Studies in Carotenogenesis

9. GENERAL CULTURAL CONDITIONS CONTROLLING CAROTENOID (SPIRILLOXANTHIN) SYNTHESIS IN THE PHOTOSYNTHETIC BACTERIUM *RHODOSPIRILLUM RUBRUM*

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The photosynthetic tissues of the higher plants synthesize α- and β-carotenes and a complex mixture of xanthophylls. They differ from non-photosynthetic tissues (e.g. carrot roots and many fruits, especially tomatoes) and from most fungi in not synthesizing, even in traces, any member of the 'Porter-Lincoln series'. This series, first observed in tomatoes (Porter & Lincoln, 1950) and later in berries (Goodwin, 1952*a*) and fungi (Goodwin, 1952*b*), consists of lycopene, neurosporene, ζ-carotene, phytofluene, phytoene and tetrahydrophytoene, each member of the series containing four hydrogen atoms less than the one following it. Porter & Lincoln (1950) considered that in tomatoes lycopene is formed via this series by the stepwise removal of hydrogen from tetrahydrophytoene, β-carotene being finally formed by isomerization of lycopene. Although this view is probably not correct (Goodwin & Jamikorn, 1952; Goodwin, Jamikorn & Willmer, 1953), the non-appearance of this series in green tissues does suggest that there may be marked differences between carotenogenesis in these and in fruit and fungi. Furthermore, fungi such as *Phycomyces* do not produce xanthophylls although these are the major carotenoid fraction in green leaves.

It was therefore decided to study carotenoid synthesis in a photosynthetic material and to compare the observations with those made under similar conditions on *Phycomyces*, concerning which a considerable amount of information has been accumulated in this laboratory.

It was decided not to use green leaves (although the important preliminary work of Bandurski (1949) on bean leaves shows that they can be successfully used) but to utilize the photosynthetic bacterium *Rhodospirillum rubrum*, because bacteria can be subjected to many laboratory techniques more readily and could be more readily compared with *Phycomyces*. Furthermore, *R. rubrum* produces essentially one carotenoid, which makes routine analysis much simpler. This carotenoid, spirilloxanthin (van Niel & Smith, 1935; Polgar, van Niel & Zechmeister, 1944) or rhodoviolascin (Karrer & Solmsson, 1936), is a xanthophyll and thus carotene production in a fungus could be compared with xanthophyll synthesis in a photosynthetic organism.

Part of this work has been presented at the 2nd International Congress of Biochemistry (Goodwin & Osman, 1952).

EXPERIMENTAL

Cultures. The cultures used were designated *C* and *P* and were obtained from Dr J. Glover; they originally came from Dr C. B. van Niel via Dr M. Kamen's laboratory. Unless otherwise stated, strain *C* was used. The cultures were maintained in stabs on nutrient agar.

Method of culture. The basic medium used throughout the investigation contained, per l., $MgSO_4.7H_2O$ (0·2 g.), anhydrous $CaCl_2$ (0·375 g.), biotin (5 μg.), L-glutamic acid (4·0 g.), DL-malic acid (3·5 g.), sodium citrate (0·8 g.), yeast extract (Difco) (0·25 g.), KH_2PO_4 (0·12 g.) and K_2HPO_4 (0·18 g.). The pH was adjusted to 7·0. Other constituents were added as indicated in the Results section. The cultures were grown anaerobically in Pyrex bottles filled completely with medium and fitted with glass stoppers held in position with adhesive plaster. Aerobic cultures were grown in 250 ml. Erlenmeyer flasks containing 100 ml. of medium. 'Light' experiments were carried out in a glass cabinet thermostatically controlled at 30° (Garton, Goodwin & Lijinsky, 1951) and illuminated on two sides by banks of 6 × 60 W. incandescent bulbs. 'Dark' cultures were grown in a normal incubator at 30°.

