which includes both phosphorylated enzyme and enzyme-CoA intermediates (7).

REFERENCES

1. Cha, S., Mourad, N., Cha, C.-J. M., and Parks, R. E., Jr., *Federation Proc.*, **24**, 362 (1965).
2. Mitchell, R. A., Butler, L. G., and Boyer, P. D., *Biochem. and Biophys. Research Communs.*, **16**, 545, (1964).
3. Kreil, G., and Boyer, P. D., *Biochem. and Biophys. Research Communs.*, **16**, 551 (1964).
4. Cha, S., and Parks, R. E., Jr., *Federation Proc.*, **22**, 361 (1963); *J. Biol. Chem.*, **239**, 1961 (1964).
5. Cha, S., and Parks, R. E., Jr., *J. Biol. Chem.*, **239**, 1968 (1964).
6. Mourad, N., and Parks, R. E., Jr., *Biochem. and Biophys. Research Communs.*, **19**, 312 (1965).
7. Upper. C. D.. Ph.D. dissertation. University of Illinois. 1964.

Synthesis of Oleic Acid by *Euglena gracilis**

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While mechanisms for the enzymatic synthesis of oleic acid and related monounsaturated fatty acids have been established in a variety of biological systems, the origin of oleic acid in plants has remained unknown. As Mudd and Stumpf have shown, the synthesis of oleic acid in extracts of avocado mesocarp requires oxygen as it does in yeast, animal tissues, and in certain bacteria (1). However, unlike other oxygen-dependent systems, plant extracts apparently cannot convert stearate or palmitate into the corresponding olefins (2). Confirming Stumpf's findings on plant systems, we have previously reported that intact cells of *Euglena gracilis* and various other algae fail to desaturate stearate or palmitate (3). Further studies with *Euglena* have, however, led to results which require a revision of the earlier conclusion that stearate is not converted to oleate in photosynthetic systems.

We have now shown that in cell-free extracts of *Euglena*, thioesters of long chain saturated fatty acids are desaturated, and we have further found that the enzyme systems from photoauxotrophic and from etiolated cells have markedly different properties. As shown in Fig. 1, an enzyme system prepared from colorless cells, grown on sucrose in the dark, readily forms unsaturated acids from the coenzyme A esters of stearic and palmitic acids. For these transformations both particulate and soluble fractions are required. Extracts from photoauxotrophic *Euglena* cells do not act on the CoA esters but they desaturate stearyl-ACP[1] to oleate (Fig. 1). Conversely stearyl-ACP (or palmityl-ACP) is a poor substrate for the enzyme system from etiolated cells.

The enzyme from green cells is soluble. It is obtained by centrifugation of broken cells at 105,000 × *g* for 1½ hours, precipitation of protein from the supernatant by 0 to 75% ammonium sulfate, and passage of the dissolved precipitate through Sephadex G-25. Two subfractions, one precipitated between 20 and 45% and the other between 45 and 70% ammonium sulfate, show desaturating activity when combined, but they are inactive when tested singly (Table I). The soluble desaturase is specific for stearyl-ACP; the ACP derivatives of palmitate or myristate are not metabolized. By requiring O_2 and TPNH for activity (Table I), the enzyme resembles the known stearyl-CoA desaturases. Ascorbate (9), FAD, FMN (10–12), or 2-amino-4-hydroxy-6-methyltetrahydropteridine (13, 14), the cofactors required by some oxygenase systems, do not stimulate the soluble *Euglena* enzyme.

The ACP used in these experiments was the heat stable protein isolated from *Escherichia coli* (15, 16). It appears from the present and other findings (17, 18), that the bacterial protein can serve as acyl carrier in plants as well.

