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Studies in Carotenogenesis

14. CAROTENOID SYNTHESIS IN THE PHOTOSYNTHETIC BACTERIUM
RHODOPSEUDOMONAS SPHEROIDES

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The general features of the non-sulphur photosynthetic bacterium *Rhodospseudomonas spheroides* have been described by van Niel (1941). Under anaerobic conditions in the light, cultures are brown but, as noted by French (1940), on allowing them access to air their colour changes to purplish red. This is also the colour obtained in cultures grown aerobically from the start. French further observed that this colour change could not be brought about by cell-free extracts, nor could it be accelerated by the addition of oxidizing agents; furthermore, the reverse process, the conversion of red cultures into brown by changing from aerobic to anaerobic conditions, was not possible. From a study of the absorption spectra of the pigment extracts, French concluded that the colour change from brown to red was mainly due to the formation of a pigment with an absorption maximum at 550 m μ . (in carbon disulphide); he did not consider this pigment to be a carotenoid.

A more detailed study of the pigments of *R. spheroides* was later carried out by van Niel (1947) (the abbreviation *R.* will be used in this paper for *Rhodospseudomonas*). On chromatography of the pigments remaining after bacteriochlorophyll had been removed, two main carotenoid pigments, one yellow (*Y*) and one red (*R*) were obtained, neither of which could be identified with previously described carotenoids. Associated with *R* and *Y* were a number of minor components, most of which appeared to be *cis* isomers of the parent pigments. Van Niel (1947) also demonstrated that the brown $\rightarrow$ red (anaerobic $\rightarrow$ aerobic) change was characterized by the disappearance of *Y* accompanied by the appearance of an equivalent amount of *R*. This change also took place in the dark, although not to

the same extent as in the light, but, in agreement with the observations of French (1940), it did not occur in cell-free extracts. Furthermore, van Niel (1947) confirmed that the reverse change red $\rightarrow$ brown could not be brought about.

The present investigation was undertaken to extend the observations of van Niel on carotenoid synthesis by *R. spheroides* in the light of present knowledge of carotenogenesis in general (Goodwin, 1953), and in another photosynthetic bacterium, *Rhodospirillum rubrum* (*Rsp. rubrum*) in particular (Goodwin & Osman, 1953, 1954).

EXPERIMENTAL

Cultures. The *R. spheroides* used throughout this investigation was a strain kindly supplied by Dr R. Y. Stanier, Department of Bacteriology, University of California, Berkeley. It was maintained in stab cultures on an agar medium. The liquid culture medium used was almost identical with that recommended by van Niel (1947): (NH₄)₂SO₄ (1.0 g.), MgCl₂ (0.5 g.), K₂HPO₄ (3.0 g.), sodium malate (3.0 g.), yeast extract (Oxoid) (1.0 g.), water 1000 ml. The final solution was adjusted to pH 6.8–7.0 with 6*N*-NaOH. The only difference from van Niel's medium is that 'Yeast autolysate (1.0 ml.)' (source unstated) has been replaced by the yeast extract. Inoculation of the liquid media was carried out with 1 ml. of a 5- to 6-day-old liquid culture. Anaerobic cultures were grown as previously described for *Rsp. rubrum* (Goodwin & Osman, 1953, 1954) in completely filled Pyrex bottles (100 ml.) fitted with ground-glass stoppers. Aerobic cultures (100 ml.) were grown in Erlenmeyer flasks (250 ml.). All cultures were shaken vigorously by hand twice a day. They were incubated at 30° either in the dark in a conventional electrically heated incubator or in the light in an all glass incubator illuminated on two sides with two banks of 4 $\times$ 60 w incandescent lamps (Goodwin & Osman, 1953).

Washed suspensions were obtained using the same technique as described previously for *Rsp. rubrum* (Goodwin & Osman, 1954).

Analytical procedures

Dry weight determinations. Direct dry wt. determinations were carried out as described for *Rsp. rubrum*. (Goodwin & Osman, 1953). An attempt was made to relate the E (1μ) value of suspensions with their dry wt. as was done with *Rsp. rubrum*, but, contrary to the experience with this organism, the results obtained in preliminary experiments were not satisfactory and the direct determination of dry wt. was always carried out.

Extraction, separation and determination of the carotenoids. The extraction procedure used was that first described for *Rsp. rubrum* by Polgar, van Niel & Zechmeister (1944) and slightly modified by Goodwin & Osman (1953); it was further slightly revised to suit *R. spheroides*. The treatment

of the methanol extract with aqueous KOH, to remove bacteriochlorophyll, was carried out for 2 min. at room temp. instead of at the b.p. This reduced to a minimum the *cis*→*trans* isomerism of the pigments, which in the case of *Y* and *R* can occur very easily. This method of extraction was, in our hands, more satisfactory than the method originally described for *R. spheroides* by van Niel (1947).

