quinone and carotenoid. If the azide-sensitive step were not the rate-limiting step, then its effect in yellow cells and membranes should be significantly less than in white, where there is probably impaired regulation and maximum synthesis. If azide inhibited the regulatory step, its effect in yellow and white would be similar. In yellow membranes the fall in incorporation of [2-^{14}C] mevalonic acid into quinone was 30 times, which is three to four times less than in white.

The low levels of quinone obtained by incubation with azide are increased in the presence of malate, although there is still very low incorporation of [2-^{14}C] mevalonic acid into quinone. In S. lutea Erickson and Parker (1969) found that the cytochrome b was more oxidised than cytochromes a and c, due to its ability to autoxidise. Therefore, as azide inhibits the respiratory chain away from cytochrome b, at cytochrome a, azide addition would leave the cytochrome b, and therefore the quinone, in a relatively oxidised state. Malate addition would reduce the quinone. It appears that quinone is more stable in the reduced state. There is another explanation however, that malate or the reduced state of quinone encourages quinone synthesis from unlabelled intermediates. The situation is complicated by azide inhibition of incorporation of [2-^{14}C] mevalonic acid into quinone and other lipids. The inhibition is great, but the quinone levels do not fall correspondingly,

indicating that azide must also inhibit breakdown or the bacterium utilizes unlabelled intermediates for quinone synthesis, in the presence of azide.

A possibly less complicated situation involved the use of HOQNO, which blocks before cytochrome b in the respiratory chain (Erikson and Parker 1969). So,unlike azide, HOQNO addition would result in the quinone being mostly reduced. HOQNO addition resulted in the highest quinone levels. Malate plus HOQNO, however,reduced these levels. Using the HOQNO results, as HOQNO does not appear to have a direct effect on synthesis, it was found that when quinone is mostly reduced (HOQNO addition) it is the most stable. Fully reducing the quinone (malate and HOQNO addition) decreases levels and alternate oxidation-reduction (malate addition) reduces the levels further. (It should be noted that no magnesium was added in these experiments). The lowest levels of quinone were obtained when it was mostly oxidised (membranes alone). The change in quinone levels in different oxidation-reduction states, could be due to alterations in the rate of breakdown, or utilization to differing extents of unlabelled intermediates.

4. DISCUSSION.

A. DIFFERENCES BETWEEN WILD-TYPE AND WHITE-CAROTENOIDLESS STRAINS OF MICROCOCCUS LYSODEIKTICUS.

One of the major proposed functions of carotenoid is as a protector of quinone against photo-oxidation in air. Anwar and Prebble (1977) found that quinone was one of three photosensitive sites in the respiratory chain of S. lutea (a closely related organism), and that carotenoid could protect it.

In this study, differences were found between wild-type and carotenoidless-mutant strains of M. lysodeikticus, when they were grown in the dark. Previously, differences between wild-type and carotenoidless-mutant strains, of various bacteria, were observed only when the cells were exposed to light and oxygen. Stanier and his colleagues (Griffiths et al, 1955, Stanier and Cohen-Bazire, 1957) first proposed that carotenoid could protect bacteria, both photosynthetic and non-photosynthetic, against lethal oxidations. Since then, the protection by carotenoids has been characterised fairly extensively, but only under conditions when the bacteria were exposed to light and oxygen.

(a) Quinone levels and viability.

Quinone levels were low in the white strain, while the rate of synthesis was high, suggesting that quinone is protected by carotenoid, even in the dark. In the presence of light and oxygen quinone might be destroyed by singlet oxygen. However if this in fact happens, then it need not be limited to the presence of light, as singlet oxygen could be produced in the dark. Certain cell reactions e.g. those of the respiratory chain, are believed to produce the superoxide radical

(Tyler 1975) and this radical can dismutate to form singlet oxygen (Pederson and Aust 1973, Khan 1970).

In four carotenoidless-mutant strains, there were low quinone levels, which shows that low quinone is associated with lack of carotenoid. This interpretation is further supported by experiments with diphenylamine. Diphenylamine inhibits coloured carotenoid synthesis and results in a white culture which has a low level of quinone, together with increased biosynthesis. Low quinone levels lead to a decrease in the activity of those membrane-bound respiratory enzymes, which utilize quinone in the oxidation of their substrates. This loss of respiratory activity could lead to the observed loss of viability.

(b) Respiratory enzyme activity.

