THE PATH OF CAROTENOID SYNTHESIS IN A PHOTOSYNTHETIC BACTERIUM*

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INTRODUCTION

The carotenoid pigments form a strikingly homogeneous class of naturally occurring compounds, which are synthesized *de novo* by all green plants, and by many bacteria and fungi. The distinctive common structural features of these pigments suggest that they may be formed biologically by a single primary sequence of reactions, the minor structural differences which characterize the individual members of the class being produced by terminal divergences from the common primary pathway. The general biochemical problems of carotenoid formation are: the nature of the condensations which give rise to the unique C_{40} skeleton; the mechanism for the formation of the extensive system of conjugated double bonds; and lastly, in the case of alicyclic carotenoids, the mechanism of ring closure. In spite of the large amount of work which has been done on carotenoid formation in plants and microorganisms, none of these general problems has yet been solved. In the present paper we shall describe a series of experiments which establish the nature of the terminal step-reactions in the formation of aliphatic carotenoids by the photosynthetic bacterium, *Rhodospirillum rubrum*.

The carotenoid pigments of *R. rubrum* have been extensively studied. The first investigations by VAN NIEL AND SMITH[1] and POLGAR, VAN NIEL AND ZECHMEISTER[2] were conducted on cells from old cultures, and showed that one compound, spirilloxanthin, accounted for about 95 % of the carotenoid present. Spirilloxanthin is an aliphatic pigment which contains 13 double bonds in conjugation and two methoxyl groups, presumably attached in the 3 and 3′ positions[2,3]. Many years later, DUYSENS[4] observed that the absorption spectrum of intact cells changes with the age of the culture in a manner which suggested that spirilloxanthin might not be the major pigment in young populations. This inference was confirmed by VAN NIEL, GOODWIN AND SISSINS[5], who analyzed the pigment system of *R. rubrum* at various stages of development, and found no less than five additional carotenoids in rapidly growing cells. These compounds are lycopene, lycoxanthin (3-hydroxy-lycopene), demethylated spirilloxanthin, and two pigments of undetermined structure which were originally discovered in another photosynthetic bacterium and designated as P481 and hydroxy P481[6]. Lycopene and lycoxanthin are aliphatic carotenoids which contain 11 double

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bonds in conjugation. The spectrum of P481 suggests that it is also aliphatic, and contains 12 double bonds in conjugation.

GOODWIN AND OSMAN[7,8] investigated the effects of diphenylamine on *R. rubrum*, and found that at appropriate concentrations it prevents normal carotenoid synthesis without arresting growth. In their first paper, they reported that no unusual carotenoids accumulate in cells which have been grown with diphenylamine, but this claim was withdrawn in their second paper, where they stated that such cells contained "traces" of phytoene and other relatively saturated carotenoids. GOODWIN AND OSMAN made one additional observation of cardinal importance for the study of carotenoid synthesis: namely, that when cells of *R. rubrum* which have been depleted of normal carotenoids by growth in the presence of diphenylamine are washed free of the inhibitor and resuspended in buffer, they are able to perform an extensive endogenous synthesis of spirilloxanthin. GOODWIN AND OSMAN did not identify the intracellular substances which provide the carbon for this endogenous synthesis, and assumed that they were non-specific reserve materials.

TERMINOLOGY

We shall use the word *carotenoid* to describe any C_{40} polyene which has the carotenoid skeleton. Phytoene, phytofluene and hydroxyphytofluene, the three colorless compounds of this class with which we shall be concerned, will accordingly be termed colorless or more saturated *carotenoids*. We shall refer to a carotenoid hydrocarbon and its hydroxy derivatives as a *group*; thus, the *lycopene group* comprises lycopene and hydroxylycopenes.

The abbreviation DPA will be used for diphenylamine. *DPA-grown cells* are cells of *R. rubrum* which have been grown photosynthetically in the presence of DPA for a time sufficient to produce a substantial reduction (90 % or greater) in the level of the normal carotenoid pigments. *Normal cells* are cells of *R. rubrum* which have been grown photosynthetically in the absence of DPA, and consequently contain a normal complement of carotenoids.

MATERIALS AND METHODS

Bacterium

Rhodospirillum rubrum strain 1.1.1, originally obtained from the collection of Professor C. B. VAN NIEL.

Medium

All cultures were grown in the modified medium of HUTNER[9]. When DPA was employed as an inhibitor of normal carotenoid synthesis, it was added several hours after the inoculation of the medium as a solution in 95 % ethanol. The final concentration of DPA in the medium was varied in different experiments between 6 and $7 \cdot 10^{-5}$ *M*. The concentration of the alcoholic solution was such that the final concentration of ethanol in the medium never exceeded 0.25 %.

Cultivation

Liquid stock cultures were grown in cotton-stoppered Florence flasks of 50 ml capacity, filled to the neck with medium. After inoculation, the flasks were incubated in a light cabinet at a temperature of $30 \pm 2°$. For experiments, cultures were grown in Roux bottles containing 750 ml of medium, and closed with rubber stoppers fitted with gas inlet, gas outlet and sampling tubes. After inoculation, bottles were placed as close as possible to the entrance window of a rectangular glass water bath, maintained at a temperature of 30°, which was evenly illuminated with 300-W tungsten lamps. The light intensity at the surface of the entrance window was 1000 foot-candles. When the medium contained DPA, a yellow filter (Corning 3484), transmitting only wavelengths

longer than 507 mμ, was placed in front of the entrance window, in order to prevent the photochemical destruction of DPA. Cultures were continuously aerated with N_2 containing 5% CO_2.

Bottle cultures were inoculated with exponentially growing cells, to give an initial optical density at 680 mμ of 0.004 (equivalent to $2 \cdot 10^6$ cells per ml). They were harvested in the exponential phase of growth when the E_{680} had reached 0.300–0.400. Under the growth conditions employed, normal cells attain this density 18–20 h after inoculation, and DPA-grown cells 60–70 h after inoculation.

Resting cell suspensions

Bottle cultures were chilled, after which the cells were harvested by centrifugation. They were then washed once and resuspended in a chilled solution of the modified HUTNER medium[9] devoid of ammonium malate and casein hydrolysate. This solution, which cannot support growth, will be referred to hereafter as "Hutner phosphate buffer".

Measurements on cell suspensions

Cell mass was determined by measurement of optical density at 680 mμ[9]. A suspension of *R. rubrum* with an E_{680} of 1.000 contains about $5 \cdot 10^8$ bacteria per ml, equivalent to a dry weight of 1.0 mg/ml. The extraction and measurement of bacteriochlorophyll were performed as earlier described[9]. Rough measurements of total carotenoid were performed on the same extracts, by determining the optical density at 485 mμ, corrected for light absorption by bacteriochlorophyll at this wavelength. The protein content of cell suspensions was determined by the biuret method[10], with two minor modifications. The cells were first sedimented and extracted with an acetone–methanol mixture (7:2 v/v) in order to remove the pigments, which interfere with the measurement of the biuret color. After the biuret reaction had been performed on the extracted cells, the sample was centrifuged before the spectrophotometric reading was made, in order to remove light-scattering cellular debris which is not dissolved by the reagent.

Carotenoid extraction

Quantitative carotenoid analyses were performed on cell suspensions with a volume of 300–500 ml, and an E_{680} of 0.300–0.400. Duplicate 10-ml aliquots were set aside for protein and bacteriochlorophyll determinations. The volume of the remaining solution was accurately measured, after which the cells were sedimented in the cold (2–5°) by centrifugation, washed once with cold Hutner phosphate buffer, and resedimented. The bacterial pellet was resuspended in a minimal volume of water, and the pigments were extracted with successive portions of 7:2 acetone–methanol, followed, in the case of samples with a high content of spirilloxanthin, by pure acetone. The combined extract was saponified for 5 min at room temperature with methanolic KOH. A sufficient quantity of 25% KOH in methanol was added to the extract to provide a final alkali concentration of 5%. A small volume of petroleum ether was then added to the saponified mixture, and the carotenoids were transferred to the petroleum ether layer by dilution of the acetone–methanol phase with sufficient concentrated aqueous NaCl solution to reduce the concentration of acetone and methanol to 10%. The extraction was repeated once, after which the combined petroleum ether extract was washed free of acetone and methanol, and dried over anhydrous Na_2SO_4.

Chromatography and quantitative estimation of carotenoids

The dried petroleum ether extract, containing about 300 μg of carotenoids (apart from phytoene) was concentrated *in vacuo* to a volume of 5 ml. The pigments were separated on a 1 cm × 15 cm column of Woelm neutral aluminum oxide, activity grade 2 (ref.[11]). The column was packed by adding successive small portions of the adsorbent suspended in petroleum ether, each portion being allowed to settle before the next addition. No pressure or suction was applied during the chromatography, which took about 4 h. The column was developed first with pure petroleum ether (b.p. 30–50°), followed in turn by petroleum ether–diethyl ether mixtures, petroleum ether–acetone mixtures and petroleum ether–methanol mixtures. The petroleum ether had been previously freed of benzene by passage through a column of SiO_2[2]. Table I shows the development of a typical chromatogram.

