EFFECTS OF LIGHT ON PIGMENTED AND NON-PIGMENTED STRAINS OF *SARCINA LUTEA*

A thesis presented by

ABU MUHAMMAD SHAMSUL HUDA

in part fulfilment of the requirements for the degree of Doctor of Philosophy in the University of London

1970

Department of Biochemistry,
Bedford College,
London, N.W.1.

Abstract

A comparative study of the photosensitization of isolated membranes of pigmented and non-pigmented Sarcina lutea in the presence and absence of an exogenous photosensitizer has been made. Illumination of membranes at low light intensities with toluidine blue resulted in photoinactivation of malate, succinate, lactate and ascorbate oxidases. Methylene blue and DCPIP reductions, cytochrome c and ascorbate-TMPD oxidase activities also showed photoinactivations. Photoinactivation was always greater in non-pigmented than in pigmented membranes. 20 minutes' illumination caused reversible inactivation, while 60 minutes' illumination caused irreversible photoinactivation of malate oxidase activity in the non-pigmented membranes.

5 minutes' illumination at high light intensity, without exogenous photosensitizer, caused reversible photoinactivation of malate and succinate oxidases and malate : DCPIP and succinate : DCPIP reductions in pigmented membranes. Non-pigmented membranes showed irreversible photoinactivation of these enzyme activities. Photoinactivation of malate : vitamin K reductase was reversible in all membranes.

15 minutes' illumination irreversibly photoinactivated malate and succinate oxidases, but reversibly photoinactivated malate and succinate : DCPIP reductions in the pigmented membranes. Non-pigmented membranes showed irreversible photoinactivation of these enzyme activities. Malate and succinate : vitamin K reductases of both strains showed reversible photoinactivation.

25 minutes' illumination with ultraviolet-filtered light showed no photoinactivation of PMS or methylene blue reductions, but showed reversible photoinactivation of malate oxidase activity in the pigmented strain and irreversible photoinactivation in non-pigmented cells and membranes. There was irreversible photo-inactivation of malate : DCPIP reduction in both strains. Permanent photoinactivation of NADH oxidase activity was observed in pigmented membranes, while NADH : DCPIP reduction showed no photoinactivation.

Malate : vitamin K reductase activity in both strains showed reversible photoinactivation. The malate oxidase and malate : vitamin K reductase activities of two other non-pigmented mutants showed identical properties.

It is concluded that carotenoid reduces photoinactivation generally in the presence of an exogenous photosensitizer, but protects a specific site in the respiratory chain in the absence of added dye.

Contents

CHAPTER 1

HISTORICAL INTRODUCTION

1. General introduction

Sarcina lutea Schroeter, a non-photosynthetic, non-pathogenic, common air-contaminant, forms coarsely granular, yellowish pigmented, circular colonies when grown on nutrient agar. The yellow compounds belong to the group carotenoids (1). The carotenoid pigments most commonly found in bacteria belong to the following groups: (i) hydrocarbons or carotenes such as betacarotene, (ii) alcohols or xanthophylls, (iii) esters and (iv) carotenoid acids. Carotenoid pigments are rather widely distributed in the microbial world. They are present invariably in all photosynthetic organisms but are also produced by some fungi and non-photosynthetic bacteria. These pigments have been widely studied during the last few decades owing to their interesting chemical structure and also to their possible biological and physiological importance.

However, at the present time comparatively little is known about the precise physiological functions of the carotenoids other than as precursors of vitamin A in animal systems. In the photosynthetic bacteria the carotenoid pigments have been found to protect these organisms against lethal photooxidations in which the intracellular bacteriochlorophyll is the photosensitizer (2, 3). It has been found that the carotenoid pigments of non-photosynthetic bacteria such as Sarcina lutea or Corynebacterium poinsettiae have a similar protective function (4, 5). Unlike the photosynthetic bacteria, photodynamic killing of carotenoidless mutants of these organisms normally occurred at low light intensities only in the presence of an added photosensitizer and oxygen, while pigmented strains were unaffected. At high light intensities (e.g. direct sunlight) and in the absence of an exogenous photosensitizer, carotenoidless mutants were killed in the presence of oxygen while the pigmented strains escaped photodynamic action (6). These results suggest the presence

within the cells of substances which act as photosensitizers at high light intensities in the photodynamic killing of carotenoidless cells. The carotenoid pigments prevent the lethal action of the exogenous as well as of endogenous photosensitizers. The chemical nature of the endogenous photosensitizer is, however, not known.

The role which carotenoids play in bacterial photosynthesis, other than their protective one, is far from being understood. Owing to the ubiquitous association of carotenoids with all photosynthetic systems, their direct participation in photosynthesis has been regarded as one of their principal functions. Carotenoids participate in photosynthesis as auxiliary absorbers of radiant energy which, in turn, may be transferred to chlorophyll and converted to chemical energy. The efficiency of this energy trapping, however, varies with different carotenoids and organisms (7). Chlorophyll plays an essential role in photosynthesis since, in its absence, photosynthesis cannot take place. By contrast, the role of carotenoids is of a more supplementary nature since photosynthesis can proceed in their absence (8). Carotenoids may also play other roles in photosynthesis. There is a probability that they might participate in the transport of electrons in photosynthesis. Electrons could be transferred from photoexcited chlorophyll to a carotenoid, with the latter then playing some essential role in "mediating" an electron-transfer from a donor to an acceptor molecule (9). In this connection several workers have observed absorption changes during photosynthesis believed to be due to carotenoids (10).

Oxygen-evolving phototrophs usually contain epoxidic carotenoids, and Blass et al. (11) have advanced the hypothesis that reversible removal of epoxidic oxygen may be part of the oxygen evolution sequence in photosynthesis. In photosynthetic bacteria, where epoxidic carotenoids are not present, oxygen evolution does not occur during photosynthesis. Krinsky et al. (12) have pointed out that the epoxidic carotenoid, neoxanthin, is virtually absent in dark grown Euglena gracilis, and that formation of neoxanthin upon illumination of the same organism, previously grown in dark, paralleled the development of photosynthetic competence. However, no direct

evidence for this hypothesis has been obtained.

As noted above, carotenoids play an important role in the protection of cells against photodynamic killing. However, the precise way in which this protective function is performed is still unknown. Since these pigments are found in the membranes (13, 14), the present study was planned to investigate the properties of the cell membrane in relation to the carotenoid pigments of Sarcina lutea. The study comprises preliminary experiments with cell-suspensions of wild and a non-pigmented mutant of S. lutea which were subjected to illumination in the presence and absence of an external photosensitizer, toluidine blue. Membranes from pigmented and non-pigmented S. lutea were illuminated initially in the presence of toluidine blue and finally without any added photosensitizer. These latter experiments were designed to investigate the properties of endogenous photosensitizers. So far no investigations have been made on the protective function of carotenoids using isolated cell membranes. This study of the role of carotenoids in S. lutea involves work with isolated membranes and provides an opportunity to gain a fuller understanding of the functions of these pigments in a non-photosynthetic bacterium.

2. Carotenoids

(a) General.

Carotenoids have attracted the curiosity of scientists since the beginning of organic chemistry. In the classical period the structural determinations of the carotenoids, derived chiefly from plant sources, were carried out by semi-micro methods, by means of chemical degradation. Various types of oxidative degradations, catalytic hydrogenation, determination of side-methyl groups etc. were used, and the structure of many of the common C-40 skeleton of carotenoids was established. This early work was elegantly reviewed by Karrer and Jucker (15) in 1943. Physical methods were partly used at that time, but the subsequent period has been characterized by development of improved micro-techniques and new methods, particularly spectroscopic and chromatographic ones. In the last ten to

fifteen years, considerable progress has also been made in the field of biosynthesis of the coloured 40-carbon pigments.

(b) Carotenoid chemistry.

Carotenoids consist of a long chain of conjugated hydrocarbons substituted at certain positions by methyl groups and generally terminated by two partially saturated rings. They may be regarded as composed of isoprene residues, usually eight, arranged in such a way that nearest to the centre of the molecule two methyl groups are present in 1:6 positions, while the lateral methyl groups occupy 1:5 positions with respect to each other (see Fig. 1).

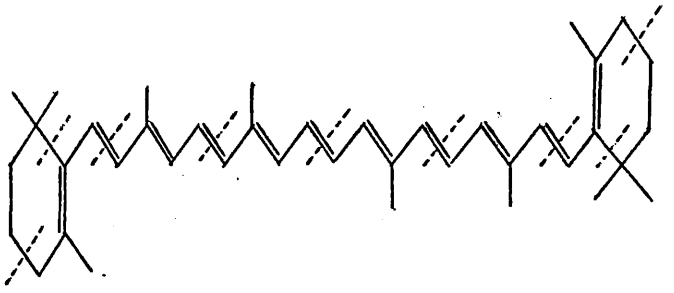


Fig. 1. Isoprene units in β-carotene.

Many of the physical and chemical properties of carotenoids result from characteristic features of their molecular structure. In particular the delocalization of the π-electrons over the entire carbon chain readily permits polarization of the molecule (16), and the energetic values of the highest occupied and lowest empty molecular orbitals suggest that these compounds should be excellent electron donors and acceptors (17).

(c) Bacterial carotenoids.

Carotenoid pigments are widely distributed in the microbial world. They are invariably present in all photosynthetic micro-organisms and are also present in a variety of non-photosynthetic bacteria (13). Early work by Sobin and Stahly (1) showed Sarcina lutea to contain only two carotenoids, while S. flava had only one,

identical with one of those found in S. lutea. Thirkell and Strang (19) reinvestigated pigments in S. lutea and S. flava and isolated seven fractions, all apparently with the same chromophoric group. These fractions were hydrocarbons or polar carotenoids. There was no evidence for any simple carotenoid esters. Strang (20) studied the spectral characteristics of the carotenoids of S. lutea and S. flava and found six pigments. Most carotenoids reported to occur in bacteria have 40 carbon atoms, but Jensen and Weeks (21) reported a C_{50} carotenoid in Flavobacterium dehydrogenans. Thirkell et al. (22) have now reported the existence of an apparently identical C_{50} carotenoid in S. flava and suggested the possible occurrence of the same in S. lutea.

3. Photosensitization and bacterial carotenoids.

In a biological system exogenous dyes can bring about damage or even complete destruction when subjected to artificial light of low intensity. Similar damage or destruction could be produced at very high light intensities (e.g. direct sunlight) without any added photosensitizer. In the latter case the endogenous pigments in the cells (e.g. cytochromes, flavins) presumably act as the photosensitizing agent. It is well known that some of the amino acid residues in a protein, such as histidine, tryptophan, methionine and tyrosine, can specifically be oxidised by irradiation with visible light in the presence of a suitable photosensitizer (23). It has also been found in vitro that when solutions of a number of proteins were irradiated with visible light in the presence of various dyes like methylene blue (24) and proflavin (25), oxygen was taken up and oxidative destruction of histidine, tyrosine and tryptophan and oxidation of side-chains containing sulphur took place. The precise nature of this photodynamic action, however, is not known. Blum (26) defined photodynamic action as "the sensitization of a biological system to light by a substance which serves as a light absorber for photochemical reactions in which molecular oxygen takes part." The dye appears to act catalytically and only undergoes destruction in side reactions.

The commonly proposed mechanisms for photodynamic action found in the biological literature may be summarised as follows (27):

(I) $S + h\nu \rightarrow S^* \rightarrow S'$; $S' + RH_2 \rightarrow S + RH_2^*$; $RH_2^* + O_2 \rightarrow$ products

(II) $S + h\nu \rightarrow S^* \rightarrow S'$; $S' + RH_2 \rightarrow SH_2 + R$; $SH_2 + O_2 \rightarrow S + H_2O_2$

(III) $S + h\nu \rightarrow S^* \rightarrow S'$; $S' + O_2 \rightarrow S + O_2^*$; $RH_2 + O_2^* \rightarrow$ products

(IV) $S + h\nu \rightarrow S^* \rightarrow S'$; $S' + O_2 \rightarrow SOO$; $RH_2 + SOO \rightarrow$ products + S

(V) $S + RH_2 \rightarrow SRH_2$; $SRH_2 + h\nu \rightarrow SRH_2^* \rightarrow SRH_2'$; $SRH_2' + O_2 \rightarrow S +$ product

Where S is the sensitizing dye, hν is a quantum of light, S^* is the first excited singlet state of the dye, S' is the triplet state of the dye, RH_2 is the substrate, RH_2^* is an excited form of the substrate, "products" represents the oxidised form(s) of the substrate, R is an oxidised (dehydrogenated) form of the substrate, SH_2 is a photo-reduced form of the dye, O_2^* is an excited or reactive form of oxygen, SOO is a reactive dye-oxygen complex (regarded as a "moloxide," or as a free radical, the oxyradical etc.), SRH_2 is a dye-substrate complex, and SRH_2^* and SRH_2' are excited forms of the dye-substrate complex.

It is to be noted that the ground state sensitizer is ultimately regenerated in all the proposed reaction schemes. In mechanisms (I) and (II), substrate is the primary reactant with the excited sensitizer, while in mechanisms (III) and (IV) oxygen is the primary reactant. Mechanism (V) requires a binding of sensitizer to substrate prior to light absorption. Although a number of reaction mechanisms have been proposed to account for photodynamic action, it must be admitted that the mechanisms involved, especially in more complex systems, are little understood. More than one mechanism can probably occur with a given substrate, depending on the dye and the reaction conditions used.

4. Photodynamic action in photosynthetic bacteria

Carotenoid pigments of the bacterial cells can act as protective agents against lethal photosensitized oxidations. This was first demonstrated in photosynthetic bacteria by Griffiths et al. (8). Their observations were based on studies with a blue-green carotenoid-less mutant strain of the non-sulphur purple bacterium Rhodopseudomonas spheroides. In this mutant, the coloured carotenoids were replaced by an accumulation of the colourless polyene, phytoene, which led to changes in the absorption spectrum of the organism both in the blue region of the spectrum and in the infrared. The major change, however, was noticed when these blue-green organisms were grown under aerobic conditions in the light. Under these conditions, the wild strain continued to grow at the same rate as it had under anaerobic conditions, although there was an inhibition of pigment formation. Under aerobic conditions there was cessation of bacteriochlorophyll and carotenoid synthesis, which was resumed on establishing anaerobic conditions. In the mutant strain, growth proceeded at its normal rate under either anaerobic conditions in the light or under aerobic conditions in the dark. However, when mutant cells were exposed to aerobic conditions in the presence of light, there was cessation of growth and decrease in the concentration of bacteriochlorophyll. Subsequently there was massive death of the bacterial cells as was demonstrated by a decrease in viable counts. Therefore, as neither light nor air alone caused death of the mutant cell, destruction was thought to be due to a photodynamic oxidation. Using suitable filters they further demonstrated that the light which caused the death of the blue-green mutant strain was light absorbed by bacteriochlorophyll. Since the carotenoid-containing strain of the same organism did not show such a photosensitization, they concluded that the presence of visibly coloured carotenoid within the cell prevented the killing of the cell when exposed simultaneously to oxygen and light, and this killing was produced by light absorbed by the bacteriochlorophyll in the absence of coloured carotenoids.

Cohen-Bazire and Stanier (2) produced carotenoidless cells of Rhodospirillum rubrum by using diphenylamine (DPA), a compound which selectively inhibits carotenoid biosynthesis. The diphenylamine is considered to act by inhibiting oxidation (dehydrogenation) of the colourless carotenoids (phytofluene etc.), which then accumulate. They observed similar photodynamic killing of the mutant as above and formulated a hypothesis that the carotenoid pigments acted as chemical buffers against lethal photooxidations.

The study of the blue-green mutant of R. spheroides was continued by Sistrom et al. (3), who also used DPA to inhibit carotenoid biosynthesis, and demonstrated that either genetic or physiological elimination of carotenoid pigments renders the cell extremely susceptible to lethal photooxidation by its own bacteriochlorophyll.

Feldman and Lindstrom (23) studied the effect of carotenoid pigments on photooxidations of some photosynthetic bacteria. The photooxidase activity of normal and carotenoidless mutants of R. spheroides and R. rubrum were compared. In Rhodopseudomonas carotenoids functioned as accessory pigments for photooxidations. When carotenoids were absent, more of the photooxidant was apparently available for indophenol oxidations. It seemed that the carotenoids could function as biological buffers for excess oxidising power. With Rhodospirillum, carotenoids apparently did not function as accessory pigments in photooxidations, and absence of carotenoids did not increase the specific activity of indophenol photooxidations. This apparent difference in carotenoid function probably reflected a difference in carotenoid concentration and/or in membrane structure.

5. Photodynamic action in non-photosynthetic bacteria

In the non-photosynthetic bacteria the presence of endogenous photosensitizers has not been readily observable unless very high light intensities are used. Most workers have used exogenous photosensitizers to demonstrate a photodynamic effect normally in the absence of carotenoid pigments. The photodynamic effect has occasionally been an inhibition of growth, but if the process of

photosensitization is prolonged, ultimately it leads to lethal photooxidations. It is essential to mention that not all of the studies to be discussed here fall under Blum's (26) definition of photodynamic action, which requires a role for molecular O_2. There are several photosensitizers (both natural and artificial) which apparently do not operate via an O_2- requiring step but nevertheless cause damage to the cell. This was pointed out by Mathews (29) who compared lethal photosensitisation in S. lutea using either toluidine blue or the plant furocoumarine, 8-methoxypsoralen (8-MOP). In her study, the pigmented and carotenoidless mutants of S. lutea were killed at essentially the same rate when the cell suspensions were exposed to long-wave ultraviolet light (330 - 430 nanometers in the presence of 8-MOP. 8-MOP did not cause any damage to the cell in the dark. Further, the degree of photokilling was less in the presence of air (or oxygen) than in the presence of helium. The apparent protective function of oxygen may have resulted from some destruction of psoralen by oxygen. By contrast, cells treated with toluidine blue only suffer lethal effects of light in the presence of oxygen, and carotenoid protects these cells from photo-oxidation. It has been suggested by Mathews and Sistrom that the anaerobic photosensitization of S. lutea by 8-MOP does not cause any damage to cellular protein as does toluidine blue in air, but rather causes damage to cellular deoxyribonucleic acid (DNA). This suggestion is supported by the mutagenic effects of 8-MOP in the presence of light.

Griffiths et al. (8) and Sistrom et al. (8), who studied photosensitization of photosynthetic bacterium R. spheroides, suggested that the hypothesis that carotenoids serve as protective agents against potentially lethal photosensitized oxidations might be extended to non-photosynthetic bacteria, where the carotenoid pigments are often present in cells frequently exposed to light and oxygen under natural growth conditions.

Kunisawa and Stanier (4) working with the non-photosynthetic bacterium Corynebacterium poinsettiae used a wild strain containing coloured carotenoid pigments and a white mutant obtained by ultra-

violet irradiation. C. poinsettiae is believed to form four principal carotenoid pigments: lycoxanthin, lycopene, cryptoxanthin and spirilloxanthin (30), although the presence of spirilloxanthin has since been denied (31). Non-pigmented cells in which the normal carotenoids were largely replaced by the colourless C-40 polyene, phytoene, were obtained by two methods: isolation of a white mutant and cultivation of the wild type in the presence of diphenylamine. The effect of visible light on dye-sensitized pigmented and non-pigmented cells showed that the non-pigmented cells could be killed rapidly by exposures which were without any effect on pigmented cells. Photosensitivity was produced by both physiological and genetic suppression of pigment synthesis. The rate of killing by ultraviolet light was the same in the non-pigmented mutant as in the pigmented wild strains, indicating that the carotenoid-protected photosensitivity was specific for visible light.

The protective function of carotenoid pigments against photodynamic killing of Sarcina lutea was studied by Mathews and Sistrom (5, 6). They were unable to show any killing of the mutant S. lutea, in the absence of added photosensitizer, after exposure for 4 hours to light from either tungsten lamps (3,000 ft. candles) or fluorescent lamps (1200 ft. candles). However, in the presence of toluidine blue, light and air, the colourless mutant was rapidly killed while the wild type was unaffected, as was the mutant in the absence of either light or oxygen. They further studied the effect of direct sunlight (12,000 ft. candles) on the wild type and carotenoidless mutant. The result of their experiment showed that after an exposure for 2 hours, more than 99 percent of the mutant cells were killed; in the same period the pigmented cells were not affected. The mutant cells were not killed in the absence of oxygen, thus demonstrating that the carotenoids of non-photosynthetic bacteria do protect the cells against a lethal photodynamic action which can occur in the absence of exogenous photosensitizer at high light intensities. These results indicated the presence within the cell of substances which can act as photosensitizers in the photodynamic killing of carotenoidless cells. The carotenoid pigments prevented

the lethal action of these endogenous, as well as exogenous, photosensitizers. The chemical nature of the endogenous photosensitizers, however, is not known.

Since it has been shown that the carotenoids are located in the cell membranes (14) it may be supposed that the membrane is the site of photodynamic action. Hence, it was possible that cellular death was associated with changes in the permeability of the cells. Mathews and Sistrom (6) used two methods to detect changes in cell permeability. In the first, the release of material absorbing at 260 nm was taken as an idea of change in permeability which took place at the same time as, or shortly after, the onset of killing due to photodynamic action. Release of the material from the pigmented strain was less than 3 percent of that from the same amount of non-pigmented cells exposed to light. The second method for the detection of changes in permeability made use of the properties of a dye, sodium-8-anilino-1-naphthalene sulphonate (ANSA), which is not fluorescent until conjugated to proteins. The mutant cells showed stronger fluorescence than the pigmented strain when illuminated in the presence of toluidine blue and ANSA. They suggested that a breakdown in the control of permeability might be the cause of photodynamic death. To substantiate this notion, the cells were killed by polymixin, an agent known to cause death by disrupting the permeability barrier of the cell. The increase in permeability caused by polymixin was 4 to 5 times greater than that caused by photodynamic action. Mathews and Sistrom concluded that these results did not support the suggestion that the primary lethal event in photodynamic killing was a change in permeability, although some changes in membrane permeability due to photodynamic action occurred.

It is possible that the primary lethal event in photodynamic action is inactivation of cellular enzymes. Succinate dehydrogenase and nicotinamide adenine dinucleotide oxidase were nearly totally inactivated when carotenoidless cells were exposed to light and air in the presence of toluidine blue. The rates of inactivation were temperature-independent. In the pigmented cells these enzymes

were not affected by this treatment (6).

In a comparative study of lethal photosensitization of S. lutea (20) by 8-methoxypsoralen (8-MOP) and by toluidine blue, Mathews showed that succinate dehydrogenase, reduced nicotinamide adenine dinucleotide oxidase and adenosine deaminase activities of the extracts of both the pigmented and colourless strains were not appreciably reduced by irradiation of cell suspensions in the presence of 8-MOP. However, the enzyme activities in the toluidine blue treated ones were greatly reduced in the colourless mutant only. Similarly, the succinoxidase and the pyruvic oxidase activities of pigmented and colourless mutant cell suspensions were not affected by 8-MOP treatment, while in the colourless mutant were greatly diminished by toluidine blue treatment. It is to be noted that the adenosine deaminase and pyruvic oxidase are the two enzymes located in the soluble fraction of the cell. She explained her findings by postulating that the permeability barrier of pigmented cells treated with toluidine blue is intact, and that not enough dye is allowed to enter the cells to photosensitize the enzymes. On the other hand, the permeability barrier of the dye-treated colourless cells is destroyed, and dye enters the cell and destroys the soluble enzymes. It may further be noted that the membrane-associated adenosine triphosphatase activity of the colourless Mycoplasma laidlawii was also appreciably destroyed when the cell suspension was exposed to visible light in the presence of toluidine blue (32). The carotenoid-containing pigmented cells exposed to light under the same conditions lost only a small amount of enzyme activity.

Roth (née Mathews) (33) studied the effect of 2.5×10^{-5}M acridine orange, an organic dye capable of photosensitizing cells, to determine the cellular site of lethal photosensitization and the protection offered by the carotenoids to the bacterium S. lutea. After irradiation of cell suspensions with visible light (2000 ft. candles) for 2.5 hours, cell extracts were prepared and assayed for reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase and adenosine deaminase activities, the former enzyme being associated with the cell membrane and the latter with the cytoplasm (14).

The $NADH_2$ oxidase of non-pigmented cells was destroyed, whereas that of the pigmented cells was unharmed. There was destruction of the soluble enzyme, adenosine deaminase, to approximately the same degree in both colourless and pigmented cells. Exposure of colourless and pigmented cells to light from a tungsten lamp (1000 ft. candles) in the presence of 2.5×10^{-5}M acridine orange resulted in no difference in the rate of killing of the colourless mutant as compared with the wild type. However, when the pigmented wild type was exposed to acridine orange and light, it was found that colourless mutants appeared in numbers much greater than the spontaneous mutation rate. The results of the experiment indicated that the carotenoid pigments were capable of preventing the destruction of membrane enzymes by acridine orange in the presence of light, even though they failed to prevent the lethal action of the dye. The exposure to acridine orange and light resulted in changes in the DNA of the exposed cells.

Thus, the effects of acridine orange on cells appear to be similar to those of toluidine blue: destruction of both soluble and membrane-associated proteins as evidenced by loss of enzyme activity. It also causes destruction of DNA as indicated by increased mutation rate. The primary mode of action of toluidine blue, as found above, appears to be destruction of membrane proteins and enzymes, but with acridine orange, however, the effect on protein seems to be a minor one. Carotenoids are capable of preventing the protein destruction and lethal effect of toluidine blue, but are incapable of preventing cell death from photosensitization by acridine orange, which is possibly due to alteration of cellular DNA. It is, however, interesting to note that, although the carotenoids failed to afford protection against both the lethal effect of acridine orange on the DNA and the destruction of soluble proteins, nevertheless they could prevent destruction of membrane protein as in toluidine blue photosensitization.

The effect of temperature on the protection by carotenoids against photosensitizations in S. lutea was studied by Mathews (34). Both pigmented and non-pigmented bacteria, in the presence of

toluidine blue and air, were exposed to light (1000 ft. candles) for 8 hours at 4°C and 34°C. The carotenoids of the pigmented S. lutea offered less protection at 4°C than they did at 34°C. The colourless organism was killed at the same rate at both temperatures, as had been reported previously (6). Mathews further showed experimentally that the pigmented cells of S. lutea, when exposed to high light intensities (16,000 ft. candles) without any photosensitizer, offered less protection at 4°C than they did at 34°C. However, at this light intensity, even in pigmented cells without photosensitizer, photokilling did occur after a delay of about 5 hours at 4°C and 8 hours at 34°C. The onset of photokilling in the carotenoidless cells was also delayed.

These photodynamic effects, which are modified by the presence of carotenoids, have been shown by Mathews and Krinsky (35) to be unrelated to the effects of ionizing radiations and ultraviolet light. Subjecting both the radiation-resistant Micrococcus sp. and its colourless mutant to gamma radiation, they found identical killing curves. With u.v. light similar results were obtained. When left exposed to visible light in the presence of photosensitizer toluidine blue, the pigmented wild type remained unaffected whereas there was photokilling of the colourless mutants. Similar results have been obtained with S. lutea. It was concluded that the carotenoid pigments had limited effects in protecting cells from radiation damage by visible light, and that they had no effect at all during damage to cells by ionizing radiation (gamma rays and x-rays) and u.v. light.

A purely photochemical process, photolysis of dark grown stationary-phase cells of Myxococcus xanthus, has been reported by Burchard and Dworkin (36). This process was a typical photodynamic one which involved no diffusional or enzymic steps. The cells grown in the light developed an orange carotenoid, while the cells grown in the dark did not develop any carotenoid. Dark-grown cells were lysed by a relatively low light intensity but only during the stationary-phase. This lysis was temperature-independent and oxygen-dependent. They found protoporphyrin IX to be the natural

endogenous photosensitizer.

Mathews (37) observed that Mycobacterium marinum, whose carotenoid synthesis was promoted by light (see page 26), was protected by the presence of carotenoid against lethal photo-oxidations mediated by the dye toluidine blue.

In the non-photosynthetic bacteria there is no direct evidence of how carotenoid pigments protect cells against lethal photo-oxidations. However, Krinsky (38) studied the role of carotenoid pigments as protective agents against photosensitized oxidation in chloroplasts, and suggested that the carotenoid pair antheraxanthin and zeaxanthin could act as "chemical buffers" to protect cells against lethal photooxidations. Epoxidation of zeaxanthin took place only in light and oxygen when conditions were established which could lead to photosensitization. The reductive de-epoxidation of antheraxanthin to zeaxanthin was a dark enzymic reaction. These two reactions constituted the "epoxide cycle" by which carotenoids could prevent lethal photosensitization of cells. The role of the epoxide cycle against lethal photosensitized oxidations was suggested to be as follows: chlorophyll, excited by light, is usually deactivated by the process of photosynthesis. Some of the excited chlorophyll could occasionally combine with molecular oxygen leading to photosensitized oxidations. This potentially lethal combination, however, would normally be inactivated by a carotenoid such as zeaxanthin, which would itself be oxidised to its epoxide derivative, antheraxanthin. An enzymic mechanism such as antheraxanthin de-epoxidase would then regenerate the protective substrate zeaxanthin. By this mechanism the epoxide cycle could protect cells against lethal photosensitized oxidations.

In Mycobacterium phlei it has been shown by Brodie et al. (39) and Brodie and Ballantine (40) that exposure of electron transport particles to light (360 nm) resulted in loss of the natural naphthoquinone and the ability to carry out oxidative phosphorylation, together with NAD oxidase and succinoxidase activities. Restoration of both oxidation and phosphorylation occurred with substrates linked to nicotinamide adenine dinucleotide (NAD^+) by the addition

of vitamin K_1. In contrast, neither naphthoquinone nor benzoquinones restored oxidative phosphorylation with succinate as substrate, indicating that a light-sensitive factor other than the known quinones is required for electron transport from succinate (41, 42). Since knowledge of the components and sequence of carriers of the respiratory chain is necessary for an understanding of the bio-energetic process, a study was undertaken by Murti and Prodie (43) to find the nature and role of this new light-sensitive factor required for succinoxidase activity in Mycobacterium phlei. Light damage caused by irradiation of the particles differed in two pathways. The succinoxidase pathway was more prone to damage by irradiation than the nicotinamide adenine dinucleotide pathway. Addition of a thermostable, water-soluble extract obtained from either M. phlei or from rat liver mitochondria resulted in 50 to 60 percent restoration of succinoxidase activity. Addition of other known co-factors, such as flavin adenine dinucleotide, flavin mononucleotide, benzo-and naphthoquinones and sulphydril agents, however, failed to restore succinoxidase activity after irradiation. The site of action of the water-soluble material from M. phlei appeared to be between the flavoprotein and the cytochrome b on the succinoxidase pathway. The succinoxidase factor failed to restore NADH oxidation, whereas 50 to 60 percent restoration of NADH oxidase activity could be achieved by the addition of vitamin K_1, which in the case of irradiated particles failed to restore succinoxidase activity. Whether oxygen or carotenoid are involved in this light effect is not known.

6. Photooxidation of carotenoids

Wright and Rilling (44) worked with a Mycobacterium sp. and demonstrated photokilling of the carotenoidless dark-grown cells using only endogenous photosensitizers and very bright light (7400 - 10,000 ft. candles). Death did not occur under nitrogen. They also found that the carotenoids of Mycobacterium sp. were rapidly bleached under the conditions of high light intensity which they used. Using S. lutea as a test organism, Roth and Krinsky (45)

observed general destruction or disappearance of carotenoid pigments during photodynamic killing. They were unable to demonstrate specific light-driven reactions among the carotenoids and suggested that the carotenoid pigments might play a physical role in the protection of non-photosynthetic bacteria against the harmful effects of light and oxygen, such as stabilization of the cell wall membrane complex and not a chemical or biochemical role.

Photosensitized bleaching of β-carotene with light was demonstrated by Hasegawa et al. (46). Their results showed that irradiation with a monochromatic beam of light (632.8 nm) from a continuous-wave gas laser did not affect β-carotene. In the presence of photosensitizing dye toluidine blue O (4×10^{-5}M), however, numerous changes took place in β-carotene. A considerable decrease in the absorbance of β-carotene was noted initially which was accompanied by small shifts of the absorption maxima to shorter wave lengths as well as the formation of two new peaks at 400 and 375 nm. Finally they observed a complete bleaching of the solution. Using column and thin-layer chromatography, up to 13 different compounds were shown to be formed by the photosensitization of the β-carotene. All the reactions were oxygen-dependent.

7. Photoinduction of carotenoid synthesis

Some organisms are photochromogenic, i.e. they will synthesize pigments only when exposed to light. The kinetics of carotenoid synthesis of two species of Mycobacteria have been studied by Batra and Rilling (47). The action spectrum of photoinduction for pigment formation of *Mycobacterium* sp. had maxima at 365 and 460 nm separated by a minimum at 395 nm. However, the action spectrum of photoinduction of another species *M. marinum*, had a principal maximum at 404 nm and two lesser maxima at 495 and 577 nm. Flavin was suggested to be the likely photoreceptor for the *Mycobacterium* sp. and a porphyrin for *M. marinum*. The photoinduction of carotenogenesis in *Mycobacterium* sp. was found to be depressed at an acid pH. A Mycobacterium species that synthesizes carotenoids only

after light stimulation, has been used by Rilling (43) to study the inhibition of carotenoid synthesis by a variety of compounds. In addition to diphenylamine, other compounds such as benzophenone, acridine orange, methylene blue, neutral red, toluidine blue, proflavine, acridine and 9, 10-dihydroacridine were found to inhibit the formation of the more highly unsaturated carotenoids. In all cases accumulation of phytofluene occurred when the formation of more highly unsaturated carotenoids was inhibited. Under increased oxygen tension all of these compounds, except diphenylamine and benzophenone, no longer inhibited formation of carotenoid pigments. Using molecular models, it could be demonstrated that the dihydro derivatives of benzophenone and diphenylamine had a close structural similarity to that of the relatively saturated carotenoids in the region where subsequent dehydrogenation occurs. Thus they postulated that these compounds could bind to the carotenoid dehydrogenases subsequently inhibiting carotenogenesis.

It appears from the work so far reviewed that the cell membranes are the primary sites of lethal photodynamic effects. The carotenoids are located exclusively in the membranes and they play an important role in the protection of the cells from the lethal effects of photo-oxidations. Therefore, some aspects of the bacterial cell membrane will now be discussed.

8. Bacterial cell membrane

The development of a limiting membrane is, in all probability, a fairly early phenomenon in the evolution of cells which are capable of showing growth and multiplication. Cell membranes possess a degree of biological universality enjoyed by genetic structures and the apparatus for the synthesis of protein, together with some energy yielding mechanisms.

Besides providing the cell with a protective and functional (in the permeability sense) barrier, the cell membranes may confer

surface specificity, a property which may be of considerable importance to the cell in the determination of its response to the environment. However, Salton (49) is of the opinion that, in the Gram-positive bacteria, the surface specificity is a property of capsules or the cell wall polysaccharides or teichoic acids. In the majority of bacteria, the plasma membrane appears to be much less conspicuous as a carrier of surface specificity than does the outer cell wall. Robertson (50) studied thin sections of cell membranes under the electron microscope and considered them to be "unit membranes" as seen in animal, plant and other microbial cells with an overall thickness of about 75 Å.

