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Control of Synthesis and Distribution of Acyl Moieties in Etiolated *Euglena gracilis*†

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ABSTRACT: In cell-free preparations of etiolated *Euglena gracilis* fatty acyl-coenzyme A (CoA) reductase was located mainly in the microsomes while incorporation of acetate into fatty acids occurred mostly in the 100,000*g* supernatant. The major labeled acyl chains in the wax ester fraction and the more polar lipids synthesized from [1-^{14}C]acetate by the soluble synthetase were C_{12},C_{14},C_{16}, and C_{16},C_{14},C_{12}, respectively, in the order of decreasing amounts. In the same order the small amount of labeled acyl chains formed by the mitochondria was C_{16}, C_{14}, and C_{20}. The major alcohol derived from [1-^{14}C]acetate by the soluble system was C_{16} while that formed by the mitochondria was C_{14}. Addition of 1-hexadecanol to the soluble synthetase caused a marked stimulation of incorporation of [1-^{14}C]acetate and of [2-^{14}C]-malonyl-CoA into the wax esters and an inhibition of the labeling of the polar lipids resulting in an overall increase in incorporation into total lipids. 1-Hexadecanol also changed the chain length distribution; at 0, 12.5, 25, and 50 μM added hexadecanol the major fatty acid synthesized was C_{12}, C_{10}, C_8, and C_6, respectively. Therefore, it is concluded that 1-hexadecanol, functioning as acyl acceptor, favored transfer of acyl chains formed by the synthetase to the alcohol and thus effectively competed with the subsequent condensation reaction. Cell-free preparations of *E. gracilis* readily converted C_{14} and C_{16} acids or their CoA esters into the corresponding fatty alcohols, whereas the fatty acids generated from [1-^{14}C]-acetate by the soluble synthetase were preferentially incorporated into the acyl portion of the lipids, suggesting a direct transfer of the acyl chains from the synthetase to the acyl acceptors. The microsomal preparations incorporated [2-^{14}C]malonyl-CoA into lipids. The major acyl chains formed by the microsomes were C_{16}, C_{14}, and C_{18} in order of decreasing amounts. In contrast to the soluble system, more than one-half of the label incorporated into lipids by the microsomes was in wax ester in which nearly 60% of the label was in the alcohol portion, suggesting that acyl chains which were reduced to alcohols may be synthesized by the microsomes themselves. The major labeled alcohol generated by the microsomes from malonyl-CoA was C_{16}. These results indicate that wax synthesis occurs in the microsomes by a complex of enzymes which synthesize acyl chains, reduce them, and esterify the alcohol to esters.

Regulation of fatty acid synthesis, mechanism and control of chain termination, and factors that control channeling of acyl chains into various classes of lipids including the reductive pathways involved in the synthesis of waxes and ether lipids are all poorly understood aspects of lipid metabolism. *Euglena gracilis* synthesizes many classes of lipids each containing its characteristic complement of acyl chains (Rosenberg, 1963, 1967; Guehler *et al.*, 1964). Furthermore, in this organism a large portion of acyl chains formed undergoes reduction to the alcohol. Therefore, *E. gracilis* is particularly suitable for determining the factors that control the synthesis and channeling of acyl chains into various pathways.

† From the Department of Agricultural Chemistry, Washington State University, Pullman, Washington 99163. *Received November 3, 1972.* Scientific paper no. 3911, project no. 2001 from the College of Agriculture, Washington State University. This work was supported in part by Grant GM-18278 from the U. S. Public Health Service.

Biosynthesis of fatty acids in etiolated and photoauxotrophic *E. gracilis* has been studied (Cheniae, 1963, 1964; Delo *et al.*, 1971; Ernst-Fonberg and Bloch, 1971). Etiolated cells appear to have the tight complex type synthetase while light grown cells contain, in addition to the tight complex, the plant type loose complex (Delo *et al.*, 1971). It has been demonstrated that in the photoauxotrophic cells the channeling of the acyl chains into the various lipids might depend on the nature of the thio ester (Renkonen and Bloch, 1969). However, in these studies reduction of acyl chains, which is quantitatively a major reaction in this organism, was not reported. In a previous paper from our laboratory, enzymatic reduction of acyl-CoA[1] was described and it was observed that in cell-free preparations of *E. gracilis* acyl moieties generated from acetate were reduced to a much smaller extent than the exogenous acyl chains but they were more readily incorporated into polar lipids (Kolattukudy, 1970). This preferential incorporation of acyl chains into polar lipids indicates that acyl moieties are directly transferred from acyl carrier protein of the synthetase to the hydroxyl group of the glyceryl moiety, in a manner similar to that suggested in the bacterial system (Goldfine *et al.*, 1967; Van Den Bosch and Vagelos, 1970). However, neither the mechanism by which the acyl moieties are channeled to the reductive pathway nor the factors that regulate the synthesis of various chain lengths are understood.

In this paper we describe experimental evidence which suggests that acyl chains are directly transferred from the synthetase to a variety of acyl acceptors including fatty alcohols and glycerol derivatives without involving the free acyl-CoA intermediate. As a consequence, the acyl chains generated by the soluble synthetase are not accessible to the acyl-CoA reductase which is shown to be located in the microsomes. We also present experimental results which suggest that microsomes synthesize acyl chains, reduce them to alcohols, and esterify them to give wax esters.

Materials and Methods

Organism. Euglena gracilis Z was the strain used in our earlier study (Kolattukudy, 1970). The strain was maintained on slants of nutrient agar at 25° with weekly subculturing. The cells were grown in a liquid medium containing 0.5% proteose peptone, 0.2% yeast extract, 0.1% beef extract, and 0.3% glucose in the dark at 25°. For large scale isolation, the cells first grown in separate 5-ml batches were transferred in their exponential growth phase to flasks containing 1.5 l. of the above medium and grown at 25° for 8 days with shaking.

Reagents. Dithioerythritol, CoA, ATP, NADH, NADPH, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, tripalmitin, CTP, CDP-choline, CDP-ethanolamine, *sn*-glycerol 3-phosphate, UDP-galactose, and acetyl-CoA were purchased from Sigma Chemical Co., St. Louis, Mo. [1-^{14}C]-Acetate (60 mCi/mmol), [1-^{14}C]palmitoyl-CoA (50 mCi/mmol), [1-^{14}C]myristate (5 mCi/mmol), and [2-^{14}C]malonic acid (5.6 mCi/mmol) were purchased from New England Nuclear, Boston, Mass. [1-^{14}C]Palmitate (55 mCi/mmol) was purchased from Amersham/Searle, Des Plaines, Ill. 1-Palmitoylglycerol and 1,2-dipalmitoylglycerol were purchased from Hormel Institute, Austin, Minn. [2-^{14}C]Malonyl-CoA was prepared by the method of Trams and Brady (1960) and purified on a DEAE-cellulose column as described by Kusaka and Goldman (1967).

