Metabolic Changes during Phosphate Deprivation in *Euglena* in Air and in Oxygen

By J. J. BLUM AND NICOLE BÉGIN-HEICK

Department of Physiology and Pharmacology, Duke University,
Durham, N.C. 27706, U.S.A.

(Received 4 April 1967)

1. *Euglena* cells were grown in culture media containing either 20 mM-phosphate or 20 μ M-phosphate, with ethanol or glucose as the sole source of carbon, and gassed with either air + carbon dioxide (95:5) or oxygen + carbon dioxide (95:5) at atmospheric pressure. 2. After growth in low-phosphate medium with ethanol as substrate, the cells developed signs of oxygen toxicity, as indicated by a decreased rate of respiration, a decreased net synthesis of paramylum and a failure to resume growth on replenishment of phosphate. 3. After growth in low-phosphate medium with glucose as substrate, the signs of oxygen toxicity were less apparent. 4. During phosphate deprivation the carotenoid content of *Euglena* increased more than threefold. This increase was largely prevented by exposure of the cells to oxygen + carbon dioxide (95:5) during growth. Oxygenation appears to interfere with ring closure of the common carotenoid precursor. 5. Mitochondria obtained from *Euglena* exposed to oxygen during phosphate deprivation, i.e. when signs of oxygen toxicity were evident, had greatly decreased activities of succinate dehydrogenase, succinate-cytochrome *c* oxidoreductase and NADH-cytochrome *c* oxidoreductase, compared with mitochondria obtained from *Euglena* exposed to oxygen in medium containing 20 mM-phosphate.

When *Euglena* cells are deprived of a carbon source, they utilize their internal reserves of paramylum for survival and, with prolonged starvation, also use protein and RNA (Blum & Buetow, 1963). The ability to use RNA and protein for survival is probably mediated via cytolysomes, which develop during starvation, engulf droplets of cytoplasm and subsequently digest these droplets (Malkoff & Buetow, 1964). When *Euglena* cells are deprived of phosphate, the challenge to survival is of a different kind, and it is handled differently by the cell. It has been shown that a non-specific acid phosphatase is synthesized (Price, 1962; Blum, 1965; Bennun & Blum, 1966) and appears in specific regions of the pellicle (Sommer & Blum, 1965). Concomitantly with the formation of the induced phosphatase, the cell acquires the ability to take up phosphate rapidly if phosphate is replenished (Blum, 1966). To produce large quantities of the induced enzyme, cultures of *Euglena* were grown in carboys, but it was found that the induced phosphatase would not form unless air was bubbled through the medium. When oxygen + carbon dioxide (95:5) was used instead of air, there was no apparent effect on the rate of cell growth and there was no increase in the yield of induced acid phosphatase. However, if fresh medium containing 10 mM-phosphate was supplied to oxygenated

cultures, the cells did not resume growth, in contrast with aerated cultures, which resumed growth at a normal rate after a lag of about 24 hr. (Blum, 1965). In view of our finding that oxygen inhibits the growth of *Astasia* (Bégin-Heick & Blum, 1967), a close relative of *Euglena*, it seemed possible that when *Euglena* was deprived of phosphate it also became sensitive to oxygen. It was further observed that pellets of cells that had ceased growth because of phosphate deprivation were yellower than pellets of cells grown in complete medium or of cells that had been collected in the early stages of phosphate deprivation, and that the use of oxygen + carbon dioxide (95:5) instead of air + carbon dioxide (95:5) prevented this increase in pigment formation. In this paper we present data on the changes in oxygen consumption and in the content of RNA, protein, paramylum and carotenoids during phosphate deprivation and on the effect of oxygen on these parameters, and show that certain features characteristic of oxygen toxicity develop during phosphate deprivation.

MATERIALS AND METHODS

The cells used were *Euglena gracilis* var. *bacillaris*, strain SML1 (streptomycin-bleached). Cells were grown axenically in defined medium (Blum, 1965) at constant temperature,

with stirring, in culture vessels (Blum & Padilla, 1962) and gassed with either air + CO₂ (95:5) or O₂ + CO₂ (95:5). For experiments in which glucose was the carbon and energy source instead of ethanol, the glucose was sterilized by filtration and added to the medium after autoclaving. The initial concentrations of orthophosphate were either 20 mM (high) or 20 μ M (low). Cells were counted with a Coulter counter (Coulter Co., Hialeah, Fla., U.S.A.).

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), RNA by a modified Schmidt-Thannhauser procedure (Blum & Padilla, 1962), and paramylum by the phenol-H₂SO₄ method of Dubois, Gilles, Hamilton, Rebers & Smith (1956). Phosphatase activity was measured at pH 5 with *p*-nitrophenyl phosphate as substrate and cells that had been treated with toluene and frozen before assay (Blum, 1965). O₂ consumption was measured by conventional Warburg respirometry, after careful washing of the cells by centrifugation for 2 min. at 800*g* (Buetow, 1961). Cells were always resuspended in complete medium with 10 mM-phosphate.