Murray (51) and Schlessinger et al. (52) showed that a lower degree of intracellular differentiation was one of the distinctive features of bacteria which were characterized by the absence of a nuclear membrane, of organized mitochondrial structures, and of a membranous type of reticulum carrying the polyribosomes. Thus the bacterial cell possesses fewer membrane bound organelles, and in this respect it offers a simpler system for investigation of membrane properties and functions. Anatomical studies of the bacterial cells reveal the presence of a single limiting plasma membrane, and in many cases a well developed internal membrane system which Fitz-James (53) referred to as "mesosomes" and van Iterson (54) as "chondroids." Salton (55) describes the mesosome as vesicular, lamellar or tubular packets of membrane formed by the invagination of the plasma membrane. When the presence of mesosomes became evident during the electron microscopic study of bacterial cells, many functions were attributed to them.

Fitz-James (56) showed that the mesosomes vary from species to species by their overall appearance in thin sections. Broadly speaking three types can be recognised, namely a lamellar type, a packed sac type or a long tubular type. It is not known at the present time what determines the type of mesosome seen in a particular organism (55). There is still insufficient direct experimental evidence to enable one to distinguish the functions of the plasma membrane from those of a mesosomal membrane. Salton (55) considers

mesosomes to be anatomically reminiscent of mitochondria. Van Iterson and Leene (57) provided evidence supporting the idea that mesosomes are mitochondrial equivalents. They noticed a greater concentration of tellurium deposition in the region of mesosomes in Bacillus subtilis, indicating more sites active in reduction of tellurite. However, Salton has pointed out that as the total surface area of the membrane in the mesosome is greater than that of the plasma membrane, heavier deposits of tellurium are to be expected in this region.

Bladen et al. (58) have shown that phosphotungstate freely penetrated into the mesosomes or intracellular membrane-intrusions, which suggested the absence of any permeability barrier enveloping these structures. Salton (55) is of the opinion that much still remains to be done on mesosomes before we can come to any conclusion about their function or their relationship to the rest of the cell.

Thus, membranes detected in bacteria appear to be limited to one or at the best two types: the plasma membrane, and its invaginations in the form of the mesosome. In photosynthetic bacteria Drews (59), Cohen-Bazire and Kunisawa (60) and Holt et al. (61) have shown that the membranes containing the photosynthetic pigments may, in some cases, be regarded as a special type of membrane invagination which constitutes the intracellular site of the photosynthetic apparatus and from which chromatophores arise following mechanical breakage of the cell.

9. Properties of bacterial membranes

Weibull (62), using lysozyme to digest the cell wall, obtained fractions from lysed protoplasts of Bacillus megaterium which he referred to as "ghosts." These ghosts were assumed to represent the plasma membranes of the bacterial cell. Recently Salton and Chapman (63) found that conditions normally employed for the isolation of cytoplasmic membranes from a lysozyme-sensitive organism such as Micrococcus lysodeikticus produced a total membrane preparation of plasma and mesosome membranes.

The most popular and probably the most reliable method used for the isolation of bacterial membranes involves the muralytic or cell wall degrading enzymes, followed by the separation of the membranes from the cytoplasm by differential centrifugation. A wide variety of muralytic enzymes such as lysozyme, phage muralysins, lysostapins etc. are available for the removal of the cell wall before isolation of the cell membrane. Membranes may be recovered from either totally lysed cells in dilute buffers, or by osmotic lysis of stabilized protoplasts formed by lysozyme treatment in isotonic media. Salton and Chapman (63), isolated membrane preparations by both methods from M. lysodeikticus and were unable to find any difference when viewed under the electron microscope. It has been concluded by Salton and Ehtisham-ud-din (64); Salton and Freer (65); and Salton and Schmidt (66), that if proper conditions are used it is quite possible to wash the membranes without leaching out any of the more conspicuous markers such as carotenoids, menaquinones and cytochromes. However, it should be noted that Shah and King (67) have found that the succinate dehydrogenase in the succinic oxidase system in M. lysodeikticus was inhibited when the cell was lysed with lysozyme, but was hardly inhibited at all if ultrasound was used to disrupt the cell. On treating the ultrasonic fragments with lysozyme, they observed inhibition of succinate oxidation.

The chemical composition of isolated membranes has been the subject of numerous investigations. Salton and Freer (65); Kates (68); Gilby et al. (13); Weibull and Bergstrom (69); Brown (70); Macfarlane (71), and Yudkin (72) found that the composition is very similar to that of other membrane systems in that they are composed of about 50 to 75 percent protein and 20 to 30 percent lipid. Varying amounts of carbohydrate, RNA and DNA have been reported in cell membrane preparations. At the moment it cannot be said precisely the extent to which some of the additional components are part of the membrane structure or covalently attached to membrane constituents.

A great deal is now known about the nature of the membrane

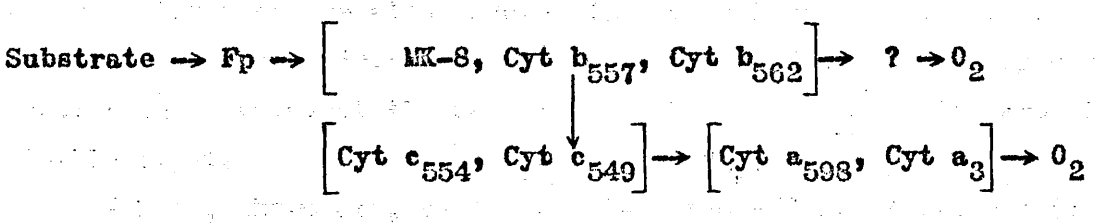
lipids. Razin et al. (73) studied the membranes from Gram-positive bacteria and found that they were devoid of cholesterol. This distinguishes these membranes from those of higher microorganisms, plants, animals and pathogenic mycoplasms which possess steroids.

Mathews and Sistrom (14) found the carotenoid pigments of the pigmented S. lutea to be located exclusively in the cell membrane, and suggested their occurrence in the form of a pigment-protein complex. Gilby et al. (13) have suggested that the carotenoid pigments in the related organism M. lysodeikticus were also bound to the cell membrane. The C_{50} carotenoid from S. flava has been shown to be an integral part of the structure of the membrane-protein fraction from which it may be isolated in the form of a carotenoid-glycoprotein complex (74). However, the presence or absence of carotenoids from the membrane has not been reported to have any effect on its structure.

The ultrastructure of non-photosynthetic carotenoid-containing bacteria was studied by Mathews (75). Her intention was to see whether any change in intracellular structure was associated with the development of carotenoid pigments in two bacteria, M. marinum and S. lutea. Examination of many thin sections showed no difference in the intracellular structure between cells of M. marinum grown in the dark (non-pigmented) and those grown in the light (pigmented). Neither was any difference found between cells of S. lutea wild type (pigmented) and cells of colourless mutant. She concluded that no new structures were made to accommodate carotenoid pigments in non-photosynthetic carotenoid-containing bacteria. Thus the pigment was either built into the membrane as an integral part of it, as in S. lutea, or was added on to pre-existing membranes, as in M. marinum.

Most bacteria possess a membrane-bound respiratory chain (an electron transport chain), i.e. a system of spatially organised dehydrogenases and cytochromes which bring about electron transport. In general the respiratory chain is regarded as a multienzyme complex which catalyzes the dehydrogenation of substrates and the transfer of reducing equivalents to oxygen. In the mammalian

respiratory chain the dehydrogenases are connected to cytochromes by hydrogen-transferring co-enzymes, NAD and ubiquinone, which then pass on the electrons to the cytochromes of groups A, B and C. The bacterial chain essentially contains the same sequence of enzyme complexes but with some differences. In bacteria, the initial stage in the oxidation of substrates often involves flavin enzymes rather than NAD-linked enzymes. Despite their similar functions, the cytochromes of groups A, B and C differ from mitochondrial cytochromes by having different absorption spectra. In addition a cytochrome O oxidase is known to occur. Very little is known about the actual pathways of electron transport or any relationship between the respiratory system and the carotenoids in S. lutea. Erickson and Parker (76) investigated the membrane-bound electron transport system of Micrococcus luteus (S. lutea). They found cytochromes a_{598}, b_{562}, b_{557}, c_{554}, c_{549}, a menaquinone (MK-8), carotenoids, NADH, malate and succinate dehydrogenases. Sensitivities to different inhibitors indicated the difference between the bacterial and mammalian systems. The M. luteus system was found to be insensitive to antimycin A and showed very low sensitivity to rotenone. Effective inhibition was shown by cyanide, pericidin A, dicoumarol, 2-n-heptyl-4-hydroxyquinoline-N-oxide and ultraviolet irradiation at 360 nm. The carotenoidless mutant exhibited identical properties to the parent organism except that no measurable succinate oxidase activity was found and there was an 8-fold increase in the malate oxidase activity of the electron transport particles. There was no difference in the action of inhibitors. They suggested the following scheme for electron transport in M. luteus:



This appears to be the only study of the electron transport system in this organism.

10. Conclusion

The non-photosynthetic bacterium Sarcina lutea contains carotenoid pigments and these pigments afford protection to photodynamic killing by very high light intensities (sunlight) without any exogenous photosensitizer or by low light intensities (artificial light) with an exogenous photosensitizer. Although the carotenoid pigments play a protective role against aerobic photosensitivity, there is no direct spectral or chemical evidence suggestive of carotenoid involvement. Several investigations involving studies of viability, permeability, enzyme lability, mutagenicity, growth, temperature effects, gas effects, light effects and ultrastructure etc. have been made in S. lutea, but at present nothing is known about the mechanism by which carotenoids protect cells under photosensitizing conditions, nor about the mechanisms involved when photodynamic death commences. The carotenoid-containing microorganism, S. lutea, though subjected to direct sunlight in nature, is not being killed, while the carotenoidless mutants of the same microorganism could be killed. Nature seems to protect these microorganisms from the deleterious effects of sunlight for the perpetuation of the species. However, the mechanism of carotenoid participation in the protective function is far from being completely understood.

Carotenoid pigments in S. lutea have been located exclusively in cell membranes in the form of a carotenoid-glycoprotein complex. Like most other bacteria, S. lutea has a respiratory chain (electron transport system) which is present in the cell membranes. Other enzyme activities are also located in the cell membrane. It is possible that the primary lethal event in photodynamic killing could involve the enzymes of the respiratory chain, many of which absorb visible light.

I have, therefore, chosen to study the effect of visible light on the respiratory chain of isolated membranes of S. lutea, using both low intensity light with a photosensitizer and high intensity light without a photosensitizer. These investigations have been

particularly aimed at determining a more specific role for carotenoids in protecting membrane enzymes from photodynamic action. As a member of the carotenoid-containing cocci, S. lutea is very easy to grow in the laboratory, and the study of the cell membrane was chosen because it is relatively easy to obtain membranes by a gentle method using lysozyme treatment which does not appear to cause much damage to the membrane enzyme activities.

CHAPTER 2

MATERIALS AND METHODS

1. Chemicals

Antimycin A, 5-bromo-2, 4-dihydroxypyridine (5-bromouracil), cytochrome c, menadione (vitamin K_3), NAD and NADH were obtained from Sigma Chemicals Company, St. Louis, Missouri, U.S.A. Toluidine blue and methylene blue were obtained from George T. Gurr Ltd., London, England. Ethyl methanesulphonate and lysozyme chloride ex egg-white cryst. were obtained from Koch Light Laboratories Ltd., Colnbrook, Bucks., England. Nutrient broth and nutrient agar were obtained from Oxoid, London, Great Britain. All other chemicals were obtained from British Drug House Chemicals Ltd., Poole, England, and were the finest grade obtainable. Manometric experiments were done with distilled water, whereas experiments on the oxygen electrode were done with deionized distilled water. Chance-Pilkington colour filter glasses were obtained from Precision Optical Instruments (Fulham) Ltd., 158 Fulham Palace Road, London W.8, England.

2. The strain of bacteria used in the present study.

Only one strain of Sarcina lutea (Micrococcus luteus) and three mutants of the same were used in the present study. The strain was obtained from Dr. A.H. Dadd, Imperial College, London, and was No. 3 in the Imperial College collection.

3. Culture and maintenance.

All strains of Sarcina lutea used in this study were maintained on nutrient agar slopes, which were stored at 5°C in dark. As it

grows luxuriantly in nutrient broth medium, the same medium was used throughout the course of the study. A loopful from a nutrient agar slope was used to inoculate 100ml. of the medium (13 g./l. nutrient broth, autoclaved at 15 lbs. pressure per square inch for 15 minutes) in a 250ml. conical flask, and the bacteria were grown with shaking in a reciprocating shaker incubator having 200 movements per minute at 26°C to late log phase. 5ml. of this pilot culture were used to inoculate 500ml, of the medium in a 1 litre Pyrex culture flask, and grown to late log phase at 26°C as above.

4. Production of mutants.

For production of the mutants of Sarcina lutea, the following three methods were employed:

A. Mutation by ultraviolet light. 0.2ml. aliquots of an overnight culture of the wild strain of Sarcina lutea were plated on sterile agar plates and irradiated with ultraviolet light. A 12 in., 7 W., mercury quartz discharge tube with an ozone-reducing filter was used to give monochromatic radiation at 253.7 mμ. (The tube was No. 0103/K obtained from Engelhard Industries Limited, Hanovia Lamp Division, Slough, England). The plates were irradiated for a period of 6 - 10 minutes at a distance of 6 inches in a sterile cabinet. The irradiated plates were left in the incubator at 26°C for 3 to 4 days, after which the mutants were selected as colourless colonies on the agar plates. Suspected mutants were examined under the phase contrast microscope for shape, size and Gram staining, and sub-cultured on agar slopes for confirmatory tests.

B. Mutation by the base-analogue 5-bromouracil. 0.5% 5-bromouracil with 1% bacteriological peptone was prepared in 10ml. of 10 times diluted nutrient broth and autoclaved at 20 lbs./in.^{2} for 30 minutes. 0.5ml. of a thickly grown overnight culture of wild Sarcina lutea was added to 10ml. of the 5-bromouracil solution in a flask, and incubated for 5 hours at 28°C with shaking,

after which cell counts were made in a counting chamber. The suspension was then diluted with nutrient broth to give a cell density of approximately 2×10^2 bacteria per ml. 0.2ml. aliquots of the diluted cell suspension, containing approximately 40 bacteria, were plated out and the plates incubated at 26°C for 2 to 3 days. The resultant white colonies, suspected of being mutants, were examined under the phase contrast microscope to check cell shape, size and Gram staining, and subcultured on agar slopes for further confirmatory tests.

C. Mutation by ethyl methanesulphonate. This mutagenic agent removes bases from DNA by acting specifically on guanine at the N_7 position, labilising the deoxyriboside linkage so that 7-alkylguanine is released from the DNA. The missing guanines might be replaced by any one of the four bases.

Saturated solutions of ethyl methanesulphonate were made in 1 ml. of sterile 0.2M-sodium phosphate buffer (pH 7.2) in duplicate test tubes. 1 ml. of the overnight culture of wild Sarcina lutea was added to one tube. Both tubes were incubated at 26°C for 20 minutes, after which 0.2ml. aliquots of two dilutions (50 and 100 times) of the bacterial suspension were plated out in triplicate. 0.2ml. aliquots from the control tube without bacteria were also plated out in triplicate, without dilution, and the plates left in the incubator at 26°C for 4 to 6 days. White colonies, believed to be non-pigmented mutants, were examined and subcultured as above.

D. Morphological and biochemical tests for the mutants.

Morphological comparisons as regards size, shape and motility of the mutants were made under the phase contrast microscope. Biochemical tests including reaction to litmus milk, gelatin liquefaction, gas production and Gram's stain were performed to confirm the properties of the mutants. All morphological and biochemical tests were carried out in comparison with the wild Sarcina lutea. These tests were primarily aimed at ensuring that contaminants were distinguished from genuine mutants.

5. Growth curve for Sarcina lutea (wild type).

An overnight culture of Sarcina lutea was obtained by the method described above, and a 1 litre flask containing medium was inoculated with 5ml. of the inoculum. A zero time measurement of the turbidity of the medium was made on a nephelometer. The flask was incubated with shaking at 26°C and, at 30 minute intervals, samples were taken for turbidity estimations. The percentage of turbidity increased after a lag phase of 30 minutes, and 16 such readings were taken. The flask was then left in the incubator overnight and the percentage of turbidity recorded on the following morning. The nephelometer was calibrated for cell density using a bacterial counting chamber. Fig. 2 gives the growth curve for Sarcina lutea under the conditions used for experiments described in this thesis.

6. Preparation of cell suspensions for experiments using standard manometric techniques and the oxygen electrode.

The bacterial cells grown by the methods described in section 3 above were harvested at room temperature by spinning at 6000 r.p.m. for 5 minutes on a bench centrifuge, collected in cold 0.02M-phosphate buffer (pH 7.2), washed four times with cold buffer, and finally suspended in the same buffer. The suspension was made uniform by means of a glass homogenizer.

7. Preparation of membranes.

An overnight culture of wild or mutant Sarcina lutea was obtained in the usual way. The cells were harvested at room temperature by centrifugation at 6000 r.p.m. for 5 minutes on a BTL bench centrifuge, and the pellet of cells suspended in a medium composed of cold sucrose (1.2M), NaCl (0.05M) and phosphate buffer (0.06M) pH 6.8. Using a glass homogenizer, the cells were mixed very gently to give a uniform suspension. Lysozyme chloride

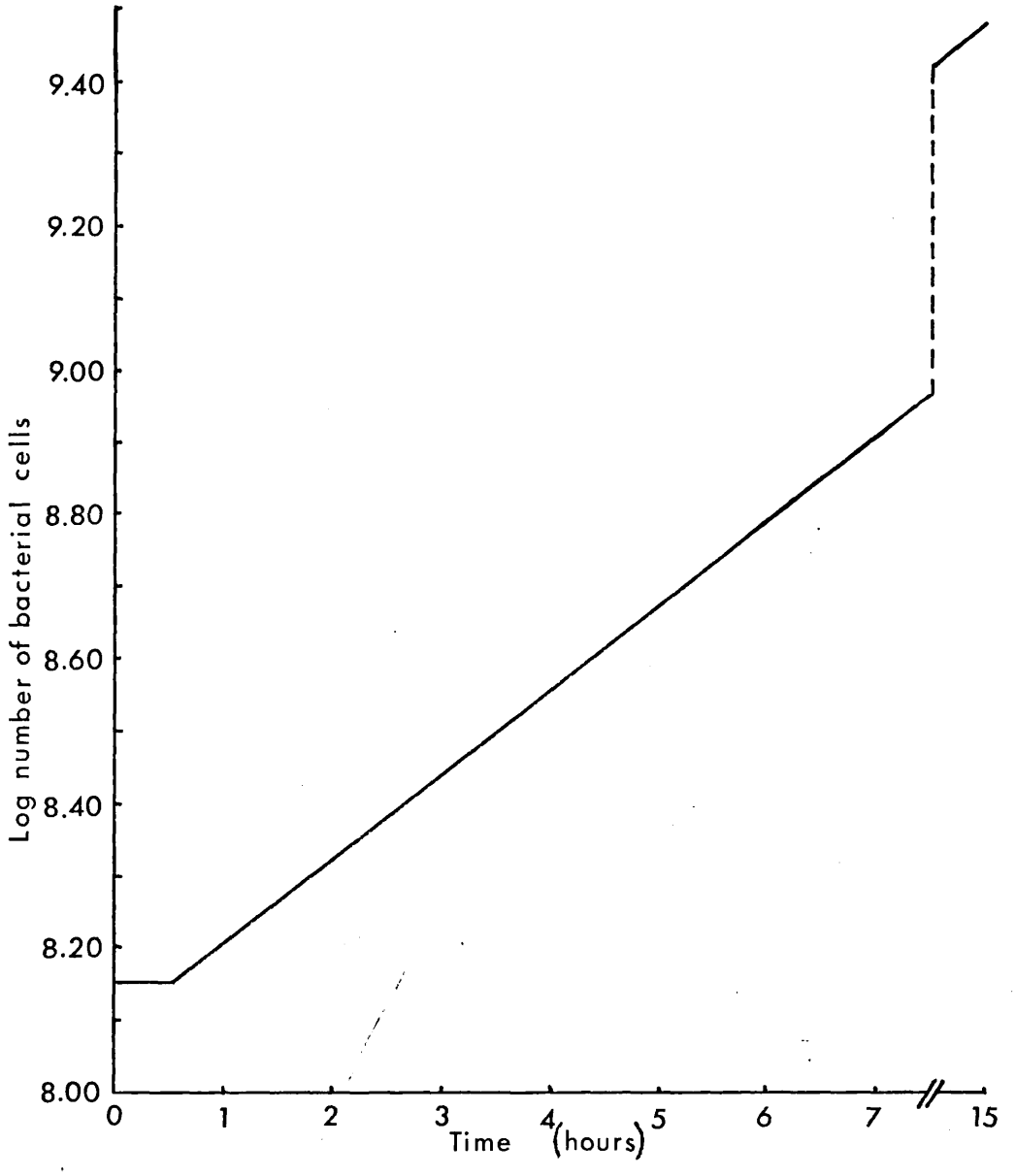


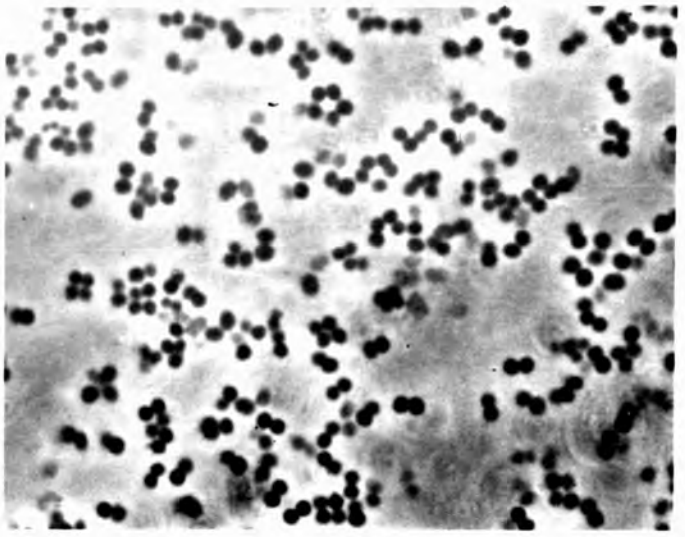
Fig.2. Standard growth curve for Sarcina lutea (wild type).

(15 mg./10 ml.) was added to the homogenate which was incubated at room temperature for 15 minutes with occasional stirring. After incubation the protoplasts were spun down at 20,000 g. at 5°. The cell protoplasts were suspended in cold phosphate buffer (0.06M) pH 6.8 containing NaCl (1%), and occasionally stirred gently to dislodge the clumped cell protoplasts for 10 minutes, keeping the tube in ice. The suspension was examined under the phase contrast microscope to ensure that ghost membranes had been obtained. The suspension was spun at 2,500 r.p.m. for 5 minutes to get rid of residual whole cells which did not undergo osmotic lysis. The supernatant fraction containing the cell membranes looking like ghost cells under the phase contrast microscope was collected in a cold plastic tube and spun at 20,000 g. for 30 minutes. The membranes were obtained as a pellet which was resuspended in phosphate buffer (0.02M) pH 7.2 containing 0.0005M $MgCl_2$, and a uniform suspension was made by gently homogenizing the pellet with a glass homogenizer. The homogenate was examined under the phase contrast microscope, and, when found to contain more than 95% membranes, experiments were carried out with this material. A comparison of the appearances of whole cells and membranes prepared by these methods may be made by reference to Plate 1.

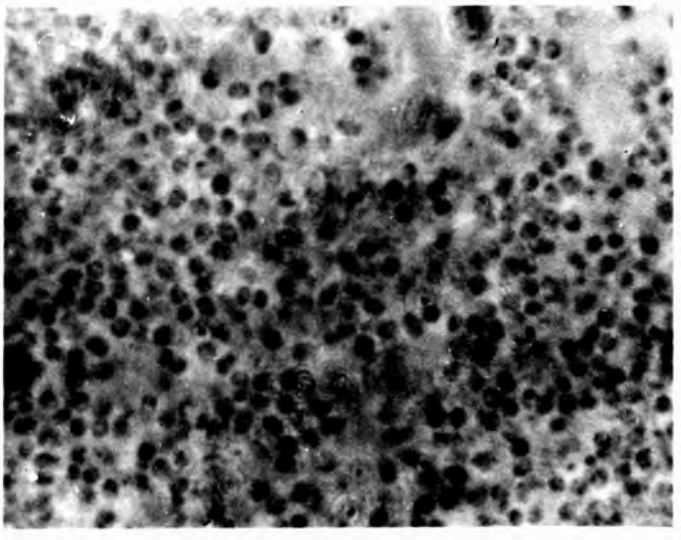
In the earlier experiments with membrane preparations the lysozyme incubation time was 45 minutes. Due to prolonged incubation with lysozyme the succinoxidase activity of the membranes was very low. Shah and King (67) have also found that lysozyme incubation for a long time affected the succinoxidase activity of bacteria. Therefore, in later experiments the period of lysozyme incubation was shortened from 45 minutes to 15 minutes, which gave an increased succinoxidase activity in membrane preparations.

8. Experiments on cell viability.

Overnight (late logarithmic phase) cultures of wild Sarcina lutea and the carotenoidless mutant (UV_4) were obtained as described earlier. Two 250ml. conical flasks containing 100ml. each of



Whole cells



Membranes

Plate 1. Comparison of the appearances of whole cells and membranes of Sarcina lutea. (Phase contrast x3900)

sterile nutrient broth were inoculated with 5ml. of wild and mutant strains of Sarcina lutea and incubated with shaking in the dark for 2 hours. When the cells were in the logarithmic phase of their growth after 30 minutes, measured amounts of the pigmented culture were put in one sterile glass cuvette while aliquots of the non-pigmented culture were placed in the other. The cuvettes were maintained at 25°C and their contents were continuously stirred by bubbling sterile air through the cultures. Sterile toluidine blue solution was added to give a final concentration of 2.5×10^{-6}M. Samples were taken from each cuvette at zero time and were diluted in sterile Na_2HPO_4-NaH_2PO_4 (0.02M) buffer pH 7.2. 0.2ml. aliquots of the diluted samples were plated out in duplicate and incubated at 26°C for 3 days for a viable colony count. The cuvettes were placed 9 inches away from four 100 W. tungsten lamps giving a light intensity of 400 lumens/sq.ft. at the front of the culture. A water filter was interposed between the cuvettes and the light source. At 15 minute intervals, three samples were collected, diluted in sterile buffer, and 0.2ml. aliquots were plated out in duplicate and left in the incubator at 26°C for 3 days, after which colony counts were made. In the case of the mutant, dilutions of the culture were increased to give a more concentrated preparation to allow for cell death caused by illumination. At the end of the experiments the temperature of the culture remaining in the cuvettes was 27°C.

9. Illumination of cell suspensions and membranes prior to enzyme assay.

The bacterial preparations were placed in two Perspex cuvettes jacketted by constant temperature water circulated from a water bath. The illuminated samples were incubated in one cuvette, while the dark sample was incubated in a second cuvette covered with blackened aluminium foil and kept away from natural and artificial light as far as practicable. Before illuminating a sample the light intensity was measured with a meter and the bacterial preparation

was placed in the cuvettes. The uniformity of suspension of the bacterial preparation was achieved by means of two stirrers which continuously kept the contents of the cuvettes stirred at a fixed speed. When the contents of the cuvettes attained the temperature of the water bath, control samples from the cuvette covered with foil were analysed. The sample to be illuminated was subjected to light from a Prinz Lowline 300 W. aircooled projector, from which the focusing lens had been removed. After illumination the cuvette was covered with blackened aluminium foil to cut off further light. Under our experimental conditions a slight amount of laboratory ceiling light could not be screened from the top of the cuvettes.

10. Manometric assay methods.

Various dehydrogenase and oxidase activities were assayed by standard manometric techniques for the measurement of oxygen uptake. The activities were expressed as microlitres of oxygen utilized per minute.

I. Malate oxidase.

Oxygen uptake was assayed manometrically at 28°C in 2ml. of medium composed of 150 mM-sodium-hydrogen malate in 0.02M-phosphate buffer (pH 7.2) and 0.0005M-$MgCl_2$.

II. Succinate oxidase.

Oxygen uptake was assayed manometrically at 28°C in 2ml. of medium composed of 150 mM-sodium succinate in 0.02M-phosphate buffer (pH 7.2) and 0.0005M-$MgCl_2$.

III. Ascorbate oxidase.

Oxygen uptake was assayed manometrically at 28°C in 2ml. of medium composed of 150 mM-ascorbic acid neutralized with NaOH, 0.02M-phosphate buffer (pH 7.2) and 0.0005M-$MgCl_2$.

IV. Lactate oxidase.

Oxygen uptake was assayed manometrically at 28°C in a medium composed of 150 mM-sodium-lactate in 0.02M-phosphate buffer (pH 7.2) and 0.0005M-$MgCl_2$.

V. Ascorbate-TMPD cytochrome oxidase.

The ascorbate-tetramethyl-para-phenylenediamine (TMPD) cytochrome oxidase activity was assayed by a manometric method at 23°C in a medium composed of 0.05M-ascorbic acid (neutralized), 10^{-4}M-TMPD, 0.02M-phosphate buffer (pH 7.2) and 0.0005M-$MgCl_2$. Control flasks recording the oxidation of substrate without enzyme gave significant oxygen uptakes.

VI. Malate : methylene blue oxidoreductase.

Methylene blue reduction was assayed manometrically at 23°C by means of the reoxidation of reduced methylene blue by oxygen. The assay medium was composed of 150mM-sodium hydrogen malate, 1 M-KCN (neutralized), 5 x 10^{-3}M-NaN_3, 0.002% methylene blue, 0.02M-phosphate buffer (pH 7.2) and 0.0005M-$MgCl_2$ in 2ml.

VII. Ascorbate-cytochrome c oxidase activity.

The ascorbate-cytochrome c oxidase activity of the membranes of pigmented and white mutant S. lutea was assayed by a manometric method at 23°C in a medium composed of 1.0ml. of membrane preparation, 150mM ascorbate (neutralized with NaOH), 0.5% cytochrome c and 0.02M phosphate buffer (pH 7.2) and 0.0005M-$MgCl_2$ in 2.0ml. Control flasks recording the oxidation of substrate without enzyme gave significant oxygen uptakes.

Substrates were put in the side arm of the Warburg flasks, while 0.2ml. of 20% KOH together with a strip of chromatography paper were placed in the centre compartment. 1.0ml. of a bacterial suspension or membrane preparation was placed in the main compartment, together with any additions (photosensitizer, electron acceptors, cofactors and inhibitors). The reaction was started by tipping the substrate into the main compartment. To ensure a uniform suspension of whole cells or membranes before pipetting into the flasks, homogeneity of the preparations was maintained by shaking the tube containing the bacterial preparation. To compensate for variations in the amount of preparation added, the initial rate of oxidation was recorded at intervals of 5 minutes for 30 minutes at 28°C preceding illumination.

A Perspex rotary Warburg apparatus (from B. Braun, Melsungen, West Germany) was illuminated by 14 x 40 W. tungsten bulbs, producing a light intensity of 660 lumens/sq. ft. at the base of each Warburg flask. Following the initial 30 minutes' assay, the flasks were

either illuminated or covered in blackened aluminium foil to exclude all light, and the manometers were read at intervals of 10 minutes. The control and the experimental flasks were in most cases duplicated.

11. Polarographic assay methods.

A. Oxygen electrode apparatus.

The malate dehydrogenase, malate oxidase, succinate and malate : vitamin K reductase, ascorbate-cytochrome c oxidase, NADH oxidase, succinate oxidase, lactate oxidase and ascorbate oxidase activities were also assayed on a Beckman 39550 O_2 electrode assembly. The oxygen sensor was designed specifically for the measurement of oxygen dissolved in samples using the Beckman 100301 Fieldlab^{TM} Oxygen Analyzer. The analyzer was connected to a Bryans chart recorder, model 27000 with variable chart speeds, via a zero suppressor and scale expander.

The essential features of the oxygen electrode and the experimental arrangement for its use in this investigation are given in Fig. 3. The electrode consisted of a sensor (A) consisting of a rhodium cathode and a silver anode. The anode was connected electrically to the cathode by a film of potassium chloride solution (B). A gas-permeable Teflon membrane (C) separated the cathode-anode assembly from the sample. The membrane fitted firmly against the cathode surface and made the electrode virtually indifferent to the composition of the medium except as the composition influenced the oxygen concentration. The tip of the sensor slipped inside a plastic sensor cap (D), which entered the reaction vessel (E), at the neck of which there was a rubber O ring (F). The reaction vessel was made of Perspex and had a central cavity accommodating 1.23ml. of fluid when stoppered. The vessel was fixed inside a Perspex water jacket (G) 44 x 44 mm., through which water from a constant temperature bath at 26°C was circulated, and the whole was mounted on a Gallenkamp magnetic stirrer with an external rheostat. A magnetic stirring bar (H) coated with PTFE was placed at the bottom of the reaction chamber. This bar, when rotating, maintained a constant flow rate past the sensor. The apparatus was calibrated with distilled water at 26°C. The analyzer scale between 5 and 8.33 ppm O_2 was expanded to give full scale deflection on the chart recorder.

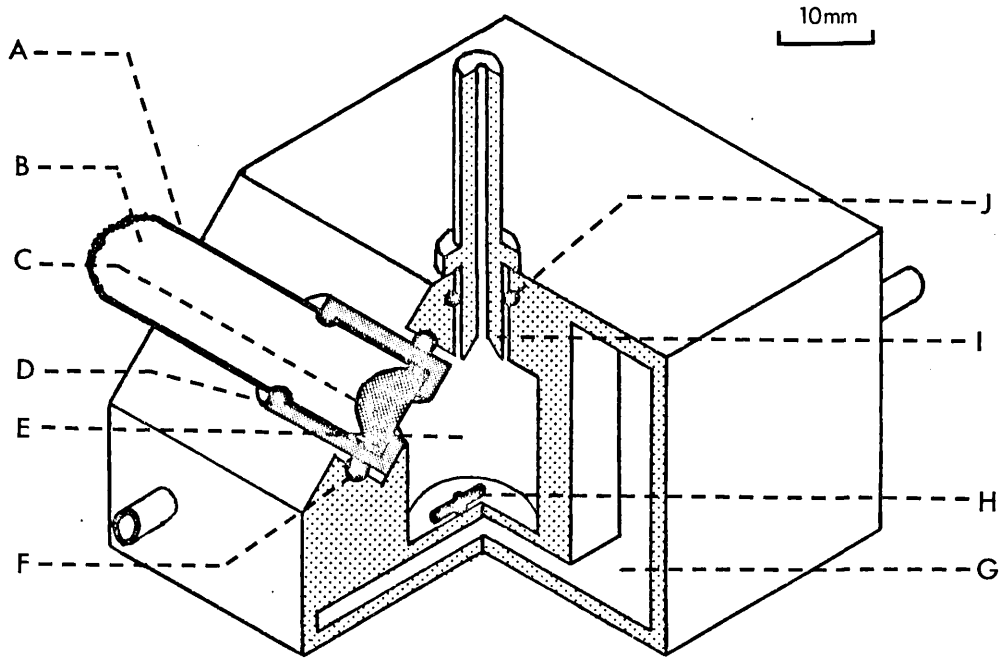


Fig. 3. The oxygen electrode.