From the experiments on enzyme activities, in white and yellow membrane preparations, some interesting observations were made. It was found that lactate and succinate oxidase activities were higher in white membranes. Succinate oxidation is believed not to require quinone in S. lutea (Anwar and Prebble 1977) and Mycobacterium phlei (Asano and Brodie 1964) and possibly in this bacterium. It seems probable that lactate oxidation is also quinone independent, although this conflicts with results obtained by Lukoyanova and Kovoleva (1975), working with M. lysodeikticus. Malate dehydrogenase activity was lower in white membranes, suggesting that malate oxidation is depressed in white membranes.

The lower activity of lactate and succinate oxidase, in yellow membranes, could be due to quinone having a regulatory role on dehydrogenase activity, higher amounts depressing oxidation, although the

quinone is probably not directly involved in the oxidation of these substrates.

(c) Carotenoid protection of other membrane constituents.

The quinone has a very important biological role, as a component of the respiratory chain,and must be protected. However,carotenoid could protect other membrane constituents,such as membrane lipids.

Carotenoid has been shown to protect lipids from peroxidation. Foote and Denny (1968) found that carotenoid can quench singlet oxygen in vitro, and that the conditions for quenching mirror the conditions e.g. chromophore length, for protection of the bacterium from photo-oxidation (Foote et al 1970), indicating that singlet oxygen may be involved.

The lack of carotenoid,of which the low quinone levels and enzyme activities may be a consequence,may result in other differences between the wild-type and carotenoidless-mutant strains of M. lysodeikticus, but of these differences, the quinone level proved to be the easiest and most reproducible one to study.

B. QUINONE TURNOVER.

In white cells and membranes there is high incorporation of [2-^{14}C]mevalonic acid into quinone, but quinone levels are low, indicating a high rate of breakdown.

(a) Factors affecting synthesis and breakdown.

It was observed that high magnesium inhibited the incorporation of [2-^{14}C]mevalonic acid into quinone. Malate and succinate reversed this, probably because they can complex magnesium. Malate complexes magnesium twice as strongly as succinate (Cannan and Kibrick 1938). Succinate increased quinone levels in the presence of 10mM magnesium but malate did not, indicating that it has other effects, probably on breakdown.

Malate could affect [2-^{14}C]mevalonic acid incorporation into quinone, by altering magnesium levels. Also it appears to slightly lower quinone levels, when added [2-^{14}C]mevalonic acid has been removed. In experiments when oscillations occurred, malate either dampened or retarded the onset of the oscillations. In other experiments, malate appeared to delay the incorporation of unlabelled intermediates into quinone and perhaps this is the mechanism by which oscillations were retarded. Malate is also a substrate for the respiratory chain, so its addition could result in the production of singlet oxygen, as previously discussed, which would increase quinone breakdown. Being a substrate, malate also affects the oxidation-reduction state of the quinone, which could alter its stability.

Malate could have several effects including:

(i) increased incorporation by lowering magnesium,

(ii) delayed incorporation of unlabelled intermediates,

(iii) promotion of singlet oxygen production, so increasing breakdown,

(iv) a change in the oxidation-reduction state of quinone.

As a consequence, interpretation of the effects of malate in several experiments is difficult.

(b) Interpretation of results from experiments utilizing radioactive precursors to study quinone breakdown.

(i) Storage of radioactive intermediates.

Studies on breakdown proved difficult,as both cells and membrane preparations are capable of storing [2-^{14}C] mevalonic acid, or labelled intermediates of quinone biosynthesis, and then utilizing them to synthesise labelled quinone, after the removal of [2-^{14}C] mevalonic acid from the external medium.

It is possible that there is a pool of quinone intermediates, part way along the pathway, although if quinone levels are low, as in white membranes, it would be expected that quinone would be synthesised as quickly as possible and a reserve pool would be less likely. Another possibility is that quinone biosynthesis involves enzyme complexes and that a pool of intermediates builds up between these complexes. The results from experiments investigating breakdown of quinone are therefore difficult to interpret,as synthesis from membrane bound precursors is also occurring.

(ii) Theoretical calculations.

In the experiments, when synthesis and breakdown are occurring, theoretical values for counts and specific activity in quinone, using varying rates of synthesis and breakdown,can be calculated. If the rate of breakdown is a function of quinone concentration, the counts in

quinone always increase, irrespective of whether the rate of synthesis or breakdown is higher. If there is a fixed rate of synthesis and breakdown, the counts in quinone increase if the rate of synthesis is greater than or equal to the rate of breakdown (Fig. 19). If the rate of breakdown is higher, the counts will rise and then fall, the curves differing for different ratios (Fig. 20). Under no conditions would the specific activity decrease. However in experiments where quinone is labelled, excess label removed and the effect of incubation of the membrane, or cell preparation, containing labelled quinone, with various substances, examined over a set time period, the specific activity sometimes fell. This indicates that the situation in the membrane cannot be as simple as the model.

