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Non-photosynthetic functions of carotenoids

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In addition to their important rôle as accessory pigments in photosynthesis, carotenoids also participate as agents which protect cells and tissues against the potentially harmful effects of visible radiation. They seem to play a unique rôle in this regard, for there are at least three separate mechanisms which can be invoked to explain the protective aspects of carotenoid function. These involve interrupting the potentially destructive photochemical reactions by quenching the triplet state of chlorophyll, physically inactivating the highly reactive singlet state of oxygen ($^1\Delta_g$) which can be formed photochemically, and finally serving as an oxidizable substrate to protect other molecules and processes from photodestruction.

These protective effects, which were first elucidated in mutant strains of photosynthetic organisms, have been shown to have an even wider rôle in nature, for many non-photosynthetic systems utilize carotenoid pigments for similar protective purposes.

INTRODUCTION

Carotenoids have been known for many years to function as accessory pigments in both plant and bacterial photosynthetic systems, transferring radiant energy to either chlorophyll or bacteriochlorophyll. It was only 20 years ago that the suggestion was made that these pigments had another function, to protect cells against the potentially harmful effects of light, oxygen and a suitable sensitizing pigment. This proposal was made by Sistrom, Griffiths & Stanier (1956) working with the wild-type and a mutant strain of the purple photosynthetic bacterium, *Rhodospseudomonas sphaeroides*. These authors isolated a mutant strain of *Rps. sphaeroides* which lacked coloured carotenoid pigments and therefore appeared blue-green in culture. The mutant strain was referred to as B-G and a comparison of the growth characteristics of the wild-type strain and the B-G strain is seen in figure 1. Under anaerobic photosynthetic conditions, both strains displayed identical growth behaviour, but in the presence of light and air the growth of the B-G strain ceased, its bacteriochlorophyll was bleached and the organism was killed. Sistrom *et al.* (1956) proposed that a photodynamic effect had been initiated in the B-G mutant and that the coloured carotenoid pigments acted as protective agents in the wild-type strain.

In the preceding paper, Cogdell (1978) has described the functions of carotenoids in the isolated reaction centres and light-harvesting antenna complexes of photosynthetic bacteria. He presents the intriguing observations that reaction centres contain unique carotenoids and that they appear to be in a *cis* configuration. I shall therefore restrict my further comments to other systems.

In non-photosynthetic bacteria, carotenoids also function as protective agents against photodynamic effects. Kunisawa & Stanier (1958) demonstrated that a carotenoidless mutant strain of *Corynebacterium poinsettiae* was killed when exposed to light and air in the presence of an exogenous photosensitizer such as toluidine blue whereas the pigmented wild-type strain was

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unaffected. Mathews & Siström (1959) proposed that carotenoid protection had ecological importance based on their experiments with *Micrococcus luteus* (*Sarcina lutea*). Using only sunlight, they demonstrated that *endogenous* photosensitizers could initiate lethal reactions in a white, carotenoidless mutant strain of *M. luteus*, whereas the carotenoid-containing wild-type strain was protected.