A - sensor; B - saturated KCl; C - teflon membrane;
D - plastic cap; E - reaction vessel; F - rubber O ring;
G - perspex water jacket; H - magnetic stirring bar;
I - perspex capillary plug for opening for reactant additions;
J - rubber O ring.

B. Calibration of apparatus.

(i) Daily calibration of oxygen electrode.

Distilled water was saturated with oxygen by mixing thoroughly on a Whirlimixer and the reaction vessel filled with this and stoppered. The scale was calibrated at 8.33 p.p.m. (i.e., 8.33 parts of O_2 in 1 million parts of fluid) at 26°C, the solubility of O_2 at 26°C. By using the zero suppressor, the scale between 5 p.p.m. and 8.33 p.p.m. was expanded so that a full scale deflection of 0 to 20 cm. on the chart recorder was equivalent to approximately 5 to 8.33 p.p.m.

(ii) Absolute calibration of O_2 electrode.

The oxygen electrode was calibrated by two independent methods. The first method made use of the known solubility of O_2 in water at a given temperature. The second method was a modification of that used by Chappell (77). Chappell used a comparison of the total amount of $NADH_2$ oxidised in a spectrophotometric assay, with the total amount of O_2 consumed in the oxidation of that $NADH_2$ as measured in an oxygen electrode apparatus. I have used a comparison of the rate of $NADH_2$ oxidation measured spectrophotometrically, with the rate of O_2 uptake associated with the oxidation of that $NADH_2$.

(iii) Method I.

From the daily calibration of the O_2 electrode as mentioned above, the difference from 5 to 8.33 p.p.m. of O_2 was equivalent to full scale deflection (= 20 cm.) of the chart recorder. The chart recorder was operated at a speed of 1 cm./min.

Since the volume of the reaction vessel was 1.23ml. and the known solubility of O_2 in water at 26°C is 8.33 p.p.m., a chart slope of 1 $\equiv \frac{8.33 \times 1.23}{20}$ $\mu g.O_2/ml./min.$

$$\equiv \frac{0.169 \times 1.23}{16} \mu g.atoms\ O_2/ml./min.$$

or, 0.0135 $\mu g.atoms\ O_2/ml./min.$

(iv) Method II.

This depends on the assay of two identical preparations using NADH

oxidase:

(a) A measure of the rate of NADH oxidation in the spectrophotometer at 340 mm.

(b) A measure of the rate of 0_2 uptake of a duplicate preparation on the oxygen electrode.

The rate of oxidase activity by spectrophotometric assay : 0.170 O.D. units/min.

The rate of oxidase activity by oxygen electrode assay : 2.00 units/min.

(N.B. These electrode units are arbitrary, being defined as 1/20 of the full scale deflection of the chart recorder per minute as described above).

$NADH_2$ has an extinction (E) of 6.22×10^6 $cm.^2$/mole, i.e. 6.22×10^6 O.D. units for 1 mole/ml. with a 1 cm. light path.

Hence, an O.D. change of 0.170/min. $\equiv \frac{0.170}{6.22 \times 10^6}$ mole/ml./min.

Since 1 mole $NADH_2 \equiv \frac{1}{2}$ mole 0_2,

$$\equiv \frac{0.170}{6.22} \text{ µg.atoms } 0_2\text{/ml./min.,}$$

$$\equiv 0.0274 \text{ µg.atoms } 0_2\text{/ml./min.}$$

This is equivalent to a slope of 2.00/min. on the chart recorder.

Hence, a slope of 1 = $\frac{0.0274}{2}$ µg.atoms 0_2/ml./min.

$$= 0.0137 \text{ µg.atoms } 0_2\text{/ml./min.}$$

Therefore, a mean value of 0.0136 has been used to calculate the rate of oxygen uptake in enzyme assays.

C. Assay of malate, succinate, lactate and ascorbate oxidases.

1.0 ml. of membrane preparation or cell suspension was added to 1.0 ml. of substrate and other additions in 0.02M-phosphate buffer (pH 7.2) in a test tube. The final substrate concentrations

were 150 mM in a final concentration of 0.02M-phosphate buffer (pH 7.2). The tube was put in a constant temperature water bath at 26°C for two minutes to attain the temperature of the assay. After thorough aeration in a Whirlimixer, 1.23ml. was placed in the electrode vessel. The oxygen uptake was followed on the chart recorder for 2 to 5 minutes. The activities were expressed as µg.atoms O_2 uptake/ml./min./mg. membrane protein, or as µg.atoms O_2 uptake/ml./min. for cell suspensions.

D. Malate/Succinate : methylene blue oxidoreductases.

Methylene blue reductions were assayed by means of the reoxidation of reduced methylene blue by oxygen, using the oxygen electrode. The assay mixture contained 1.0 ml. of membrane preparation, 0.20 ml. of 1.5M-malate or succinate, 0.10 ml. of 1M-KCN (neutralized) or 0.20 ml. of 0.05M-sodium azide, 0.20 ml. of 0.02% methylene blue in 0.02M phosphate buffer (pH 7.2) to give a final volume of 2.0 ml. The reduction was followed polarographically on the O_2 electrode as already described. The activity was expressed as µg.atoms O_2 uptake/ml./min./mg. membrane protein.

E. Malate dehydrogenase.

The malate dehydrogenase activity was assayed as above on the O_2 electrode by reduction of phenazine methosulphate (PMS). The method was a modification of the manometric method of King (73) with a constant level of phenazine methosulphate. The assay mixture contained 1.0 ml. of the membrane preparation, 0.10 ml. of 0.009M-PMS in buffer (stored frozen in dark and avoiding undue exposure to light), 0.10 ml. of 1M-KCN (neutralized), 0.20 ml. of 1.5M-malate and 0.60 ml. of phosphate buffer (0.02M) pH 7.2 to give a final volume of 2.0 ml. The enzyme assay was carried out as already mentioned. The activity was expressed as µg.atoms O_2 uptake/ml./min./mg. membrane protein.

F. Malate/succinate-vitamin K reductase.

The malate/succinate vitamin K reductase activities were

assayed by reoxidising reduced vitamin K in the O_2 electrode. The assay medium was composed of 1.0 ml. of the membrane preparation, 0.10 ml. of 1M-KCN (neutralized) or 0.20 ml. of 0.05M-NaN_3, 0.20 ml. of 1.5M malate or succinate and 0.10 ml. of 1 mM-vitamin K_3 (menadione). Phosphate buffer (0.02M) pH 7.2 was added to give a final volume of 2.0 ml. to the assay medium, and the activity was assayed on the O_2 electrode as mentioned before. The activity was expressed as µg.atoms O_2 uptake/ml./min./mg. membrane protein.

G. Ascorbate-cytochrome c oxidase.

The ascorbate-cytochrome c oxidase activity was assayed on O_2 electrode. The assay mixture contained 1.0 ml. of membrane preparation, 0.20 ml. of 0.0001 M-ascorbate (neutralized), 0.10 ml. of 2×10^{-3} M-cytochrome c and 0.70 ml. of phosphate buffer (0.02M) pH 7.2 to give a final volume of 2.0 ml. The activity was assayed as described above and expressed as µg.atoms O_2 uptake/ml./min./mg. membrane protein.

H. NADH oxidase.

The NADH oxidase activity was measured in the O_2 electrode. The assay medium was composed of 0.50 ml. of the membrane preparation in buffer, 0.20 ml. of 0.1% NADH solution in buffer (used within 4 hours of preparation) and 1.30 ml. of phosphate buffer (0.02M) pH 7.2. The activity was assayed as mentioned before and was expressed as µg.atoms O_2 uptake/ml./min./mg. membrane protein.

12. Spectrophotometric assay methods.

A Beckman model D.B. spectrophotometer was used with cuvettes having a 1 cm. light path. The reactants were equilibrated at 26° before mixing and the spectrophotometer water jacket was maintained at 26°.

A. Malate : dichlorophenol indophenol, succinate : dichlorophenol

indophenol, and NADH : dichlorophenol indophenol reduction.

Reduction of 2, 6-dichlorophenol indophenol (DCPIP) was followed spectrophotometrically at 600 nm. The reaction mixture contained 0.30 ml. of 1.5M-malate or succinate, or 0.30 ml. of 0.1% NADH, in phosphate buffer (0.02M) pH 7.2 containing 0.0005M-$MgCl_2$, 0.30 ml. of 0.00005M-DCPIP in buffer, 0.50 ml. of membranes in buffer and 0.15 ml. of 1M-KCN (neutralized) or 0.30 ml. of 0.05M-NaN_3 in buffer. The final volume was made up to 3.00 ml. with buffer. The final concentrations of phosphate buffer and $MgCl_2$ were 0.02M and 0.0005M respectively. The reaction was started by addition of enzyme (0.50 ml. of membrane preparation) to the cuvette. The absorbance reading of the experimental cuvette was set at about 0.6, against a blank which contained all the components except the enzyme. Spectrophotometric readings began at about 5 seconds after adding the membrane preparation to the mixture in the experimental cuvette. The reaction was linear with time for at least 3 minutes. The reduction of DCPIP was followed by the decrease of absorbance at 600 nm. The activity was expressed as µ moles DCPIP reduced/ml./min./mg. membrane protein.

B. NADH oxidase.

The NADH oxidase activity was assayed spectrophotometrically. The assay medium, containing 0.50 ml. of membrane preparation in buffer and 0.30 ml. of 0.1% NADH solution in buffer (used within 4 hours of preparation), was placed in a cuvette of 1 cm. optical path, and 2.20 ml. of phosphate buffer (0.02M) pH 7.2 containing 0.0005M-$MgCl_2$ was added to give a final volume of 3.00 ml. The blank was prepared without enzyme, and the oxidase activity was assayed by measuring a decrease in absorption at 340 nm. The activity was expressed as µg.atoms O_2 uptake/ml./min./mg. membrane protein.

C. Protein.

The protein content of the membrane preparations was estimated by the colorimetric method of Folin and Lowry (79).

13. Extraction, estimation of pigment concentration, and analysis of carotenoids of Sarcina lutea.

Sarcina lutea was grown in 5 litres of nutrient broth as described earlier, and harvested by centrifuging at 6000 g. for 10 minutes. The pellet of whole cells was suspended in distilled water and the volume made up to 100ml. 3.0ml. aliquots of the whole cell suspension were dried in an oven at 90° overnight to obtain an estimate of the dry weight of the suspension.

The remainder of the suspension of whole cells was spun to obtain a pellet, and repeatedly extracted with acetone until no further appreciably coloured extract was obtained. The volume of the coloured extract was noted, and the absorption spectrum of the extract was recorded.

This acetone solution was exhaustibly extracted with light petroleum (b.p. 40 - 60°), and finally with ether to remove the more polar pigments. The petroleum and ether extracts were combined, washed with water, dried over anhydrous Na_2SO_4, and evaporated to small volume under nitrogen.

The evaporated material was dissolved in light petroleum and analysed by chromatography on a zinc carbonate column containing two parts of zinc carbonate and one part of "hyflo-supercel." The series of eluting solvents for fractions 1, 2 and 3 were 10% ether in light petroleum, 2 - 10% acetone in light petroleum, and 5% ethanol in light petroleum spirit respectively. The volumes of the different fractions were noted, and the absorption spectra, with their maxima, were recorded. The percentage of carotenoids in each fraction was calculated using an $E^{1\%}_{1cm}$ value of 2500 as suggested by Davies (80).

CHAPTER 3

Experiments with Toluidine Blue

1. Introduction

The work so far reviewed in the first chapter discussed some aspects of photodynamic action in the non-photosynthetic bacterium S. lutea. Workers mostly used an external photosensitizer and demonstrated photodynamic action by cellular death, which was evidenced by viable colony count. Studies on some enzyme activities have demonstrated enzyme lability in the presence of exogenous photosensitizers and artificial light. Damage to cell permeability has been suggested as one of the causative factors in cellular death due to photodynamic action. In all these studies the assay of enzyme activities was performed after subjecting whole cells to photosensitizing conditions, and obtaining membrane fragments afterwards.

In this section, apart from the study of cellular death, some enzyme activities of cell suspensions, and cell membranes obtained after lysozyme treatment, were studied by using low light intensities (artificial light) with an exogenous photosensitizer, toluidine blue. The question of permeability is thus ruled out altogether in studies using cell membranes. Various oxidative systems of the respiratory chain have been assayed using substrates like malate, succinate, lactate and cytochrome c. Unlike previous work, the enzyme activities of cell suspensions and cell membranes were assayed during the photosensitizing conditions rather than at the end of the illumination period. The study includes enzyme assays of the entire electron transport chain, and of isolated segments of the chain. Different electron acceptors, in conjunction with inhibitors, have been used in an attempt to study the entire sequence of cellular death from the onset of damage to the terminal stages of photodynamic action. Both manometric and polarographic O_2 assay methods

have been employed. Unlike other workers who worked mostly on whole cells, the present studies were carried out mainly on cell membranes where the carotenoids and the various enzymes are located.

2. Oxidation of malate, succinate, lactate and ascorbate by whole cells and membranes of pigmented S. lutea and a carotenoidless mutant.

Initial experiments with whole cells and membranes of Sarcina lutea were designed to find a concentration of substrate which would be sufficient for manometric experiments lasting 3 to 4 hours. A suitable concentration of malate, lactate, succinate and ascorbate for oxidation studies was found to be 150 mM. Using 150 mM substrate, the oxidation of malate, succinate, lactate and ascorbate by whole cells and cell membranes of the pigmented strain of Sarcina lutea was tested. Malate, lactate and succinate are readily oxidised by whole cells.

The result showed that in pigmented membranes, while there was good oxidation of malate and moderately good oxidation of lactate, the oxidation of succinate was obviously low (see Fig. 4). The membrane preparations were made by lysozyme treatment as mentioned on page 38 . The results of the experiment conform with the observations of Shah and King (67) who found that the succinate oxidase system of M. lysodeikticus was inhibited by lysozyme treatment.

In later experiments, shorter periods of lysozyme treatment yielded membranes with moderately good succinoxidase activities. Unfortunately, initial experiments were performed with membranes obtained by lysozyme treatment for 45 minutes, while in the subsequent experiments the lysozyme treatment was only for 15 minutes.

Membranes of the pigmented strain and the carotenoidless white mutant of S. lutea were compared for their ability to oxidise malate and ascorbate-cytochrome c (mammalian cytochrome c).

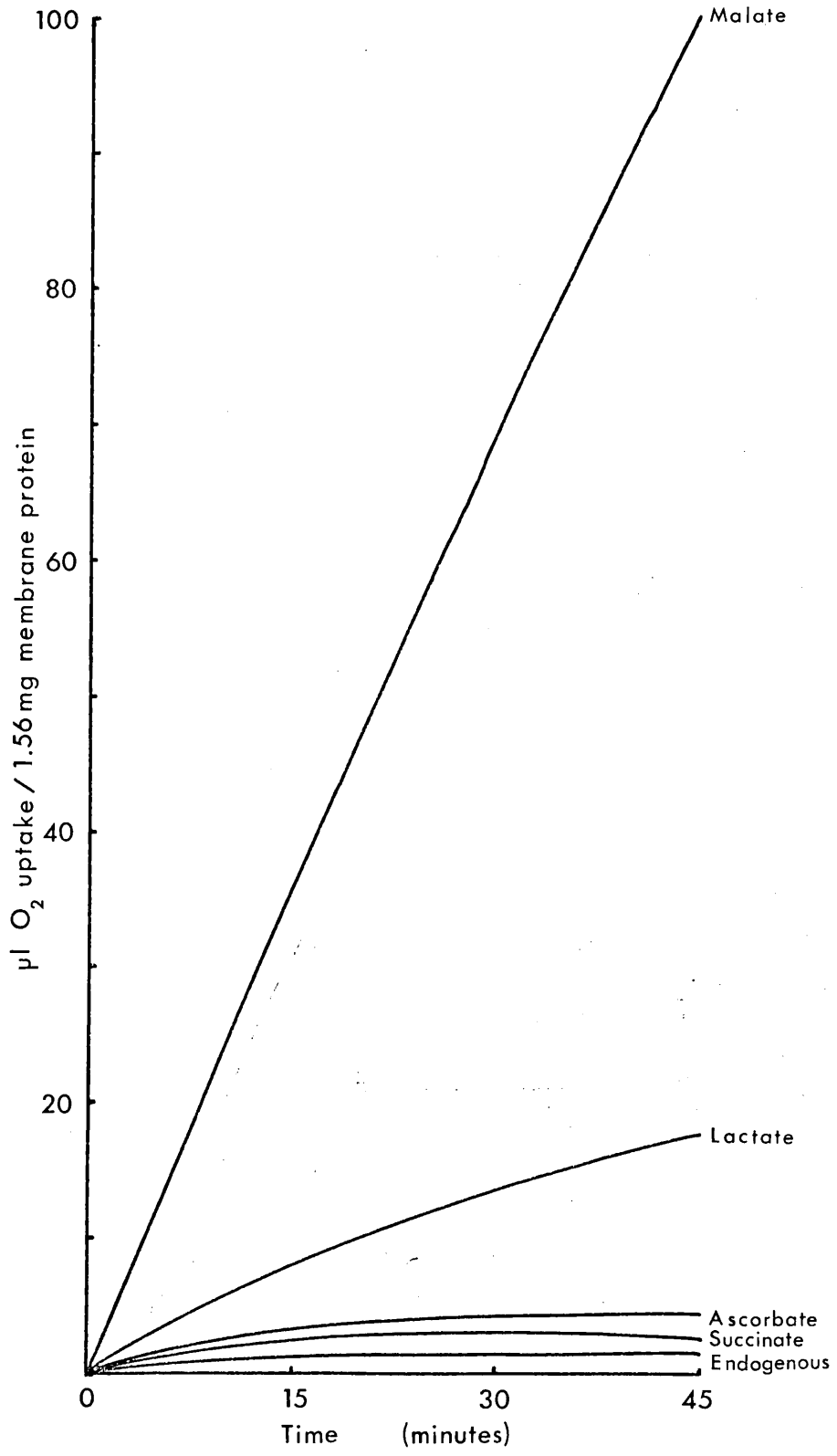


Fig. 4. Oxidation of malate, lactate, ascorbate and succinate by membranes of the pigmented strain of S. lutea.

For details of experimental procedures and malate, lactate, ascorbate and succinoxidase assays see pages 43 and 44.

The results (Fig. 5) showed that the malate oxidase system of both the pigmented and the white mutant was very active. As the rate of oxidation in both the pigmented and carotenoidless mutant was almost the same, it seems that the presence or absence of carotenoids does not influence the oxidative processes. Of the three substrates (malate, lactate and succinate), malate oxidation was the most active, and as such it was decided to use malate as the substrate for enzyme assays for most of the later experiments. There was poor oxidation of ascorbate-cytochrome c. Therefore, higher concentrations of ascorbate (150 mM) were needed which resulted in high rates of autooxidation of ascorbate. Thus assays involving ascorbate were necessarily insensitive.

3. Effect of exposure of pigmented and carotenoidless whole cells and membranes to light from tungsten lamps (620 lumens/sq.ft.) on malate oxidation.

Six experiments were performed to show the effect of exposure to light (620 lumens/sq.ft.) on the malate oxidase activities of pigmented and non-pigmented whole cells and membranes. The result of a representative experiment using an oxygen electrode appears in Table 1.

There was only a small and very variable depression of malate oxidation in either pigmented or white whole cells or membranes when subjected to illumination for 180 minutes. When Mathews and Sistrom (5) studied the protective function of carotenoid pigments in cell suspensions of the pigmented and the white mutant of S. lutea by illuminating at a higher light intensity (3000 ft. candles) for 4 hours, they showed that there was no photodynamic killing of either the pigmented or the white mutant. Therefore, the use of an exogenous photosensitizer seemed to be essential to demonstrate a photodynamic action at a lower light intensity. The remaining experiments of this chapter, therefore, have been performed in the presence of an exogenous photosensitizer, toluidine blue.

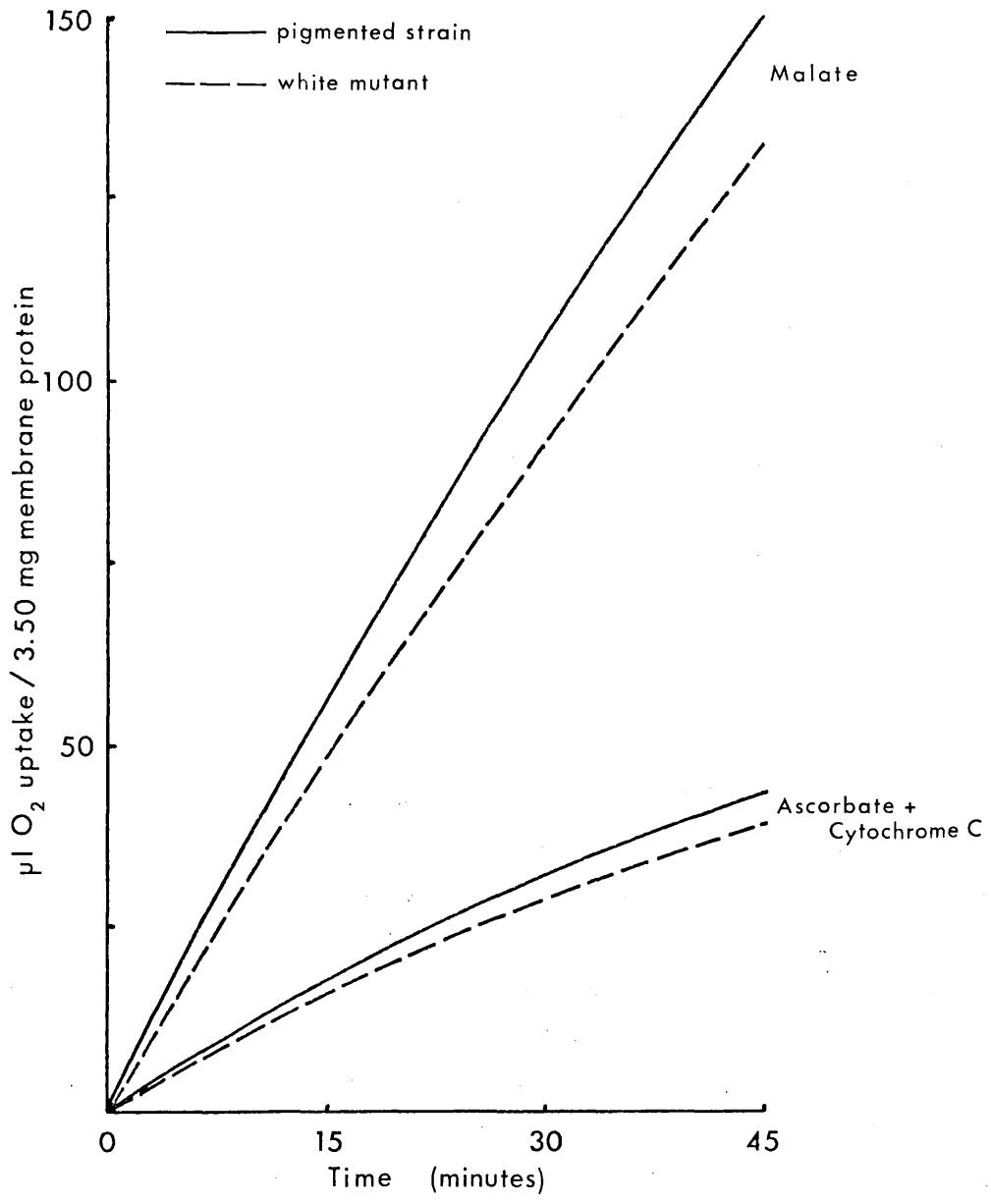


Fig. 5. Oxidation of malate and ascorbate-cytochrome c by membranes of the pigmented and white strains of S. lutea.

150mM malate, 150mM ascorbate and 0.5% cytochrome c were added to the respective flasks. The auto-oxidation of ascorbate in the blanks were subtracted from the experimental results. For details of experimental procedures and assays of malate and ascorbate-cytochrome c oxidase activities see pages 43 and 44.

Table 1. Illumination of whole cells and membranes of pigmented and a carotenoidless white mutant of *Sarcina lutea*: effect on malate oxidation.

Illumination time (mins.)	Pigmented *Sarcina lutea* Malate oxidase activity				U.V.-induced colourless mutant Malate oxidase activity			
	Rate of O_2 uptake (µg. atoms of O_2/ml./min.)				Rate of O_2 uptake (µg. atoms of O_2/ml./min.)			
	Whole cells		Membranes		Whole cells		Membranes	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light
0	0.005	-	0.028	-	0.012	-	0.013	-
20	0.010	0.009	0.035	0.030	0.014	0.012	0.013	0.011
40	0.009	0.010	0.033	0.028	0.013	0.012	0.014	0.008
60	0.010	0.011	0.032	0.027	0.014	0.013	0.014	0.012
80	0.010	0.011	0.033	0.027	0.013	0.012	0.014	0.011
100	0.009	0.012	0.036	0.028	0.013	0.012	0.014	0.014
120	0.009	0.010	0.036	0.028	0.013	0.011	0.016	0.014
140	0.010	0.010	0.036	0.022	0.014	0.013	0.019	0.015
160	0.008	0.011	0.029	0.028	0.014	0.012	0.015	0.013
180	0.010	0.010	0.032	0.028	0.014	0.013	0.017	0.015

The whole cell suspensions and membrane preparations were illuminated from 4 tungsten lamps (620 lumens/sq.ft.) at 12° C and 150 mM-malate was added immediately prior to polarographic O_2 assay. The rate of O_2 uptake has been expressed per milligram of membrane protein in the case of cell membranes, while in the case of whole cells it has been expressed relatively. For details of experimental procedures, illumination and assay of enzyme activities, see pages 45 to 48.

4. Viability of illuminated pigmented Sarcina lutea and its u.v.-induced white mutant in the presence of 2.5 μM-toluidine blue.

It has been demonstrated that the carotenoids afford protection against photodynamic killing in S. lutea. Mathews and Sistrom (5) observed that carotenoidless mutants died either at very high light intensities such as sunlight and air in the absence of an added photosensitizer, or at low light intensities such as artificial light in the presence of an added photosensitizer toluidine blue. Prior to investigations of effects of light on membrane enzymes, experiments were performed to reproduce the same type of light effect on the viability of S. lutea. When pigmented S. lutea and the white mutant were illuminated at 620 lumens/sq.ft. light intensity in the presence of 2.5 μM-toluidine blue, the carotenoidless mutants showed decreased viability with the increase of duration of illumination, whereas the pigmented variety showed no cell death at all (Table 2). This suggests that the carotenoids afforded protection against photo-killing in these strains of S. lutea. The number of viable cells of the pigmented strain showed an increase due to slight growth as the illumination progressed.

5. Effect of illumination with various concentrations of toluidine blue (1 μM, 2.5 μM and 5 μM) on the malate oxidase activity of whole cells of pigmented and white S. lutea.

It has been shown, both by other workers and above, that low light intensity with an external photosensitizer, toluidine blue, causes cell death in the carotenoidless mutant of S. lutea. Four experiments with three different concentrations of toluidine blue were performed with a view to finding the effect of illumination at 660 lumens/sq.ft. on the malate oxidase activity of the two strains of S. lutea.

The light sensitivity of malate oxidase activity of whole cells of both the pigmented and the mutant strains increased (evidenced by a depressed oxidation rate) with the increase of toluidine blue

Table 2. Viability of illuminated pigmented Sarcina lutea and an u.v.-induced white mutant of the same in the presence of 2.5 μM toluidine blue.

Illumination time (mins.)	Viable cells/ml. x 10^6	
	Pigmented strain	White mutant
0	89	840
15	99	340
30	103	117
45	130	43

For details of the experimental procedures and illumination see page 40.

concentration, but the extent of damage to the malate oxidase activity was found to be more in the carotenoidless mutant than in the pigmented strain (Figs. 6 a, b, c). These results suggested that an external photosensitizer would cause some damage to the malate oxidase system in both strains of S. lutea, though the carotenoids in the pigmented strain afforded some protection to the bacterium (evidenced by lesser depression of oxidation rate) which was lacking in the carotenoidless white mutant. However, the protection afforded by carotenoids did not last long.

6. Effect of illumination in the presence of 2.5 µM-toluidine blue on succinoxidase and lactate oxidase activities of white and pigmented cells.

The effect of illumination (660 lumens/sq.ft.) with the external photosensitizer toluidine blue (2.5 µM) on the succinoxidase and lactate oxidase activities of whole cells of the pigmented and white strains of S. lutea is shown in Fig. 7 and Fig. 8 respectively. With succinoxidase, depressed oxidation rates due to illumination were found in both the pigmented and white mutant strains as with malate oxidase, though the extent of damage was more in the mutant than in the pigmented strain. Here also the carotenoids in the pigmented strain afforded some protection to the damage caused by light with photosensitizer, toluidine blue. Experiments on the lactate oxidase activity of whole cells of the pigmented and non-pigmented strains of S. lutea showed similar results to those above.

The results of illumination at low light intensities (660 lumens/sq.ft.) with an external photosensitizer, toluidine blue, on the malate, succinate and lactate oxidase activities of the pigmented and while whole cells, suggested that there was more damage in the carotenoidless mutants than in the pigmented strain. Mathews and Sistrom (6) also found damage caused by light with photosensitizer to occur more in the white mutant than in the pigmented strain, and they suggested the site of damage to be cell

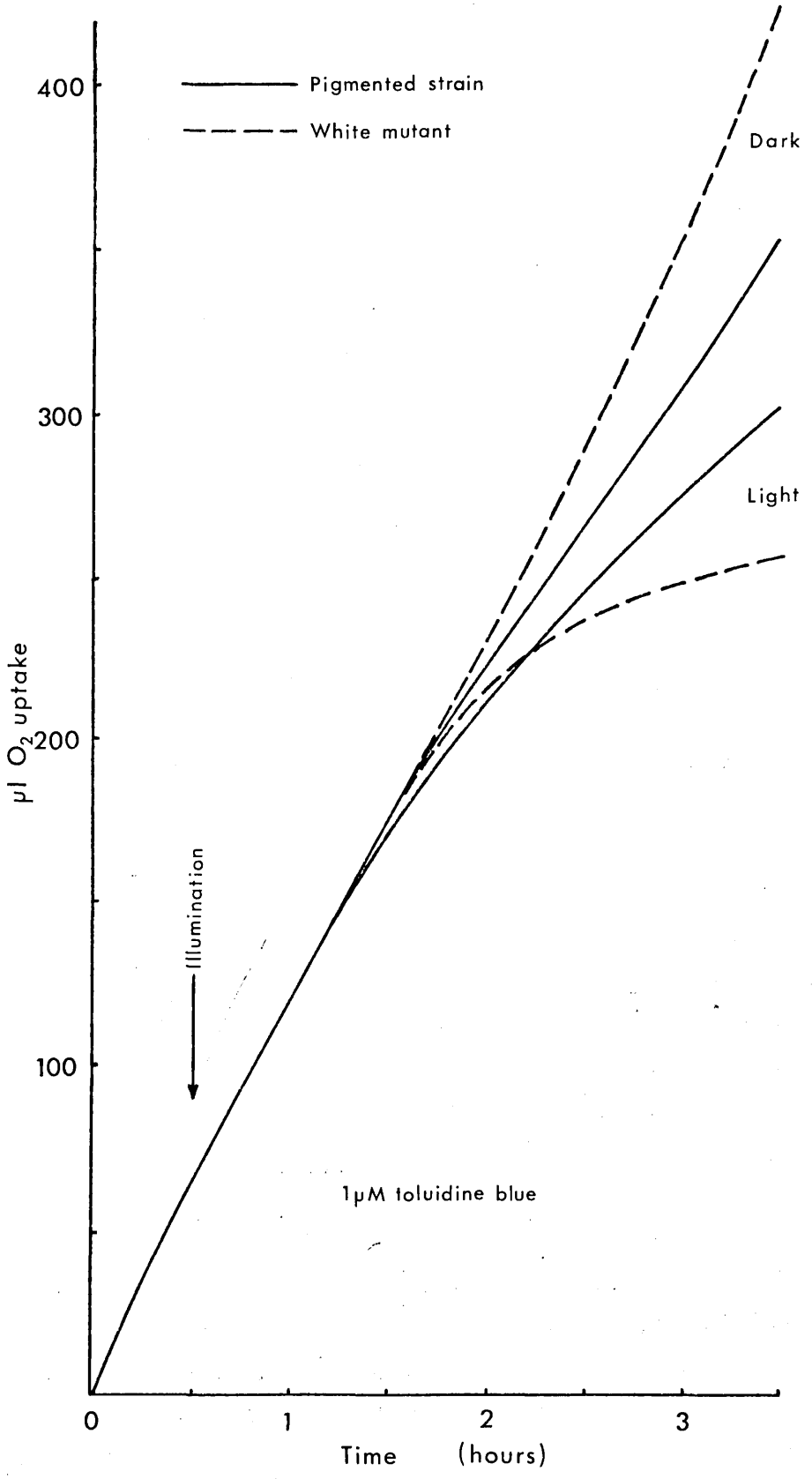


Fig. 6a. Effect of exposure to illumination (650 lumens/sq ft) with 1µM toluidine blue on the malate oxidase activity of whole cells of the pigmented and white strains of S. lutea.

For details of experimental procedures, illumination and assay of malate oxidase activity see pages 43 and 44.