The absorption spectra of the eluted fractions were measured with a Beckman recording spectrophotometer, and the carotenoid content was calculated on the basis of the extinction coefficients listed in TABLE II. For the hydroxy compounds, the extinction coefficient of the corresponding hydrocarbon was employed.

As shown in TABLE I, many of the unhydroxylated carotenoids were isolated in the form of several geometrical isomers. It cannot at present be decided whether these isomers all occur naturally, or whether some of them are formed during the isolation. In any case, they complicate the problem of quantitative measurement. The *cis* isomers of carotenoids have in general lower extinction coefficients than the all-*trans* forms, but the exact values of their extinctions are known in very few cases. Accordingly, no attempt was made to assign a specific value for the extinction

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TABLE I

CHROMATOGRAPHIC SEPARATION OF CAROTENOIDS SYNTHESIZED BY *R. rubrum* IN THE PRESENCE OF DPA

Zones in order of increasing adsorption	*Color of zone*	*Required eluant*	*Identification*
a	colorless	pure petr. ether	Phytoene
b	green fluorescence in U.V.	pure petr. ether	Phytofluene
c	yellow	pure petr. ether	ζ-Carotene – 1st isomer
d	yellow	5% ether in petr. ether	ζ-Carotene – 2nd isomer
e	yellow	5–10% ether in petr. ether	Neurosporene – 1st isomer
f	yellow	5–10% ether in petr. ether	ζ-Carotene – 3rd isomer
g	yellow	10% ether in petr. ether	P412
h	yellow	10–15% ether in petr. ether	Neurosporene – 2nd isomer
i	orange	15–20% ether in petr. ether	Lycopene – 1st isomer
j	yellow	20–25% ether in petr. ether	P450
k	orange	25% ether in petr. ether	Lycopene – 2nd isomer
l	purple	2.5% acetone in petr. ether	P481 – 1st isomer
m	purple	5% acetone in petr. ether	Spirilloxanthin – 1st isomer
n	orange	5–6% acetone in petr. ether	P481 – 2nd isomer
o	purple	6% acetone in petr. ether	Spirilloxanthin – 2nd isomer
p	yellow	5–7% acetone in petr. ether	OH-Phytofluene
q	yellow	6–7% acetone in petr. ether	OH-ζ-Carotene
r	yellow	7–8% acetone in petr. ether	OH-Neurosporene
s	yellow	7–8% acetone in petr. ether	OH-Lycopene
t	orange	8–12% acetone in petr. ether	OH-P481
u	purple	12% acetone in petr. ether	OH-Spirilloxanthin
v	yellow	4% methanol in petr. ether	di-OH-ζ-Carotene
w	yellow	25% methanol in petr. ether	di-OH-Lycopene

Additional isomers of lycopene, P481 and spirilloxanthin often occurred.

TABLE II

ABSORPTION DATA FOR THE CAROTENOIDS OF *R. rubrum*

Carotenoid	*Positions of absorption maxima in petroleum ether, mμ*			$E^{1\%}_{1\,cm}$ *for main maximum*	*Literature reference for extinction value*	*Number of conjugated double bonds*
Phytoene	274	284	296	1400	28	3
Phytofluene	332	347	367	1500	28	5
OH-Phytofluene	332	347	367			5
ζ-Carotene	376	396	418	2000	29	7
OH-ζ-Carotene	376	395	417			7
di-OH-ζ-Carotene	378	397	420			7
P412	388	412	438	2370*		8
Neurosporene	413.5	438	468	2740	13	9
OH-Neurosporene	414	437	466			9
P450	423	450	480	3100**		10
Lycopene	446	474	506	3460	12	11
OH-Lycopene	445	474	506			11
di-OH-Lycopene	441	471	501			11
P481	455	482	514	2500	6	12
OH-P481	455	482	515			12
Spirilloxanthin	464	491	524	2350	2	13
OH-Spirilloxanthin		489	523			13

* From extrapolation between extinction coefficients of ζ-carotene and neurosporene.
** From extrapolation between extinction coefficients of neurosporene and lycopene.

coefficient of each individual *cis* isomer; instead, the fixed values listed in Table II were employed for all the isomers of a given carotenoid. Calculations showed that the probable error so introduced would not materially affect the results.

Some *cis* isomers have absorption maxima at wavelengths considerably shorter than those of the all-*trans* parent compound, and can therefore be confused with isomers of carotenoids that have a less extensive conjugation system. This problem of identification arose in our experiments in connection with the numerous isomers of lycopene, P481 and spirilloxanthin. The correct assignment of such isomers could not be made simply on the basis of the order of elution from the column (*cf.* Table I). In such cases, the occurrence of *cis* peaks at characteristic wavelengths served to determine the parent carotenoid. *Cis* peaks commonly occur at a wavelength 142 $\pm$ 2 mμ shorter than the maximum at longest wavelength of the all-*trans* parent compound[12].

Identification of carotenoids

Exclusive of geometrical isomers, nearly 20 different carotenoids were present in some of the mixtures analyzed in the present work. Many of these could be identified with certainty on the basis of our own or earlier studies, but in some cases the identifications were tentative or inferential. The evidence on which the identification of each compound was based is summarized below. Letters refer to the zones on columns described in Table I and to the corresponding eluted fractions.

Hydrocarbons

a) *Phytoene.* Phytoene was identified by its ultraviolet absorption spectrum and chromatographic behavior. The presence of phytoene in DPA-grown cells of *R. rubrum* was shown earlier by GOODWIN AND OSMAN[8], who also proved its identity by mixed chromatography.

The quantitative determination of phytoene in our experiments was complicated by the presence of DPA. Phytoene and DPA have overlapping ultraviolet absorption spectra, and are similarly adsorbed on "Woelm" neutral alumina grade 2, which was otherwise found to be the most favorable adsorbent for the separation of the carotenoid mixtures encountered. A compound with a maximum at 253 mμ in petroleum ether was also partly eluted in the phytoene fractions.

Rechromatography of the phytoene-containing fractions on "Woelm" basic alumina activity grade 1 (ref.[11]) resulted in satisfactory separation from DPA and fair separation from the compound with a maximum at 253 mμ, which was more strongly adsorbed. However, this procedure introduced the error of rechromatography into the phytoene determination.

We therefore consider our values for phytoene to be at best approximations, without the quantitative reliability of the values which are reported for the other carotenoids.

b) *Phytofluene.* Phytofluene was identified by its ultraviolet absorption spectrum, its green fluorescence in ultraviolet light, and its chromatographic behavior. GOODWIN AND OSMAN[8] had previously established its presence in DPA-grown cells of *R. rubrum* by mixed chromatography.

c, d, f) *ζ-Carotene.* These fractions were tentatively identified from their absorption spectrum and chromatographic behavior as ζ-carotene isomers. GOODWIN AND OSMAN[8] have shown the presence of ζ-carotene in DPA-grown cells of *R. rubrum* by mixed chromatography.

e, h) *Neurosporene.* Zone h was identified as neurosporene. The absorption spectrum of a chromatographically purified fraction was superimposable on that of crystalline neurosporene, isolated from the green mutant of *Rhodopseudomonas spheroides*, and mixed chromatography of the two substances on alumina gave a single zone. The neurosporene from the green mutant of *R. spheroides* had been previously identified[13] with the neurosporene originally discovered in *Neurospora crassa*[14]. Fraction e shows the same absorption spectrum and is assumed to be a neurosporene isomer.

GOODWIN AND OSMAN's fraction H, which they suggested to be a carotenoid containing ten double bonds[8], has absorption maxima similar to our neurosporene.

g) *Pigment with maxima at 388, 412 and 438 mμ in petroleum ether.* This carotenoid is so far unidentified, but probably identical with GOODWIN AND OSMAN's fraction I, suggested to be an open-chain compound containing a chromophore of 8 conjugated double bonds[8]. We have designated this carotenoid as P412, from the position of the main peak in petroleum ether. This is in accordance with the nomenclature for P481 (ref.[6]).

i, k) *Lycopene.* These fractions were identified as lycopene isomers by comparison with the data of VAN NIEL, GOODWIN AND SISSINS[5] on lycopene in *R. rubrum*. Absorption spectra, chromatographic behavior and iodine-isomerization studies agreed with their findings.

j) *Pigment with maxima at 423, 450 and 480 mμ in petroleum ether.* This carotenoid is unidentified. It is probably identical with fraction G of GOODWIN AND OSMAN[8]. The absorption maxima correspond to those of an open-chain compound containing 10 conjugated double bonds. For reference purposes we have called this carotenoid P450.

l, n) *P481.* The absorption spectrum and chromatographic behavior correspond to those of P481, the presence of which in *R. rubrum* was first indicated by VAN NIEL AND SMITH[1] and later confirmed by VAN NIEL *et al.*[5]. This carotenoid was first considered to be a *cis* isomer of

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spirilloxanthin[8]; later evidence suggested an all-*trans* configuration[6]. The structure of this carotenoid is not yet established.