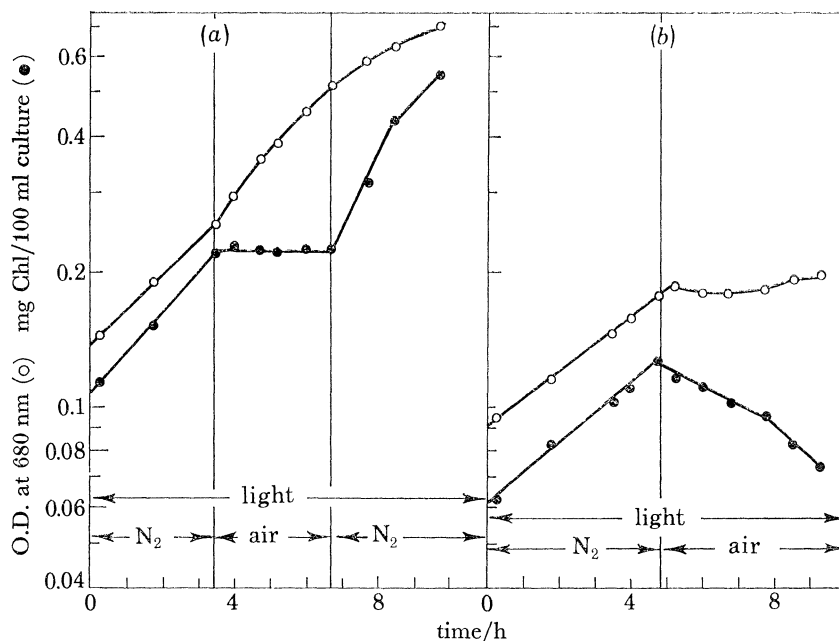


FIGURE 1. Growth and pigment characteristics of the wild-type strain (a) and the blue-green mutant strain (b) of *Rhodospseudomonas spheroides*. Cells were grown under air (95% air-5% CO₂) in light and either growth (○, cell mass) or pigment concentration (●, chlorophyll) measured (Siström *et al.* 1956).

The protective effect of carotenoid pigments has also been demonstrated in plants. Koski & Smith (1951) studied corn mutants which lacked coloured carotenoid pigments and found a normal light-induced conversion of protochlorophyll to chlorophyll, but continued illumination resulted in chlorophyll bleaching. Anderson & Robertson (1960) also demonstrated a protective action for carotenoids in corn mutants, and similar observations have been made in plants treated with herbicides which inhibit carotenoid biosynthesis (Burns, Carter & Buchanan 1971) and in algae (Claes 1954; Sager & Zalokar 1958). We can summarize these observations as demonstrating that carotenoid pigments protect plants, algae and bacteria against potentially lethal photosensitization reactions *in vivo*. To understand the mechanism of protection requires a brief discussion of the photochemical reactions involved in photosensitization.

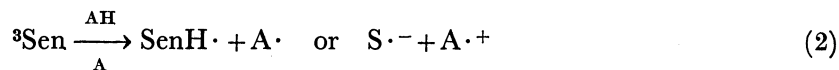
THE CHEMICAL BASIS FOR PHOTOSENSITIZATION

It is now apparent that the photochemical reactions resulting in a photodynamic effect proceed through a metastable form of the excited sensitizer, the triplet sensitizer (³Sen). This excited species has a sufficiently long lifetime to interact with other chemicals and initiate photochemical reactions.

The formation of ^3Sen from the first excited state (^1Sen) is depicted in equation 1.



The reactions initiated by ^3Sen have been classified by Gollnick & Schenck (1967) into two types. Type I reactions,



involve hydrogen abstraction or electron transfer by ^3Sen which results in the formation of radical intermediates and subsequent radical reactions. Type II reactions,



involve the direct transfer of excitation energy from ^3Sen to the ground state of oxygen, $^3\text{O}_2$, resulting in the formation of ground state sensitizer and oxygen in its first excited state, the $^1\Delta_g$ state of singlet oxygen. This species of oxygen is very reactive, combining with unsaturated fatty acids, amino acids and purines such as guanosine sufficiently rapidly to initiate extensive cellular damage (Krinsky 1976). The chemical reactions of $^1\text{O}_2$ are described in detail by Foote (1976) and the biological rôle of $^1\text{O}_2$ by Krinsky (1978).

The photochemical reactions which can be damaging to cells are therefore photo-oxidations initiated by ^3Sen . What then is the rôle of carotenoids in preventing this damage?

