In Vivo and In Vitro Biosynthesis of Free Fatty Alcohols in *Escherichia Coli* K-12

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ABSTRACT

In vivo studies have indicated that exogenous free fatty acids may serve as precursors of the free fatty alcohols of *Escherichia coli* K-12. Following disruption of the cells, the enzymatic activity capable of catalyzing the reduction of long chain fatty aldehydes to fatty alcohols was localized in the 100,000 x g supernatant fraction. Nicotinamide adenine dinucleotide phosphate, reduced form, was the required cofactor. The product of the reaction was characterized rigorously as 1-hexadecanol when hexadecanal was the substrate. Three independent, but complementary, assay methods were developed to assay the aldehyde reductase activity. By employing these methods, an equivalence between nicotinamide adenine dinucleotide phosphate, reduced form, oxidation and 1-hexadecanol synthesis was established. Two protein fractions catalyzing the reduction of fatty aldehydes to fatty alcohols were detected in the 100,000 x g supernatant fraction following ammonium sulfate fractionation and diethylaminoethyl-cellulose chromatography. Enzymatic activity (70%) applied to the diethylaminoethyl-cellulose column was eluted at a phosphate concentration of 0.115 M. The remaining 30% was eluted at a concentration of 0.23 M. Following sephadex chromatography, it was observed that the enzyme eluting at 0.115 M phosphate had an apparent mol wt of 250,000 Daltons while that eluting at 0.23 M had an apparent mol wt of 62,000 Daltons. The enzymes were similar with respect to substrate specificity, pH optima, ionic strength optima, and stability with respect to thiol inhibitors, suggesting different sized aggregates of similar subunits.

INTRODUCTION

Long chain, nonisoprenoid fatty alcohols have been characterized in bacteria (1-5) and their biosynthesis studied (1, 5-13). However, the biosynthesis of the fatty alcohols in *Escherichia coli* has not been investigated.

This article provides information concerning the partial purification and characterization of an nicotinamide adenine dinucleotide phosphate, reduced form, (NADPH) linked aldehyde reductase from *E. coli* catalyzing the synthesis of long chain fatty alcohols from free fatty aldehydes. Evidence is presented to establish that this enzyme is not the alcohol dehydrogenase previously reported in this microorganism (14).

METHODS

Materials: [1-C^{14}] Hexadecanoyl CoA and [1-C^{14}] hexadecanoic acid were purchased from New England Nuclear, Boston, Mass. [1-C^{14}] Hexadecanal was synthesized from [1-C^{14}] hexadecanoic acid (15) and the specific activity adjusted with unlabeled hexadecanal. Gas liquid chromatography (GLC) analysis of the product purified by thin layer chromatography (TLC) (5) showed the major component was hexadecanal, greater than 95% total, with small amounts of octadecanal. All of the unlabeled aldehyde substrates, except acetaldehyde and butyraldehyde, were synthesized from the corresponding alcohol (16) and purified by TLC. GLC analysis of the unlabeled aldehydes showed that 95-100% total was one chain length. Acetaldehyde and butyraldehyde were generated from ethanol and butanol using yeast alcohol dehydrogenase (17). This reaction was carried out for 10 min at 23 C. At the end of this time period, an aliquot was removed to measure NADH absorption at 340 nm, and then the reaction mixture was adjusted to pH 5. This latter step served to stop the reaction and also prevented the yeast alcohol dehydrogenase from reoxidizing any ethanol or butanol which might be generated by the reductase studied here. After the pH 5 precipitation, the reaction mixture was kept at 4 C, and 0.2 ml aliquots from this solution were used immediately as substrates. [1-C^{14}] Hexadecanol was synthesized by $LiAlH_4$ reduction of [1-C^{14}] hexadecanoic acid. The [1-C^{14}] hexadecanol was purified by TLC. GLC analysis of this compound showed no other contaminants.

The pyridine nucleotides used were purchased from Sigma Chemical Co., St. Louis, Mo. All solvents were the highest grade com-

mercially available and, with the exception of methanol, were redistilled prior to use.

Bacteria: *E. coli* K-12 were grown on trypticase soy broth (TSB), as described in previous publications (5). For experiments measuring incorporation of [1-C^{14}] hexadecanoic acid, the acid was solubilized with albumin (18), sterilized by ultrafiltration, and 5.5 μmoles (1.5 x 10^6 dpm) added to each culture flask immediately before inoculation.

Lipid extraction, purification, and analysis: Lipids were isolated, purified, and analyzed, as described previously (5). In this study, fatty alcohols were quantitated via GLC (19). A linear relationship was observed between the amount of material injected into the gas chromatograph and the detector response over a range of 0.25-1.0 nmoles 1-hexadecanol. Peak areas were measured by triangulation. When 0.25 nmoles hexadecanol were injected, the average peak area observed was 99.2-100.4% individual peak areas obtained in 5 separate injections. The sensitivity of the gas chromatograph was such that 1 nmole hexadecanol corresponded to a full scale deflection on a 1 millivolt recorder.

