0·0033 M. An experiment carried out with citrate concentrations between 0·001 and 0·0033 M showed non-competitive inhibition. Fig. 2 illustrates this experiment. Though it would appear possible to combine Figs. 1 and 2, this is not permissible as they were obtained from different experiments.

DISCUSSION

Elliot & Kalnitsky (1950) showed a competitive inhibition by fluoroacetate of citrate oxidation by rabbit-kidney preparations. They claim that the oxidation of *cis*-aconitate was not inhibited by fluoroacetate and in fact was accelerated. This result is difficult to explain in view of the findings of Peters & Wilson (1952), who used a soluble aconitase together with a purified sample of the active inhibitor (fluorocitrate) and showed a competitive inhibition of this enzyme by the method of Lineweaver & Burk (1934). They observed that a non-competitive type of inhibition might occur if time were not allowed for the enzyme, substrate and inhibitor to come to equilibrium before measuring the initial velocity of the reaction. We have observed competitive inhibition between citrate concentration of 0·01 and 0·0033 M, whereas below the latter concentration inhibition was non-competitive. We are at present unable to explain this result.

SUMMARY

1. The effect of fluoroacetate on citrate oxidation by mitochondrial systems has been investigated.

2. The apparent competitive nature of this inhibition has been demonstrated with citrate concentrations of 0·01–0·0033 M.

3. Below 0·0033 M citrate concentration the inhibition is non-competitive.

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Studies in Carotenogenesis

11. CAROTENOID SYNTHESIS IN THE ALGA *HAEMATOCOCCUS PLUVIALIS*

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Haematococcus pluvialis is a unicellular, motile, green alga of the class Chlorophyceae. Like that of most flagellates its resting stage is characterized by the production of cysts, the formation of which is generally regarded as a response to unfavourable environmental conditions, although this may not always be so (see Fritsch (1935) for a full discussion). Formation of cysts in algae is very often accompanied by a change in colour from green to orange or red, and it was in the naturally occurring (red) encysted form of *H. pluvialis* that astaxanthin (3:3′-diketo-4:4′-dihydroxy-β-carotene) was first found in plant tissues (Tischer, 1937, 1944); it was accompanied by small amounts of α- and β-carotenes and lutein. Until that time astaxanthin had been found only in animal tissues, but since then it has also been found in the, presumably encysted, algae, *Brachiomonas simplex* and *Protosiphon botryoides* (Bielig, 1947). (It should be noted, however, that Strain (1951) believes that there are two ketonic carotenoids in *P. botryoides*, neither of which is astaxanthin.)

Another well-known example of a red-encysted alga is *Trentepholia aurea*, the resting stage of

which is very rich in β-carotene (Tischer, 1936; Lederer, 1938; Heilbron, 1942). This pigment was first observed in *T. aurea* by Cohn (1861) who called it haematochrome. This name has also generally been applied to the orange-red pigment from *H. pluvialis* and to the red pigment in the eye spot (stigma) of many flagellates. As we now know that the pigment of *T. aurea* is different from that of *H. pluvialis* and, as there is no direct evidence that the eye-spot pigment is a carotenoid, it is probable that the term haematochrome has served its purpose and should be discarded.

No quantitative observations have been made on the synthesis of astaxanthin by *H. pluvialis*, but it is generally assumed that nitrogen deficiency is one of the major controlling factors (Pringsheim, 1914; Chodat & Mayer, 1927; Meier, 1929; Dr M. Droop, personal communication). On the other hand, Lwoff & Lwoff (1930) found that addition of acetate to a mineral-peptone medium stimulated the synthesis of the red pigment (astaxanthin), although the culture was growing rapidly. Lwoff & Lwoff (1930) also found that no astaxanthin was synthesized in the dark except in the eye spot but, as indicated above, it is not legitimate, at the moment, to conclude that the pigment of the eye spot is a carotenoid.

The work to be described here was undertaken to find out the nature of the carotenoids in the green form of *H. pluvialis*, and to follow quantitatively the synthesis of astaxanthin in the light of present knowledge concerning carotenogenesis in other organisms (Goodwin, 1953).