(iii) A reserve pool of quinone, or intermediates in quinone biosynthesis, as an explanation for the specific activity results obtained.

In the experiments described here several trends were obtained, three when the specific activity rose and three when it fell. With an increase in specific activity there was also either:

(a) an increase in counts in the quinone fraction, with a decrease in quinone levels ; or

(b) an increase in counts and quinone levels, the quinone increasing to a lesser extent; or

(c) a decrease in counts and quinone levels, the decrease in quinone levels being greater.

These observations, when the specific activity increases, fit in with the theoretical calculations. In (a) and (c) there is a fixed rate of synthesis and breakdown, the rate of breakdown being greater. In

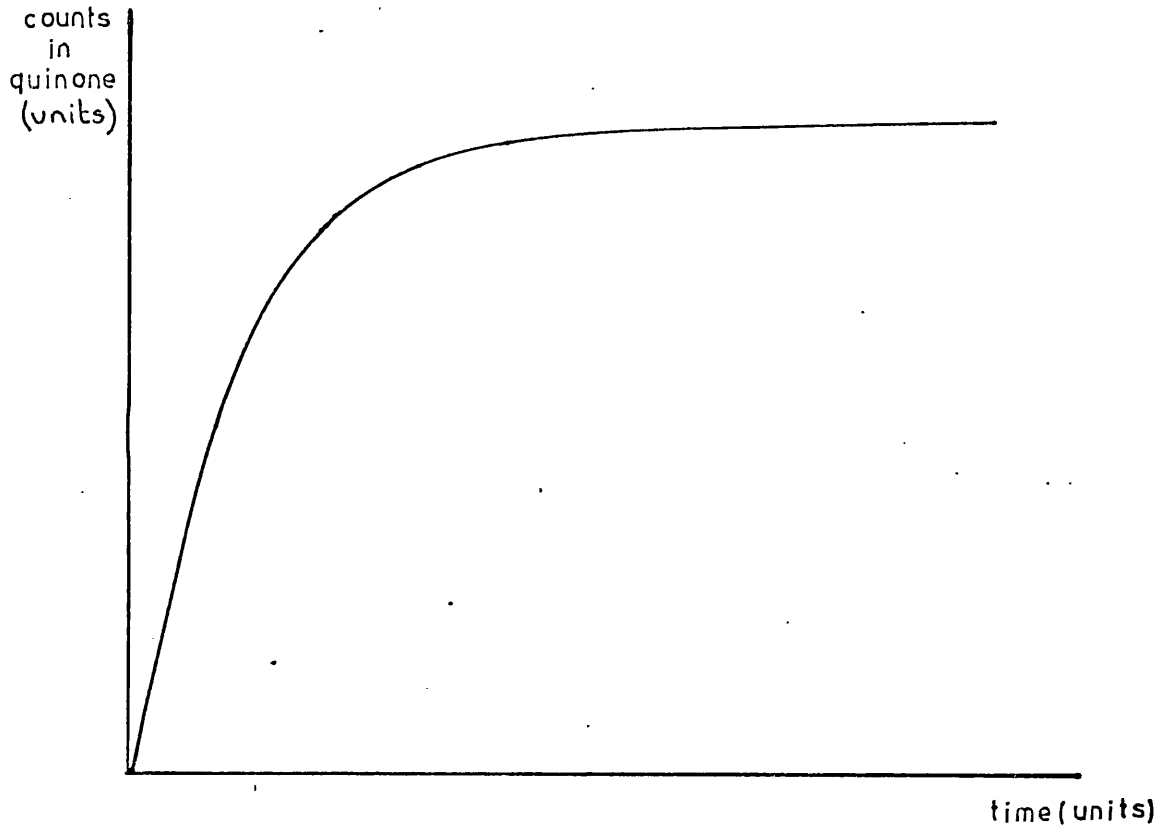
Figure 19

Theoretical counts in quinone, under varying conditions of synthesis and breakdown.

(a) when the rate of synthesis is constant, but the rate of breakdown depends on the level of quinone, irrespective of which rate is higher.

(b) when both rates are constant, but the rate of synthesis is greater than or equal to the rate of breakdown.