When stream splitting was carried out on the gas chromatograph, the split ratio of the detector to splitter port was 1:3 as determined with [1-C^{14}] hexadecanol.

Preparation of cell free system: All procedures were carried out between 0-4 C. *E. coli* cells were suspended in 0.075 M potassium phosphate buffer (pH 7.4) containing 5 mM mercaptoethanol; cells:buffer = 1:4 (w/v). The cells were sonicated for fifteen 2 min periods with 3 min cooling intervals and the sonicate centrifuged at 12,000 x g for 10 min to remove unbroken cells and debris. The particulate fraction was sedimented from the 12,000 x g supernatant using a Beckman L2-65B ultracentrifuge at 100,000 x g for 60 min. The protein concentration of the supernatant was 19-20 mg/ml. Throughout this article, the 12,000 x g supernatant will be referred to as the crude sonicate, the 100,000 x g supernatant as supernatant, and the 100,000 x g particulate as particulate.

The supernatant was adjusted to a protein concentration of 12-13 mg/ml with 0.075 M potassium phosphate buffer containing 5 mM mercaptoethanol. Ammonium sulfate was added to give 55% saturation, and, after standing 15 min, the precipitated protein was sedimented at 12,000 x g for 20 min. The pellet was redissolved in 0.01 M potassium phosphate buffer containing 5 mM mercaptoethanol and stored at -20 C. The activity remained stable for a period of 3 months.

Enzyme assays: Three independent methods were developed for assaying the reduction of hexadecanol to 1-hexadecanol. Methods A and B are based upon measuring the formation of 1-hexadecanol, and method C involves spectrophotometric determination of NADPH utilization. Identical assay conditions were used for all three methods, and, unless otherwise indicated, the concentration of assay components in a final volume of 2.0 ml were: 50 mM potassium phosphate buffer (pH 7.4), 0.125 mM NADPH, 0.100 mM hexadecanal dispersed in Tween 20 (final concentration = 1 μg/ml) and an appropriate amount of protein. The reactions were carried out in 15 ml glass-stoppered conical tubes or 3.0 ml glass cuvettes for 8 min at 37 C.

Method A: In this method, 1-hexadecanol formation was measured by determining the radioactivity incorporated into the fatty alcohols from the labeled substrates. The assays were carried out as described and the incubation mixture extracted according to the method of Bligh and Dyer (20). Carrier 1-hexadecanol had been added to the extracting solvent, such that ca. 0.5 μmole was added to each assay at the time of extraction. The extracted lipid was concentrated under N_2 in a 30 ml centrifuge tube and chromatographed by TLC using two solvent systems (I and II) to purify the 1-hexadecanol. I. Hexane:Chloroform:Methanol (73:25:2) II. Hexane:Ethyl Ether:Acetic Acid (30:70:1).

The purified 1-hexadecanol was dissolved in 0.3 ml n-heptane; 0.1 ml was radioassayed and 1 μliter analyzed by quantitative GLC to determine carrier recovery. Carrier recovery was estimated from the ratio of the peak area of 1-hexadecanol in the sample to the peak area of the 1-hexadecanol originally present in the extracting solvent. The latter area was determined by adding the same amount of carrier hexadecanol to the same volume of solvent as used in the assay tube, concentrating and examining the lipid residue in exactly the same manner as the lipid from the assay. In this instance, the peak area of 1-hexadecanol was considered to represent 100% recovery. Nmoles of 1-hexadecanol synthesized were calculated by correcting the raw cpm for carrier recovery and counting efficiency and dividing total dpm calculated by the specific activity of the substrate.

Method B: This method measured 1-hexadecanol formation directly, i.e. without carrier addition, by GLC. The technique involved extracting the assay mixture as before, except 20 nmoles 2-octadecanol were added as an internal standard to the extracting solvent. The

lipid residue was dissolved in 30 μliter $CHCl_3$, immediately transferred to a capillary tube to prevent evaporation and 1 μliter analyzed by GLC. The amount of 1-hexadecanol formed was determined by triangulation of the peak area generated by 1-hexadecanol and then relating this value to a calibration curve. Losses incurred upon extracting were estimated by measuring the recovery of 2-octadecanol in the same way as 1-hexadecanol recovery in method A. The value measured for the amount of 1-hexadecanol then was corrected for losses using recovery of 2-octadecanol.

For methods A and B, an assay mixture incubated under the same conditions as the sample, but in the absence of protein or NADPH, served as a control.