Chromatography. Except as otherwise specified the details of the chromatographic techniques are essentially similar to those used previously (Kolattukudy, 1970).

Disruption of the Cells and the Isolation of Cellular Fractions. Prior to disruption the cells were washed with 0.1 M phosphate buffer (pH 6.5) containing 0.3 M sucrose, 5×10^{-4} M dithioerythritol, and 10^{-3} M $MgCl_2$. The washed cells were suspended in the same medium and disrupted in a French Laboratory Press (American Instrument Co.) at 8000 psi. The slurry of the ruptured cells was subjected to differential centrifugation at 500*g* (5 min, washed once), 15,000*g* (20 min, washed twice), and 100,000*g* (60 min, washed twice). These fractions were designated as cellular debris, mitochondria, microsomes, and supernatant, respectively. All the fractions were made to the volume of the original homogenate in the same medium. All these operations were done at 0–4°.

Assays. The fatty acid reducing activity was measured as described earlier (Kolattukudy, 1970) with the exception that the concentration of dithioerythritol was lowered to 5×10^{-4} M.

For acetate incorporation studies the reaction mixture in a final volume of 2.0 ml contained 0.6 μmol of CoA, 1.1 μmol of NADH, 1.1 μmol of NADPH, 9 μmol of ATP, 30 μmol of glucose 6-phosphate, 5 units of glucose-6-phosphate dehydrogenase (Sigma, type XI), 3 μmol of $MnSO_4$, 40 μmol of sodium bicarbonate, 0.17 μmol of [1-^{14}C]sodium acetate (20×10^6 cpm), and enzyme in 0.1 M potassium phosphate buffer (pH 6.5) containing 10^{-3} M $MgCl_2$ and 5×10^{-4} M dithioerythritol. Usually the reaction mixtures were incubated with shaking for 60 min at 30° and the reaction was stopped by the addition of a 2:1 mixture of chloroform and methanol. When labeled malonyl-CoA was used the reaction mixture contained 0.5 μmol of NADH, 0.5 μmol of NADPH, 4.5 μmol of ATP, 15 μmol of glucose 6-phosphate, 2.5 units of glucose-6-phosphate dehydrogenase, 1 μmol of $MnSO_4$, 70 nmol of [2-^{14}C]malonyl-CoA (6×10^5 cpm), 40 μmol of acetyl-CoA, and the enzyme preparation in a total volume of 1.0 ml of the buffer medium used above.

Analysis of Lipid Fractions from Acyl-CoA Reductase and Fatty Acid Synthetase Systems. The total lipids were recovered from the chloroform and methanol solution according to the method of Folch *et al.* (1957) and the chloroform solutions were washed with water and then evaporated to dryness under reduced pressure. They were fractionated on analytical plates of silica gel G (0.50 mm) with known standards, using hexane–ethyl ether–formic acid (40:10:1, v/v) as the solvent system (Kolattukudy, 1967). Radioactive regions were matched with the added standards and the silica gel from these regions was scraped and assayed for ^{14}C as described before (Kolattukudy, 1970).

The wax ester fraction was isolated by preparative thin-layer chromatography. It was then refluxed with 10 ml of 14% BF_3 in methanol for 2–3 hr. The alcohol and methyl ester fractions were isolated by thin-layer chromatography on silica gel G with the solvent system mentioned above, and the isolated alcohol fraction was acetylated by treatment with a 2:1 mixture of acetic anhydride and pyridine overnight at room temperature. The acetylated alcohol was purified by thin-layer chromatography. The methyl ester and alcohol acetate fractions were further analyzed by radio gas–liquid chromatography with authentic standards. Quantitation was done by the triangulation method.

The polar lipid fraction which remained at or just above

[1] Abbreviations used are: CoA, coenzyme A; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

the origin in the above thin-layer chromatography was isolated and refluxed with 14% BF_3 in methanol. The methyl esters isolated from this reaction mixture were then analyzed as described for wax ester fraction. For separation of mono- and diglycerides from the phospholipids, hexane–ethyl ether–methanol (14:8:1, v/v) was used as the developing solvent.

Other Estimations. Protein was estimated by the method of Lowry *et al.* (1951) after precipitation with 10% trichloroacetic acid and removal of the pigments with 80% acetone.

Results and Discussion

Effect of Carbon Source on Acyl-CoA Reductase Level in E. gracilis. Extracts of cells grown in glucose were found to have two-to-fourfold higher fatty acyl-CoA reductase activity than those from acetate or mannose grown cells. About 0.3% glucose in the medium resulted in cell-free extracts with the maximum specific activity for acyl-CoA reductase and therefore glucose grown cells were routinely used for further experiments.

Incorporation of [1-^{14}C]Acetate and [1-^{14}C]Myristic Acid into Lipids by Cell-Free Preparations. In order to investigate the factors that control the channeling of acyl chains into acyl lipids and reductive pathways, incorporation of [1-^{14}C]acetate and labeled fatty acids into the various lipids by cell-free preparations of *E. gracilis* was studied. The results from a typical experiment are reported in Table I.

The 500*g* supernatant from extracts of *E. gracilis* incorporated acetate into a variety of classes of lipids. The highest incorporation was into the polar lipids, followed by wax esters and the triglycerides. Exogenous [1-^{14}C]myristate also underwent incorporation into various lipid classes but unlike acetate the incorporation into wax esters was more than that into polar lipids. When the polar lipid fractions from both systems were treated with BF_3–CH_3OH reagent, all the radioactivity in the polar lipids was in the methyl ester fraction. A similar treatment of the wax ester fraction followed by thin-layer chromatographic analysis of the products showed that with [1-^{14}C]myristate nearly 60% of the ^{14}C in the wax was recovered in the alcohol fraction while less than 18% of the wax derived from [1-^{14}C]acetate was in the alcohol portion. The results obtained with [1-^{14}C]palmitate were similar to those recorded for [1-^{14}C]myristate. Time-course experiments showed that incorporation of labeled acetate and myristate into the various lipid classes was fairly linear up to about 60 min beyond which the rates began to level off. However, the relative distribution of label among the various lipid classes remained the same irrespective of the experimental period used from 2.5 to 90 min and protein concentration from 0.5 mg to 4 mg/ml except that the proportion of ^{14}C in the alcohol portion of the wax was slightly higher with the shorter experimental periods when myristate was the substrate.