(a)


counts in quinone (units)
time (units)

(b)

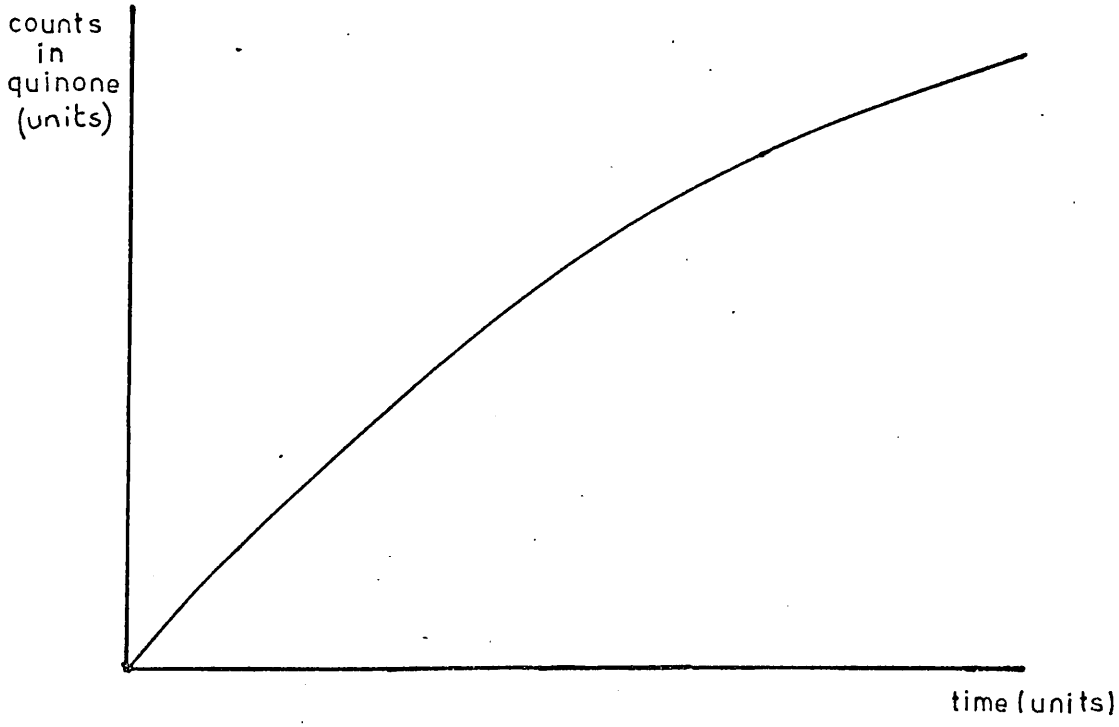

counts in quinone (units)
time (units)