P481 shows great steric lability. It crystallizes in the all-*trans* form. The absorption spectrum of a fresh hexane solution shows sharp resolution into narrow bands, suggesting an open chain carotenoid. The positions of the absorption maxima indicate a chromophore of 12 conjugated double bonds. A hexane solution of P481 isomerizes within a few minutes at room temperature in diffuse daylight, with appearance of a double *cis* peak at 355 and 373 mμ. Iodine isomerization is spontaneous. It is accompanied by the normal spectral shifts characteristic for *trans-cis* isomerization.

m, o) *Spirilloxanthin.* Absorption spectrum, chromatographic behavior, iodine isomerization and solubility properties agreed with the data given by various authors[1,2,5,8] for spirilloxanthin.

OH-derivatives

p) *OH-phytofluene.* Absorption spectrum and fluorescence are similar to those of phytofluene, but the compound is more strongly adsorbed on the column. It is epiphasic in the partition test between petroleum ether and 90% methanol. These data suggest the presence of one OH-group.

Such a compound has been found in DPA-grown cells of other purple bacteria[6].

q) *OH-ζ-carotene.* The suggestion of a mono-OH-ζ-carotene is based on absorption spectrum, chromatographic behavior and appearance in the epiphase in partition between petroleum ether and 90% methanol.

GOODWIN AND OSMAN[8] described a compound with the same properties in DPA-grown cells of *R. rubrum*.

Carotenoids containing allyllic OH-groups generally react with chloroform containing HCl, to yield a longer conjugated chain by splitting off a molecule of water[15]. With this carotenoid, HCl-chloroform treatment gave no other product. The OH-group is therefore probably not in α-position to the conjugated chain.

r) *OH-neurosporene.* This fraction seems to bear the same relationship to neurosporene as fraction q does to ζ-carotene. Absorption spectrum, chromatographic behavior and epiphasic appearance in the partition test with 90% methanol again suggest a mono-OH-compound.

A chromatographically purified fraction did not react with HCl-chloroform. The OH-group is therefore again probably not in α-position to the conjugated chain.

s) *OH-lycopene.* Absorption spectrum, chromatographic behavior and behavior in the partition test resemble those of lycoxanthin (3-hydroxylycopene), earlier found in *R. rubrum*[8].

t) *OH-P481.* The absorption spectrum is indistinguishable from that of P481. In view of its stronger adsorption on alumina and its behavior in the partition test (equal distribution between petroleum ether and 95% methanol; epiphasic to 90% methanol), GOODWIN AND LAND[6] designated this carotenoid OH-P481.

u) *OH-spirilloxanthin.* Adsorption and partition test behavior (epiphasic to 90% methanol) indicate the presence of one free OH-group. GOODWIN AND OSMAN[8] suggested that this compound was a mono-OH-spirilloxanthin or a demethylated spirilloxanthin. We have called it OH-spirilloxanthin. A crystalline specimen showed a less defined absorption spectrum than spirilloxanthin, with no *cis* peak and maxima at slightly shorter wavelengths. The compound is spontaneously isomerized by iodine, with the usual spectral changes.

Absorption maxima in CS_2:

all-*trans*-spirilloxanthin	500,532,568 mμ
all-*trans*-OH-spirilloxanthin	529,565 mμ

v) *di-OH-ζ-carotene.* The absorption spectrum is similar to that of ζ-carotene. The compound is equally distributed between petroleum ether and 95% methanol. In distribution between petroleum ether and 90% methanol, 66% of the pigment was found in the hypophase. The carotenoid is presumed to be a di-OH-ζ-carotene.

w) *di-OH-lycopene.* The properties resemble those of lycophyll (3,3-di-OH-lycopene)[16]. Its absorption spectrum is similar to that of lycopene, and it is hypophasic in partition with 90% methanol. A similar compound has been found in the purple bacterium *Chromatium* sp.[6].

RESULTS

Interconversions of carotenoids in normal cells

A culture of *R. rubrum* in the course of exponential photosynthetic growth was chilled, and the cells were then harvested by centrifugation. They were washed once with cold Hutner phosphate buffer, and resuspended in 2 l of the same buffer. This suspension was equally distributed between 3 Roux bottles, which were wrapped in

aluminum foil to exclude light. The bottles were placed in an illuminated water bath held at 32°C, and continuously aerated with N_2 containing 5 % CO_2. After temperature equilibrium had been attained, the experiment was started by removing the aluminum foil from the bottles, thus exposing the bacteria to illumination. Samples of the suspension (comprising equal volumes withdrawn from each of the three bottles) were analyzed for pigment content after 0, 3, 6 and 24 hours. The results are shown in Table III and Fig. 1.

TABLE III

TRANSFORMATIONS OF CAROTENOIDS IN CELLS OF *R. rubrum* HARVESTED DURING EXPONENTIAL GROWTH, AND SUBSEQUENTLY INCUBATED IN BUFFER UNDER ANAEROBIC CONDITIONS IN THE LIGHT

Carotenoids	*Time of incubation*			
	Initial	*3 h*	*6 h*	*24 h*
	μg of carotenoid per 100 mg protein			
Lycopene	15.2	3.1	3.0	3.2
OH-Lycopene	8.3	1.7	traces	0.0
di-OH-Lycopene	4.1	2.1	2.3	2.1
P481	151.1	155.2	127.0	32.4
OH-P481	16.2	18.4	30.4	28.0
Spirilloxanthin	43.2	64.9	82.3	175.1
OH-Spirilloxanthin	5.9	4.5	2.0	3.8
Total carotenoids	244.0	249.9	247.0	244.6
	mg per 100 ml suspension			
Bacteriochlorophyll	0.350	0.347	0.352	0.348
Protein	22.0	20.2	19.5	20.5

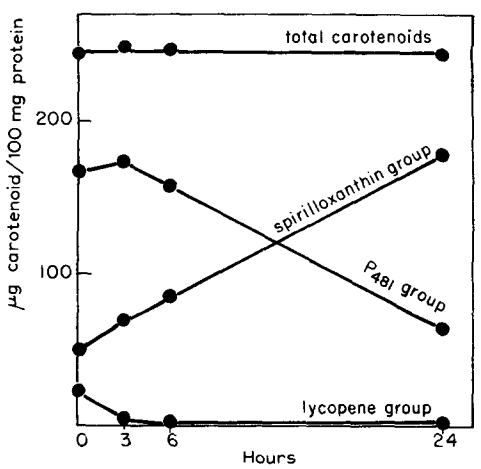

Fig. 1. Interconversions of the normal carotenoid pigments in exponential cells of *R. rubrum*, after resuspension in buffer and incubation at 30° in anaerobiosis and light.

The protein and chlorophyll contents of the suspension remained essentially unchanged throughout the experiment. The total amount of carotenoid pigment also did not change, within the limits of experimental error; but the relative amounts of

the various carotenoid pigments changed greatly. The sample taken at the start of the experiment contained the mixture of carotenoids characteristic of exponentially growing cells[5]: lycopene, P481, spirilloxanthin, and their monohydroxy derivatives, together with traces of dihydroxylycopene. The P481 group was predominant, representing 68.5 % of the total. After 3 h, the lycopene group had virtually disappeared, with corresponding increases in the P481 and spirilloxanthin groups. During the subsequent 21 h the amount of P481 decreased steadily, while the amount of spirilloxanthin continued to increase, finally reaching 73 % of the total carotenoids. The correspondence between the disappearance of P481 and the formation of spirilloxanthin during this period was striking: 113 μg of P481 disappeared, and 109.5 μg of spirilloxanthin were formed, per 100 mg of bacterial protein.

This experiment demonstrates that spirilloxanthin can be synthesized at the expense of lycopene and P481 in cells which cannot perform a net synthesis of carotenoid pigments, as a result of suspension in a medium which contains no carbon source. The conversion of lycopene to P481 is more rapid than the conversion of P481 to spirilloxanthin. These changes in the carotenoid pigment system do not occur if the cells are maintained under anaerobic conditions in the dark, a fact which suggests that the reactions concerned require energy, generated in illuminated cells by photophosphorylation. The role of the hydroxy derivatives in these transformations is not yet clear; the only conclusion which can be drawn with certainty from the data is that hydroxy P481 is formed from P481, and not directly from hydroxylycopene (*cf.* Table III).