In order to determine whether the concentration of the CoA ester can influence the incorporation pattern into different lipid classes, a 15,000*g* supernatant fraction was assayed with different concentrations of palmitoyl-CoA. At all concentrations of palmitoyl-CoA examined up to 1×10^{-4} M, incorporation into the wax ester fraction was higher than that into the polar lipids (Table I). Higher concentrations inhibited wax synthesis while incorporation into polar lipids showed further increase. In the wax ester fraction the fatty alcohol moiety contained 45–50% of the label at the different concentrations of [1-^{14}C]palmitoyl-CoA used. These results are similar to those obtained with [1-^{14}C]myristic acid and [1-^{14}C]palmitic acid. Therefore, it is concluded that the distribution of label observed with exogenous acyl chains is not due to the high concentrations of CoA esters that might be generated from them.

TABLE I: Incorporation of [1-^{14}C]Acetate, [1-^{14}C]Myristic Acid, and [1-^{14}C]Palmitoyl-CoA into Lipid Fractions by Cell-Free Homogenates from Etiolated *E. gracilis*.[a]

	nmol Incorporated		
		Wax Ester	
Substrate	Polar Lipids	Acyl	Alcohol
[1-^{14}C]Acetate	12.1	8.2	1.7
[1-^{14}C]Myristic acid	33.8	16.9	24.4
[1-^{14}C]Palmitoyl-CoA (nmol)			
25	6.2	3.6	3.4
50	6.4	7.4	6.1
100	8.6	13.0	9.3
200	10.2	15.2	14.1
400	27.6	8.8	7.5

[a] The 500*g* supernatant was used as the enzyme source with [1-^{14}C]acetate and [1-^{14}C]myristic acid, while with [1-^{14}C]-palmitoyl-CoA a 15,000*g* supernatant was used. The reaction mixtures with [1-^{14}C]acetate and [1-^{14}C]myristic acid contained 2.5 mg of protein per ml and the appropriate cofactors described under Methods. With palmitoyl-CoA the reaction mixture contained 1.1 μmol of NADH and 3.0 mg of protein in a final volume of 2 ml of the buffer medium used with acetate. All the reactions were run at 30° for 1 hr. Total lipids from these systems were extracted with chloroform and methanol (2:1) and fractionated on silica gel G plates in hexane–ethyl ether–formic acid (40:10:1, v/v). The wax ester fraction isolated from this chromatography was transesterified with BF_3–CH_3OH reagent and the fatty alcohol and methyl esters of the fatty acids were isolated by thin-layer chromatography on silica gel G in the above solvent system. In the experiment with [1-^{14}C]acetate, 0.3 and 5.9 nmol were incorporated into free fatty acids and triacylglycerols, respectively.

It is possible that the chain length of the acids from [1-^{14}C]-acetate is not suitable for fatty acyl-CoA reductase which has been shown to be specific for C_{14}–C_{16} acids (Kolattukudy, 1970). In order to test this possibility methyl esters from polar lipids and wax esters derived from experiments with [1-^{14}C]acetate and [1-^{14}C]myristate were further analyzed for their chain length distribution with radio gas–liquid chromatography. In the case of myristate, virtually all the radioactivity was in C_{14} in both polar lipids and wax esters.

The acyl moieties of these two classes of lipids derived from [1-^{14}C]acetate constituted very characteristic mixtures of chain lengths. In the polar lipids the major radioactive acids were C_{16}, C_{14}, and C_{12} in decreasing amounts of ^{14}C, whereas in the wax ester fraction the major radioactive acids were C_{12}, C_{14}, and C_{16} in the same order. The ^{14}C distribution among the acyl moieties of the polar lipids derived from [1-^{14}C]-acetate in the cell-free preparations is very similar to that obtained with intact cells. The major labeled alcohol derived from [1-^{14}C]acetate was C_{16} with small amounts of C_{14} and C_{12}, whereas [1-^{14}C]myristate gave only C_{14} alcohol as ex-

TABLE II: Apparent Localization of Acyl-CoA Reductase and Fatty Acid Synthesizing Activities in Etiolated *E. gracilis*.[a]

Cell Fractions	Acyl-CoA Reductase 10^5-cpm Alcohol/mg of Protein	% Act.	Fatty Acid Synthetase 10^5-cpm Fatty Acid/mg of Protein	% Act.
Crude homogenate	4.89	(100)	3.52	(100)
Cellular debris	0.09	4.4		
Mitochondria	0.81	19.2	5.38	1.2
Microsomes	13.35	54.3	0.08	0.5
Soluble supernatant	0.12	10.8	6.71	118.9

[a] Subcellular fractions were collected as described in the text. The reaction mixture for the acyl-CoA reductase assay contained 1.1 μmol of NADH, 50 nmol of [1-^{14}C]palmitoyl-CoA (2×10^6 cpm), and enzyme in a final volume of 2 ml of 0.1 M phosphate buffer (pH 6.5) containing 10^{-3} M $MgCl_2$ and 5×10^{-4} M dithioerythritol. The fatty alcohol fraction was isolated by thin-layer chromatography after treatment of the total lipids with BF_3–CH_3OH reagent. Fatty acid synthetase activity was assayed as described under Methods. The reaction was stopped by adding 12 ml of 10% KOH in ethanol. The contents were then refluxed for 2–3 hr under nitrogen, acidified with HCl, and extracted with chloroform.

TABLE III: Effect of Various Cofactors on the Activity of Soluble and Mitochondrial Fatty Acid Synthesizing Systems from Etiolated *E. gracilis*.[a]

Conditions	10^4-cpm Fatty Acids/mg of Protein: Soluble	Mitochondrial
All Cofactors	64.54	1.40
−NADH	29.93	0.64
−NADPH and regenerating system	39.96	2.01
−ATP, CoA	3.66	0.07
$-H_2CO_3$	54.22	1.00
$-Mn^{2+}$	62.80	3.19
−All	0.88	0.05
+Avidin	56.00	

[a] The mitochondrial and the 100,000*g* soluble supernatant fractions were collected as described in the text. The complete reaction mixture contained all the cofactors and was the same as that described under Methods. With avidin (6.5 units) the enzyme was preincubated for 10 min in cold before the addition of other reactants. The products were isolated as described under Table II.

pected (Kolattukudy, 1970). When the triglyceride fraction was analyzed for the fatty acid chain lengths it was found to have C_{12} and C_{14} with a predominance of the former.