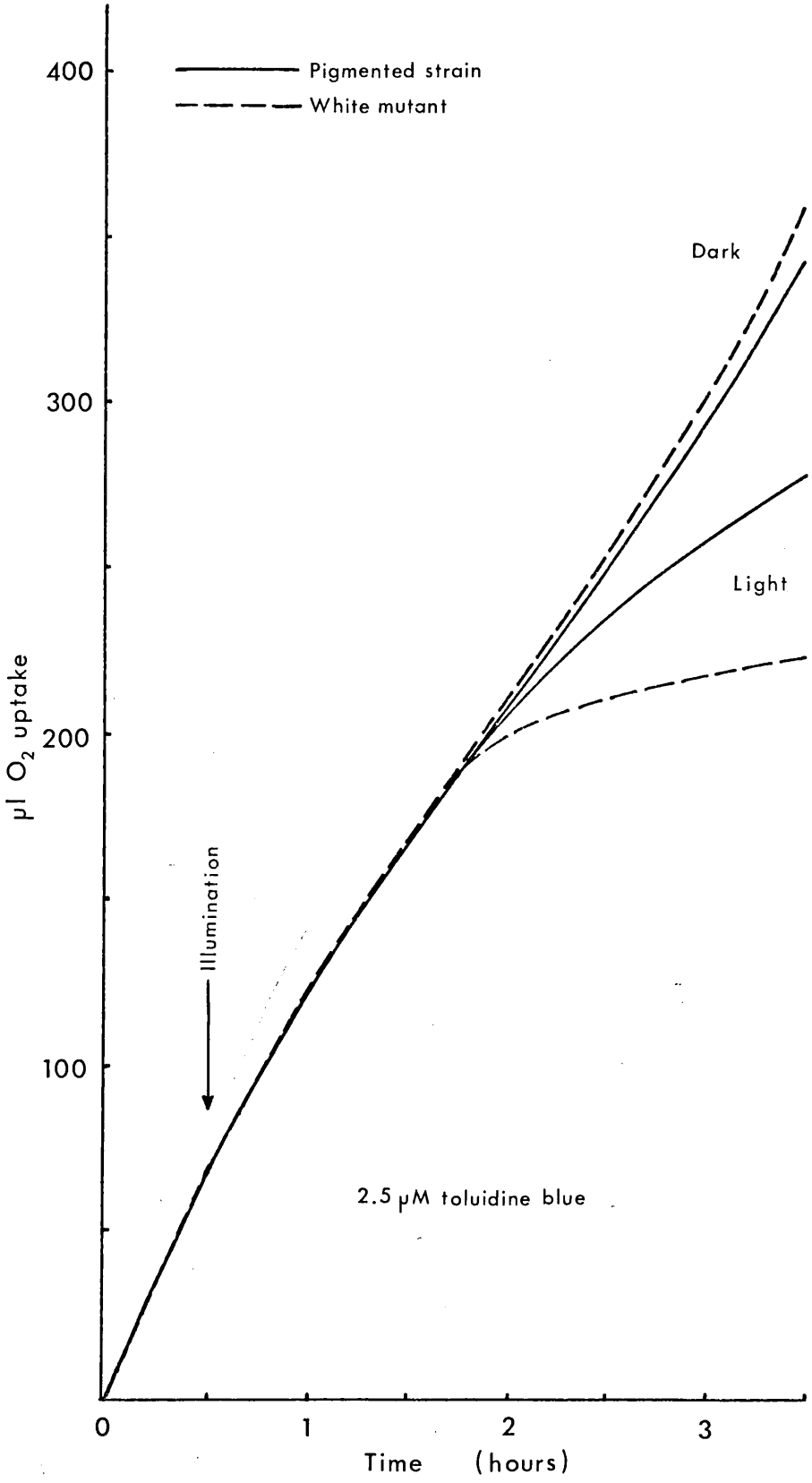


Fig. 6b. Effect of exposure to illumination (650 lumens/sq ft) with 2.5μM toluidine blue on the malate oxidase activity of whole cells of the pigmented and white strains of S. lutea.

For details of experimental procedures, illumination and assay of malate oxidase activity see pages 43 and 44.

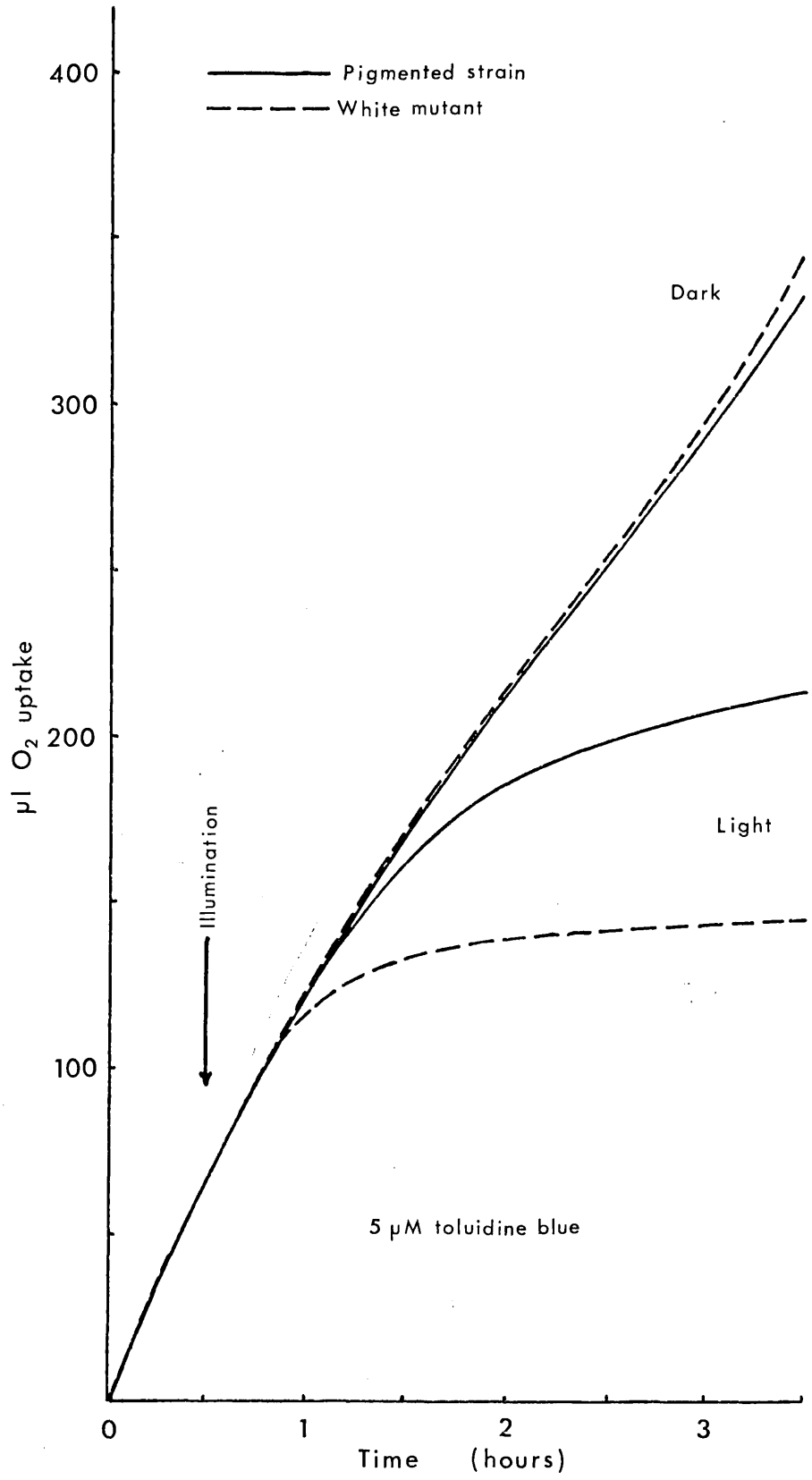


Fig. 6c. Effect of exposure to illumination (650 lumens/sq ft) with 5µM toluidine blue on the malate oxidase activity of the whole cells of the pigmented and white strains of S. lutea.

For details of experimental procedures, illumination and assay of malate oxidase activity see pages 43 and 44.

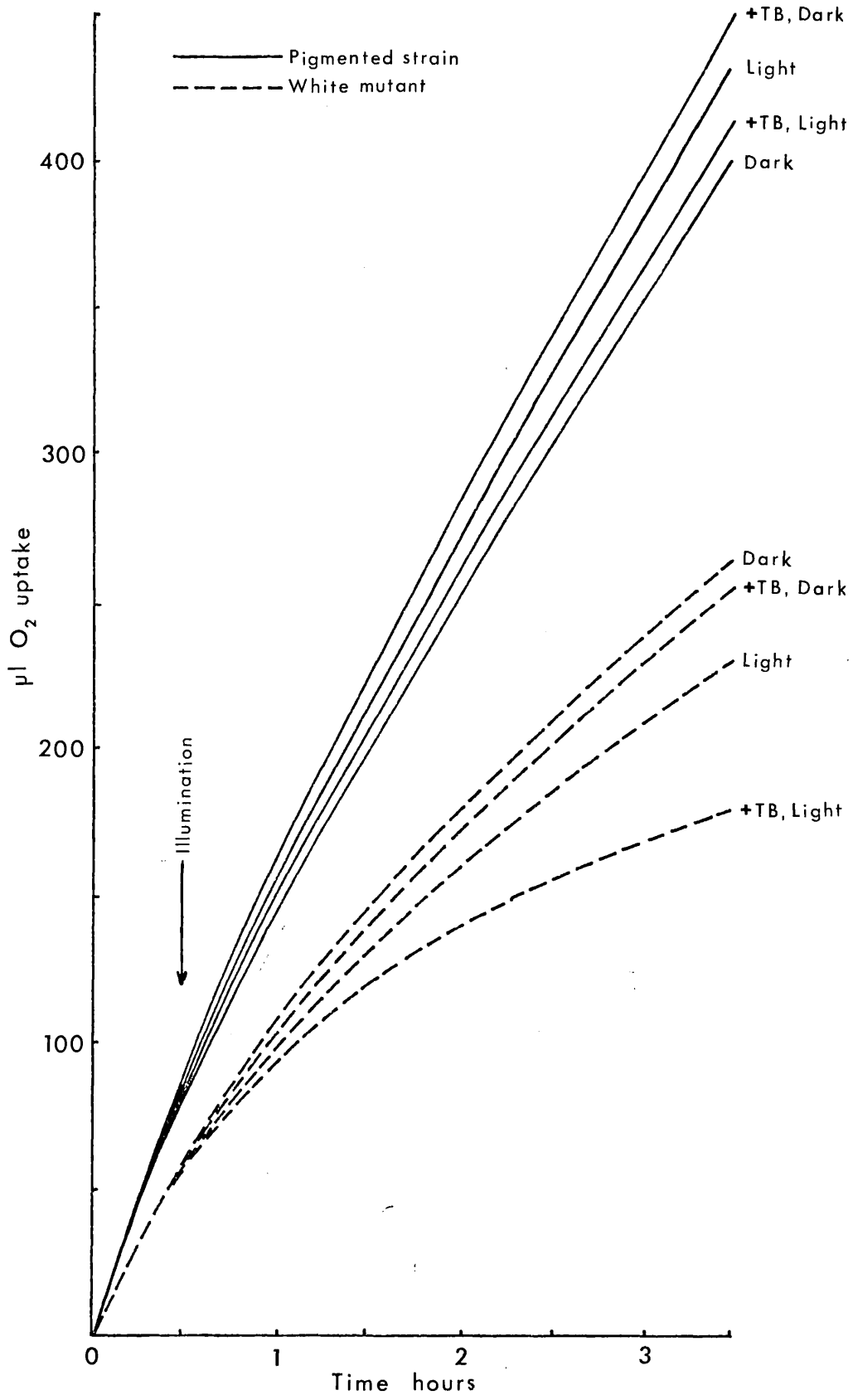


Fig. 7. Effect of exposure to illumination (660 lumens/sq ft) with 2.5µM toluidine blue on the succinoxidase activity of whole cells of the pigmented and white strains of S. lutea.

150mM sodium succinate and 2.5µM toluidine blue were added to flasks together with cell suspensions in buffer. No toluidine blue was added to the control flasks. For details of the experimental procedures, illumination and assay of succinoxidase activity see pages 43 and 44.

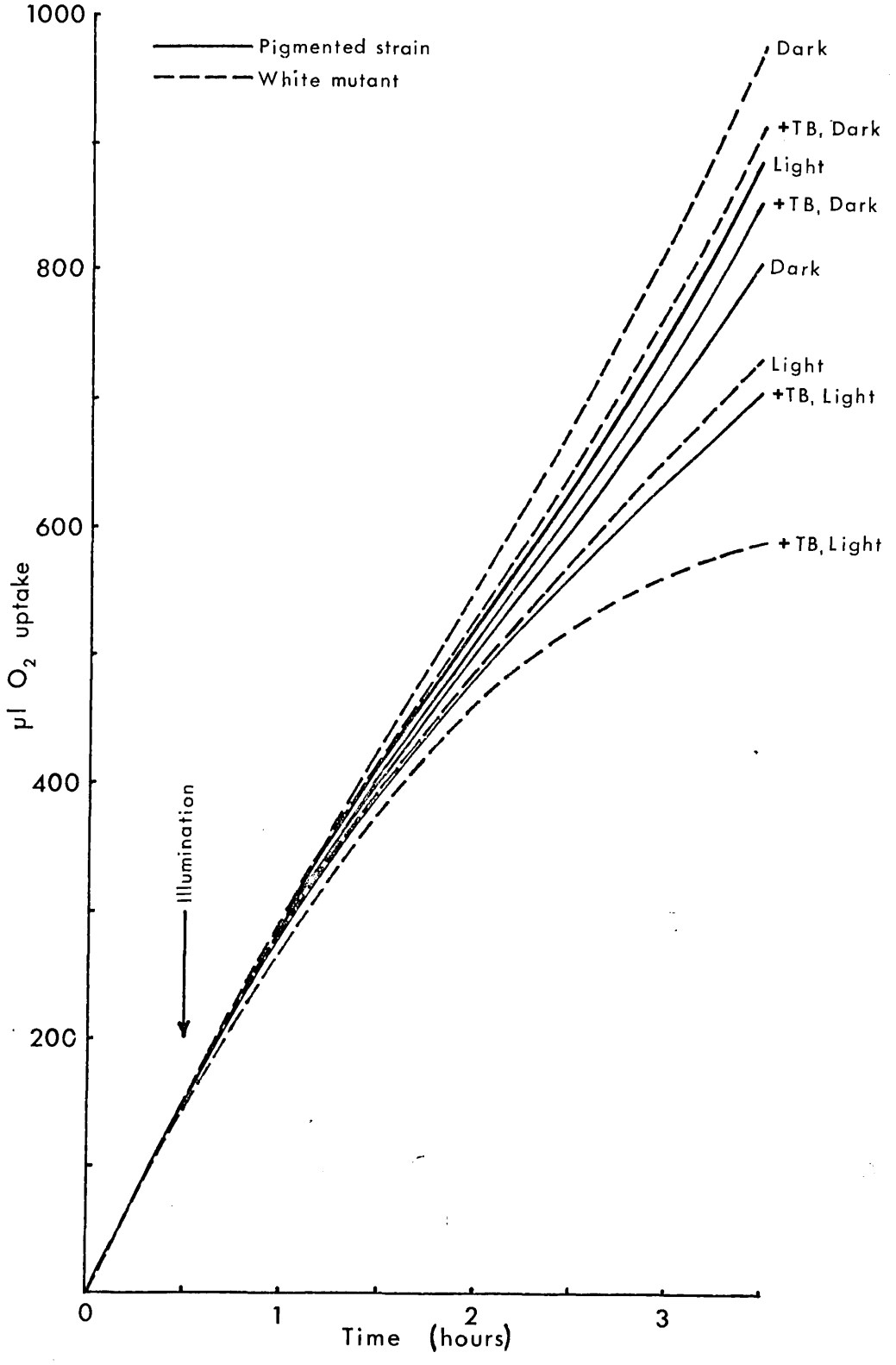


Fig. 8. Effect of exposure to illumination (660 lumens/sq ft) with 2.5µM toluidine blue on the lactate oxidase activity of whole cells of he pigmented and white strains of S. lutea.

150mM sodium lactate and 2.5µM toluidine blue were added to flasks together with cell suspensions in buffer. No toluidine blue was added to the control flasks. For details of the experimental procedures, illumination and assay of lactate oxidase activity see pages 43 and 44.

membranes. As carotenoids are located in the cell membranes, and most of the respiratory enzyme systems are also located in the membrane, it seemed quite probable that these enzyme activities were damaged by illumination with an external photosensitizer which, if prolonged, could bring about complete inactivation of the respiratory activity leading to cell death. Carotenoids seemed to delay the onset of cell death by affording some protection from photokilling in the case of the pigmented strain, while the carotenoidless mutants were more vulnerable to damage. Therefore, this work was directed towards cell membranes which were obtained by lysozyme treatment. Previous workers (6) had irradiated whole cells and considered the possibility that a permeability effect was the primary cause of cellular death; this they later discounted. The study of isolated membranes made it further possible to eliminate any permeability effect.

7. Effect of various concentrations of toluidine blue in the light on malate oxidation by cell membranes of the white mutant of S. lutea.

Experiments were performed to find the effect of illumination (600 lumens/sq.ft.) on the malate oxidase activity of the membranes from the white mutant. Various concentrations of the external photosensitizer, toluidine blue, were used. Fig. 9 shows that the carotenoidless membranes were also sensitive to toluidine blue in the presence of light, like the whole cells. The sensitivity increased (evidenced by depressed oxidation rate) with the increase in the concentration of photosensitizer.

Having observed the effects of light with various concentrations of toluidine blue on the malate oxidase activities of carotenoidless membranes, malate oxidation in the light of both pigmented and white membranes was compared. The concentration of toluidine blue used was that employed by Mathews and Sistrom (6), namely 2.5 μM. The results of a typical experiment are shown in Fig. 10.

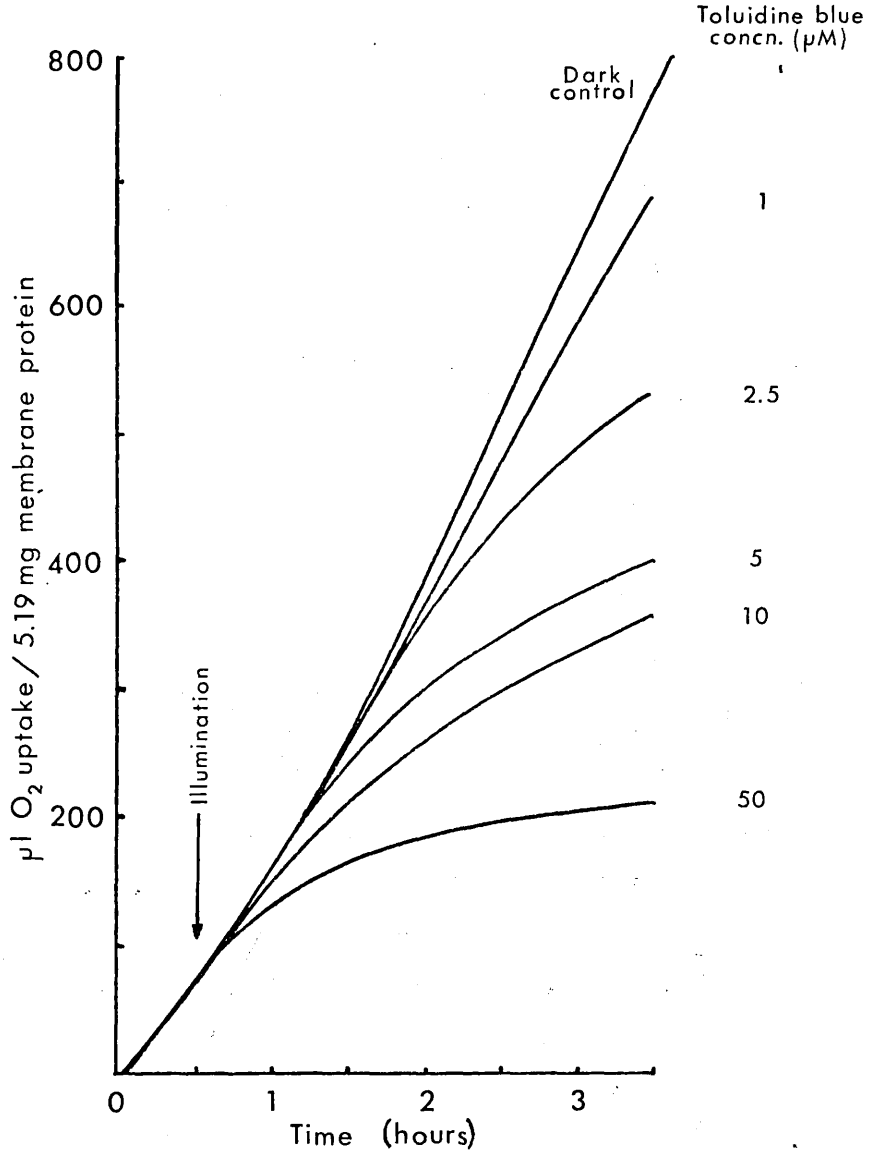


Fig.9. Effect of various concentrations of toluidine blue on malate oxidation in the light (660 lumens/sq ft) by membranes of the white strain of S. lutea.

For experimental procedures, illumination and assay of malate oxidase activity see pages 43 and 44.

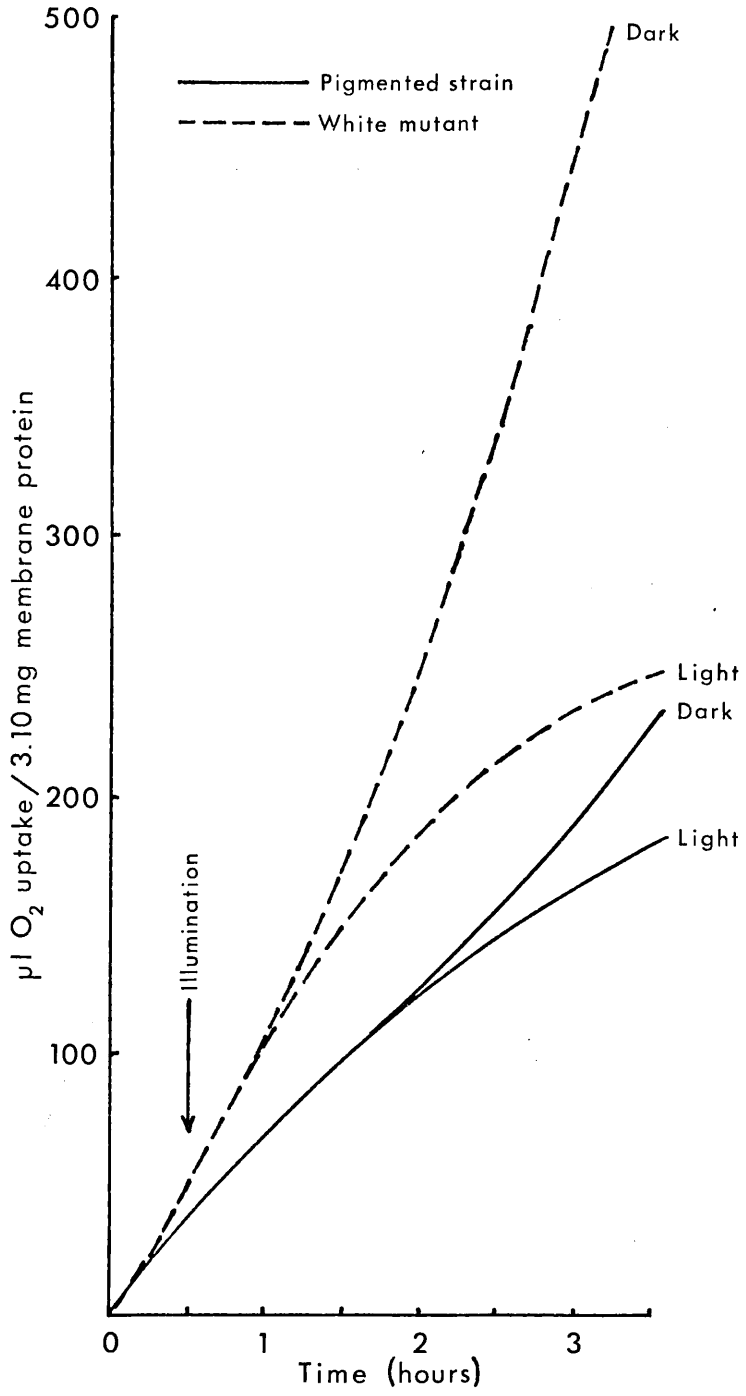


Fig. 10. Effect of 2.5μM toluidine blue on malate oxidation in the light (660 lumens/sq ft) by the membranes of the pigmented and white strains of S. lutea.

For details of experimental procedures, illumination and assay of malate oxidase activity see pages 43 and 44.

The malate oxidase activity of the pigmented strain of S. lutea was slightly sensitive to light with 2.5 μM-toluidine blue (evidenced by less depressed rate of malate oxidation), but the mutant variety showed a much greater sensitivity. The results also suggested that there was some protection from light damage in the carotenoid containing strain, whereas protection was lacking in the carotenoid-less white mutant.

Therefore, it appeared that in the process of illumination at a toluidine blue concentration between 10^{-6}M and 10^{-5}M, the sensitivities of the pigmented and non-pigmented membranes increased with the increase in the sensitizer concentration, but the non-pigmented membranes showed much greater light sensitivity with toluidine blue particularly at higher concentrations.

3. Effect of light with toluidine blue and exogenous histidine on malate oxidation by the membranes of pigmented and white S. lutea.

It is well known that some of the amino acid residues such as histidine, tryptophan, methionine and tyrosine in a protein were specifically oxidised by irradiation with visible light in the presence of a suitable photosensitizer (23), and that such a reaction did not cause cleavage of the peptide bond (81). Various reagents have been used for the oxidative modifications of proteins, but most of them showed a low degree of specificity. They generally attacked sulphydryl and disulphide groups, thio ether, indole, phenol, imidazole and other groups. It has also been found that when solutions of a number of proteins were irradiated with visible light in the presence of various dyes like methylene blue (24) and proflavin (25), oxygen was taken up, and oxidative destruction of histidine, tyrosine and tryptophan nuclei and oxidation of side chains containing sulphur took place. The precise nature of the products formed during the reactions was, however, unknown.

In the present experiment toluidine blue was used. It seemed probable that prolonged illumination of membranes with higher concentrations of toluidine blue (10^{-5}M) might cause damage to

histidine residues in the proteins of the membranes. Further experiments were planned to see if there was any protection of the histidine residues from photooxidation with toluidine blue. The results of one of the four experiments performed is shown in Fig. 11.

In the irradiated white mutant, damage to the malate oxidase activity commenced after 30 minutes' illumination, and inactivation continued progressively. By the addition of exogenous histidine to the mutant, the onset of light damage to the malate oxidase activity was delayed by one hour, after which the damaging action continued progressively, suggesting that exogenous histidine afforded some protection. In the pigmented membranes, the onset of light inactivation was slightly more delayed than in the non-pigmented mutant membranes, but in the pigmented membrane the malate oxidase activity was fully protected from inactivation by the addition of exogenous histidine. The results suggested that while light with toluidine blue caused irreversible damage to the mutant, damage to the pigmented strain could be protected fully by the addition of exogenous histidine.

The results of this experiment led to the idea that toluidine blue normally used at a higher concentration (10^{-5}M) in the presence of light would probably attack amino acid residues in membrane proteins. If illumination was allowed to continue for up to 3 - 4 hours, irreversible changes occurred rendering almost complete inactivation of the membranes. Therefore it was decided to see whether a shorter period of illumination with toluidine blue could produce a partial and reversible inactivation of the membranes in the carotenoidless mutant strain of S. lutea.

9. Effect of various durations of illumination with 10^{-5}M-toluidine blue on the malate oxidase activity of membranes of the white mutant.

Four experiments were performed to see the effect of 10, 20, 30, 60 and 210 minutes' illumination at 660 lumens/sq.ft., followed by a period of dark incubation, on the malate oxidase activity of the membranes of the white mutant of S. lutea. The results of one

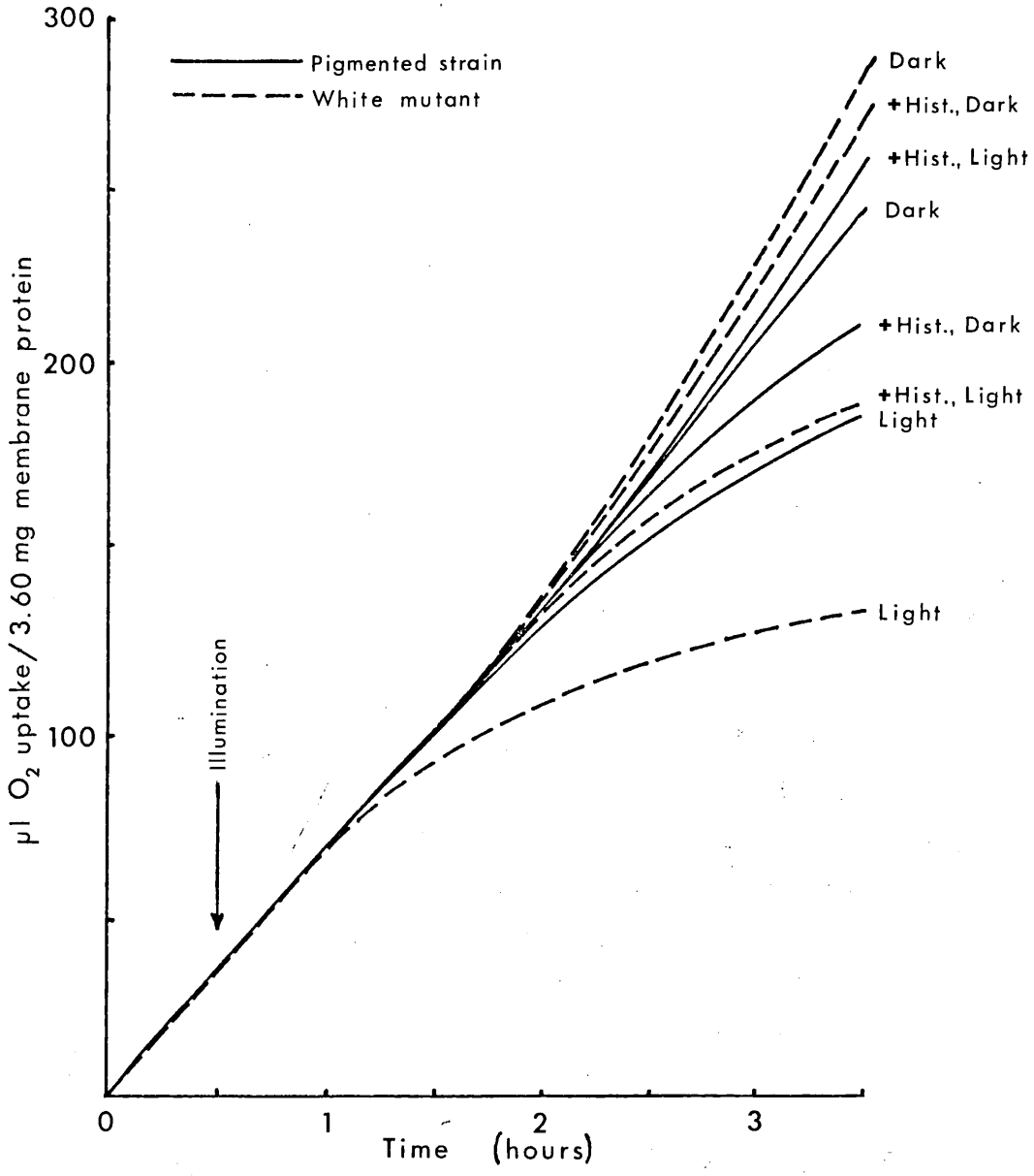


Fig. 11. Effect of light (650 lumens/sq ft) with 10^{-5}M toluidine blue and exogenous histidine on malate oxidation by membranes of the pigmented and white strains of S. lutea.

$5x10^{-3}$M histidine hydrochloride (Hist.) was added to the respective flasks together with 10^{-5}M toluidine blue, 150mM malate and the membrane preparations. For experimental procedures, illumination and assay of malate oxidase activity see pages 43 and 44.

experiment are shown in Fig. 12.

Short illumination of 10 minutes did not cause any damage to the malate oxidase system as compared with the dark control. 20 minutes' illumination caused slight damage to the oxidase activity (evidenced by slight depression of malate oxidation) which recovered almost completely when kept in the dark after illumination. 30 minutes' illumination caused more damage which, when kept in the dark, recovered about 50 percent of its activity. Illumination for longer than 60 minutes produced almost permanent inactivation, rendering inactivation of the oxidase completely irreversible. Thus, longer illumination with toluidine blue at higher concentration caused almost permanent inactivation to the malate oxidase activity in the membranes of the white mutant of S. lutea, while illumination for less than 30 minutes caused slight inactivation which recovered completely when kept in the dark.

The results of experiments done so far suggested that the malate, succinate and lactate activities of both the pigmented and the non-pigmented strains of S. lutea were damaged by light with toluidine blue, though the damage was less in the former than in the latter which lacked in carotenoids. Mathews and Sistrom (5, 14) suggested that light with toluidine blue caused damage to the protein structure in carotenoidless mutants, while some amount of protection from photodynamic action was afforded to the pigmented strain by the carotenoids. Mathews (29) studied the lethal photosensitization of S. lutea with toluidine blue, and suggested that the primary site of photokilling was the protein of the cell membrane as manifested by the destruction of the membrane enzyme activity and the regulation of permeability. However, no-one has demonstrated experimentally the exact site of damage by light with a photosensitizer. Since the pigments of the respiratory chain might act as photosensitizing agents in the absence of an exogenous photosensitizer, it seemed probable that they could be the site of carotenoid protection as well.

