PHOTOREGULATED CAROTENOID BIOSYNTHESIS IN NON-PHOTOSYNTHETIC MICROORGANISMS

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ABSTRACT

Photoregulated carotenoid biosynthesis consists of an initiating photoactivation which bestows carotenogenic competence upon photochromogenic microorganisms. Subsequent carotenoid biosynthesis is light independent but requires microbial growth. Experimental systems have been developed for several photochromogenic microorganisms to separate the primary photoact from the following carotenogenic reactions. Investigations with *Flavobacterium dehydrogenans* have shown that photoactivated carotenogenesis is one aspect of a photoresponse which includes RNA-controlled protein biosynthesis, qualitative changes in lipids and fatty acids and a general metabolic shift-down. It is probable that the fundamental photoresponse is a readjustment of the bacterial physiology to allow existence in light and this centres upon a need to biosynthesize carotenoid for photoprotection against photodynamic damage of the cell.

INTRODUCTION

Light-dependent biological reactions are diverse and commonplace in Nature[1] and light-dependent carotenoid biosynthesis is among these. Such a phenomenon occurs in both photosynthetic and non-photosynthetic organisms and is an example of a photoregulated biosynthetic process. There is no reason to believe that the basic mechanism is other than an absorption of electromagnetic energy by certain molecules and that this somehow causes a carotenogenic response. This paper will consider the phenomenon as it occurs in non-photosynthetic miroorganisms, especially the bacterium *Flavobacterium dehydrogenans.*

OCCURRENCE IN NATURE

The natural occurrence of photoregulated carotenoid biosynthesis has been included in a recent review of carotenoid biosynthesis[2], in a review of algal carotenoids[3] and its occurrence and characteristics in non-photosyn-

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thetic microorganisms, the subject of a comprehensive review by Batra[4]. It is sufficient in this paper to consider only a few illustrations of its occurrence in Nature in an effort to emphasize that photoregulation may operate at different control points in different biological systems. It is quite possible that although there may be different control points, the explanation of the phenomenon relates to the photoprotective role of carotenoids[5] and that photoactivated carotenogenesis is only one aspect of a more profound photoresponse.

Dark-grown etiolated seedlings contain only traces of xanthophylls and exposure of the plants to light is followed by *de novo* carotenoid biosynthesis[6,7]. Light-activated carotenogenesis in this instance occurs as part of the photoregulated biosynthesis of all pigments of the chloroplast which itself develops in response to light[8]. Phytochrome may participate in the light-stimulated pigment biosynthesis as was shown from studies of maize seedlings[9,10]. Light does regulate carotenogenesis in green tissues but the event is only one aspect of a complex photoregulation.

In tomato fruit light does not appear to initiate carotenogenesis. The fruit will develop its complement of carotenoids in the dark but light gives a quantitative stimulus to the process. Raymundo, *et al.*[11] found, for example, that tomato fruit ripened in light contained six times the amounts of carotenoid found in dark-ripened fruit. Their data suggested that light stimulation occurred at a biosynthetic point after the formation of phytoene.

A similar, stimulatory role for light was observed in soluble preparations derived from acetone powders prepared from spinach-leaf chloroplasts[12]. The preparations converted 15,15'-^{3}H-lycopene to cyclic carotenoids more efficiently in light than dark. A similar preparation from tomato-fruit plastids was not effected by light[13]. The enzymes involved in the light-stimulated reaction have not been purified enough to know the reason for light participation. There are other examples of enzyme reactions which have light as a cofactor requirement and some of these are not well known. The well-known enzyme systems needing light include phytochrome[52] and the visual process[14]. Less well known are the urocanase from *Pseudomonas putida*[15], an algal deoxyribonucleic acid photoreactivating enzyme[16], and ribulose diphosphate carboxylase[17].

Photoregulated carotenoid biosynthesis occurs commonly in algae[3] and resembles the event as it occurs in higher plants. To illustrate, *Euglena* grown heterotrophically in the dark does not form chloroplasts and the cells are essentially free of carotenoid[18]. In contrast, *Chlorella* grown in the dark produces apparently normal chloroplasts with the usual carotenoids[19]. For the *Euglena*, exposure to light of the dark-grown cells, initiated chloroplast development including carotenoid biosynthesis, thus paralleling the circumstance in etiolated seedlings. A seemingly different carotenoid photoregulating system occurs in certain mutant strains of *Chlorella* studied by Claes[20,21]. The mutants formed chloroplasts in the dark in which carotenoid biosynthesis was stopped with formation of hydrolycopenes. Phytoene, phytofluene, δ-carotene, proneurosporene and prolycopene accumulated in the dark. Exposure of the alga to light resulted in conversion of the accumulated carotenoids into β-carotene, α-carotene and α-zeaxanthin. Light was required for the cyclization step to occur, insertion of oxygen to form the xanthophyll occurred in the dark. The mutants resemble both tomato fruits

and the cell-free preparations from spinach chloroplasts in having a light requirement for biosynthetic reactions in the post-phytoene sequence.

Photoregulated carotenogenesis also occurs in a variety of non-photosynthetic microorganisms and especially in certain fungi and non-photosynthetic bacteria[4]. This event has been studied especially in *Neurospora crassa*[22,23], in *Fusarium aquaeductuum*[24,25], in a *Mycobacterium* sp.[26,27], *Mycobacterium marinum*[28,29], *Myxococcus xanthus*[30] and *Flavobacterium dehydrogenans*[31,32]. In some of the microorganisms appreciable amounts of phytoene occur in dark-grown systems and illumination is followed by formation of coloured carotenoids[22]. In others, notably *F. dehydrogenans*, the dark-grown populations seem devoid of all carotenoid[33].