Although the major acyl chains derived from [1-^{14}C]acetate were well within the specificity of the reductase the amount of ^{14}C in alcohols was only less than 7% of that in acyl chains, while the amount of ^{14}C in alcohols was nearly half of that in acyl lipids with exogenous [1-^{14}C]myristate. These results indicate that acyl chains synthesized from [1-^{14}C]acetate in the cell-free preparations are not accessible to the reductase. One possible reason for inaccessibility is that the acyl chains formed by fatty acid synthetase are directly transferred from acyl carrier protein (ACP) to the hydroxyl groups of the acyl acceptors such as fatty alcohol or a glycerol derivative. The chains that reach final stages of elongation, namely C_{16}, can also be transferred, at least to a small extent, to CoA and the resulting CoA esters may be reduced to the alcohol.

Apparent Subcellular Localization of Acyl-CoA Reductase and Fatty Acid Synthesizing Activities. It has been reported that in buffer extracts of photoauxotrophic (Cheniae, 1963) and etiolated *E. gracilis* (Delo *et al.*, 1971) the major portion of the fatty acid synthesizing activity was found in the soluble fraction. However, in such studies isotonic conditions were not used. Since the accessibility of fatty acyl chains generated from acetate to the reductase may also be influenced by the subcellular localization of fatty acid synthetase and acyl-CoA reductase, we attempted fractionation of the cell-free preparations. Experimental results summarized in Table II show that virtually all the fatty acid synthesizing activity from [1-^{14}C]acetate was located in the 100,000*g* supernatant while microsomes contained the major part of the acyl-CoA reductase. The mitochondrial fraction contained a substantial portion of the reductase activity and measurable amounts of fatty acid synthesizing activity. The 100,000*g* supernatant contained more than 10% of the total reductase. Since many attempts to solubilize the microsomal reductase with detergents, sonication, and combinations of these, etc., failed we conclude that the reductase found in the 100,000*g* supernatant is not derived from the microsomes during subcellular fractionation procedures. In the perputial gland tumor of the mouse, fatty acid-CoA reductase was found to be in the microsomes (Snyder and Malone, 1971) and while this paper was in preparation Wang *et al.* (1972) reported that the 105,000*g* particulate fraction from *Mycobacterium tuberculosis* catalyzed fatty acyl-CoA reduction.

The removal of particulate material from the crude homogenate appeared to enhance fatty acid synthetase activity resulting in a 20% increase in recovery of the activity. Usually the specific activity of the 100,000*g* supernatant was 1.9–2.5 times that of the crude homogenate. The microsomal fraction did not incorporate substantial amounts of [1-^{14}C]acetate into fatty acids.

Cofactor Requirement for the Incorporation of [1-^{14}C]-Acetate into Fatty Acids by the Soluble and Mitochondrial Fractions. Incorporation of [1-^{14}C]acetate into fatty acids by both soluble and the mitochondrial fractions required ATP and CoA (Table III). Both NADH and NADPH were needed for maximal activity at least for the soluble system. White *et al.* (1971) has indicated that both NADH and NADPH function synergestically in the multienzyme fatty acid synthetase complexes of *Mycobacterium phlei*, *Euglena gracilis*, and *Saccharomyces cerevisiae*. However, the mitochondrial activity showed a preference for NADH since the omission of the NADPH regenerating system resulted in at least a 1.6-fold increase in activity. A similar requirement for NADH and inhibition by NADPH has been previously observed with beef heart mitochondrial preparation (Dahlen and Porter, 1968). Omission of bicarbonate and Mn^{2+} or addition of avidin inhibited acetate incorporation into fatty acids but the inhibition was not very severe. Incorporation of acetate into fatty acids by extracts of light grown cells also was relatively insensitive to avidin and HCO_3^- (Cheniae, 1963). The in-

TABLE IV: Incorporation of [1-^{14}C]Acetate into the Lipid Classes by the Soluble and the Mitochondrial Fractions and Their Chain Length Distributions.[a]

Lipid Fraction	Radioact. (10^4 cpm/mg of protein)	Chain Length (%)						
		10	12	14	15	16	18	20
		Soluble Supernatant						
Polar lipids	30.5	4.5	15.4	34.0	3.9	37.2	4.0	1.0
Wax ester								
Acyl	7.0	11.3	32.8	26.1	6.2	23.6		
Alcohol	2.0			10.2		83.0	6.8	
Triacylglycerol	12.5	19.0	49.6	13.6		9.0	8.1	
Free fatty acid	0.6	14.3	47.2	12.6		19.7	6.1	
		Mitochondrial						
Polar lipids	3.0		0.6	34.1		40.8	4.2	20.3
Wax ester								
Acyl	0.6		18.2	28.4		35.8	17.6	
Alcohol	0.4			84.2		15.8		

[a] The mitochondrial and the 100,000*g* soluble supernatant fractions were prepared as described in the text. The assay conditions and the processing of the sample were the same as those under Methods except that with the mitochondrial preparation Mn^{2+}, NADPH, and the regenerating system were omitted. Chain lengths of the isolated lipid fractions were determined by radio gas–liquid chromatography after converting the acyl portion into methyl esters. The alcohol fraction was acetylated as described in the text. The chromatography was done on coiled stainless steel column (117 × 0.61 cm o.d.) packed with 3% OV-1 on 80–100 mesh Gas Chrom Q at 160° for methyl ester and 190° for acetylated alcohols with 90 cm^3/min of argon. Quantitation was done by triangulation.

sensitivity to avidin is probably a reflection of the insensitivity of the acetyl-CoA carboxylase of this organism to avidin rather than differences in the fatty acid synthesis *per se*.

From preliminary experiments with the cell-free system an optimal dithioerythritol concentration for the system was found to be 5×10^{-4} M. Dithioerythritol was found to be better than dithiothreitol or mercaptoethanol. The optimal amounts of NADPH and glucose 6-phosphate under the present experimental conditions were about 1 and 30 μmol, respectively.

Distribution of Acyl Chains Synthesized by the Soluble and Mitochondrial Fractions among the Different Classes of Lipids. Thin-layer chromatographic analysis of labeled lipids isolated from the soluble and mitochondrial preparations revealed differences in the distribution of incorporated radioactivity among the different lipid classes (Table IV).

With the soluble system the maximum incorporation was into the polar lipids followed by triglycerides, wax esters, and to a small extent into the free fatty acid fraction. In the mitochondrial system the radioactivity was mainly in the polar lipid and wax ester fractions. The major labeled acyl chains in the polar lipids synthesized by the soluble system were C_{16}, C_{14}, and C_{12} in the order of decreasing amounts of ^{14}C while those in the wax ester fraction were C_{12}, C_{14}, and C_{16}. Virtually all the ^{14}C in the wax alcohol fraction was contained in C_{16}. Mitochondria incorporated acetate into acyl chains quite different from those obtained with the soluble system (Table IV). The major labeled acyl chains in the polar lipid fraction were C_{16}, C_{14}, and C_{20} in the order of decreasing amounts of radioactivity with detectable amounts of label in C_{18}, C_{12}, and C_{22}. The major labeled acyl chains in the wax esters were C_{16} and C_{14}. Unlike that found in the soluble system, the labeled alcohol in the mitochondrial system was mostly C_{14} but appreciable quantities of C_{16} were also present. The longer acyl chains found in the mitochondrial systems may be due to the presence of an elongation system in the mitochondria rather than *de novo* synthesis. The differences in the cofactor requirements also suggest that the two systems differ.