Determination of dry weight of bacteria. As the bacteriochlorophyll-spirilloxanthin-protein complex does not absorb light of wavelength greater than 850 mμ., it was thought that the light absorbed (scattered) at about 1 μ. would indicate the growth of the cultures. This was tested by carrying out dry weight determinations and measuring *E* (1 μ.) values in a 1 cm. cell on numerous cultures. It was found that when these two parameters were plotted against each other a straight line resulted over a considerable range of *E* values. In all further experiments, therefore, the *E* (1 cm., 1 μ.) values of the cultures were measured in a Beckman photoelectric spectrophotometer and the dry weights of the cultures were read off the calibration curve reproduced in Fig. 1.

Carotenoid determination. The culture was well shaken, a measured volume centrifuged and the supernatant removed. The pigment was then extracted from the bacterial mass using a method based on that of Polgar *et al.* (1944). The cells were thoroughly mixed with about 10 ml. of methanol; this dehydrates the bacteria and also removes

almost all the bacteriochlorophyll, whilst extracting only minute traces of the carotenoid. The dehydrated residue was then extracted with three to four successive portions of benzene; these were combined and shaken with about 15 ml. of aqueous Na_2CO_3 to remove final traces of bacteriochlorophyll and then made up to a suitable volume for

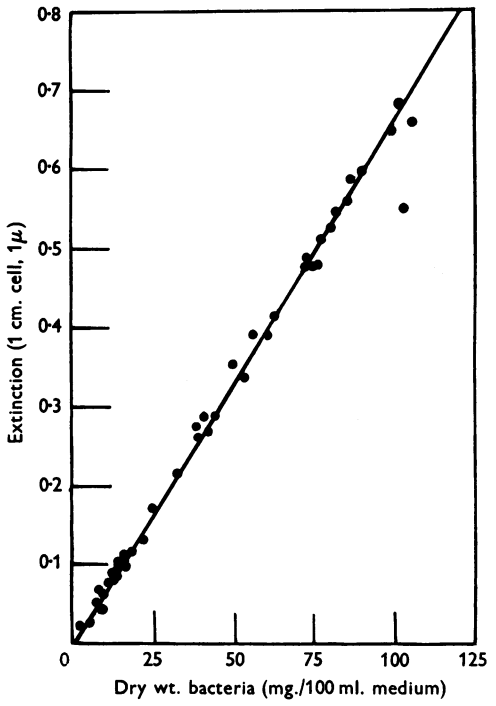

Fig. 1. The calibration curve for converting $E_{1\,cm.}$, 1 μ. of a suspension of *R. rubrum* into dry weight of bacteria.

measurement of the extinction of the solution at 510 mμ., the main maximum of absorption of pure spirilloxanthin (Polgar *et al.* 1944). From this the amount of pigment in the extract can be calculated assuming the $E^{1\%}_{1\,cm.}$ for pure spirilloxanthin to be 2360 (Polgar *et al.* 1944). Little error is introduced into the calculation by assuming the pigment to be completely *all-trans*-spirilloxanthin for, as will be seen below, only traces of the *cis* forms are present and the occurrence of other carotenoids cannot be demonstrated.

Chromatographic analysis of pigments. The pigment extracts were chromatographed on weakened alumina (Goodwin, 1952*a*) using benzene as developer.

RESULTS

Polyenes produced by R. rubrum. Chromatography of the carotenoid extract of *R. rubrum*, cultured under either aerobic or anaerobic conditions, yielded identical results. On chromatography, two coloured zones were formed. The upper zone was by far the greater, representing about 98 % of the total pigment present. This agreed with the results obtained by Polgar *et al.* (1944), and an examination of the spectral properties of the two pigments showed that the major component was spirilloxanthin, whilst the minor zone represented a mixture of *cis* isomers of spirilloxanthin. A colourless zone exhibiting bright blue fluorescence ran straight through the column. The benzene was removed from this fraction which was redissolved in light petroleum (b.p. 40–60°) and rechromatographed. A number of small fluorescent zones were separated, each giving ill-defined absorption spectra with no marked maxima. These substances have not been identified. None of the more saturated polyenes such as phytofluene, ζ-carotene, etc., which occur in tomatoes and other fruit and in *Phycomyces*, could be detected in *R. rubrum*.