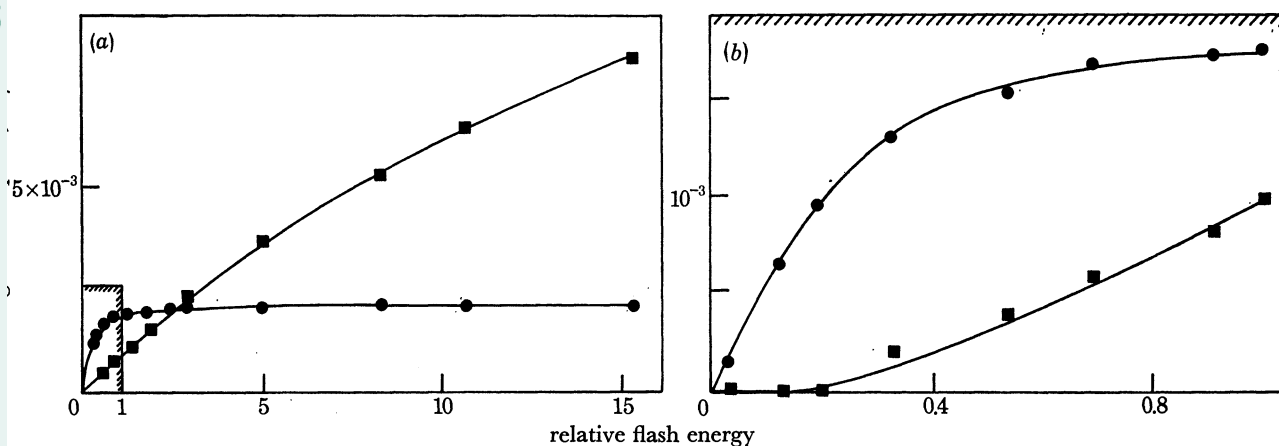


FIGURE 2. Formation of triplet carotenoid in chloroplasts as a function of light intensity. In (a), the extent of absorption change at 515 nm, indicative of the formation of ^3Car (■), increases almost linearly with increasing light intensity, whereas photosynthesis (●) saturates quite rapidly; (b) depicts an enlarged version of the hatched area of (a) (Witt 1971).

CAROTENOID PROTECTION AGAINST PHOTOSENSITIZATION

As mentioned above, it is the ^3Sen which initiates potentially harmful photochemical reactions. Over 20 years ago, Fujimori & Livingston (1957) demonstrated that carotenoid pigments had the capacity to quench the triplet state of chlorophyll (^3Chl) *in vitro*, and it is now known that the reaction involves a triplet-triplet energy transfer from chlorophyll to carotenoids, occurring both *in vitro* and *in vivo* (Mathis 1969; Witt 1971). The formation of ^3Car ,



occurs only at light intensities which are saturating for photosynthesis, for it is only under such circumstances that ^3Chl would accumulate.

The phenomenon of ^3Car formation as a function of light intensity in chloroplasts is shown in figure 2. Similar observations in photosynthetic bacteria have been reviewed in the preceding paper (Cogdell 1978). Foote (1976) has estimated that 90 % of all ^3Chl formed *in vivo* would be quenched by this energy transfer process.

In addition to quenching ^3Chl directly, carotenoids also react very readily with $^1\text{O}_2$ in a spin-conserved reaction, regenerating ground state oxygen and forming ^3Car . This reaction,



was first postulated by Foote & Denny (1968) who demonstrated the ability of β -carotene to quench $^1\text{O}_2$ reactions. The reaction is an energy-transfer reaction, and only a small fraction (0.1 %) of the carotenoids quenching $^1\text{O}_2$ react chemically to yield oxidation products (Foote, Chang & Denny 1970). Foote (1976) has calculated that 99.999 % of the $^1\text{O}_2$ formed from ^3Chl *in vivo* would be quenched by carotenoids before reacting with other $^1\text{O}_2$ acceptors.

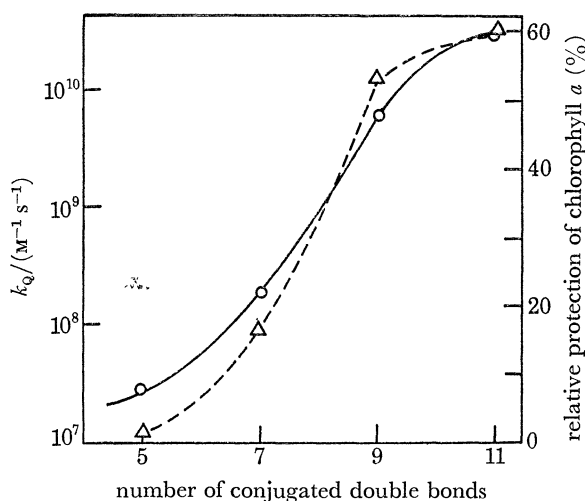


FIGURE 3. The relation between the number of conjugated double bonds and either $^1\text{O}_2$ quenching rates (O) (Foote *et al.* 1970) or protection against photobleaching of chlorophyll *a* (Δ) (Claes 1960; Claes & Nakayama 1959).