In both photosynthetic and non-photosynthetic organisms it is reasonable to relate photoregulated carotenoid biosynthesis to the need for carotenoid to protect cells from photodynamic sensitization[5]. This is especially so for the photosynthetic where light-activated chlorophyll is at once necessary for photosynthesis and is capable of destroying the organism without the intercession of carotenoids. It is almost to be anticipated that biological systems would have evolved in which there was the luxury of a mechanism to turn on carotenoid biosynthesis when need for photoprotection arose, and that this would be regulated by light. In photosynthetic systems this would be whenever photosynthesis was functional, which suggests that a carotenoidless state would be evolutionary folly. This is not the circumstance for non-photosynthetic microorganisms even though photodynamic sensitization may occur and be lethal, as for *Myxococcus xanthus*[34].

EXPERIMENTAL SYSTEM

It has been known from the time of the initial studies of photoinduced carotenogenesis in non-photosynthetic microorganisms that the mechanism consists of a primary photoact which gives carotenogenic competence to the system, and secondary biosynthetic reactions which express this competence as carotenoid product[23,24,27,28]. Only the primary reaction requires light, the carotenoid product is formed in a biochemical series of dark reactions and presumably these are the usually accepted ones for carotenogenesis[35]. It has also been apparent from the start that expression of photoactivated carotenogenic competence requires conditions which allow the microorganisms to grow. The two-fold aspect makes possible separation of the initial photoact from subsequent reactions of carotenoid biosynthesis and provides an experimental system for study of the phenomenon. The research techniques which have been used by the different investigators do not differ in major detail[4] and most differences relate to individuality of a microorganism. The objectives of most studies have been to search for the molecular species involved in the photoact and the means by which photoactivation is translated into carotenogenesis.

Separation of the primary photoact is experimentally simple and consists of illuminating the test biological system under controlled, standardized conditions which minimize microbial growth and metabolism generally. Since the photoinduction apparently is the result of a primary photochemical reaction, it is independent of temperature and on-going metabolic reactions.

This means that photoactivation may take place at low temperature in a microbial suspension in non-nutritive buffer such as a phosphate or Tris system. The essential precautions are those which insure that the microbial system is not damaged during photoactivation, not so much by the activating light as by being held in buffer for whatever time the experimentation requires. The carotenogenic competence resulting from photoactivation is a lasting state. Rilling[26] found that his mycobacterial system, once it had been illuminated, was able to produce carotenoid during dark incubation after storage for 3 months at $-15°C$. Saleh[31] reported that competence was retained for at least 50 h at 5°C in *F. dehydrogenans* and Rau[50], that both *Fusarium aquaeductum* and *Neurospora crassa* retained their competence for at least 48 h. Batra[4] has suggested that the photoactivated state is irreversible and attributed this to an hypothetical, stable photoproduct which would confer carotenogenic competence. Whatever the explanation, stability of the carotenogenic competence in the absence of microbial growth is a great convenience for the investigator.

Expression of photoactivated carotenogenic competence depends upon incubation of the biological system under conditions which permit the microorganisms to grow. At conclusion of this incubation, carotenogenic competence is appraised in terms of the carotenoid formed, usually after extraction and analysis in the conventional manner[33]. These requirements can introduce experimental complications. One of these is the need to know total carotenoid as well as the distribution of this among whatever individual carotenoids the microorganism produced in the growth medium used. In the experimental systems which have been used, the carotenoid product most often seems to contain not only the principal coloured compounds but also the hydrolycopene series which defines carotenogenesis[35]. For example, *M. marinum*[36] and the *Mycobacterium* sp. used by Rilling[27], showed 7 and 9 chromatographically separable fractions in the extracted carotenoid, respectively. *F. dehydrogenans* offers an advantageous experimental system in these respects.

F. dehydrogenans may be grown in a variety of culture media and both quality and quantity of the carotenoids produced will vary widely in the different media[33]. The highest total carotenoid content is 10–12 μg/mg cell-N and this has been found in a glucose, NH_4Cl medium supplemented with either a mixture of B-complex vitamins or yeast extract. In these conditions, which include continuous illumination during growth of the bacterium, the total carotenoid is essentially one product which has the trivial name, decaprenoxanthin[39]. In other media such as nutrient broth, as many as 10 carotenoids make up the total carotenoid. The carotenoids of *F. dehydrogenans* have been characterized and the structures are reasonably well proved[37–39]. The qualitative variations represent effects of nutritional conditions, which either allow carotenogenesis to proceed without interruption from phytoene to the final product, decaprenoxanthin, or there is a nutritional imbalance which somehow slows the biosynthetic processes and allows precursor carotenoids to accumulate[40]. The relative amounts of decaprenoxanthin vary from 24 to 99 per cent of the total carotenoid in different media[33]. There is an experimental advantage in being able to measure the effects of photoactivation with a microorganism

which may be grown so that its total carotenoid consists of a single compound.

Analysis of the carotenoid product requires measurement of whatever carotenoids there may be in the microorganism before it is photoactivated. There are no recorded instances in which measurable carotenoid has not been found. This may be because of technical difficulties in the exclusion of all light during experimentation, or because of a low level of carotenogenesis occurring in the non-photoactivated state. Rilling[27] made a special effort to exclude light in his studies of a *Mycobacterium* sp. but the bacterium produced between 8–10 μg total carotenoid/g bacteria, mostly phytoene, under the most rigorous experimental conditions. The effect of photoactivation was readily measured since the total carotenoid increased to about 38 μg/g and was distributed among 9 chromatographic fractions. A similar situation exists in *Neurospora crassa* where the dark-grown, non-photoactivated cultures contained 24 mg/100 g mycelium, mostly phytoene[23]. When these populations were placed in light for 1 h and then incubated for 24 h in the dark, the total carotenoid increased to about 70 μg/100 g mycelium of which 38 μg were phytoene. *Mycobacterium marinum* contains about 6 μg total carotenoid/g bacteria, 80 per cent phytoene, and after photoactivation and dark incubation this increased to 57 μg, 68 per cent of which was β-carotene and only 15 per cent was phytoene[36]. *F. dehydrogenans* contains from 0.003 to 0.03 μg total carotenoids/mg cell-N in the dark-grown, non-photoactivated state and this increased to between 2 and 6 μg following photoactivation and dark-incubation[33]. Decaprenoxanthin was the only carotenoid detected in either system. If the various specific amounts of carotenoid are recomputed to approximately the same units, it is apparent that the bacterial systems all produce about the same amounts of total carotenoid in the non-photoactivated state. This is in the range 0.005 and 0.1 μg/mg cell weight. The *Neurospora* contained much more, *c.* 250 μg/mg cell weight. *F. dehydrogenans* distinguished itself in not producing detectable phytoene and in giving a greater carotenogenic response to photoactivation. Fifty-fold increases in total carotenoid following photoactivation were common with *F. dehydrogenans*, and the other microorganisms did not have more than ten-fold increases. The most probable explanation for these differences may be in the growth characteristics of *F. dehydrogenans* and not in the photoregulatory process.