EXPERIMENTAL

Cultures. The culture of *H. pluvialis* used was obtained from the Type-Culture Collection of Algae and Protozoa, Botany School, Cambridge. Two culture media were used. *A* was 'soil-extract' medium made by extracting soil (1 kg.) with boiling water (1500 ml.) for 15 min. *B* was the medium described by Ondratschek (1940) as giving good growth; it contains (all %, w/v): K_2HPO_4, 0·001; $MgSO_4.7H_2O$, 0·001; $CaCl_2$, 0·001; $FeSO_4.7H_2O$, 0·0001; KNO_3, 0·1 and sodium acetate ($C_2H_3O_2Na.3H_2O$), 0·2; the pH was adjusted to 7·6 with 0·1 N-NaOH.

Cultures were grown in 25 ml. of medium in 100 ml. Erlenmeyer flasks held at 25°, either in a conventional incubator (in the dark), or in a thermostatically controlled water bath illuminated at a distance of 20 cm. by two 80 w 'daylight' fluorescent tubes. Inoculation of the flasks was carried out with 1 ml. of a 3-day 'green' culture grown on the soil-extract medium. It was interesting to find that such inocula would grow when transferred either to a soil extract or KNO_3:acetate medium, but that inocula from a KNO_3:acetate culture would not grow easily when transferred to a soil-extract medium, although it would do so when transferred to a KNO_3:acetate medium.

Extraction, separation and determination of pigments. After a number of trials the following method was found best for extracting carotenoids from *H. pluvialis* and other algae. The cultures were harvested by centrifuging and the residual medium discarded. Aqueous KOH (1 ml. of 60 %, w/v) and then ethanol (10 ml.) were added, the centrifuge tube was placed in a water bath at 40° and the mixture constantly stirred and ground with a flattened glass rod. After 5 min. the residue was allowed to settle and the supernatant decanted. The procedure was repeated twice with ethanol (10 ml.) only, when all the pigment was extracted leaving only a very pale yellow residue. The ethanol extracts were combined in a separating funnel and an equal volume of ethyl ether added, followed by water, slowly, until two layers were formed. All the carotenoids except astaxanthin, which had been oxidized to astacin by the treatment just described, were extracted into the ether layer. The potassium salt of astacin accumulated at the ether–aqueous ethanol interface as a fluffy brownish red deposit and the partly degraded chlorophyll was in the aqueous phase.

The ether layer was collected, washed free from alkali with successive small quantities of warm water, dried by the addition of anhydrous Na_2SO_4, separated from this by filtration, and then taken to dryness *in vacuo* at 30°. The residue was then dissolved in a small volume of light petroleum (light petroleum b.p. 40–60° was used throughout this investigation) ready for chromatographic analysis.

The insoluble potassium salt of astacin was collected, dissolved in a small volume of glacial acetic acid and extracted into ethyl ether. After being washed free from traces of acetic acid, the ethereal solution of astacin was treated in the same way as described for the neutral carotenoids.

The neutral carotenoids were chromatographed on either activated alumina (grade 'O'; P. Spence and Co., Widnes) or deactivated alumina, made by treating grade 'O' material with methanol (Goodwin, 1952*a*). Quantitative determinations of the separated pigments were carried out by dissolving the pigment fractions in known volumes of light petroleum and measuring the $E_{1\,cm.}$ value at the appropriate $\lambda_{max.}$. By comparing these values with the known $E^{1\%}_{1\,cm.}$ values for the pure pigments (Goodwin, 1952*b*), the amounts present could easily be determined.