The quenching of $^1\text{O}_2$ by carotenoids could only proceed efficiently if the triplet energy level of the carotenoid pigment were below that of the $^1\Delta_g$ state of $^1\text{O}_2$. The latter species lies 22.5 kcal/mol (94 kJ/mol) above ground state oxygen. Farmilo & Wilkinson (1973) demonstrated that the triplet energy level of β -carotene was below 23 kcal/mol (96 kJ/mol) and Mathis & Kleo (1973) estimated this value to be 17.5 kcal/mol (73.2 kJ/mol). Apparently, ^3Car can dissipate this excitation energy directly to the solvent, and therefore function in a cyclic fashion.

Mathis & Kleo (1973) calculated the triplet energy of various polyenes and concluded that polyenes with nine or more conjugated double bonds would be effective quenchers of $^1\text{O}_2$ whereas polyenes containing seven or fewer conjugated double bonds would not be effective. These calculations reflect precisely the observations made in biological systems (Claes & Nakayama 1959; Claes 1960, 1961) and in systems *in vitro* (Foote *et al.* 1970). In figure 3, the relation between the length of the conjugated polyene chain in carotenoids and either the

quenching of $^1\text{O}_2$ or the protection of chlorophyll photobleaching has been plotted. As can be seen, carotenoids containing nine or more conjugated double bonds are very effective in these systems, whereas carotenoids containing seven or fewer conjugated double bonds are quite ineffective.

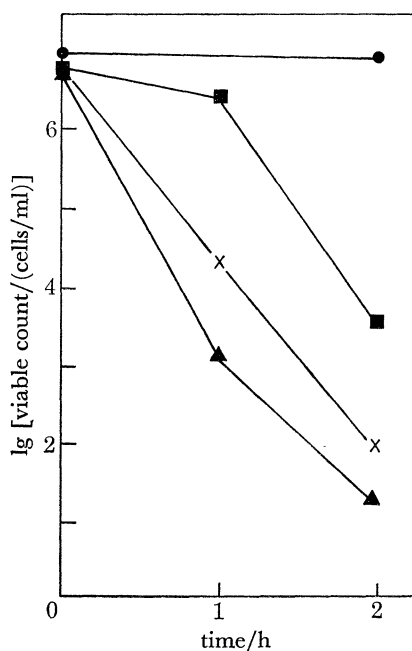


FIGURE 4. The loss of viability of wild-type and mutant strains of *Micrococcus luteus* (*Sarcina lutea*) as a function of irradiation time in the presence of the photosensitizer toluidine blue. The strains used were wild-type (●), 4b (■), 7 (▲) and the colourless 93a (×). (Mathews-Roth & Krinsky 1970).

These observations were extended by Mathews-Roth & Krinsky (1970), working with mutant strains of *M. luteus*. Among these strains was one (strain 7) in which the major carotenoid pigments contain only eight conjugated double bonds. As can be seen in figure 4, mutant strain 7 was killed at the same rate as the carotenoidless white mutant, 93a. However, mutant 7 contains less pigment per cell than does the wild-type strain and therefore viability data were also obtained for mutant 4b, which contains the same pigments as the wild-type strain but at a lower concentration, comparable to the concentration found in mutant 7. As can be seen, mutant 4b shows some protection, but considerably less than the wild-type strain. In an *in vitro* system, Mathews-Roth, Wilson, Fujimori & Krinsky (1974) observed that the major carotenoid pigments of *M. luteus*, which contain nine conjugated double bonds, are as effective as β -carotene, lutein or isozeaxanthin in quenching $^1\text{O}_2$. The carotenoid pigment from mutant strain 7, containing only eight conjugated double bonds, was significantly less effective in quenching $^1\text{O}_2$. Based on these observations, Mathews-Roth *et al.* (1974) suggested that the factors which determine the ability of carotenoid pigments to protect cells against the harmful effects of a photodynamic effect should include concentration of carotenoid, conjugated double bond length, and possibly the location of carotenoid with respect to the location of the endogenous photosensitizer(s).