The methods used for the preparation of mitochondria and the assay of succinate dehydrogenase, succinate-cytochrome *c* oxidoreductase and NADH-cytochrome *c* reductase activities are described in detail by Bégin-Heick & Blum (1967). The units of acid phosphatase activity used were μ moles of P_i released/min./10⁶ cells.

Carotenoids were extracted from washed cells first with ethanol, then with acetone and finally with ethanol. The combined extract was freed of any cells by centrifugation and evaporated to dryness in a stream of warm air. The residue was dissolved in ethanol, and clarified by centrifugation if necessary, and its extinction at selected wavelengths was measured in a Gilford spectrophotometer, with cuvettes of 1 cm. path-length. The carotenoids were further characterized by thin-layer chromatography. Samples of known carotenoids to serve as standards were generous gifts from Dr N. I. Krinsky of Tufts University School of Medicine. The absorption spectra of the carotenoids isolated from thin-layer chromatograms were compared with values in the literature (Goodwin, 1955*a, b*; Krinsky & Goldsmith, 1960).

RESULTS

Changes in growth rate and in cell composition during phosphate deprivation in air and in oxygen when ethanol is the carbon source. *Euglena* usually grew at about the same rate whether oxygen + carbon dioxide or air + carbon dioxide was bubbled through the culture (Fig. 1), although in some cultures oxygenation decreased the rate of growth. If complete medium containing high phosphate was added after cell division had stopped because of phosphate deprivation, the cells resumed growth at the normal generation time after a lag of about 24 hr. whether they had been supplied with air via diffusion (cf. Blum, 1965) or with air + carbon dioxide (Fig. 1). If the culture was gassed with oxygen + carbon dioxide, however, growth did not resume for at least 44 hr. (Fig. 1). Thus during exposure to oxygen in the absence of phosphate the cells acquired a deficit, which was revealed as a

Fig. 1. Effect of oxygen on the growth of cells in ethanol-containing medium in the presence and absence of phosphate. The broken lines in (c) show the growth curves of two cultures of *Euglena* in ethanol-containing medium with 20 mM-phosphate. At 42 hr. after the arbitrary zero time, the gas mixture bubbled through one of the cultures was changed from air + CO₂ (○---○) to O₂ + CO₂ (●---●), while the other culture (□---□) was continued on the air + CO₂ mixture. The solid lines show the growth (c), RNA content (b) and respiration rate (a) of a pair of cultures grown in ethanol-containing medium with an initial concentration of 20 μ M-phosphate. At 1 hr. after the arbitrary zero time, the gas bubbled through the culture represented by ●---● was changed from air + CO₂ to O₂ + CO₂, the other culture (□---□) remaining on air + CO₂. At 97 hr. both cultures were diluted with medium containing ethanol and 20 mM-phosphate ('Dil.').

failure to resume growth when phosphate was resupplied. During the period of phosphate deprivation, the RNA content of the cells, ordinarily about 45 μ g./10⁶ cells, decreased to about 10 μ g./10⁶ cells, thus demonstrating the ability of the cell to survive even with RNA contents of less than 25% of the normal value. It is not established whether the decrease in RNA content was a result of degradation of the RNA and utilization of its components or of dilution during the last two divisions before growth ceases. That some degradation occurred is indicated by the results of an experiment in which RNA was isolated from *Euglena* by a combination of the methods of Scherrer & Darnell (1962) and Brawerman, Hufnagel & Chargaff (1962) at various times during phosphate deprivation, and examined after sedimentation on a linear 5–20% (w/v) sucrose gradient

Fig. 2. Changes in phosphatase activity (a), respiration rate (b), paramylum content (c) and growth rate (d) in ethanol-grown phosphate-deprived cultures of *Euglena*. Fig. 2(d) shows the growth rate of two cultures of *Euglena* in a medium initially containing 20 μ M-phosphate. At 75 hr. after the arbitrary zero time, the gas bubbling through one of the cultures was changed from air + CO₂ (○—○) to O₂ + CO₂ (●—●), the other culture (□—□) remaining on air + CO₂. At 188 hr. both cultures were diluted with medium containing ethanol and 20 mM-phosphate ('Dil.'). The units of phosphatase activity are μ moles/min./10⁶ cells.

(Rake & Graham, 1964). In agreement with the results of Horiuchi (1959), working with phosphate-starved *Escherichia coli*, it was found that the ratio heavy RNA : light RNA greatly decreased during phosphate deprivation. The presence of oxygen instead of air somewhat decreased the rate of decline of the RNA, but the effect of oxygen on RNA utilization was small. On replenishment of the phosphate most of the RNA was resynthesized during the lag period before cell division resumed. Again, the effect of oxygen on this resynthesis of RNA was small (Fig. 1). Oxygen had an important effect on the respiration of *Euglena* soon after the onset of phosphate deprivation. During phosphate deprivation with air + carbon dioxide bubbling, the rate of oxygen consumption decreased from about 55 to 35 μ l./hr./10⁶ cells in the control (air + carbon dioxide) culture. On replenishment of phosphate, the rate of respiration immediately started increasing towards the initial value. In the oxygenated culture, the rate of respiration decreased from 55 to 25 μ l./hr./10⁶ cells during phosphate deprivation,

and when phosphate was replenished there was no recovery of respiration for at least 44 hr. (Fig. 1).