Method C: The third method involved following NADPH oxidation in a Gilford 2400 spectrophotometer by continuously monitoring the decrease in absorbance at 340 nmeters. Nmoles of NADPH oxidized was calculated using the observed absorbancy and an extinction coefficient for NADPH = 6.25×10^3 L/mole cm. An assay system incubated in the absence of hexadecanal served as a control.

Radioactivity measurements: The scintillation fluid used consisted of Liquifluor with toluene mixed to a concentration of 0.4% 2,5 diphenyloxazole and 0.1% 1,4 bis-2 (5'phenyloxazole) benzene. The radioactive sample was dissolved in 0.1-0.5 ml n-heptane and added to 10 ml scintillation fluid. Radioactivity was assayed with a Packard Tri Carb liquid scintillation counter, model 3320 (efficiency = 84%).

Protein determination: Protein was measured using the biuret method with crystalline bovine serum albumin as a standard or in more dilute solutions by determining the A_{280}/A_{260} (21, 22).

Diethylaminothyl (DEAE)-cellulose chromatography: DEAE cellulose (Whatman DE32, microgranular) was prepared for chromatography by treating with 0.5 N HCl, then with 0.5 N NaOH, and washed with water until the pH of the supernatant was 7.4. The cellulose was stored at 4 C with 0.001 M sodium azide until needed. Just prior to column chromatography, CO_2 was removed from the cellulose suspension by vacuum, and the cellulose poured into a column 2.5 x 80 cm. The packed column was washed with 0.01 M phosphate buffer (pH = 7.4) containing 5 mM mercaptoethanol until the pH of the effluent was 7.4. The phosphate content of the eluate from this column was determined colorimetrically (23).

Sephadex chromatography: Sephadex G-200 was equilibrated with 0.075 M potassium phosphate buffer (pH = 7.4 and 5 mM mercaptoethanol) in a column 2.6 x 80 cm. D-1 enzyme (4 Dml) (9 mg/ml protein) was layered onto the G-200 bed as a 10% sucrose solution. Elution was carried out with the above buffer at a pressure of 10 cm. Fractions (5 ml) were collected and assayed spectrophotometrically for enzyme activity. Protein was assayed in each fraction by determining the optical density at 280 nmeters. The void volume of the column was 120 ml, as determined with Blue Dextran-2000. The column was calibrated by measuring the elution volume of aldolase (158,000), ovalbumin (45,000), chymotrypsinogen (25,000), and ribonuclease (13,700). A plot of log M.W. vs. V_e/V_o, where V_e = elution volume of protein and V_o = void volume, was constructed. A separate column, 2.5 x 40 cm, containing sephadex G-100 was prepared and calibrated in a similar manner. The void volume of this column was 72 ml, as determined by aldolase. The enzyme mol wt was determined using the specific calibration curve and the observed V_e of the enzyme. D-2 enzyme (4.0 ml) (4 mg/ml protein) was layered onto the column bed as a 10% sucrose solution and the column developed as before. Fractions (2.5 ml) of the effluent were collected and assayed for enzyme activity and protein content as before.

RESULTS

In vivo incorporation of fatty acids into E. coli lipids: The lipids extracted from *E. coli* grown in the presence of [1-C^{14}] hexadecanoic acid were resolved by column chromatography and the column fractions radioassayed. In two separate experiments, 80-82% radiolabel, 7.3 x 10^6 dpm, initially added to the incubation medium was detected in the phospholipid fraction and 1.5-2% in the neutral lipids. Of the total radioactivity in the neutral lipid fraction, 70-76% was detected in the free fatty acids, none in the free fatty aldehydes, and 4-6% in the free fatty alcohols after purification of these lipid types by TLC. The results of the analysis indicate that exogenous fatty acid is incorporated into the intracellular free fatty acid pool and also is reduced to free fatty alcohols. While the free fatty aldehydes did not contain detectable radioactivity, they were present in trace amounts (0.142 μmoles/100 mg lipid). GLC of the free fatty acid methyl esters performed in conjunction with stream splitting indicated that all of the 7×10^3 dpm recovered were eluted as methyl hexadecanoate ruling out extensive catabolism of the fatty acid. Radiopurity of the free fatty alcohols was assessed in a similar fashion after conversion of the alcohols to the acetoxy alkanes (24). Following

TABLE I

In Vitro Synthesis of Fatty Alcohols by *Escherichia Coli* K-12

Substrate	Protein mg	Fatty alcohol dpm	Fatty alcohol synthesized nmoles
1-C^{14}-Hexadecanoic[a,c]	8.04	213	0
acid	0	200	0
1-C^{14}-Hexadecanoyl[b,c]	0.50	240	0.06
CoA	0	121	0
1-C^{14}-Hexadecanal[c]	0.43	115,600	21.3
	0	4,200	0
1-C^{14}-Hexadecanal[c]	0.43	105,100	19.5
	0	4,500	0

[a]Adenosine 5'-triphosphate (4.7 mM), coenzyme A (CoA) (1.2 mM), Mg^{+2} (1 mM), and reduced form of glutathione (3.5 mM) were included in this assay. Concentration of 1-C^{14}-hexadecanoic acid (1.5×10^6 dpm/μmole) = 4.4×10^{-4} M. Final volume of reaction mixture was 2.0 ml. Incubations were carried out at 37 C for 8 min.

