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Studies in Carotenogenesis

10. SPIRILLOXANTHIN SYNTHESIS BY WASHED CELLS OF *RHODOSPIRILLUM RUBRUM*

By T. W. GOODWIN AND H. G. OSMAN

Department of Biochemistry, The University of Liverpool

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It has been known for some time that the photo-synthetic bacterium *Rhodospirillum rubrum* synthesizes, in addition to bacteriochlorophyll, a single carotenoid spirilloxanthin (Polgar, van Neil & Zechmeister, 1944). This pigment appears to be identical with rhodoviolascin isolated by Karrer & Solmsen (1938) from a mixed culture of *Rhodovibrio*, and to have the following structure

Recently, Goodwin & Osman (1952, 1953a) have investigated the general conditions governing spirilloxanthin synthesis in growing *Rsp. rubrum*. The present paper reports an investigation into the synthesis of this pigment in washed cells. Part of this work has already been reported briefly (Goodwin & Osman, 1953b).

EXPERIMENTAL

Cultures. The strain of *Rsp. rubrum* used was the same as that examined previously (Goodwin & Osman, 1953a). 'Normal' cultures were grown on our standard medium, which is very similar to that used by Gest & Kamen (1949), containing per litre: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.20 g.), CaCl_2 (0.0375 g.), biotin (5 μg .), L-glutamic acid (4.0 g.), DL-malic acid

(3.5 g.), sodium citrate, $2\text{H}_2\text{O}$ (0.8 g.), yeast extract (Difco) (0.25 g.), KH_2PO_4 (0.18 g.). The pH was adjusted to 7.0. 'Diphenylamine' cultures were grown on the same medium containing diphenylamine at a level of 1/140 000. The cultures were grown at 30° in a glass incubator (Garton, Goodwin & Lijinsky, 1951) illuminated with two banks of 6 $\times$ 75 w tungsten incandescent lamps. In order to get a more exact temperature control, some of the transfer experiments were carried out at 30° in a similarly illuminated, thermostatically controlled water bath.

Except in the case of those cultured in the presence of a $\text{NaHCO}_3/\text{CO}_2$ buffer, anaerobic cultures were grown in glass-stoppered Pyrex bottles (200 ml.) completely filled with medium. When buffering with $\text{NaHCO}_3/\text{CO}_2$ was required, the cultures (100 ml.) were placed in 250 ml. Erlenmeyer flasks in a large desiccator which was flushed with an argon: CO_2 (95:5, v/v) mixture. A valve was inserted in the bung of the desiccator in order to allow for the expansion of the gas mixture when the desiccator was placed in the incubator. Aerobic cultures were grown in 250 ml. Pyrex conical flasks containing 100 ml. of medium.

Washed suspensions were obtained by centrifuging 3- to 4-day-old growing cultures, washing the bacterial mass with sterile distilled water and then re-centrifuging. After the washing had been twice repeated, these cells were ready for resuspension in new media under conditions similar to those described for growing cultures. The density of the suspensions was adjusted to between 20 and 50 mg. dry wt. of cells/100 ml.

Analytical procedures. Dry weights were determined turbidimetrically by measuring the $E_{1\text{cm}}$ of the bacterial suspension at 1 μ . and reading off the weights on a standard curve (Goodwin & Osman, 1953a).

Spirilloxanthin was extracted from the bacterial mass with benzene, after first treating the cells with methanol (10–15 ml./40 ml. of suspension) (Polgar *et al.* 1944; Goodwin & Osman, 1953a); in normal cultures this serves the double purpose of dehydrating the cells and quantitatively removing the bacteriochlorophyll without removing more than traces of spirilloxanthin. (In diphenylamine cultures, however, some phytofluene, etc., appears in this fraction, see later.) The amount of the pigment present was determined from the E value of the benzene extract at 510 $m\mu$, since $E_{1\%}^{1\text{cm}}$ for the pure pigment is known to be 2360 (Polgar *et al.* 1944). All spectroscopic measurements were made using a Beckman photoelectric quartz spectrophotometer.

