Reduction of Fatty Acids to Alcohols by Cell-Free Preparations of *Euglena gracilis**

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ABSTRACT: Etiolated *Euglena gracilis* Z contained wax esters as a major lipid component and exogenous labeled acetate was readily incorporated into both the alcohol and acid moieties of the wax. The major radioactive alcohols of the wax ester were straight chain C_{15}, C_{14}, C_{16}, and C_{13} in the order of decreasing amounts with detectable amounts of C_{12}, C_{17}, and C_{18}. In the same order the major labeled acids of the wax ester were C_{14}, C_{13}, C_{15}, C_{16}, and C_{12} with detectable amounts of C_{18} and C_{17}. The major labeled fatty acids in the polar lipids were C_{16}, C_{14}, C_{15}, and C_{18} in the order of decreasing amounts with some C_{12} and C_{17}. Enzymatic reduction of fatty acids to primary alcohols was demonstrated with a partially purified preparation from etiolated cells. The necessary cofactors were adenosine triphosphate, coenzyme A, and reduced nicotinamide–adenine dinucleotide but not reduced nicotinamide–adenine dinucleotide phosphate. The enzyme system had a pH optimum near 6.5 and apparent K_m of 1.6×10^{-5} M and 2.4×10^{-4} M for myristic acid and reduced nicotinamide–adenine dinucleotide, respectively. It was specific for C_{14} to C_{16} fatty acids consistent with the chain lengths of the alcohol moieties found in *Euglena* wax. The enzyme was strongly inhibited by *N*-ethylmaleimide and *p*-chloromercuric benzoate suggesting the presence of an essential thiol group. Aldehyde intermediate could be trapped by the inclusion of phenylhydrazine hydrochloride in the reaction mixture. Synthetic labeled hexadecanal was reduced by the same preparation with reduced nicotinamide–adenine dinucleotide or to a lesser extent with reduced nicotinamide–adenine dinucleotide phosphate as cofactor. The product alcohol was esterified with exogenous myristic acid only in the presence of adenosine triphosphate and coenzyme A, showing that the esterification process required activated fatty acid.

Free and esterified fatty alcohols of chain lengths C_{12} to C_{32} are widely distributed in nature (Eglinton and Hamilton, 1967; Kolattukudy, 1969b; Nicolaides, 1965). Although the surface lipids appear to be the most widespread source of fatty alcohols, in certain marine organisms fatty alcohols constitute a major component of the total lipids of the organism (Nevenzel, 1969). Long chain alcohols even exhibit hormone-like biological activity in animals (Levin, 1963) as well as plants (Crosby and Ulitos, 1959). Fatty alcohols are of further significance as they appear to be precursors of glyceryl alkyl ethers (Snyder *et al.*, 1969). However, little is known about their biosynthesis except for some observations on the incorporation of C_2 to C_{18} fatty acids (Nicolaides *et al.*, 1955; Kolattukudy, 1965, 1966; Nevenzel, 1969; Mazliak, 1968; Piek, 1964) into alcohols *in vivo*. In connection with plasmalogen synthesis some *in vivo* tracer studies have indicated possible interconversion of fatty acids, aldehydes, and alcohols (Baumann *et al.*, 1965; Hagen and Goldfine, 1967; Ellingboe and Karnovsky, 1967), but enzymatic fatty acid reduction was not reported. In biosynthetic studies on sphingosine with a particulate system from brain a palmityl–CoA dependent oxidation of reduced pyridine nucleotide was observed (Brady and Koval, 1958), but the product was not identified.

Euglena gracilis, when grown in the dark, contains wax esters as a major (50%) lipid component (Rosenberg, 1963; Guehler *et al.*, 1964) which appears to serve as an energy reserve (Rosenberg, 1967). In spite of the large amounts of wax esters found and the extensive studies on the lipid metabolism of this organism (Cheniae, 1963, 1964; Cheniae and Kerr, 1965; Nagai and Bloch, 1967; Bloch *et al.*, 1967), the mechanism of fatty alcohol synthesis remains unknown. It has been suggested that the aldehydes produced by α-oxidation of fatty acids give rise to the alcohols (Rosenberg, 1963). However, a direct reduction of the carboxyl group of a fatty acid appeared to be a plausible mechanism. In order to make the reduction of a carboxyl group energetically feasible it must be activated prior to reduction, and three different activated forms of carboxyl groups are known to undergo reduction. Acyl phosphate in aspartate semialdehyde synthesis (Black and Wright, 1955a,b), acyl-AMP in α-amino adipic acid reduction involved in lysine synthesis (Sagisaka and Shimura, 1962a,b), and acyl-CoA in hydroxymethylglutaryl-CoA reduction involved in mevalonic acid synthesis (Knappe *et al.*, 1959; Ferguson *et al.*, 1959) are examples.

In the present paper a cell-free preparation from etiolated *Euglena* which reduces fatty acids to alcohols is described and evidence is presented which shows that the fatty acyl-CoA is the substrate for the reduction, with aldehyde as the intermediate and NADH as the reductant. Some characteristics of the soluble enzyme involved are also described. It is also shown that the esterification of alcohol requires fatty acyl-CoA.