Chromatographic separation of the pigments. This was carried out according to the method of Goodwin & Osman (1953, 1954) using alumina (grade 'O': P. Spence and Co., Widnes) deactivated with methanol (Goodwin & Srisukh, 1949).

Quantitative determination of the pigments. The amounts of the two major components (*Y*) and (*R*) were calculated according to the equations of van Niel (1947) after measuring the E values of the solution of the mixed pigments in benzene at 500 and 530 $m\mu$. This method is based on the assumption that there are no other pigments present; this is not strictly true but as the other pigments represent only a small percentage of the whole, the error caused by their presence is not large.

Fig. 1. Absorption spectrum in light petroleum (b.p. 40–60°) of pigments *Y* (---) and *R* (—).

RESULTS

The pigments present in *Rhodopseudomonas spheroides*

A number of extracts of anaerobic cultures were examined chromatographically on deactivated alumina. The separations obtained were always the same, but the relative amounts of the components tended to vary slightly from culture to culture. A typical separation is illustrated in Table 1 and corresponds closely with that obtained by van Niel (1947). Zones 2 and 5 are the most abundant fractions and are identical with pigments *Y* and *R* of van Niel. Their absorption spectra in light petroleum are recorded in Fig. 1. The remaining zones represent traces of pigment only, and could not be examined in great detail. Zone 1 was also noted by van Niel, who suggested that it might be flavorhodene (Karrer & Solmssen, 1936); the shape of its absorption spectrum appears, however, to

Table 1. *Chromatographic separation of Rhodopseudomonas spheroides carotenoids*

Adsorbent weak alumina; pigments listed in order of increasing adsorptive power. Anaerobic culture.

Zone	Description	Absorption spectra λ_{max} ($m\mu$) in light petroleum	Ether in light petroleum (% v/v) required to elute pigment from column
1	Yellow; trace	443, 470	2
2	Yellowish brown; major zone	428, 453, 485	5–10
3	Pink; trace	455, 483, 515	10
4	Yellow; trace	429, 452, 484	10
5	Pink; second most abundant zone	459, 488, 522	20
6	Trace of pink	462, 492, 525	20
7	Brownish pink; trace	Ill-defined maxima 482	25
8	Trace; yellowish brown	428, 453, 483	40
9 and 10	Trace of two separate dirty yellow zones with ill-defined maxima	—	60

preclude this possibility. Zone 4 is probably a *cis* isomer of *Y*. Zones 6–10 correspond to a zone described by van Niel as ‘at least 6 small fractions of purplish, red and yellow adsorbates’, and which he did not describe further. Zones 7, 9 and 10 have very indeterminate spectra and are probably oxidative artifacts; zone 6 might be spirilloxanthin (Polgar *et al.* 1944) it shows the correct chromatographic behaviour and absorption maxima, but the persistence of the bands is less than that of spirilloxanthin. Zone 8 has a spectrum identical in shape and position with *Y*. Because of its chromatographic properties, however, it must contain one hydroxyl group and is therefore probably monohydroxy *Y*. No colourless polyenes, such as phytoene or phytofluene, were detected.

Chromatography of the pigment extract of cultures of *R. spheroides* grown aerobically gave the same qualitative picture as that obtained with extracts of anaerobic cells. Quantitatively the relative amounts of *Y* and *R* were approximately reversed.

Pigment synthesis in growing cultures

In his investigations on *R. spheroides*, van Niel (1947) examined only the change in pigment distribution when mature cultures were transferred from anaerobic to aerobic conditions. He did not relate pigment production to growth (dry weight) of the bacterium, nor did he follow the change in pigment production with time. Table 2 gives the results of one of many experiments carried out to obtain this further information. Anaerobically, growth is complete after 5 days with the pigment concentration remaining almost constant throughout, the amount of *Y* present being just over twice that of *R*. Under aerobic conditions full growth is achieved at 4–5 days and in young cultures the concentrations of *R* and *Y* are almost exactly the reverse of those in anaerobic cultures. A further important difference between anaerobic and aerobic cultures is that whilst in the former the concentration of *Y* falls only slightly with increasing age, it drops much more rapidly in aerobic cultures; e.g. *Y* has almost

Table 2. Dry weight and pigment production by *Rhodopseudomonas spheroides*

Standard liquid medium; temp. 30°; amounts produced/100 ml. medium; each value represents the mean of at least two determinations; the variation between individual determinations is of the order ±2% for dry wt. and ±5% for pigments.