The lipid content of the cells was determined by grinding the cells (from 200 ml. of suspension) with anhydrous Na_2SO_4 until a dry powder was obtained. This was then extracted four times with successive portions of diethyl ether, the ether was then removed from the combined extracts, the residue blown dry in a stream of N_2 , and finally dried to constant weight in a vacuum desiccator.

Chromatography of the polyenes. In the case of diphenylamine cultures but not normal cultures, the methanol extract (see above) contains colourless polyenes and carotenoids in traces, as well as bacteriochlorophyll. The polyenes were separated from the chlorophyll by adding KOH (1 ml. of 60% (w/v) to each 10 ml. of methanol extract), boiling for 2 min., diluting with an equal volume of water, and extracting the polyenes with diethyl ether. After washing until free from alkali, the diethyl ether was removed, the residue dissolved in a small volume of light petroleum (light petroleum, b.p. 40–60°, was used throughout this investigation), and chromatographed on alumina (grade '0', supplied by P. Spence and Co., Widnes). The polyenes were separated and examined according to the method previously described (Goodwin, 1952).

Oxygen uptake. This was measured using the conventional Warburg apparatus.

RESULTS

Spirilloxanthin synthesis by normal washed cells

The results of experiments in which washed cells of *Rsp. rubrum* were incubated anaerobically in the light with various metabolizable carbon substrates are given in Table 1. It will be seen that in no case was there a clear demonstration of pigment synthesis. Similar results were obtained when the organisms, suspended in the same media, were incubated aerobically both in the light and in the dark; they are, therefore, not recorded here. When normal cells were transferred to the standard medium, both growth and pigment synthesis occurred (Figs. 1 and 2). Growth and pigment synthesis were also obtained when the cells were transferred to a solution of glutamate, but with two other amino acids tested, L-leucine and glycine, neither growth nor pigment synthesis was observed (the slight decrease with glycine is not considered significant). Presumably glutamate provides both a carbon and a nitrogen source, whilst the other amino acids do not. These observations confirm the

Table 1. *Spirilloxanthin synthesis by washed suspensions of Rsp. rubrum*

(Cells grown 3–4 days; on normal medium; re-suspended in various media, incubated anaerobically in the light and analysed at different times; different cell suspensions were used in each experiment.)

Medium	Concn. pigment ($\mu\text{g./ml. suspension}$)			
	0 hr.	6 hr.	9 hr.	24 hr.
Normal medium	1.32	1.11	1.32	1.48
without glutamate				
Sodium acetate (0.025 M)	1.96	1.85	1.80	2.04
Sodium acetate (0.025 M)*	1.41	—	1.45	1.48
Malate (0.025 M)	2.07	2.09	2.04	2.22
Malate (0.025 M)	2.20	—	2.22	2.25
+ citrate (0.005 M)				
Phosphate buffer	1.11	1.11	—	[0.96]†

* Buffered to pH 7.0 with $\text{NaHCO}_3/\text{CO}_2$ (0.02 M- NaHCO_3 /gas phase (gas phase = CO_2 :argon, 5:95, v/v)). All other media (except the first) buffered with 0.067 M phosphate buffer ($\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$) to pH 7.0. The first medium was unbuffered. All phosphate buffers used in this investigation contained K_2HPO_4 and KH_2PO_4 .

† Measured 48 hr. after resuspension.

earlier work of van Niel (1944) who stated that 'glycine and leucine give rise at best to slight growth', and that glutamate will serve as a source of both carbon and nitrogen.

Spirilloxanthin synthesis by diphenylamine cells

When washed diphenylamine cells were examined, the situation was entirely different. Resuspension of these cells anaerobically, in the light, in phosphate buffer alone, resulted in an immediate synthesis of spirilloxanthin (and bacteriochlorophyll); furthermore, the inclusion of a metabolizable carbon source did not increase the synthesis (Fig. 3). (It is of interest that the work of Glover & Kamen (1952) has shown that malonate is used for growth by *Rsp. rubrum*.) Fig. 4 shows the results of similar experiments in which diphenylamine cells, resuspended in phosphate buffer, were incubated aerobically in the dark and in the light, and anaerobically under similar conditions. These experiments, which were continued for 72 hr., show that in all cases synthesis is essentially complete after 24 hr., and that the aerobic, dark cultures synthesize as much pigment as the illuminated, anaerobic cultures.