We have had an opportunity to investigate the protective action of carotenoid pigments in an artificial membrane system. Anderson & Krinsky (1973) added carotenoid pigments to liposomes prepared from phosphatidylcholine, cholesterol and dicetyl phosphate; these liposomes

were irradiated, as well as control liposomes lacking carotenoids, in the presence of toluidine blue. As can be seen in figure 5, the presence of the carotenoid canthaxanthin maintained the membrane integrity of these liposomes as well as preventing the burst of lipid peroxidation, as measured by malondialdehyde formation, during irradiation with white light. A similar protection against $^1\text{O}_2$ action initiated by radiofrequency discharge was also reported (Anderson, Krinsky, Stone & Clagett 1974).

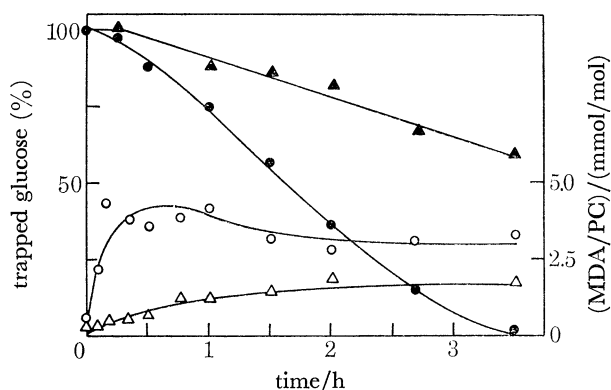


FIGURE 5. The protective effect of carotenoid pigments in liposomes. Membrane integrity was measured by the release of trapped glucose from irradiated control (\bullet) and canthaxanthin-containing liposomes (\blacktriangle) and the malondialdehyde (MDA) production from control (\circ) and canthaxanthin-containing liposomes (Δ) (Anderson & Krinsky 1973).

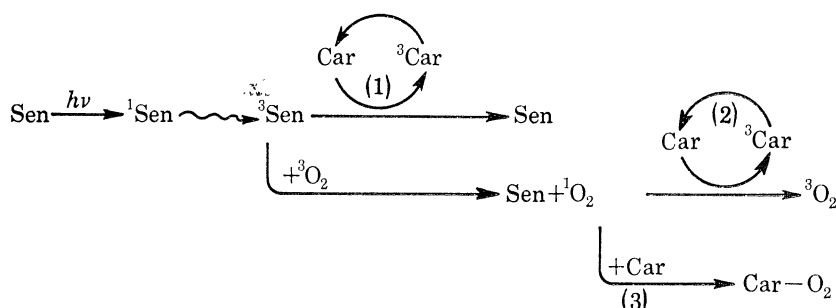


FIGURE 6. Mechanisms of carotenoid protection. A sensitizer molecule (Sen) is excited by light to its first excited state (^1Sen) and then undergoes intersystem crossing to form the triplet species (^3Sen). Carotenoids can now function in three protective reactions: (1) ^3Sen can be quenched directly by carotenoids forming ground state sensitizer and ^3Car . (2) The ^3Sen which is not quenched by carotenoids can interact with oxygen, reforming ground state sensitizer and $^1\text{O}_2$. This latter species is rapidly quenched by carotenoids, regenerating ground state oxygen and ^3Car . (3) The $^1\text{O}_2$ which is not physically quenched can undergo a chemical reaction with carotenoids resulting in oxidized carotenoids.