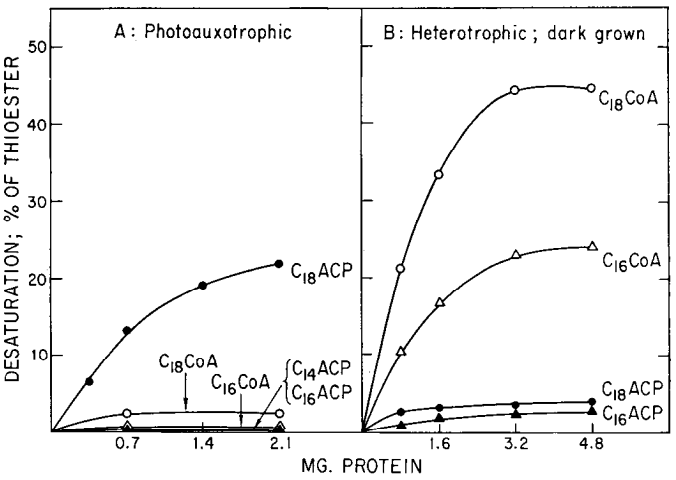

Fig. 1. *A*, the source of enzyme was *Euglena gracilis* Z grown with constant illumination on a mineral medium (4) for 4 to 6 days at room temperature. A gas mixture of 5% CO_2 in air was continuously passed through the culture. The harvested cells were washed and suspended in 5 volumes of a 0.5 M sucrose solution containing 0.01 M phosphate buffer, pH 7.2, 0.01 M NaCl, and 0.001 M EDTA. The cells were disrupted in a French pressure cell at 3000 psi, cell debris was removed by low speed centrifugation, and the supernatant fraction centrifuged at $10^5 \times g$ for 90 min. Ammonium sulfate was added to the resulting supernatant to 75% saturation, the precipitated protein was dissolved in a minimum volume of 0.5 M sucrose solution containing phosphate, NaCl and EDTA, and desalted on a Sephadex G-25 column, previously equilibrated with the same sucrose solution. Incubation mixtures contained enzyme protein in the quantities indicated, 0.2 μmole of TPNH, 50 μmoles of triethanolamine-HCl buffer, pH 7.2, 10 μmoles of mercaptoethanol, and the 1-^{14}C-acyl ACP thioesters in the following amounts in millimicromoles: stearyl, 0.084; palmityl, 0.1; and myristyl, 0.21. These values are based on the quantity of ^{14}C-fatty acid released from the acyl-ACP by reaction with 0.7 M hydroxylamine for 1 hour at 30°. The quantity of 1-^{14}C-stearyl-CoA or palmityl-CoA also based on thioester determination was 0.04 mμmole. The total volume was 0.5 ml, and the time of incubation (in air) was 10 min. The *E. coli* ACP, prepared according to Majerus, Alberts, and Vagelos (5), and electrophoretically pure (prepared by Dr. P. Gold and Mrs. J. Gussin), was acylated by reaction with the mixed anhydrides of the ^{14}C-fatty acids in the presence of mercaptoethanol.[2] From 0.25 to 1.0 mole of ^{14}C-fatty acid was incorporated per mole of ACP. The percentage of ^{14}C-fatty acid present as thioester as indicated by hydroxamate formation varied from 10 to 28%. The remainder of the radioactivity is released by strong alkali. After incubation with enzyme, the reaction mixtures were saponified and separated into saturated and unsaturated fatty acids as described (6). The data given are percentage of added acyl-thioester converted to unsaturated fatty acid. In other experiments, it was found that with 1.4 mg of enzyme protein, desaturation was linear up to a concentration of 1 mμmole of stearyl-ACP per 0.5 ml. The identity of the enzymatic product as oleic acid was established by permanganate-periodate degradation (7). *B*, the source of enzyme was *Euglena* grown with shaking on an organic medium (8) for 6 to 8 days in the dark. The harvested cells were washed and disrupted as described for the photoauxotrophic cells and the crude extract (16 mg of protein per ml) used without further fractionation. The components of the incubation mixture and all other conditions were the same as described for *A*.

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[1] The abbreviation used is: ACP, acyl carrier protein.

[2] Private communication from Dr. P. R. Vagelos.

TABLE I

Requirements for desaturation of stearyl-ACP by enzyme from photoauxotrophic Euglena

The composition of the reaction mixture and the experimental conditions were the same as described in the legend for Fig. 1*A*.

Enzyme and additions	Desaturation
	%
0 to 75% $(NH_4)_2SO_4$ fraction, 0.9 mg of protein	
TPNH	15.2
TPNH (boiled enzyme)	0.4
DPNH	5.2
TPN^+	0.5
TPNH, anaerobic*	0.4
TPNH, air†	11.8
20 to 45% $(NH_4)_2SO_4$, 0.45 mg of protein (A)	
TPNH	2.1
45 to 75% $(NH_4)_2SO_4$, 0.26 mg of protein (B)	
TPNH	1.6
A + B, 0.71 mg of protein	
TPNH	19.7

* Incubations were carried out in Thunberg tubes. Air was replaced by flushing three times with helium followed by evacuation.

† This experiment served as a control. After flushing with He and evacuation, air was admitted and the tube was incubated for 10 min.

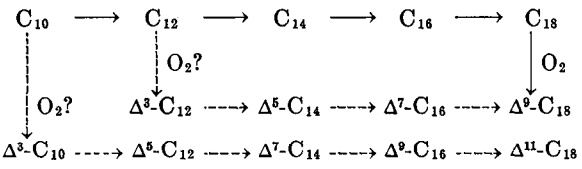

FIG. 2. Postulated pathways to unsaturated fatty acids in photoauxotrophic *E. gracilis*. The ACP derivatives are assumed to be the substrates in all of the indicated transformations.