Little synthesis is achieved anaerobically in the dark, and a combination of aerobiosis and light tends to inhibit synthesis.

It is of interest that the pigment concentration of resuspended diphenylamine cells incubated aerobically in the dark or anaerobically in the light reaches the same level as that found in normal 3- to 4-day cultures grown anaerobically in the light (Table 2). The high value obtained with transferred,

Fig. 1. Growth of 3- to 4-day-old *Rsp. rubrum*, washed and transferred to various nitrogen-containing media. ●—●, normal medium (unbuffered); ○—○, glutamate only, 0.4% (w/v), buffered with 0.067M phosphate to pH 7.0; ●—●, leucine only, 0.3% (w/v), buffered with 0.067M phosphate to pH 7.0; ○—○, glycine only, 0.5% (w/v), buffered with 0.067M phosphate to pH 7.0; ▲—▲, glycine only, 0.5% (w/v), buffered with $\text{NaHCO}_3/\text{CO}_2$ to pH 7.0.

Fig. 2. Spirilloxanthin production by 3- to 4-day-old *Rsp. rubrum*. The cultures and experimental conditions were the same as in Fig. 1.

Fig. 3. Spirilloxanthin synthesis by washed diphenylamine cultures of *Rsp. rubrum* re-suspended in various media anaerobically in the light. ●—●, 0.067M phosphate buffer (pH 7.0) alone, cell concentration (dry wt.) 30 mg./100 ml.; ○—○, 0.067M phosphate buffer + malate (0.025M), cell concentration (dry wt.) 29 mg./100 ml.; ●—●, 0.067M phosphate buffer + malonate (0.025M), cell concentration (dry wt.) 28 mg./100 ml.; ○—○, 0.067M phosphate buffer + succinate (0.025M), cell concentration (dry wt.) 32 mg./100 ml.; ▲—▲, 0.067M phosphate buffer + citrate (0.025M), cell concentration (dry wt.) 32 mg./100 ml.

Fig. 4. Spirilloxanthin synthesis by washed diphenylamine cultures of *Rsp. rubrum* re-suspended in phosphate buffer. The cell suspension contained 24 mg. dry wt. cells/100 ml.; ●—●, anaerobic, illuminated; ○—○, aerobic, dark; ○—○, aerobic, illuminated; ●—●, anaerobic, dark.

Table 2. *Spirilloxanthin concentration in Rsp. rubrum cultured under various conditions*

Conditions of culture	Concentration of spirilloxanthin (mg./g. dry wt.)
Diphenylamine, 3-4 days	0.12
Diphenylamine, 3-4 days, washed and transferred to phosphate buffer for 48 hr.	
(a) anaerobic, illuminated	2.56
(b) anaerobic, dark	0.80
(c) aerobic, illuminated	1.72
(d) aerobic, dark	2.48
Normal, 3-4 days	
(a) anaerobic, illuminated	2.8
(b) aerobic, illuminated	1.8
(c) aerobic, dark	0.8

aerobic, dark cultures is specially significant, for normally under these conditions synthesis is very feeble (Goodwin & Osman, 1953a).

Gest (1951) observed that washed cells of *Rsp. rubrum* would metabolize organic compounds under dark, anaerobic conditions in the presence of a suitable electron acceptor such as potassium ferricyanide. It was thought that in the presence of this acceptor, washed diphenylamine cells would synthesize increased amounts of pigment under anaerobic, dark conditions. Using 200 μ moles of potassium ferricyanide, no increased pigment synthesis was observed in anaerobic, dark cultures. In the presence of the same amount of electron acceptor and a metabolizable substrate (malate, 200 μ moles), the results obtained again showed no significant stimulation of the synthesis to spirilloxanthin.