The several reactions whereby carotenoid pigments can protect cells and tissues against potentially harmful photosensitized reactions are depicted in figure 6. These protective reactions may well represent a unique situation in which a single class of compounds utilizes three separate mechanisms to carry out a single biological function. The function is survival in an environment in which cells contain photosensitizing pigments and are exposed to light and oxygen. These are, of course, the conditions which define aerobic life on Earth. Certainly during earlier periods of evolution, before oxygen became available, carotenoids were not necessary for survival. However, it is safe to conclude that once oxygen entered the atmosphere, carotenoids became essential components for cellular survival.

OTHER CAROTENOID FUNCTIONS

In addition to acting as accessory pigments in photosynthesis and acting as protective agents, numerous other functions have been attributed to carotenoids. Many of these have been reviewed recently (Krinsky 1971) but experimental data supporting these functions are still very weak. In certain instances, the newer data discount the possible participation of carotenoids as functional molecules. For example, carotenoids had been postulated to function as the photoreceptor pigments in phototropism for the last 40 years. Recent evidence of Presti, Hsu & Delbrück (1977), among others, have now removed carotenoids from such a rôle. These authors worked with mutants of *Phycomyces* which contain no detectable β -carotene (less than 4×10^{-5} of wild-type amount) but exhibit normal phototropic responses.

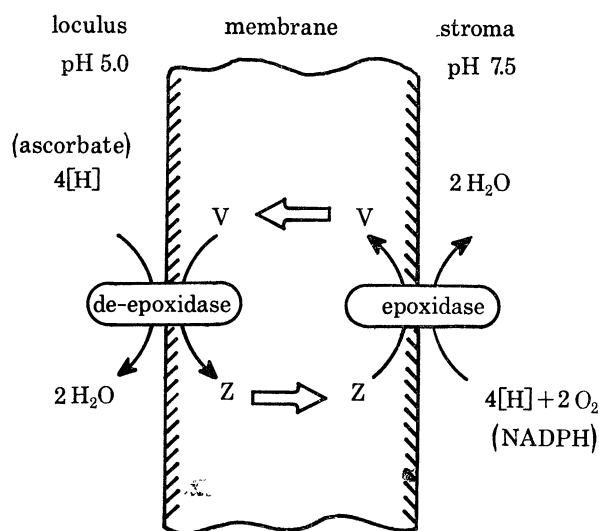


FIGURE 7. Transmembrane model of the violaxanthin cycle in chloroplasts. V, Violaxanthin; Z, zeaxanthin; an intermediate, antheraxanthin, is not shown (Siefermann & Yamamoto 1975).

Several workers have demonstrated that plant and algal carotenoids undergo epoxidation and de-epoxidation reactions. One of the earliest suggestions was that these reactions in algae were another aspect of the protective function of carotenoids (Krinsky 1966), but this view no longer appears tenable (Hager 1967). In higher plants the two reactions have been characterized better and appear to form a cycle shown in figure 7 and referred to as the violaxanthin cycle. The violaxanthin de-epoxidase reaction is favoured by light, which lowers the pH of the loculus (Hager 1969). The epoxidation of zeaxanthin appears to function as an external mono-oxygenase requiring reduced pyridine nucleotides (NADPH or NADH) and oxygen (Siefermann & Yamamoto 1975). Despite the excellent work which has gone into characterizing these reactions, there is still no understanding of their possible functional significance.

Closely related to the violaxanthin cycle is the observation that the isolated envelope of spinach chloroplasts is yellow, being enriched in violaxanthin in comparison with the entire chloroplast (Douce, Holtz & Benson 1973). Even more intriguing is the fact that the carotenoids in the chloroplast appear to take part in the violaxanthin cycle, being enriched in violaxanthin when prepared in the dark and enriched in zeaxanthin when prepared in the light (Jeffrey, Douce & Benson 1974). Again, we must await additional experiments to try to deduce a functional rôle of these carotenoids.

Several years ago a number of investigators were able to inhibit photosynthetic reactions by an organic solvent extraction of isolated chloroplasts, and restore activity by adding back various lipid components, such as β -carotene and plastoquinone. Reports using these techniques were occasionally conflicting, and this field appears to still be in a state of flux. Knaff, Malkin, Myron & Stoller (1977) have reported recently on their experiments in reconstituting photosystem II activity in solvent-extracted chloroplast fragments. They concluded that plastoquinone acts as the primary electron acceptor in this system, and not β -carotene. This reaction was monitored by measuring the low-temperature photooxidation of P680. However, the addition of plastoquinone and β -carotene was required to observe an additional spectral change, designated the C550 photoreduction. Unfortunately, the exact chemistry of this latter reaction, as well as its possible function in photosynthesis, remains to be elucidated.