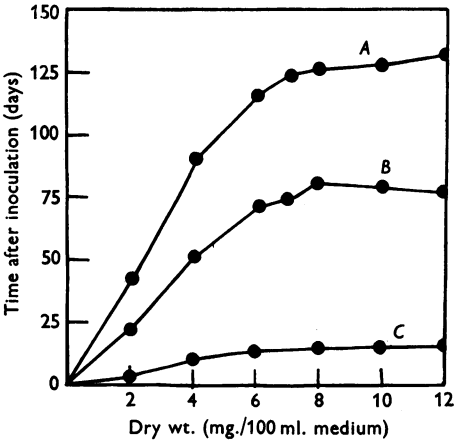

Fig. 2. Growth of *R. rubrum* on the standard medium at 30°. *A*, anaerobically in light; *B*, aerobically in light; *C*, aerobically in dark.

Spirilloxanthin synthesis on the standard medium. *R. rubrum* was cultured aerobically in the light and dark and anaerobically in the light (the organism will not grow anaerobically on the standard medium in the dark) and the dry weight and spirilloxanthin production were determined over a 12-day period. Typical results for growth are given in Fig. 2. In all cases growth was complete within 4–6 days of inoculation. The dry weight production under aerobic conditions was about 6 times greater in the light than in the dark, and in light was nearly twice greater under anaerobic than under aerobic conditions.

The concentrations of spirilloxanthin in these cultures are given in Fig. 3. In all cases the concentration of pigment did not reach its maximum until about 4 days after inoculation; thus it is clear that in the early stages of growth, the organism is preferentially synthesizing cell material rather than carotenoid. The pigment concentration in aerobic cultures in the light was almost twice that in the

dark, and that in the anaerobic cultures almost twice that of the 'light' aerobic cultures.

Strain *P* was examined and found to synthesize rather less spirilloxanthin, both aerobically in the light and anaerobically, than did strain *C*, although the growth was the same in both cases. In aerobic cultures in the dark, strain *P* grew normally but was completely colourless, failing to synthesize either bacteriochlorophyll or spirilloxanthin. The reason for the difference between the two strains is not yet known, but it is important to note that it was found that strain *P* does not accumulate any colourless polyenes which might be considered precursors of spirilloxanthin.

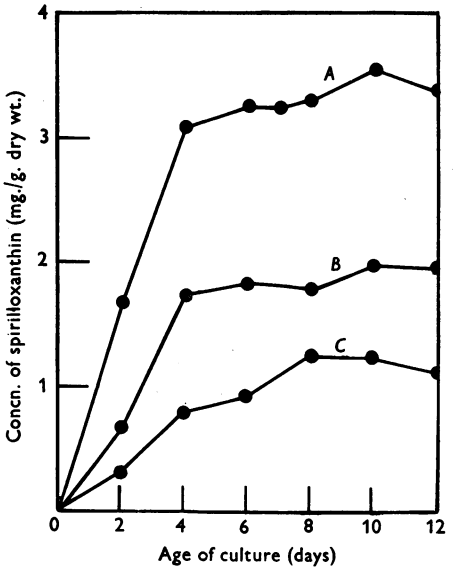

Fig. 3. Spirilloxanthin synthesis by *R. rubrum* cultured on the standard medium at 30°. *A*, anaerobically in light; *B*, aerobically in light; *C*, aerobically in dark.

Effect of varying the nitrogen source of the medium. Goodwin & Lijinsky (1951) showed that L-leucine could, under certain circumstances, stimulate carotenogenesis in *Phycomyces*. Experiments were therefore carried out in which the normal nitrogen source (glutamic acid) in the basal medium for *R. rubrum* was replaced either wholly or partly by L-leucine. Cultures in the light, either aerobically or anaerobically, with L-leucine as the sole nitrogen source grew to a lesser degree and also produced cells containing a lower concentration of spirilloxanthin (Figs. 4, 5). Aerobic cultures in the dark, on the other hand, grew to the same extent irrespective of whether the nitrogen source was glutamate or L-leucine or a mixture of the two; furthermore, the synthesis of spirilloxanthin on the L-leucine medium was slightly but consistently greater than