RESULTS

The carotenoids present

In the green stage. The carotenoid extract from 5-day 'soil-extract' cultures were first examined by chromatography on deactivated alumina, and the resulting separation is described in Table 1. It will be seen that only two main zones exist, *A* and *D*. From its adsorptive power and absorption spectrum *D* appeared to be almost certainly lutein. This was confirmed by comparison in a mixed chromatogram with an authentic specimen of lutein; no separation of the pigments could be obtained. Pigments *B* and *C* were present in traces (less than 1 % of the total) and could not be identified. Pigment *B*, however, did show some resemblance to echinenone. Fraction *E* (about 5 % of the total) is, from its absorption spectrum and high adsorptive power, possibly violaxanthin. There are, however, a number of very strongly adsorbing xanthophylls with very similar absorption spectra (see Strain, 1951). As

Table 1. *Chromatography of carotenoids from green* H. pluvialis

Adsorbent, weakened alumina. Zones in order of increasing adsorptive power. Developer, light petroleum containing various concentrations of ethyl ether. Alga (5 days) cultured at 25° in soil extract.

Zone	Colour	Approx. amount (% of total)	Values of $\lambda_{max.}$ in light petroleum (mμ.)	Concn. of ether (v/v) required to elute zone	Identification
A	Brownish	10–20	~425, 488, 475	0	Mainly β-carotene
B	Pink	<1	453	10	?
C	Yellow	<1	—	15	—
D	Lemon-yellow	75–80	424, 443, 471	80	Lutein
E	Yellow	5	415, 438, 467	100	Violaxanthin?

~ denotes an inflexion.

these pigments are only separable with difficulty and after considerable 'trial and error' chromatography, much more material would have been required for unequivocal identification than was at our disposal.

Fraction *A*, which ran straight through a column of deactivated alumina, was rechromatographed on activated alumina. It consisted essentially of one pigment, β-carotene, identified in the usual manner. Small amounts of *neo*-β-carotene *B* were also observed, but this pigment was probably an artifact, for it has been noticed repeatedly that even when great precautions are taken to eliminate *trans* → *cis* isomerization, it is almost impossible not to produce traces of *neo*-β-carotene *B* when manipulating a solution of 'pure' β-carotene. No α-carotene was present and, furthermore, no colourless polyenes such as phytofluene or phytoene could be detected in the amounts of material examined.

In the red stage. The neutral carotenoids extracted from red *H. pluvialis* were identical with those found in the green cultures but astaxanthin was also present. It was converted into astacin by alkali, and this pigment had an absorption spectrum identical with that of authentic astacin (Goodwin & Srisukh, 1949). As stated in the introduction, however, the presence of this pigment in red *H. pluvialis* has previously been unequivocally identified by isolation in the crystalline state from large naturally occurring encysted cultures.

In the brown stage. The half-way stage between the green and red cultures was examined because of the possibility that it might yield intermediates in the synthetic pathway to astaxanthin; no such pigments had been observed in the red cultures. The same results were, however, obtained in the brown as in the red cultures, except that less astaxanthin was present. No intermediates were noted. It is, of course, more than probable that brown cultures are so coloured because not all the cells have encysted and that the colour is due to a mixture of green and red cells, rather than that they represent an intermediate stage in the physiological process of encystment.

Quantitative experiments

The synthesis of the various pigments during the development of cultures grown on soil extract is illustrated by Table 2. It will be seen that the transition from green to red is accompanied by a considerable synthesis of astaxanthin but that this pigment is not produced at the expense of the neutral pigments which, in fact, also increase in older cultures. In red cultures, the astaxanthin represents about 50 % of the total pigment present. It was found that if green cultures were centrifuged, washed with sterile water, and resuspended in water only, they were still capable of changing into their red form.

Using the KNO_3:acetate medium, the results recorded in Table 3 show that the synthesis of astaxanthin is less in this medium than on the soil-extract medium, although the synthesis of the neutral pigments is increased; astaxanthin accounts for only between 10 and 15 % of the total pigments present. Onset of synthesis of astaxanthin on the KNO_3:acetate medium was delayed until the 11th compared with the 5th day on the soil-extract medium, and was not complete until 14 days after inoculation. Although no dry-weight determinations were carried out, the increased pigment production on the KNO_3:acetate medium appeared, from visual inspection of the cultures, only to be a reflexion of stimulated growth, and not due to a specific effect on pigmentation. As in the case of soil-extract cultures, green cultures washed and resuspended in water only, synthesized astaxanthin.