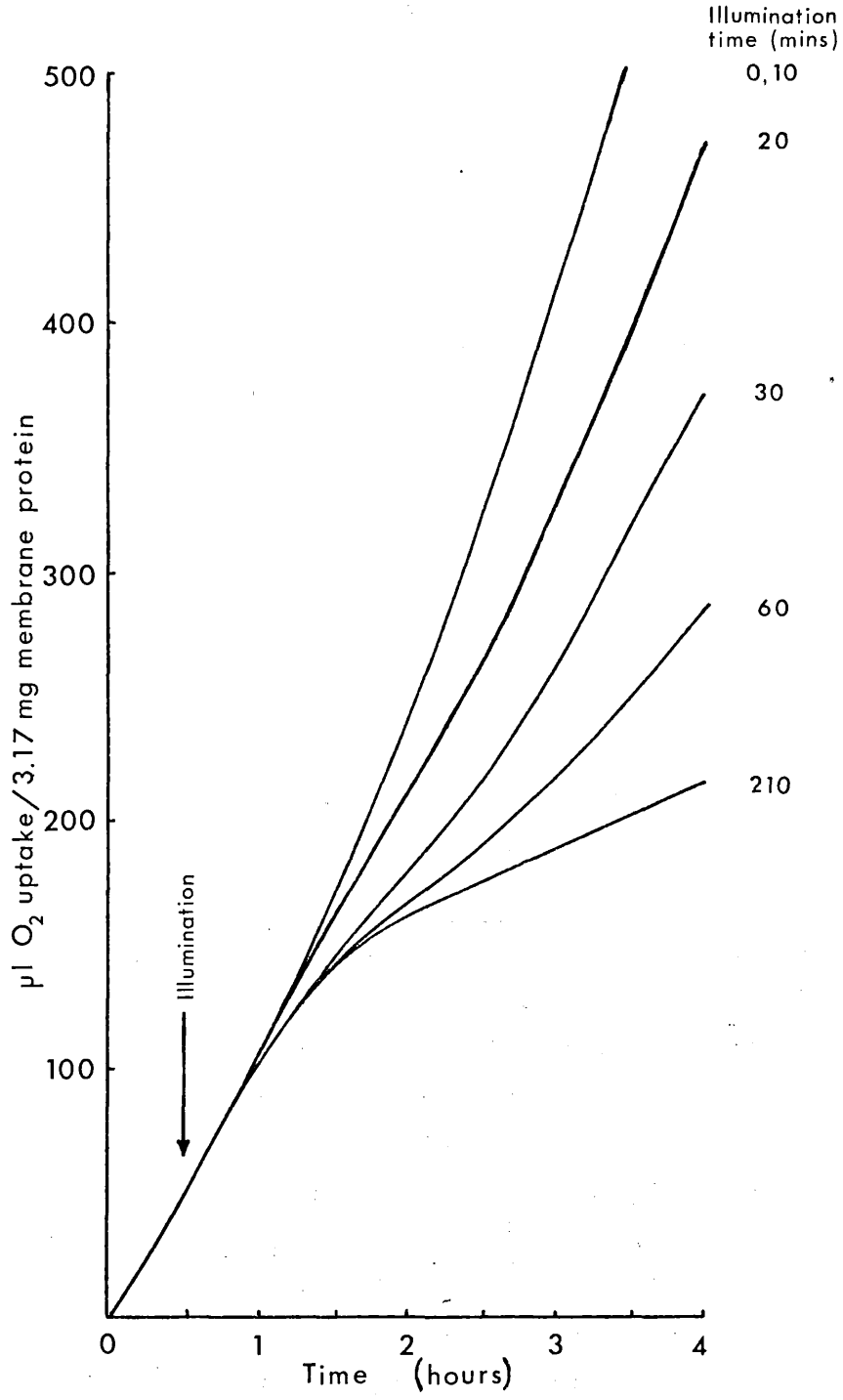


Fig. 12. Effect of various durations of illumination at 660 lumens/sq ft with 10^{-5}M toluidine blue on malate oxidation by membranes of the white strain of S. lutea.

For experimental procedures, illumination and assay of malate oxidase activity see pages 43 and 44.

An electron can enter the respiratory chain through various dehydrogenases which are firmly bound to the whole enzyme complex of the chain. Thus, the first stage in the oxidation of some substrates is not necessarily their dehydrogenation to reduced nicotinamide adenine dinucleotide (NADH); the process can start with the flavin enzymes which transfer an electron directly to the respiratory chain.

Light with toluidine blue caused damage to the protein and membranes of bacteria, which may include damage to the respiratory apparatus which is located in the membranes. It was therefore decided to study the electron transport chain of S. lutea and its mutants, with a view to finding an exact site of damage caused by light with the photosensitizer, toluidine blue, and to investigate the particular role played by the carotenoids in affording protection against photokilling. The flavoprotein enzymes transfer electrons to the cytochromes in bacteria. Therefore the next step in the study of the electron transport chain was to see whether the flavoproteins were affected. Malate could be oxidised anaerobically at the expense of artificial electron acceptors such as methylene blue. The enzyme is malate : methylene blue oxidoreductase. Therefore experiments were designed to see the effect of light with toluidine blue (10^{-5}M) on the malate oxidation and methylene blue reduction by membranes of pigmented and non-pigmented S. lutea.

10. Effect of light with toluidine blue (10^{-5}M) on malate oxidation and methylene blue reduction by pigmented and white membranes.

Six manometric experiments were performed to observe the effect of light with toluidine blue (10^{-5}M) on malate oxidation and methylene blue reduction by pigmented and non-pigmented membranes. The results (see Figs. 13a and 13b) showed that the rate of reduction of methylene blue was depressed by light with toluidine blue in both the pigmented and the non-pigmented strains of S. lutea almost to the same extent, suggesting equal damage to the flavoproteins in both strains. The carotenoid pigments failed to afford

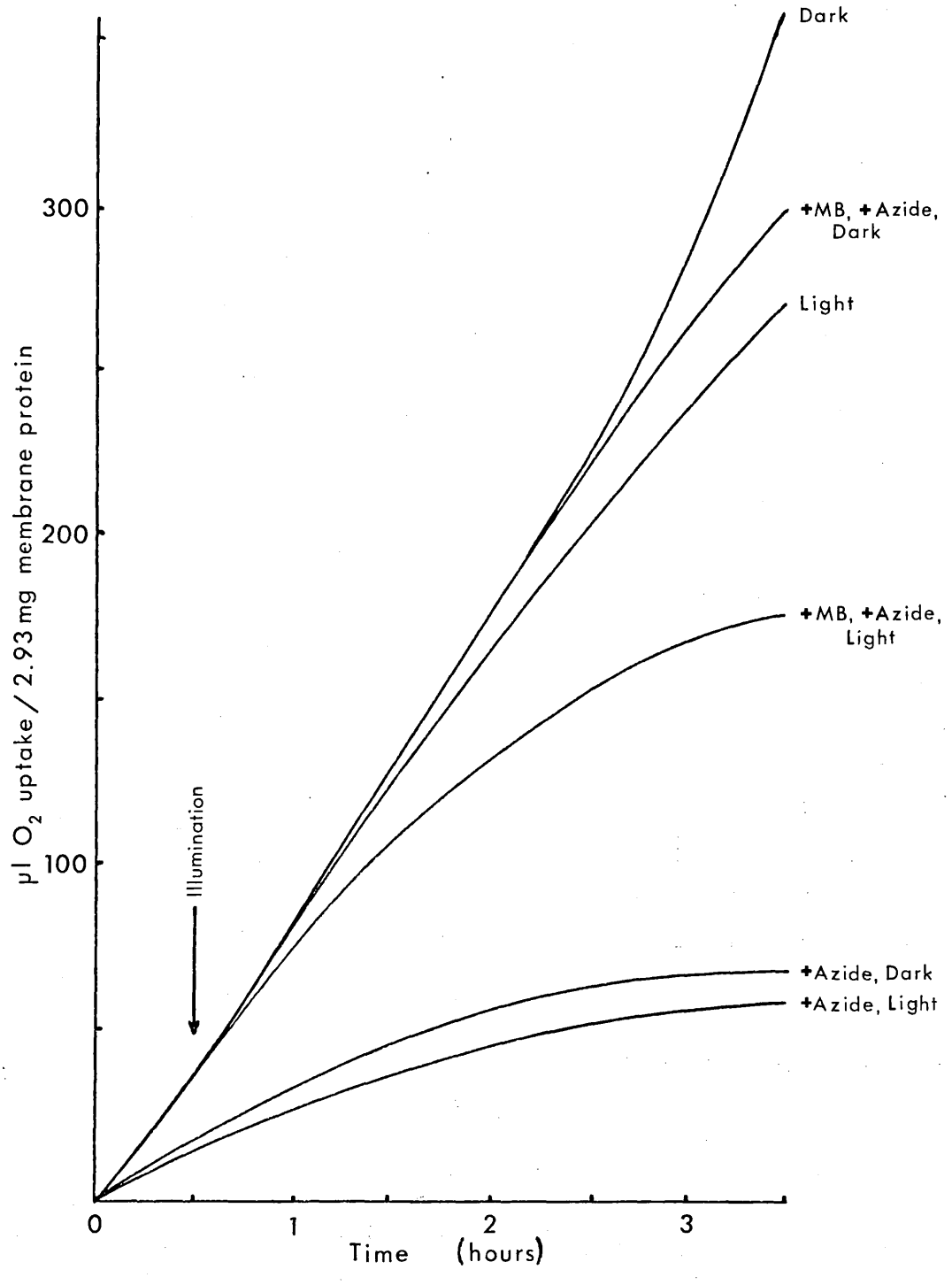


Fig. 13a. Effect of light (650 lumens/sq ft) with 10^{-5}M toluidine blue on malate oxidation and methylene blue (MB) reduction by membranes of the pigmented strain of S. lutea.

For details of experimental procedures, illumination and assays of malate oxidase and malate-methylene blue oxidoreductase activities see pages 43 and 44.

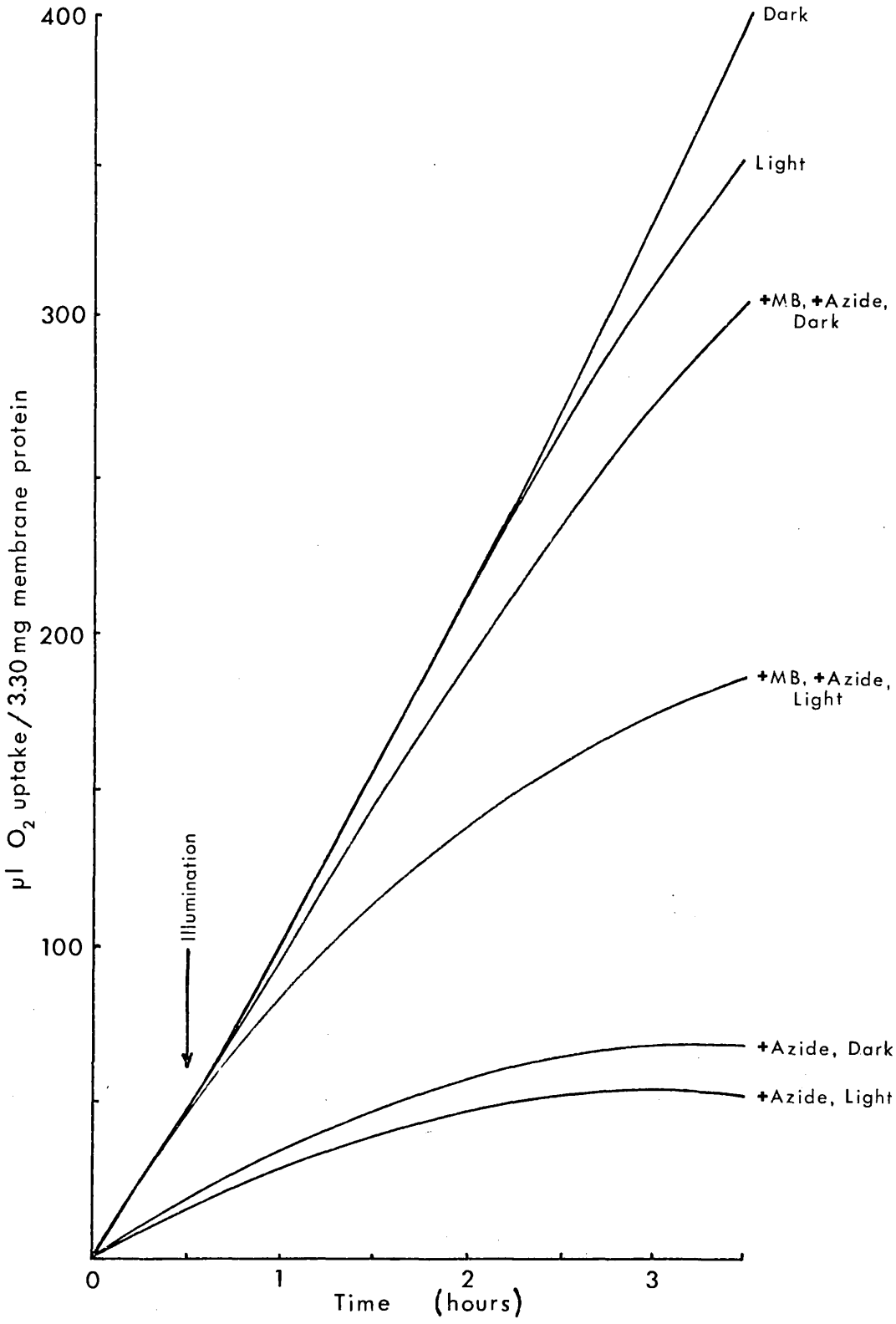


Fig. 13b. Effect of light (650 lumens/sq ft) with 10^{-5}M toluidine blue on malate oxidation and methylene blue (MB) reduction by membranes of the white strain of S. lutea.

For details of experimental procedures, illumination and assays of malate oxidase and malate-methylene blue oxidoreductase activities see pages 43 and 44.

any protection to the pigmented strain. Therefore it was evident that light with toluidine blue caused some damage to the early part of the bacterial electron transport chain - the flavoproteins.

Having found one site of damage by light with toluidine blue at the flavoprotein level, experiments were planned to examine the electron transport chain of pigmented and white mutant of S. lutea beyond the flavoproteins. 2, 6-Dichlorophenol indophenol (DCPIP) probably accepts electrons at the cytochrome c level. Therefore, if there is any damage by light with toluidine blue between the flavoprotein and the cytochrome c, there will be a greater depression of the rate of DCPIP reduction than of methylene blue reduction.

11. Effect of light with toluidine blue (2.5 μM) on malate oxidation, methylene blue reduction, 2, 6-Dichlorophenol indophenol (DCPIP) reduction and cytochrome c oxidation by pigmented and non-pigmented membranes.

Four experiments were performed to compare the effects of light with toluidine blue on malate oxidation, methylene blue reduction and cytochrome c oxidation by membranes of pigmented and the colourless mutant of S. lutea.

The results of one of the experiments (Table 3) showed that the rates of malate oxidation were depressed in both the pigmented and non-pigmented membranes after 60 minutes' illumination. The depression in the non-pigmented membranes was more than in the pigmented membranes. Similarly the rates of reduction of methylene blue and dichlorophenol indophenol were also depressed after illumination, but again the effect was more in the white mutant than in the pigmented membranes. The magnitude of depression of reduction of dichlorophenol indophenol was more than that of methylene blue. This suggests the possibility of another light effect on the electron transport chain beyond the flavoprotein and up to the cytochrome c

Table 3. Effect of light (650 lumens/sq.ft.) and toluidine blue (2.5 μM) on malate oxidation, methylene blue (M.B.) reduction, dichlorophenol indophenol (DCPIP) reduction and cytochrome c oxidation by membranes of pigmented and non-pigmented Sarcina lutea.

Illumination time (mins.)	Pigmented Sarcina lutea				White mutant of Sarcina lutea			
	Malate oxidation rate (% of rate of dark control)	M.B. reduction rate (% of rate of dark control)	DCPIP reduction rate (% of rate of dark control)	Ascorbate - cytochrome c oxidation	Malate oxidation rate (% of rate of dark control)	M.B. reduction rate (% of rate of dark control)	DCPIP reduction rate (% of rate of dark control)	Ascorbate - cytochrome c oxidation
0	100	100	100	100	100	100	100	100
30	87				66			
60	56	47	35	33	38	40	25	19

For details of experimental procedure, illumination, and assay of ascorbate - cytochrome c oxidase, malate oxidase, reduction of methylene blue and reduction of dichlorophenol indophenol, see pages 45 to 50.

level. The oxidation of ascorbate-cytochrome c was also depressed more in the white mutant than in the pigmented membranes. However, the rate of oxidation of ascorbate-cytochrome c was very low (0.00224 µg. atoms O_2 uptake/ml./min./mg. membrane protein). The cytochrome c used in these experiments was a mammalian cytochrome c. It has been shown by several workers (32, 83, 84, 85) that the oxidases of a few bacterial species oxidise soluble mammalian cytochrome c rapidly, but most do not oxidise it at all or do so at a very low rate. The oxidases of S. lutea appear to oxidise mammalian cytochrome c at a very low rate. These results showed inactivation of up to three sites of the respiratory chain in both strains by light with toluidine blue, firstly at the flavoprotein, secondly between the flavoprotein and cytochrome c, and thirdly at the oxidase region. The results using polarographic assay of oxygen uptake showed slight differences as regards the reduction of methylene blue from those shown in the previous experiment using manometric technique. These differences, which could not be observed readily in manometric experiments, were apparently more obvious here due to the greater sensitivity of the polarographic method.

In studies of the respiratory chain, artificial electron donors have long been used. By this means segments of the respiratory chain can be isolated for sequential transfer of electrons. The site of action of the artificial electron donors is often not specific, and therefore results obtained by these means must be interpreted with care. On the findings of the experiments done before, it was thought that some damage could be caused by light with toluidine blue to the cytochromes of the respiratory chain. For studies of the isolated segments of respiratory chain between cytochrome c and oxygen, Jacobs and Sanadi (86) used reduced cytochrome c. With the introduction of N, N, N', N'-tetramethyl-para-phenylene diamine (TMPD) as a mediator of electrons from the ascorbate, an efficient electron donor was

available for the cytochrome c stage (87, 88). It was therefore decided to observe the effect of light with toluidine blue on the ascorbate-TMPD cytochrome oxidase system of the membranes of pigmented and white mutant of S. lutea.

12. Effect of light with toluidine blue (2.5 μM) on the ascorbate-TMPD cytochrome oxidase activities of the membranes of pigmented and white mutant of S. lutea.

Several experiments were performed and the results of two of the experiments are represented by Figs. 14 and 15 . The rate of oxidation of ascorbate-TMPD was depressed by light with 2.5 μM toluidine blue in both the pigmented and the white mutant of S. lutea, though depression in the pigmented membrane was less than in the white mutant membranes. Thus it appears that the carotenoid pigments were able partially to protect the cytochrome oxidase system from photoinactivation. The experiments with TMPD were not carried further because of the high level of autooxidation of ascorbate-TMPD, especially at high concentrations. Lower concentrations gave very little oxidation.

Naphthoquinones and ubiquinones are found in bacterial cells. Bishop et al. (89) investigated the quinones in bacteria and showed that Gram-positive bacteria contained only naphthoquinones, while the Gram-negative bacteria contained both naphthoquinones and ubiquinones (mainly vitamin K_2). The role of ubiquinones and naphthoquinones in the bacterial respiratory chain has been intensely studied. Asano and Brodie (42) have made an extensive study of the electron transport systems in bacterial particles. The bacterial particles oxidised malate, succinate and NADH, and the oxidations of all three substrates were stopped by irradiation with light at 360 mm. By subsequent addition of vitamin K the oxidation of malate and NADH could be restored. The agent responsible for the restoration was regarded as a possible participant in the particular

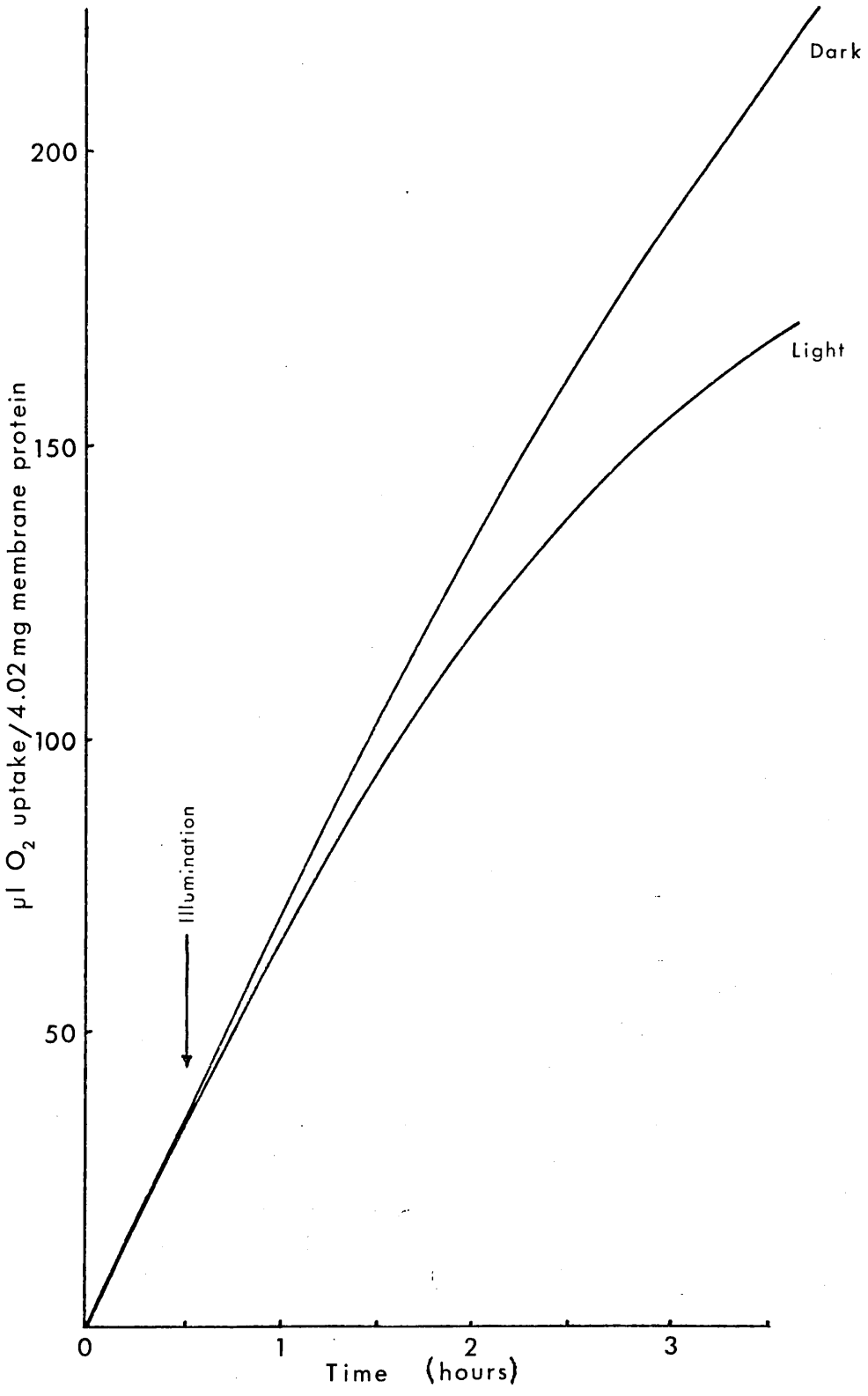


Fig. 14. Effect of light (660 lumens/sq ft) with 2.5µM toluidine blue on the ascorbate-TMPD cytochrome oxidase activity of the membranes of the pigmented strain of S. lutea.

150mM ascorbate with 10^{-4}M tetramethyl-p-phenylene diamine (TMPD) were added to flasks containing 1ml of membrane preparation in 2.5µM toluidine blue. The autooxidation of ascorbate-TMPD of blanks without membranes were subtracted from the experimental results. For details of experimental procedures, illumination and assays see page 44.

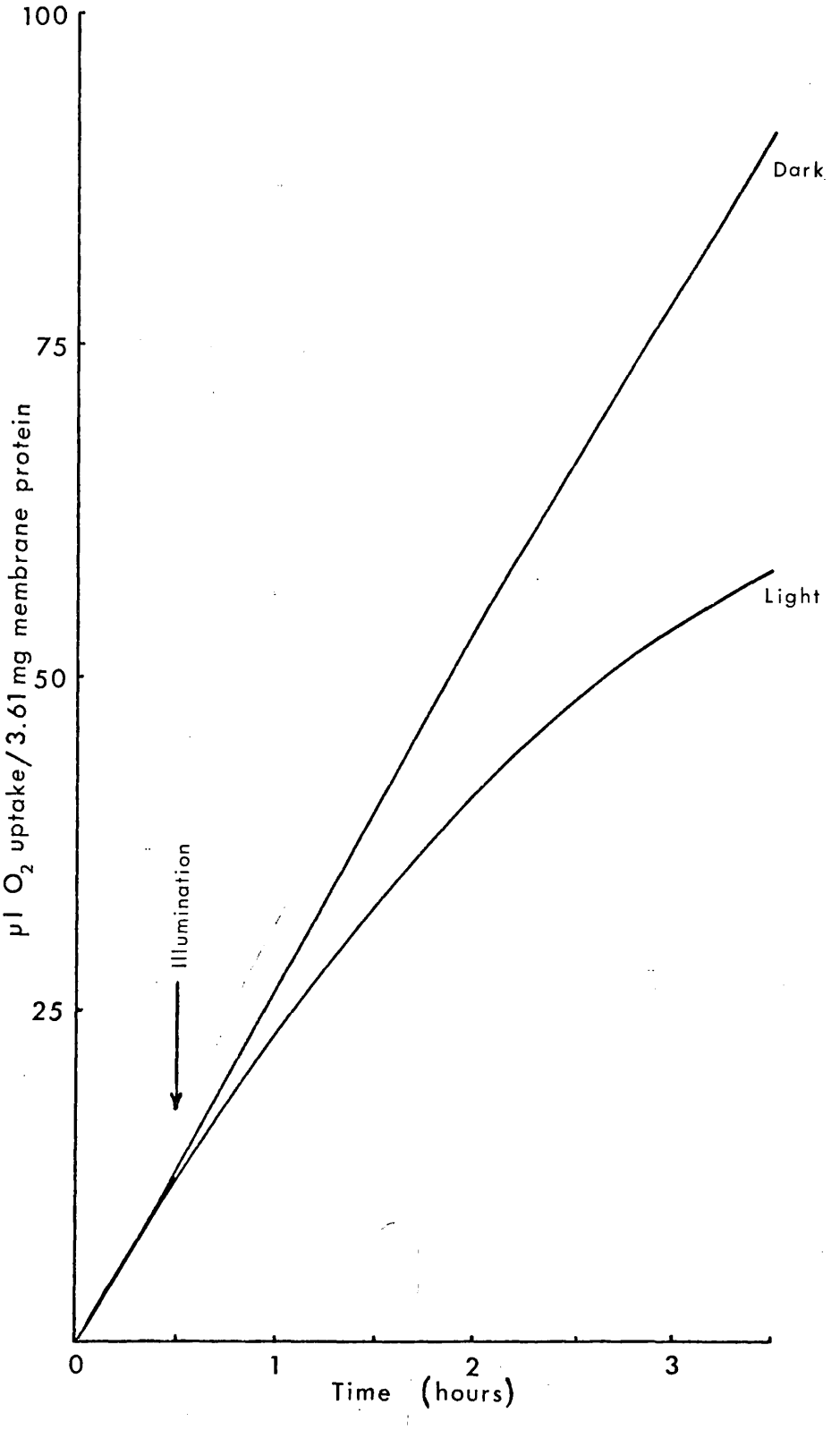


Fig. 15. Effect of light (660 lumens/sq ft) with 2.5µM toluidine blue on the ascorbate-TMPD cytochrome oxidase activity of membranes of the white strain of S. lutea.

150mM ascorbate with 10^{-4}M tetramethyl-p-phenylene diamine (TMPD) were added to flasks containing 1ml of membrane preparation in 2.5µM toluidine blue. The autooxidation of ascorbate-TMPD of blanks without membranes were subtracted from the experimental results. For details of experimental procedures, illumination and assay see page 44.

respiratory chain, especially if the activating quinone was similar to the natural one.

In addition to the firmly bound dehydrogenases which are not dependent on NAD for their activities, bacteria contain weakly bound dehydrogenases such as malate : vitamin K reductase. In the bacterial respiratory chain, vitamin K could be reduced not only by the action of several dehydrogenase components of the chain, but also by the specific enzymes - malate : vitamin K reductase and NADH : vitamin K reductase. These enzymes are bound weakly with the respiratory chain and easily pass into the soluble state. M. phlei has a special malate : vitamin K reductase which introduces an electron from malate into the respiratory chain (42, 90, 91). From their study, Asano and Brodie postulated that the electron transport from malate dehydrogenase and NADH dehydrogenase was effected through vitamin K to the cytochromes. It was therefore decided to see whether the malate : vitamin K reductase activity was destroyed by irradiation in the presence of toluidine blue.

13. Effect of light with 2.5 μM-toluidine blue on the malate : vitamin K reductase activity of membranes of the pigmented and white mutant of S. lutea.

One of four experiments performed is represented by Tables 4 and 5. Prolonged illumination for 90 minutes by light (775 lumens/sq.ft.) with 2.5 μM-toluidine blue of membranes of pigmented and white strains caused depressed malate : vitamin K reductase activities in both strains, although the extent of damage to the pigmented strain was less than in the carotenoidless mutant strain. Reduction of DCPIP was similarly affected.

Results of another experiment where illumination with toluidine blue was carried on for 120 minutes (see Table 6) also showed similar results to the above.

It cannot necessarily be asserted that this irradiation caused damage to the vitamin K in the presence of toluidine blue. Damage to the flavoproteins could effect a failure to reduce

Table 4. Effect of light (775 lumens/sq.ft.) with toluidine blue (2.5 μM) on malate oxidase, malate:Vitamin K reductase, and DCPIP reduction in the membranes of the white mutant of Sarcina lutea.

Illumination time (mins.)	μg. atoms O_2 uptake/ml./min./g. membrane protein: Malate oxidase activity		Malate:Vitamin K reductase activity		μ moles DCPIP reduced/ml./min./g. membrane protein	
	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample
0	21	-	87	-	27	-
20		13		62		23
50		6		53		14
90	25	3	102	34	31	7

The membranes with 2.5 μM toluidine blue were incubated at 8° C. Additions of 10 mM - Menadione (Vitamine K_3), 5 x 10^{-3} M-sodium azide, 150 mM - sodium hydrogen malate and 0.00053 M-DCPIP were made before assay.

For details of the experimental procedure, illumination, assay of malate oxidase and malate: vitamin K reductase and reduction of DCPIP see pages 45 to 50.

Table 5. Effect of light (775 lumens/sq.ft.) with toluidine blue (2.5 μM) on malate oxidase, malate: vitamin K reductase, and DCPIP reduction by membranes of the pigmented strain of Sarcina lutea.

Illumination time (mins.)	μg. atoms O_2 uptake/ml./min./g. membrane protein				μ moles DCPIP reduced/ml./min./g. membrane protein	
	Malate oxidase activity		Malate: vitamin K reductase activity			
	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample
0	28	-	81	-	26	-
20		21		69		20
50		20		78		20
90	22	8	70	29	27	16

The membranes with 2.5 μM toluidine blue were illuminated at 8° C. Before assay, additions of 10 mM - menadione (Vitamin K_3), 5 x 10^{-3} M - NaN_3, 150 mM - Na-H-Malate and 0.00053 M-DCPIP were made. For details of illumination, experimental procedure, assay of malate oxidase and malate: vitamin K reductase, and reduction of DCPIP see pages 45 to 50.

Table 6. Effect of illumination (775 lumens/sq.ft.) for 120 minutes with 2.5 μM toluidine blue on the malate : vitamin K reductase activity of membranes of the pigmented and white strain of S. lutea.

Illumination time (mins.)	Malate : vitamin K reductase activity μg.atoms O_2 uptake /ml./min./mg. membrane protein			
	Pigmented strain		White mutant	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.252	-	0.396	-
15		0.252		0.442
30		0.255		0.366
45		0.255		0.305
60		0.239		0.331
75		0.255		0.305
90		0.231		0.290
105		0.191		0.183
120	0.279	0.207	0.473	0.183

The membranes with 2.5 μM toluidine blue were illuminated at 8°C. Prior to assay, additions of 10 mM menadione (vitamin K_3), 5×10^{-3}M-NaN_3, and 150 mM sodium hydrogen malate were made. For details of illumination, experimental procedure and assay of malate : vitamin K reductase activity, see pages 45 to 49.

vitamin K, which in turn reflected the depressed malate : vitamin K reductase activity in both the pigmented and non-pigmented membranes. Thus a possible second site of inactivation of the membrane enzymes due to illumination with toluidine blue could not be suggested.

14. Effect of illumination for 90 minutes with 2.5 µM toluidine blue on the malate dehydrogenase and malate : vitamin K reductase activities of the membranes of pigmented and the white mutant of S. lutea.

Prolonged illumination in the presence of toluidine blue caused damage to the malate : vitamin K reductase activities in both the pigmented and the non-pigmented membranes of S. lutea. Initially, malate dehydrogenase passes electrons to vitamin K reductase, which in turn reduces the cytochromes. Therefore the effect of prolonged illumination with toluidine blue on the malate dehydrogenase and the malate : vitamin K reductase activities of the membranes of pigmented and non-pigmented S. lutea were compared.

Four experiments were performed and the results of one of the experiments are shown in Tables 7 and 8. The malate dehydrogenase activities, assayed by reduction of phenazine methosulphate (PMS), and malate : vitamin K reductase activities, were depressed to the same extent due to prolonged irradiation in the presence of toluidine blue in both the pigmented and the white mutant of S. lutea.

Thus the results of these experiments suggest only one place in the vitamin K reductase system which is affected by illumination with toluidine blue, namely the flavoprotein.

15. Summary

i. Illumination of whole cells of pigmented and non-pigmented

Table 7. Effect of illumination for 90 minutes (775 lumens/sq.ft.) with 2.5 µM toluidine blue on malate dehydrogenase and malate : vitamin K reductase activities of the membranes of pigmented S. lutea.

Illumination time (mins.)	µg.atoms O_2 uptake /ml./min./mg. membrane protein			
	Malate dehydrogenase activity		Malate : vitamin K reductase activity	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.151	-	0.454	-
15		0.108		0.432
30		0.097		0.389
45		0.080		0.303
60		0.065		0.243
75		0.043		0.173
90	0.151	0.038	0.407	0.167

The membranes with 2.5 µM toluidine blue were illuminated at 8°C. 0.009M-phenazine methosulphate (PMS), 5×10^{-3}M-NaN_3, 150 mM-malate and 10 mM menadione (vitamin K_3) were added prior to the assay of enzyme activities. For details of illumination, experimental procedure, and assay of malate dehydrogenase and malate : vitamin K reductase activities see pages 45 to 49.

Table 8. Effect of illumination for 90 minutes (775 lumens/sq.ft.) with 2.5 μM toluidine blue on malate dehydrogenase and malate : vitamin K reductase activities of the membranes of white mutant of S. lutea.

Illumination time (mins.)	μg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Malate dehydrogenase activity		Malate : vitamin K reductase activity	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.233	-	0.840	-
15		0.151		0.575
30		0.124		0.497
45		0.073		0.311
60		0.039		0.249
75		0.039		0.137
90	0.259	0.031	0.622	0.109

The membranes with 2.5 μM toluidine blue were illuminated at 8°C. 0.000M-PMS, 5×10^{-3}M-NaN_3, 150 mM malate and 10 mM menadione (vitamin K_3) were added prior to the assay of enzyme activities. Details of experimental procedures, illumination, and assay of malate dehydrogenase and malate : vitamin K reductase activities appear on pages 45 to 49.

Sarcina lutea at low light intensities in the presence of an exogenous photosensitizer, toluidine blue, showed decreased viability in the carotenoidless white mutant, while the pigmented strain escaped photodynamic killing.

ii. When the cell membranes were irradiated at low light intensities in the presence of a photosensitizer, toluidine blue, there was widespread inactivation of the enzyme activities of the membrane-bound respiratory chain. The malate oxidase activities of cell suspensions and membranes of both the pigmented and non-pigmented strains were depressed. The depression was more in the carotenoidless mutant than in the pigmented strain.

iii. The succinoxidase, lactate oxidase and ascorbate oxidase activities were also affected in the same manner as above.

iv. In the study of the electron transport chain of isolated membranes, it was found that visible light with toluidine blue caused damage to the early part of the electron transport chain - the flavoproteins. Both methylene blue reduction and vitamin K reduction showed a depressed rate in both strains, which was more in the carotenoidless mutant than in the wild type.

v. The rate of reduction of dichlorophenol indophenol was also depressed by light with toluidine blue but the inactivation appeared to be greater.

vi. Cytochrome c oxidase and ascorbate-TMPD cytochrome oxidase activities were also similarly affected, suggesting a further site at the cytochrome c-cytochrome oxidase level which is affected by visible light.

vii. Illumination of shorter duration (10 minutes) with toluidine blue, followed by dark incubation, in the white mutant membranes caused no damage to the malate oxidase activity as compared with the dark control. 20 minutes' illumination caused slight damage which recovered almost completely after dark incubation. Illumination of more than 60 minutes produced permanent damage, rendering the oxidase activity completely irreversible.