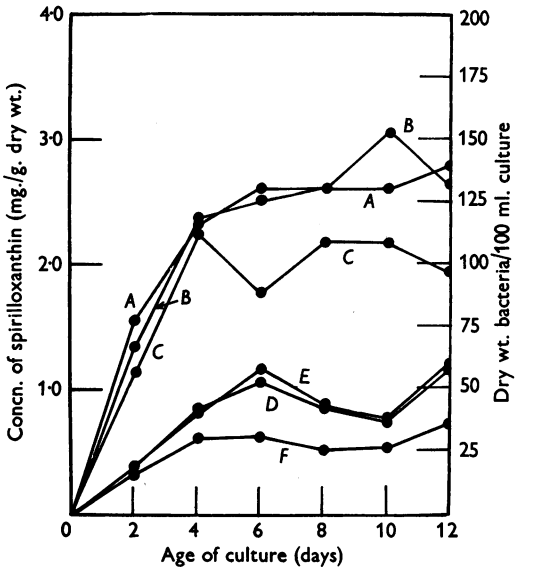

Fig. 4. Growth and spirilloxanthin production by *R. rubrum* grown anaerobically at 30° on (i) the standard medium, (ii) the standard medium in which all the glutamate was replaced by L-leucine, and (iii) the standard medium in which one-half the glutamate was replaced by L-leucine. *A*, growth, all glutamate; *B*, growth, glutamate + leucine; *C*, growth, all leucine; *D*, spirilloxanthin, all glutamate; *E*, spirilloxanthin, glutamate + leucine; *F*, spirilloxanthin, all leucine.

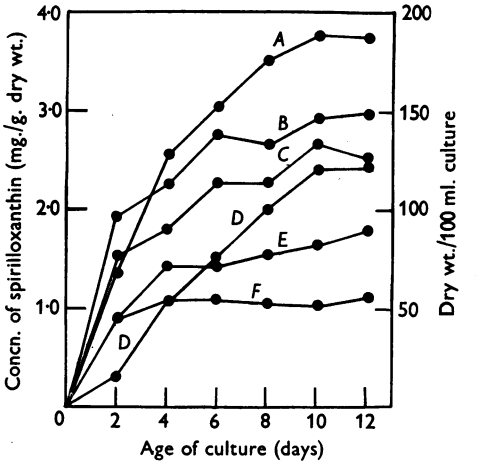

Fig. 5. Growth and spirilloxanthin production by *R. rubrum* grown aerobically in the light at 30°, under conditions outlined in Fig. 4 and with the same significance attached to the letters.

on glutamate (Fig. 6). All cultures containing L-leucine produced a characteristic odour, suggesting that part of the leucine had been decarboxylated rather than deaminated.

The effect of diphenylamine on the synthesis of spirilloxanthin. The synthesis of β-carotene in the fungus *Phycomyces blakesleeanus* is inhibited by the presence of diphenylamine, whilst the production of the more saturated polyenes (phytofluene, etc.) is stimulated (Goodwin, 1952*a*). When diphenylamine was tested on *R. rubrum* under aerobic conditions, it was found that over the concentration range 0–7·15 μg./ml. (1/140000) diphenylamine inhibits the synthesis of spirilloxanthin whilst having very little effect on growth (Fig. 7). The synthesis of bacteriochlorophyll was also inhibited. At a concentration of 14·3 μg./ml. (1/70000) there is complete inhibition of the synthesis of both pigments, the organism is completely colourless and growth is reduced to the level normally observed in cultures grown aerobically in the dark (Fig. 2).

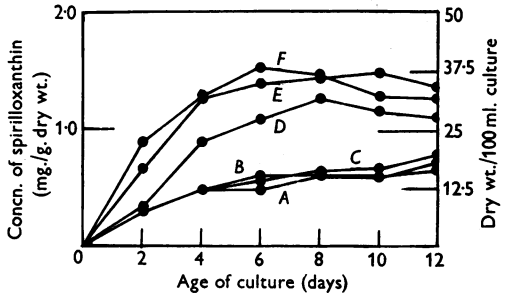

Fig. 6. Growth and spirilloxanthin production by *R. rubrum* grown aerobically in the dark at 30°. Other conditions as in Figs. 4 and 5.