In a similar experiment a pair of cultures was grown in medium initially at 20 μ M-phosphate until the phosphatase activity had risen from the constitutive value of 5 μ moles/min./10⁶ cells to about 15 μ moles/min./10⁶ cells (Fig. 2). At this time (about 75 hr. after the arbitrary zero time of the experiment) the gas being supplied to one of the cultures was changed from air + carbon dioxide to oxygen + carbon dioxide, while the other culture continued to be supplied with the air + carbon dioxide mixture. Growth during the period of phosphate deprivation was essentially the same for both cultures, but after phosphate was replenished the control culture resumed growth after the usual lag period, while the oxygen-treated culture did not (Fig. 2). In this experiment there was some uncertainty about the growth pattern of the oxygen-treated cells owing to the pronounced tendency of cells that had been deprived of phosphate for over 180 hr. to sink to the bottom and stick to the walls of the culture vessel for about a day after phosphate replenishment. Since in all experiments where phosphate deprivation was of somewhat shorter duration regrowth of oxygenated cultures did not resume for at least 48 hr. (cf. Fig. 1 and Table 2), it is reasonable to assume that regrowth did not begin in this culture either. The analytical data to be discussed now were taken during the period 90–201 hr. from the start of the experiment and were not affected by the uncertainty in cell counts of the culture.

During phosphate deprivation the acid phosphatase activity rose to about 32 units in the aerated culture, but only to about 22 units in the oxygenated culture, and the activity declined to about 17 units with further oxygenation (Fig. 2). Similar data were obtained for the experiment shown in Fig. 1. Thus oxygenation partially inhibited, but did not prevent, the formation of the induced acid phosphatase activity. As in the previous experiment (cf. Fig. 1), the respiration of the oxygenated cells was considerably more inhibited than that of the aerated cells and did not increase after phosphate replenishment. In cultures that were deprived of an energy and carbon source, the paramylum of the cells was used for survival (Blum & Buetow, 1963). In cultures that were deprived of phosphate, paramylum continued to be synthesized after cell division stopped, and the cells attained paramylum concentrations of over 1.1 mg./10⁶ cells. At first, oxygenation did not decrease the net rate of paramylum synthesis, but after about 2 days of oxygen treatment the net synthesis of paramylum stopped (Fig. 2).

In a separate experiment the protein concentration of a phosphate-deprived culture was measured.

Fig. 3. Changes in carotenoids in ethanol-grown phosphate-deprived cultures of *Euglena*. The data refer to the two cultures described in Fig. 2, and the symbols have the same significance as in Fig. 2. The E_{424} values shown on the ordinate of (c) were computed for the carotenoids of 10^9 cells extracted into 1 ml. of ethanol.

At the time when bubbling with oxygen + carbon dioxide began, the protein content was $317 \mu\text{g.}/10^6$ cells, a value typical of normal *Euglena*. During the next 4 days of oxygenation, while the RNA steadily decreased from 37 to $16 \mu\text{g.}/10^6$ cells, the protein content was 297, 277, 306 and $326 \mu\text{g.}/10^6$ cells. Thus oxygenation of phosphate-deficient cultures did not result in protein utilization.

Changes in carotenoid content during phosphate deprivation when ethanol is the carbon source. In the same experiment as shown in Fig. 2, cells were extracted at various times with ethanol and acetone as described above and the extinctions at 424, 447 and $477 \text{ m}\mu$ were measured, these being the principal absorption peaks of some of the carotenoids found in *Euglena*. It was shown by Blum & Buetow (1963) that during carbon starvation, when the cells were utilizing paramylum, RNA and protein for survival, the carotenoid content of the cell increased almost twofold. There was also a

Fig. 4. Changes in paramylum content (a), carotenoid content (b), respiration rate (c) and growth rate (d) of *Euglena* in phosphate-deficient glucose medium. At 3 hr. after the arbitrary zero time, the gas bubbling through the culture shown by $\bullet$ was changed from air + CO_2 to O_2 + CO_2 , while the control culture was continued on the air + CO_2 mixture ($\square$). At 79 hr., both cultures were diluted with glucose medium containing 20 mm-phosphate ('Dil.'). In (b), solid lines refer to E_{424} values, and broken lines to the E_{424}/E_{447} values. In (a), broken lines indicate that more data would be required to determine the shape of these curves.

change in the types of carotenoids present, as indicated by a slight change in E_{424} relative to E_{447} (Blum & Buetow, 1963). When these cells were deprived of phosphate, there was more than a doubling of the carotenoid content as judged by the increase in E_{424} (Fig. 3). In the oxygenated culture, however, the rise in carotenoid content was almost entirely prevented. During phosphate deprivation there was little if any qualitative change in the types of carotenoids, as shown by the constancy of E_{424} relative to E_{447} and by the almost constant epoxide ratio (Fig. 3). Oxygenation in addition to preventing the increase in carotenoid content also altered the carotenoid profile, as

indicated by the decrease in the epoxide ratio (about 0.12, relative to aerated cells) and the increase in the E_{424}/E_{447} ratio (Fig. 3). The epoxide ratio has been defined by Krinsky (1964) as the ratio of E_{477} values in the absence and presence of a trace of hydrochloric acid. A decreasing epoxide ratio indicates a decrease in the mono-epoxide and diepoxide forms of the carotenoids relative to the less oxidized forms.