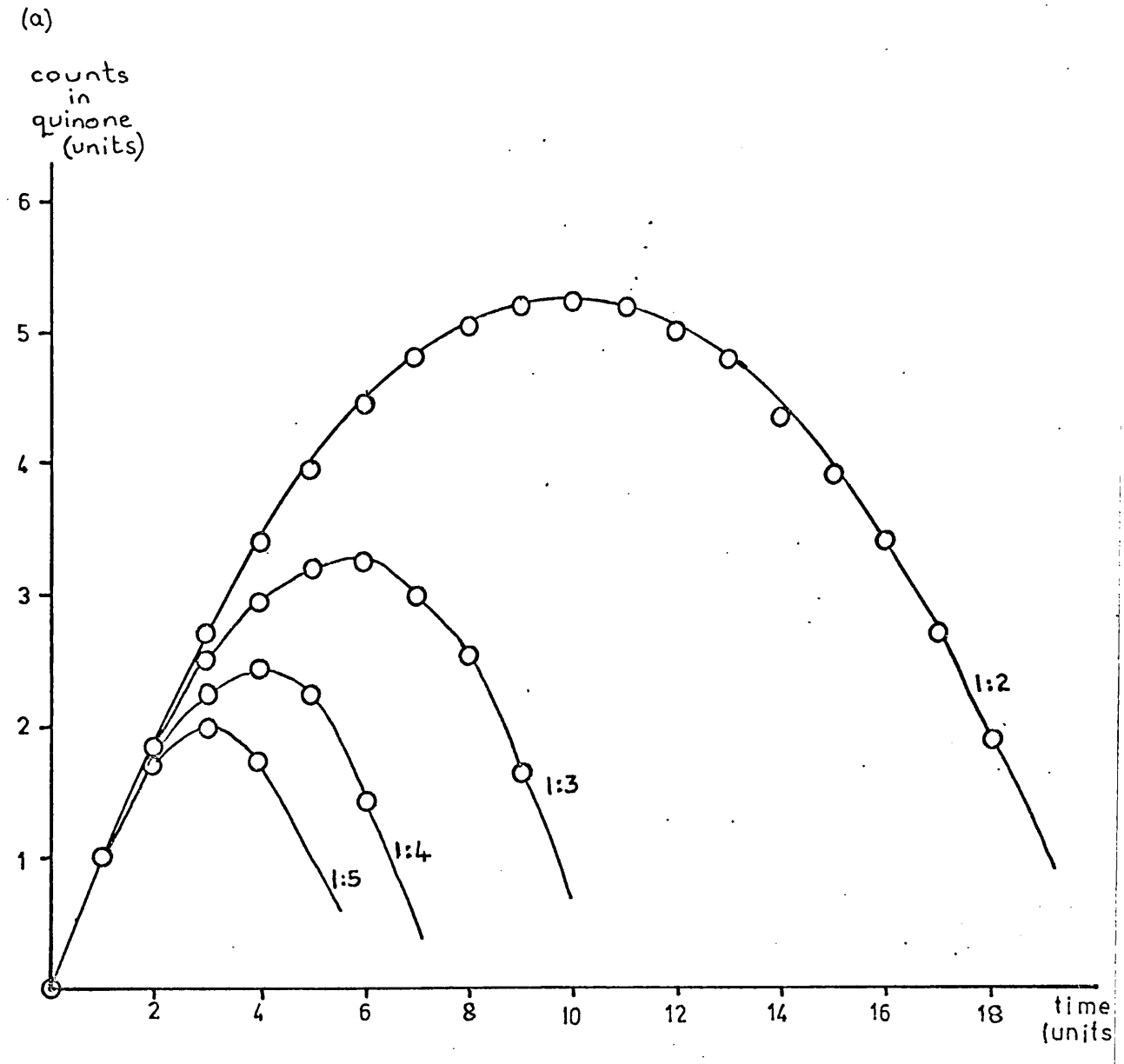
Figure 20

Theoretical counts in quinone, when the rate of breakdown is greater than the rate of synthesis.

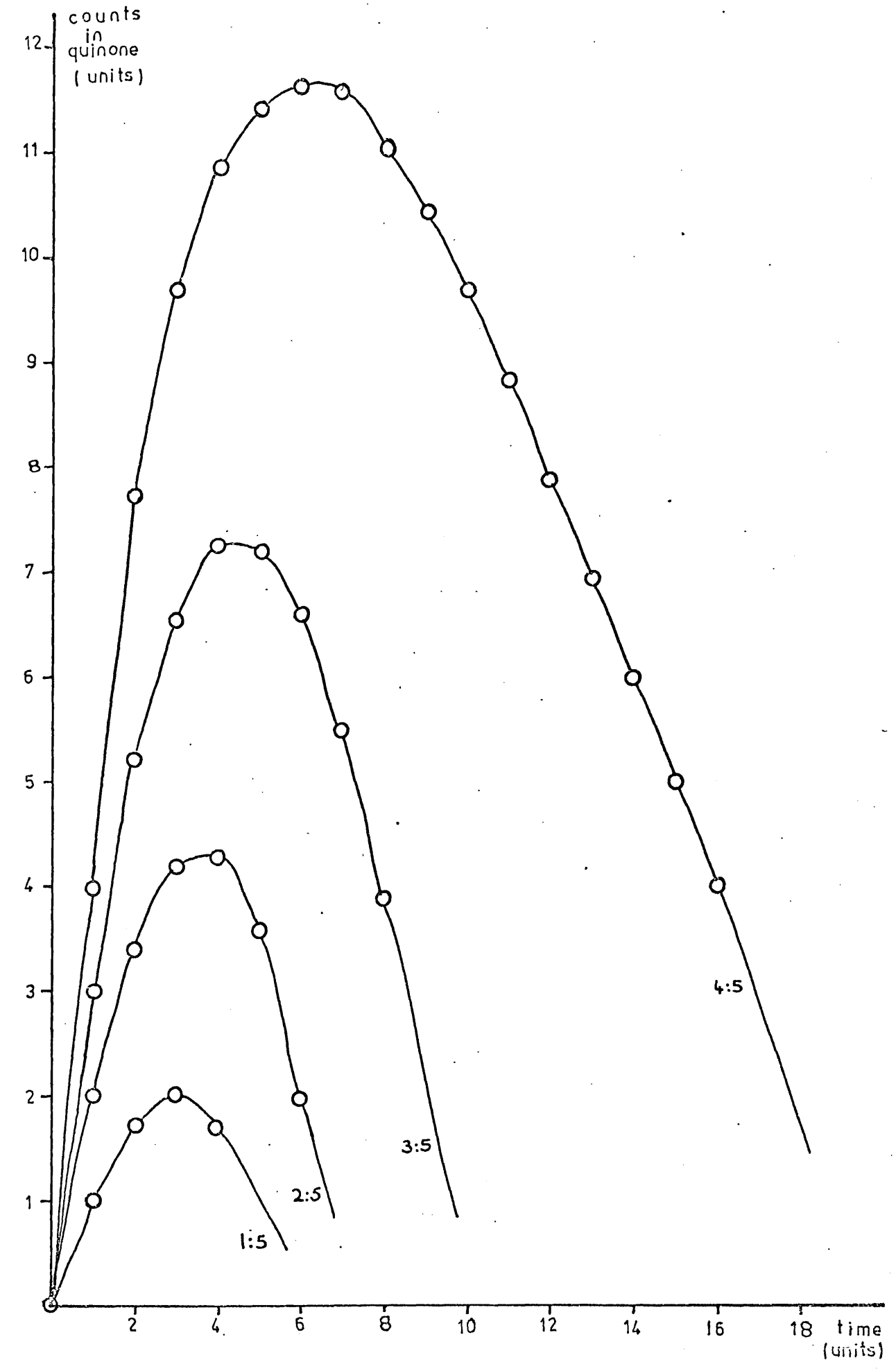
(a) the effect of changing the rate of breakdown, with a constant rate of synthesis.