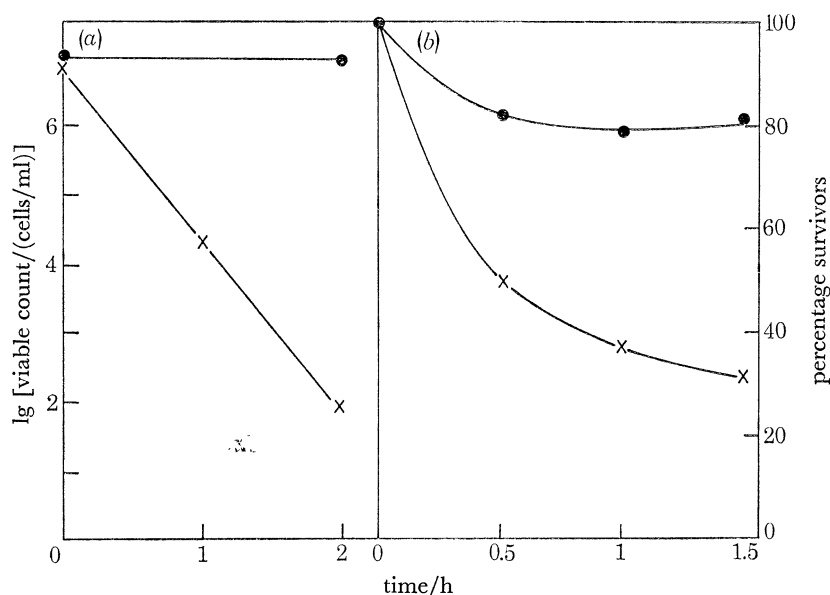


FIGURE 8. Differential killing of the wild-type (●) and a carotenoidless (×) strain of *Micrococcus luteus* (*Sarcina lutea*). (a) Photokilling induced by light, air and toluidine blue (Mathews-Roth & Krinsky 1970). (b) Microbicidal activity of human polymorphonuclear leucocytes (Krinsky 1974).

There have been several reports suggesting that carotenoids may act as electron acceptors and donors in photosynthesis, but these reports remain primarily theoretical (Arnold 1976). There is, however, evidence for these reactions *in vitro* (Mathis & Vermiglio 1972; Beddard, Davidson & Trethewey 1977) and as such may have a functional rôle in some aspects of photosynthesis. At the moment, however, these functions must be considered speculative.

CONCLUSIONS

We are left therefore with strong evidence for carotenoid protection against photodynamic damage, and, apart from energy transfer in the form of accessory photosynthetic pigments, no other substantiated function.

However, the carotenoid protective function may extend beyond photochemically induced damage, since carotenoids are also very effective quenchers of 1O_2 , regardless of how it is made. A number of years ago, Allen, Stjernholm & Steele (1972) proposed that polymorphonuclear

leucocytes produce $^1\text{O}_2$, which in turn serves as a microbicidal agent. To test this hypothesis, Krinsky (1974) exposed human polymorphonuclear leucocytes to either the wild-type strain or the carotenoidless strain of *M. luteus*. These two strains had been shown earlier (Mathews-Roth & Krinsky 1970) to differ in their sensitivity to visible light in the presence of a sensitizer, presumably due to the ability of the carotenoid pigments in the wild-type strain to quench $^1\text{O}_2$. As can be seen in figure 8*b*, the wild-type strain showed no significant killing after a 90 min incubation in the presence of polymorphonuclear leucocytes, whereas the carotenoidless mutant strain was killed rapidly under these circumstances. These results are similar to those obtained with photosensitized killing (figure 8*a*) and suggest that similar mechanisms may be operating in both systems to kill some strains of bacteria and protect other strains.

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