Effects of phosphate deprivation and of oxygenation on cells growing in glucose medium. When glucose instead of ethanol was the sole source of carbon and energy, the cells were much less sensitive to oxygen (Fig. 4). Cells treated with oxygen reached a higher concentration in the phosphate-deficient medium than the aerated controls and, on replenishment of phosphate, they resumed growth at about half the rate of the aerated controls, compared with oxygenated ethanol-grown cells, which failed to resume growth on phosphate replenishment. The rate of respiration of cells grown in glucose was considerably lower than that of cells grown in ethanol and, unlike the cells grown in ethanol, these cells showed no significant effect of oxygenation on the respiratory rate throughout the experiment. Paramylum content increased during growth in phosphate-deficient glucose-containing medium, but oxygenation had a much smaller effect on the paramylum content of glucose-grown cells compared with ethanol-grown cells. The increase in carotenoid content during phosphate deprivation also occurred when glucose was the carbon source, but to a lesser extent than when ethanol was the carbon source (compare Fig. 3 with Fig. 4). As with the ethanol-grown cells, oxygenation largely prevented the increase in carotenoid content and caused a qualitative change in the nature of the carotenoids, as indicated by an increase in E_{424} relative to E_{447} . The increase in this ratio in the glucose-grown cells was, however, less than in the ethanol-grown cells.

Effects of phosphate deprivation and of oxygenation on the carotenoid profile of Euglena. To examine the effects of phosphate-deprivation and of oxygenation on the individual carotenoids of *Euglena*, pooled extracts of the air-ethanol-, oxygen-ethanol-, air-glucose-, and oxygen-glucose-grown cells were subjected to thin-layer chromatography (Table 1). The purified compounds were assigned tentative identifications on the basis of their R_F values, their absorption maxima and minima and the effect of hydrochloric acid or borohydride on their spectra. The data on a lipid believed to be a fatty acid ester of ergosterol are included in Table 1. This lipid was present at a much lower concentration in oxygen-ethanol-grown cells than in the air-ethanol-grown controls. The extinction data shown in Table 1 are not necessarily a quantitative reflection of the

carotenoid content of the cells, although some precautions were taken against losses caused by oxidation or light. The data reflect some average composition taken throughout each phosphate-deprivation experiment and are primarily intended for comparative purposes. Within these limitations, however, several important generalizations can be made. First, the carotenoids responded differently to oxygenation, as might have been predicted on the basis of the change in E_{424} (Figs. 3 and 4). Canthaxanthin, phytoene, ζ -carotene, and compound 16 either did not change or actually increased in amount on oxygenation of ethanol-grown cells. The other carotenoids all decreased, the amounts in many cases becoming too small to measure. With compound 11, tentatively identified as antheraxanthin furanoid oxide, the decrease caused by oxygen treatment was about 200-fold. The compound originally present was probably antheraxanthin, since the furanoid oxides are not believed to be present in cells. The change to the furanoid oxide form probably occurred as a result of chromatography on silica gel since standard antheraxanthin was very sensitive to light and oxygen when on the silica-gel plate. Secondly, the carotenoid profile in air-glucose-grown cells was different from the profile in air-ethanol-grown cells. Thus phytoene, the carotenoid present in largest quantity in the ethanol-grown cells, was effectively absent from the glucose-grown cells, whereas β -carotene and zeaxanthin were still present in easily measurable amounts. Thirdly, as predicted from the smaller change in the E_{424}/E_{447} ratio (cf. Figs. 3 and 4), there was a smaller effect of oxygen on the relative contents of the various carotenoids of the glucose-grown cells compared with the ethanol-grown cells. It should be noted that ζ -carotene was the only carotenoid that appeared to increase as a result of oxygen treatment in both ethanol-grown and glucose-grown cells.

Effects of exposure to oxygen on the mitochondrial enzymes of Euglena grown in high- and low-phosphate medium. In cells grown in a medium with ethanol as the sole carbon source, respiration is inhibited under conditions of phosphate deprivation. Because of the striking effects of exposure to oxygen + carbon dioxide during growth on the mitochondria of *Astasia* (Bégin-Heick & Blum, 1967), the effects of oxygen on the mitochondria of *Euglena* were investigated. Cells grown in high-phosphate medium (Expt. I of Table 2) were gassed with air + carbon dioxide until the cell concentration reached about 5×10^5 cells/ml., when half of the culture was collected and mitochondria were prepared and assayed. An equivalent amount of fresh medium (containing 20 mM-phosphate and ethanol) was then added to the culture vessel and the cells were allowed to grow for about 24 hr. with