[b]Concentration of 1-C^{14}-hexadecanoyl CoA (1×10^6 dpm/μmole) = 1×10^{-4}M.

[c]Both nicotinamide adenine dinucleotide, reduced form, and nicotinamide adenine dinucleotide phosphate, reduced form, were present in 1 mM concentration. Hexadecanal present in 1×10^{-4} M concnetration (specific activity = 5.2×10^6 dpm/μmole).

acetylation, all of the radioactivity initially present as the fatty alcohol migrated as the acetoxy alkane during TLC. GLC of the acetoxy alkanes and stream splitting of the effluent indicated again that all of the radioactivity injected was associated with the component eluting as 1-acetoxy hexadecane.

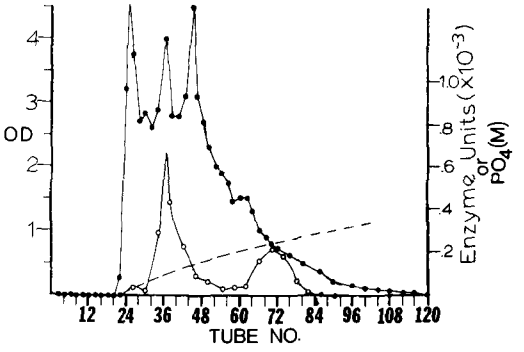

FIG. 1. Diethylaminoethyl-cellulose chromatography of the 0-55% ammonium sulfate fraction. Protein (1.5-1.6 g) in 0.01 M phosphate buffer pH 7.4, containing 5.0 mM mercaptoethanol, was applied to the column and the column washed with the same buffer until no UV absorbing material was detected in the effluent. At this point, a gradient was started with 0.075 M phosphate and 0.50 M phosphate. Both buffers were at pH 7.4 and contained 5 mM mercaptoethanol. The flow rate was 50 ml/hr; 10 ml fractions were collected. Protein concentration (—•—•—) was measured by determining the absorbance 280 nm. Phosphate content of the eluate (---) was assayed colorimetrically (23). Enzyme activity (-O O-) assessed by methods B and C in the same assay mixture. Tubes containing activity were combined; the protein was precipitated with $(NH_4)_2SO_4$; and precipitate was redissolved in 0.075 M potassium phosphate buffer (pH 7.4 and 5 mM mercaptoethanol). OD = outside diameter.

In vitro biosynthesis of free fatty alcohols: A crude sonicate of *E. coli* K-12 was prepared and assayed according to the radioassay method. The data presented in Table I show that this extract was unable to catalyze the reduction of [1-C^{14}] hexadecanoic acid or [1-C^{14}] hexadecanoyl CoA to hexadecanol but that hexadecanal served as a substrate. These data are not consistent with the findings of the in vivo experiments that indicated reduction of a fatty acid to a fatty alcohol. However, the in vitro experiments are not considered contradictory for reasons discussed later.

Product characterization: Radio purity of the radio labeled fatty alcohols isolated from the previous incubation experiments was established in two ways. First, a known amount of the radio labeled fatty alcohol was converted to the acetoxy alkane (24), and this derivative was purified by TLC using solvent system II. The lipids migrating as known 1-acetoxy hexadecane were eluted from the chromatographic plate and radioassayed. In two separate experiments, 96 and 98% radioactivity initially present as a fatty alcohol, 51 x 10^3 dpm, was recovered as the acetoxy alkane derivative. The radio purity of [1-C^{14}] hexadecanol was evaluated further by GLC. Unlabeled fatty alcohols tetra, penta, hepta, and octadecanol were added to the C^{14} labeled fatty alcohols and the column effluent stream split, collected, and radioassayed for each component. The amount of radio label in the 1-hexadecanol peak was 90% total radioactivity recovered. It was concluded,

TABLE II

Pyridine Nucleotide Requirement for Fatty Aldehyde Reductase in 0-55% $(NH_4)_2SO_4$ Fraction[a]

