reactive in the native enzyme (Harris & Polgar, 1965). The amino acid sequences of the pig and ox enzymes are known to be very similar indeed (Perham, 1964) and hence, on the reasonable assumption that a sequence of 14 residues does not occur twice in a chain of about 330 residues, an amino acid sequence containing the reactive cysteine and lysine residues of pig glyceraldehyde 3-phosphate dehydrogenase may be deduced to be: Lys-Ile-Val-Ser-Asp(NH₂)-Ala-Ser-CySH-Thr-Thr-Asp(NH₂)-CySH-Leu-Ala-Pro-Leu-Ala-Lys-Val-Ile-His-Asp-His-Phe-Gly-Ile-Val-Glu-Gly-Leu-Met-Thr-(Thr,Val,His)-Ala-Ile-Thr-Ala-Thr-Glu(NH₂)-Lys-Thr-Val-Asp-Gly-Pro-Ser-Gly-Lys-Leu. Further experiments on the pig enzyme support this conclusion (J. I. Harris & R. N. Perham, unpublished work). The specific acetylation of the ε-amino group of lysine-42 during reaction of the NAD-free pig enzyme with *p*-nitrophenyl acetate appears to occur by intramolecular migration of acetyl groups from cysteine-12, where they are initially bound in thio ester linkage, indicating a close proximity of these two amino acid residues in the three-dimensional structure of the NAD-free enzyme (Harris & Polgar, 1965). This proximity could occur either by folding of the individual polypeptide chain or by suitable orientation of the four polypeptide chains in the active enzyme so that the reactive cysteine and lysine residues of different monomers are then juxtaposed.

The amino acid sequences around the reactive cysteine residues in glyceraldehyde 3-phosphate

dehydrogenase from ox and pig have been shown to be identical for at least 32 residues. It seems likely that this identity will extend even further and stands in marked contrast with the amino acid replacements found in the alcohol dehydrogenases (Harris, 1964).

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A Hydroxy Fatty Acid Dehydrogenase in *Euglena gracilis*

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A requirement for NADPH and molecular oxygen in the enzymic desaturation of fatty acyl-CoA or fatty acyl-ACP* esters in a number of organisms (Bloomfield & Bloch, 1960; Marsh & James, 1962; Davidoff, 1964; Nagai & Bloch, 1965) has led to the proposition that such desaturations might proceed by way of an oxygenase

type of reaction, involving hydroxy acid intermediates. Testing this hypothesis, Light, Lennarz & Bloch (1962) failed to obtain conversion of 9- or 10-hydroxystearic acid into oleic acid either in extracts or in growing cells of the yeast *Saccharomyces cerevisiae*. Marsh & James (1962) obtained some evidence for a transformation of 9- or 10-hydroxystearic acid into oleate by rat-liver homogenates, but the conversion of the hydroxy acids

* Abbreviation: ACP, acyl carrier protein.

was very slight compared with the overall conversion of stearate into oleate. Elovson (1964) observed that labelled 9- and 10-hydroxystearic acids were very rapidly metabolized in the intact rat. Although label appeared in oleic acid, the distribution of radioactivity in the carbon chain of oleic acid indicated that such activity was due to complete degradation of the hydroxy acid and resynthesis from acetate. Similarly, Davidoff (1964) found that growing cells of the cellular slime mould *Dictyostelium discoideum* did not directly convert added 9(10)-hydroxystearic acid into oleate whereas stearate was readily desaturated. The findings by Nagai & Bloch (1965) that soluble enzymes from photoauxotrophic *Euglena gracilis* readily desaturate stearoyl-ACP to oleate but do not act on the corresponding CoA ester led us to re-examine the role of hydroxystearate in oleate formation and specifically to test whether the ACP derivative of the hydroxy acid could serve as an intermediate.

The outcome of these experiments was negative. Extracts of *Euglena gracilis* that were active in the desaturation of stearoyl-ACP to oleate (Nagai & Bloch, 1965) failed to convert the ACP ester of D(-)-9-hydroxystearic acid into oleate (Table 1). 9-Acetoxystearoyl-ACP, *cis*-9,10-epoxystearoyl-ACP and DL-9(10)-hydroxystearoyl-ACP also failed to yield oleate in this system.

It became apparent during this work that, although no unsaturated fatty acids were produced from D(-)-9-hydroxystearoyl-ACP, radioactivity

consistently appeared in the 'saturated' fatty acid fraction as defined by silicic acid chromatography. The non-polar radioactive product was isolated and identified as 9-oxostearate by vapour-phase chromatography. D(-)-9-Hydroxystearoyl-CoA and the free fatty acid were converted into the keto acid equally as well as the ACP ester. NAD⁺ and NADP⁺ serve as cofactors for the oxidation with about the same efficiency. The reaction seems to be relatively unspecific with respect to chain length and position of the hydroxyl group, as DL-3-hydroxydecanoic acid was oxidized to a similar extent. The enzyme lost activity rapidly on storage for several days at -20° or after repeated freezing and thawing. The reversibility of the reaction was tested by incubating 9-oxostearic acid in the same system with either NADH or NADPH as cofactor (Table 1).

The fact that hydroxystearoyl-ACP is readily converted into oxostearate, but not dehydrated, by extracts that efficiently desaturate stearoyl-ACP to oleate may be regarded as one more piece of evidence against a mechanism involving hydroxy acids as intermediates in this reaction. It should be stressed, however, that the ACP used in these experiments was from *Escherichia coli* and is obviously not the natural acyl carrier for *Euglena gracilis* fatty acid transformations; *Euglena gracilis* ACP, which has not yet been isolated, may be a much more suitable carrier for the 'intermediate' even though the overall reaction proceeds with *Escherichia coli* ACP. Marsh & James (1962) found that

Table 1. *Effect of Euglena gracilis* desaturating enzyme on various substrates

Conditions of incubations are given in the text. Enzyme fractions are as follows: I, 100000g supernatant; II, 20-45% + 45-75% saturated (NH₄)₂SO₄ precipitates; III, 75% saturated (NH₄)₂SO₄ precipitate.