Table 1. *Effect of growth conditions on carotenoids of Euglena*

The ethanol-acetone extracts of the cells used to get the extinction data shown in Fig. 3 were pooled and the solvent evaporated under nitrogen. The lipids were redissolved in known volumes of ethanol and each was applied as a band on 0.5-mm.-thick silica-gel plates and chromatographed in solvent I (R_F values are given in parentheses after the solvent numbers). The indicated regions were scraped off the plate and extracted with ethanol three times; the ethanol was then removed by evaporation under N_2 . The fractions were redissolved in ethanol, rechromatographed on 0.25-mm.-thick thin-layer plates once or twice more in the solvents shown; each compound was finally extracted into ethanol, dried and dissolved in 1 ml. of ethanol. The spectrum was then determined with a Beckman DBG recording spectrophotometer (1 cm. light-path). The wavelengths at which absorption maxima (the λ_{max} . value for the highest maximum is denoted with an asterisk) and minima occurred are listed in the Table. In some cases a few crystals of NaBH ₄ (compounds 9, 10, 13, 14, 15, and 16) or 0.05 ml. of 0.12 <i>N</i> -HCl (compounds II and III) were added; the positions of the absorption maxima or shoulder (indicated by a parenthesis) after reduction or acidification are shown in the fourth column. The solvents used for chromatography contained, (v/v): solvents I and II, methylene dichloride-ethyl acetate (8 : 2 and 6 : 4, respectively); solvents III and IV, hexane-ethyl acetate (9 : 1 and 8 : 2, respectively); solvent V, hexane. The extinctions shown are computed for 10 ⁸ cells extracted into 1 ml. of ethanol; 'a' means that the E value was less than 0.01/10 ⁸ cells/ml. of ethanol; (x) next to the name of a compound indicates that the absorption spectrum is characteristic of that compound, but the R_F values suggest that the compound has an unknown substituent on it; 'b' indicates that for the glucose-grown cells compounds 3 and 4 were eluted together from the thin-layer chromatogram after running in solvent III.										
Compound no.	Tentative identification	Solvent systems and R_F values	Absorption maxima and minima		λ_{max} , (m μ) or HCl	R_F values of standards	Ethanol		Glucose	
			λ_{max} , (m μ)	λ_{min} , (m μ)			Air	O ₂	Air	O ₂
1	Ergosterol	I (1-0)→III (1-0)→V (0-05)	291, 282*, 271, (260)	276, 230	—	—	12.8	1.2	0.58	0.33
2	β -carotene	I (1-0)→III (0.75-0.95)→IV (0.95)	(482), 455*, 430	472	—	I (1-0), III (0.90)	2.0	0.49	0.37	0.04
3	ζ -carotene	I (1-0)→III (1-0)→V (0.35)	430*, 400, 380	418, 392	—	—	4.5	6.9	0.06 ^b	0.2 ^b
4	—	I (1-0)→III (1-0)→V (0.48)	(478), 450*, (425)	—	—	—	3.5	a	—	—
5	Phytoene	I (1-0)→III (1-0)→V (0.48)	(295), 285*, 270	—	—	—	7.1	4.2	a	a
6	Trollein-like	I (1-0)→III (0.50-0.75)→IV (0.90)	(485), 460*, 435	—	—	—	1.7	a	a	a
7	—	I (1-0)→III (0.35-0.50)→IV (0.85)	(480), 455*, (420)	—	—	—	0.43	a	a	a
8	Echinenone	I (1-0)→III (0.35-0.50)→IV (0.67)	460*	—	NaBH ₄ : (480), 450, (420)	I (1-0), III (0.43)	0.34	a	a	a
9	Hydroxy-echinenone	I (1-0)→III (0.35-0.50)→IV (0.64)	463*	—	NaBH ₄ : (485), 460, (430)	—	0.89	a	0.06	0.02
10	Canthaxanthin	I (1-0)→III (0.25-0.35)→IV (0.75)	475*	—	NaBH ₄ : (480), 455, (425)	I (1-0), III (0.15)	0.88	0.71	0.05	0.02
11	Antheraxanthin	I (0.3-0.6)→II (0.45)	458, 430*, (411)	445	HCl: no change	I (0.31), II (0.50)	4.9	a	0.11	0.05
12	Furanoid oxide	I (0.1-0.3)→II (0.49)	(480), 452*, (423)	—	—	I (0.47), II (0.55)	1.8	a	0.58	0.14
13	Zeaxanthin	I (0.6-1.0)→II (0.55)	475*	—	NaBH ₄ : (480), 455, (425)	—	0.85	0.17	0.16	0.04
14	Canthaxanthin (x)	I (0.1-0.3)→II (0.54)	460*	—	NaBH ₄ : (480), 452, (423)	—	0.49	a	a	a
15	Echinenone (x)	I (0.3-0.6)→II (0.22)	460*	—	NaBH ₄ : (482), 455, (425)	—	0.76	a	a	a
16	Hydroxy-echinenone (x)	I (0.3-0.6)→II (0.33)	(480), 455*, 430, (405)	440	NaBH ₄ : no change	—	1.0	1.7	a	a
17	—	I (1.0)→III (0.15)→IV (0.30)	(480), 458*, 425	—	HCl: no change	—	2.3	a	a	a