Photoactivation is accomplished by illuminating a suspension of microorganisms, or a fungal mat, with polychromatic light or light of selected spectral quality. The procedure used in our studies of *F. dehydrogenans* is generally illustrative[31,32]. All manipulations, except for the actual photoactivation, were done in the dark or under a red light which did not photoactivate the bacterium. Cultures of dark-grown, non-photoactivated *F. dehydrogenans* were incubated until mid or late logarithmic growth phase which is between 12 and 16 h at 30°C. At this time the cells were recovered by centrifugation and resuspended to produce a stock suspension. This was used to prepare the test system which usually was a 50 ml volume in 0.03 M phosphate buffer, pH 7, and equivalent to an optical density of 1 at 580 nm. The test system was chilled to about 5°C and illuminated with light of known intensity and spectral quality for a predetermined time, usually 1–2 min. The

sample was contained in a beaker and stirred magnetically during illumination. Following this the photoactivated cell suspension was diluted with phosphate buffer containing sufficient amounts of the ingredients of the medium (Medium YE[32]) to produce 200 ml volume, and this then became a culture system to be incubated in the dark, 4 h, 30°C, for expression of photoactivated carotenogenic competence. The optical densities of the bacterial suspensions were always measured at the time of photoactivation and at the start and finish of dark incubation. The latter two measurements monitored growth during dark incubation and the final measurement was used in making computations of specific carotenoid since OD values have been related to bacterial cell-nitrogen for the bacterium[33]. Dilution of the bacterial suspension prior to the start of dark incubation was a necessary and critical step. The maximum populations of *F. dehydrogenans* in the medium used are equivalent to between 1.0 and 1.5 OD units at 580 nm. The duration of reproductive growth of a bacterial population is greatly influenced by the magnitude of the initial population and since it is possible to choose a starting population of almost any magnitude by varying the optical density of the inoculum, the choice will decide whether there are many, a few or no generations. The dilutions usually gave between 0.25–0.35 OD units at the start of dark incubation and this level allowed a maximum of three reproductive cycles before the maximum population was reached, assuming all the bacteria divided. Berry[32] estimated that about 35 per cent of the bacteria in these experimental systems divided during any one generation. In most of our studies the dark incubation time was restricted to 4 h which allows an average of one reproductive cycle to occur. *Figure 1* shows a typical photoactivated carotenogenic response during 4 h of dark incubation. Following the dark incubations the bacterial cells were recovered by centrifugation and the carotenoids extracted. Since the bacterium produced only decaprenoxanthin in the medium used, all that was necessary was to remove the carotenoid from the methanol extracts by phase separation into diethyl ether and measure the carotenoid directly by the spectrophotometric method[33]. The electronic absorption spectrum of decaprenoxanthin is distinctive and the presence of

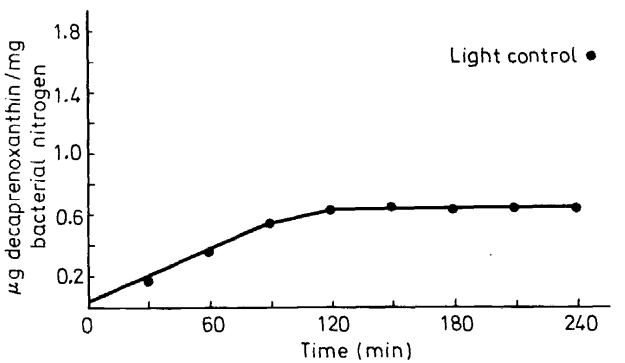

Figure 1. Specific carotenoid biosynthesis by *Flavobacterium dehydrogenans* in Medium YE during one generation time. Dark-grown populations were photoactivated for 15 min, *c.* 5000 lx heterochromatic light and subsequently incubated in the dark, 30°C, with continuous shaking and the carotenoid was determined at the incubation times shown[32]

any other of the coloured carotenoids produced by the bacterium is readily apparent from the absorption curve.

The usual aseptic procedures were used to culture dark-grown, non-photoactivated *F. dehydrogenans* in amounts adequate for experimentation but commencing with centrifugal harvesting of these cultures, and for the remainder of an experiment including dark incubation, non-sterile techniques could be used and usually were. This greatly simplified experimentation and was a perfectly feasible procedure. The time required to photoactivate a series of experimental systems seldom exceeded 2 h and the test systems were either held at low temperature or dark incubation started without delay. The short dark-incubation time (4 h) and the initially high OD at the start of the incubation both negate the influence of casual contamination which if it has occurred, has been of no obvious consequence during more than four years of experimentation.