Materials and Methods

Organism. E. gracilis Z was purchased from the Culture Collection of Algae, Department of Botany, Indiana Univer-

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sity, and grown in the dark in 1.5-l. batches of medium containing 0.5% proteose peptone, 0.2% yeast extract, and 0.1% sodium acetate (Brawerman and Chargaff, 1959). After 5 or 6 days of growth, cells were harvested by centrifugation. Phase contrast microscopy was used to check for contamination of the culture and only uncontaminated ones were used for the preparation of the cell free extracts.

Reagents. Dithioerythritol, CoA, ATP, NADH, and NADPH were purchased from Sigma Chemical Co., St. Louis, Mo. [1-^{14}C]Decanoic acid (specific activity 16.4 mCi/mmole), [1-^{14}C]dodecanoic acid (specific activity 21.0 mCi/mmole), [1-^{14}C]palmitic acid (specific activity 55.2 mCi/mmole), and [1-^{14}C]stearic acid (specific activity 48.4 mCi/mmole) were purchased from Amersham/Searle, Des Plaines, Ill. [1-^{14}C]Palmitaldehyde was synthesized from [1-^{14}C]palmitic acid by the Rosenmund reduction (White *et al.*, 1967). [1-^{14}C]Palmitic acid (0.5 mCi, 20 mCi/mole) was refluxed with 1 ml of thionyl chloride for 1 hr and then the excess reagent was evaporated off under reduced pressure. The resulting labeled palmitoyl chloride was dissolved in 1 ml of acetone freshly distilled from Drierite. The reaction vessel consisted of a 50-ml erlenmeyer flask tightly closed with a serum cap. The flask contained 200 mg of 5% Pd on $BaSO_4$ suspended in 10 ml of acetone. Hypodermic needles served as inlet and outlet for hydrogen gas bubbled through the reaction mixture which was being stirred with a magnetic stirrer. After thus activating the catalyst for 30 min the labeled palmitoyl chloride solution was injected into the reaction vessel. After bubbling hydrogen through the reaction mixture for 30 min water was added and the crude aldehyde extracted with chloroform. The labeled aldehyde was purified by preparative thin-layer chromatography on silica gel G plates with hexane–ether–formic acid (40:10:1) as the developing solvent. Radiochemical yield of pure aldehyde was better than 80% when all operations were done with as little exposure to air as possible. The substrates were dispersed in appropriate buffer with the aid of Tween 20 as described earlier (Kolattukudy, 1967a).

Chromatography. Silica gel G plates were prepared, activated, and developed in lined tanks as described before (Kolattukudy, 1965). Gas–liquid chromatography was performed with a Perkin-Elmer 811 gas chromatograph equipped with a flame ionization detector and effluent splitter. About 85% of the effluent was continuously passed through a Barber–Colman radioactivity monitor. The fatty acids as methyl esters and fatty alcohols as such were chromatographed on 6 ft × 0.25 in. o.d. coiled copper columns packed with 15% stabilized diethylene glycol succinate on 60–70 mesh Anakrom A B (Analabs, North Haven, Conn.). Column temperature was 190° and flow rate of carrier gas argon was 90 cc/min.

Determination of Radioactivity. All samples were assayed for ^{14}C by a liquid scintillation counter as described earlier (Kolattukudy, 1965). Internal standards were used to determine the efficiency which was usually 62% and all assays were done with a standard deviation not greater than 1%.

Experiments with Whole Cells. Washed cells were suspended in 0.1 M potassium phosphate buffer containing 10^{-3} M magnesium chloride. The number of cells was determined by blood cell counter. The desired amount of cells was transferred to test tubes or Warburg flasks containing the substrate and incubated at 30° with shaking either under 1500-ft candles of light or darkness. At the end of the incubation period the total lipids were extracted with a 2:1 mixture of chloroform and methanol (Folch *et al.*, 1957). The chloroform solution of the lipids was evaporated to dryness under nitrogen and dissolved in 0.5 ml of chloroform and aliquots were analyzed by thin-layer chromatography often after dilution with unlabeled pure lipids. The ^{14}C in the various lipids on the plate was determined as described before (Kolattukudy, 1965).

The wax ester fraction was isolated by preparative thin-layer chromatography and then it was refluxed with excess of 14% BF_3 in methanol for 2 hr. After diluting the reaction mixture with water, the products were extracted with chloroform, the solvent was removed under nitrogen, and the wax alcohol and methyl ester fractions were isolated by thin-layer chromatography on silica gel G with hexane–ethyl ether–formic acid (40:10:1) as the solvent system (Kolattukudy, 1967b). Each fraction was then subjected to radio gas–liquid chromatography.

The polar lipid fraction isolated by thin-layer chromatography was also treated with BF_3–methanol and methyl esters were isolated and analyzed as described above for wax ester fraction.

Preparation of the Cell-Free Extract. Cells were washed with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.005 M mercaptoethanol and 0.001 M magnesium chloride. The washed cells from one batch were suspended in 20 ml of the washing medium and subjected to sonication (with a Biosonic II, at 90% power), 6 × 10 sec with a minute's cooling time between each treatment. Microscopic examination showed that most cells were broken by this treatment. Whole cells and debris were removed by centrifugation at 500*g* for 5 min and then the supernatant was centrifuged at 15,000*g* for 20 min. This supernatant was then centrifuged at 100,000*g* for 60 min.