Oxygen uptake by washed cells

It was found that the oxygen uptake by normal, washed cells, resuspended in phosphate buffer, was negligible. In the case of washed, resuspended, diphenylamine cells, there was considerable uptake, that in the dark always being greater than that in the light. Typical Q_{O_2} (μ l. O_2 /mg. dry wt./hr.) values for dark and illuminated cultures were -9.7 and -5.7, respectively. In this respect diphenylamine cells are similar to normal cells, in which it has previously been found that light inhibits oxygen uptake (see, for example, van Niel, 1944).

Polyenes other than spirilloxanthin in diphenylamine cultures

In the first report on the inhibition of spirilloxanthin synthesis by diphenylamine (Goodwin & Osman, 1953a), it was stated that this inhibition was

not accompanied by the synthesis of more-saturated polyenes. It has now been found that traces of such polyenes are synthesized by diphenylamine cultures of *Rsp. rubrum*, and it is important to note the reason for the failure to detect these traces in the earlier investigation. The first step in extracting the pigments from the cells is to treat with methanol (Polgar *et al.* 1944); this removes the bacteriochlorophyll quantitatively, but only extracts about 1% of the spirilloxanthin. The spirilloxanthin is then extracted from the dehydrated cells with benzene. Examination of this benzene extract from diphenylamine cultures showed that none of the more saturated polyenes was present; the methanol extract was not examined in the first experiments because it was assumed that, as in normal cultures, it would contain only traces of spirilloxanthin. In later experiments this fraction was examined and found to contain small amounts of a complex mixture of polyenes more saturated than spirilloxanthin. The reason for the virtual 100% extraction of these polyenes by the methanol is not known. A number of such methanol extracts was accumulated, the bacteriochlorophyll removed by treatment with alkali and the polyenes were prepared for chromatographic analysis as described under Methods. The chromatogram described in Table 3 was obtained.

Of the 15 polyenes noted in Table 3, five have been identified by spectrophotometric and mixed chromatographic tests as known compounds, namely: *all-trans*-spirilloxanthin (*D*), *cis*-spirilloxanthin (*E*), ζ -carotene (*M*), phytofluene (*N*) and phytoene (*O*). Most of the other fractions appear to be new polyenes, for similar compounds have not been found described in the literature. (*L*) probably contains nine conjugated double bonds and is quite similar to one of the polyenes obtained by Koe & Zechmeister (1952) on treating phytofluene with *N*-bromosuccinimide. (*J*) from its absorption spectrum (not recorded) is very closely related to ζ -carotene, and may be a methylated monohydroxy derivative of the parent. (*I*) is quite new, and corresponds to a system containing eight conjugated double bonds (Fig. 6). (*H*) and (*K*) are very closely related, and their absorption spectra (Fig. 5) would correspond with systems containing ten conjugated double bonds. From their adsorptive power and their absorption spectra, (*C*) and (*B*) would appear to be monohydroxy derivatives of the seven and ten conjugated polyene systems, respectively. (*A*), which occurs only in very minute traces, may be a demethylated *cis*-spirilloxanthin somewhat similar to the pigment found by Lederer (1938) in *Bacterium halobium*. The sharp resolution into narrow bands of the absorption spectra of all these unknown compounds except *G* (Fig. 6) strongly suggests that they are all open-chain compounds.

Table 3. *The chromatographic separation of the polyenes synthesized by Rsp. rubrum grown on a diphenylamine medium*

(Methanol fraction; adsorbent, fully deactivated alumina; fractions noted in order of decreasing adsorption.)

Fraction no.	Relative* amounts	Description	Absorption spectrum maxima (m μ .) (in light petroleum)	Provisional identification	Percentage ether (v/v) in light petroleum required to separate zone from that immediately above
A	0.8	Pink	453, 481, 514	Demethylated <i>cis</i> -spirilloxanthin	50
B	19	Yellow	420, 441, 458	Monohydroxy compound	50
C	5	Yellow	377, 397, 420	Monohydroxy compound	40
D	10	Purple	464, 490, 524	<i>all-trans</i> -Spirilloxanthin	20
E	15	Brownish pink	456, 482, 515	<i>cis</i> -Spirilloxanthin	20
F	9	Yellow	416, 439, 468	—	10
G	6.5	Brownish yellow	427, 453, 484	—	5
H	9	Yellow	417, 440, 470	—	5
I	4.5	Yellow	393, 414, 442	—	4
J	3.5	Lemon yellow	375, 395, 418	—	2
K†	10	Yellow	375, 395, 418, 433, 466	Mixture containing J	2
L	0.3	Yellow	407, 433, 462	—	2
M	10	Yellow	376, 396, 418	ζ -Carotene	2
N	18	Colourless green fluorescence in u.v.	331, 349, 368	Phytofluene	0
O	129	No fluorescence	275, 287, 297	Phytoene	0