The ethanol-acetone extracts of the cells used to get the extinction data shown in Fig. 3 were pooled and the solvent evaporated under nitrogen. The lipids were redissolved in known volumes of ethanol and each was applied as a band on 0.5 mm.-thick silica-gel plates and chromatographed in solvent I (R_F values are given in parentheses after the numbers). The indicated regions were scraped off the plate and extracted with ethanol three times; the ethanol was then removed by evaporation under N₂. The fractions were redissolved in ethanol, rechromatographed on 0.25 mm.-thick thin-layer plates once or twice more in the solvents shown; each compound was finally extracted into ethanol, dried and dissolved in 1 ml. of ethanol. The spectrum was then determined with a Beckman DBG recording spectrophotometer (1 cm. light-path). The wavelengths at which absorption maxima (the λ_{max} value for the highest maximum is denoted with an asterisk) and minima occurred are listed in the Table. In some cases a few crystals of NaBH₄ (compounds 8, 9, 10, 13, 14, 15, and 16) or 0.05 ml. of 0.12 N-HCl (compounds 11 and 16) were added; the positions of the absorption maxima or shoulder (indicated by a parenthesis) after reduction or acidification are shown in the fourth column. The solvents used for chromatography contained, (v/v): solvents I and II, methylene dichloride-ethyl acetate (8:2 and 6:4 respectively); solvents III and IV, hexane-ethyl acetate (9:1 and 8:2, respectively); solvent V, hexane. The extinctions shown are computed for 10⁸ cells extracted into 1 ml. of ethanol; 'a' means that the E value was less than 0.01/10⁸ cells/ml. of ethanol; (x) next to the name of a compound indicates that the absorption spectrum is characteristic of that compound, but the R_F values suggest that the compound has an unknown substituent on it; 'b' indicates that for the glucose-grown cells compounds 3 and 4 were eluted together from the thin-layer chromatogram after running in solvent III.

Table 2. *Effects of oxygen on the activity of some respiratory-chain enzymes in the mitochondria of Euglena*

In Expt. I the cells were grown in medium containing 20 mM-phosphate and exposed either to air + CO₂ (95 : 5) or O₂ + CO₂ (95 : 5) for the 24 hr. interval preceding the collection of the cells and preparation of mitochondria. In Expt. II, cells were grown in a medium initially containing 20 μ M-phosphate. From zero time, one culture was gassed with air + CO₂ (95 : 5) and one with O₂ + CO₂ (95 : 5), and at 70.2–71.2 hr. both cultures were diluted with fresh medium containing 20 mM-phosphate. Methods of enzyme assay are given in the Materials and Methods section. The activity units are m μ moles of substrate reduced/min./mg. of protein.

Expt. no.	Time (hr.)	10 ⁻³ × no. of cells		Succinate dehydrogenase activity		Succinate-cytochrome c oxidoreductase activity		NADH-cytochrome c oxidoreductase activity	
		Air	O ₂	Air	O ₂	Air	O ₂	Air	O ₂
I	—	—	—	64	66	160	146	216	169
II	46	421	367	50	23	73	55	79	55
	70	480	420	57	29	78	50	127	83
	71.5	253	234	—	—	—	—	—	—
	94	261	248	40	19	102	45	213	47
	102	322	256	—	—	—	—	—	—
	118	514	349	—	—	—	—	—	—

exposure to oxygen + carbon dioxide. There was little effect of oxygen treatment on the activity of the respiratory-chain enzymes measured, (Table 2), as might be expected from the lack of signs of oxygen toxicity on *Euglena* grown in high-phosphate medium. When parallel cultures were grown in low-phosphate medium with exposure to oxygen + carbon dioxide or air + carbon dioxide, as described in the legend to Table 2, there was little inhibition of growth rate during oxygenation, but considerable delay in the resumption of growth on replenishment of phosphate (Table 2). The succinate dehydrogenase, succinate-cytochrome c oxidoreductase and NADH-cytochrome c reductase activities of the aerated cells decreased progressively in low-phosphate medium but, except for succinate dehydrogenase, they returned towards normal levels within 24 hr. after phosphate replenishment. In the cells grown under oxygen the decrease in the activities of these respiratory-chain enzymes was more pronounced during growth in low-phosphate medium, and instead of returning towards the normal levels after phosphate replenishment these activities were further decreased.

The mitochondrial fraction of *Euglena* was yellow and contained the same kinds of carotenoids as those reported above for the whole cell. The *Euglena* mitochondrial fraction also contained a violet pigment, presumably rhodoquinone-45, described in more detail by Bégin-Heick & Blum (1967).

DISCUSSION

Very few studies have been made of the physiological and biochemical changes that occur in organisms during prolonged periods of phosphate

deprivation. Horiuchi (1959) found that the RNA, DNA and protein content per bacterium (*Escherichia coli* B) decreased, although certain specific proteins such as alkaline phosphomonoesterase and ribonuclease increased considerably. Holme & Palmstierna (1956) noted that the glycogen content of *Escherichia coli* B increased during phosphate starvation. The effects of phosphate deprivation on *Euglena* depend on the carbon source present and also on the partial pressure of oxygen in the gas mixture supplied to these cells. There appears to be no significant change in the protein content of *Euglena* during phosphate deprivation, although there may be large changes in individual proteins such as the pellicular acid phosphatase. The RNA content of *Euglena* decreases about 75% during prolonged phosphate deprivation without any noticeable loss of viability, as judged from the resumption of growth at a normal rate after a lag of about one generation time after the addition of phosphate. In both *Escherichia coli* B and *Aerobacter aerogenes*, however, phosphate deprivation causes a marked decrease in viable counts (Mallette, Cowan & Campbell, 1964; Postgate & Hunter, 1962). Oxygenation has little effect on the rate of loss of RNA during the period of phosphate deprivation in ethanol-grown *Euglena*, nor is there any appreciable effect of oxygenation on the rate of resynthesis of RNA after phosphate replenishment.