(b) the effect of changing the rate of synthesis, with a constant rate of breakdown.

1:5 = ratio of the rate of synthesis to the rate of breakdown is 1:5


(a)
counts in quinone (units)
6
5
4
3
2
1
2
4
6
8
10
12
14
16
18
time (units)
1:2
1:3
1:4
1:5

(b)


counts in quinone (units)
12
11
10
9
8
7
6
5
4
3
2
1
1:5
2:5
3:5
4:5
2
4
6
8
10
12
14
16
18
time (units)

(b) the rate of synthesis is greater than that of breakdown.

Three trends were also observed when the specific activity fell, either:

(d) increased quinone level with decreased counts in quinone; or

(e) decreased quinone level, with a greater decrease in counts; or

(f) increased counts in quinone, and a greater increase in quinone levels.

There are two explanations for the fall in specific activity. One is that there is a reserve pool of intermediates, and the other, that there is a reserve pool of short-chain quinone, both being in the membrane.

(a) mevalonic acid ⟶ Quinone ⟶ breakdown

If quinone levels low ↑ ↓ If quinone levels high

Quinone (reserve)

(b) mevalonic acid ⟶ A ⟶ Quinone ⟶ breakdown

If quinone levels low ↑ ↓ If quinone levels high

A (reserve)

Postulation of a pool of reserve quinone is probably not acceptable, as it would have to be non-extractable. This is unlikely, even for quinone of shorter chainlength. In both cases i.e. reserve pool of quinone or intermediates, the same explanation can be used. If the pool was high when the labelled mevalonic acid was added, then the labelled precursor would be incorporated directly into the quinone. Then, when the labelled precursor was removed and the levels of quinone fell, any further quinone would be made from unlabelled

intermediates and so the specific activity would fall. Whether the quinone levels increased or decreased would depend on whether the rate of synthesis was greater than the rate of breakdown. However, if there is a reserve pool,it would also operate under the conditions when the specific activity rose.

Under these conditions,it must be assumed that the reserve pool was low and so it was filled up, almost totally,with labelled intermediate. When[2-^{14}C]mevalonic acid was removed, the quinone was made from labelled intermediates and so the specific activity would rise. This hypothesis assumes that the pool is not normally accessible to products of mevalonate metabolism.

Gibbons (1977) has found that there are reserve pools of intermediates in cholesterol biosynthesis,in rat liver. If biosynthesis is driven at a high rate, by adding a high concentration of precursor e.g. 20 micro Curie mevalonic acid, which is past the first rate-limiting step, then there is synthesis from endogenous intermediates, which are in a compartment, metabolically isolated if there is low synthesis. When biosynthesis is high, the intermediate pools are expanded and spill into the isolated pool, connecting them. If the active pool is labelled, then connection with the reserve pool will result in a large dilution of radioactivity. Gibbons believes that these pools are necessary to satisfy a need for a change in cholesterol biosynthesis, which must be more rapid than can be provided by a longer term change in the capacity of the primary rate-limiting step.

The ideas of Gibbons (1977) can be applied to quinone synthesis in Micrococcus lysodeikticus. For the specific activity to fall, the rate of synthesis must only be high enough to result in the reserve

isolated pool mingling with the intermediates near to the time when [2-^{14}C]mevalonic acid was removed, otherwise they would be similarly labelled. Then,with no more labelled precursor, the intermediate pool would be largely unlabelled and so the specific activity in quinone would fall. For the specific activity to rise, the pools must merge fairly early after the addition of[2-^{14}C]mevalonic acid, or not merge at all. The quinone levels themselves would depend on the relative rates of synthesis and breakdown.