CHAPTER 4

Experiments without an exogenous photosensitizer

1. Introduction

Results of the experiments described so far suggested several places in the electron transport chain of S. lutea which were affected by illumination in the presence of the exogenous photosensitizer, toluidine blue. The first inactivation of the respiratory chain was at the flavoprotein level, the second was between the flavoprotein and cytochrome c, and the third in the cytochrome oxidase region. In these experiments it was evident that the damage by light to the enzyme activities was complicated by the presence of toluidine blue. Toluidine blue at any of the concentrations used did not affect the oxidation rate unless it was subjected to illumination. It is not known with absolute certainty the precise mode of action of toluidine blue, but it was evident from the results so far obtained that toluidine blue caused widespread damage to a variety of enzymes, possibly by attacking the histidine residues of the proteins or some other amino acid residues in the membrane. Besides, there are pigments in the membranes such as flavins, cytochromes etc., which could also act as endogenous photosensitizers in the presence of visible light, and could produce photooxidative damage. It was, therefore, decided to omit toluidine blue from further experiments, and to use a much higher light intensity of the order of 1000 to 1500 lumens/sq.ft. All experiments involving the assay of O_2 uptake described in this chapter were performed with an oxygen electrode.

2. Effect of high light intensity on the malate oxidase activity and the activities of segments of the respiratory chain in pigmented and non-pigmented membranes of S. lutea.

Four experiments were performed to examine the effect of high light intensity, without the photosensitizer toluidine blue, on the malate oxidase activities of membranes of the pigmented and non-pigmented strains of S. lutea. Results of one of the experiments are represented by Table 9.

Illumination for 60 minutes at higher light intensities without toluidine blue caused damage to the malate oxidase system (evidenced by depressed oxidation rate of malate) in both the pigmented and the white mutant of S. lutea. The depression was more in the white mutant than in the pigmented strain. This suggested that, in the absence of the exogenous photosensitizer toluidine blue, some photooxidation took place due to the presence of endogenous photosensitizers which acted at higher light intensities. The carotenoid pigments offered some protection against photo-inactivation in the pigmented membranes which was lacking in the carotenoidless membranes.

Having found that the malate oxidase activity could be damaged by illumination at high light intensities in the absence of an exogenous photosensitizer, toluidine blue, it was decided to examine which segments of the electron transport system of S. lutea could be damaged at higher light intensities without any exogenous photo-sensitizer. Comparative studies were also made to observe whether the effect of illumination on the succinoxidase system differed from that on malate oxidase.

Four experiments were performed to compare the effect of high light intensities (1240 lumens/sq.ft.) without toluidine blue on the malate : vitamin K reductase activity, succinate : vitamin K reductase activity, and malate and succinate oxidase activities of membranes of the white mutant of S. lutea. Table 10 represents the results of one of the experiments.

The malate : vitamin K reductase and succinate : vitamin K

Table 9. Effect of high light intensity (1240 lumens/sq.ft.) without toluidine blue on the malate oxidase activity of the membranes of pigmented and white strains of S. lutea.

Illumination time (mins.)	Malate oxidase activity μg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Pigmented strain		White mutant	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.080	-	0.076	-
15		0.067		0.046
30		0.047		0.031
45		0.033		0.023
60	0.073	0.023	0.075	0.021

The membranes were illuminated at 8°C and, prior to assay of the malate oxidase activity, 150 mM malate was added to the membranes. For experimental details, illumination, and malate oxidase assay see pages 45 to 48.

Table 10. Effect of high light intensity (1240 lumens/sq.ft.) without toluidine blue on the malate : vitamin K reductase and succinate : vitamin K reductase activities, and malate and succinate oxidase activities of membranes of the white mutant of S. lutea.

Illumination time (mins.)	µg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Malate : vitamin K reductase activity		Succinate : vitamin K reductase activity	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.222	-	0.089	-
15		0.162		0.087
30		0.162		0.085
45		0.162		0.078
60		0.175		0.070
90	0.203		0.089	

	µg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Malate oxidase activity		Succinate oxidase activity	
	Dark control	Illuminated sample	Dark control	Illuminated sample
70	0.063	0.013	0.057	0.012

Membranes were illuminated at 8°C and additions of 10 mM menadione (vitamin K_3), 5 x 10^{-3}M-NaN_3, 150 mM-malate and 150 mM-succinate were made prior to assay. For details of experimental procedure, illumination and assay of malate and succinate : vitamin K reduction activities, malate and succinate oxidase activities, see pages 45 to 49.

reductase activities were depressed by illumination, and the malate and succinate oxidase activities were also depressed. The malate oxidase system seemed to be more active than the succinate oxidase system. These results were similar to those obtained in the presence of an exogenous photosensitizer, toluidine blue. Therefore, in the absence of any exogenous photosensitizer, endogenous pigments appeared to act as photosensitizers in the membranes at higher light intensities.

The effects of high light intensities (1240 lumens/sq.ft.) on DCPIP reduction by the membranes of pigmented and white S. lutea are shown in Table 11. A depressed rate of reduction of DCPIP caused by prolonged illumination of up to 120 minutes was observed in both the pigmented and non-pigmented strains, and the extent of damage to both seemed to be equal in most experiments. These results suggest some damage to the malate-cytochrome c reductase activity of the respiratory chain.

It appears, from the results of the experiments done so far without any exogenous photosensitizer, that various partial reactions of the electron transport chain are damaged by high light intensities. It is, however, not known what causes this damage, nor whether, as in the toluidine blue system (see page 71), reversible changes precede irreversible changes brought about by illumination at high light intensities. Therefore, it was decided to perform some experiments at high light intensities without any photosensitizer for a variable duration of time, to test for the reversibility of various enzyme activities.

3. Effect of a short exposure to illumination at high light intensity on the malate oxidase and succinate oxidase activities, and activities of segments of the respiratory chain, in pigmented and non-pigmented membranes of S. lutea.

Four experiments were performed to see the effect of short

Table 11. Effect of high light intensity (1240 lumens/sq.ft.) without toluidine blue on DCPIP reduction by the membranes of pigmented and white strains of S. lutea.

Illumination time (mins.)	μ moles DCPIP reduced/ml./min./mg.membrane protein			
	Pigmented strain		White mutant	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.067	-	0.054	-
15		.060		0.043
30		.055		0.046
45		.047		0.037
60		.047		0.036
75		.047		0.037
90		.048		0.037
105		.037		0.033
120	0.065	.033	0.057	0.026

The membranes were illuminated at 8°C, and 0.00053M-DCPIP, 5×10^{-3}M NaN_3 and 150 mM-malate were added prior to assay. For details of experimental procedures, illumination and assays of enzyme activity see page 50.

exposure to illumination at high light intensity (950 lumens/sq.ft.) on malate oxidase activity and on DCPIP reduction by the membranes of pigmented S. lutea. The results of one experiment, represented by Table 12, showed that exposure to illumination for 5 minutes caused slight depression of the enzyme activities, which reversed completely during the following 45 minutes' incubation in the dark at 8°. There is no apparent difference between the effects on oxidase activity and on DCPIP reduction.

When these experiments were repeated using non-pigmented membranes (see Table 13), similar results were obtained. However, the reversibility of malate oxidase activity and the reduction of DCPIP were not complete even after one hour of dark incubation.

Four experiments were performed to compare the effects of short exposures (5 minutes) to illumination at high light intensities on malate oxidase activity and DCPIP reduction by membranes of pigmented S. lutea. The results of one experiment are shown in Table 14.

The results showed that exposures to illumination for 5 minutes caused depressed malate oxidase activity, which reversed completely during the following 75 minutes' incubation in the dark at 8°. The malate : DCPIP and succinate : DCPIP reductions also showed similar effects, and there was no apparent difference between the oxidase activities and the DCPIP reduction activities. The recovery time was slightly longer than in the previous experiment because of the higher light intensity used, which caused slightly more damage to the system.

The effects of 5 minutes' illumination on the malate and succinate oxidase activities and on DCPIP reduction by the membranes of non-pigmented S. lutea are shown in Table 15. The results showed similar depressions in all the enzyme activities assayed due

Table 12. Effect of 5 mins. exposure to illumination at high intensity (950 lumens/sq. ft.) on the malate oxidase activity and DCPIP reduction by membranes of pigmented S. lutea.

Time (mins.)	Malate oxidase activity		DCPIP reduction	
	µg.atoms O_2 uptake/ml./min./mg. membrane protein		µ moles DCPIP reduced/ml./min./mg. membrane protein	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.106		0.070	
5		0.099		0.062
15		0.101		0.062
30		0.101		0.067
45		0.106		0.070
60		0.106		0.070
75		0.106		0.068
90	0.106	0.106	0.067	0.067

The membranes were irradiated at 8°C by light (950 lumens/sq. ft.) for 5 minutes and then kept in the dark away from the light source by covering with blackened aluminium foil. Additions of 150 mM-malate, 0.00053M-DCPIP and 5 x 10^{-3}M-NaN_3, were made prior to assay. For further details of experimental procedure, illumination and assays of malate oxidase and DCPIP reduction see pages 45 to 50.

Table 13. Effect of 5 minutes' exposure to illumination at high intensity (1240 lumens/sq. ft.) on the malate oxidase activity and DCPIP reduction by membranes of the white mutant of S. lutea.

Time (mins.)	Malate oxidase activity		DCPIP reduction	
	µg.atoms O_2 uptake/ml./min./mg. membrane protein		µ moles DCPIP reduced/ml./min./mg. membrane protein	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.117		0.073	
5		0.102		0.069
15		0.097		0.076
30		0.097		0.076
45		0.117		0.073
60	0.123	0.117	0.080	0.073

The membranes were irradiated at 8°C by light (1240 lumens/sq.ft.) for 5 minutes and then kept away from light source covered by blackened aluminium foil. Additions of 150 mM-malate, 0.00053M-DCPIP and 5 x 10^{-3}M NaN_3 were made prior to assay. For details of experimental procedures, illumination and assay of malate oxidase and DCPIP reduction, see pages 45 to 50.

Table 14. Effect of 5 minutes' illumination at high light intensity (1240 lumens/sq.ft.) on malate oxidase activity and DCPIP reduction by membranes of pigmented S. lutea.

Time (mins)	Malate oxidase activity		Malate : DCPIP reduction	
	µg.atoms O_2 uptake/ml./min./mg. membrane protein		µ moles DCPIP reduced/ml./min./mg. membrane protein	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.186		0.143	
5		0.179		0.127
15		0.177		0.143
30		0.173		0.143
45		0.176		0.139
60		0.177		0.139
75		0.187		0.167
90	0.184	0.184	0.167	0.167

Time (mins.)	Succinate : DCPIP reduction	
	µ moles DCPIP reduced/ml./min./mg. membrane protein	
	Dark control	Irradiated sample
0	0.063	
5		0.056
15		0.057
30		0.059
45		0.063
60		0.060
75		0.060
90	0.063	0.063

The membranes were irradiated at 8°C by light (1240 lumens/sq.ft) for 5 minutes and then kept away from light source covered by blackened aluminium foil. Additions of 150mM malate and 150mM-succinate, 0.00053M-DCPIP and 5 x 10^{-3}M-NaN_3, were made prior to assay. For details of experimental procedures, illumination and assays of enzyme activities see pages 45 to 50.

Table 15. Effect of 5 minutes' illumination at high light intensity (1240 lumens/sq.ft.) on malate and succinate oxidase activities, and DCPIP reduction, by membranes of the white mutant of S. lutea.

Time (mins.)	Malate oxidase activity μg.atoms O_2 uptake/ml./min./mg. membrane protein		Malate : DCPIP reduction μ moles DCPIP reduced/ml./min./mg. membrane protein	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.176		0.107	
5		0.167		0.095
15		0.157		0.095
30		0.183		0.097
45		0.173		0.105
60		0.173		0.107
90	0.220	0.183	0.126	0.106

Time (mins.)	Succinate oxidase activity μg.atoms O_2 uptake/ml./min./mg. membrane protein		Succinate : DCPIP reduction μ moles DCPIP reduced/ml./min./mg. membrane protein	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.057		0.052	
5		0.052		0.047
15				0.052
30				0.044
45				0.044
60				0.044
90	0.070	0.053	0.052	0.042

The membranes were irradiated at 8°C by light (1240 lumens/sq.ft.) for 5 minutes and then kept away from light source covered by blackened aluminium foil. Additions of 150mM-malate and 150mM-succinate, 0.0005M-DCPIP and 5×10^{-3}M NaN_3 were made prior to assay. For details of experimental procedures, illumination and assays of malate and succinate oxidase activities and reduction of DCPIP see pages 45 to 50.

to illumination for 5 minutes. However, the reversibility of malate and succinate oxidase activities, and of DCPIP reduction, were not complete even after 90 minutes of dark incubation.

When the succinoxidase and malate oxidase activities were compared using pigmented membranes of S. lutea after illuminating at high light intensities for 5 minutes, there were depressed oxidase activities with both malate and succinate (see Table 16), which reversed completely after dark incubation.

The effects of short exposure to illumination at high light intensities on the malate oxidase and malate : vitamin K reductase activities by the membranes of pigmented S. lutea are shown in Table 17. Both the malate oxidase and malate : vitamin K reductase activities were depressed by illumination of short duration, and the activities were completely restored during the following 75 minutes' incubation in the dark at 8°.

The results of all experiments done so far without any exogenous photosensitizer showed that short illumination for 5 minutes at high light intensities caused some damage to several enzyme activities, such as malate oxidase, succinate oxidase, DCPIP reduction and malate : vitamin K reductase activity, in pigmented and non-pigmented S. lutea. The inactivation of enzyme activities, which was caused by illumination of the pigmented membranes, was slight and all reversed completely when incubated in the dark for periods which varied according to the intensities of light. The higher the light intensity, the longer was the time required to achieve complete restoration of activities. In the case of the carotenoidless mutants, the depressions in enzyme activities were slightly more in comparison with the pigmented strain, and a complete restoration of enzyme activities could not be achieved. These results suggested that the onset of the damaging effect was fairly rapid, but permanent inactivation was not achieved during

Table 16. Effect of 5 minutes' illumination at high light intensity (1240 lumens/sq.ft.) on malate oxidase and succinate oxidase activities of membranes of pigmented S. lutea.

Time (mins.)	Malate oxidase activity μg.atoms O_2 uptake/ml./min./mg. membrane protein		Succinate oxidase activity μg.atoms O_2 uptake/ml./min./mg. membrane protein	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.167		0.063	
5		0.152		0.061
15				
25		0.152		
30		0.152		
45		0.157		
60		0.157		
75		0.157		
90				0.064
105		0.154		
135		0.162		
150	0.162	0.162		
165			0.066	0.066

The membranes were irradiated at 8°C by light (1240 lumens/sq.ft.) for 5 minutes and then kept away from the light source, covered by blackened aluminium foil. Additions of 150mM malate and 150mM succinate were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities, see pages 45 to 48.

Table 17. Effect of 5 minutes' illumination at high light intensities (1240 lumens/sq.ft.) on malate oxidase and malate : vitamin K reductase activities by the membranes of pigmented S. lutea.

Time (mins)	µg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Malate oxidase activity		Malate : vitamin K reductase activity	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.211		0.341	
5		0.196		0.320
15		0.196		0.320
30		0.206		0.320
45		0.206		0.330
60		0.209		0.336
75		0.211		0.341
90	0.211	0.211	0.341	0.341

The membranes were irradiated at 8°C by light (1240 lumens/sq.ft.) for 5 minutes, after which they were kept in the dark away from the light source by covering with blackened aluminium foil. Additions of 150mM malate, 10mM menadione (vitamin K_3) and 5×10^{-3}M NaN_3 were made prior to enzyme assays. For details of experimental procedures, illumination and assay of enzyme activities, see pages 45 to 49.

that short period. Therefore further experiments were performed to see the effects of a longer duration of illumination at high light intensities.

4. Effect of 15 minutes' illumination at high light intensities on the malate and succinate oxidase activities and the activities of segments of the respiratory chain in pigmented and non-pigmented membranes of S. lutea.

Four experiments were performed to see the effect of 15 minutes' illumination at high light intensity (1240 lumens/sq.ft.) on the malate and succinate oxidase activities and on DCPIP reduction by the pigmented membranes of S. lutea. Table 18 represents the results of one experiment.

The results showed depressed malate and succinate oxidase activities, but these did not recover during dark incubation for the following 90 minutes. The succinoxidase activity seemed to be less affected than the malate oxidase activity. The DCPIP reduction was also depressed by illumination, but the extent of inactivation seemed to be less than in the malate and succinate oxidase systems. There was complete restoration of DCPIP reduction activity during the following 90 minutes of incubation in the dark at 3°. This suggested the possibility of some irreversible damage to the cytochrome oxidase system if it was assumed that DCPIP received its electrons from cytochrome c.

When these experiments were repeated using non-pigmented membranes (see Table 19), similar results were obtained. The magnitude of inactivation was more in the non-pigmented membranes than in the pigmented membranes, and the malate oxidase system was more affected than the succinate oxidase system. Complete restoration of activities was not achieved in any case, and damage to the DCPIP-reducing system seemed to be less than to the malate and succinate oxidase systems.

Table 18. Effect of 15 minutes' illumination at high light intensity (1240 lumens/sq.ft.) on malate and succinate oxidase activities, and DCPIP reduction, by membranes of pigmented S. lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Malate oxidase activity		Succinate oxidase activity	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.161		0.047	
15		0.130		0.045
30		0.111		0.039
45		0.136		0.022
60		0.124		0.022
75		0.124		0.045
90	0.143	0.124	0.052	0.045

Time (mins.)	µ moles DCPIP reduced/ml./min./mg. membrane protein			
	Malate : DCPIP reduction		Succinate : DCPIP reduction	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.134		0.059	
15		0.121		0.052
30		0.125		0.056
45		0.121		0.056
60		0.143		0.059
75		0.144		0.063
90	0.133	0.133	0.063	0.063

The membranes were irradiated at 8°C by light (1240 lumens/sq.ft.) for 15 minutes, after which they were kept in the dark away from the light source by covering with blackened aluminium foil. Additions of 150mM malate, 150mM succinate, 0.00053M-DCPIP and 5×10^{-3}M-NaN_3 were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 50.

Table 19. Effect of 15 minutes' illumination at high light intensity (1240 lumens/sq.ft.) on malate and succinate oxidase activities, and DCPIP reduction, by membranes of the white mutant of S. lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Malate oxidase activity		Succinate oxidase activity	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.118		0.051	
15		0.073		0.047
30		0.063		0.046
45		0.071		0.047
60		0.055		0.051
75		0.063		0.051
90	0.113	0.063	0.055	0.050

Time (mins.)	µ moles DCPIP reduced/ml./min./mg. membrane protein			
	Malate : DCPIP reduction		Succinate : DCPIP reduction	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.227		0.087	
15		0.174		0.079
30		0.143		0.087
45		0.135		0.090
60		0.195		0.090
75		0.233		0.090
90	0.264	0.195	0.100	0.090

The membranes were irradiated at 8°C by light (1240 lumens/sq.ft.) for 15 minutes, after which they were kept in the dark away from the light source by covering with blackened aluminium foil. Additions of 150mM-malate, 150mM-succinate, 0.00053M-DCPIP and 5×10^{-3}M-NaN_3 were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 50.

The effects of 15 minutes' illumination at 1240 lumens/sq.ft. on the malate oxidase and malate : vitamin K reductase activities of pigmented membranes are shown in Table 20.

The malate oxidase activity was depressed by illumination, and complete restoration of activity did not occur even after 105 minutes' incubation in the dark following illumination. There was also damage to the vitamin K reductase activity, but complete restoration of activity was achieved during the following 90 minutes' incubation in the dark at 8^{0}.

Table 21 represents one of four experiments performed to observe the effect of 15 minutes' illumination by light (1240 lumens/sq.ft.) on the succinate oxidase and succinate : vitamin K reductase activities by pigmented membranes.

The succinoxidase activity and the succinate : vitamin K reductase activity were both depressed by illumination, and complete restoration of succinate : vitamin K reductase activity was achieved following 90 minutes' incubation in the dark at 8^{0}. However, the succinoxidase activity did not reverse completely.

Similar experiments were performed to compare the malate oxidase, malate : vitamin K reductase and succinate : vitamin K reductase activities in the non-pigmented membranes of S. lutea. The results showed (see Table 22) depressed malate oxidase activity, which did not show complete restoration after 90 minutes' incubation in the dark following illumination. However, the malate : vitamin K reductase and succinate : vitamin K reductase activities were also depressed, but complete restoration of the activities was achieved after 90 minutes' incubation in the dark following illumination.

Therefore, the results of experiments done so far to find the effects of short illumination of 5 minutes showed that the malate oxidase activity of the pigmented membranes of S. lutea was depressed,

Table 30. Effect of 15 minutes' illumination at high light intensity (1240 lumens/sq.ft.) on the malate oxidase and malate : vitamin K reductase activities of the membranes of pigmented S. lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Malate oxidase activity		Malate : vitamin K reductase activity	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.054		0.032	
15		0.029		0.061
30		0.032		0.056
45		0.026		0.051
60		0.032		0.051
75		0.034		0.051
90		0.034		0.056
105	0.051	0.034	0.056	0.056

The membranes were irradiated at 8°C by light (1240 lumens/sq.ft.) for 15 minutes, after which they were kept away from the light source covered by blackened aluminium foil. Additions of 150mM-malate, 10mM-menadione (vitamin K_3) and 5 x 10^{-3}M-NaN_3 were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 49.

Table 21. Effect of 15 minutes' illumination at high light intensity (1240 lumens/sq.ft.) on succinate oxidase and succinate : vitamin K reductase activities of the membranes of pigmented S. lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Succinate oxidase activity		Succinate : vitamin K reductase activity	
	Dark Control	Illuminated sample	Dark control	Illuminated sample
0	0.015		0.034	
15		0.013		0.029
30		0.013		0.024
45		0.013		0.024
60		0.014		0.029
75		0.014		0.029
90	0.014	0.014	0.031	0.031

The membranes were irradiated at 3°C by light (1240 lumens/sq.ft.) for 15 minutes, after which they were kept away from the light source covered by blackened aluminium foil. Additions of 150mM-succinate, 10mM-menadione and 5 x 10^{-3}M-NaN_3 were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 49.

Table 22. Effect of 15 minutes illumination at high light intensity (1240 lumens/sq.ft.) on malate oxidase, malate: vitamin K reductase, and succinate: vitamin K reductase activities of membranes of the white mutant of Sarcina lutea.

Illumination time	Malate oxidase activity		Malate: vitamin K reductase activity		Succinate: vitamin K reductase activity	
(mins.)	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample
	μg. atoms O_2 uptake/ml./min./mg. membrane protein					
0	0.044		0.101		0.070	
15		0.027		0.052		0.052
30				0.080		0.052
45				0.084		0.059
60				0.091		0.060
75				0.080		0.060
90	0.047	0.034	0.105	0.105	0.077	0.077

The membranes were irradiated at 8° C by light (1240 lumens/sq.ft.) for 15 minutes, after which they were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150 mM malate, 150 mM- succinate, 10 mM- menadione (vitamin K_3) and 5×10^{-3} M- NaN_3 were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities, see pages45 to 49.

and complete restoration took place after 45 minutes of dark incubation. DCPIP reduction was also depressed, and complete restoration of activity was achieved after 45 minutes of dark incubation (Table 12). Similar experiments on non-pigmented membranes showed loss of malate oxidase activity and DCPIP reduction activity, but there was no complete restoration of activity even after one hour of dark incubation (Table 13). Succinoxidase activities showed the same effects of illumination as did malate oxidase. Malate oxidase activity and malate : vitamin K reductase activity showed depression in pigmented membranes, which recovered completely after 75 minutes of incubation in the dark. These results suggested the possibility of two damaging effects of light on the respiratory system of S. lutea: one at the beginning of the respiratory system, and the other at the cytochrome oxidase system.

When the illumination time was prolonged to 15 minutes there was damage to the malate and succinate oxidase systems in pigmented membranes, which did not reverse completely (Table 18). The DCPIP reduction activities, however, reversed after 60 minutes of incubation in the dark, suggesting that there was an irreversible inactivation outside the region between malate and the site of DCPIP reduction. The same experiment on non-pigmented membranes showed more damage to the malate oxidase, succinate oxidase, malate : DCPIP reduction and succinate : DCPIP reduction activities (Table 19), but all of them were damaged irreversibly. Malate : vitamin K reductase and succinate : vitamin K reductase activities were also depressed in both the pigmented and non-pigmented membranes, but there was complete restoration of activities after 90 minutes' incubation in the dark (Tables 20, 21 and 22), suggesting that inactivation of vitamin K reductase activities was completely restored later. This suggested the possibility that the initial damaging effect on the respiratory chain, possibly the flavoprotein, was reversible even in non-pigmented membranes.

In view of the several possible sites for types of light damage on the respiratory chain of the bacterium S. lutea, at least one in the flavoprotein region and possibly one in the cytochrome oxidase region, it was thought possible that the various sites of photodynamic action might be inactivated by different wave lengths of visible light. It was therefore decided to filter out certain wave lengths from tungsten light by using colour filter glasses, and experiments were planned to observe the effects of filtered light on the enzyme activities of S. lutea. As the light intensities were diminished due to insertion of colour filter glasses, the duration of illumination was increased from 15 minutes to 25 minutes.

5. Effect of 25 minutes' illumination with an OY 13 and OY 10 filter on malate oxidase activity, and on methylene blue reduction by pigmented membranes.

Four experiments were performed to observe the effect of 25 minutes' illumination with an OY 13 yellow filter glass at high light intensity (1426 lumens/sq.ft.) on malate oxidase activity and on reduction of methylene blue by the membranes of pigmented S. lutea. The results of one of the experiments are shown in Table 23.

The malate oxidase activity was depressed after 25 minutes' illumination with the filtered light, but activity was completely restored in the following period of dark incubation. However, the malate : methylene blue oxidoreductase activity was not affected at all by the illumination.

The OY 13 light orthochromatic colour filter glass gives 0% transmission at 400 nm and a peak transmission (75%) from 500 nm; it thus cuts off the ultraviolet and violet regions of white light from the tungsten lamp (see page 160). Since the flavoproteins absorb light in the near ultraviolet and blue regions of the spectrum, it was presumed that when the OY 13 filter was used, the flavoproteins would be unaffected. As the artificial electron acceptor methylene blue presumably accepts electrons from the flavoproteins, the malate : methylene blue oxidoreductase is not

Table 23. Effect of 25 minutes' illumination with an OY 13 colour filter glass at high light intensity (1246 lumens/sq.ft.) on malate oxidase activity and methylene blue reduction in the membranes of pigmented S. lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Malate oxidase activity		Methylene blue reduction	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.032		0.029	
25		0.013		0.020
45		0.014		
60		0.012		
75		0.020		
90		0.021		
105		0.018		
120		0.026		
135		0.021		
150		0.029		
165		0.033		
180		0.030		
190	0.029			
220			0.030	0.030

The membranes were irradiated at 20°C by light (1426 lumens/sq.ft.) with OY 13 colour glass filter. Before inserting the colour filter glass, the light intensity was measured by the light meter, and after 25 minutes' illumination the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150mM-malate, 0.002% methylene blue and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illuminations and assay of enzyme activities see pages 45 to 49.

inactivated by the OY 13 filtered light. Hence a second light reaction, sensitive to OY 13 filtered light, is demonstrated in the assay of malate oxidase activity. This inactivation appears to be reversible.

When these experiments were performed with membranes from the white mutant (see Table 24), the inactivation of malate oxidase was not completely reversible. Methylene blue reduction again did not show any light effect at all after 25 minutes of illumination.

These results suggested that the flavoproteins in both the pigmented and non-pigmented membranes escaped the damaging effect of filtered light, and the early part of the electron transport chain was unaffected. The malate oxidase activity of the pigmented membranes showed reversible damage, while that of the non-pigmented membranes showed irreversible damage. Therefore, it could be assumed that the flavoproteins are damaged by the ultraviolet and violet regions of white light, while a second site beyond the flavoproteins is damaged by visible light from 500 nm onwards. The carotenoid pigments appeared to be associated with complete recovery of the malate oxidase activity in the pigmented membranes, while there was irreversible inactivation of the malate oxidase activity in the mutant membranes which lacked carotenoids.

The effect of 25 minutes' illumination with OY 13-filtered light on DCPIP reduction was compared with the effect on malate oxidase activity in pigmented membranes, as shown in Table 25.

There was complete restoration of the malate oxidase activity after irradiation, but not of the DCPIP reduction, suggesting permanent damage to the cytochrome system by OY 13-filtered light.

By comparison, in the white mutant membranes OY 13-filtered light produced inactivation of the malate oxidase and the DCPIP reduction which was irreversible (see Table 26).

Table 24. Effect of 25 minutes' illumination with an OY 13 colour filter glass at high light intensity (1395 lumens/sq.ft.) on malate oxidase activity and methylene blue reduction in membranes of the white mutant of S. lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Malate oxidase activity		Methylene blue reduction	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.032		0.033	
25		0.013		0.040
45		0.017		
60		0.015		
75		0.021		
90		0.019		
105		0.024		
120		0.023		
135		0.019		
150		0.023		
165		0.024		
180		0.019		
190	0.030			
220			0.040	0.040

The membranes were irradiated at 20°C by light (1395 lumens/sq.ft.) with the OY 13 colour glass filter. Before inserting the colour filter glass, the light intensity was measured by the light meter, and after 25 minutes' illumination the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150mM-malate, 0.002% methylene blue and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 49.

Table 25. Effect of 25 minutes' illumination with an OY 13 colour filter glass at high light intensity (1395 lumens/sq.ft.) on malate oxidase activity and DCPIP reduction by the membranes of pigmented S. lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min./mg. membrane protein		µ moles DCPIP reduced/ml./min./mg. membrane protein	
	Malate oxidase activity		DCPIP reduction	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.1376		0.6314	
25		0.1645		0.5904
45		0.1377		
50	0.1376			
60		0.1402		
75		0.1634		
90		0.1569		
100	0.2067			
105		0.1760		
120		0.1336		0.5904
135		0.1376		0.6132
150	0.1376	0.1376	0.7336	0.5904

The membranes were irradiated at 20°C by light (1395 lumens/sq.ft.) with an OY 13 colour glass filter. Before inserting the colour filter glass the light intensity was measured by the light meter, and after 25 minutes' illumination the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150mM-malate, 0.00053M-DCPIP, and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 50.

Table 26. Effect of 25 minutes' illumination with an OY 13 colour filter glass at high light intensity (1364 lumens/sq.ft.) on malate oxidase activity and DCPIP reduction by membranes of the white mutant of S. lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min./mg. membrane protein		µ moles DCPIP reduced/ml./min./mg. membrane protein	
	Malate oxidase activity		DCPIP reduction	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.034		0.180	
25		0.071		0.155
45		0.069		
60	0.031	0.065		
75		0.067		
90		0.054		
105		0.065		
120		0.073		
135		0.071		
150		0.071		
165		0.065		
170				
180		0.060		0.162
190	0.032		0.201	

The membranes were irradiated at 20°C by light (1364 lumens/sq.ft.) with the OY 13 colour filter glass. Before inserting the colour filter glass, the light intensity was measured by the light meter, and after 25 minutes' illumination the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150mM-malate, 0.00053M-DCPIP and 1 M-KCN were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 50.

Therefore, the results of the above experiments showed that photoinactivation of the malate oxidase activity was completely reversible in the membranes of pigmented S. lutea after 25 minutes of illumination with OY 13 colour filter glass at high light intensities. In the non-pigmented mutant, inactivation of the malate oxidase activity was not reversible. Methylene blue reduction was completely unaffected by light in both pigmented and non-pigmented strains. The inactivation of DCPIP reduction was not reversible in either strain, suggesting some more permanent damage to the cytochrome system by the filtered light.

An OY 10 (haze) filter was available, giving 0% transmission at 350 nm and peak transmission (90%) from 450 nm over the remainder of the visible spectrum (see page 160). In view of the success gained with the use of the OY 13 filter, it was thought worth while to test the effect of illumination with the OY 10 filter at high light intensities on malate and NADH oxidase activities, methylene blue reduction and DCPIP reduction by the membranes of pigmented S. lutea.

Four experiments were performed to test the effect of 25 minutes' illumination with the OY 10 filter at high light intensities on malate and NADH oxidase activities, methylene blue reduction and DCPIP reduction by the membranes of pigmented S. lutea. Tables 27a and 27b show the results of one of the experiments.

There was considerable damage by light with the OY 10 filter to all the enzyme activities assayed. Malate and NADH oxidase, methylene blue reduction and DCPIP reduction all showed photo-inactivation in the pigmented strain. Thus, the flavoproteins were affected by light transmitted by this filter. As the DCPIP reduction showed irreversible inactivation, this suggested some damage beyond flavoproteins and up to the cytochrome c level in the electron transport chain of S. lutea.

Table 27a. Effect of 25 minutes illumination with an OY 10 colour filter glass at high light intensity (1395 lumens/sq.ft.) on the malate oxidase and NADH oxidase activities and on methylene blue reductase by the membranes of pigmented Sarcina lutea.

Illumination time (mins.)	µg. atoms O_2 uptake/ml./min./mg. membrane protein					
	Malate oxidase activity		NADH oxidase activity		Methylene blue reduction	
	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.114		0.609		0.197	
25		0.031		0.282		0.105
60		0.035		0.253		0.053
90		0.031		0.267		0.039
120		0.031		0.297		0.048
150		0.026		0.297		0.048
180	0.055	0.022	0.580	0.297	0.096	0.039

The membranes were irradiated at 20° C by light (1395 lumens/sq.ft.) with an OY 10 colour filter glass. Before inserting the colour filter glass, the light intensity was measured and, after 25 minutes illumination, the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150 mM-malate, 0.1% NADH, 0.002% methylene blue and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 50.

Table 27b. Effect of 25 minutes' illumination with an OY 10 colour glass filter at high light intensity (1395 lumens/sq.ft.) on DCPIP reduction by the membranes of pigmented S. lutea.

Time (mins.)	µ moles DCPIP reduced/ml./min./mg. membrane protein			
	Malate : DCPIP reduction		NADH : DCPIP reduction	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.743		1.39	
25		0.337		1.00
60		0.342		1.04
90		0.342		1.15
120		0.356		1.04
150		0.372		1.04
180	0.723	0.336	1.34	1.03

The membranes were irradiated at 20°C by light (1395 lumens/sq.ft.) with an OY 10 colour filter glass. Before inserting the colour filter glass, the light intensity was measured, and after 25 minutes' illumination the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150mM-malate, 0.00053M-DCPIP, 0.1% NADH and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 50.