Component varied	Hexadecanol formed/8 min nmoles
Complete	42.6
NADPH and NADH omitted	3.0
NADPH omitted	3.5
NADH omitted	40.8
Protein omitted	1.9
Boiled protein	2.0
Heated protein	21.0

[a]Reactions were assayed according to method B. The complete assay mixture included: 0.7 mg protein, 250 nmoles each of nicotinamide adenine dinucleotide, reduced form, (NADH) and nicotinamide adenine dinucleotide phosphate, reduced form, (NADPH), 200 nmoles hexadecanal and 100 μmoles potassium phosphate buffer (pH 7.4) in a final volume of 2.0 ml. Incubations were carried out at 37 C for 8 min. The boiled protein was boiled for 5 min, and the heated protein was heated at 55 C for 5 min.

therefore, that the reduction [1-C^{14}] hexadecanal to hexadecanol did take place.

The product identified as 1-C^{14} hexadecanol was characterized further by combined GLC-mass spectrometry (MS). Mass spectra were taken on either side and center of the 1-hexadecanol peak. The fragmentation patterns were all identical to the spectra of known 1-hexadecanol (25) and contained no indications of impurities. In brief, the key fragmentations involve loss of H_2O (m/e 224) coupled with the loss of ethylene (m/e 196) and cleavage between carbon atoms 1 and 2 to give an ion at m/e 31. The hydrocarbon fragmentation is that of the corresponding olefin. This occurs because the parent molecule readily loses H_2O, generating the olefinic ion, which then fragments (25).

Effect of time and protein concentration upon rate of fatty alcohol biosynthesis: The relationship between time of incubation and nmoles of fatty alcohol synthesized was evaluated over an incubation interval varying from 1-14 min with two different assay procedures. It was observed that fatty alcohol biosynthesis was linear with respect to time of incubation over the interval studied and that similar results were obtained by either the GLC or radioassay procedure.

The relationship between the amount of supernatant protein incubated and the nmoles of fatty alcohol synthesized was evaluated using the GLC and radioassay procedures. In this instance, fatty alcohol synthesis was linear with respect to protein concentration over a range 0.05-0.30 mg/ml and no difference was observed with respect to the assay procedure employed.

Ammonium sulfate fractionation and cofactor requirements: Using method B, cofactor requirements and the effect of heating were determined for a 0-55% $(NH_4)_2SO_4$ fraction containing 80-85% activity initially present in the crude sonicate. The results from these studies are presented in Table II. The enzymatic activity had an absolute requirement for NADPH. Boiling completely destroys the enzymatic activity, while heating at 55 C for 5 min reduces the total activity, as well as protein, twofold. Thus, heating does not serve as a purification step, although some degree of heat stability is present.

Correlation of NADPH oxidation and 1-hexadecanol formation: The amount of NADPH oxidized, as measured spectrophotometrically (method C), was compared to the amount of fatty alcohol produced (method B), using the 0-55% $(NH_4)_2SO_4$ fraction as the enzyme source. The spectrophotometric assay was performed, as described in "Methods." At the end of 8 min incubation intervals, the contents of the cuvette were extracted with organic solvent, and the amount of fatty alcohol synthesized was evaluated via GLC. From the data presented in Table III, it is apparent that similar results are obtained when either NADPH oxidation or 1-hexadecanol formation is employed to assess enzyme activity.

DEAE-cellulose chromatography: Figure 1 shows the results that were obtained following DEAE-cellulose chromatography of the protein participitating at 0-55% saturation with $(NH_4)_2SO_4$. These results indicate that 95-100% activity applied to the column was recovered in two separate fractions. One protein fraction eluted with 0.115 M phosphate (D-1 enzyme) contained 70% recovered activity, the remaining 30% activity was found in a

TABLE III

Quantitative Correlation of Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form, (NADPH) Oxidation with 1-Hexadecanol Formation[a]

Experiment	Nmoles NADPH oxidized	Nmoles 1-hexadecanol formed
1	38.0	40.0
2	37.0	39.5

[a]Assay mixtures contained 0.40 mg protein, 250 μmoles NADPH, 200 nmoles hexadecanal, and 100 μmoles potassium phosphate buffer (pH 7.4) in a final volume of 2.0 ml. NADPH oxidation was monitored continuously at 340 nm; after an incubation interval of 8 min, the contents of the cuvette were extracted and the 1-hexadecanol content measured according to method B.

protein fraction that eluted with 0.23 M phosphate (D-2 enzyme). The aldehyde reductase activity in each fraction was established by measuring NADPH oxidation spectrophotometrically and 1-hexadecanol formation via GLC in the same assay mixture. The two independent assay methods were found to give essentially the same values for aldehyde reductase activity. A summary of the degree of purification of the reductase studied here is presented in Table IV.