* Based on *all-trans*-spirilloxanthin as 10; semi-quantitative assessment made assuming $E_{1\text{cm}}^{1\%}$ to be the same for all fractions.

† The absorption spectrum maxima of purified K are 418, 439, 468 m μ .

Fig. 5. The absorption spectra in light petroleum of various polyenes from diphenylamine cultures of *Rsp. rubrum*. (For description of fractions see Table 3.) Curve A, fraction K; B, fraction B; C, fraction H.

Fig. 6. The absorption spectra in light petroleum of various polyenes from diphenylamine cultures of *Rsp. rubrum*. (For description of fractions see Table 3.) Curve A, fraction L; B, fraction I; C, fraction G.

The only peculiarity about the fractions in Table 3 is that *L* (nine conjugated double bonds) is adsorbed below *I* and *J* (eight and seven conjugated double bonds, respectively). It is possible that *L* is a hydrocarbon, and that *I* and *J* are methylated monohydroxy derivatives (cf. spirilloxanthin).

The quantitative changes in the major fractions of these polyenes which occurred when washed diphenylamine cells were resuspended in phosphate buffer and incubated anaerobically in the light are given in Table 4. For this experiment no attempt was made to separate the numerous small fractions present in the 'pre-spirilloxanthin, post-phytofluene' fraction. The $E_{1\text{cm}}^{1\%}$ values used in calculating the amounts present were: phytoene, 1100 (Porter & Lincoln, 1950); phytofluene, 1540 (Koe & Zeichmeister, 1952); and spirilloxanthin, 2360 (Polgar *et al.* 1944); for the unidentified pigments $E_{1\text{cm}}^{1\%}$ (λ_{max}) was assumed to be 2250.

Table 4 shows that the concentrations of these polyenes were very small in comparison with the normal concentrations of spirilloxanthin (2.8 mg./g. dry wt.). Furthermore, although during the period of this experiment the main (benzene-extractable) fraction of spirilloxanthin increased about tenfold (Fig. 4), there was no concomitant decrease in the levels of these more saturated polyenes, as would have been reasonable to expect were they precursors of spirilloxanthin. Most of these fractions remained constant throughout, although phytofluene and *C* decreased slightly. It is interesting that the traces of spirilloxanthin and its derivate (*A*) found in the methanol extract increased in the same way as the benzene-extractable spirilloxanthin, but not to quite the same extent.

Table 4. The amount of different polyenes in methanol extract of diphenylamine cultures of *Rsp. rubrum* before and after transference to phosphate buffer

(Note: methanol extraction removes all the more saturated polyenes, but only traces of spirilloxanthin.)

Polyene	Concentration (mg./g. dry wt.)		
	At transfer	24 hr. after transfer	48 hr. after transfer
Phytoene	0.165	0.182	0.174
Phytofluene	0.050	0.044	0.036
†Mixed fractions <i>F-M</i> *	0.081	0.072	0.073
Spirilloxanthin (<i>cis</i> - and <i>trans</i> -)	0.029	0.052	0.050
Fraction <i>C</i> *	0.032	0.013	0.007
Fraction <i>B</i> *	0.045	0.043	0.043
Fraction <i>A</i> *	0.006	0.013	0.015

* See Table 3, for description of fractions.

† No attempt was made to separate fractions *F-M* for this experiment.

Fig. 7. Spirilloxanthin synthesis by *Rsp. rubrum* cultured on normal medium in the presence of diphenylamine and possible diphenylamine antagonists. ●—●, normal medium; ●---●, normal medium + 1/140 000 diphenylamine; ○---○, *B* + 200 µg. $K_3Fe(CN)_6$; ○—○, *B* + 200 µg. $FeSO_4 \cdot 7H_2O$.