Fractionation. To the 15,000*g* or 100,000*g* supernatant (100 ml with about 10 mg of protein/ml) 7.1 g of crystalline ammonium sulfate was slowly added with stirring. After stirring for an additional 30 to 40 min the precipitate was collected by centrifugation. This treatment was repeated four additional times and the precipitate collected each time by centrifugation. All protein fractions were dissolved in 5 ml each of 0.1 M potassium phosphate buffer containing 10^{-3} M magnesium chloride and 5×10^{-3} M mercaptoethanol and aliquots were assayed for fatty acid reduction as described in the following section. Assay for protein was done by the Biuret reaction (Gornall *et al.*, 1949). All operations from harvesting the cells to the enzyme assays were all done at about 4°.

Assay for Fatty Acid Reducing Activity. The reaction mixture containing usually 1–5 mg of protein, 0.5 mg of coenzyme A, 1 mg of NADH, 3 mg of ATP, and 200 mμmoles (1.2×10^6 cpm) of labeled fatty acid in a total volume of 2 ml of 0.1 M phosphate buffer or citrate–phosphate buffer (pH 6.5) was incubated at 30° with shaking for 15 to 60 min. The buffer usually contained 10^{-3} M magnesium chloride and 5×10^{-3} M mercaptoethanol or dithioerythritol. At the end of the incubation period 10- to 50-fold excess of a 2:1 mixture of chloroform and methanol was added and lipids were extracted as described by Folch *et al.* (1957).

The lipids were then refluxed with 10 ml of 14% BF_3 in methanol for 2 hr. This reaction mixture was diluted with water and lipids were extracted with chloroform. After evaporating the solvent off under N_2 the products were dissolved in

0.3 ml of chloroform; 50 μl of this solution was applied to 0.25-mm thick layers of silica gel G activated overnight at 110° and developed with hexane–ethyl ether–formic acid (40:10:1) in a lined tank. The lipids were located with a spray of 2′,7′-dichlorofluorescein; the silica gel from the area of primary alcohols was scraped into a counting vial and the scintillation fluid added mixed well and assayed for ^{14}C in a liquid scintillation spectrophotometer. Usually the product was diluted with unlabeled alcohol to aid in the localization of it on the thin-layer plate.

Trapping of the Aldehyde Intermediate. About 5 mg of protein was incubated with 150 mμmoles of [1-^{14}C]palmitic acid or [1-^{14}C]myristic acid, 1.25 mg of NADH, 0.5 mg of CoA, and 3 mg of ATP and varying concentrations of phenylhydrazine hydrochloride in a total volume of 2 ml of 0.1 M citrate–phosphate buffer (pH 7.0) containing 5×10^{-3} M dithioerythritol for 45 min at 30°. Then the reaction mixture was heated in a boiling water bath for 5 min followed by treatment with an excess of pyruvic acid or α-ketoglutaric acid. The regenerated aldehydes were extracted with chloroform and the solvent was removed under reduced pressure or under nitrogen. The recovered lipid was applied to 0.25-mm silica gel G thin-layer plates after diluting the product with unlabeled aldehyde. The plates were developed with benzene or hexane–ethyl ether–formic acid (40:10:1) and sprayed with 2′,7′-dichlorofluorescein and radioactivity on various regions determined as described previously (Kolattukudy, 1965).

The aldehyde fraction was isolated by thin-layer chromatography and a methanol solution of it treated with excess sodium borohydride at room temperature for 5–10 min. After diluting the reaction mixture with water the reduction products were isolated by extraction with chloroform. The solvent was removed with a stream of nitrogen and the lipids were analyzed by thin-layer chromatography with benzene or hexane–ethyl ether–formic acid (40:10:1) as the solvents.

The alcohol derived from the trapped aldehyde was subjected to gas–liquid chromatography, the effluent being continuously monitored for ^{14}C.

Aldehyde Reduction. About 6 mg of protein was mixed in a test tube with 1.5 mg of NADH and palmitaldehyde in a total volume of 2 ml of citrate–phosphate buffer containing 5×10^{-3} M dithioerythritol. Immediately the tube was stoppered with a serum cap and the gas phase replaced with nitrogen, hypodermic needles serving as inlet and outlet for the gases. The reaction mixtures were incubated at 30° for 30 min, and then the products were extracted with chloroform. After removing the solvent under reduced pressure the products were diluted with unlabeled alcohol and wax ester, and then subjected to thin-layer chromatography.

Results and Discussion

Experiments with Whole Cells. Etiolated cells readily incorporated labeled acetate (up to one-third of administered) into lipids; about 50% of the label was in wax ester fraction, the rest being in polar lipids. Saponification of the wax esters and thin-layer chromatographic analysis of the products showed that both acid and alcohol moieties of the wax ester contained approximately equal amounts of radioactivity. The major radioactive alcohols were C_{15}, C_{14}, C_{16}, and C_{13} in the order of decreasing amounts with smaller quantities of C_{17}, C_{18}, and C_{12} (Figure 1). In the same order the major