The pigment extracts obtained from diphenylamine cultures were examined chromatographically. No evidence was obtained of the synthesis of the more saturated polyenes, phytofluene, etc., as was the case with *Phycomyces*. There was, however, a somewhat increased production of the fluorescent materials which are less strongly adsorbed than spirilloxanthin.

Anaerobic cultures of *R. rubrum* were similarly affected by diphenylamine. The results of a typical experiment are recorded in Table 1. It will be seen that a concentration of 7·15 μg./ml. (1/140000) of diphenylamine somewhat inhibits growth in anaerobic cultures, but the inhibition of pigment synthesis is very much more pronounced.

Adenosine-5-phosphate (AMP) and riboflavin which (separately) can counteract the inhibition of β-carotene production by diphenylamine in *Phycomyces*, were also tested for this effect in *R. rubrum*. Neither of these was effective in reversing the diphenylamine inhibition.

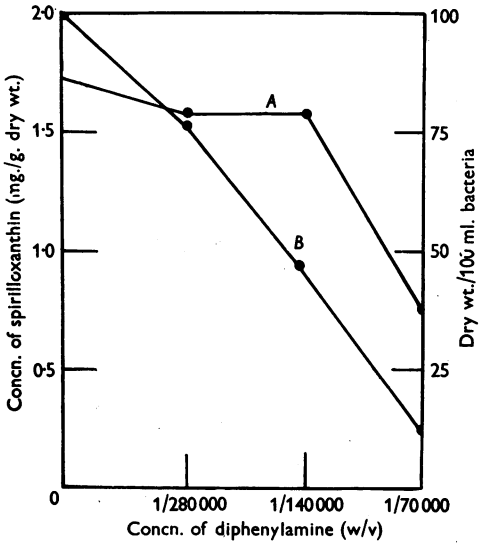

Fig. 7. The effect of varying concn. of diphenylamine on growth and spirilloxanthin synthesis by *R. rubrum* cultured aerobically in the light at 30°. *A*, growth; *B*, spirilloxanthin.

The effect of streptomycin on spirilloxanthin synthesis in R. rubrum. Streptomycin was found to be very active in inhibiting growth of *R. rubrum* both in aerobic and anaerobic cultures, a concentration of 100 μg./ml. being sufficient to cause almost complete inhibition. Over a small range of concentrations near the maximum tolerated by the organism, it was found that streptomycin had a specific inhibitory action on pigment synthesis. In the case of aerobic cultures, at a streptomycin level

Table 1. *The effect of diphenylamine* (7·15 μg./ml., 1/140000) *on growth and spirilloxanthin production by* R. rubrum *grown anaerobically in the standard medium*

	Without diphenylamine		With diphenylamine	
Age of culture (days)	Wt. of dry bacteria (mg./100 ml. culture)	Concn. of spirilloxanthin (mg./g. dry wt.)	Wt. of dry bacteria (mg./100 ml. culture)	Concn. of spirilloxanthin (mg./g. dry wt.)
6	62*	3·15	77	1·67
8	101	3·65	86	1·62
10	121	3·84	76	1·74
12	121	3·79	89	1·97

* This is a lower value than that normally obtained (see Fig. 3).

Table 2. *The effect of streptomycin on growth and spirilloxanthin production by* R. rubrum *cultured aerobically in the standard medium*

Concn. of streptomycin (w/v)	Four-day cultures		Eight-day cultures	
	Growth (mg. dry wt./100 ml.)	Spirilloxanthin (mg./g. dry wt.)	Growth (mg. dry wt./100 ml.)	Spirilloxanthin (mg./g. dry wt.)
0	74	1·68	89	2·19
10^{-2}	0	0	0	0
10^{-3}	10	0	13	0
10^{-4}	9	0	12·0	0
10^{-5}	34	0·95	25	1·21

Table 3. *The effect of streptomycin on growth and spirilloxanthin production by* R. rubrum *cultured anaerobically in the standard medium*