THE PHOTOACT

In light-induced carotenogenesis the photoact may be defined as the activation by light of some molecular species which in its photoactivated state is able to initiate carotenoid biosynthesis. Once an experimental system has been arranged, search for the molecular species may commence with measurement of photoaction spectra. This has been done for *Neurospora crassa*[23], *Fusarium aquaeductuum*[41], *Mycobacterium* sp. and *Mycobacterium marinum*[29], *Myxococcus xanthus*[34] and *Flavobacterium dehydrogenans*[31]. The data suggest that the molecular species forming the photoreceptor may be of two types, flavin or porphyrin. Since most of the microorganisms do synthesize low levels of carotenoids in the non-photoactivated state, these also have been considered as possible photoreceptors[4]. For the two fungi, light in the wavelength range 360–520 nm was effective and the photoaction spectra generally resembled flavins. The effective spectral range was extended to 600 nm in the two *Mycobacterium* species and the photoaction spectrum was like a porphyrin. Photoactivation in both *Myxococcus xanthus* and *Flavobacterium dehydrogenans* occurred in the range 360–635 nm and the photoaction spectra were porphyrin-like although Saleh[31] suggested that a flavin–porphyrin complex could be the photoreceptor for the *Flavobacterium*. Attempts have been made to isolate the photoreceptor for both *Myxococcus xanthus* and *Mycobacterium marinum*[34,42]. The compounds isolated resembled protoporphyrin IX and either mesoporphyrin or coproporphyrin, respectively.

The energy requirement of the photoact has been estimated by several investigators. The energy requirement at about one-half saturating light dose was: *Mycobacterium* sp. 5.3×10^4 ergs/cm^2 at 445 nm[42]; *Mycobacterium marinum*, 3.6×10^5 ergs/cm^2 at 404 nm[4]; *Neurospora crassa*, 10^5 ergs/cm^2 at 465 nm[23]; and *Flavobacterium dehydrogenans*, 2.6×10^4 ergs/cm^2 at 436 nm and 4×10^3 ergs/cm^2 at 546 nm. *Figure 2* shows the energy requirements of *Flavobacterium dehydrogenans* for carotenoid responses at selected wavelengths[31]. Light intensities were measured by solution-phase actinometry[43] and spectral quality was controlled with second-order interference filters. The two most effective spectral regions are 360–450 nm and 500–570

nm. In its efficiency and sensitivity photoactivated carotenogenesis approaches such photoresponses as flowering and phototropism[44].

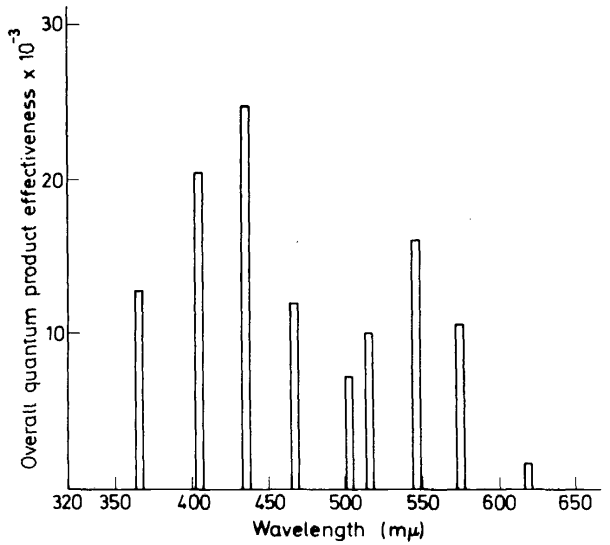

Figure 2. Photoaction spectrum for *Flavobacterium dehydrogenans* expressed as quantum product effectiveness (moles decaprenoxanthin/Einsteins). Dark-grown populations were photoactivated, 2 min, with light controlled by second-order interference filters and the light intensity measured by solution-phase actinometry. Cultures were incubated, 4 h, 30°C, in the dark following photoactivation and then the cells were analysed for total carotenoid[31]

THE CAROTENOGENIC RESPONSE

The mechanism by which the photoact becomes a carotenogenic response is not known and the concepts which have been proposed have been reviewed by Batra[4]. It would be very useful to know whether the entire carotenoid biosynthetic system is latent in the non-photoactivated state or whether the system is partially functional, although this would not necessarily decide the basis for photoregulation. Among the probable mechanisms would be photoregulated repression–depression and a light controlled regulatory protein (enzyme). Photoregulated repression–derepression mechanisms have most often been proposed[45,46] and seem attractive especially when based upon the histidine model.

The major event during dark incubation following the photoact is a burst of protein biosynthesis before carotenoids appear. In some species there is a delay before carotenoids are detectable and this has been termed the lag period[4]. In time this corresponds to 40–90 min for *Mycobacterium* sp. and *Neurospora crassa*[26,47] and extends up to 4 h for *Mycobacterium marinum*[32] and *Fusarium aquaeductuum*[48]. There is no apparent lag period for *Flavobacterium dehydrogenans*[32]. The reason for the lag is not known but it is during this period that the light-initiated protein biosynthesis occurs. The event has been studied indirectly using metabolic inhibitors known to effect

protein biosynthesis, or DNA or RNA regulation of the process. The compounds used included chloramphenicol[29, 32, 36], puromycin[32, 36], actinomycin D[32] and cycloheximide[49, 50]. In most instances the inhibitors prevented carotenogenesis following photoactivation when they were added prior to or at the time of the photoactivation, but not always when they were added at later times. Inhibition of carotenogenesis was not due to inhibition of growth. Berry's studies of the effects of chloramphenicol have shown that the development of carotenogenic competence in *F. dehydrogenans* is completed within 15 min of photoactivation[32]. This is shown in *Figure 3*