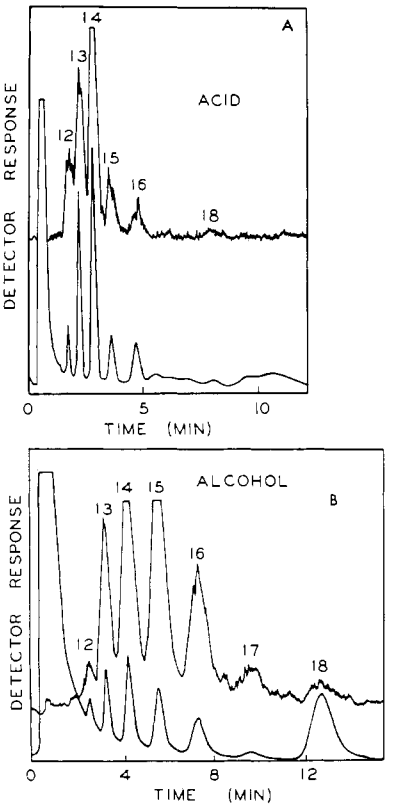

FIGURE 1: Gas–liquid chromatogram of wax acids (A) and wax alcohols (B) from etiolated *Euglena gracilis* which metabolized [1-^{14}C]acetate for 2 hr. In each case the top tracing shows the radioactivity monitor response and the bottom tracing the flame ionization detector response. The number on each peak represents the chain length. The experimental conditions are described under Materials and Methods.

radioactive acids in the wax were C_{14}, C_{13}, C_{15}, C_{16}, and C_{12} with much smaller amounts of C_{18} and C_{17} acids. Although both alcohol and acid moieties were of similar chain lengths, the relative proportions of various chains were not identical in the two moieties. The specific activities of all components of wax were similar. The major labeled fatty acids in the polar lipids were C_{16}, C_{14}, C_{15}, and C_{18} in the order of decreasing amounts with smaller amounts of C_{12} and C_{17} (Figure 2). Chemical composition of the lipids was in general agreement with that reported by others (Rosenberg, 1963; Guehler *et al.*, 1964). Exogenous palmitic acid was also incorporated into cellular lipids, the major part (about 85%) being in polar lipids. About 15% was found in wax esters.

Cells grown in the light incorporated exogenous labeled acetate into the lipids in the light most rapidly (about 32% of administered), the etiolated cells being about half as efficient (about 17% of ^{14}C fed) in incorporating exogenous acetate into lipids. When the incorporation was done in the dark, however, etiolated cells incorporated acetate into lipids at least twice as rapidly (about 10% of fed ^{14}C) as did the light grown cells. Presumably the fatty acid synthetase of light grown cells is organized to draw the energy and reduced nucleotides from photosynthetic reactions whereas in the etiolated cells fatty acid synthetase utilizes nonphotosynthetic reactions for its ATP and reducing power needs. Such preliminary

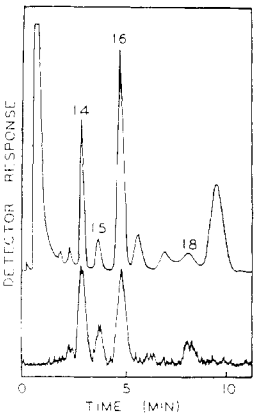

FIGURE 2: Gas–liquid chromatogram of fatty acids from polar lipids of etiolated *Euglena gracilis* which metabolized [1-^{14}C]acetate for 2 hr. Experimental conditions and description of the figure are the same as in Figure 1.

incorporation studies and thin-layer chromatographic analysis of the labeled lipids produced under various conditions indicated that etiolated cells incorporated in the dark more exogenous substrates into waxes than under the other conditions examined. Furthermore, in the etiolated cells wax esters constituted a major lipid component. Thus etiolated cells were chosen for enzyme studies on wax synthesis and in particular fatty acid reduction to the alcohol.

Preliminary Studies on Cell-Free Preparations. Sonicated preparations incorporated acetate into lipids in the presence of ATP, CoA, NADPH, and NADH, major part (92%) of this activity being in the 15,000*g* supernatant. However, the major part (about 97%) of the incorporated label was found in the polar lipids with small amounts in free fatty acids and wax ester. Even when 10–15% of the label was in

TABLE 1: Cofactor Requirements for Fatty Acid Reduction by *Euglena* Extracts.[a]

Additions	Alcohol Formed (cpm $\times 10^{-5}$)
ATP, CoA, NADH, and NADPH	2.30
None	0.09
CoA, NADH, and NADPH	0.25
ATP, NADH, and NADPH	0.30
ATP, CoA, and NADPH	0.33
ATP, CoA, and NADH	2.80
Boiled enzyme control	0.08

[a] *Euglena* extract containing about 6 mg of protein was incubated with 0.5 mg of CoA, 3 mg of ATP, 1 mg of NADH, 1 mg of NADPH, and 150 mμmoles (1.8×10^6 cpm) of [1-^{14}C]myristic acid in a total volume of 2 ml of 0.1 M phosphate buffer (pH 7.0) containing 10^{-3} M magnesium chloride and 5×10^{-3} M mercaptoethanol, for 60 min at 30°. The lipid products were isolated alcohols liberated and assayed as described in the experimental section.