Age of culture (days)	Anaerobic cultures			Aerobic cultures		
	Dry wt. (mg.)	Pigment (mg./g. dry wt.)		Dry wt. (mg.)	Pigment (mg./g. dry wt.)	
		<i>R</i>	<i>Y</i>		<i>R</i>	<i>Y</i>
2	90	0.86	2.40	69	2.11	0.88
3	105	1.05	2.36	—	—	—
4	108	0.99	2.09	106	2.35	0.69
5	149	0.88	1.94	—	—	—
6	—	—	—	104	2.54	0.73
7	140	0.70	1.78	—	—	—
8	—	—	—	118	2.49	0.23
10	142	0.75	1.57	100	2.51	0.10

Table 3. Change in pigment distribution on transferring *Rhodopseudomonas spheroides* from anaerobic to aerobic conditions

Five-day cultures transferred for 24 hr. in light and 48 hr. in dark; except where stated otherwise. Temp. 28°; washed suspensions in 0.067M-KH₂PO₄-K₂HPO₄ buffer.

Concentration of pigments (mg./g. dry wt.)								
Before transfer			After transfer					
			In dark			In light		
<i>R</i>	<i>Y</i>	Total	<i>R</i>	<i>Y</i>	Total	<i>R</i>	<i>Y</i>	Total
Washed suspensions								
0.95	2.38	3.33	1.36	1.59	2.95	1.82	0.74	2.56
0.59	4.98	5.57	1.94	2.55	4.49	3.15	0.95	4.10
0.97	2.55	3.52	1.72	1.72	3.44	—	—	—
0.65	1.99	2.64	1.18	0.93	2.11	1.69	0.30	1.99
Unwashed suspensions								
1.37	4.31	5.68	(a)* 2.55 (b)* 2.36	3.45 3.40	6.00 5.76	3.23 3.33	2.91 2.58	6.14 5.91

* (a) are 24 hr. transfers and (b) 48 hr. transfers.

completely disappeared from 8-day aerobic cultures. Anaerobic cultures are occasionally encountered with very high pigment concentration, especially of *Y* (see, for example, some of the experiments quoted in Table 3).

Observations on the anaerobic → aerobic pigment change

Four-day anaerobic cultures (100 ml.) were transferred to 250 ml. Erlenmeyer flasks either as such or after washing and resuspension in 0.067 M- K_2HPO_4 - KH_2PO_4 buffer at pH 6.8. The results obtained (Table 3) confirm those of van Niel (1947) using unwashed cells, in demonstrating that admission of oxygen to anaerobic cultures in either darkness or light results in increased synthesis of *R* and the disappearance of *Y*.

In van Niel's experiments the amount of *Y* disappearing equalled the amount of *R* appearing; in the great majority of our experiments (including many not recorded) there was some loss of total pigment. An experiment recorded with unwashed cells is one of the few in which this did not occur.

Table 3 further shows that washed cells are also capable of bringing about this conversion and that conversion in the dark proceeds to a lesser extent than in the light. This is not just a matter of speed, for 48 hr. dark transfers differ very little from 24 hr. transfers.

Effect of diphenylamine

Diphenylamine, a specific inhibitor of carotenogenesis in a number of organisms (Goodwin, 1953), was tested on *R. spheroides*. Results under aerobic and anaerobic conditions (Table 4) were very similar. At a level of 1/35 000 diphenylamine com-

pletely inhibits growth, whilst at levels of 1/140 000 and 1/280 000, the only effect is that growth rate is slightly reduced. A concentration of 1/70 000, however, inhibits growth very considerably for 6-7 days and then, quite suddenly, the cultures produce normal growth. During the period when growth is inhibited, synthesis of pigment *R* is generally reduced slightly whilst that of *Y* is considerably reduced. Chromatography of the polyene extracts from young diphenylamine cultures did not reveal the presence of any of the more saturated polyenes (such as the phytofluene series).

Action of other inhibitors

Well-known metabolic inhibitors have been investigated for their effect on the anaerobic (yellow) → aerobic (red) pigment change both in light and in the dark and the effect compared with their action on growth. The results of a number of light experiments are recorded in Table 5; except for hydroxylamine, discussed below, results in the dark were exactly the same as in the light and are not recorded. The buffering capacity of the medium was adequate except in the presence of 10^{-2} M- and 10^{-1} M-KCN, when the pH rose to 9.7 and 10.6 respectively. Control experiments, however, showed that raising the pH to 10.6 had no effect on the pigment change.