(c) Factors affecting quinone breakdown.

(i) Oxygen involvement in breakdown?

Low oxygen levels, an effect produced by not shaking a suspension of cells or membranes,results in an increase in quinone levels compared to no addition in both white and yellow cells and membranes. The synthesis from [2-^{14}C] mevalonic acid could not account for all the increase, which suggests that quinone is being broken down less rapidly. However evidence for this is not strong,as no experiments were carried out in completely oxygen-free conditions. The fact that levels rise in the yellow strain, suggests that the quinone may not be completely protected by carotenoid.

(ii) Oscillations in quinone levels.

Oscillations in quinone levels were observed in white membranes but never in yellow membranes, which suggests that they are associated with lack of carotenoid. The oscillations were observed with and without malate and, it must be pointed out, depend on a single point. But, as there are large differences in values and oscillations were observed in several different experiments, they are probably genuine. The oscillations could be a product of a synthetic system not fully under control.

(iii) Oxidation-reduction state of quinone.

In experiments when magnesium was not used, there were indications that the oxidation-reduction state of quinone affects its level in the membrane. The highest levels of quinone were observed in the presence of azide and malate, or HOQNO. Levels were lower when quinone was fully reduced (in the presence of malate and HOQNO), or alternatively, when oxidised and reduced (in the presence of malate) and mostly oxidised (membranes alone). Either the oxidation-reduction state of the quinone influences breakdown, or it influences synthesis, probably from unlabelled intermediates. Malate, as previously discussed, however, may be having other effects.

Azide, another inhibitor of the respiratory chain, has a direct effect on quinone and carotenoid synthesis, so results obtained using azide are more complex, but here again, reduced quinone gives higher quinone levels.

(iv) Glycine and acetate.

Glycine increases quinone levels in cells and membranes from the yellow strain, but quinone is decreased in white cells and membranes. Acetate lowers quinone levels, in white and yellow membranes, but has no effect in cells of either strain. This is not unexpected, as cofactors for acetate utilization are probably not present in membranes, whereas in cells there are probably permeability problems.

(d) Factors affecting quinone synthesis.

[2-^{14}C] Mevalonic acid is incorporated into quinone in the white and yellow strains, and into polar lipids in the wild-type. Isopentenyl pyrophosphate was not incorporated significantly into either strain. For the large incorporation of [2-^{14}C] mevalonic acid into quinone,

observed in white membranes, there must be a supply of ring structure or intermediates stored in the membrane, as nothing was added from which ring structures could be synthesised.

(i) Effect of succinate.

Succinate increases incorporation of [2-^{14}C] mevalonic acid into quinone, in cells. This could be because succinate is acting as a substrate for quinone synthesis, or for oxidative phosphorylation. Further, ATP production may reduce the magnesium concentration in the cell, or succinate itself could chelate the magnesium ions, both of which factors would promote quinone synthesis from [2-^{14}C] mevalonic acid.

(ii) Menadione.

Menadione also increases the incorporation of [2-^{14}C] mevalonic acid into quinone. There are conflicting reports as to whether it is an intermediate in quinone biosynthesis (Guerin et al 1970, Goldenbaum et al 1975). It increases incorporation to a greater extent if it is incubated with the growing cells, overnight. This suggests that the menadione is being modified, before use as a ring structure. Or it could stimulate quinone synthesis without being involved, as it is very like possible ring structure intermediates. Alternatively, it could be stored and only used when the ring intermediate was no longer available. There must be large amounts of ring precursors stored in the membrane, as there is a large amount of incorporation of [2-^{14}C] mevalonic acid into quinone, in membranes, which would not occur without a source of ring structure.

(iii) Menaquinone-7.

Menaquinone-7 decreases incorporation of [2-^{14}C] mevalonic acid

into quinone. It is very similar to the natural quinone of M.lysodeikticus. The decrease in quinone biosynthesis, in the presence of added quinone, indicates end product inhibition. Menaquinone-7 also decreases counts in the "phytoene" fraction. Possibly it represses an enzyme just before the branch point of quinone and carotenoid biosynthesis, although this seems unlikely as then, irrespective of its level in the membrane, carotenoid could not be synthesised if there was already sufficient quinone in the membrane.

(iv) Magnesium.

Magnesium levels, in cells and membrane preparations, could affect synthesis,as high levels (10mM) have been shown to inhibit synthesis. Free magnesium levels in the cell could depend,to some extent,on the ratio of ATP to ADP, as ATP binds magnesium much more strongly than ADP. Quinone would then be related to the energy state of the cell.