Incorporation of [1-^{14}C]Acetate and [1-^{14}C]Myristate into Lipids by a Combination of Microsomes with the Soluble Supernatant. Results discussed in a previous section suggested that the fatty acyl chains generated by the soluble synthetase could be directly transferred to glyceride type acceptors without involving a free CoA ester intermediate. If this conclusion is valid, addition of microsomes to the soluble preparation should not cause a marked increase in the amount of fatty alcohol formed from *de novo* generated acyl chains. In order to test this possibility recombination experiments were done and the results are summarized in Table V.

The radioactivity distribution among the lipid classes in the soluble synthetase system was more or less similar to that reported in a preceding section. The microsomal system did not incorporate appreciable amounts of acetate into lipids. Upon combination of the soluble synthetase and microsomal reductase no appreciable change in the total incorporation of [1-^{14}C]acetate was observed. However, the combined system showed a decrease in the ^{14}C incorporated into the polar lipids with an equivalent increase in that found in the wax ester fraction. This increase in the label was mainly in the acyl moieties and not in the alcohol portion. When the proportion of the microsomal protein in the reaction mixture was much higher than that found in the cells the total incorporation of acetate into lipids decreased with no increase in the proportion of the label in the fatty alcohols. On the other hand, with [1-^{14}C]myristic acid addition of microsomes to the soluble preparation resulted in increased production of labeled fatty alcohol. Thus, even in the presence of added active fatty acyl-CoA reductase acyl moieties formed by the fatty acid synthetase were not channeled into the reductive

TABLE V: Incorporation of [1-^{14}C]Acetate and [1-^{14}C]Myristic Acid in Microsomal, Soluble Supernatant, and Recombined System from Etiolated *E. gracilis*.[a]

	Radioactivity (10^5 cpm)		
		Wax Ester	
	Polar Lipids	Acyl	Alcohol
With [1-^{14}C]Acetate			
Soluble supernatant	28.76	3.62	1.23
Microsomes + soluble supernatant	24.04	6.87	1.34
With [1-^{14}C]Myristic Acid			
Microsomes	0.30	2.28	2.84
Soluble supernatant	3.02	0.23	0.17
Microsomes + soluble supernatant	3.04	0.76	0.90

[a] The microsomal and the soluble supernatant fractions were isolated as described in the text. In recombination of the microsomes and soluble supernatant equal volumes of both the fractions were used. The reaction mixtures and the other experimental procedures were the same as those under Methods. The amounts of protein used in these assays were: 5 mg with soluble supernatant and 1 mg with microsomes.

pathway. These results once again demonstrate that the *de novo* synthesized acyl moieties were not freely accessible to the reductase.

Control of Fatty Acid Synthesis and Distribution by Acceptors. Since experimental results thus far discussed indicated that acyl chains formed by the fatty acid synthetase may be directly transferred to acyl acceptors it is possible that the presence and concentration of acceptors have significant influence on the amount and type of fatty chains formed by the synthetase. Furthermore, since each lipid class contains its characteristic chain length distribution pattern, the acyl acceptors might also influence the chain lengths of the acyl moieties generated by the synthetase. If such is the case, exogenous acceptors should be able to change distribution of radioactivity among the various classes of lipids as well as chain length distribution of acyl moieties derived from [1-^{14}C]acetate. In order to investigate this possibility, various acceptors were added to the regular synthetase reaction mixture. Addition of glycerol phosphate (5 μmol) slightly stimulated (20–30%) the incorporation of [1-^{14}C]acetate into the polar lipids but inhibited (about 30%) that into the wax ester fraction. Upon further analysis of the wax esters it was found that the inhibition of the incorporation of label into the fatty alcohol portion was nearly 60% while that into the acyl portion was inhibited by about 20%. Addition of CDP-choline, CDP-ethanolamine, CTP, and UDP-galactose did not alter the radioactivity distribution among the various classes of lipids to any appreciable extent. Addition of monopalmitin to the supernatant also resulted in a slight stimulation (20–25%) of [1-^{14}C]acetate incorporation into total lipids. Upon fractionation of lipids synthesized in the presence of 7.5×10^{-5} M monopalmitin it was observed that the added monopalmitin slightly inhibited incorporation of labeled acetate into phospholipid and wax ester fractions but caused an increase in the label found in the diglyceride (threefold), free fatty acid (twofold), and the triglyceride (1.5-fold) fractions. Addition of chimyl alcohol also resulted in qualitatively similar changes indicating that the monoglyceride effect was not due to the hydrolysis products derived from the monoglycerides. These results indicated that if the acyl acceptor is a glyceride it favors channeling into glyceride type of lipids quite effectively.

Our attempts to use glycerol phosphate as an exogenous acceptor did not channel the acyl moieties into polar lipids appreciably. Since relative concentrations of the endogenous acceptors might be playing an important part in the partitioning of the acyl chains into various lipids, removal of endogenous acceptors should allow us to use defined concentrations of various acceptors. In order to remove water-soluble acceptors such as glycerol phosphate or dihydroxyacetone phosphate, dialysis against 0.05 M phosphate buffer (pH 6.5) containing 10^{-3} M $MgCl_2$ and 5×10^{-4} M dithioerythritol was attempted. However, this treatment severely inhibited the acetate incorporating activity of the preparation. The effect of added glycerol phosphate on the dialyzed preparation was quantitatively and qualitatively similar to that observed with undialyzed preparations. It is possible that the acyl acceptor responsible for the incorporation of acyl chains into polar lipids is a lipid which is not easily dialyzed. Our attempts to remove the lipid type acyl acceptors by extraction with diethyl ether also resulted in partial loss of the acetate incorporating activity of the preparation. The effect of addition of glycerol phosphate was similar to that observed with untreated supernatant.

Addition of hexadecanol on the other hand resulted in a marked stimulation in the incorporation of [1-^{14}C]acetate into total lipids. Analysis of the products from such a reaction showed that the amount of label in the polar lipids was only about 5% of that found in control experiments with no added hexadecanol, while the amount of label in the wax ester fraction increased six-to-eightfold. Significant amounts of radioactivity could not be detected in the triglyceride fraction isolated from the reaction mixtures containing added hexadecanol, whereas about 16% of the label incorporated into lipids was found in the triglyceride fraction in the absence of added hexadecanol. These results clearly show that exogenous hexadecanol effectively competes with endogenous acyl acceptors. Addition of hexadecanol to dialyzed and ether-extracted preparations showed marked stimulation of incorporation of acetate into the wax ester fraction at the expense of polar lipids just as the untreated supernatant.