As many of the experiments were carried out at different times, the results are expressed as the percentage of the concentration of pigment *R* compared with the control level (100%); the disappearance of *Y* always parallels the increase in *R* and the *Y* values are therefore not recorded. The most important observation is the comparative immunity of the colour change to relatively

Table 4. *Effect of diphenylamine on growth and carotenoid synthesis by Rhodopseudomonas spheroides*

Amounts produced per 100 ml. culture, temp. 30°; 1:35 000 completely inhibits growth both in aerobic and anaerobic conditions.

Time (days)	Concentration of diphenylamine ... 0		1:70 000		1:140 000		1:280 000	
	Pigment concn. (mg./g. dry wt.)		Pigment concn. (mg./g. dry wt.)		Pigment concn. (mg./g. dry wt.)		Pigment concn. (mg./g. dry wt.)	
	Dry wt. (mg.)	<i>R</i> <i>Y</i>	Dry wt. (mg.)	<i>R</i> <i>Y</i>	Dry wt. (mg.)	<i>R</i> <i>Y</i>	Dry wt. (mg.)	<i>R</i> <i>Y</i>
Anaerobic growth								
3	102	1.32 2.33	4.75	0.46 0.15	69	1.11 2.21	90	0.97 2.55
5	140	0.77 1.89	5.25	0.84 0.55	119	0.97 1.64	137	0.93 1.97
7	140	0.70 1.78	10.75	0.54 0.65	139	0.53 1.20	148	0.66 1.39
10	141	0.70 1.74	104	0.51 1.42	152	0.69 1.67	145	0.74 1.52
Aerobic growth								
2	69	2.11 0.88	6.5	1.11 0.10	45	2.04 0.63	67	2.03 0.90
4	106	2.35 0.67	19.5	1.62 0.06	96	2.79 0.57	101	2.41 0.82
6	104	2.54 0.73	23	1.25 0.22	102	2.47 0.55	112	2.39 0.54
8	118	2.49 0.23	90	— —	116	2.44 0.21	112	2.52 0.27
10	100	0.51 0.10	83	2.02 0.13	92	2.81 0.10	104	2.75 0.12

massive concentrations of the inhibitors; only with 10^{-2} M sulphanilamide and 10^{-1} M-KCN was marked inhibition achieved, and in these cases it was only because the cells had been killed. This was demonstrated by testing the viability of these cultures which showed inhibition by washing them and inoculating them into fresh medium. With 10^{-3} M hydroxylamine, inhibition has been consistently observed in the dark but not in light. A further important fact which emerges is that the inhibitors which, at certain concentrations, completely inhibit growth, never have any effect on the pigmentary changes.

DISCUSSION

R. spheroides is one of two photosynthetic bacteria in which carotenogenesis has been examined in detail,

and it is instructive to compare it with the other organism, *Rsp. rubrum* (Goodwin & Osman, 1953, 1954); see Table 6. Although much more information on the general metabolism of these two organisms is required before the problem of carotenogenesis is finally solved, it appears that pigment synthesis is very different in each case. Especially important is the reaction of the two organisms to diphenylamine. With a concentration of 1/70 000 diphenylamine, young cultures of *R. spheroides* produce less pigment than do normal cultures; this is the same as in *Rsp. rubrum*, although no saturated polyenes are synthesized in *R. spheroides*. After 6 days treatment with diphenylamine, growth and pigment synthesis in *R. spheroides* suddenly become normal; no such phenomenon occurs with *Rsp. rubrum*.

Table 5. *The effect of various inhibitors on anaerobic growth and the anaerobic → aerobic colour change in Rhodospseudomonas spheroides*

Inhibitor	Concn.* (M)	Effect on colour change; amount of <i>R</i> produced compared with control (%)	Effect on growth
Arsenite	3×10^{-3}	112	Complete inhibition
2:4-Dinitrophenol	1×10^{-4}	85	Complete inhibition
Sulphanilamide	1.5×10^{-2}	88	Almost complete
Sulphanilamide	5×10^{-2}	No change (cells killed)	Complete inhibition
NH ₂ NH ₂	10^{-2}	88	Complete inhibition
NH ₂ OH.HCl†	10^{-3}	99	Complete inhibition
NaN ₃	10^{-2}	100	Complete inhibition‡
Iodoacetate	10^{-2}	103	Complete inhibition
KCN	10^{-2}	102	Complete inhibition
KCN	10^{-2}	84	
KCN	10^{-1}	31	
NaF	10^{-1}	91	
Arsenate	10^{-1}	113	Complete inhibition
Malonate	4×10^{-2}	101	No effect
Diphenylamine	8.5×10^{-5}	108	No effect (See Table 4)

* Only the highest concentrations examined are reported.