(e) Control of quinone synthesis.

Quinone synthesis could be induced by:

(a) a low level of quinone (due to a high rate of breakdown); or

(b) the oxidation-reduction state of quinone; or

(c) the level of magnesium.

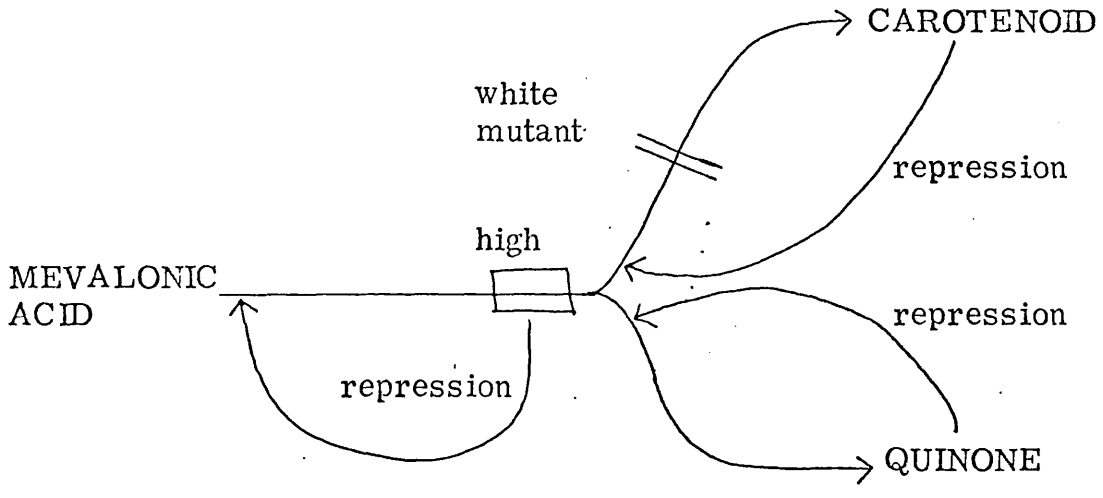
From the experiments with menaquinone-7,there is probably feedback inhibition on synthesis by quinone. Although menaquinone-7 also decreased counts in the "phytoene" fraction, it seems more likely that quinone affects an enzyme just after, rather than before, the branch point of quinone and carotenoid synthesis. There must be optimum levels of quinone and carotenoid in the membrane, so carotenoid

probably controls its own synthesis, also affecting an enzyme just after the branch point. The level of the intermediates prior to the branch point , therefore, may control the early part of the chain, when they reach a higher than normal level. The proposed hypothesis is shown in Fig. 21. Some regulation of the synthesis of the ring structure also seems essential.

Not only are quinone and carotenoid linked by the protective activity, but they also share the isoprenoid biosynthetic pathway, involving mevalonic acid and isopentenyl pyrophosphate. So the situation exists, that one arm of the pathway produces quinone; important to the bacterium as an integral part of the respiratory chain and which is susceptible to destruction by oxidation, and the other arm of the pathway produces carotenoid, which protects the quinone from destruction.

Figure 21

Proposed scheme for the control of quinone and carotenoid synthesis.


CAROTENOID
white
mutant
repression
high
MEVALONIC
ACID
repression
repression
QUINONE

Several conclusions can be drawn from this thesis:

1. There are significant differences between wild-type and carotenoidless-mutant strains of Micrococcus lysodeikticus, when they are grown in the dark.
2. Lack of carotenoid results in lower quinone levels, lower respiratory activity and lower viability.
3. Both cells and also membrane preparations can incorporate [2-^{14}C]mevalonic acid into quinone and also into carotenoid in the yellow strain.
4. There is a membrane-bound system for quinone and carotenoid synthesis in Micrococcus lysodeikticus.
5. There is high breakdown in the white strain and a form of oxygen is probably involved in the breakdown.
6. In the yellow strain the quinone is protected by carotenoid.
7. There is a reserve pool of intermediates in the shared pathway of quinone and carotenoid biosynthesis.
8. Control of quinone synthesis, in the white strain, is probably deficient, which may lead to the oscillations observed only in the white strain.
9. Quinone appears to regulate its own synthesis.
10. Carotenoid protects quinone, not only from photo-oxidations but also from other, possibly oxygen-mediated, destruction in the dark.
11. The oxidation-reduction state of quinone affects its level in the membrane.
12. Azide directly inhibits quinone and carotenoid biosynthesis, probably not at the regulatory step.

13. There must be a supply of ring structure or intermediates in ring structure synthesis in the membrane.

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