Effect of Hexadecanol Concentration on Fatty Acid Synthesis and Distribution. When the wax ester fraction isolated from the incubation mixtures containing exogenous hexadecanol was treated with BF_3–CH_3OH, the recovery of the radioactivity in the products was very low indicating that some of the radioactivity in this fraction was contained in very short chain acids. These results suggested that exogenous fatty alcohol might have influenced the chain length of the acyl chains formed by the fatty acid synthetase. Therefore, we examined this effect in greater detail at different concentrations of hexadecanol and the results are summarized in Table VI. Addition of 25 nmol of hexadecanol caused about 80% inhibition of the incorporation of label into polar lipids and a three- to fourfold increase in the labeling of wax esters. As the concentration of hexadecanol increased there was a progressive increase in the amount of incorporation of ^{14}C into the wax ester fraction accompanied by a further decrease in the synthesis of polar lipids. As the amount of hexadecanol added increased from 100 nmol, progressively larger quantities of labeled hexadecyl acetate were also formed. In order to examine the chain

TABLE VI: Effect of Hexadecanol Concentrations on the Distribution of Acyl Moieties Synthesized from [1-^{14}C]Acetate by the Soluble Supernatant System from *E. gracilis*.[a]

Hexadecanol Added (nmol)	Radioactivity (10^5 cpm)			
	Polar Lipids	Triacyl-glycerol	Hexadecyl Acetate	Wax Ester
None	5.84	1.04	ND	2.4
25	1.20	ND	ND	7.4
50	0.89	ND	ND	10.8
100	0.68	ND	4.3	12.7
200	0.58	ND	14.6	15.8
300	0.50	ND	27.3	19.5
500	0.40	ND	44.9	15.3

[a] The soluble supernatant (100,000*g*) was obtained as described in the text. The incubation mixture was the same as that described under Methods; the protein concentration was 1.5 mg/assay. Hexadecanol was dispersed in deionized water by sonication with a Biosonic III needle probe at full speed for 3 min. Total lipids were isolated and analyzed as described under Methods. In the solvent system used hexadecanol acetate moved well below wax esters but above triacylglycerol fraction. ND, not detected.

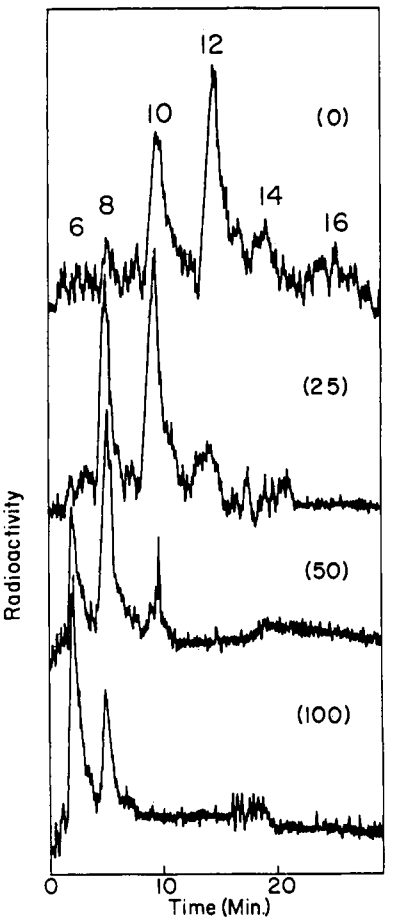

FIGURE 1: Radio gas-liquid chromatogram of the labeled fatty acid butyl esters from the wax ester fraction derived from [1-^{14}C]acetate by the 100,000*g* supernatant in the presence of different concentrations of added hexadecanol (figures in parentheses indicate nanomoles of the alcohol added). Butyl esters were made by refluxing the wax ester fraction with 14% BF_3 in butanol for 3–4 hr and isolating them from silica gel G plates (1.0 mm) developed in benzene. Radio gas–liquid chromatography was done on coiled stainless steel column (117 × 0.61 cm o.d.) packed with 5% OV-1, on 80–100 mesh Gas Chrom Q with 90 cm^3/min of argon. The column was temperature programmed from 110 to 200° with 4°/min as the program rate. Synthetic butyl esters of authentic standards were used for identification. The number on each peak represents the chain length of the acyl moiety.

length of the acyl moieties without loss due to volatility, the wax esters were treated with BF_3–butanol reagent. This technique gave us good recoveries of the acyl chains as butyl esters which were subsequently analyzed by radio gas–liquid chromatography (Figure 1). In the absence of added hexadecanol the acyl chains of the wax esters were C_{12}, C_{10}, C_{14}, and C_{16} in the order of decreasing amounts of radioactivity. With 25 nmol of added hexadecanol there was a dramatic shift in the chain length distribution of the acyl moieties of the wax in that C_{10} became the dominant labeled acyl moiety followed by C_8 with C_{12} and C_{14} being minor components. As the concentration of the exogenous hexadecanol increased the labeled acyl moieties formed became shorter. The dominant labeled acyl moieties in the wax ester were C_8 and C_6 with 50 and 100 nmol of added hexadecanol, respectively. A further increase in hexadecanol concentration resulted in a further decrease in C_8 and an increase in shorter acids. However, due to the volatility of the butyl esters of shorter acids we did not examine acyl moieties shorter than C_6. Thus, it is clear that relatively low concentrations of hexadecanol, which is a normal metabolite in *E. gracilis*, have a dramatic influence on the rate of synthesis as well as the chain length of acyl chains formed by the fatty acid synthetase. Sumper *et al.* (1969) offered a kinetic explanation for the formation of particular chain lengths by the fatty acid synthetase of yeast. According to their hypothesis, relative rates of condensation and transfer reactions determine at what length the fatty chain is terminated. If *de novo* synthesized acyl chains are directly transferred to acyl acceptors such as fatty alcohol, increasing concentrations of hexadecanol favor the transfer reaction in preference to condensation with another malonyl moiety and thus bring about a shortening of the acyl chains formed by the synthetase. The experimental results in Figure 1 clearly demonstrate such a process and strongly support the kinetic explanation of chain termination proposed by Sumper *et al.* (1969). On the other hand we cannot rule out the possibility that hexadecanol alters the conformation of the fatty acid synthetase in such a way that chain termination occurs earlier than usual.