† This concentration of hydroxylamine inhibits the colour change in the dark; all other inhibitors have the same effect in light and darkness.

‡ Complete inhibition of growth was obtained at 10^{-3} M-NaN₃.

Table 6. *A comparison of carotenogenesis in Rhodospirillum rubrum and Rhodospseudomonas spheroides*

Experimental conditions	<i>Rsp. rubrum</i>	<i>R. spheroides</i>
Main pigments produced	One; Spirilloxanthin (<i>S</i>)	Two- <i>R</i> and <i>Y</i>
Effect of growth	Low concns. in young cultures	Same concn. throughout growth
Anaerobic culture	High concn. of <i>S</i>	High concn. of <i>Y</i> , low of <i>R</i>
Aerobic cultures	Lower concn. of <i>S</i>	Low concn. of <i>Y</i> , high of <i>R</i> Total same as under anaerobic conditions
Anaerobic washed cells suspended in phosphate buffer	No change in <i>S</i> content	Decrease in <i>Y</i> and increase in <i>R</i>
Action of diphenylamine (1/70 000)	Inhibits <i>S</i> production; stimulates production of more saturated polyenes	Inhibits growth and pigment production in early stages, but after about 6-7 days both approach normality. No detectable synthesis of the more saturated precursors

Conversion of pigment Y into R

From his original investigations on *R. spheroides*, van Niel (1947) concluded that *Y* was converted into *R*. Although this conclusion may be correct, there are indications that another explanation is probably true. Table 2 shows that in growing aerobic cultures *Y* is decreasing although *R* is not increasing; that is, under one condition at least, *Y* undergoes a change other than a possible conversion into *R*. It is possible therefore, that in the anaerobic $\rightarrow$ aerobic change, synthesis of *R* from small molecules may suddenly be stimulated. This type of carotenogenesis has recently been observed in the green alga *Haematococcus pluvialis*; when the green cells of this alga are resuspended in phosphate buffer alone, large amounts of astaxanthin are synthesized *de novo* and not at the expense of the carotenoids already present (Goodwin & Jamikorn, 1954).

Furthermore, the change from *Y* to *R*, if it occurs, must be structurally complicated. The shifting of the main maxima of a carotenoid from 453 to 480 m μ ., must essentially entail the production of 3-4 conjugated double bonds, that is, the removal of 6-8 hydrogen atoms. In this case, that cannot be all, for a profound difference in the shape of the absorption spectra of *Y* and *R* exists (Fig. 1). The sharpness of the bands of *Y* suggest that it has a completely open-chain structure similar to that of lycopene (compare the definition of the bands of lycopene with that of β -carotene and of *Y*; Goodwin, 1952). The shape of the spectrum of *R*, in which the fine structure has almost completely disappeared, suggests either the appearance of one carbonyl group (the chromatographic behaviour of the pigment confirms this) or the ring closure of both ends of the open-chain compound. The addition of an hydroxyl or, possibly, a carbonyl group is required to account for the chromatographic behaviour of *R*. Thus, although the structures of *Y* and *R* are not yet known, it is obvious that they must differ considerably and that the postulated change *Y* $\rightarrow$ *R* cannot be a simple dehydrogenation.

If the change *Y* $\rightarrow$ *R* does take place, it is difficult to envisage it occurring in one step, and up to the present it has not been possible to detect any 'intermediates' in this postulated transformation.

SUMMARY

1. The presence of two major carotenoids (one yellow, *Y*, and one red, *R*), together with six minor components in *Rhodospseudomonas spheroides* has been confirmed. One of the minor fractions appears to be a monohydroxy derivative of *Y*. *Y* predominates in anaerobic and *R* in aerobic cultures. *Y* but not *R* disappears from aerobic cultures soon after they are fully grown.

2. The synthesis of *R* and the disappearance of *Y* when air is admitted to anaerobic cultures has been confirmed. The same reaction occurs in washed anaerobic cells resuspended in phosphate buffer.

3. Diphenylamine (1/70 000) inhibits growth and pigment synthesis for 5-6 days; then suddenly growth and carotenogenesis become normal. No saturated polyenes are synthesized on diphenylamine-containing media.

4. Many compounds which inhibit growth of *R. spheroides* do not, at the same concentrations, affect the yellow $\rightarrow$ red pigmentary change; this change is very insensitive to inhibitors.

5. Reasons are given for assuming that in the yellow $\rightarrow$ red change, pigment *Y* may not be converted into pigment *R*.

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