Fig. 8. Growth of *Rsp. rubrum* cultured on the normal medium in the presence of diphenylamine antagonists. (The same cultures as for Fig. 7.) The legends have the same significance as those used for Fig. 7.

Effect of potassium ferricyanide and ferrous sulphate on spirilloxanthin synthesis in diphenylamine cultures

Turian (1950) stated that in *Mycobacterium phlei*, ferrous sulphate reversed the inhibitory action of diphenylamine on carotenogenesis, but this does not appear to be so in *Phycomyces blakesleeanus* (Goodwin, Jamikorn & Willmer, 1953). It will be seen from Figs. 7 and 8 that ferrous sulphate overcame the slight inhibitory action of diphenylamine on growth of *Rsp. rubrum*, and also very slightly stimulated carotenoid production; it did not, however, increase synthesis to the normal level. Potassium ferricyanide was tested in the same way with very similar results (Figs. 7, 8).

Lipid synthesis by Rsp. rubrum

The lipid contents of both normal and diphenylamine cultures of *Rsp. rubrum* at various stages of growth are recorded in Table 5. It will be seen that at all stages of growth the lipid contents of diphenylamine cultures were consistently slightly lower than those of normal cultures.

The changes in lipid content of washed, diphenylamine cultures, resuspended in phosphate buffer were measured in a number of experiments: in no case was a significant change observed after resuspension.

Bacteriochlorophyll synthesis by washed cells

Although this investigation was not primarily concerned with chlorophyll synthesis, one experiment was carried out to see if washed diphenylamine cells could synthesize bacteriochlorophyll. Two equal amounts of diphenylamine cells were extracted with 10 ml. of methanol, the one before the other, after resuspension in phosphate buffer and incubation anaerobically in the light for 48 hr. It was found that the *E* value at 770 m μ . (the wavelength of one of the bands of bacteriochlorophyll (Wassink, Katz & Darrenstein, 1939)) increased by 45% as the result of resuspension and incubation in the light. This indicated a synthesis of bacteriochlorophyll. As the colour of the methanol fraction also changed on resuspension, there may also occur qualitative changes in the bacteriochlorophyll fraction. This was not investigated.

Table 5. *The lipid content of normal and diphenylamine cultures of Rsp. rubrum*

Age of cultures (days)	Lipid concentration (%)	
	Normal cultures	Diphenylamine cultures
4	7.78	7.60
6	7.12	4.57
8	6.59	5.57
10	6.29	3.63
12	4.67	2.57

DISCUSSION

It appears from the results given in Figs. 3 and 4, that diphenylamine cultures of *Rsp. rubrum* contain a colourless material, which, when the diphenylamine is washed out, can be converted into spirilloxanthin. For full conversion energy is required, and this can be obtained photochemically under anaerobic conditions, or by respiratory processes in unilluminated cultures. The identity of the material providing the energy is not known, but this also must be accumulated in diphenylamine cultures, for normal washed suspensions have an almost negligible endogenous metabolism, whereas washed diphenylamine cells consume a considerable amount of oxygen. This energy source does not appear to be lipid, but this possibility cannot be ruled out completely, because such very small quantities are concerned. A trace of a specific, lipid-like material might appear in diphenylamine cultures, and disappear on resuspension without being detected by the method for lipid determination used in this investigation.

The very small amount of pigment synthesized by washed, diphenylamine cells resuspended anaerobically in the dark (Fig. 4) can be explained by the absence of an active, energy-producing process. The production of reduced pigment in aerobic, illuminated cultures is somewhat more difficult to account for; it is possible that the combined action of light and oxygen tends to destroy the pigment as it is synthesized. A more subtle explanation, however, may eventually emerge, because it was found that oxygen uptake is reduced in illuminated, compared with unilluminated, diphenylamine cultures. It might be suggested that normal washed cells do synthesize pigment, but that owing to its rapid turnover there is no net synthesis; this remains to be decided, using isotopes.