This experiment makes it possible to interpret in biochemical terms the findings of VAN NIEL, GOODWIN AND SISSINS[5] that spirilloxanthin is a relatively minor carotenoid constituent in young cultures of *R. rubrum*, but practically the only carotenoid present in old cultures. The mixture of pigments present in exponentially growing cells reflects the existence of a dynamic equilibrium between the terminal members of the biosynthetic chain. This equilibrium shifts increasingly in favor of the end-product, spirilloxanthin, as the flow of carbon into the biosynthetic pathway declines with the approach of the population to the stationary phase of growth.

The reaction sequence for spirilloxanthin synthesis can be represented schematically as:

$$\text{precursors} \xrightarrow[1]{} \text{lycopene} \xrightarrow[2]{} \text{P481} \xrightarrow[3]{} \text{spirilloxanthin}$$

In growing cells, reaction 1 must be very rapid relative to reactions 2 and 3, since precursors of lycopene are not normally detectable. The possibility of detecting such precursors is, of course, a function of the sensitivity of the analytical techniques employed. We have found that small quantities of lycopene precursors are detectable provided that sufficient quantities of cells (of the order of 100 g wet weight) are analyzed. In one such analysis, cells harvested during exponential growth were found to contain phytofluene, ζ-carotene and neurosporene, corresponding respectively to 0.03, 0.2 and 0.4 % of the total carotenoid present. Direct evidence that these three compounds are biosynthetic precursors of lycopene will be presented in a later section of this paper.

The effect of DPA on carotenoid synthesis by growing populations of R. rubrum

DPA at a final concentration of $7 \cdot 10^{-5}$ *M* was added to a culture of *R. rubrum* which was in the course of exponential photosynthetic growth under steady-state

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conditions of chlorophyll and carotenoid synthesis*. Gross effects were measured by periodic determinations of cell mass, protein, bacteriochlorophyll and total carotenoid. At a few points after the addition of DPA, large samples (300 ml) were withdrawn for detailed analysis of the carotenoid pigments.

The gross effects of DPA on growth and pigment synthesis are shown in Fig. 2.

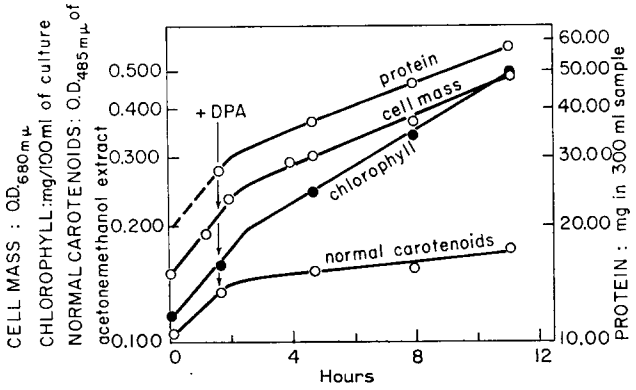

Fig. 2. The effect of DPA ($7 \cdot 10^{-5}$ *M*) on growth, protein synthesis, and pigment synthesis in a culture of *R. rubrum* growing exponentially under anaerobic conditions in the light.

At the concentration employed, DPA is somewhat toxic, as evidenced by the sharp declines in the rates of growth and chlorophyll synthesis that follow its introduction into the culture. However, as already reported[17], we have been wholly unable to confirm the allegation of GOODWIN AND OSMAN[7] that DPA causes a *specific* inhibition of bacteriochlorophyll synthesis in *R. rubrum*. Fig. 2 is typical of the results which we have obtained in numerous experiments. After the introduction of DPA, the rates of growth and chlorophyll synthesis are reduced to exactly the same extent: eventually, if the experiment is continued for a sufficient time, the rate of chlorophyll synthesis increases relative to the growth rate. This is a consequence of overshadowing, and reflects the fact, established earlier[9], that the differential rate of chlorophyll synthesis by purple bacteria is an inverse function of light intensity. Chlorophyll synthesis accordingly proceeds in a normal fashion in the presence of DPA. Carotenoid synthesis, on the other hand, is drastically affected by the introduction of DPA. The data for "normal carotenoid" in Fig. 2 were obtained by measuring the extinction at 485 mμ of acetone–methanol extracts containing the mixed carotenoid pigments, corrected for light absorption by chlorophyll at this wavelength. The method is crude; and the apparent slight increase in normal carotenoids after the addition of DPA is not real. Quantitative analysis reveals that the increase of extinction at 485 mμ following the addition of DPA actually results from interconversions of the pigments already present in the cells.

The changes in the carotenoid pigment system which follow the introduction of DPA are shown in Table IV. These changes reflect two distinct biochemical events, readily evident when the data are somewhat simplified and presented in graphical form (Fig. 3).

* Under these conditions, the rates of growth and of pigment synthesis are identical.

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TABLE IV

CAROTENOID SYNTHESIS BY *R. rubrum* DURING EXPONENTIAL PHOTOSYNTHETIC GROWTH AFTER ADDITION OF $7 \cdot 10^{5}$ *M* DPA

Carotenoid	*Time after addition of* DPA *to culture*					
	0 h		*3 h*		*9.25 h*	
	μg of carotenoid in sample (300 ml)					
Phytoene (measured)*	0.0		38.5		134.5	
Phytoene (corrected)**	0.0		27.0		94.0	
Phytofluene	0.0		4.8		21.0	
OH-Phytofluene	0.0		0.0		0.0	
ζ-Carotene	0.0		5.1	7.1	18.4	24.1
OH-ζ-Carotene	0.0		2.0		5.7	
Neurosporene	0.0		1.8	3.4	1.1	9.8
OH-Neurosporene	0.0		1.6		8.7	
Lycopene	2.1	20.2	traces	4.9	2.3	2.3
OH-Lycopene	18.1		4.9		0.0	
P481	16.1	49.7	23.6	54.2	20.9	41.7
OH-P481	33.6		30.6		20.8	
Spirilloxanthin	3.0	3.0	13.6	13.6	24.2	24.2
OH-Spirilloxanthin	0.0		traces		0.0	

* Determined from extinction of phytoene-containing eluate at 284 mμ, without rechromatography.

** Corrected for estimated absorption at 284 mμ by DPA.

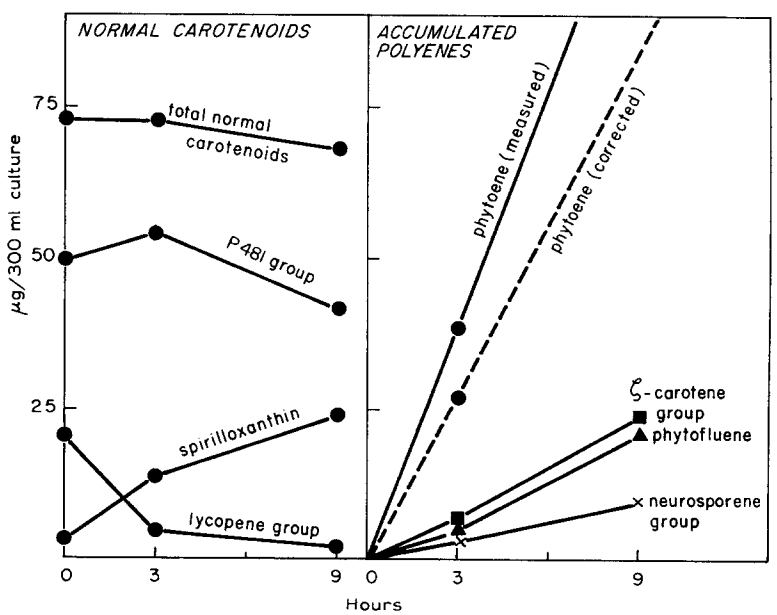

Fig. 3. Changes in the carotenoid pigment system of *R. rubrum* during photosynthetic growth in the presence of $7 \cdot 10^{-5}$ *M* DPA (added at zero time). Data from the experiment portrayed in Fig. 2.

1. The net synthesis of the normal carotenoids is completely arrested, but spirilloxanthin continues to be synthesized at the expense of the lycopene and P481 groups which were already present in the cells. Insofar as the normal pigment system of *R. rubrum* is concerned, the effect of DPA resembles the effect of eliminating the exogenous carbon source, which has already been described.

2. A series of more saturated carotenoids starts to accumulate in the cells. Phytoene is formed in very large amounts, accompanied by lesser quantities of phytofluene, ζ-carotene, neurosporene and their hydroxy derivatives. In this particular experiment, no hydroxyphytofluene was detected; but it can always be found in cells which have grown for several generations in the presence of DPA. Apart from phytoene, the ζ-carotene group accumulates most rapidly; the phytofluene group is formed a little more slowly, and the neurosporene group most slowly of all. It is thus evident that DPA blocks totally the flow of carbon from precursors into lycopene. Since the lycopene-P481-spirilloxanthin conversions continue in the presence of DPA, the inhibitor evidently has little or no effect on the terminal step-reactions of normal carotenoid synthesis.