The use of the OY 13 and OY 10 filters gave very interesting results with reactions involving the flavoproteins; and, in view of this, the effect of illumination with various other colour filter glasses filtering near ultraviolet and blue violet light was tested on methylene blue reduction by the membranes of pigmented S. lutea. As the light intensity was much higher than in the previous experiments (1433 lumens/sq.ft.), the illumination time after insertion of the colour filter glass was shortened to 15 minutes instead of 25 minutes.

6. Effect of illumination with various colour filter glasses on methylene blue reduction by membranes of pigmented S. lutea.

Four experiments were performed to observe the effect of 15 minutes' illumination with different colour filters at high light intensities on methylene blue reduction by membranes of pigmented S. lutea. The results of one experiment are shown in Table 23. The OGr 3 green and OB 14 colour filter glasses produced a light effect (evidenced by a depressed rate of methylene blue reduction) while the OY 13 light orthochromatic, OX 1 ultraviolet and OV 1 purple colour filter glasses did not produce any light effect at all.

The OX 1 ultraviolet colour filter gives 0% transmission at 230 nm and a peak transmission (73%) at 350 nm. The OV 1 purple colour filter glass gives 0% transmission at 340 nm and a peak transmission (72%) between 370 and 380 nm (see page 161). Light from these two filters did not produce any inactivation of methylene blue reduction, and consequently may be presumed to have no effect on the flavoproteins.

The OB 14 colour filter glass gives 0% transmission at 270 nm, and the peak transmission (85%) is between 350 and 400 nm. The OGr 3 green colour filter glass gives 0% transmission at 400 nm and a peak transmission (73%) between 500 and 550 nm (see page 160). The results suggested that OB 14 and OGr 3 filtered-light could produce some inactivation of flavoproteins, though the inactivation of the former was more than that of the latter. Other colour

Table 28. Effect of 15 minutes illumination with various colour filter glasses at high light intensity (1488 lumens/sq.ft.) on methylene blue reduction by the membranes of pigmented Sarcina lutea.

Time (mins.)	Methylene blue reduction µg. atoms O_2 uptake/ml./min./mg. membrane protein									
	OB 14 Filter		OY 13 Filter		OX 1 Filter		OV 1 Filter		OGr 3 Filter	
	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.066		0.066		0.066		0.066		0.066	
15		0.050		0.066		0.071		0.077		0.063
30	0.066	0.044	0.066	0.066	0.066	0.071	0.066	0.071	0.066	0.049

The membranes were irradiated at 20° C by light (1488 lumens/sq.ft.) with the respective colour filter glass. Before inserting the colour filter glasses, the light intensity was measured and, after 15 minutes irradiation, the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150 mM-malate, 0.002% methylene blue and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 49.

filters failed to demonstrate any damage to the flavoproteins. Therefore, it appears that the flavoproteins may be sensitive mainly to 350 - 400 nm, and slightly sensitive to beyond 400 nm of the visible spectrum. It is possible that the total energy transmitted by OY 1 is inadequate to produce an effect on the flavoproteins.

Weber et al. (92) have studied the possible role of vitamin K in electron transport, and their data indicate that vitamin K transfers electrons between NADH and the cytochromes. It was therefore decided to examine the NADH oxidase system of S. lutea.

7. Effect of 25 minutes' illumination with OY 13 colour filtered light on NADH oxidase activities and on NADH : DCPIP reduction activities by membranes of pigmented and non-pigmented S. lutea.

Four experiments were performed to test the effect of 25 minutes' illumination with OY 13 filtered light on NADH oxidase activity and on NADH : DCPIP reduction by the membranes of pigmented S. lutea. The results of one of the experiments are represented by Table 29. There was inactivation by illumination of the NADH oxidase system (evidenced by depressed rate of NADH oxidation), but no inactivation of the NADH : DCPIP reduction activity. The damage to the NADH oxidase appeared to be permanent.

Similar results were obtained when the white membranes were examined for these enzyme activities (see Table 30).

Therefore, illumination with the OY 13 filter at high light intensities showed inactivation of the NADH oxidase systems of both the pigmented and non-pigmented strains of S. lutea, but no inactivation was evident in the NADH : DCPIP reduction activities in either strains. By contrast it is interesting to note that malate oxidase activity in the pigmented membranes showed complete reversibility, while the malate : DCPIP reduction activity did not

Table 29. Effect of 25 minutes illumination with an OY 13 colour filter glass at high light intensity (1395 lumens/sq.ft.) on malate oxidase and NADH oxidase activities, and NADH:DCPIP reduction by the membranes of pigmented Sarcina lutea.

Illumination time (mins.)	µg. atoms O_2 uptake/ml./min./mg. membrane protein				µ moles DCPIP reduced/ml./min./mg. membrane protein	
	Malate oxidase activity		NADH oxidase activity		NADH:DCPIP reduction	
	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.033		0.095		0.372	
25		0.031		0.068		0.397
45		0.031		0.057		
60		0.029		0.068		
90		0.029		0.061		
120	0.029	0.029	0.074	0.061		
150		0.027		0.061		
180	0.029	0.029	0.074	0.061		

The membranes were irradiated at 20° C by light (1395 lumens/sq.ft.) with the OY 13 colour filter glass. Before inserting the colour filter glass, the light intensity was measured and, after 25 minutes illumination, the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150mM-malate, 1% NADH, 0.00053M-DCPIP and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 50.

Table 30. Effect of 25 minutes illumination with an OY 13 colour filter glass at high light intensity (1457 lumens/sq.ft.) on malate oxidase and NADH oxidase activities and NADH:DCPIP reduction by membranes of the white mutant of Sarcina lutea.

Illumination time (mins.)	µg. atoms O_2 uptake/ml./min./mg. membrane protein				µ moles DCPIP reduced/ml./min./mg. membrane protein	
	Malate oxidase activity		NADH oxidase activity		NADH:DCPIP reduction	
	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.069		0.162		0.586	
25		0.065		0.130		0.586
45		0.065		0.122		
60	0.082	0.056	0.155	0.122		
90		0.056		0.115		
120	0.054	0.037		0.119		
150		0.043		0.115		
180	0.056	0.043	0.132	0.115		

The membranes were irradiated at 20° C by light (1457 lumens/sq.ft.) with an OY 13 colour filter glass. Before inserting the colour filter glass, the light intensity was measured and, after 25 minutes illumination, the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150 mM-malate, 1% NADH, 0.00053 M-DCPIP and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 50.

reverse. In the non-pigmented membranes, neither the malate oxidase activity nor the malate : DCPIP reduction activity showed complete reversibility. The NADH oxidase system was inactivated, while there was no inactivation of NADH : DCPIP reduction activity in the mutant membranes. Therefore it seemed that while there was irreversible NADH oxidase photoinactivation in both strains, there was no inactivation of the NADH : DCPIP reduction at all in either strain. This suggested that there was some irreversible damage to the NADH oxidase system beyond the site from where DCPIP receives its electrons. It was now decided to observe the effect of illumination with OY 13 filter on vitamin K reductase activity.

8. Effect of 25 minutes' illumination with the OY 13 filter at high light intensity on the malate : vitamin K reductase activity of pigmented and non-pigmented membranes.

Four experiments were performed to see the effect of 25 minutes' illumination with the OY 13 filter on malate oxidase and malate : vitamin K reductase activities by the pigmented membranes of S. lutea. The results of one of the experiments are shown in Table 31. Both the malate oxidase and malate : vitamin K reductase activities showed inactivation by OY 13-filtered light, and both showed complete reversibility, suggesting that the damage caused to the oxidase system and the vitamin K reductase activity was not permanent.

When these activities, together with malate : methylene blue oxidoreductase activity, were examined in non-pigmented membranes, there was similar inactivation of the malate oxidase and malate : vitamin K reductase activities, but there was complete recovery of the vitamin K reductase activity while the malate oxidase activity did not recover completely (see Table 32). The methylene blue reduction, however, was not affected at all. The complete reversibility of the inactivation of malate : vitamin K reductase activity, even in the non-pigmented membranes, suggested that there

Table 31. Effect of 25 minutes' illumination with an OY 13 colour filter glass at high light intensity (1426 lumens/sq.ft.) on the malate oxidase and malate : vitamin K reductase activities of membranes of pigmented S. lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min./mg. membrane protein			
	Malate oxidase activity		Malate : vitamin K reductase activity	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.056		0.112	
25		0.045		0.054
45		0.041		0.045
60	0.050	0.047	0.074	0.087
90		0.041		0.087
120	0.043	0.047	0.056	0.068
150		0.037		
180	0.045	0.045	0.081	0.081

The membranes were irradiated at 20°C by light (1426 lumens/sq.ft.) with an OY 13 colour filter glass. Before inserting this, the light intensity was measured, and after 25 minutes' illumination the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150mM-malate, 1mM-menadione (vitamin K_3) and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 49.

Table 32. Effect of 25 minutes illumination with an OY 13 colour filter glass at high light intensity (1426 lumens/sq.ft.) on malate oxidase and malate:vitamin K reductase activities, and on methylene blue reduction, by membranes of the white mutant of Sarcina lutea.

Illumination time	μg. atoms O_2 uptake/ml./min./mg. membrane protein					
	Malate oxidase activity		Malate:vitamin K reductase activity		Methylene blue reduction	
(mins.)	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.093		0.071		0.086	
25		0.052		0.054		0.086
60		0.058		0.060		
90		0.080		0.060		
120	0.089	0.060	0.071	0.063		
150		0.071		0.070		
180	0.083	0.065	0.075	0.080	0.089	0.089

The membranes were irradiated at 20° C by light (1426 lumens/sq.ft.) with an OY 13 colour filter glass. Before inserting the colour filter glass, the light intensity was measured and, after 25 minutes of illumination, the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150 mM-malate, 1 mM-menadione (vitamin K_3), 0.002% methylene blue and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 49.

was some damage between the flavoproteins and the cytochromes due to illumination with the OY 13-filtered light, which reversed completely when incubated in the dark. This suggests another site in the electron transport chain between the flavoproteins and the cytochromes which is attacked by OY 13-filtered light.

It is possible that the effects of light described above were due to some mechanical injury done while obtaining the membranes by lysing the cells osmotically. If this was not true, then the above results should be reproduced with whole cells. Therefore, the effect of OY 13-filtered light on malate oxidase activities in whole cells of the pigmented and non-pigmented strains was examined.

9. Effect of 25 minutes' illumination with OY 13-filtered light on the malate oxidase activity of pigmented and non-pigmented cell suspensions.

Four experiments were performed to investigate the effect of OY 13-filtered light at high intensity on the malate oxidase activity of whole cells of pigmented S. lutea. The results of one of the experiments are represented in Table 33, where complete reversal of photoinactivation of malate oxidase activity in whole cells may be seen.

In the white mutant, irreversible photoinactivation of malate oxidase activity was obtained (see Table 34).

These results on malate oxidase activities of the pigmented and non-pigmented whole cells of S. lutea showed exactly the same type of light effect as was observed in the cases of the pigmented and non-pigmented membranes of S. lutea. Therefore, whatever damage was caused to the membranes was not due to any injury done while preparing the membranes.

Further, it was decided to confirm the effect of 25 minutes'

Table 33. Effect of 25 minutes' illumination with an OY 13 colour filter glass at high light intensity (1395 lumens/sq.ft.) on malate oxidase activity of whole cells of pigmented S. lutea.

Time (mins.)	μg.atoms O_2 uptake/ml./min. Malate oxidase activity	
	Dark control	Illuminated sample
0	0.0226	
25		0.0272
45		0.0190
60	0.0231	0.0231
75		0.0140
90		0.0231
105		0.0253
120	0.0272	0.0253
135	0.0201	0.0217

The whole cell suspension was irradiated at 20°C by light (1395 lumens/sq.ft.) with an OY 13 colour filter glass. Before inserting the colour filter glass the light intensity was measured, and after 25 minutes' illumination the cell suspension was kept in the dark away from the light source covered by blackened aluminium foil. Addition of 150mM-malate was made prior to assay. For details of experimental procedures, illumination and assay of enzymeactivity see pages 45 to 48.

Table 34. Effect of 25 minutes' illumination with an OY 13 colour glass filter at high light intensity (1457 lumens/sq.ft.) on malate oxidase activity of whole cells of the white mutant of S.lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min. Malate oxidase activity	
	Dark control	Illuminated sample
0	0.014	
25		0.003
45		0.005
60	0.014	0.010
80		0.010
100		0.009
120	0.012	0.003
140		0.003
160		0.005
180	0.009	0.005

The whole cell suspension was irradiated at 20°C by light (1457 lumens/sq.ft.) with an OY 13 colour filter glass. Before inserting the colour filter glass the light intensity was measured, and after 25 minutes' illumination the cell suspension was kept in the dark away from the light source covered by blackened aluminium foil. Addition of 150mM-malate was made prior to assay. For details of experimental procedures, illumination and assay of enzyme activity see pages 45 to 48.

illumination with OY 13-filtered light on the malate dehydrogenase activity of pigmented membranes of S. lutea, using phenazine methosulphate in place of methylene blue.

10. Effect of 25 minutes' illumination with OY 13-filtered light at high intensity (1395 lumens/sq.ft.) on the malate oxidase and malate dehydrogenase activities of membranes of pigmented S. lutea.

The effect of 25 minutes' illumination with OY 13-filtered light on the malate oxidase and malate dehydrogenase activities of pigmented membranes of S. lutea are shown in Table 35. The malate dehydrogenase activity was assayed by reduction of phenazine methosulphate (PMS).

There was complete reversal of the inactivation of malate oxidase, while there was no inactivation of malate dehydrogenase activity at all. This confirmed that the flavoproteins were not damaged at all by the OY 13-filtered light. However, there was expected damage to the malate oxidase system which reversed completely.

11. Effect of 25 minutes' illumination with OY 13-filtered light at 4°C and 20°C on the malate oxidase activity of pigmented membranes of S. lutea.

Mathews (34) performed experiments in which pigmented and non-pigmented strains of S. lutea, in the presence and absence of toluidine blue, were exposed to light at 4°C and 34°C for 8 hours. The results of those experiments indicated that the ability of the carotenoid pigments to protect cells from lethal photooxidations, by both exogenous and endogenous photosensitizers, was decreased at low temperatures. Some experiments with membrane preparations of pigmented S. lutea were performed to examine the effect of 25 minutes' illumination with OY 13-filtered light at 4°C and 20°C at high intensity (1426 lumens/sq.ft.) on the malate oxidase activity of the bacterium.

The results of one experiment are shown in Table 36. There

Table 35. Effect of 25 minutes' illumination with an OY 13 colour filter glass at high light intensity (1395 lumens/sq.ft.) on malate oxidase and malate dehydrogenase activities in the membranes of pigmented S. lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min./mg. protein			
	Malate oxidase activity		PMS reduction	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.057		0.204	
25		0.031		0.204
60		0.043		
90	0.091	0.092		
120		0.105		
150				
180	0.076	0.076	0.187	0.187

The membranes were irradiated at 20°C by light (1395 lumens/sq.ft.) with an OY 13 colour filter glass. Before inserting this the light intensity was measured, and after 25 minutes' illumination the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150mM-malate, 0.009M-phenazine methosulphate (PMS) and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 49.

Table 36. Effect of 25 minutes' illumination with an OY 13 colour filter glass at 4°C and 20°C by high light intensity (1426 lumens/sq.ft.) on malate oxidase activity of membranes of pigmented S. lutea.

Time (mins.)	µg.atoms O_2 uptake/ml./min./mg. membrane protein Malate oxidase activity			
	20°C		4°C	
	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.072		0.075	
25		0.056		0.054
45		0.047		0.045
60	0.066	0.035	0.054	0.041
75		0.053		0.052
90		0.053		0.052
105		0.053		0.052
120	0.063	0.053	0.052	0.057
135		0.053		0.059
150		0.053		0.066
165		0.053		0.059
180	0.055	0.055	0.052	0.054

The membranes were irradiated at 20°C and 4°C by light (1426 lumens/sq.ft.) with an OY 13 colour filter glass. Before inserting the colour filter glass the light intensity was measured, and after 25 minutes' illumination the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Addition of 150mM-malate was made prior to assay. For details of experimental procedure, illumination and assay of malate oxidase activity see pages 45 to 48.

was no appreciable difference between the two temperatures in the protection afforded by carotenoids to the bacterium. There was complete reversal of activities at both temperatures.

12. Effect of 25 minutes' illumination with OY 13-filtered light on malate oxidase, malate : vitamin K reductase and malate : methylene blue oxidoreductase activities of two other mutant membranes.

The experiments performed so far were with wild Sarcina lutea containing carotenoid pigments, and an ultraviolet induced white mutant (UV_4) which was carotenoidless. The ultraviolet irradiation caused a block in the synthesis of carotenoid pigments. The difference between the results obtained with the carotenoid-containing wild strain and with the carotenoidless mutant strain of S. lutea could be accounted for in terms of the presence and absence of carotenoid pigments. If the presence or absence of carotenoid pigments did matter in the differences of the activities so far assayed, then it could be expected that, in any mutant of Sarcina lutea where carotenoid pigments were absent, the activities in response to illumination would be the same. Mutants of S. lutea were produced by three different methods (see page 36). All three mutants, namely: UV_4, $5BU_7$ and EMS_{15}, looked similar: non-pigmented and white. As there was no carotenoid pigment in any of the three mutants, it was expected that they would behave similarly in response to illumination. Therefore, it was decided to examine the membrane properties of the other two mutants.

Four experiments for each of the two mutants of S. lutea - $5BU_7$ and EMS_{15} - were performed. Tables 37 and 38 represent the results of one of the experiments on each strain.

The results showed complete reversibility of the malate : vitamin K reductase activities. There was irreversible inactivation of the malate oxidase activity, and the malate : methylene blue oxidoreductase activities were not affected at all by OY 13-filtered light. Therefore, all three mutants of Sarcina lutea - UV_4,

Table 37. Effect of 25 minutes illumination with an OY 13 colour filter glass at high light intensity (1426 lumens/sq.ft.) on the malate oxidase and malate:vitamin K reductase activities and on methylene blue reduction by the membranes of the white mutant, 5BU7.

Illumination time	Malate oxidase activity		Malate : vitamin K reductase activity		Methylene blue reduction	
	µg. atoms O_2 uptake/ml./min./mg. membrane protein					
(mins.)	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.019		0.088		0.022	
25		0.010		0.057		0.031
60		0.009		0.057		
90		0.010		0.062		
120		0.010		0.062		
150		0.010		0.075		
180	0.016	0.006				
200			0.076	0.079	0.022	0.025

The membranes were irradiated at 20° C by light (1426 lumens/sq.ft.) with an OY 13 colour filter glass. Before inserting the colour filter glass, the light intensity was measured and, after 25 minutes illumination, the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150 mM-malate, 1 mM-menadione (vitamin K_3), 0.002% methylene blue and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities see pages 45 to 49.

Table 38. Effect of 25 minutes illumination with an OY 13 colour filter glass at high light intensity (1426 lumens/sq.ft.) on the malate oxidase and malate : vitamin K reductase activities and on methylene blue reduction by the membranes of the white mutant EMS_{15}.

Illumination time	Malate oxidase activity		Malate : vitamin K reductase activity		Methylene blue reduction	
(mins.)	Dark control	Illuminated sample	Dark control	Illuminated sample	Dark control	Illuminated sample
0	0.050		0.071		0.042	
25		0.025		0.042		0.042
60		0.017		0.038		
90		0.021		0.033		
120		0.021		0.063		
150		0.017		0.046		
180			0.050	0.050		
200	0.029	0.015			0.038	0.042

The membranes were irradiated at 20° C by light (1426 lumens/sq.ft.) with an OY 13 colour filter glass. Before inserting the colour filter glass, the light intensity was measured and, after 25 minutes illumination, the membranes were kept in the dark away from the light source covered by blackened aluminium foil. Additions of 150 mM-malate, 1 mM-menadione (vitamin K_3), 0.002% methylene blue and 1 M-KCN (neutralized) were made prior to assay. For details of experimental procedures, illumination and assay of enzyme activities, see pages 45 to 49.

$5BU_7$ and EMS_{15} - showed similar properties.

13. Summary

I. Illumination at high light intensities without any exogenous photosensitizer caused damage to malate oxidase activity. Damage was greater in the non-pigmented than in the pigmented membranes.

II. The malate and succinate : vitamin K reductase activities, and the succinoxidase activity of non-pigmented membranes, were also damaged.

III. The malate : DCPIP reduction activity was damaged equally in both the pigmented and the non-pigmented strains.

IV. 5 minutes' illumination followed by incubation in the dark caused partial inactivation of malate oxidase, succinoxidase, malate : DCPIP reduction and succinate : DCPIP reduction in the pigmented membranes. There was complete reversibility of the inactivation after 45 minutes' dark incubation. No difference was apparent between malate oxidase and malate : DCPIP reduction. In the non-pigmented membranes, however, there was inactivation of the enzyme activities, which seemed to be permanent as there was only partial recovery after 60 - 90 minutes' dark incubation. The malate : vitamin K reductase activity in pigmented membranes was partially inactivated, but there was complete recovery after 75 minutes' dark incubation.

V. 15 minutes' illumination caused permanent inactivation of malate and succinate oxidase activities in the pigmented membranes. The inactivation was slightly less in succinoxidase activity than in malate oxidase activity. There was partial inactivation of malate : DCPIP reduction and succinate : DCPIP reduction which reversed completely after 90 minutes' dark incubation.

In the non-pigmented membranes the above activities were permanently inactivated, and the magnitude of damage was more than that of the pigmented membranes. Complete recovery was not seen in the activities even after 90 minutes' dark incubation.

The malate : vitamin K reductase and the succinate : vitamin K reductase activities of both the pigmented and the non-pigmented membranes were depressed after illumination, but there was complete reversibility of the activity after 90 minutes' dark incubation.

VI. 25 minutes' illumination with a yellow (OY 13-filtered) light showed reversible inactivation of malate oxidase activity of the pigmented membranes. There was no inactivation at all of malate : methylene blue oxidoreductase activity or phenazine methosulphate reduction activity.

In the non-pigmented membranes the malate oxidase activity showed irreversible inactivation, while there was again no effect on methylene blue reduction.

The malate : DCPIP reduction activity in both the pigmented and the non-pigmented membranes showed irreversible inactivation.

VII. Illumination with ultraviolet-filtered (OY 10) light showed irreversible inactivation of malate and NADH oxidase activities, methylene blue reduction and DCPIP reduction activities in the pigmented membranes.

Illumination with various filters showed inactivation of malate : methylene blue oxidoreductase activities of pigmented membranes only with OB 14 and OGr 3 filters.

VIII. 25 minutes' illumination with OY 13-filtered light showed inactivation of NADH oxidase activity in the pigmented membranes which did not reverse completely, but the NADH : DCPIP reduction activity showed no inactivation at all.

Malate : vitamin K reductase activity in both the pigmented and the non-pigmented strains showed inactivation, but there was complete reversibility in both strains.

IX. 25 minutes' illumination with OY 13-filtered light on the malate oxidase activity of whole cells of pigmented S. lutea showed inactivation which reversed completely. The non-pigmented cells, however, showed irreversible inactivation of malate oxidase activity.

X. No differences were observed between illumination at 4°C and 20°C.

XI. Two further white mutants, $5BU_7$ and EMS_{15}, behaved identically to UV_4 in illumination experiments involving assay of malate oxidase and malate : vitamin K reductase activities.

CHAPTER 5

Carotenoids

A number of non-photosynthetic bacteria synthesize carotenoids which are usually of a highly polar nature (93). Chargaff and Dieryck (94) suggested that S. lutea contained two carotenoids, from the evidence of partition between 90% methanol and light petroleum (b.p. 60 - 80°). They assumed that the epiphase consisted of one compound which they considered to be a hydrocarbon (sarcinene). The presence of a more polar hypophasic carotenoid (probably a xanthophyll) was inferred by them. Nakamura (95) suggested that a single esterified pigment was present in S. lutea whereas Takeda and Ōta (96), from the same bacterium, obtained a crystalline compound which they considered to be a xanthophyll (sarcinaxanthin). Sobin and Stahly (1) reported that S. lutea contained two carotenoids (carotenols). They could find no esters or carotenoid acids in the bacterium. Since these results were inconclusive, Thirkell and Strang (19) reinvestigated the pigments of S. lutea and found seven fractions which they designated hydrocarbons, or polar materials. The bacterium appeared to synthesize pigments which could be complexed *in vivo* to different proteins or to different amounts of protein. I, therefore, have extracted the carotenoids from the strain of S. lutea discussed here, and noted their spectral characteristics.

Extraction of carotenoids from S. lutea grown on nutrient broth medium yielded a yellow solution with a maximum absorption in acetone at 440 nm. Assuming an $E^{1\%}_{1cm}$ value of 2500, the carotenoid content of this strain of S. lutea was found to be 12.55 µg./g. dry weight of bacteria.

Chromatography of the extracted pigment on a zinc carbonate column with varying eluting solvents gave three fractions listed in

Table 30 . The absorption maxima of the individual pigment fractions were recorded, and the amount of carotenoid in each fraction, together with their percentages, were calculated. The absorption spectrum of fraction 2 is shown in Fig.16 . The spectra of all three fractions were almost identical, and these spectra were similar to those obtained by Thirkell and Strang (19).

There is insufficient information to correlate the fractions obtained here with the pigments identified by Thirkell and Strang (19). Nevertheless, from the absorption maxima, it seems that almost identical pigments have been obtained and that this strain is probably not dissimilar in its pigments from other strains of S. lutea and may well have the C_{50} carotenoids as suggested by Thirkell et al. (22). The last two or three pigments could not be extracted in my experiment because of the difference in the eluting solvents.

No coloured carotenoids could be extracted from the ultraviolet-induced mutant (UV_4).

Table 39. Chromatography of the total pigment extract of *Sarcina lutea* on a zinc carbonate column containing two parts of zinc carbonate and one part of "hyflo-supercel".

Fractions	Eluted from zinc carbonate and "hyflo-supercel" column in	Absorption maxima	Number of µg. of carotenoid in each fraction	% carotenoid in each fraction
1	10% ether in light petroleum spirit	412, 436, 465	2·40	13
2	2-10% acetone in light petroleum spirit	412, 437, 466	6·40	27
3	5% ethanol in light petroleum spirit	413, 438, 465	9·88	60

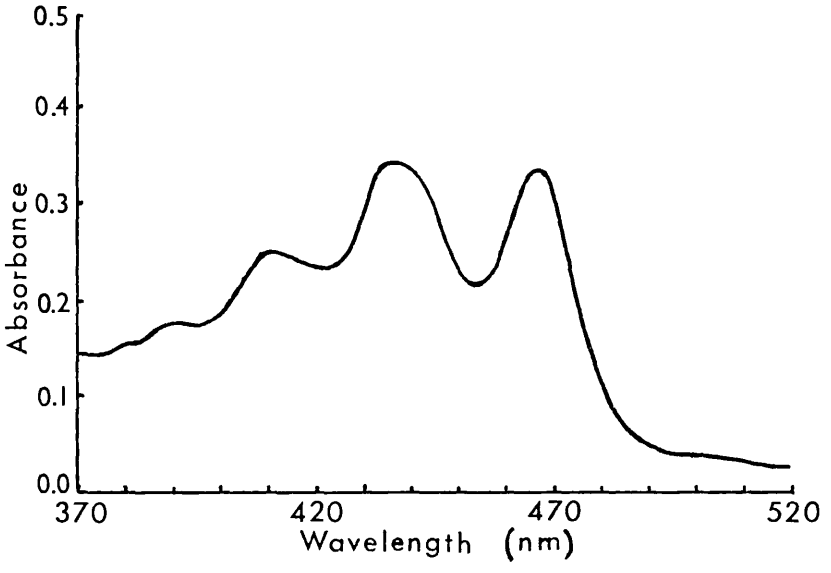


Fig. 16. Absorption spectrum of carotenoids of fraction No.2 in 5% acetone in petroleum spirit (0.80 mg carotenoids per ml).

CHAPTER 6

Discussion

1. Malic dehydrogenase of S. lutea

Bacteria can oxidise an enormous number of organic and inorganic compounds to furnish energy. Many microorganisms can manage with only one compound as a substrate in the growth medium. This suggests that among the different bacterial species there exists a wide variety of dehydrogenases. The majority of these are soluble enzymes in the cytoplasm of the bacterial cell and are connected with the respiratory chain only through NAD, the universal hydrogen carrier. In addition to NADH dehydrogenase and succinate dehydrogenase, bacteria may contain numerous dehydrogenases which are firmly bound to the membrane. It appears that none of the membrane-bound dehydrogenases reduce pyridine nucleotides. There is evidence that those of NADH, malate, succinate, lactate and formate are flavoproteins in bacteria (42, 97, 98). Only the membrane-bound dehydrogenases are directly active in electron transport, and in the absence of an added cofactor can reduce a number of dyes and ferricyanide (97).

A characteristic feature of many bacteria is the simultaneous presence of two dehydrogenases which effect dehydrogenation of the same substrate. One of them is soluble and requires NAD for its activity; the other is firmly bound in the respiratory chain and does not require NAD. The malic dehydrogenases of Micrococcus lysodeikticus, which is closely related to S. lutea, provide a good example of this (99, 100).

Sarcina lutea seemed to have membrane-bound flavoproteins (dehydrogenases) which did not require NAD for their activity. In preliminary experiments with whole cells and membranes of pigmented S. lutea it has been observed that malate is very actively

oxidised. Succinate and lactate are also moderately oxidised. On addition of NAD to membrane preparations there was no change whatsoever in the rate of oxidation of malate, suggesting that the membrane-bound dehydrogenases in S. lutea, like those of M. lysodeikticus, are not NAD linked. Moreover, the NADH oxidase pathway differs from that of the malate oxidase pathway in the presence of illumination. Hence malate is not oxidised via NAD.

2. Reversible damage to malic oxidase in the presence of toluidine blue.

In experiments where reversibility of the inactivation of malate oxidase activity of membranes of the white mutant was demonstrated after illumination in the presence of 2.5 μM toluidine blue (Fig. 12), it was observed that illumination of short duration (10 - 20 minutes) produced very little permanent inactivation of the enzyme activity. 30 minutes' illumination caused more permanent damage to the oxidase activity, which recovered only up to 50% when incubated in the dark following irradiation. There was permanent inactivation of the enzyme activity beyond 60 minutes' illumination.

There are two possible explanations of these observations. Firstly, it is possible that the onset of photodynamic action was a slow process which proceeded until complete inactivation was achieved at a single site. Illumination of shorter duration produced a depression in the rate of oxidation, but complete restoration of activity was achieved when the photosensitizing conditions ceased.

Alternatively, it is possible that initially some reversible photoinactivation takes place at one part of the electron transport chain, while prolonged illumination produces a second inactivation, which is irreversible, at another site in the electron transport chain. Therefore, it could be the photolability of different segments of the chain which really accounts for these observations. The second alternative is supported by the existence of several sites of photoinactivation as discussed below.

3. Sites of light inactivation in the presence of toluidine blue.

The results of experiments on methylene blue (Figs. 13a and 13b) suggest that illumination caused damage to the early part of the electron transport chain - the flavoproteins - in both pigmented and non-pigmented membranes of S. lutea. In manometric experiments the carotenoids did not show much protection against photodynamic action. This was possibly because the manometric technique of assay is less sensitive than the polarographic one. However, experiments on methylene blue and DCPIP reductions and on cytochrome c oxidation in both pigmented and non-pigmented membranes measured by polarographic methods (Table 3) showed interesting results. There was a difference in the rate of reduction of methylene blue between the pigmented and the non-pigmented membranes; the rate was depressed more in the latter than in the former. This reflected some protective role of carotenoids against photodynamic action on the flavoproteins. DCPIP reduction showed a greater depression of rate than methylene blue reduction. This increased damage to DCPIP reduction rate can be interpreted not only in terms of inactivation of the flavoproteins in the early part of the chain, but also in terms of another effect of light between the flavoproteins and cytochrome c. The oxidation of cytochrome c was also depressed by illumination, suggesting some damage at the cytochrome oxidase level. Further, results of the experiments on ascorbate-TMPD cytochrome oxidase activity (Figs. 14 and 15) support the view that there is a site of damage by light at the cytochrome c-cytochrome oxidase level. Therefore, illumination with an exogenous photosensitizer, toluidine blue, causes damage to the flavoproteins, to the cytochrome c-cytochrome oxidase level, and to the cytochrome oxidase level of the electron transport chain of S. lutea.

4. Action of toluidine blue and light

The use of dyestuffs like methylene blue, toluidine blue etc., as hydrogen acceptors is marred by the fact that they may exert

toxic effects on enzyme systems. They may also lack the ability to combine in an appropriate manner at the enzyme centre so that reduction will occur. Toluidine blue is a basic dye. It is known that the basic dyes may be easily reduced by enzyme systems (101, 102). The acid dyes of similar redox potential, however, may be reduced very slowly. This is due to the fact that the basic dyes are easily accessible to, or easily adsorbed by, the active centres of the enzymes, whereas the acid dyes are not. The adsorption of the basic dyestuff may be so large that irreversible inactivation of the enzymes may take place (103). This has been noted with hydrogen acceptors such as methylene blue and toluidine blue. In the above experiments the enzyme activities were assayed after addition of the dyes to the bacteria, but these systems were not subjected to illumination. In my experiments the dye, toluidine blue, was added to the bacterial preparation at a lower concentration than was used in the above work, and was then subjected to illumination. Under these conditions, addition of toluidine blue did not alter the rate of oxidation before the systems were subjected to illumination.

It is also well known that some of the amino acid residues such as histidine, tryptophan, methionine and tyrosine, in a protein are specifically oxidised by irradiation with visible light in the presence of a suitable photosensitizer (23), and such a reaction does not cause cleavage of the peptide bond (81). The results of the experiment with exogenous histidine (Fig. 11) suggest some damage to the histidine residues and possibly to some other amino acid residues in the protein due to illumination in the presence of toluidine blue. Therefore, in the experiments with toluidine blue, it is reasonable to suppose that the amino acid residues of membrane proteins have suffered photooxidation. The finding that all segments of the electron transport chain examined show some damage would support this view.