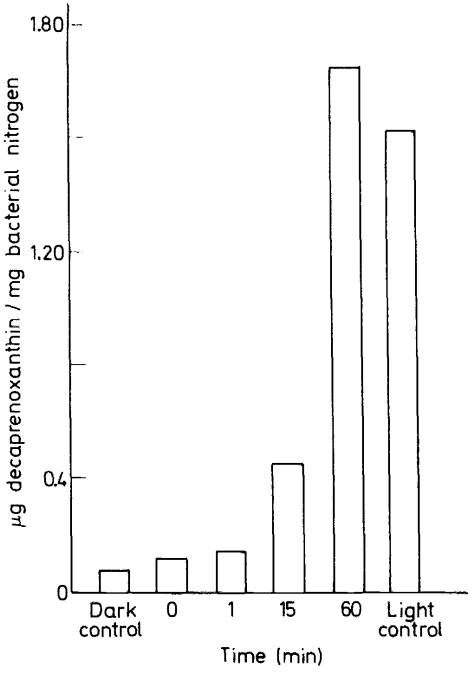

Figure 3. The effect of chloramphenicol on biosynthesis of carotenoid by *Flavobacterium dehydrogenans*. Chloramphenicol (15 μg/ml culture) added to each culture, except controls, at 0, 1, 15 and 60 min from start of incubation, 4 h, 30°C. All cultures were illuminated continuously during incubation except the dark control[32]

where the individual histograms represent specific amounts of decaprenoxanthin formed in replicate cultures to which chloramphenicol was added at the times shown, commencing immediately before the systems were lit (zero time). The experimental systems commenced with dark-grown non-photoactivated inocula and the carotenoid formed was assayed in all the cultures after 4 h incubation in the light. The specific amounts of decaprenoxanthin in cultures to which chloramphenicol was added before the lights were turned on and 1 min following, are essentially the same as the dark control which contained no chloramphenicol. The amount formed in the culture incubated 15 min before addition of the inhibitor was greatly increased and is about 25 per cent of that in the light control. This increase indicates that the enzymic

apparatus to express photoactivated carotenogenic competence is functional within 15 min of the photoact. The lower specific carotenoid amount quite probably is due to delays with which individual bacteria commence their reproductive cycle, rather than partial inhibition of carotenoid biosynthesis. Since conditions of growth synchrony did not exist in the test systems, the zero times of individual cells would not necessarily be the same as the experimental zero time. Assuming this is the explanation, the photoregulated protein biosynthesis was completed within a very short time.

Photoactivated carotenogenesis may be viewed in the perspective of a photoregulated protein biosynthesis and a question asked. Is this protein biosynthesis related only to the photoregulated carotenogenesis, or is it part of a larger phenomenon analogous, for example, to carotenoid biosynthesis occurring as part of light-regulated chloroplast development? There are illustrations of photoregulated protein biosynthesis in Nature. An example which is pertinent is the role of light in timing the cell-life cycle through control of RNA biosynthesis and therefore through proteins[51]. A second important illustration is phytochrome control of protein biosynthesis[52]. Some of the work we have done with *F. dehydrogenans* suggests that photoregulated carotenoid biosynthesis in this bacterium is part of a larger photoregulated event.

PHOTORESPONSES OF *FLAVOBACTERIUM DEHYDROGENANS*

Berry observed[32] that actinomycin D, added to the experimental system prior to photoactivation, prevented carotenogenesis in *F. dehydrogenans*. This suggested that there had been a critical interference with DNA-directed mRNA biosynthesis[53] and that this might have prevented development of photoactivated competence. Actinomycin D is known to bind to guanidine residues of DNA and prevent attachment of RNA polymerase to an initiation site[54]. Berry devised an experimental system to determine whether there was any influence of light upon the RNA system. This consisted of measuring the differential incorporation of 2-^{14}C-uracil and ^{3}H-thymidine by non-photoactivated and photoactivated, dark-grown suspensions of the bacterium. The effect of light upon incorporation of 2-^{14}C-uracil is shown in *Figure 4*. The two experimental systems were identical aliquots taken from a stock bacterial suspension in 0.02 M phosphate buffer (pH 7) which had been prepared from a 16-hour-old culture of dark-grown, non-photoactivated *F. dehydrogenans*. The two test systems were stirred magnetically and at zero time, 10 μCi of 2-^{14}C-uracil plus 11 μg uracil, were added to each. Point sampling commenced after 1 min with simultaneous removal of 0.5 ml from each sample and this procedure was continued for the 15 min of the experiment. Immediately following the first sampling, one of the systems was illuminated for 2 min and then the light turned off. The data show that the lit system incorporated more uracil than the dark one and that the differential became apparent with the first sample taken after illumination started. The maximum differential incorporation was 46 per cent at 6 min and at 15 min the differential had almost disappeared. A second experiment showed that actinomycin D added to one of two identical, photoactivated systems abolished nearly 60 per cent of the uracil incorporated. The incorporation of

^{3}H-thymidine by photoactivated and non-photoactivated experimental systems was also tested and no differential incorporation was found (*Figure 5*). These data support the concept of a photoregulated RNA process in which DNA biosynthesis is not required. It is tempting to accept the result as support for photoregulated repression–derepression control of carotenogenesis