It is possible that fatty alcohol controlled the chain length of the acyl chains formed from [1-^{14}C]acetate by the synthetase only indirectly by affecting the acetyl-CoA carboxylase resulting in an altered acetyl-CoA:malonyl-CoA ratio (Smith and Dils, 1966; Bartley *et al.*, 1967; Sumper *et al.*, 1969; Nandedkar *et al.*, 1969; Smith and Abraham, 1970). In order to test this possibility we used [2-^{14}C]malonyl-CoA as a substrate instead of [1-^{14}C]acetate with a malonyl-CoA to acetyl-CoA ratio that generally favors longer chains. The influence of added hexadecanol on this system is illustrated in Table VII. In the control the incorporation pattern of malonyl-CoA was similar to that obtained earlier with acetate. Addition of 50 nmol of hexadecanol caused a fourfold increase in the incorporation of [2-^{14}C]malonyl-CoA into the wax ester fraction and a dramatic decrease in that into the polar lipid fraction. Radio gas–liquid chromatographic

TABLE VII: Effect of Hexadecanol on the Distribution of Acyl Moieties Synthesized from [2-^{14}C]Malonyl-CoA by the Soluble Supernatant from *E. gracilis*.[a]

	Radioactivity (10^3 cpm)				Wax Ester Acyl Chain Length (%)					
	Polar Lipids	Free Fatty Acid	Triacyl-glycerol	Wax Ester	6	8	10	12	14	16
Control	60.2	9.0	21.0	40.0			30.8	44.6	20.4	4.2
Hexadecanol added (50 nmol)	14.7	2.3	2.0	161.5	5.9	56.3	32.6	5.2		

[a] The soluble supernatant was obtained as described in the text. The reaction system in a final volume of 1 ml contained 0.5 μmol of NADH, 0.5 μmol of NADPH, 4.5 μmol of ATP, 15 μmol of glucose 6-phosphate, 2.5 units of glucose-6-phosphate dehydrogenase, 1 μmol of $MnSO_4$, 70 nmol of [2-^{14}C]malonyl-CoA (6×10^5 cpm), 40 nmol of acetyl CoA, and enzyme protein (4.0 mg) in 0.1 M phosphate buffer (pH 7.0) containing 10^{-3} M $MgCl_2$ and 5×10^{-4} M dithioerythritol. The reaction was run for 1 hr at 30°. Processing and the fractionation of the total lipids were done as described under Methods. Butyl esters of the wax ester fraction were made as described under Figure 1, and subjected to radio gas–liquid chromatography under the conditions described in Figure 1. Quantitation was done by triangulation.

analysis (Table VII) of the acyl chains (as butyl esters) of the wax ester fraction showed that the control contained C_{12}, C_{10}, C_{14}, and C_{16} in the order of decreasing amounts of ^{14}C while the corresponding fraction formed in the presence of 50 nmol of added hexadecanol contained C_8, C_{10}, C_6, and C_{12} acids in the same order. These results are very similar to those observed with [1-^{14}C]acetate and demonstrate that the hexadecanol effect on chain length distribution is probably not due to its effect on acetyl-CoA carboxylase. Therefore, these results strongly support our contention that hexadecanol accepts the acyl chains from the synthetase and thus prevents further condensation with another malonyl moiety.

Source of Acyl Chains for the Microsomal Acyl-CoA Reductase. The results discussed so far demonstrate that the acyl chains built by the soluble system are directly transferred to the acceptors (such as hexadecanol) present in the soluble system and are not accessible for the microsomal acyl-CoA reductase. Therefore, the question arises how one out of four acyl chains formed in the organism is reduced to alcohol by the microsomal reductase. In order to answer this question we first examined whether some metabolite can trigger transfer of acyl chains synthesized by the soluble synthetase to CoA or water. The CoA ester could then undergo reduction by the microsomal reductase. It was found that addition of monopalmitin resulted in a change in the product distribution of soluble synthetase in favor of free fatty acids (and glycerides). For example, addition of 7.5×10^{-5} M of monopalmitin to the soluble synthetase assay gave two times as much radioactivity in the free fatty acids as that observed with no added monopalmitin. Addition of microsomes to the soluble system in the presence of monopalmitin should give increased amounts of fatty alcohol if the free fatty acids released by the effect of monopalmitin are converted into alcohols by the microsomes. In fact, addition of 7.5×10^{-5} M of monopalmitin to the 15,000*g* supernatant with [1-^{14}C]acetate and the necessary cofactors gave 1.8–2.5 times as much ^{14}C in alcohols as that obtained without the addition of monopalmitin. These results indicated that concentrations of glycerides and possibly other metabolites that represent nutritional abundance can trigger a transfer of acyl chains from the soluble fatty acid synthetase to CoA or water so that these chains would become accessible to the microsomal fatty acyl-CoA reductase.

The total amount of acyl chains that are diverted to the alcohols even in the presence of monopalmitin was not anywhere near as high as one finds in the whole cells. Therefore, we searched for alternate or additional means by which acyl chains are provided for fatty alcohol synthesis. One such possibility is that microsomes might synthesize their own acyl chains which might be used primarily for wax synthesis. The results in Table VIII showed that with [2-^{14}C]malonyl-CoA the microsomal system synthesized acyl chains. The incorporation of malonyl-CoA per milligram of protein was higher in the microsomal fraction than that in the soluble supernatant, although the latter system contained nearly two-thirds of the total activity. A thin-layer chromatographic analysis of the total lipids synthesized by the microsomes showed that 50–60% of the label was in wax esters in which nearly 60% of the ^{14}C was contained in the alcohol portion. Radio gas–liquid chromatographic analysis of the acyl chains of polar lipid and wax ester fractions as methyl esters showed the predominance of C_{16}. The major labeled alcohol of the wax ester was also C_{16} with much smaller quantities of C_{14} and C_{18}. Thus, it is clear that the microsomal fraction can indeed build acyl chains from malonyl-CoA and these acyl chains are readily converted into fatty alcohols and subsequently to wax esters.

The nature of microsomal fatty acid synthesizing activity and factors that control the channeling of carbon into the microsomal system is yet to be defined. From the results thus far obtained, the possibility that the microsomes catalyze an elongation type reaction sequence rather than a *de novo* type cannot be ruled out. However, the fact that [2-^{14}C]-malonyl-CoA was incorporated mainly into C_{16} even in the presence of added C_{12} acid (data not shown) suggests that the microsomes do not simply add one C_2 unit to the C_{12} acid.