Effect of light. When 5 to 6-day soil-extract cultures or 10 to 11-day KNO_3:acetate cultures were placed in the dark the colour did not change from green to brown or red. Quantitative experiments on a number of such KNO_3:acetate cultures showed that they had actually lost on the average 67 % of their neutral carotenoids 9 days after being placed in the dark.

Effect of variation in acetate concentration. In an experiment carried out to test the effect on pigment synthesis of the concentration of acetate in the

Table 2. *Carotenoid synthesis by developing* H. pluvialis

Pigment produced per flask containing 25 ml. of soil extract. Cultures from four flasks were combined for each analysis. Incubation at 25°.

Age of culture (days)	Colour	Total neutral carotenoids (μg.)	β-Carotene (μg.)	Astaxanthin (μg.)	Astaxanthin as % of total pigments
5	Green	3·5	0·4	0	0
8	Brownish red	4·4	0·4	6·3	58·9
11	Red	7·0	0·4	9·1	56·5
14	Red	8·2	0·7	9·3	53·1
17	Red	11·3	0·6	8·7	43·5
20	Red	10·5	1·0	9·3	47·0
23	Red	10·7	1·0	10·4	49·3

Table 3. *Carotenoid synthesis by developing* H. pluvialis

Pigment produced per flask containing 25 ml. of KNO_3: acetate medium. Cultures from four flasks combined for each analysis. Incubation at 25°.

Age of culture (days)	Colour	Total neutral carotenoids (μg.)	β-Carotene (μg.)	Astaxanthin (μg.)	Astaxanthin as % of total pigments
5	Green	3·6	0·7	0	0
8	Green	13·8	2·4	0	0
11	Brown	17·8	2·8	1·6	8·3
14	Brownish red	25·8	3·2	4·6	15·1
17	Brownish red	25·6	5·0	2·9	10·3
20	Brownish red	22·3	4·5	3·2	12·6
33	Brownish red	22·7	3·7	3·4	13·0

Table 4. *The effect of acetate concentration on carotenoid production by* H. pluvialis

KNO_3: acetate medium with the acetate concentration varied; concentrations above 0·4% up to 3·0% (w/v) did not support growth. Pigment produced per flask containing 25 ml. of medium. Cultures from four flasks combined for each analysis. Incubation at 25°.

	Green, 8-day cultures			Brown-red, 15-day cultures		
		Astaxanthin			Astaxanthin	
Concn. of acetate (%, w/v)	Total neutral carotenoids (μg.)	μg.	% of total pigments	Total neutral carotenoids (μg.)	μg.	% of total pigments
0	Too small to measure			4·6	<1	—
0·2	8·4	0	0	23·0	4·5	16·3
0·4	2·7	0	0	2·7	<1	10·0

KNO_3: acetate medium it was found (Table 4) that 0·2% (w/v) gave best growth and that above 0·4% no growth was obtained and the cultures appeared to be dead; at 0° little if any growth occurred. Concentrations of acetate between 0 and 0·2% were not examined.

Effect of diphenylamine. Diphenylamine has been shown to be a potent and specific inhibitor of carotenogenesis in *Mycobacterium phlei*, *Phycomyces blakesleeanus* and *Rhodospirillum rubrum* (Turian, 1951; Goodwin, 1952*a*; Goodwin, Jamikorn & Willmer, 1953; Turian & Haxo, 1952); it was, therefore, tested on *H. pluvialis*. This organism was extremely sensitive to diphenylamine and it was not possible to obtain growth even when the diphenylamine concentration was reduced to 1/560 000, a concentration 8–16 times less than normally found effective in the other organisms.