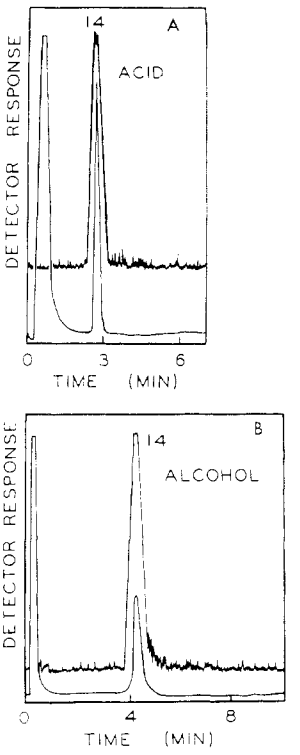

FIGURE 3: Gas–liquid chromatogram of acid (A) and alcohol (B) moieties of the wax isolated from *Euglena* extracts incubated with [1-^{14}C]myristic acid, CoA, ATP, and NADH. The top tracing shows radioactivity and bottom tracing flame ionization detector response. The samples were diluted with unlabeled methyl myristate and myristyl alcohol. Experimental conditions are described under Materials and Methods.

wax almost 90% of it was in the acid moiety of the wax. Thus, very little radioactivity could be found in the nonsaponifiable lipids, an observation consistent with the results reported by other workers (Cheniae, 1963; Bloch *et al.*, 1967). On the other hand, when [1-^{14}C]myristic acid was used as the substrate the homogenate produced labeled wax with 60% of its radioactivity in the alcohol moiety. Fractionation of the homogenate by centrifugation at 15,000*g* and 100,000*g* showed that the activity was in the 100,000*g* supernatant. Therefore the homogenate obtained by sonication was centrifuged at 100,000*g* or for convenience at about 24,000*g* and the supernatant was routinely used as the crude cell-free preparation.

Product of Reduction. Thin-layer chromatography of the lipids isolated from the reaction mixture at the end of the incubation with [1-^{14}C]myristic acid showed that the major product was wax ester with smaller amounts of ^{14}C in polar lipids. Hydrolysis showed that the acid moiety of the ester contained as much as 40% of the radioactivity contained in the wax; further analysis by gas–liquid chromatography showed that the ^{14}C in the acid moiety of the wax represented incorporation of labeled intact myristic acid molecules into the wax (Figure 3). Therefore, in order to measure fatty acid reduction the alcohol moiety of the wax had to be separated and this was routinely done by transesterification with BF_3–methanol reagent. Since aldehydes did not contain significant radioactivity only the alcohol had to be isolated. In order to

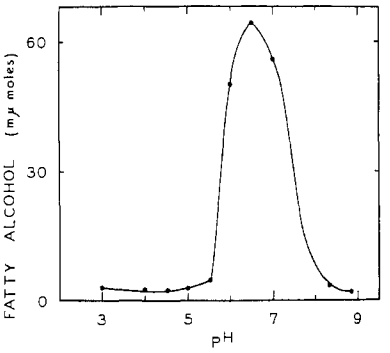

FIGURE 4: Effect of pH on fatty acid reduction by *Euglena* extracts. About 6 mg of protein, 1 mg of NADH, 0.5 mg of CoA, 3 mg of ATP, and 200 mμmoles of [^{14}C]myristic acid in a total of 2.6 ml of 0.1 M citrate–phosphate buffer up to pH 7.0, 0.1 M pyrophosphate buffer (pH 8.3), or 0.1 M borate buffer (pH 8.8), all containing 5×10^{-3} M dithioerythritol were incubated at 30° for 20 min. Isolation and assay of myristyl alcohol are described under Materials and Methods.

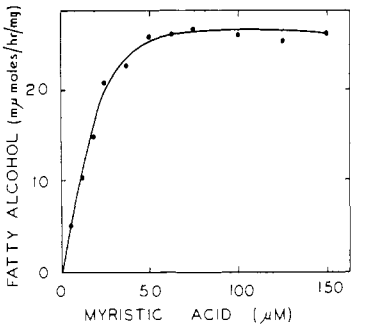

FIGURE 5: Effect of myristic acid concentration on the rate of its reduction by *Euglena* extracts. About 4 mg of protein, 0.5 mg of CoA, 3 mg of ATP, 1 mg of NADH, and various amounts of [1-^{14}C]myristic acid shown in the figure in a total volume of 2 ml of citrate–phosphate buffer (pH 6.5) containing 5×10^{-3} M dithioerythritol were incubated for 15 min at 30°. Isolation and assay of the product are described under Materials and Methods.

show that the ^{14}C in the alcohol represented a simple reduction of the acid and not extensive degradation and resynthesis, the labeled alcohol isolated from the wax was subjected to gas–liquid chromatography (Figure 3). Since only labeled C_{14} alcohol was formed from C_{14} acid the ^{14}C in the alcohol fraction represented only direct reduction of the acid. Higher amounts of ^{14}C in the alcohol moiety as compared to the acid moiety of the wax ester indicates some endogenous source of acyl moieties. Participation of much larger proportions of endogenous acyl moiety in wax formation was observed in the *Brassica oleracea* system (Kolattukudy, 1967a).

Cofactors. From the data shown in Table I it is clear that reduction of fatty acids to alcohols required ATP, CoA, and NADH, showing that the actual substrate for the reductase was most probably fatty acyl-CoA. Therefore, this system is mechanistically similar to the sphingosine synthesis (Brady and Koval, 1958) and the hydroxymethylglutarate reductase (Knappe *et al.*, 1959; Ferguson *et al.*, 1959) except for the aldehyde intermediate discussed later. Reductions of the thioesterified carboxyl groups in yeast and brain required specifically NADPH. But the fatty acyl-CoA reduction in *E. gracilis* is specific for NADH (Table I). Preliminary studies on a fatty acyl-CoA reductase from *B. oleracea* showed that it also preferred NADH (Kolattukudy, 1969b).