Characterization of D-1 and D-2 enzyme fractions: The D-1 and D-2 enzyme fractions both exhibited an absolute requirement for NADPH. Nicotinamide adenine dinucleotide, reduced form, (NADH) was neither active nor inhibitory at the same concentration as NADPH.

The substrate specificity of the D-1 and D-2 enzymes was measured using aldehyde substrates of different chain lengths and degrees of unsaturation. The spectrophotometric procedure was employed to assay enzyme activity, and all substrates were present at the concentration previously established to give their optimal activity (zero order kinetics). In the case of the unsaturated aldehydes, GLC determination of alcohol formation also was employed to evaluate the possibility of double bond reduction. This effect was not observed with either enzyme fraction. Figure 2 shows that D-1 and D-2 enzymes catalyzed the reduction of a number of aldehydes, saturated and unsaturated. However, it is significant to note that acetaldehyde

TABLE IV

Summary of Purification of Fatty Aldehyde Reductase from *Escherichia coli*

Fraction	Protein mg	Activity[a]	Recovery	Specific activity[b]	Fold purification
10^5 x g Supernatant	2600	13,500	100	5.2	---
Ammonium sulfate (0-55%)	1765	13,410	99.3	7.6	1.46
Diethylaminoethyl-cellulose					
D-1	478	7887[c]	58.4	16.5	3.17 (5.4)[d]
D-2	84	3427[c]	25.4	40.8	7.84 (31.0)[c]
Sephadex G-200					
(D-1)	65	7887	58.4	121	23.0 (39.9)[d]

[a]Unit of activity = That amount of enzyme which catalyzes formation of 1 nmoles of hexadecanol or oxidation of 1 nmole of nicotinamide adenine dinucleotide phosphate, reduced form, in 1 min.

[b]Specific activity = units/mg protein.

[c]The values do not indicate the total number of units of D-1 and D-2 recovered in their respective fractions. Some tubes containing low activity were not included.

[d]Actual specific activities of D-1 and D-2 cannot be measured separately in the 100,000 x g supernatant. The value measured is the combined specific activity of enzyme(s) D-1 and D-2. The theoretical specific activities of D-1 and D-2 can be calculated by multiplying the total units of enzyme activity measured in the supernatant by the fraction that the activity observed in either D-1 or D-2 represents of the sum of the individual activities in both fractions. The resulting values will give the theoretical total units of D-1 and D-2. The theoretical specific activities then can be calculated using these values. The number in parenthesis is the fold purification calculated on the basis of the theoretical specific activity, assuming the same fractional distribution of both enzymes in the 100,000 x g supernatant.

gave no measurable reaction, nor is there any remarkable difference in the specificity of the two enzyme fractions. Separate experiments have shown that there was no enzyme activity when 100 μM 2-pentadecanone was used as the substrate for the D-1 or D-2 enzyme. It was not possible to measure the kinetic parameters of these enzymes with the various substrates or NADPH, since the reaction rate was linear for only a very short time interval at low reactant concentrations, making it difficult to measure the initial velocity accurately.

The enzymatic activity at different pHs was determined for both the D-1 and D-2 enzymes. Both enzymes were active over the pH range, 5.5-10.0. The D-1 fraction had two pH optima at 7.5 and 9.0, while the D-2 enzyme also had two pH optima at 8.0 and 9.5. Considering the enzyme preparations are still impure, a difference in pH optima of 0.5 units was not considered significant.

The effect of ionic strength of the buffer upon the aldehyde reductase activity also was investigated. There was no observed change in the activity of the D-1 or D-2 enzymes when the ionic strength was varied between 0.005 M phosphate and 0.50 M phosphate (pH = 7.4).

The activities of both aldehyde reductases were measured in the presence of thiols and thiol inhibitors. Addition of 5 mM mercaptoethanol of 5 mM glutathione to the assay mixture had no effect upon the activity of either enzyme. However, the activity of both enzyme fractions was inhibited by preincubation with mercuric ions, and this inhibition was reversed when mercaptoethanol was added to the protein heavy metal preparation (Table V). Preincubation of the enzyme fraction with NADPH or hexadecanal failed to protect against inhibition by the metal ions. Similar