Concn. of streptomycin (w/v)	Four-day cultures		Eight-day cultures	
	Growth (mg. dry wt./100 ml.)	Spirilloxanthin concn. (mg./g. dry wt.)	Growth (mg. dry wt./100 ml.)	Spirilloxanthin concn. (mg./g. dry wt.)
0	102	3·21	98	3·80
10^{-4}	19	1·33	42	2·31
10^{-5}	63	3·07	89	3·86

of 10 μg./ml., not only was growth reduced, but the concentration of spirilloxanthin was considerably lowered, indicating a specific inhibition (Table 2). Similar results were obtained in anaerobic cultures (Table 3). It will be seen that anaerobic cultures are somewhat less sensitive to streptomycin, a concentration of 1 mg./ml. being required before complete inhibition occurred. At a level of 100 μg./ml. growth was reduced and pigment synthesis specifically inhibited. At a level of 10 μg./ml. streptomycin acts by reducing rate of growth rather than the amount finally achieved; pigment synthesis was not inhibited at this concentration. In both cases, as with diphenylamine, the synthesis of bacteriochlorophyll was also inhibited by streptomycin.

DISCUSSION

A comparison of carotenoid synthesis in *R. rubrum* and in *Phycomyces blakesleeanus* reveals interesting differences which shed some light on the general problem of carotenogenesis.

Growth and carotenogenesis. In *Phycomyces* only small amounts of β-carotene are produced during the growth of the mycelium; the major portion is synthesized after growth is complete (Goodwin & Willmer, 1952). In *Rhodospirillum* also growth tends to occur before pigment production (Fig. 3), but the effect is not so marked and synthesis becomes maximal before the cultures have completed growth.

Effect of light and oxygen. As in the case of *Phycomyces* (Garton *et al.* 1951), light appears to exert a direct stimulatory action on carotenogenesis in *Rhodospirillum*. No experiments on the effect of oxygen tension on pigment synthesis by *Phycomyces* have been carried out and this organism cannot grow anaerobically. The reason for the great stimulation of pigmentation in *Rhodospirillum* grown anaerobically is at present obscure.

Effect of L-*leucine.* On media containing 1% (w/v) glucose, the presence of L-leucine considerably stimulates carotenogenesis in *Phycomyces* (Goodwin & Lijinsky, 1951). Replacement of glutamate by L-leucine in the standard medium used for culturing *Rhodospirillum* resulted in decreased synthesis of spirilloxanthin in the light. In aerobic-dark cultures, however, a slight but marked stimulation of synthesis was observed in the presence of L-leucine. It is possible that in the dark L-leucine can supply in small amounts an intermediate, which is readily produced photosynthetically and which is not the limiting factor in cultures grown in the light.

Effect of inhibitors. Diphenylamine inhibits the synthesis of β-carotene in *Phycomyces* and stimulates that of the more saturated polyenes (phytofluene, ζ-carotene, etc.) (Goodwin, 1952*a*, *b*). Synthesis of spirilloxanthin in *Rhodospirillum* is similarly reduced by diphenylamine, but the appearance of more saturated polyenes was not observed. It has been suggested that carotenoids are built up via more saturated compounds (Porter & Lincoln, 1950) and thus the accumulation of such compounds in the presence of diphenylamine could be due to the inhibition of their dehydrogenation. The failure to observe more saturated polyenes in 'diphenylamine-cultures' of *Rhodospirillum* suggests that their increased production in similar cultures of *Phycomyces* is not related to the inhibition of the synthesis of β-carotene. This is further evidence to that already obtained (Goodwin *et al.* 1953; Goodwin & Jamikorn, 1952), indicating that the fully unsaturated carotenoids (β-carotene, etc.) are not synthesized via phytofluene, etc

Whilst either AMP or riboflavin counteracts the inhibition of β-carotene synthesis by diphenylamine (Goodwin *et al.* 1953), neither substance has any such effect on the formation of spirilloxanthin. It is thought that this difference is due to variations in the basic metabolic processes of the two organisms, from which the polyene building unit is obtained, rather than to any fundamental difference in the method of integration of the building units. This conclusion is borne out when the action of streptomycin on the two organisms is considered. In *Phycomyces* grown on a glucose medium, streptomycin has no effect on growth but specifically inhibits carotenogenesis (Goodwin & Griffiths, 1952); the growth of *Rhodospirillum*, on the other hand, is strongly inhibited by low concentrations of streptomycin, indicating different basic metabolic processes in the two organisms. There is, however, a small range of concentrations of streptomycin, over which the synthesis of spirilloxanthin is much more affected than is general growth, thus bringing the effect on carotenogenesis *per se* into line with that observed in *Phycomyces*.