Fractionation of the Cell-Free Preparation. Preliminary fractionation showed that fatty acid reducing activity could be precipitated by 50% saturation with ammonium sulfate. Stepwise increase in ammonium sulfate concentration showed that about 80% of the activity was contained in the protein precipitated at 20–40% saturation with ammonium sulfate. Gel filtration by a 40 × 2 cm column of Sephadex G-100 showed that the fatty acid reducing activity was contained in the protein front, associated with the largest proteins in the extract. However, since the activity could not be easily resolved into acyl-CoA reductase and aldehyde reductase, unlike the *B. oleracea* enzyme system (Kolattukudy, 1969b), further fractionation was not attempted. The protein precipitated at 20–40% saturation with ammonium sulfate was used for further studies.

Time Course and Effect of Protein Concentration. Rate of alcohol formation was linear with respect to the protein concentration at least up to 2 mg/ml. Fairly linear rates of formation of fatty alcohols were observed up to at least 30 min and in most studies 15-min incubation periods were used so that the measurements were within the linear portion of the time-course curve.

pH Dependence of Fatty Acid Reduction. There was very little reduction of fatty acids up to pH 5.5 beyond which there was sharp increase in activity (Figure 4). The enzyme activity sharply declined as the pH was raised from 7 to 9 showing a pH optimum near 6.5. Such a pH optimum may also reflect the activation step and therefore it should not be taken as the pH optimum for the reduction step itself.

Substrate Saturation. There was a linear increase in the rate of C_{14} alcohol formation as the concentration of myristic acid was increased, giving rise to a typical substrate saturation curve (Figure 5) with an apparent K_m of 1.6×10^{-5} M with respect to myristic acid. Since the substrate was dispersed with a detergent the terms, concentration and apparent K_m, should be taken with usual precautions. In any case the saturation curve shows fairly good rates at moderately low concentrations. Linear increase in the rate of reaction was

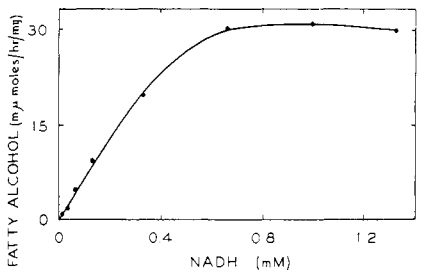

FIGURE 6: Effect of NADH concentration on the rate of fatty acid reduction by *Euglena* extracts. About 4 mg of protein, 0.5 mg of CoA, 3 mg of ATP, 150 mμmoles of [1-^{14}C]myristic acid, and the amounts of NADH shown in the figure in a total volume of 2 ml of citrate–phosphate buffer (pH 6.5) containing 5×10^{-3} M dithioerythritol were incubated at 30° for 15 min. The labeled myristyl alcohol formed was determined as described under Materials and Methods.

TABLE II: Sedimentation Coefficients, Intrinsic Viscosities, and Molecular Weights of IgG and Its Fragments at Neutral and Acidic pH.

Protein	Solvent (M)	pH	$s^0_{20,w}$ (S)	$[\eta]^a$	Mol Wt^b
Partially reduced and alkylated normal IgG	NaCl (0.1)–Na-phosphate (0.01)	7.0	6.70	0.083	170,000
Partially reduced and alkylated normal IgG	HCl (0.01)	2.2	2.42^c	0.210	58,000
Fab(t) fragment	NaCl (0.1)–Na-Phosphate (0.01)	7.0	3.64	0.048	51,000
Fab(t) fragment	HCl (0.01)	2.2	1.98^c	0.120	33,000
Fc(t) fragment	NaCl (0.1)–Na-phosphate (0.01)	7.0	3.61	0.050	51,000
Fc(t) fragment	HCl (0.01)	2.2	1.46	0.198	26,000
Fc(t) fragment	HCl (0.01)–NaCl (0.1)	2.2	2.3,^{d} 6.5	0.097	

^{a} Expressed in 100 ml/g. ^{b} Calculated by the equation of Scheraga and Mandelkern (1953). ^{c} The peak was skewed with fast-sedimenting components. ^{d} Two peaks of similar size were observed.

5–7). The change in negative ellipticity at 200 mμ of the Fab(t) fragment in acid was of the same order of magnitude as the changes in reduced and alkylated normal IgG and myeloma IgG. The Fc(t) fragment showed the highest degree of disorganization of the proteins examined.

In the near ultraviolet region, the circular dichroism bands of all the proteins were reduced significantly by the acid and they were only partially recovered if the measurements were made 30–60 min after neutralization. The rate of diminution and recovery of these bands is likely to be parallel to the change at 202 mμ. The renaturation of all the proteins occurred in almost the same way as of the Fc(t) fragment. Normal IgG and myeloma IgG in which the disulfide bonds were intact precipitated by reneutralization; but the supernatant of the precipitate showed renaturation.

The general circular dichroism patterns observed in acid denaturation of IgG and its fragments, *i.e.*, the shift of the positive band at 202 mμ to a negative band at 200 mμ and relatively little change in the 217-mμ band, were very similar for all of the proteins.