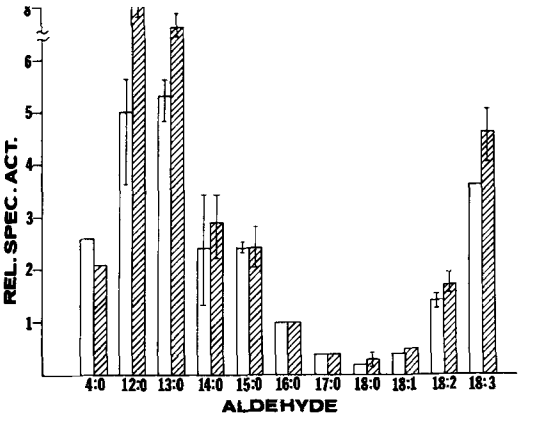

FIG. 2. Histogram depicting the relative specific activities of the D-1 and D-2 enzymes with aldehydes substrates of different chain lengths and degree of unsaturation. Enzyme activity was assessed spectrophotometrically. The range of specific activities obtained from three separate assays is indicated by the vertical bar (I). If no bars appears, then there was no variation. The shorthand notation used for the aldehyde can be interpreted as follows: number of carbon atoms = number of double bonds. The unsaturated 18 carbon aldehydes were oleyl, linoleyl, and linolenyl aldehyde. □ D-1 ▨ D-2

results were observed when the D-1 and D-2 enzyme preparations were preincubated with Zn^{+2} ions. In this instance, a 10-fold higher zinc concentration was required to achieve the same degree of inhibition.

The D-1 and D-2 protein fractions were chromatographed on Sephadex G-200 and G-100, respectively, as described in the "Methods." All of the D-1 enzyme activity applied to the Sephadex G-200 column was eluted in a volume of buffer that gave a V_e/V_o value of 1.5. After plotting this value on the calibration curve obtained by standardizing this column

TABLE V

Effect of Thiol Inhibitiors upon Activity of D-1 and D-2 Enzymes[a]

Hg^{+2} mM	D-1 NADPH oxidized nmoles	D-1 Inhibition %	D-2 NADPH oxidized nmoles	D-2 Inhibition %
0	6.7	0	10.3	0
.01	2.7	60	7.2	30
.025	0	100	0.9	91
.10	0	100	0	100
.025 + .025 mM SH	6.7	0	9.7	6

[a]Inhibitiors were preincubated for 15 min at 37 C with 0.25 mg D-1 enzyme or 0.27 mg D-2 enzyme in the concentrations given in the table. The inhibition of the enzyme activity was reversed by incubating an aliquot of the inhibited protein with mercaptoethanol for 15 min at 37 C. 0.1 M Tris Buffer was used in this experiment because the heavy metals formed insoluble salts with phosphate. The assays were carried out in glass cuvettes for 8 min at 37 C, and nicotinamide adenine dinucleotide phosphate, reduced form, (NADPH) oxidation was measured spectrophotmetrically (3). Each assay mixture contained 0.25 mg D-1 enzyme or .27 mg D-2 enzyme, 200 nmoles hexadecanal, 125 nmoles NADPH, and 200 μmoles Tris Buffer (pH 7.4) in a final volume of 2.0 ml. The control sample included all of the components except hexadecanal.

TABLE VI

Oxidation of Fatty Alcohol Substrates Using the 1 x 10^5 xg Supernatant Fraction[a]

Protein mg	dpm	Corrected dpm	Hexadecanoic acid/8 min nmoles
0	766	0	0
.2	4025	3259	0.34
.4	7322	6556	0.68

[a]The reactions were carried out for 15 min at 37 C. Activity was assessed using method A. The assay mixtures included: 1 μ oxidized form and nicotinamide adenine dinucleotide, 100 nmoles 1-C^{14}-hexadecanol (9.6 x 10^6 dpm/μmole) and 100 μmoles potassium phosphate buffer (pH 7.4). Fatty acids were isolated by thin layer chromatography.

with ribonuclease, chymotrypsin, ovalbumin, and alsolase, a mol wt of 250,000 was calculated for the D-1 protein.

The D-2 enzyme activity was eluted from the Sephadex G-100 column in a volume of buffer that gave a V_e/V_o value of 1.25. Using the calibration curve established for this column, a mol wt of 62,000 was calculated for the D-1 protein.

It should be noted here that only 25% aldehyde reductase activity applied to the G-100 column was detected in the active fraction. The lost activity could not be restored upon recombination of any of the column fractions. Furthermore, this loss in activity was not due to mechanical or absorptive losses of the protein since 95% protein applied to the G-100 column was recovered in the effluent, as judged by 280/260 ratios (21). It was concluded from these results that the D-2 enzyme was unstable upon gel filtration.

Reversibility of reaction: Method A was used to investigate the oxidation of 1-hexadecanol to hexadecanal and hexadecanoic acid by the supernatant fraction. The assay mixture contained 1 mM NADP and 1 mM nicotinamide adenine dinucleotide (NAD) and 1.0 mM [1-C^{14}] hexadecanol $\frac{9.6 \times 10^6 \text{ dpm}}{(\mu\text{moles})}$ and 0.2 or 0.4 mg protein in a final volume of 2.0 ml. Samples were incubated for 8 min at 37 C and extracted, as described previously. Carrier hexadecanal, hexadecanoic acid, and hexadecyloctadecanoate were added to the extract and these moieties isolated by TLC and radioassayed. Radioactivity was found in only the hexadecanoic acid (Table VI).