An explanation of the failure of normal, washed cells to synthesize spirilloxanthin can be based on the fact that the pigment occurs as a protein complex in the chromatophores (Pardee, Schachman & Stanier, 1952). If it is assumed that in normal cultures the protein moiety of the chromoprotein is 'saturated' with pigment, then one would not expect more pigment to be formed in washed cells; on the other hand, in the presence of nitrogenous compounds, when protein is being synthesized, pigment formation is to be expected; that this is so is demonstrated in Fig. 1. This is in contrast to the situation with *Phycomyces*, which produces large amounts of β -carotene under vegetative conditions (Goodwin & Willmer, 1952); in this case, however, the pigment is dispersed in oil droplets and not conjugated with a protein.

The synthesis of spirilloxanthin by washed diphenylamine cells becomes explicable if one

assumes that diphenylamine has little if any effect on the synthesis of the protein part of the chromoprotein, and that there exists in these cells 'colourless' chromatophores; this is a reasonable assumption, because diphenylamine has very little effect on growth of *Rsp. rubrum* (Goodwin & Osman, 1953a). As soon as the diphenylamine is removed, the material from which spirilloxanthin is formed is converted into spirilloxanthin, which is then attached to its specific protein. It is, of course, possible that this material is itself already in combination with this protein, and that the conversion into the chromoprotein takes place *in situ*. As the concentration of spirilloxanthin in diphenylamine cells rises to that of normal cells after resuspension in phosphate buffer and incubation under appropriate conditions (Table 2), the suggestions made above would also explain why the addition of metabolizable carbon substrates to the phosphate buffer does not lead to an increase in the amount of pigment synthesized.

It is obvious that the diphenylamine cultures contain an active colourless spirilloxanthin precursor. The difficult problem of identifying this remains to be solved; it may be an 'immediate' precursor, e.g. acetate, or it may be reserve material such as polysaccharide or lipid; it does not, however, appear to be any of the more saturated polyenes, such as those suggested by Porter & Lincoln (1950) to be carotene precursors in tomatoes. Diphenylamine certainly stimulates the synthesis of small amounts of these compounds (Table 4), but they do not disappear in resuspended cultures from which diphenylamine has been removed, and in which spirilloxanthin is being actively synthesized. Similar observations have been made with *Phycomyces* (Goodwin *et al.* 1953); diphenylamine stimulates the synthesis of the Porter & Lincoln series whilst inhibiting that of β -carotene. Addition of adenosine 5'-phosphate (AMP), reverses the inhibition of β -carotene synthesis, but does not reduce that of the Porter & Lincoln series to its normal low level. AMP, however, will not reverse the inhibition in *Rsp. rubrum* (Goodwin & Osman, 1953a).

It is interesting to find that the synthesis of phytoene by *Rsp. rubrum* is stimulated by diphenylamine. Goodwin (1952) appeared to find considerable increases in phytoene synthesis in diphenylamine cultures of *Phycomyces*. Since phytoene and diphenylamine have similar spectral and adsorptive properties it was possible that the apparent increased synthesis of phytoene was spurious and due to the presence of traces of diphenylamine in the mycelium. As the washed, resuspended, diphenylamine cultures of *Rsp. rubrum* synthesized spirilloxanthin, there cannot be any diphenylamine present in these cultures, and

thus the material absorbing at 285 m μ . must be phytoene.

The mode of action of diphenylamine in inhibiting carotenogenesis still remains obscure. As the synthesis of both spirilloxanthin and bacteriochlorophyll is inhibited in *Rsp. rubrum*, it is possible that diphenylamine functions either (a) far back in the synthetic process, perhaps in the utilization of a common precursor (possibly acetate), or (b) in a mechanism common to both syntheses, but involving different substrates.

SUMMARY

1. Washed cells of *Rhodospirillum rubrum* do not synthesize spirilloxanthin when transferred to a metabolizable source of carbon.