Interconversions of carotenoids in DPA-grown cells after removal of the inhibitor

Cultures of *R. rubrum*, inoculated at an E_{680} of 0.004, were incubated under photosynthetic growth conditions. 3 h later, DPA was added to the growing cultures. After 70 h of photosynthetic development in the presence of DPA, the cultures were chilled, centrifuged in the cold, washed with cold Hutner phosphate buffer, and resuspended in 2 l of the same buffer*. The suspension was evenly distributed between 3 Roux bottles, and continuously aerated with N_2 containing 5% CO_2. The bottles were wrapped in aluminum foil, and placed in a water bath at 30°C until temperature equilibration had occurred. The experiment was then initiated by exposing the suspensions to continuous illumination, maintained for a period of approximately 24 h. At zero time and at intervals thereafter, samples (comprising equal volumes from each bottle) were withdrawn for analysis. Each sample was chilled immediately after withdrawal, in order to minimize possible changes in the carotenoids during the manipulations prior to pigment extraction.

Several experiments of this nature were performed, and complete data for two of them are presented in Table V. In Expt. 1 of Table V, the concentration of DPA during growth was $7 \cdot 10^{-5}$ *M*, just sufficient to cause a total inhibition of normal carotenoid synthesis. In Expt. 2, a slightly lower concentration of DPA was employed ($6.5 \cdot 10^{-5}$ *M*), and the inhibition of normal carotenoid synthesis was not quite complete. Before the observed interconversions are discussed, a few comments on the carotenoid constitution of the cells at zero time must be made. The distribution of carotenoids in these two initial samples is typical of cells which have been grown for several generations in the presence of DPA, and accords well with the distribution to be expected on the basis of the kinetic experiment on DPA effects already described (Table IV). The content of normal carotenoids (lycopene, P481 and spirilloxanthin groups) is very low: in the cells used for Expt. 1, it represented about 4% of the total,

* Preliminary experiments had shown changes in the carotenoid composition of DPA-grown cells occur extremely rapidly after removal of the inhibitor. In order to minimize such changes, it is essential to carry out the operations of washing and resuspension at 0–5°. Once the cells have been resuspended, changes can be prevented, even at higher temperatures, by keeping the suspension under strictly anaerobic conditions in the dark[17].

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TABLE V

ENDOGENOUS CAROTENOID SYNTHESIS BY WASHED, DPA-GROWN CELLS OF *R. rubrum* RESUSPENDED IN BUFFER AND INCUBATED ANAEROBICALLY IN THE LIGHT

The cells used for Expt. 1 had been grown in the presence of $7 \cdot 10^{-5}$ *M* DPA; those used for Expt. 2, in the presence of $6.5 \cdot 10^{-5}$ *M* DPA.

Compound	*Experiment 1*				*Experiment 2*			
	Time of incubation				*Time of incubation*			
	Initial	*20 min*	*2 h*	*23 h*	*Initial*	*20 min*	*3.75 h*	*23.7 h*
	μg carotenoid/100 mg protein				*μg carotenoid/100 mg protein*			
Phytoene*	322	287	358	306	—	—	—	—
Phytofluene	98.2	67.8	55.0	19.7	77.0	70.5	22.2	4.2
OH-Phytofluene	12.4	17.3	13.4	tr.	9.2	9.3	0.8	0.0
	110.6	85.1	68.4	19.7	86.2	79.8	23.0	4.2
ζ-Carotene	82.0	69.1	71.8	42.7	81.3	76.9	107.2	21.5
OH-ζ-Carotene	31.5	22.9	28.6	18.5	34.9	35.2	19.8	4.2
	113.5	92.0	100.4	61.2	116.2	112.1	127.0	25.7
di-OH-ζ-Carotene	4.3	4.0	10.4	9.7	4.2	4.0	3.8	4.2
P_{412}	10.6	11.5	7.3	6.2	16.0	12.2	8.6	5.4
Neurosporene	25.7	32.9	23.2	15.7	34.0	41.5	19.6	5.2
OH-Neurosporene	23.5	51.6	55.5	31.6	29.2	35.6	6.0	4.6
	49.2	84.5	78.7	47.3	62.2	77.1	25.6	9.8
P_{450}	14.7	8.3	14.2	9.9	9.0	6.1	14.6	4.2
Lycopene	0.0	3.2	10.3	13.0	6.4	8.3	14.2	27.7
OH-Lycopene	0.0	1.4	27.8	15.0	6.4	32.6	92.1	33.2
	0.0	4.6	38.1	28.0	12.8	40.9	103.6	60.9
P_{481}	6.9	9.7	11.3	63.2	11.4	6.0	27.8	65.9
OH-P_{481}	7.9	7.4	6.0	89.5	4.6	4.0	15.4	104.1
	14.8	17.1	17.3	152.7	16.0	10.0	43.2	170.0
Spirilloxanthin	7.8	5.0	9.6	11.9	3.7	2.0	8.0	65.0
OH-Spirilloxanthin	0.0	0.0	0.0	3.7	0.0	0.0	0.0	9.4
	7.8	5.0	9.6	15.6	3.7	2.0	8.0	74.4
Total, excluding phytoene	325.5	312.0	345.4	350.3	327.3	344.5	360.5	358.8
Chlorophyll, μg per mg protein	27.9	27.8	31.7	28.6	29.2	29.6	29.4	30.4

* Measured after rechromatography on basic alumina. Phytoene was not determined in Expt. 2.

and lycopene was entirely absent. Normal carotenoids were slightly more abundant in the cells used for Expt. 2, and included some lycopene; these minor differences reflect the lessened severity of DPA inhibition during the preceding period of growth. Phytoene was not determined in Expt. 2. Apart from this, the relative and absolute abundances of the carotenoids more saturated than lycopene were very similar in the two lots of DPA-grown cells. It may be noted that four minor constituents not detected in the course of the experiments on DPA inhibition were discovered in these cells: hydroxyphytofluene, dihydroxy ζ-carotene, P412 and P450. Only very small quantities of these four compounds are formed, which no doubt explains our failure to detect them during the early stages of carotenoid synthesis in the presence of DPA. Excluding phytoene, the concentration of carotenoids in DPA-grown cells is very similar to that in normal cells which have been grown under otherwise comparable conditions. Including phytoene, it is more than twice as great.

When washed, DPA-grown cells are resuspended in buffer and incubated anaerobically in the light for 24 h, their content of bacteriochlorophyll and protein does not change significantly. We have been unable, despite numerous repetitions of such experiments, to confirm the reports of GOODWIN AND OSMAN[8] that a net synthesis of chlorophyll can occur under these conditions. Excluding phytoene, for which the quantitative data are unreliable, there is usually a very slight apparent net synthesis of carotenoids in such experiments: it amounted to 7 % in Expt. 1, and 10 % in Expt. 2. In view of the extreme complexity of the analyses, such increases are of dubious significance, and it may well be that no net synthesis takes place. Over a period of 24 h, however, the qualitative composition of the carotenoids in the cells changes enormously. The gross transformations that take place can be summarized as follows: the quantity of the phytofluene, ζ-carotene and neurosporene groups steadily decreases, with a concomitant increase in the quantity of normal pigments. There is, furthermore, a stoichiometric equivalence between the disappearance of the former carotenoids and the formation of the latter ones. This fact is illustrated in Fig. 4, which is based on the data of Expt. 2. It is evident that under the conditions of such experiments a synthesis of normal carotenoids is proceeding largely, if not entirely, at the expense of the more saturated carotenoids which had been accumulated within the cells during preceding growth in the presence of DPA. For reasons which have been discussed in the section on MATERIALS AND METHODS, the values for phytoene are not quantitatively reliable, and the apparent fluctuations of these values shown in Expt. 1 are probably all within experimental error. A minor net decrease of phytoene would certainly have escaped detection; but the data of Fig. 4 indicate that if the accumulated phytoene contributes at all to the formation of the normal carotenoids, its contribution must be negligible.