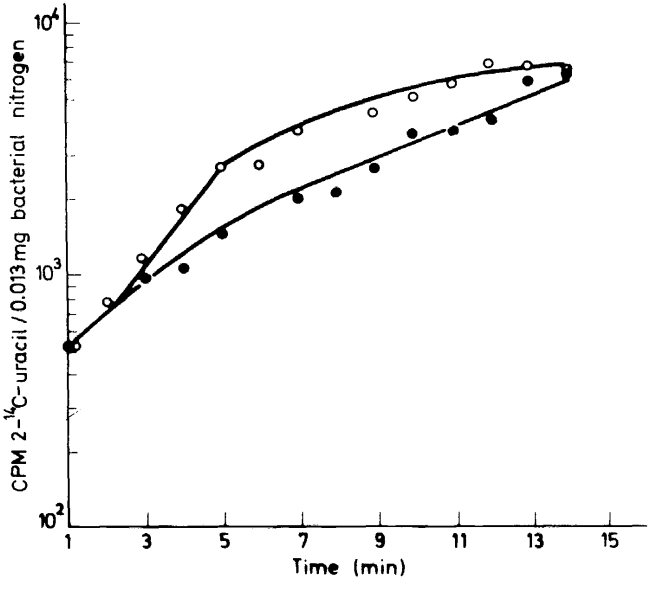

Figure 4. Incorporation of 2-^{14}C-uracil by photoactivated and non-photoactivated *Flavobacterium dehydrogenans* suspended in 0.02 M phosphate buffer, pH 7[32]. Identical aliquots were prepared from 16-hour-old, dark-grown, non-photoactivated cultures and 10 μCi, 2-^{14}C-uracil + 11 μg uracil, added to each at zero time. After 2 min one of the aliquots was photoactivated (2 min, *c.* 11 000 lx, heterochromatic light) and then returned to dark conditions. Point sampling to establish incorporation of 2-^{14}C-uracil was done at 1 min intervals. Photoactivated suspensions, O—O; non-photoactivated suspensions, ●—●

in *F. dehydrogenans*, but other studies with the bacterium suggest this would be premature. Berry's data are similar, however, to a much more detailed study of a photoregulated protein biosynthesis done with maize seedlings[55]. The effects of illumination of etiolated maize seedlings included increased incorporation of ^{14}C-leucine by ribosomes. One very interesting feature of the data reviewed by Mans[55], was that the light-stimulated incorporation of the leucine persisted for 11 h in the dark. He proposed that either the mRNA formed in response to light was much longer-lived than that of bacteria, which is known to have a very short existence, or that synthesis of mRNA continued in the dark after commencing as a photoresponse. Carotenogenic competence which follows photoactivation in the non-photosynthetic microorganisms also persists for long periods in the dark, i.e. the photoactivated competence continues to be expressed in the dark once the state has been attained.

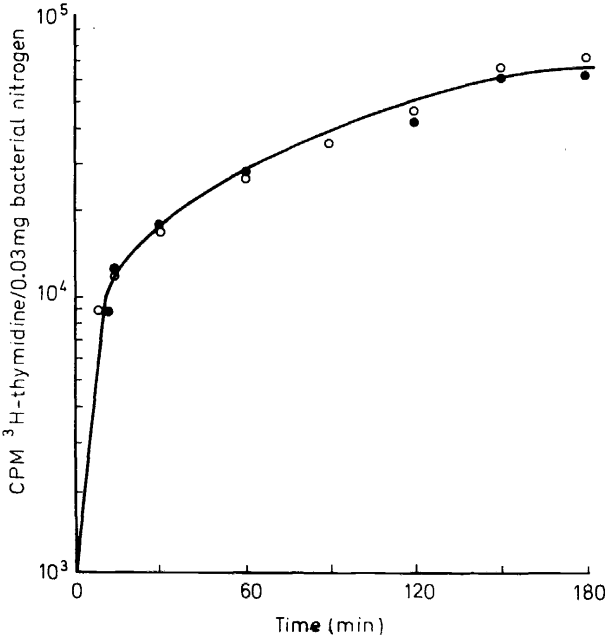

Figure 5. Incorporation of ^{3}H-thymidine by photoactivated and non-photoactivated *Flavobacterium dehydrogenans* suspended in 0.02 M phosphate buffer, pH 7[32]. Conditions as in *Figure 4* except 100 μCi ^{3}H-thymidine + 100 μg thymidine added instead of the uracils. Photoactivated suspensions, O--O; non-photoactivated suspensions, ●—●

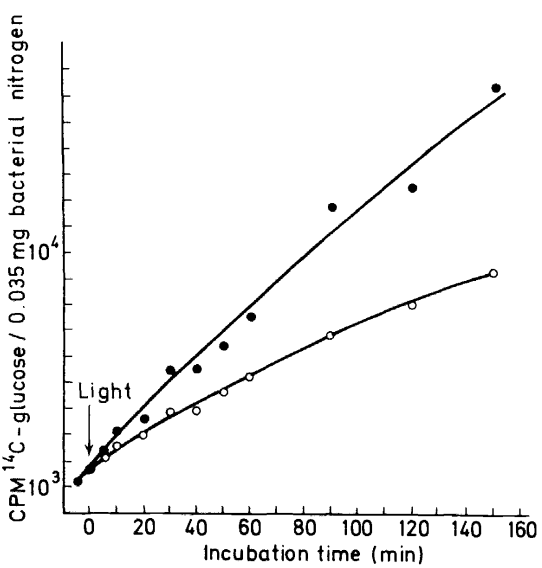

Figure 6. The effect of light on the incorporation of uniformly labelled ^{14}C-glucose into the total extractable lipid of *Flavobacterium dehydrogenans*[58] (see text). Light incubation, O--O; dark incubation, ●—●

Light-regulated carotenogenesis and uracil incorporation in *F. dehydrogenans* could either be unrelated responses or aspects of a single event. There is a third type of photoresponse which involves the lipids of *F. dehydrogenans* grown in light or dark conditions, which has been shown in some very recent studies by Wirahadikusumah[58]. His investigations were of the lipids of dark- and light-grown populations. There were no quantitative differences in the weights of total lipid extracted from the two populations. Chromatographic analyses (t.l.c.) of the total lipid extracts showed no qualitative differences in the major classes of lipids, with the exception of an unidentified phospholipid which was only present in the dark-grown populations. Studies of the fatty acid components showed that more dramatic qualitative and quantitative differences existed. The analysis of the fatty acids as methyl esters (g.l.c.) showed nine fatty acids in the lipid extracts and apparently the same compounds occurred in both of the bacterial populations. Four of the compounds, those with the least retention times, were not identified and made up less than 12 per cent of the total fatty acids in the light-grown populations and almost 25 per cent in the dark-grown populations. The remaining five compounds were identified as palmitate, palmitoleate, stearate, oleate and linoleate. He found greater amounts of palmitate, palmitoleate, stearate and oleate and lesser amounts of the compounds with shorter g.l.c. retention times, in the light-grown populations than in the dark-grown populations. Distribution of the two categories in light were 88 and 12 per cent, and in the dark, 72 and 28 per cent. Lipid biosynthesis in *F. dehydrogenans* grown in light favoured formation of fatty acids with 16 C-atoms or more, at the expense, seemingly, of fatty acids with shorter C-chains. Changes in the fatty acids of photosynthetic tissues occur as a photoresponse, which has been associated with photophosphorylation and consequent production of NADPH and ATP[60]. Mudd and McManus have shown that the requirement for photophosphorylation could be replaced by added glucose-6-phosphate[61]. The data obtained by Wirahadikusumah leave no doubt that light influences lipid biosynthesis in *F. dehydrogenans*. This might be related to changes in the bacterial membranes and eventually to photoactivated carotenogenesis.