5. Endogenous photosensitizer.

Evidence exists that there are pigments in the membranes such

flavins, cytochromes etc., which could act also as endogenous photosensitizers in the presence of visible light (6). At very high light intensities (e.g. direct sunlight), and in the absence of any exogenous photosensitizer, these pigments could produce photooxidative damage. This photooxidative damage could be prevented if the carotenoid pigments were present. The results in chapter 4 suggest that there are endogenous photosensitizers present in the membranes of S. lutea which could act as agents to bring about photooxidative damage to the membrane-bound enzyme activities of the bacterium. The light intensity in these experiments was relatively lower than that used by other workers (6). The malate oxidase, succinate oxidase and NADH oxidase activities are all affected by illumination at relatively high light intensities without any exogenous photosensitizer. Illumination of short duration (5 minutes) caused photoinactivation of malate and succinate oxidase activities, and malate and succinate : DCPIP reductase activities, which reversed completely during dark incubation following illumination. Illumination for 15 minutes caused permanent inactivation of malate and succinate oxidase activities only in the pigmented membranes. When illuminated for 25 minutes with OY 13-filtered light, there was partial inactivation of the malate oxidase activity of the pigmented membrane which reversed completely following dark incubation. The NADH oxidase activity of the pigmented membranes on the other hand showed permanent inactivation following 25 minutes' illumination with OY 13-filtered light (Table 29). It therefore appears that the NADH oxidase pathway is different from that of the malate oxidase pathway in S. lutea. The site of damage to the NADH pathway seems to be different from that of the malate pathway, and photoinactivation also takes place much earlier than in malate oxidase inactivation.

6. Effect of light on flavoproteins

The malate : methylene blue oxidoreductase activity of both pigmented and non-pigmented membranes was inactivated by white

light, but was unaffected by 25 minutes' illumination with OY 13 filtered light which cuts off the ultraviolet and violet regions of white light from the tungsten lamp. Similarly the phenazine methosulphate reductase activity of the pigmented membranes was unaffected by 25 minutes' illumination with OY 13-filtered light. The flavoproteins are the sites from which these two artificial electron acceptors pick up electrons. Thus it seems probable that the flavoproteins, which normally absorb light in the near ultra-violet and blue regions of the spectrum, escape photoinactivation when these shorter wave lengths are filtered out. The malate : methylene blue oxidoreductase activity was, however, damaged by OY 10 and OB 14-filtered light. Therefore it is possible to suggest that the flavoproteins are mainly sensitive to light at 350 - 400 nm and just beyond 400 nm of the visible spectrum. Normally the flavoproteins show an absorption curve with peaks at 370 and 450 nm. It seems that illumination of shorter duration would cause partial inactivation of the flavoproteins whose activity would then be restored completely in the following dark incubation; but, if the process of illumination is continued indefinitely, there will be permanent inactivation leading to the irreversible stage. Therefore, one site at the flavoprotein level was established which was damaged by light.

7. Vitamin K reductase.

The malate : vitamin K reductase activity of pigmented membranes was partially inactivated by 5 minutes' illumination. The malate and succinate : vitamin K reductase activities of both pigmented and non-pigmented membranes showed photoinactivation when illuminated for 15 minutes, but there was complete restoration of the enzyme activities following dark incubation. Damage to the flavoproteins could be reflected in a depressed activity of the malate and succinate : vitamin K reductases. However, with OY 13-filtered light (which does not affect the flavoproteins), inactivation of enzyme activity was still observed, but this was restored in the

following dark incubation, even in non-pigmented membranes. Thus it appears that this photoinactivation was partial and was at a site between the flavoproteins and the quinones.

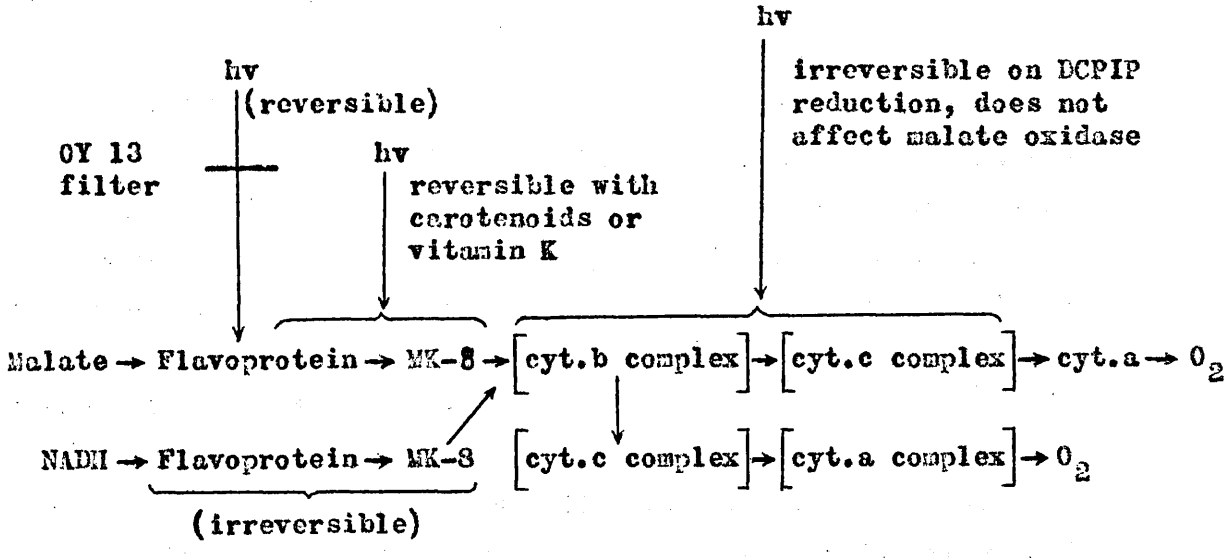
3. DCPIP reduction.

The malate and succinate : DCPIP reductase activities showed photoinactivation by light. The inactivation in both pigmented and non-pigmented strains was completely reversible when illuminated for 5 minutes. 15 minutes' illumination caused reversible photo-inactivation of these activities only in the pigmented strain. 25 minutes' illumination with OY 13-filtered light always caused irreversible photoinactivation in both pigmented and non-pigmented strains. Thus, with malate as substrate, DCPIP is not accepting electrons from the same site as methylene blue, but after the site from where vitamin K is reduced. Since malate oxidase is reversibly inactivated by OY 13-filtered light, it is necessary to postulate that DCPIP accepts electrons from a point in the electron transport chain which is beyond vitamin K but not directly on the pathway to oxygen, and which is irreversibly photoinactivated. The scheme for electron transport proposed for S. lutea by Erickson and Parker (see page 32) would allow for such a system. With OY 13-filtered light, however, NADH : DCPIP reductase activity showed no photo-inactivation when illuminated for 25 minutes; but, with OY 10-filtered light, there was permanent inactivation of both the malate and NADH : DCPIP reductase activities in the pigmented membranes. This suggests a second site at the flavoprotein level from which DCPIP could accept electrons. Therefore, it seems that DCPIP accepts electrons either from a site close to the NADH dehydrogenase or from a site close to the cytochrome chain. It may also be concluded that an additional site for photoinactivation occurs in part of the cytochrome chain. This site is not directly on the pathway to oxygen.

9. Sites of light inactivation

It appears that light causes damage at a minimum of three different sites in the electron transport chain of S. lutea: firstly at the flavoprotein level, secondly between the flavoproteins and the quinones, and thirdly in the cytochrome region; while a fourth site affected is in the region of the NADH dehydrogenase. The presence or absence of carotenoids does not affect the photosensitivity of the flavoprotein site.

Erickson and Parker (76) investigated the electron transport chain of S. lutea and suggested a scheme for electron transport in this bacterium (see page 32). My work differs from theirs in various respects. Their mode of obtaining the electron transport particles is different. The carotenoidless mutant produced by them showed no detectable succinate oxidase activity, while in my experiments there was reasonable succinate oxidase activity present in the mutant membranes. There was an 8-fold increase in the malate oxidase activity of their mutant strain, while there were almost identical malate oxidase activities in both the wild and mutant strains used in my experiments. However, the scheme suggested by them could also operate in the strain of S. lutea investigated here. The following scheme shows the different light effects observed in this study.



10. Relationship between photoinactivation with exogenous and endogenous photosensitizers.

The results obtained with the exogenous photosensitizer, toluidine blue, suggested damage to various enzyme activities of S. luteo, and the study of the electron transport chain showed damage to the flavoproteins and to other sites beyond the flavoproteins. This exogenous photosensitizer can be regarded as a mobile photosensitizer, and as such it is able to act over the entire surface of the membrane. Thus, toluidine blue may have caused photooxidation of some amino acid residues of membrane protein, and thereby produced widespread photooxidative damage. By contrast, the endogenous photosensitizers are rather fixed photosensitizers. They are situated in the membrane at fixed places and presumably catalyse photochemical reactions locally. As carotenoids are present in the membranes in the form of a glycoprotein complex, it is conceivable that at least one endogenous photosensitizer may be located in the vicinity of one of these complexes. Therefore it is not likely that the exogenous and endogenous photosensitizers would cause photoinactivation of enzyme activities in a similar manner. Similarly the protective role of carotenoids would be exercised in different ways.

11. Endogenous photosensitizers.

The flavoproteins themselves may act as endogenous photosensitizers when illuminated at relatively high light intensities. Illumination of short duration (5 minutes) produced initially a reversible photoinactivation of enzyme activity. This suggests that the damaging effects caused by an endogenous photosensitizer must be localized. Only after prolonged illumination does an irreversible reaction occur.

It seems probable from the results of the reversibility experiments, in which 5 minutes' illumination produced photoinactivation of the malate and succinate oxidase activities, and

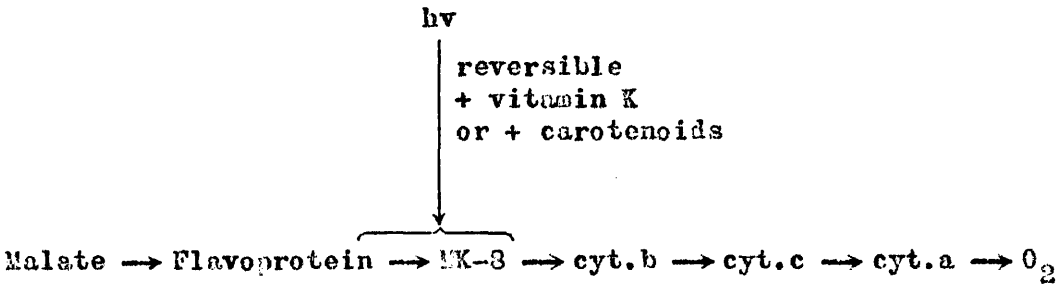
of vitamin K reductase activities, that the primary event in the production of photoinactivation may involve a respiratory pigment. The onset of the reaction is quite rapid, but the recovery time from the deleterious effects of light is 45 minutes. It is possible that amino acid residues such as histidine, cysteine etc., are attacked, as is probably the case with exogenous photosensitizers. Photoinactivation set in fairly rapidly but was not complete, as complete recovery took place within 45 minutes of dark incubation. It may be that the photooxidative process does not take place all over the protein structure simultaneously. More labile sites away from the active centres are attacked first, and the sequence of events spreads all over the entire protein structure and takes some time to produce widespread damage. The process of photoinactivation stops when illumination ceases, and the system is reactivated by some sort of rearrangement of the structure of the membrane.

12. Role of carotenoids in the protection against photodynamic action.

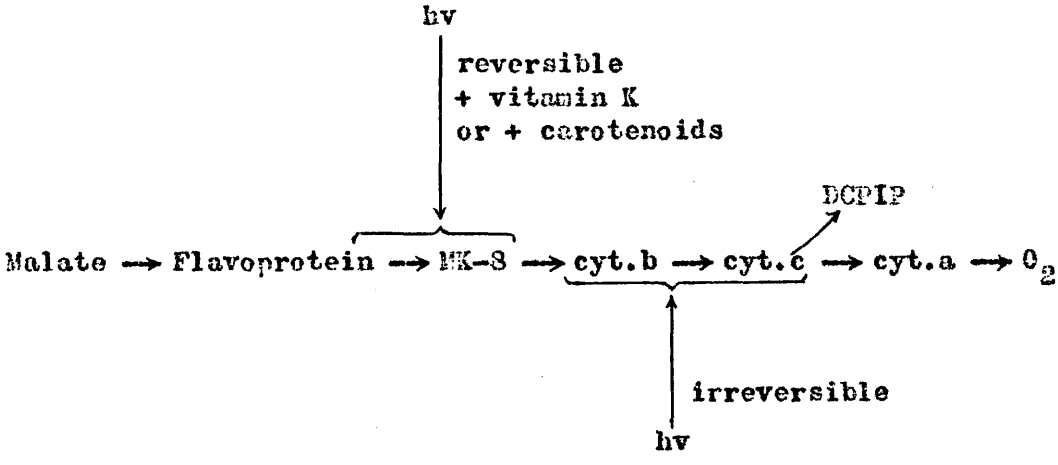
The role of carotenoids in the protection against photodynamic destruction varies with the photosensitizer used. Mathews (29) has shown that in the presence of 8-MOP the rate of photokilling of the pigmented strain was essentially the same as that of the colourless strain. This stands in contrast to earlier findings (6) concerning photosensitization by toluidine blue, which killed the colourless strain but not the pigmented strain. Mathews (29) further showed in a comparative study of the photosensitization of S. lutea by 8-MOP and toluidine blue that various enzyme activities, such as succinic dehydrogenase, $NADH_2$ oxidase and adenosine deaminase activities of the cell extracts, and succinoxidase and pyruvic oxidase activities of whole cells, were all damaged by toluidine blue but were unaffected by 8-MOP. The permeability barriers of the cell membranes were also damaged by toluidine blue. In my experiments the possibility of damage to the permeability of the cell membrane does not arise at all because the study is mainly

concerned with isolated cell membranes. Despite these facts, in all my experiments with or without any photosensitizer it has been observed that the carotenoidless mutant strain showed greater sensitivity to photooxidation than the pigmented strain containing carotenoids. This suggests a protective role of carotenoids against photooxidative damage. In earlier studies of photosensitization of S. lutea by toluidine blue, Mathews and Sistrom (6) found no loss in protection by carotenoids in up to 2 hours' exposure to dye and light at 6.5°C. Later, Mathews (34) performed further experiments in which the pigmented bacteria, in the presence of toluidine blue, were exposed to light at 4°C and 34°C for a longer duration than in previous studies. She showed that the carotenoids of the pigmented S. lutea offered less protection at 4°C than they did at 34°C. Similar results were obtained when the experiment was performed without any exogenous photosensitizer, except that death occurred somewhat later. The results of my experiments performed at two temperatures with pigmented membranes of S. lutea showed no difference in protection by carotenoids (see Table 30). However, these experiments were performed with cell membranes instead of cell suspensions, and the duration of illumination was also much shorter than that of the other worker. At the electron transport level when 25 minutes' illumination are used, however, recovery does not seem to be temperature dependent. In the presence of light, carotenoids seem partially to inactivate photooxidation by toluidine blue. Carotenoids seem to protect the flavoproteins of the electron transport chain of S. lutea. Their mode of action could be the oxidation of an oxygen-toluidine blue complex formed in the light (see page 15). In the presence of toluidine blue the carotenoids seem to act randomly, while in the absence of any exogenous photosensitizer they seem to act specifically. 25 minutes' illumination with ultraviolet filtered light showed inactivation at a site beyond the flavoproteins and up to the vitamin K level, which was reversible in a vitamin K reductase assay or in a malate oxidase assay in the presence of carotenoids. This means that the oxidase activity with malate reversed only in

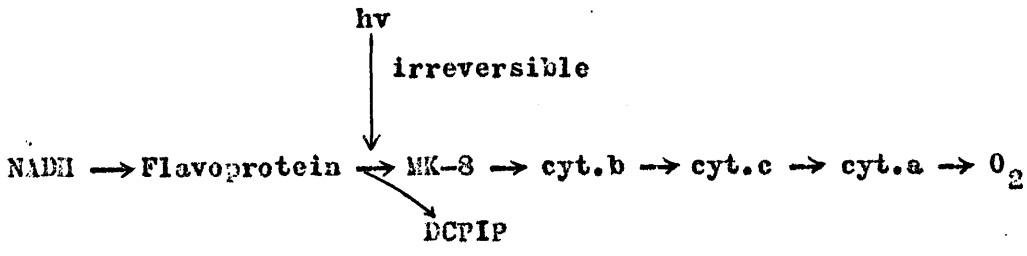
the presence of carotenoids or when vitamin K was added for a vitamin K reductase assay. It can be represented diagrammatically as follows:



There may be another alternative interpretation of these results which postulates that there is a reversible photoinactivation at a site between the flavoproteins and vitamin K, together with another irreversible photoinactivation before the site from which DCPIP accepts electrons. Diagrammatically it could be represented as follows:



In the case of NADH, there is irreversible photoinactivation at the early part of the chain as shown below:



Thus, carotenoids act either by promoting a recovery of oxidase activity after inactivation or by protecting a specific site, probably in the cytochrome region, from irreversible photo-inactivation.

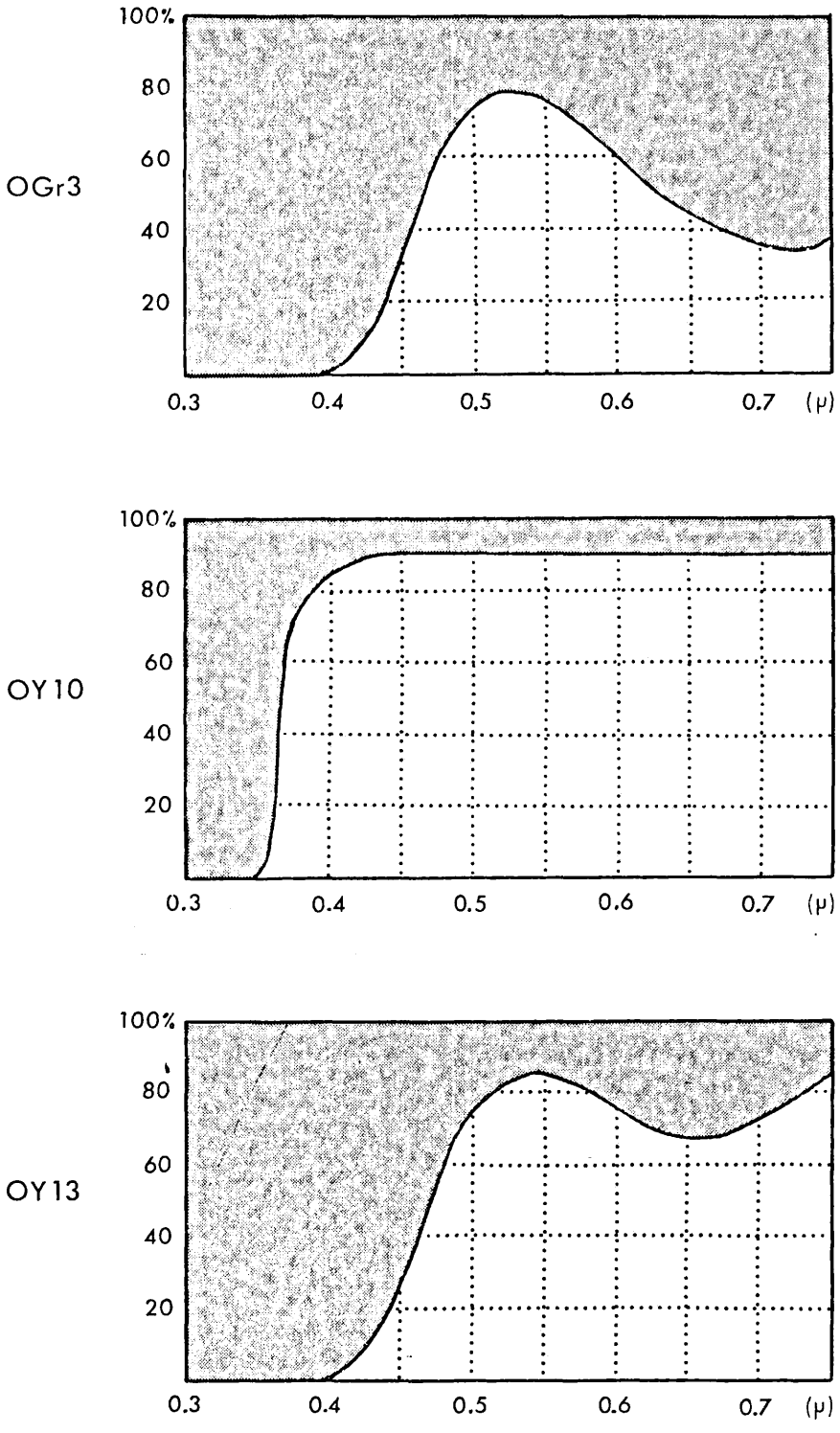


Fig. 17. Transmission characteristics of Chance colour glass filters (2mm thickness).

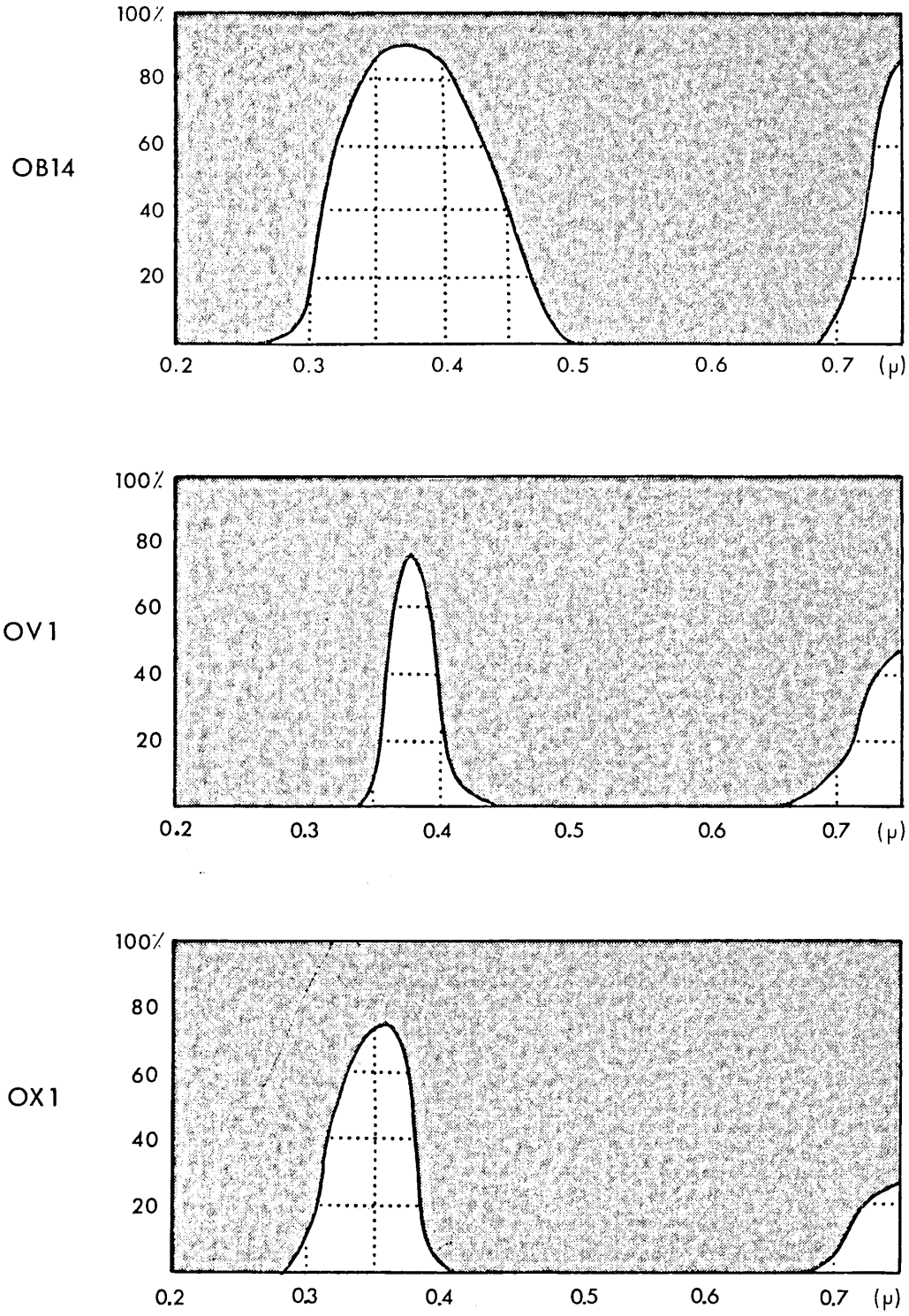


Fig. 17 (continued).

References

Number

1. Sobin, B. and Stahly, G.L. (1942). J. Bacteriol. 44, 265.
2. Cohen-Bazire, G. and Stanier, R.Y. (1953). Nature, Lond. 131, 250.
3. Sistrom, W.R., Griffiths, M. and Stanier, R.Y. (1956). J. Cell. Comp. Physiol. 48, 459.
4. Kunisawa, R. and Stanier, R.Y. (1953). Arch. Mikrobiol. 31, 146.
5. Mathews, M.M. and Sistrom, W.R. (1959 b). Nature, Lond. 184, 1302.
6. Mathews, M.M. and Sistrom, W.R. (1960). Arch. Mikrobiol. 35, 139.
7. Blinks, L.R. (1954). Ann. Rev. Plant Physiol. 5, 93.
8. Griffiths, M., Sistrom, W.R., Cohen-Bazire, G. and Stanier, R.Y. (1955). Nature, Lond. 176, 1211.
9. Platt, J.R. (1959). Science. 129, 372.
10. Smith, L. and Ramirez, J. (1959). Arch. Biochem. Biophys. 79, 233.
11. Blass, U., Anderson, J.M. and Calvin, M. (1959). Plant Physiol. 34, 329.
12. Krinsky, N.I., Gordon, A. and Stern, A.I. (1964). Plant Physiol. 39, 441.
13. Gilby, A.R., Few, A.V. and McQuillen, K. (1958). Biochim. Biophys. Acta. 29, 21.
14. Mathews, M.M. and Sistrom, W.R. (1959 a). J. Bacteriol. 78, 778.
15. Karrer, P. and Jucker, E. (1950). Carotenoids. English edition, Amsterdam, London, New York, Brussels : Elsevier Publishing Co. Inc.

Number

16. Bingle, J.T. and Lucy, J.A. (1968). Biol. Rev. 40, 422.

17. Pullman, B. and Pullman, A. (1965). Quantum Biochemistry. New York : Interscience.

18. Jensen, S.L. (1965). Ann. Rev. Microbiol. 19, 163.

19. Thirkell, D. and Strang, R.H.C. (1967). J. gen. Microbiol. 46, 53.

20. Strang, R.H.C. (1969). Phytochem. 8, 431.

21. Jensen, S.L. and Weeks, O.B. (1966). Norweg. J. Chem. Mining Met. 26, 130.

22. Thirkell, D., Strang, R.H.C. and Chapman, J.R. (1967). J. gen. Microbiol. 49, 157.

23. Rippa, M. and Pontremoli, S. (1968). Biochemistry. 7, 1514.

24. Weil, L. and Buchert, A.R. (1951). Arch. Biochem. Biophys. 34, 1.

25. Slyterman, L.A.Ae. (1962). Biochim. Biophys. Acta. 60, 557.

26. Blum, H.F. (1941). "Photodynamic action and diseases caused by light." Reinhold, New York.

27. Spikes, J.D. and Glad, B.W. (1964). Photochem. Photobiol. 3, 471.

28. Feldman, L.A. and Lindstrom, E.S. (1964). Biochim. Biophys. Acta. 79, 266.

29. Mathews, M.M. (1963 a). J. Bacteriol. 85, 322.

30. Starr, M.P. and Saperstein, S. (1953). Arch. Biochem. 43, 157.

31. Jensen, S.L. (1965 b). Phytochem. 4, 925.

32. Rottem, S., Gottfried, L. and Razin, S. (1968). Biochem. J. 109, 707.

33. Roth (née Mathews), M.M. (1967). J. Bacteriol. 93, 506.

34. Mathews, M.M. (1964 a). Photochem. Photobiol. 3, 75.

Number

35. Mathews, M.M. and Krinsky, N.I. (1965). Photochem. Photobiol. 4, 813.

36. Burchard, R.P. and Dworkin, M. (1966). J. Bacteriol. 91, 535.

37. Mathews, M.M. (1963 b). Photochem. Photobiol. 2, 1.

38. Krinsky, N.I. (1966). Biochemistry of Chloroplasts. Ed. T.W. Goodwin, p.423. Lond. and New York : Academic Press.

39. Brodie, A.F., Wever, M.M. and Gray, C.T. (1957). Biochim. Biophys. Acta. 25, 443.

40. Brodie, A.F. and Ballantine, J. (1960). J. biol. Chem. 235, 228.

41. Brodie, A.F. (1959). J. biol. Chem. 234, 398.

42. Asano, A. and Brodie, A.F. (1964). J. biol. Chem. 239, 4230.

43. Murti, K. and Brodie, A.F. (1969). Science. 164, 302.

44. Wright, L.J. and Rilling, H.C. (1963). Photochem. Photobiol. 2, 339.

45. Roth, M.M. and Krinsky, N.I. (1967). Unpublished observation.

46. Hasegawa, K., MacMillan, J.D., Maxwell, W.A. and Chichester, C.O. (1969). Photochem. Photobiol. 9, 165.

47. Datra, P.P. and Rilling, H.C. (1964). Arch. Biochem. Biophys. 107, 435.

48. Rilling, H.C. (1965). Arch. Biochem. Biophys. 110, 39.

49. Salton, M.R.J. (1964). The Bacterial Cell Wall. Amsterdam : Elsevier.

50. Robertson, J.D. (1959). Biochem. Soc. Symp. Cambridge, Engl. 16, 3.

51. Murray, R.G.E. (1962). Soc. gen. Microbiol. Symp. 12, 119.

52. Schlessinger, D., Marchesi, V.T. and Kwan, H.C.K. (1965). J. Bacteriol. 90, 456.

53. Fitz-James, P.C. (1960). J. Biophys. Biochem. Cytol. 8, 507.

Number

54. Van Iterson, W. (1965). Bacteriol. Rev. 29, 299.

55. Salton, M.R.J. (1967). Ann. Rev. Microbiol. 21, 417.

56. Fitz-James, P.C. (1965). Soc. gen. Microbiol. Symp. 15, 369.

57. Van Iterson, W. and Leene, W. (1964). J. Cell. Biol. 20, 377.

58. Bladen, H.A., Nylen, M.U. and Fitzgerald, R.J. (1964). J. Bacteriol. 88, 768.

59. Drews, G. (1960). Arch. Mikrobiol. 36, 99.

60. Cohen-Bazire, G. and Kunisawa, R. (1963). J. Cell. Biol. 16, 401.

61. Holt, S.C., Conti, S.F. and Fuller, R.C. (1966). J. Bacteriol. 91, 311.

62. Weibull, C. (1953). J. Bacteriol. 66, 696.

63. Salton, M.R.J. and Chapman, J.A. (1962). J. Ultrastruct. Res. 6, 439.

64. Salton, M.R.J. and Ehtisham-ud-din, A.F.M. (1965). Australian J. Exptl. Med. Sci. 43, 255.

65. Salton, M.R.J. and Freer, J.H. (1965). Biochim. Biophys. Acta. 107, 531.

66. Salton, M.R.J. and Schmidt, M.D. (1967). Biochim. Biophys. Acta. 135, 196.

67. Shah, S.D. and King, H.K. (1965 a). Biochem. J. 94, 13p.

68. Kates, M. (1966). Ann. Rev. Microbiol. 20, 13.

69. Weibull, C. and Bergstrom, L. (1953). Biochim. Biophys. Acta. 30, 340.

70. Brown, J.W. (1961). Biochim. Biophys. Acta. 52, 363.

71. Macfarlane, M.G. (1962). Nature, Lond. 196, 136.

72. Yudkin, M.D. (1966). Biochem. J. 98, 923.

Number

73. Razin, S., Argaman, M. and Avigan, J. (1963). J. gen. Microbiol. 33, 477.

74. Thirkell, D., Hunter, M.I.S., Crawford, J. and Fracassini, A.S. (1969). J. gen. Microbiol. 56, 109.

75. Mathews, M.M. (1966). J. Bacteriol. 91, 1369.

76. Erickson, S.K. and Parker, G.L. (1969). Biochim. Biophys. Acta. 180, 56.

77. Chappell, J.B. (1964). Biochem. J. 90, 225.

78. King, T.E. (1963). J. biol. Chem. 238, 4032.

79. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.L. (1951). J. biol. Chem. 193, 265.

80. Davies, B.H. (1965). Analysis of carotenoid pigments. In Chemistry and Biochemistry of Plant Pigments. Ed. by T.W. Goodwin. London and New York : Academic Press.

81. Kenkare, U.W. and Richards, F.M. (1966). J. biol. Chem. 241, 3197.

82. Butt, W.D. and Lees, H. (1953). Nature, Lond. 132, 732.

83. Vernon, L.P. and White, F.G. (1957). Biochim. Biophys. Acta. 25, 321.

84. Kamen, M.D. and Vernon, L.P. (1955). Biochim. Biophys. Acta. 17, 10.

85. Pokallus, R.S. and Pramer, D. (1964). Arch. Biochem. Biophys. 105, 208.

86. Jacobs, E. and Sanadi, D.R. (1960). Biochim. Biophys. Acta. 38, 12.

87. Conover, T.E., Danielson, L. and Ernster, L.(1963). Biochim. Biophys. Acta. 67, 259.

88. Howland, J.L. (1963). Biochim. Biophys. Acta. 77, 419.

Number

89. Bishop, D., Pandya, K. and King, H. (1962). Biochem. J. 83, 606.

90. Asano, A. and Brodie, A.F. (1963). Biochem. Biophys. Res. Commun. 13, 416.

91. Asano, A. and Brodie, A.F. (1965 b). J. biol. Chem. 240, 4002.

92. Weber, M.M., Brodie, A.F. and Morselis, J.E. (1956). Science. 123, 806.

93. Goodwin, T.W. (1954). Carotenoids, p.113. New York : Chemical Publishing Co.

94. Chargaff, E. and Dieryck, J. (1932). Die Pigmente der S. lutea, Naturwissenschaften, 20, 872.

95. Nakamura, T. (1936). Bull. chem. Soc. Japan, 11, 176.

96. Takeda, Y. and Ōta, T, (1941). Hoppe-Seyler's Z. physiol. Chem. 268, 1.

97. White, D.C. (1964). J. biol. Chem. 239, 2055.

98. Benziman, M. and Gallanter, Y. (1964). J. Bacteriol. 88, 1010.

99. Cohn, D.V. (1956). J. biol. Chem. 221, 413.

100. Cohn, D.V. (1958). J. biol. Chem. 233, 299.

101. Quastel, J.H. and Wooldridge, W.R. (1927). Biochem. J. 21, 143.

102. Dixon, M. (1926). Biochem. J. 20, 703.

103. Quastel, J.H. and Wheatley, A.H.M. (1931). Biochem. J. 25, 629.

ACKNOWLEDGEMENTS

It gives me great pleasure to acknowledge my deep sense of gratitude and indebtedness to Dr. J.N. Prebble for giving me constant guidance, encouragement and many hours of discussion throughout the course of this investigation.

My thanks are also extended to Mr. E. Hawkes for his technical assistance, and to members of the Department of Biochemistry at Bedford College for their friendship.