Substrate	Enzyme	Cofactor	Percentage desaturation of thio ester	Percentage conversion	
				Into non-polar fatty acid	Into polar fatty acid
Stearoyl-ACP	II	NADPH	7	—	—
D(-)-9-Hydroxystearoyl-ACP	II	None	0	5	—
	II	NADPH	<0.7	14	—
	I	NADP	0	32	—
D(-)-9-Hydroxystearoyl-CoA	II	NADPH	<0.4	20	—
D(-)-9-Hydroxystearic acid (free fatty acid)	II	NADPH	—	31	—
<i>cis</i> -9,10-Epoxystearoyl-ACP	II	NADPH	0	1	—
Stearoyl-ACP	III	NADPH	8	—	—
9-Acetoxystearoyl-ACP	III	NADPH	<0.6	20	—
9-Oxostearic acid (free fatty acid)	I	None	—	—	2
	I	NADPH	—	—	31
	I	NADH	—	—	14

liver homogenates converted 13% of added stearic acid into oleate whereas less than 4% of hydroxystearate was dehydrated. They concluded that the hydroxy acid could be an intermediate only if it were irreversibly bound to the enzyme. Our present results suggest that, in *Euglena gracilis* at least, any hydroxy acid substrate would be converted into oxostearate, hence channelling off the 'intermediate' into an unfruitful pathway. In view of the requirement of the desaturating enzyme for NADPH and oxygen, typical of oxygenase reactions, the mechanism remains unique and obscure. One might postulate a rather more complicated intermediate, perhaps a hydroperoxide, or alternatively a direct abstraction of two hydrogen atoms from the chain without the formation of a carbon-oxygen bond. In either case, no obvious experiments to test the hypothesis suggest themselves.

Methods. The enzyme was extracted from photoauxotrophic cultures of *Euglena gracilis* as described by Nagai & Bloch (1965). Either the supernatant fraction obtained by centrifuging broken cells at 100 000g for 90 min. or $(\text{NH}_4)_2\text{SO}_4$ precipitates prepared from this fraction (Nagai & Bloch, 1965) were used as indicated in Table 1. Incubation mixtures contained 0.7–1.0 mg. of protein, 0.2 μmole of NADPH, NADH or NADP^+ as indicated in Table 1, 50 μmoles of triethanolamine-HCl buffer, pH 7.2, and 10 μmoles of mercaptoethanol. Substrate amounts were: $[1-^{14}\text{C}]$ -stearoyl-ACP, 0.2 μmole ; $\text{D}(-)$ -9-hydroxy[12,13- $^3\text{H}_2$]stearoyl-ACP, 0.4 μmole ; $\text{D}(-)$ -9-hydroxy[12,13- $^3\text{H}_2$]stearoyl-CoA, 0.6 μmole ; 9-acetoxy[12,13- $^3\text{H}_2$]stearoyl-ACP, 0.7 μmole ; *cis*-9,10-epoxy[1- ^{14}C]stearoyl-ACP, 0.85 μmole ; these values represent μmoles of thio ester based on the quantity of labelled fatty acid released from acyl-ACP or acyl-CoA by reaction with 0.7 M-hydroxylamine for 1 hr. at 30°. The concentrations of free fatty acids were: $\text{D}(-)$ -9-hydroxy[12,13- $^3\text{H}_2$]stearic acid, 0.5 μmole ; 9-oxo[12,13- $^3\text{H}_2$]stearic acid, 7.5 μmoles . The total volume of the incubation mixture was 0.5 ml. and the time of incubation was 1 hr. at 30°. The *Escherichia coli* ACP was prepared by Dr P. Gold and Mrs J. Gussin in this Department by the method of Majerus, Alberts & Vagelos (1964) and was electrophoretically pure. The ACP was acylated by reaction with the mixed anhydrides of the labelled fatty acids in the presence of mercapto-

ethanol (P. R. Vagelos, personal communication). From 0.20 to 1.0 mole of labelled fatty acid was incorporated/mole of ACP. The percentage of labelled fatty acid present as thio ester, as indicated by hydroxamate formation, varied from 9 to 22%. After incubation with the enzyme, the reaction mixtures were saponified and the methyl esters of fatty acids separated into saturated and unsaturated fractions by silicic acid chromatography of the mercuric adducts (Goldfine & Bloch, 1961). Fractions were eluted from a column of silicic acid (1 g.) as follows: saturated fatty acids (stearate and oxostearate) by 20 ml. of 5% (v/v) ether in pentane; polar fatty acids (hydroxy acids) by 20 ml. of ether; unsaturated fatty acids by 10 ml. of 1% (v/v) acetic acid in methanol. Vapour-phase chromatography of the methyl esters was performed on a column of 10% diethylene glycol succinate on Chromosorb W at 195° in a Research Specialties model 600 gas chromatograph. Retention times (relative to methyl stearate) were: 9-hydroxystearate, 9.0; 9-oxostearate, 6.0. $\text{D}(-)$ -9-Hydroxy[12,13- $^3\text{H}_2$]stearic acid was prepared by the catalytic reduction of naturally occurring 9-hydroxyoctadec-12-enoic acid. 9-Acetoxy[12,13- $^3\text{H}_2$]stearic acid was synthesized by a method described by Light *et al.* (1962) and *cis*-9,10-epoxy[1- ^{14}C]stearic acid by the method of Julietti, McGhie, Rao & Ross (1960).

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