The transformations which follow the removal of DPA are exceedingly complex: a large number of step-reactions proceed more or less simultaneously. A detailed picture of the transformations which occur would be obtainable only by very frequent analyses. Our kinetic experiments were severely limited by the difficulty of the analytical techniques, which precluded the performance of compiete carotenoid determinations on more than four samples in a single experiment. The data in Table V therefore represent a few cross-sections of the carotenoid composition of the cells, widely spaced in time. These experiments were made even more difficult by the variability of the biological material. The cells must be cultivated in the presence of a

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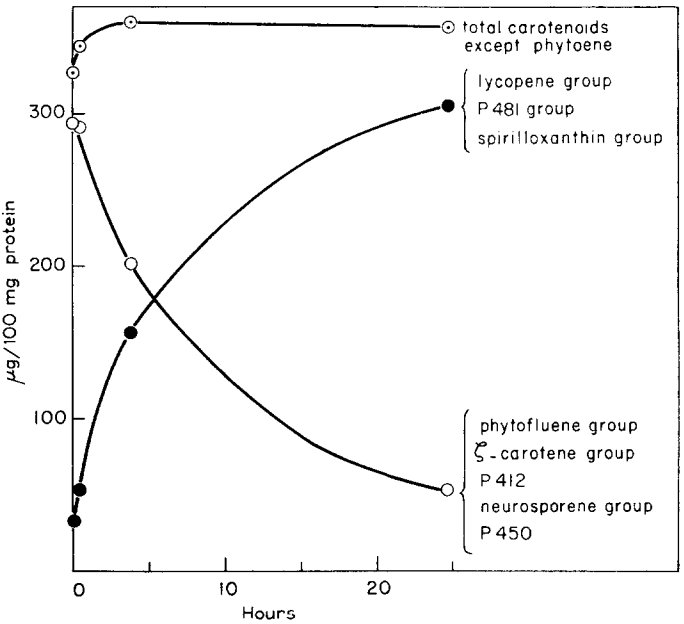

Fig. 4. Endogenous synthesis of normal carotenoids from more saturated precursors in DPA-grown cells of *R. rubrum*, washed free of the inhibitor and incubated in buffer under anaerobic conditions in the light. Plotted from the data of Expt. 2 (Table V).

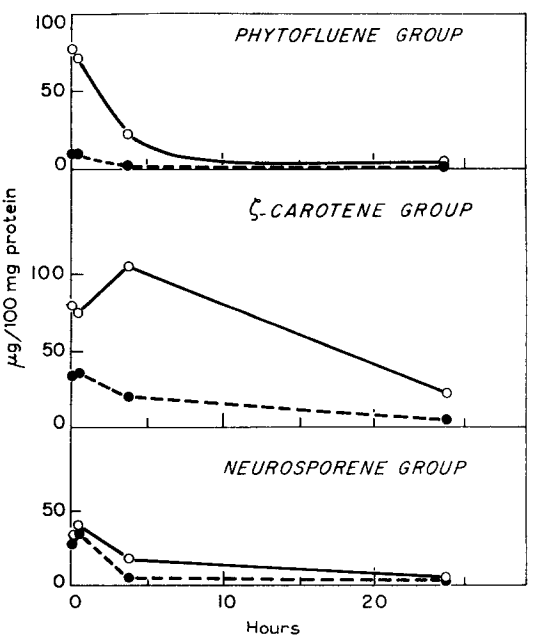

Fig. 5. Transformations of the phytofluene, ζ-carotene and neurosporene groups during endogenous synthesis of normal carotenoids by *R. rubrum*. Solid lines: hydrocarbons. Dashed lines: monohydroxy derivatives.

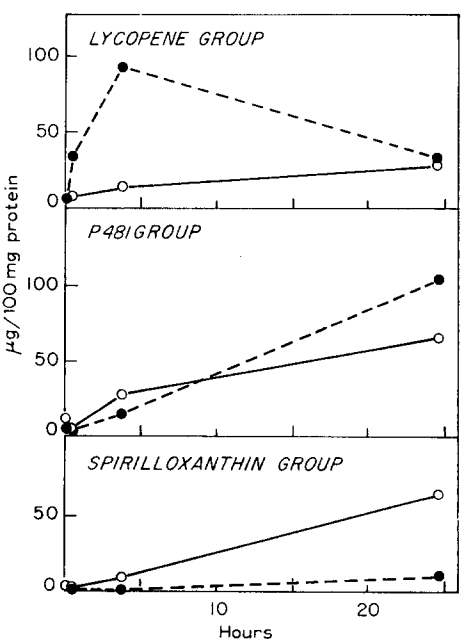

Fig. 6. Transformations of the lycopene, P481 and spirilloxanthin groups during endogenous synthesis of normal carotenoids by *R. rubrum*. Solid lines: unhydroxylated compounds. Dashed lines: monohydroxy derivatives.

concentration of DPA that is close to the bacteriostatic level; however carefully external variables are controlled, the rate of the transformations which follow removal of the inhibitor is never exactly reproducible from one experiment to the next. The same general reaction sequence can be inferred, however, from the results of each experiment. The effect which a difference of less than 10% in the concentration of DPA during growth can have on the course of subsequent endogenous synthesis is evident from a detailed comparison of the results obtained in Expts. 1 and 2 (Table V).

The transformations of the individual carotenoid groups observed in Expt. 2 are presented graphically in Figs. 5 and 6. The earliest detectable events of endogenous synthesis are interconversions of the more saturated carotenoids, as evidenced by appreciable spectral changes in the crude pigment extracts during the first 10 or 15 min[17]. The earliest time at which quantitative analyses were performed was 20 min; these data (Table V and Fig. 5) reveal that the phytofluene and ζ-carotene groups are undergoing conversion to the neurosporene group. The accumulation of the neurosporene group after 20 min in the experiment illustrated in Fig. 5 was not nearly so pronounced as in other experiments; but in this case, synthesis of the lycopene group also began very rapidly, and presumably the neurosporene pool was quickly diminished as a result. The changes observed during the first 20 min of Expt. 1 are more typical: here there was negligible lycopene synthesis, and the flow from the earlier precursors into neurosporene was much more pronounced.

After its rapid initial accumulation, the neurosporene pool diminishes as synthesis of the normal carotenoids gets under way. The phytofluene group continues to diminish steadily. As a rule, the ζ-carotene group increases slightly after 2 to 4 h, presumably because the flow into it from the phytofluene group is at this time more rapid than the outflow to the neurosporene group. This increase is a transient one, and after 24 h the ζ-carotene group has always diminished again to a relatively low level.

Of the normal carotenoids, the lycopene group is always formed first (Fig. 6). This group reaches a maximal level within 2–4 h, and then falls again as the P481 group rises. The spirilloxanthin group appears last of all. It is still negligible several hours after the initiation of endogenous synthesis, and in some experiments (for example, Expt. 1 in Table V) does not appear in appreciable quantities even after 24 h. In other experiments (Expt. 2, Table V and Fig. 6) substantial quantities of spirilloxanthin are produced at the end of 24 h, although the P481 group is always the predominant component of the pigment system at this time. The relatively limited synthesis of spirilloxanthin in these experiments may reflect the fact that the formation of this pigment from lycopene involves the addition of two methoxyl groups to the C_{40} skeleton. This portion of the biosynthetic sequence accordingly differs from the preceding conversions; between phytofluene and lycopene, there are no changes in the carbon skeleton. During endogenous synthesis, therefore, the formation of spirilloxanthin must be limited by the availability of intracellular methyl donors, which can supply the carbon for the two methoxyl groups. Since the P481 group is always formed in large amounts during endogenous synthesis, it seems likely that the introduction of the two methoxyl groups characteristic of spirilloxanthin occurs after the synthesis of P481 from lycopene, P481 itself being a C_{40} compound:

$$\text{lycopene} \longrightarrow \text{P481} \xrightarrow{+\,2\,\text{MeO}} \text{spirilloxanthin}$$

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The role of the monohydroxy derivatives in the biosynthetic sequence must now be considered. Monohydroxy derivatives of all carotenoids from phytofluene to neurosporene accumulate along with the parent hydrocarbons during DPA inhibition; furthermore, the monohydroxy derivatives of lycopene, P481 and spirilloxanthin are formed during normal carotenoid synthesis. As shown in Table V and Figs. 4 and 5, the monohydroxy derivatives of all these compounds undergo marked transformations during endogenous synthesis; and, as a rule, their transformations broadly parallel the concomitant transformations of the parent hydrocarbons. It is therefore evident that the monohydroxy compounds participate actively in carotenoid synthesis. Three alternative biochemical interpretations of the role of these compounds can be entertained. Designating successive hydrocarbons in the biosynthetic sequence as A, B and C, and the corresponding hydroxy compounds as A-OH, B-OH and C-OH, we can represent these alternative hypotheses as follows:

1. A ⟶ B ⟶ C ⟶ ··· ⟶ Spirilloxanthin
   ⇅ ⇅ ⇅
   A-OH B-OH C-OH

2. A ⟶ A-OH ⟶ B ⟶ B-OH ⟶ C ⟶ C-OH ⟶ ··· ⟶ spirilloxanthin

3. A ⟶ B ⟶ C → ··· ↘
   A-OH ⟶ B-OH ⟶ C-OH → ··· ↗ spirilloxanthin

Hypothesis 1 states that the monohydroxy compounds constitute side-products of the main biosynthetic sequence, which proceeds through the carotenoid hydrocarbons. In order to account for the biochemical lability of the monohydroxy compounds, they must be assumed to be freely interconvertible with the parent hydrocarbons. Hypothesis 2 states that the monohydroxy compounds are intermediates between successive hydrocarbons in the biosynthetic sequence. Hypothesis 3 states that there are two parallel biosynthetic pathways, one for the hydrocarbons and one for the hydroxy compounds; since spirilloxanthin is the unique ultimate biosynthetic product, the two parallel pathways must converge at this point. Only the third hypothesis can be definitely eliminated on the basis of the available data. During endogenous synthesis, there is at all times an excellent stoichiometric balance between the disappearance of the more saturated carotenoids, and the formation of the normal carotenoids (Fig. 4). If hypothesis 3 were correct, stoichiometry should be maintained also for the hydrocarbons as a group, and for the hydroxy derivatives as a group. Expt. 1 is particularly instructive in this respect, since virtually no synthesis of spirilloxanthin occurred. Calculation shows that during the course of endogenous synthesis in this experiment there was a progressive fall in the total quantity of carotenoid hydrocarbons (excluding phytoene) and a progressive increase in the total quantity of their hydroxy derivatives. It therefore follows that reactions of the type A → A-OH occur, although it cannot yet be decided whether they lie on the main path of carotenoid synthesis.