Other possible inhibitors were not tested in great detail because of difficulties in producing cultures on a large scale, but the following (in the concentrations stated) were lethal to washed 7 to 9-day-old suspensions of *H. pluvialis*: arsenite, 3×10^{-4}M; arsenate, 10^{-4}M; NaF, 10^{-4}M; KCN, 10^{-4}M; NaN_3, 10^{-3}M; hydroxylamine, 10^{-5}M; hydrazine, 10^{-3}M; malonate, 4×10^{-4}M; 2, 4-dinitrophenol and sodium iodoacetate, 10^{-4}M.

DISCUSSION

The pigments identified in the green stage of *H. pluvialis* show that in this respect the organism is typical of the Chlorophyceae (Goodwin, 1952*a*), producing only β-carotene and a very simple mixture of xanthophylls (mainly lutein and one other, possibly violaxanthin). These observations

give further confirmation of the generalization that the xanthophylls of the Chlorophyceae are very much less complex than those in the green leaves of higher plants.

No colourless polyenes such as phytofluene and phytoene were observed in this investigation, although their presence in minute traces must remain a possibility if it is assumed that the Chlorophyceae are closely related, from the pigment point of view, to green tissues of higher plants. Recently, colourless polyenes have been observed in traces in leaves using extremely large amounts of starting material (Rabourn & Quackenbush, 1953; Eny, 1953; Zechmeister & Karmakar, 1953). The demonstration of the presence of β-carotene and lutein, as well as astaxanthin in the encysted form of *H. pluvialis* confirms the earlier observations of Tischer (1944). Tischer, however, reported that β-carotene and lutein were present in traces only. This may be so in the naturally occurring encysted forms examined by Tischer, but in our laboratory cultures growing on a nutritionally poor medium (soil extract) the amount of astaxanthin was never more than 60% of the total carotenoids, although the cultures were bright red. The disposition of the astaxanthin in the cell must be such that it masks the yellow of the neutral carotenoids and, incidentally, chlorophyll which we also found in the encysted forms.

The demonstration that the synthesis of astaxanthin does not occur at the expense of the neutral carotenoids indicates that it must be formed from either a colourless precursor already present in the green cultures or from carbon dioxide photosynthetically. At the moment there is no means of deciding which is correct, but the observation that the synthesis of astaxanthin does not take place in the dark indicates that if a precursor is already present, insufficient energy is available from the respiration of the cells to convert it into the pigment, and that this energy must be provided photochemically. In this respect it is interesting to compare the synthesis of astaxanthin in *H. pluvialis* with that of spirilloxanthin in *Rsp. rubrum* originally grown in the presence of diphenylamine (Goodwin & Osman, 1954). These diphenylamine cells, which are almost colourless, will, when washed free from diphenylamine and resuspended in phosphate buffer, synthesize spirilloxanthin provided energy is available. This can be provided photochemically in anaerobic cultures or by respiration in dark-aerobic cultures.

As stated previously, it is now generally assumed that encystment and astaxanthin production is a result of a lack of assimilable nitrogen. The present results, which show that in the nitrogen-poor medium (soil extract) *H. pluvialis* produces relatively more astaxanthin than in the nitrate: acetate medium *B*, tend to confirm this view. There are, however, other factors, such as the nature of the sources of nitrogen, which may eventually also be shown to be important.

It would be unwise to conclude from the results shown in Table 4 that the addition of acetate to the basic nitrate medium (medium *B* without acetate) specifically stimulated synthesis of neutral carotenes and astaxanthin, because growth, as judged by the appearance of the culture and the size of the centrifuged cell mass, was also stimulated considerably; in fact, very little growth occurred in the absence of acetate. It would also be unwise to compare the present results, obtained using nitrate as the source of nitrogen, with the early qualitative observations of Lwoff & Lwoff (1930) that addition of acetate stimulated astaxanthin synthesis when peptone was the source of nitrogen. Our *H. pluvialis* would not grow on medium *B* in which potassium nitrate was replaced by peptone.

SUMMARY

1. Green cultures of *Haematococcus pluvialis* contain β-carotene, lutein and small amounts of a third pigment, possibly violaxanthin. Traces of two other pigments were observed but no colourless polyenes could be detected. Lutein represents about 75–80% and β-carotene 10–20% of the total carotenoids.