DISCUSSION

In vivo studies reported here indicate that free fatty acids and alcohols are metabolically interconvertible. Free fatty aldehydes, while present in trace amounts in *E. coli*, were not radio labeled, when either [1-C^{14}] palmitate or [1-C^{14}] cetyl alcohol were substrates. This finding contrasts to studies with *Clostridium butyricum* and *M. tuberculosis* in which the free fatty aldehyde was a transient intermediate (12, 13). However, the inability to isolate radio labeled fatty aldehyde is not sufficient evidence to exclude the aldehyde as an intermediate in this reaction since it may have remained bound to the enzyme complex in vivo. In other in vitro enzymatic systems catalyzing the reduction of fatty acids to fatty alcohols, the aldehyde intermediate could not be isolated, unless it was trapped as the hydrazone (26). When similar in vitro trapping studies were attempted at the pH of the incubation medium (pH 7.4), the hydrazone did not form with fatty aldehyde concentrations less than 5 nmoles/ml. With the small amount of endogenous fatty aldehyde present here, such a trapping experiment was unsuccessful. In separate in vitro experiments, unlabeled octadecanal was incorporated into the lipid extracting solvent and added to the medium at the end of the incubation period. Fatty aldehydes were isolated and the presence of hexadecanal evaluated by the GLC and radioassay procedures. Hexadecanal was not detected by either method. Considering the sensitivity of the assay procedures employed, these results are not sufficient to exclude the fatty aldehyde as an intermediate, especially since the exogenous aldehyde is metabolized readily.

The fact that [1-C^{14}] palmitate or [1-C^{14}] palmitoyl coenzymee A (CoA) was not reduced to a fatty alcohol in vitro does not indicate an absence of appropriate enzymes. Previous studies have shown that the enzymatic reduction of B-hydroxy- B-methyl glutaryl CoA to mevalonic acid or aspartic acid to homoserine requires an initial activiation of the carboxyl group (27-30). *E. coli* grown as described here have a low acyl CoA synthetase activity (31-33). Since these cells also have a high thioesterase activity (34-36), one would expect

a cell-free system to contain only a small fraction of any exogenous fatty acid in the activiated form. A similar difficulty in demonstrating the reduction of exogenous fatty acyl CoA in the presence of a high thioesterase activity has been noted before in plants (26).

Evidence that fatty aldehydes are metabolically active in *E. coli* is based upon their rapid reduction to the corresponding fatty alcohol when added in vitro. Two protein fractions catalyzing this process have been purified partially from the 100,000 x g supernatant fraction and characterized.

At present, it is not possible to decide with certainty whether the two protein fractions designated D-1 and D-2 represent separate enzymes or whether one is an aggregate composed of a greater number of subunits than the other. The similarities in the characteristics of each protein fraction argues for the concept that the D-2 enzyme is a dissociation product of the D-1 enzyme.

The observation that acetaldehyde is not a substrate for either enzyme and that NADH was noot a required cofactor indicates that this enzyme is not identical with the alcohol dehydrogenase of *E. coli* (14).

The high enzyme activities observed when the polyunsaturated fatty aldehyes were employed as substrates were unexpected, since the corresponding fatty alcohols were not detected in *E. coli*. The similarity in the activities observed with the polyunsaturated 18 carbon aldehydes and dodecanal and tridecanal may be explained by the structures of the polyunsaturated chains. The *cis*-double bonds in the latter moieties lead to a shortening of the effective chain length. Because of this effect, the length of linolenyl aldehyde will be similar to dodecanal; however, the polyunsaturated moiety is fatter because of the bend in the molecule and more polar. This latter effect may account for the fact that linolenyl aldehyde is not quite as active a substrate as dodecanal.

The observation that 2-pentadecanone is not a substrate for either the D-1 or D-2 enzyme fractions indicates that these enzymes are not involved in the biosynthesis of the secondary alcohols in *E. coli* and that the carbonyl group must be present as such for enzyme activity.

Previous studies from this laboratory have indicated that the fatty alcohols of *E. coli* are both 1- and 2-alkanols (5). While a direct isolation of the secondary alcohols was not attempted, it is considered that only primary fatty alcohols were formed in vivo from exogenous fatty acids or in vitro from palmitaldehyde. This is based upon the observation that the secondary alcohols of *E. coli* are all less than 16 carbon atoms in chain lengths, and radioactivity was detected in only hexadecanol when the alkyl acetates were analyzed via GLC and the effluent stream split.

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