Hydrodynamic Properties of Normal IgG and Its Fragments. The sedimentation coefficients, intrinsic viscosities, and molecular weights of normal IgG and its fragments are listed

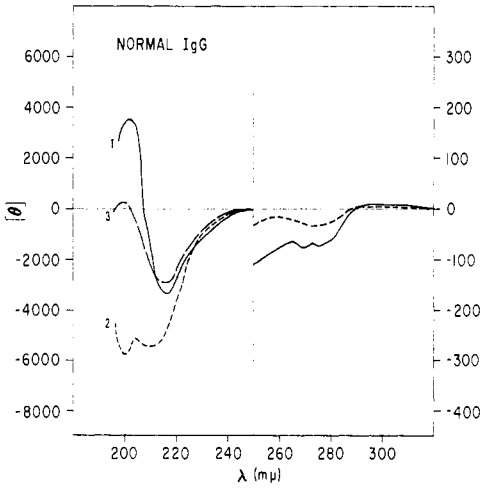

FIGURE 5: The circular dichroism spectra of normal IgG in the far and near ultraviolet. The native state (—, 1) is compared with the pattern in acid (---, 2) and after neutralization of the acid (— — —, 3). The initial neutral solutions contained 0.4–0.6 mg/ml protein in 3 ml of 0.02 M NaCl. The pH of the solutions was adjusted to 2.2 by 0.1 N HCl. The reneutralization was accomplished by adding 100 μl of 0.1 M Na-phosphate, (pH 8.0), and 0.1 N NaOH. The circular dichroism spectra were taken approximately 30 min after preparation of the solutions. The near ultraviolet spectra were measured at 0.5- or 1.0-cm optical path length and the far ultraviolet spectra were recorded at 0.05-cm optical path. Since IgG was precipitated after neutralization, the supernatant solutions were used; the optical path in the deep ultraviolet of these very diluted solutions was 1.0 or 0.5 cm.

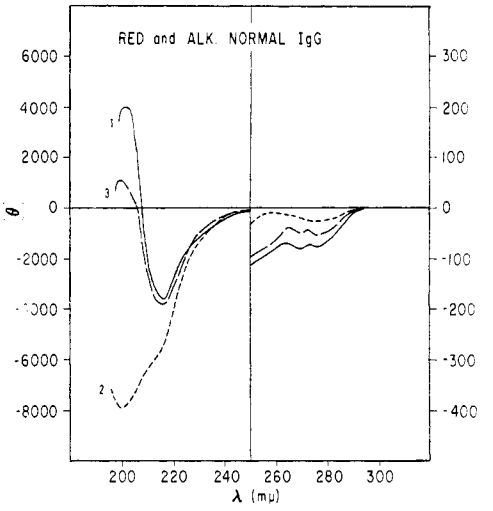

FIGURE 6: Circular dichroism spectra of reduced and alkylated normal IgG in the far and near ultraviolet zones. Designation of the curves and conditions of experiment are the same as in Figure 5.

TABLE III: Reduction of [1-^{14}C]Palmitaldehyde by a *Euglena* Enzyme Preparation.[a]

Expt	Addition	Product (cpm × 10^{-5}) Free Alcohol	Wax
1	None	0.23	0.09
	NADH	8.70	1.13
	Boiled control	0.20	0.10
2	None	0.26	0.05
	NADPH	9.60	0.37
	NADH	12.3	0.75
	NADH + myristic acid	12.6	0.68
	NADH + myristic acid + ATP + CoA	3.1	10.4[b]

[a] In expt 1, 4 mg of protein was incubated with 3.5 × 10^6 cpm [1-^{14}C]palmitaldehyde and, where indicated, 1.5 mg of NADH in a total volume of 2 ml of citrate–phosphate buffer (pH 6.0) containing 5 × 10^{-3} M dithioerythritol, at 30° for 30 min in an atmosphere of nitrogen. In expt 2, 6 mg of protein was incubated with 2 × 10^6 cpm of [1-^{14}C]palmitaldehyde and, where indicated, 1.5 mg of NADH or NADPH, 200 mμmoles of myristic acid, 3 mg of ATP, and 0.5 mg of CoA in a total volume of 2 ml of 0.1 M citrate phosphate buffer (pH 6.0) containing 5 × 10^{-3} M dithioerythritol at 30° for 10 min. Then the lipids were extracted and analyzed by thin-layer chromatography as described in the experimental section. [b] The wax ester on transesterification with BF_3–methanol reagent for 3 hr gave alcohols and methyl esters. Thin-layer chromatography showed that 98% of the radioactivity of the wax eser was in the alcohol.