2. The change in cells of *H. pluvialis* from green to brown or red is accompanied by the formation of considerable amounts of astaxanthin. This synthesis is not at the expense of the pigments already present in the green stage.

3. Relatively more astaxanthin is formed on a soil extract medium than on a nitrate:acetate medium.

4. Light is necessary for the formation of astaxanthin.

5. Diphenylamine completely inhibits growth of *H. pluvialis* at a concentration of 1/560000.

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The Effect of 3:5-Dinitro-*ortho*-cresol on Phosphocreatine and the Adenosine Phosphate Compounds of Rat Tissues

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A distinctive feature of poisoning by dinitro-*o*-cresol (DNOC) in man (Luijt, 1947) and in experimental animals (Parker, Barnes & Denz, 1951) is the extreme muscular rigidity which develops at or very soon after death. Stoner, Threlfall & Green (1952) have shown that this muscular change in DNOC poisoning is accompanied by a decrease in the concentration of polyphosphates, phosphocreatine and glycogen, and drew attention to the similarity of these changes to those described by Bate-Smith & Bendall (1949) and Bendall (1951). In the present study preliminary experiments indicated that in certain circumstances, death of DNOC-poisoned rats could occur when relatively normal amounts of energy-rich phosphate compounds were present in the muscle. At the same time these animals exhibited the typical rapid onset of rigor. In consequence, this point was investigated more fully and, as changes in phosphate metabolism might well have been expected in other tissues, the observations were extended to the heart, brain and diaphragm.

METHODS

Procedure

In the experiments involving the analysis of muscle it was necessary to minimize the death struggles of the animals, as such movements increase the rate of breakdown of high-energy phosphate compounds. This purpose was achieved by the use of the muscle-relaxing drug, α:β-dihydroxy-γ-(2 methylphenoxyl)propane (Myanesin, British Drug Houses Ltd.), following the suggestion of Bate-Smith & Bendall (1948). The animals (unless otherwise stated the animals used were albino female rats of 200 g. wt.), were given an intraperitoneal injection of Myanesin (200 mg./kg. body wt.) 10 min. before administration of DNOC. At the time of injection of DNOC the animals were completely relaxed. Normal values for phosphate compounds were obtained from untreated animals. These rats were given an injection of Myanesin sufficient to produce relaxation in 10 min. (200 mg./kg. body wt.) and then killed 5–15 min. after this time by a blow on the head, or, if the brain was required, by immersion in liquid O_2. A group of eleven rats was used for muscle analysis (group IV in Tables 1 and 3) and six of these were used for brain analysis. The values of phosphate compounds of heart and diaphragm were obtained from a second group (group VII in Tables 2 and 3) of rats treated similarly to the first group.

DNOC was administered by intraperitoneal injection of a 1% (w/v) solution of the sodium salt adjusted to pH 7·4.

Immediately after death, the treated animals were held head downwards in liquid O_2. The head and shoulders were immersed, but freezing of the rest of the body was avoided in order to ease the removal of the heart and diaphragm. After the initial strong ebullition had subsided, the animal was removed from the liquid O_2 and decapitated by a single blow from a broad-bladed chisel. The head was immediately returned to the liquid O_2. It was found that if the head was left in liquid O_2 for at least 3 or 4 min. the skull usually cracked in an anterior-posterior direction. This enabled the skull to be split into two roughly equal portions each containing half of the brain. Immediately after decapitation, samples of muscle weighing between 1 and 2 g. were cut from one of the hind limbs and dropped into liquid O_2. The heart was removed whole and washed in ice-cold water before being placed in the liquid O_2. The diaphragm was removed as completely as possible, washed quickly in ice-cold water and then frozen.

Preparation of extracts

Skeletal muscle. The frozen muscle was weighed and ground with washed sand in 3 × 5 ml. of 3% (w/v) perchloric acid. The filtered extracts were combined and neutralized with N-NaOH. The solution was refiltered and made up to 50 ml. with distilled water.