Wirahadikusumah[58] also observed an effect of light upon amounts of ^{14}C-glucose and ^{14}C-acetate incorporated into extractable lipids of *F. dehydrogenans* (*Figures 6* and 7). The same experimental procedure was used in both test systems. A non-photoactivated inoculum was used to commence a culture in Medium YE, and this was incubated in the dark. The need to remove samples for extraction of lipid at the start was satisfied by using a large enough inoculum to produce an initially large bacterial density (0.5 OD). Radio-labelled compound was added shortly after incubation commenced and 10 min later a sample was taken to measure the incorporation of isotope into lipid. Following this the culture was divided into two equal aliquots and dark incubation continued long enough to take a second sample from each aliquot. After this, one aliquot was incubated in light for the remainder of the experiment, about 2 h. The second aliquot was incubated in the dark continuously. Results are summarized in *Figure 6* for the incorporation of ^{14}C-glucose and in *Figure* 7, for ^{14}C-acetate. The salient feature in both instances is the reduction in substrate incorporation into lipid in the illuminated cultures. The differential incorporation became apparent in each

instance shortly after illumination commenced and the maximum was 42 per cent for the glucose and 10 per cent for the acetate. Decreased incorporation in light is not the result of substrate being shunted from lipid biosynthesis into carotenoid since the amounts of glucose and acetate involved greatly exceed the amounts of carotenoid formed. A more reasonable explanation is that there was an alteration in the general metabolism of *F. dehydrogenans* as the result of exposure to light, which might have necessitated a physiological adjustment to the requirements imposed by the new environment. A shift-down in the metabolism of a bacterium may be followed in the rates of RNA and protein biosynthesis, and eventually in terms of DNA replication. Berry commenced direct measurements of this and his preliminary results indicate a decrease in RNA biosynthesis immediately after dark-growing populations were placed in light[32]. Light-induced shifts in metabolic rates in algae have been reported[51].

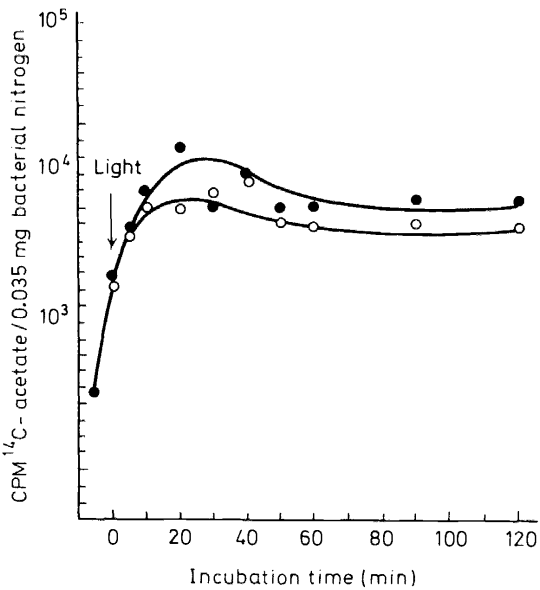

Figure 7. The effect of light on the incorporation of ^{14}C-acetate into total extractable lipid in *Flavobacterium dehydrogenans*[58] (see text). Light incubation, O—O; dark incubation, ●--●

If the photoresponses observed in *F. dehydrogenans* are related to physiological adjustment required for growth of populations in light, photoregulated carotenoid biosynthesis could occur as part of the development of the population cycle since the event seems to require reproducing populations. The experimental system used in studies of photoactivated carotenogenesis restricts the event to one generation of the population cycle (*Figure 1*). If dark incubation following photoactivation is extended beyond the usual 4 h and bacterial growth is measured as well, it is apparent that the burst of carotenoid biosynthesis is restricted to the first generation following the photoact (*Figure 8*). The decrease in specific carotenoid observed after the

first 4 h is due to the diluting effect of successive generations of non-photoactivated, and therefore non-pigmented, progeny. Photoactivated carotenogenic competence is not passed from the first to successive generations of *F. dehydrogenans*. The effect seems to be confined to the bacteria which receive light. Support for this concept comes from experiments which show that periodic photoactivation during the population cycle results in a constant amount of specific carotenoid[31] and continuous illumination, in increasing amounts[33]. The coincidence of growth and photoactivation of carotenogenesis could be the result of a need to add carotenoid to the developing membranes of the bacterium, in response to a requirement for protection against photodynamic sensitization[5]. Reproductive growth is not always a requirement in photoactivated carotenogenesis. *Myxococcus xanthus* does not commence this until its populations have reached the maximum stationary phase and active reproduction has ceased[34]. The inclusion of carotenoids in bacterial membranes is well established[57].