During carbon starvation in *Euglena* the paramylum is used for survival. During phosphate deprivation in either ethanol- or glucose-containing media, the cells continue to synthesize paramylum until the content is about twice the usual value. In ethanol-containing medium, oxygen considerably decreases the ability of the cells to continue

synthesizing paramylum, but in glucose-containing medium the effect of oxygenation is not so marked. This is not unexpected if one of the primary effects of oxygenation is at the level of the mitochondria.

Although the carotenoids of streptomycin-bleached *Euglena* have no known function, it is noteworthy that during carbon starvation, when RNA, protein and paramylum are consumed for survival, the carotenoid content per cell increases and the relative amounts of the different carotenoids are altered (Blum & Buetow, 1963). Smillie & Rigopoulos (1962) also observed a rise in the carotenoid content of a streptomycin-bleached *Euglena gracilis* strain when cell division ceased at the end of the exponential phase of growth or when cells were deprived of a carbon source. When *Euglena* cells are deprived of phosphate there is also an increase in the carotenoid content and a change in the relative amounts of the carotenoids present. This change is evident not only from the decrease of the epoxide ratio during phosphate deprivation, but from the following considerations as well. Goodwin & Gross (1958) reported that the major polyene carotenes of strain SML1 were β -carotene and a ζ -carotene-like fraction, and noted that in all the bleached strains they examined the concentrations of xanthophylls were considerably decreased compared with the green organism. In dark-bleached *Euglena gracilis* var. *bacillaris*, Krinsky, Gordon & Stern (1964) reported that the major xanthophyll was antheraxanthin, with smaller amounts of hydroxyechinenone and trollein also present. In strain SML1 grown on ethanol, we find large amounts of phytoene as well as ζ - and β -carotene, and a variety of xanthophylls, with antheraxanthin predominating. Cells grown in a glucose-containing medium have a much lower carotenoid content than do cells grown in an ethanol-containing medium, zeaxanthin being the major xanthophyll component and β -carotene the major polyene carotene.

The effect of oxygenation on carotenoid synthesis is similar in the ethanol-grown and the glucose-grown cells. Oxygen prevents the increase in carotenoid synthesis that occurs during phosphate deprivation. Oxygen also increases the E_{424}/E_{447} ratio, and, in the ethanol-grown cells at least, decreases the epoxide ratio. Of the non-cyclized polyenes (e.g. phytoene, phytofluene, ζ -carotene and lycopene) we find some decrease in phytoene and an increase in ζ -carotene as a result of oxygenation, but the sum stays roughly the same as for the cells grown in air. It is clear that oxygenation does not primarily interfere with the metabolism of the non-cyclized polyenes. Those carotenoids that are formed by ring closure of the common C_{40} precursor (Goodwin, 1952), e.g. γ -carotene, β -carotene,

zeaxanthin, trollein, antheraxanthin, echinenone, hydroxyechinenone and canthaxanthin, are greatly decreased in amount. It appears that one action of 95% oxygen may be to inhibit ring closure. It is not likely that the effect of oxygen on carotenoid production is responsible for the development of the signs of oxygen toxicity, since the carotenoids have no known function in strain SML1 and since the closely related naturally-bleached euglenoid, *Astasia longa*, is virtually devoid of carotenoids (Blum, Sommer & Kahn, 1965) but exhibits signs of oxygen toxicity (Bégin-Heick & Blum, 1967).

The effects of phosphate deprivation on the respiration of *Euglena* depend on the carbon source and on the percentage of oxygen in the gas mixture supplied to these cells. In ethanol-grown cells, the respiration rate declines to almost half the value characteristic of cells in high-phosphate medium, but this decrease is much less drastic than the virtual cessation of respiration during deprivation of a carbon source (Blum & Buetow, 1963). If the cells are gassed with oxygen + carbon dioxide during phosphate deprivation in ethanol-containing medium, the respiration rate is further decreased and approaches the endogenous level, i.e. that characteristic of normal cells when transferred to a medium containing no carbon source. Danforth and his colleagues have analysed the respiration of *Euglena* and have concluded that there are two oxidative pathways in this organism, an endogenous pathway for which paramylum is the proximal carbon source and a pathway for the oxidation of exogenous acetate, ethanol and other substrates (Wilson & Danforth, 1958; Danforth & Wilson, 1961; Marzullo & Danforth, 1964). According to Cook (1966) the endogenous respiration is essentially unchanged by temperature in the range 15–29°, whereas oxygen consumption in the presence of acetate increases linearly with temperature in this range. The effect of oxygen treatment on phosphate-deficient *Euglena* growing with ethanol as the sole carbon source is to decrease the respiration rate towards the endogenous value and to prevent the recovery of respiration rate on phosphate replenishment. These changes in cell respiration are paralleled by decreases in the activities of some respiratory-chain enzymes in the mitochondria obtained from these cells. In the presence of glucose, phosphate deprivation does not decrease the endogenous rate, and the respiration of the phosphate-deficient cells is insensitive to treatment with oxygen + carbon dioxide. The present results can be explained without recourse to a compartmentalization hypothesis, since the maximum inhibition of mitochondrial respiratory-chain activity was about 70% and the residual activity could account for the endogenous respiration. An alternative explanation, however, is that