The activity of the stearyl-ACP specific enzyme from photoauxotrophic *Euglena* accounts for the formation of oleic acid, but it fails to explain the presence of Δ^{11}-octadecenoic acid, and of the Δ^7- and Δ^9-isomers of hexadecenoic acid in photoauxotrophic *Euglena* (19, 20). Double bond isomers of this type are also found in *Clostridium butyricum* and in other bacteria which synthesize long chain unsaturated fatty acids anaerobically by chain-elongation of unsaturated acids, *e.g.* Δ^3-decenoate or Δ^3-dodecenoate (21, 6, 22). On structural grounds, it therefore seems likely that *Euglena* and perhaps other photosynthetic organisms employ a mechanism of the bacterial type, in addition to stearyl-ACP desaturation. Evidence in favor of this assumption is the ability of cell-free extracts of photoauxotrophic *Euglena* to elongate the ACP-derivatives of octanoate, decanoate, and dodecanoate to long chain saturated and unsaturated acids.[3] Analogous transformations occur in extracts of *E. coli* with octanoyl-ACP.[3] However, unlike the bacterial system, the *Euglena*-elongating system produces long chain unsaturated acids only in the presence of oxygen.[3] These findings are consistent with the sequence of reactions shown in Fig. 2, a scheme already proposed on the basis of other evidence (23). The principal and so far speculative feature of this scheme is the oxidative introduction of the double bond at the C_{10} or C_{12} level. The presence of Δ^5- and Δ_7-tetradecenoic acids in green *Euglena*, as shown by Korn, further supports this mechanism (19).

[3] Unpublished experiments.

From the present findings, we conclude that the photoauxotrophic form of *Euglena* synthesizes monounsaturated fatty acids by modifications of two mechanisms occurring in non-photosynthetic organisms. One of the enzymes, a soluble desaturase acts on stearyl-ACP but not on shorter chain ACP derivatives or on CoA esters of fatty acids. The second enzyme system of green *Euglena*, for which the evidence is still incomplete, appears to employ the principle of elongating already unsaturated acids as do bacteria with the difference that in *Euglena*, one of the steps requires molecular oxygen. By contrast, *Euglena* grown heterotrophically in the dark and lacking chloroplasts desaturates acyl-CoA thioesters apparently by the same mechanism that is widely distributed in nonphotosynthetic organisms. These findings further emphasize the fundamentally different patterns of lipid biosynthesis in photoauxotrophic and heterotrophic *Euglena* (20).

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REFERENCES

1. MUDD, J. B., AND STUMPF, P. K., *J. Biol. Chem.*, **236,** 2602 (1961).
2. STUMPF, P. K., AND JAMES, A. T., *Biochim. et Biophys. Acta*, **70,** 20 (1963).
3. ERWIN, J., HULANICKA, D., AND BLOCH, K., *Comp. Biochem. Physiol.*, **12,** 191 (1964).
4. CRAMER, M., AND MYERS, G., *Arch. Mikrobiol.*, **17,** 384 (1952).
5. MAJERUS, P., ALBERTS, A. W., AND VAGELOS, P. R., *Proc. Natl. Acad. Sci. U. S.*, **51,** 1231 (1964).
6. GOLDFINE, H., AND BLOCH, K., *J. Biol. Chem.*, **236,** 2596 (1961).
7. VON RUDLOFF, E., *Can. J. Chem.*, **34,** 1413 (1956).
8. HUTNER, S. H., BACH, M. K., AND ROSS, G. I. M., *J. Protozool.*, **3,** 101 (1956).
9. LEVIN, E. Y., LEVENBERG, B., AND KAUFMAN, S., *J. Biol. Chem.*, **235,** 2080 (1960).
10. CONRAD, H., DUBUS, R., AND GUNSALUS, I. C., *Biochem. and Biophys., Research Communs.*, **6,** 293 (1961).
11. KATAGIRI, M., YAMAMOTO, S., AND HAYAISHI, O., *J. Biol. Chem.*, **237,** PC2413 (1962).
12. FULCO, A. J., AND BLOCH, K., *J. Biol. Chem.*, **239,** 993 (1964).
13. KAUFMAN, S., *J. Biol. Chem.*, **239,** 332 (1964).
14. TIETZ, A., LINDBERG, M., AND KENNEDY, E. P., *J. Biol. Chem.*, **239,** 4081 (1964).
15. ALBERTS, A. W., GOLDMAN, P., AND VAGELOS, P. R., *J. Biol. Chem.*, **238,** 557 (1963).
16. LENNARZ, W. J., LIGHT, R. J., AND BLOCH, K., *Proc. Natl. Acad. Sci. U. S.*, **48,** 840 (1962).
17. OVERATH, P., AND STUMPF, P. K., *J. Biol. Chem.*, **239,** 4103 (1964).
18. BROOKS, J. L., AND STUMPF, P. K., *Biochim. et Biophys. Acta*, **98,** 213 (1965).
19. KORN, E. D., *J. Lipid Research*, **5,** 352 (1964).
20. HULANICKA, D., ERWIN, J., AND BLOCH, K., *J. Biol. Chem.*, **239,** 2778 (1964).
21. SCHEUERBRANDT, G., GOLDFINE, H., BARONOWSKY, P. E., AND BLOCH, K., *J. Biol. Chem.*, **236,** PC70 (1961).
22. BARONOWSKY, P. E., Ph.D. dissertation, Harvard University, 1963.
23. BLOCH, K., in J. K. GRANT (Editor), *The control of lipid metabolism*, Academic Press, Inc., New York, 1963, p. 1.

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