The endogenous synthesis of spirilloxanthin in *R. rubrum* after the removal of DPA was discovered by GOODWIN AND OSMAN[8]. They concluded that spirilloxanthin is formed from non-specific cellular reserve materials, not from the accumulated store of more saturated carotenoids. Since this conclusion was based on an experiment identical in principle to the experiments on endogenous synthesis which have just

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been described, it is necessary to discuss briefly the differences in experimental procedure which made such diametrically opposed interpretations possible. In the first place, changes of carotenoid composition were measured by GOODWIN AND OSMAN only after 24 and 48 h of endogenous synthesis, and they thus overlooked completely the very striking changes which take place during the first few hours of the process. In the second place, their analyses were not at all detailed; the compounds eluted from the column between phytofluene and spirilloxanthin were not separated and individually estimated. Lastly, their carotenoid extractions were incomplete, so that the stoichiometric aspects of endogenous synthesis could not be ascertained. Their failure to assess correctly the biochemical significance of endogenous synthesis is, therefore, fully understandable.

Intracellular location of carotenoids in DPA-grown cells

The photosynthetic pigments (carotenoids and bacteriochlorophyll) of the purple bacteria are localized within the cell in cytoplasmic particles known as chromatophores, which can be separated from other cellular constituents by differential high-speed centrifugation of extracts prepared by the mechanical disruption of the cells[18]. An experiment was therefore undertaken in order to ascertain the intracellular location of the carotenoids which accumulate in *R. rubrum* when normal carotenoid synthesis is blocked by DPA. DPA-grown cells were mechanically disrupted, and the resulting extract was separated by centrifugation into the sedimentable chromatophore fraction and the supernatant "soluble" fraction. Each fraction was then analyzed for its content of protein and bacteriochlorophyll, and the carotenoids were extracted and transferred into petroleum ether. The technical details of such an experiment are described elsewhere[19].

Spectrophotometric examination of petroleum ether extracts showed at once that the more saturated carotenoids of the phytofluene, ζ-carotene and neurosporene groups were associated exclusively with the chromatophore fraction. Owing to the presence of other substances that absorb light in the same ultraviolet region, the presence of phytoene in crude petroleum ether extracts cannot be safely established by spectrophotometry. The extracts were accordingly subjected to chromatography, and the phytoene content was determined quantitatively in the eluates. The results, presented in Table VI, indicate that phytoene is also almost entirely associated with the chromatophore fraction.

This experiment shows that the synthesis of normal carotenoids from more saturated precursors which occurs after the removal of DPA must take place entirely in

TABLE VI

INTRACELLULAR DISTRIBUTION OF PHYTOENE AND BACTERIOCHLOROPHYLL IN DPA-GROWN CELLS OF *R. rubrum*

Material analyzed	*Phytoene* μg/mg of protein*	*Bacteriochlorophyll, μg/mg protein*
Whole cells	5.0	31.0
Chromatophores	8.9	57.0
Soluble fraction	0.225	0.0

* Isolated by chromatography on Woelm basic alumina activity grade 1, which gives a complete separation from DPA.

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the chromatophores. The carotenoids are all wholly insoluble in water, and it is therefore understandable that the ultimate steps in their biosynthesis should be spatially localized in this fashion. Presumably at some point in the biosynthetic sequence, intermediates are incorporated into the photosynthetic unit at the sites which will ultimately be occupied by the normal carotenoid pigments.

The intracellular location of the phytoene is of particular interest. The experiments on endogenous synthesis show that if phytoene contributes at all to the formation of the normal carotenoids, its contribution must be a negligible one, since the disappearance of the other saturated carotenoids accounts in a quantitatively satisfactory manner for the amount of normal pigments formed. Several interpretations of the inactivity of the phytoene in these experiments can be entertained. Firstly, it is possible that phytoene is not a member of the normal biosynthetic sequence, but rather a side-product that accumulates when the normal biosynthetic process is interfered with. This argument can also, of course, be used to explain the accumulation of phytoene in mutants of many different organisms with genetic blocks in the path of carotenoid synthesis. In our opinion, however, it would be premature to dismiss the possibility that phytoene is an intermediate in carotenoid formation. Its apparent inactivity in *R. rubrum* may be explicable on other grounds. The terminal stages of carotenoid synthesis take place in a highly organized structure, the chromatophore, which is the site of the primary reactions of photosynthesis. If the accumulated phytoene were not in the proper intracellular location, it might well be physically inaccessible to enzyme action. The simplest form of physical inaccessibility would, of course, be an intracellular location outside the chromatophore, but the data presented in Table VI definitely exclude this interpretation. The hypothesis of physical inaccessibility to enzyme action must therefore be formulated in a more subtle fashion. In DPA-grown cells the synthesis of the more saturated carotenoids *exclusive of phytoene* proceeds at a rate that is roughly equivalent to the rate of synthesis of normal carotenoids in the absence of DPA. It can be readily imagined that in such a structure as a chromatophore or a chloroplast there are a fixed and limited number of sites at which functionally active carotenoids can be accommodated. Since the later stages of carotenoid synthesis occur within the chromatophore, these sites must already be occupied during chromatophore formation by the C_{40} precursors which are destined to give rise to the end-products of the biosynthetic sequence. However, the biosynthetic rate in DPA-grown cells is such that the compounds of the phytofluene-ζ-carotene-neurosporene series can fill all available sites. The surplus phytoene, even though it is accumulated in the chromatophores, may be excluded from participation in endogenous conversion for this reason. The question of its normal role in carotenoid synthesis must therefore remain open.

DISCUSSION

The synthesis of carotenoids in purple bacteria

In the past few years, a survey of the carotenoid composition of purple bacteria has been conducted by GOODWIN and his collaborators[20,21]. The results of this study show that five principal carotenoids, together with their hydroxy derivatives, occur in various combinations in the species so far examined. These five pigments are lycopene, spirilloxanthin, Y, R and P481. The structures of the first four (Fig. 7) are

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established with reasonable certainty[2,3,22,23]. They are all aliphatic and (with the exception of lycopene) carry methoxyl groups. As demonstrated in the present paper, P481 is a precursor of spirilloxanthin, and is therefore almost certainly also aliphatic. An alicyclic carotenoid has been found in only one species, *Rhodomicrobium vannielii*, which contains traces of β-carotene together with much larger quantities of aliphatic carotenoids[24]. The four major pigments of known structure can be separated into two sub-groups on chemical grounds: pigments Y and R are derivatives of dihydrolycopene, and thus have a central conjugation system which is different from that of lycopene and spirilloxanthin. Examination of the data assembled by GOODWIN[21] shows that in the genus *Rhodospirillum*, only the lycopene, P481 and spirilloxanthin groups are found. In most species of the genus *Rhodopseudomonas*, on the other hand, the Y and R groups are preponderant, although traces of lycopene are sometimes also present.

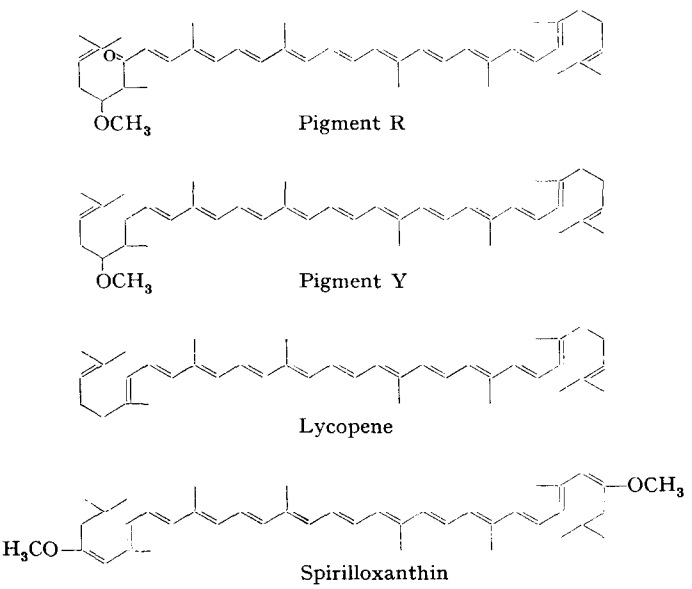

Fig. 7. Major carotenoids of known structure which occur in the purple bacteria.