SUMMARY

1. The dry weight of cultures of *Rhodospirillum rubrum* can be determined by measuring the extinction of the suspension at 1 μ. wavelength.

2. Spirilloxanthin, together with traces of its *cis* isomers, is the only carotenoid synthesized by *R. rubrum*. Phytofluene, ζ-carotene, neurosporene or related polyenes were not present.

3. On a malate-glutamate-salt medium, maximal growth occurred when cultures were between 4 and 6 days old. Mean maximal dry weight production by both strains *C* and *D* was 75, 10 and 125 mg./100 ml. for light-aerobic, dark-aerobic and anaerobic (light) cultures, respectively.

4. The rate of spirilloxanthin synthesis is lower in young cultures than in old, reaching its maximum about 4 days after inoculation. Mean maximal concentrations in strain *C* were 1·8, 1·1 and 3·5 mg./g. (dry weight) for light-aerobic, dark-aerobic and anaerobic cultures, respectively. The corresponding values for strain *P* grown in the light were slightly lower. This strain does not synthesize spirilloxanthin in the dark.

5. Substitution of leucine for glutamine resulted in reduced growth and pigment production in 'light' cultures. In dark-aerobic cultures, normal growth was maintained and pigment production slightly increased.

6. Diphenylamine up to a concentration of 7·15 μg./ml. (1/40 000) inhibits synthesis of spirilloxanthin and bacteriochlorophyll without affecting growth. At higher concentrations pigmentation is completely inhibited and growth is reduced. Adenosine-5-phosphate and riboflavin had no counteracting effect on diphenylamine inhibition. No polyenes more saturated than spirilloxanthin were observed in 'diphenylamine' cultures.*

7. In both aerobic and anaerobic cultures streptomycin at concentrations of 100–1000 μg./ml. almost completely inhibits growth. At a concentration of 10–100 μg./ml. inhibition of pigmentation is greater than that of growth.

We wish to thank Prof. R. A. Morton, F.R.S., for his continued interest in this work; Dr J. Glover for a gift of the strains of *R. rubrum* used and for much valuable advice concerning their culture; and the Medical Research Council for a grant towards laboratory expenses.

* *Note in proof.* Recently, minute traces of phytofluene have been noted.

REFERENCES

Bandurski, R. S. (1949). *Bot. Gaz.* **111**, 95.
Garton, G. A., Goodwin, T. W. & Lijinsky, W. (1951). *Biochem. J.* **48**, 154.
Goodwin, T. W. (1952*a*). *Biochem. J.* **50**, 550.
Goodwin, T. W. (1952*b*). *Biochem. J.* **51**, 458.
Goodwin, T. W. & Griffiths, L. A. (1952). *Biochem. J.* **51**, xxxiii.
Goodwin, T. W. & Jamikorn, M. (1952). *Nature, Lond.*, **170**, 104.
Goodwin, T. W., Jamikorn, M. & Willmer, J. S. (1953). *Biochem. J.* **53**, 531.
Goodwin, T. W. & Lijinsky, W. (1951). *Biochem. J.* **50**, 268.
Goodwin, T. W. & Osman, H. G. (1952). *2nd Int. Congr. Biochem. Abstr.* p. 210.
Goodwin, T. W. & Willmer, J. S. (1952). *Biochem. J.* **51**, 213.
Karrer, P. & Solmsson, U. (1936). *Helv. chim. acta*, **19**, 1019.
Polgar, A. van Niel, C. B. & Zechmeister, L. (1944). *Arch. Biochem.* **5**, 243.
Porter, J. W. & Lincoln, R. E. (1950). *Arch. Biochem.* **27**, 390.
van Niel, C. B. & Smith, J. H. C. (1935). *Arch. Mikrobiol.* **6**, 219.