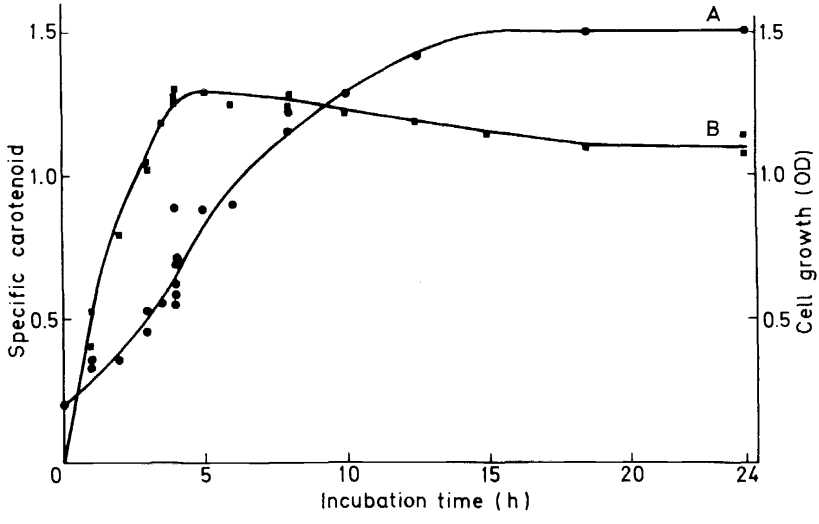

Figure 8. Carotenogenic response of dark-grown, non-photoactivated cultures of *Flavobacterium dehydrogenans* following photoactivation, 15 min, *c.* 55 000 lx, heterochromatic light[31]. The photoactivated cultures were incubated in the dark following illumination and growth measured as optical density at 580 nm, at the indicated time intervals. Carotenoid content of the bacterial cells was measured at the same times. A, growth curve; B, carotenoid response

We have demonstrated that *F. dehydrogenans* will not survive the lethal effects of photodynamic sensitization[56] when toluidine blue is added as an exogenous photosensitizer[58]. The protective effects of the carotenoid are shown in *Figure 9*. The data show the effects of photosentization by toluidine blue upon suspensions of carotenoidless (dark-grown) and carotenoid-containing (light-grown) *F. dehydrogenans* when suspensions of the two populations are held in light and dark conditions. The number of surviving bacteria at the time intervals were counted by the plating procedure. The data are typical for such studies[5] with one exception. Carotenoid-containing

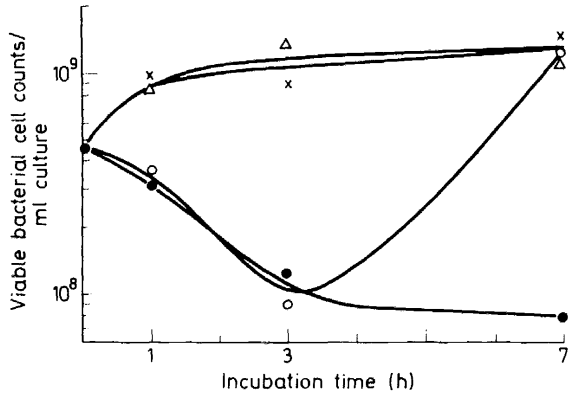

Figure 9. Effect of toluidine-blue-mediated photosensitization on the survival of non-pigmented and pigmented *Flavobacterium dehydrogenans*[58]. Toluidine-blue-containing, dark-grown incubated in the dark, △—△; toluidine-blue-containing, dark-grown incubated in the light, ●—●; toluidine-blue-containing, light-grown incubated in light, ○—○; light-grown incubated in light, no toluidine blue added. × — ×

F. dehydrogenans was not protected during the first 3 h of testing but after this the number increased greatly. This result has been verified and seems to indicate that lethal photodynamic sensitization occurs unless the pigmented populations are growing. Wirahadikusumah also showed that an externally added photosensitizer was not necessary to demonstrate occurrence of photodynamic damage in carotenoidless populations of *F. dehydrogenans*. In these studies (*Figure 10*) the carotenoidless (dark-grown) and carotenoid-containing (light-grown) populations grew equally well in the dark, but

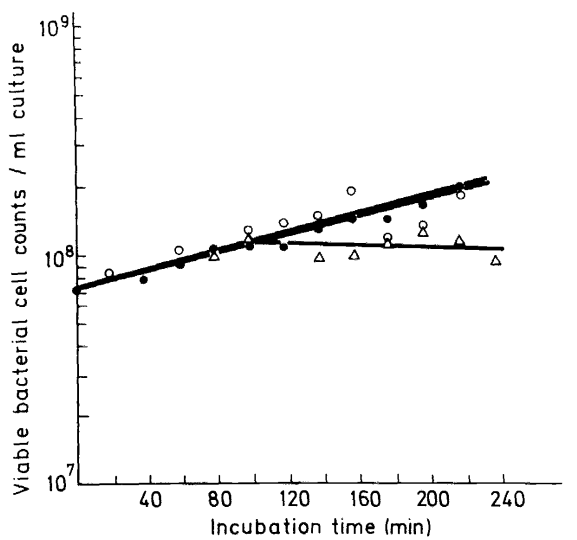

Figure 10. The effect of visible light on the survival of pigmented and non-pigmented *Flavobacterium dehydrogenans*[58]. Dark-grown cells (carotenoidless) incubated in the dark, ●—●; dark-grown cells (carotenoidless) incubated in light, △—△; light-grown (carotenoid-containing) cells incubated in light, ○—○

exposure of the dark-grown population to light resulted in a cessation of growth after about 100 min. This could indicate that an adjustment was being made to permit growth in light since there was no apparent decrease in the numbers.

In summary, photoregulation of carotenoid biosynthesis in *F. dehydrogenans* probably is the central aspect of a general photoresponse of this non-photosynthetic bacterium. Data support the belief that the events which have been observed represent a re-ordering of the bacterial metabolism when populations growing in the dark are shifted to light. The result of the adjustment may be a modification of the bacterial membranes to contain carotenoid which serves a protective role against photodynamic sensitization and permits the bacterium to grow in light.

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