showed no significant amounts of aldehyde or free alcohol (Figure 8). The aldehyde intermediate must have undergone rapid reduction to the alcohol which must have been esterified as soon as it was formed. Although inclusion of semicarbazide or phenylhydrazine in the reaction mixture caused some inhibition of fatty acid reduction, some aldehyde intermediate was trapped by these carbonyl reagents. Regeneration of the aldehyde by an exchange reaction with α-ketoglutaric acid or pyruvic acid gave a radioactive product with an R_f corresponding to aldehydes in 2 solvent systems, benzene or hexane–ethyl ether–formic acid (40:10:1) (Figure 9). This product, isolated by means of thin-layer chromatography, when treated with sodium borohydride gave a product with an R_f identical with that of hexadecanol in the same two solvent systems mentioned above. The labeled alcohol thus obtained, when subjected to gas–liquid chromatography on a diethylene glycol succinate column, showed a retention time identical with that of hexadecanol, the sole radioactivity peak coinciding exactly with the mass peak of the coinjected unlabeled hexadecanol (Figure 9). Thus the trapped aldehyde is the direct reduction product of the substrate C_{16} acid. As the concentration of phenylhydrazine in the reaction mixture increased from 0.1 mM to 5 mM the relative proportion of

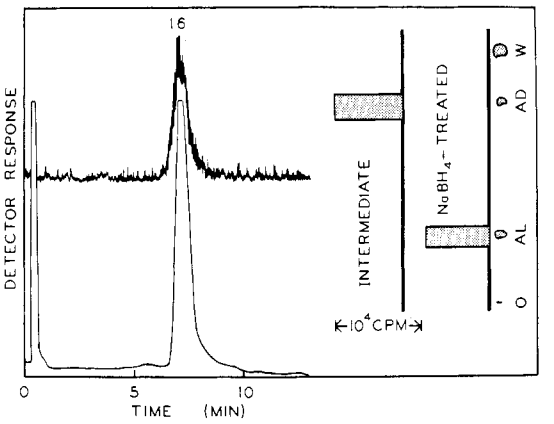

FIGURE 9: The thin-layer chromatograms (right) of the aldehyde isolated from an incubation mixture shown in Figure 8 and its $NaBH_4$ reduction product. Chromatography on 0.25 mm silica gel G with benzene as the solvent system. Bars show radioactivity and the spots show positions of unlabeled palmitaldehyde (AD), cetyl alcohol (AL), and cetyl palmitate (W) which were added before chromatography. Gas–liquid chromatogram (left) of the $NaBH_4$ reduction product on 15% diethylene glycol succinate column operated at 190° with a carrier gas flow of 90 cc/min. The top tracing shows radioactivity and the bottom tracing, the flame ionization detector response for the coinjected cetyl alcohol.

^{14}C in the trapped aldehyde as compared with that in the wax ester increased (Figure 8).

The trapping of the radioactive intermediate did not occur when the reaction mixture did not contain NADH (Figure 8) or when a usual reaction mixture was boiled after a 40-min incubation at 30° and then incubated for 30 min with 1 mM phenylhydrazine hydrochloride. With such control experiments and the evidence to show that the trapped material was aldehyde corresponding to the substrate acid it appears that the reduction of fatty acyl-CoA to the alcohol did proceed *via* the aldehyde.

If aldehyde is an intermediate in the alcohol synthesis, the enzyme system may be expected to catalyze the reduction of exogenous aldehydes to the alcohol using NADH as the reductant. Results in Table III show that synthetic palmitaldehyde was in fact reduced to the alcohol with a requirement for NADH, boiled enzyme control showing no activity. The small amounts of ^{14}C in the wax ester fraction probably represent esterification of some labeled alcohol with a little endogenous pool of acyl moiety which was already suggested to occur in this system from the uneven ^{14}C distribution in the acid and alcohol moieties of the wax. Aldehyde intermediate could not be trapped and the exogenous aldehyde underwent little reduction in the case of hydroxymethylglutaryl-CoA reductase (Knappe *et al.*, 1959; Ferguson *et al.*, 1959). In the case of palmitoyl-CoA reduction observed in the brain system in connection with sphingosine synthesis, aldehyde was suggested to be the intermediate and exogenous aldehyde did apparently substitute for the endogenous intermediate (Brady and Koval, 1958; Brady *et al.*, 1958). In the *Euglena* system aldehyde intermediate could be trapped and exogenous aldehyde did undergo reduction, suggesting an aldehyde intermediate. The reduction of acyl-CoA required specifically NADH but reduction of exogenous aldehyde did not show such an absolute specificity. These results may mean

that the first step of the reduction process is specific for NADH while the second is not. However, the enzyme used could also have contained an aldehyde reductase similar to the mevaldate reductase (Schlesinger and Coon, 1961; Donninger and Popjak, 1966) besides the enzyme that reduced acyl-CoA to the alcohol. A fatty aldehyde reductase has been partially purified from *B. oleracea* (Kolattukudy, 1969b).

Exogenous fatty acid was readily converted into alcohol whereas the fatty acids formed from labeled acetate were converted mostly into polar lipids. One possible explanation is that the former is activated to the CoA derivative which is the substrate for the reductase, whereas the latter, being probably acyl carrier protein-bound (Bloch *et al.*, 1967), underwent esterification into polar lipids without involving the CoA derivative (Ailhaud and Vagelos, 1967).

The results presented in this paper show that fatty acyl-CoA is converted to fatty alcohol in a two-step reduction involving aldehyde as intermediate. The specificity studies indicate that the enzyme studied here is probably involved in the wax synthesis observed in etiolated *Euglena*. In the case of alcohols containing odd numbers of carbon atoms α-oxidation is likely to be involved. The α-oxidation system may provide the aldehyde (Stumpf, 1956) for further reduction to alcohol or it may lead to the formation of odd chain acids which may be reduced to alcohol by the enzyme system described here.

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