Many years ago, VAN NIEL[25] showed that an irreversible stoichiometric conversion of Y to R can take place in *Rhodopseudomonas spheroides*. The observations which we have made on the interconversions of the normal carotenoids in washed cells of *Rhodospirillum rubrum* likewise establish the biosynthetic relationships between lycopene, P481 and spirilloxanthin. The terminal steps in the formation of the five major carotenoids characteristic of purple bacteria can therefore be formulated as:

$$? \longrightarrow \text{lycopene} \longrightarrow \text{P481} \longrightarrow \text{spirilloxanthin},$$

and

$$? \longrightarrow \text{Y} \longrightarrow \text{R}$$

It may be noted that the analytical data of GOODWIN[21] on the distribution of carotenoids in three species of the genus *Rhodospirillum* fit perfectly with the first of these formulations. *R. rubrum* contains the lycopene, P481 and spirilloxanthin groups;

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R. photometricum contains the lycopene and P481 groups; *R. molischianum* contains the lycopene group alone. In terms of biosynthetic capabilities, these distributions imply that *R. photometricum* lacks the ability to convert P481 to spirilloxanthin, and that *R. molischianum* lacks the ability to convert lycopene to P481.

Let us now consider the earlier steps in these two biosynthetic sequences. The studies of DPA effects on *R. rubrum* which are reported in the present paper establish the nature of the precursors for the lycopene-P481-spirilloxanthin series:

$$\text{phytoene} \xrightarrow{?} \text{phytofluene} \longrightarrow \zeta\text{-carotene} \longrightarrow \text{neurosporene} \longrightarrow \text{lycopene.}$$

The same technique cannot be used to explore the biosynthesis of Y and R, since DPA does not specifically inhibit carotenoid formation in the species that produce these two pigments[21]. Some evidence concerning the formation of Y and R has been obtained, however, by the study of mutant phenotypes of *Rhodopseudomonas spheroides* with derangements of carotenoid synthesis[19]. The brown phenotype forms greatly reduced quantities of R, and accumulates neurosporene in addition to Y[26]. The green phenotype produces neither Y nor R, and accumulates principally neurosporene and dihydroxyneurosporene, together with much smaller quantities of ζ-carotene and phytofluene[26]. The blue-green phenotype contains only one carotenoid, phytoene[19]. These facts suggest the following pathway for the synthesis of Y and R:

$$\text{phytoene} \xrightarrow[1]{} \text{phytofluene} \longrightarrow \zeta\text{-carotene} \longrightarrow \text{neurosporene} \xrightarrow[2]{} \text{Y} \xrightarrow[3]{} \text{R}$$

The blue-green and green phenotypes are presumed to be completely blocked in the performance of reactions 1 and 2, respectively; the brown phenotype is presumed to be partly blocked in the performance of reaction 3. The resemblances between this reaction sequence, inferred on the basis of biochemical genetic data, and the reaction sequence for the synthesis of spirilloxanthin, established by the analysis of the DPA effect, are quite evident. The initial steps in both sequences are identical, a divergence occurring after the formation of neurosporene:

$$\text{common precursors} \longrightarrow \text{neurosporene} \begin{cases} \nearrow \text{Y} \longrightarrow \text{R} \\ \searrow \text{lycopene} \longrightarrow \text{P481} \longrightarrow \text{spirilloxanthin} \end{cases}$$

The general interpretation of DPA effects

Since the discovery by TURIAN[17] in 1950 that DPA specifically inhibits carotenoid synthesis in mycobacteria, there have been many reports of the effect of DPA on other micro-organisms. It is a very toxic substance, and its action is sometimes non-selective: as the concentration of DPA is increased, growth is arrested before any effects on carotenoid synthesis can be detected. However, in those cases where it does act as a selective inhibitor of normal carotenoid synthesis, growth in its presence is invariably accompanied by the intracellular accumulation of more saturated carotenoids, characteristically phytoene, phytofluene, ζ-carotene and neurosporene. Heretofore, two alternative interpretations of the observed accumulations have been possible. It could be assumed that the blockage of carotenoid synthesis causes a diversion of the normal biosynthetic intermediates to side-products (the more saturated carotenoids) which are not normally formed at all by the cell. Alternatively, it could be assumed that the more saturated carotenoids are normal biosynthetic intermediates which accumulate in the cell when the terminal steps of the biosynthetic

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sequence are inhibited by DPA. Our present observations on DPA effects in *R. rubrum* provide decisive evidence in favor of the second of these two alternative interpretations. Phytofluene, ζ-carotene and neurosporene are clearly the biosynthetic precursors of lycopene, P481 and spirilloxanthin; only the status of phytoene in the reaction sequence remains somewhat questionable. Accordingly, the accumulation of these compounds in other organisms when normal carotenoid synthesis is prevented by DPA takes on a new significance: it indicates the existence of one general pathway for the synthesis of a wide variety of carotenoids, both aliphatic and alicyclic.

The mode of action of DPA

The selective inhibition of carotenoid synthesis by DPA was discovered accidentally, and the mechanism of its action is still not understood. The experiments reported in this paper at least permit a more precise specification of the step-reactions which are affected by DPA. One is evidently the conversion of neurosporene to lycopene: when DPA is added to a culture, the net synthesis of the lycopene, P481 and spirilloxanthin groups ceases, and the neurosporene group starts to accumulate. A second is the conversion of the ζ-carotene group to the neurosporene group: after the removal of DPA, there is an immediate burst of neurosporene formation. Conceivably other steps, such as the conversion of the phytofluene group to the ζ-carotene group, are also affected by the inhibitor, but the evidence for additional sites of action is less clear. At all events, we may conclude that DPA does not simply act at a single step in the reaction sequence. Since it is known to be an anti-oxidant, a plausible supposition is that it affects, to a greater or lesser extent, the succession of oxidative step-reactions which occur in the transformation of phytoene to colored carotenoids. However, a definitive interpretation in chemical terms is not at present possible, in view of the uncertainty which surrounds the structures of the more saturated carotenoids.

SUMMARY

Exponentially growing cells of the photosynthetic bacterium *Rhodospirillum rubrum* contain a mixture of carotenoids: lycopene, P481, spirilloxanthin, and their monohydroxy derivatives. When such cells are resuspended in buffer and incubated anaerobically in the light, the total amount of carotenoid pigment in the cells remains constant, but there is a net synthesis of spirilloxanthin at the expense of the other carotenoids originally present. This synthesis proceeds from lycopene through P481. Added to a photosynthetically growing culture of *R. rubrum* at a final concentration of $7 \cdot 10^{-5}$ *M*, diphenylamine causes a complete arrest of normal carotenoid synthesis, accompanied by a rapid accumulation of carotenoids more saturated than lycopene. Phytoene, phytofluene, hydroxyphytofluene, ζ-carotene, hydroxy-ζ-carotene, neurosporene and hydroxyneurosporene are the principal accumulating compounds. If the diphenylamine is removed and the cells are resuspended in buffer and incubated anaerobically in the light, an endogenous synthesis of normal carotenoids takes place at the expense of all the accumulated precursors with the probable exception of phytoene. The kinetic analysis of this endogenous synthesis reveals the sequential relationships between the participating carotenoids.

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GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE, ITS PURIFICATION AND APPLICATION*

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The presence of an enzyme, galactose-1-phosphate uridyl transferase in galactose-adapted yeast[1] and in calf and rat liver[2] has been reported by KALCKAR *et al.* This enzyme catalyzes Reaction 1 and, together with uridine diphosphogalactose-4-epimerase[3,4,5] which catalyzes Reaction 2, it accomplishes the reversible conversion of α-galactose-1-phosphate§ to α-glucose-1-phosphate as shown by the sum of equations 1 and 2.

$$\text{UDPG} + \text{Gal-1-P} \rightleftharpoons \text{UDPGal} + \text{G-1-P} \quad (1)$$

$$\text{UDPGal} \rightleftharpoons \text{UDPG} \quad (2)$$

$$\text{Sum: Gal-1-P} \rightleftharpoons \text{G-1-P} \quad (3)$$

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§ The following abbreviations are used: Gal-1-P, α-galactose-1-phosphate; G-1-P, α-glucose-1-phosphate; G-6-P, glucose-6-phosphate; 6-PG, 6-phosphogluconate; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; UDPG, uridine diphosphoglucose; UDPGal, uridine diphosphogalactose.