2. Cells grown in the presence of diphenylamine, when washed and transferred to phosphate buffer alone, under aerobic conditions in the dark, or anaerobic conditions in the light, synthesized spirilloxanthin. The amount of pigment synthesized approached that found in normal cells. Little synthesis occurred aerobically in the dark, and only small amounts in aerobic, illuminated cultures. The presence of a metabolizable carbon source did not increase the amount of pigment synthesized.

3. Suspensions of normal cells in phosphate buffer took up little or no oxygen, whereas diphenylamine cells consumed considerable amounts of oxygen when unilluminated; less, when illuminated.

4. Potassium ferricyanide and ferrous sulphate counteracted the slight growth-inhibitory effect of diphenylamine, and also slightly stimulated pigment synthesis.

5. Small amounts of polyenes (including phytoene, phytofluene and ζ -carotene) were found in diphenylamine cultures but not in normal cultures. The amounts of these polyenes did not alter significantly when washed, diphenylamine cells were resuspended in phosphate buffer.

6. The lipid levels in cells growing normally, and in diphenylamine cells were measured and found to be slightly lower in the latter. On resuspension, the lipid levels of washed, diphenylamine cells did not alter significantly.

7. Washed diphenylamine cells, resuspended in phosphate buffer, synthesized bacteriochlorophyll.

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Specificity of Bond Fission during the Acid Hydrolysis of Insulin

By G. L. MILLS

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London

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The object of the experiments described here has been to determine the nature and number of the bonds broken in the insulin molecule during its digestion with cold, concentrated hydrochloric acid. In this way some information has been gained concerning the relative ease with which various bonds are hydrolysed and the positions of susceptible bonds in the molecule. These data have also been correlated with the loss of activity of the insulin.

Acid digests of insulin have frequently been examined, either in structural studies (Sanger & Tuppy, 1951; Sanger & Thompson, 1953), or in attempts to correlate physiological activity with the structure of the molecule (for example Phillips, 1952). But although the recent work of Desnuelle & Bonjour (1951) on globin, and of Acher, Jutisz & Fromageot (1950) on lysozyme has shown that different peptide bonds may be hydrolysed at greatly differing rates, few accounts have been published of experiments on either proteins or peptides of known structure. The work on globin and lysozyme has demonstrated that the fission of bonds involving a serine or threonine amino group is much more rapid than that of other bonds. Elliott (1952) has further shown that, by treatment with anhydrous acidic reagents, these bonds are transformed from an *N*-acyl to an *O*-acyl form via the intermediate formation of an oxazoline ring. Leach (1953) has reviewed data on hydrolysis rates of peptide bonds.

It is clear from an examination of the previous work that a short period of digestion of insulin in cold acid would bring about the following changes: (1) Some bonds involving serine and threonine amino groups would be attacked. (2) Some ammonia

would be removed from glutamine and asparagine residues. (3) According to Partridge & Davis (1950), a few bonds adjacent to aspartic or glutamic acid residues might be broken. (4) Any bonds made peculiarly labile by their position in the molecule would be destroyed. All these effects have been demonstrated under the experimental conditions described here.

After digestion of insulin in 10·5*N* hydrochloric acid for a prescribed time, as described in the Experimental section, the protein precipitated from the hydrolysate was dialysed for 2 days to ensure that all small peptides were removed. The non-diffusible product will be referred to as H-protein. The free amino groups of the H-protein were labelled by reaction with fluorodinitrobenzene (FDNB) and estimated chromatographically after hydrolysis of the dinitrophenyl protein (DNP-protein). The original terminal amino acids of the intact peptide chains (DNP-glycine and DNP-phenylalanine, Sanger, 1945), were thus always present. Also found were: DNP-serine, DNP-aspartic acid, DNP-glutamic acid and DNP-alanine. DNP-threonine was found only in certain experiments described below.

Sanger (1949*b*) has shown that the only threonine residue present in bovine insulin forms part of the terminal sequence -Thr.Pro.Lys.Ala (using the nomenclature of Brand & Edsall, 1947). The corresponding peptide was isolated, after conversion into its DNP derivatives, from the acid liquor remaining after the H-protein had been filtered off. As this tetrapeptide also accounts for the only lysine residue in insulin, the number of threonine bonds hydrolysed could be determined by estimating the