the endogenous pathway is insensitive to oxygen + carbon dioxide.

A primary difference between *Astasia* and *Euglena* in their sensitivity to oxygen + carbon dioxide at atmospheric pressure is that the absence of phosphate appears to sensitize the mitochondria of *Euglena* to the damaging effects of oxygen, whereas the mitochondria of *Astasia* are damaged even when the *Astasia* cells are grown in high-phosphate medium. The detailed mechanism of the sensitizing effect of phosphate deprivation is unknown.

We are indebted to Mr Alvernon Hayes and Mrs Betty Horton for excellent technical assistance, and to Dr N. I. Krinsky of Tufts University School of Medicine for providing us with samples of carotenoids and for several valuable suggestions on the identification of the carotenoids. This work was supported by Grants GB-2788 and GB-5554 from the National Science Foundation, and by Grant Rol-HD-01269 from the National Institutes of Health. J. J. B. is the recipient of Research Career Development Award 5-K3-GM-2341 from the National Institutes of Health.

REFERENCES

- Bégin-Heick, N. & Blum, J. J. (1967). *Biochem. J.* **105**, 813.
 Bennun, A. & Blum, J. J. (1966). *Biochim. biophys. Acta*, **128**, 106.
 Blum, J. J. (1965). *J. Cell Biol.* **24**, 223.
 Blum, J. J. (1966). *J. gen. Physiol.* **49**, 1125.
 Blum, J. J. & Buetow, D. E. (1963). *Exp. Cell Res.* **29**, 407.
 Blum, J. J. & Padilla, G. M. (1962). *Exp. Cell Res.* **28**, 512.
 Blum, J. J., Sommer, J. R. & Kahn, V. (1965). *J. Protozool.* **12**, 202.
 Brawerman, G., Hufnagel, D. A. & Chargaff, E. (1962). *Biochim. biophys. Acta*, **61**, 340.
 Buetow, D. E. (1961). *Analyt. Biochem.* **2**, 242.
 Cook, J. R. (1966). *Biol. Bull., Woods Hole*, **181**, 83.
 Danforth, W. F. & Wilson, B. W. (1961). *J. gen. Microbiol.* **24**, 95.
 Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, R. (1956). *Analyt. Chem.* **28**, 350.
 Goodwin, T. W. (1955a). *Advanc. Enzymol.* **21**, 295.
 Goodwin, T. W. (1955b). In *Modern Methods of Plant Analysis*, vol. 3, p. 272. Ed. by Paech, K. & Tracey, M. V. Berlin, Gottingen and Heidelberg: Springer-Verlag.
 Goodwin, T. W. (1952). *The Comparative Biochemistry of the Carotenoids*, p. 65. London: Chapman and Hall Ltd.
 Goodwin, T. W. & Gross, J. A. (1958). *J. Protozool.* **5**, 292.
 Holme, T. & Palmstierna, H. (1956). *Acta chem. scand.* **10**, 1553.
 Horiuchi, T. (1959). *J. Biochem., Tokyo*, **46**, 1467.
 Krinsky, N. I. (1964). *Biochim. biophys. Acta*, **88**, 487.
 Krinsky, N. I. & Goldsmith, T. H. (1960). *Arch. Biochem. Biophys.* **91**, 271.
 Krinsky, N. I., Gordon, A. & Stern, A. I. (1964). *Plant Physiol.* **39**, 441.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Malkoff, D. B. & Buetow, D. E. (1964). *Exp. Cell Res.* **35**, 58.
 Mallette, M. F., Cowan, C. I. & Campbell, J. R. (1964). *J. Bact.* **87**, 779.
 Marzullo, G. & Danforth, W. F. (1964). *J. gen. Microbiol.* **34**, 9.
 Postgate, J. R. & Hunter, J. R. (1962). *J. gen. Microbiol.* **29**, 233.
 Price, C. A. (1962). *Science*, **135**, 46.
 Rake, A. V. & Graham, A. F. (1964). *Biophys. J.* **4**, 267.
 Scherrer, K. & Darnell, J. E. (1962). *Biochem. biophys. Res. Commun.* **7**, 486.
 Smillie, R. M. & Rigopoulos, N. (1962). *J. Protozool.* **9**, 149.
 Sommer, J. R. & Blum, J. J. (1965). *J. Cell Biol.* **24**, 235.
 Wilson, B. W. & Danforth, W. F. (1958). *J. gen. Microbiol.* **18**, 535.