In the *E. gracilis* system discussed in this paper the presence of active acyl-CoA reductase served as an indicator of the release of acyl chains from the fatty acid synthetase. The preferential incorporation of acyl chains generated from [1-^{14}C]acetate into polar lipids and the acyl portion of the wax ester rather than into the alcohols and the results of the recombination studies indicate that in etiolated *E. gracilis* acyl chains built by the soluble synthetase are directly transferred to the various acyl acceptors. In bacterial systems it has been shown that acyl chains may be transferred from acyl carrier protein to glycerol phosphate without involving CoA ester

(Van Den Bosch and Vagelos, 1970; Ailhaud *et al.*, 1967). It is possible that the concentration of acyl acceptors plays a regulatory role on the rate of synthesis and the nature of acyl chains in these systems. For example, the decreased rates of synthesis of fatty acids observed in glycerol-deprived glycerol-requiring auxotrophs of *Bacillus subtilis* (Mindich, 1972) may also be explained on the basis of a regulation by decreased levels of acyl acceptors. In the animal systems (Bressler and Wakil, 1962; Phillips *et al.*, 1970) the thioesterase has been suggested to be involved in chain termination while in yeast acyl transfer to CoA is apparently involved (Lynen, 1967; Sumper *et al.*, 1969; Schweizer *et al.*, 1970). Tight complex type fatty acid synthetases thus far examined were reported to transfer the acyl chains to CoA or water. However, our results indicate that in etiolated *E. gracilis*, which appears to have tight complex synthetase (Delo *et al.*, 1971), acyl transfer directly to an acceptor other than CoA or water occurs. Specificity of thioesterases (Barnes and Wakil, 1968; Barnes *et al.*, 1970; Bonner and Bloch, 1972) has been suggested to be a possible factor that controls the chain length. In yeast, where specificity of the transferase is too broad to be of significance in controlling chain length (Schweizer *et al.*, 1970), a kinetic explanation based on relative rates of condensation and transfer was offered to explain the control of chain length (Sumper *et al.*, 1969). Our results indicate that in *E. gracilis* the concentrations of acceptors can determine the chain length of the acyl moieties formed by the synthetase by favoring transfer to the acyl chain at different stages of elongation.

The type of compartmentalization and control of acyl chain synthesis observed in the present system may be relevant to other organisms, particularly those which synthesize wax esters such as marine copepods (Nevenzel, 1970) and sebacious glands of animals (Nicolaides, 1965). For example, experiments with cell-free preparations of the uropygial glands of goose indicated that acyl chains generated from [1-^{14}C]acetate and [1-^{14}C]propionate were preferentially incorporated into acyl lipids but not into alcohols which constitute a major component of the gland lipids (Buckner and Kolattukudy, 1973[2]). On the other hand our preliminary experimental results indicated that in cell-free preparations of etiolated *Chlorella vulgaris*, which does not normally synthesize very much wax ester, addition of hexadecanol did not influence the chain length of the acyl chains generated from acetate. Thus, chain length regulation by alcohol may be relevant only to tissues which synthesize wax esters, although in other cases other acyl acceptors might play a similar role.

The exact mechanism by which acyl acceptors and possibly other metabolites regulate the channeling of the acyl chains generated by fatty acid synthetase into various classes of lipids is not clear. Although fatty alcohols appear to be functioning as direct acyl acceptors the possibility that they directly interact with the synthetase to produce conformational changes which modify the rate and stage of chain termination cannot be ruled out. In order to more clearly understand these aspects the effect of acyl acceptors on purified synthetase must be elucidated and work along these lines is in progress in this laboratory.

Acknowledgments

We thank Dr. Milton Zucker for a critical reading of the manuscript and Linda Brown for technical assistance.

[2] Buckner, J. S., and Kolattukudy, P. E. (1973), unpublished results.

TABLE VIII: Relative Incorporation of [2-^{14}C]Malonyl-CoA into Lipids in Soluble and Microsomal Preparations and the Distribution of Acyl Moieties in the Microsomes.[a]

	Lipid Fraction	Incorp per mg of Protein (cpm × 10^3)	Chain Length (%) 14	16	18
Soluble supernatant	Total lipids	28.2 (61.2)[b]			
Microsomes	Total lipids	82.7 (37.9)			
	Wax ester				
	acyl	18.5	21.5	65.0	13.5
	alcohol	25.8	Tr	90.6	9.4
	Phospholipids	13.6			
	Monoacylglycerol	2.5			
	Diacylglycerol	12.8	27.2	63.4	9.4
	Free fatty acid	9.7			
	Triacylglycerol	2.1			

[a] Soluble supernatant and the microsomal fractions were isolated as described in the text. The reaction mixture was the same as that described under Table VII and the fractionation of the total lipids was as described in the text. For chain length determination total lipids were fractionated into polar and wax ester fractions by thin-layer chromatography in benzene. Both the fractions were transesterified with BF_3–CH_3OH reagent and the purified methyl esters were used for radio gas–liquid chromatography. The wax alcohol fraction was acetylated as described in the text. Radio gas–liquid chromatography was done on coiled stainless steel column (117 × 0.6 cm o.d.) packed with 5% SE-30, on 80–100 mesh Gas Chrom Q at 170° with 90 cm^3/min of argon. Quantitation was done by triangulation. [b] Figures in parentheses indicate the percentage enzyme activity in the cellular fraction.

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Composition and Structure of the O-Specific Side Chain of Endotoxin from *Serratia marcescens* 08†

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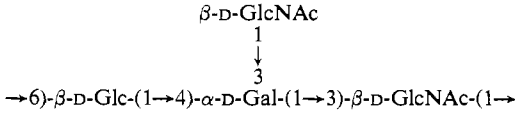
ABSTRACT: The endotoxin complex of *Serratia marcescens* 08 was hydrolyzed by 1% acetic acid, and the O-specific side chain was isolated from the hydrolysate by ion-exchange chromatography on Dowex 1 and gel filtration on Sepharose 4B. The determinations of chemical composition and molecular weight indicated that the purified O-specific side chain was a polysaccharide consisting of D-glucose, D-galactose, and *N*-acetyl-D-glucosamine in a molar ratio of 1:1:2. On the basis of evidence obtained from periodate oxidation, methylation, mass spectrometry, infrared spectroscopy, hydrazinolysis, and partial acid hydrolysis, it was concluded that the O-specific side chain is a polysaccharide consisting of repeating units of a branched tetrasaccharide. The average number of repeating units in the O-specific side chain was estimated to be 17. The structure and anomeric configuration of the repeating unit were identified as



Recently, we demonstrated (Wober and Alaupovic, 1971; Wang and Alaupovic, 1973) that the polysaccharide moieties of endotoxins from a chromogenic and a nonchromogenic strain of *Serratia marcescens* consist of the same structural entities found in *Salmonella* (Lüderitz, 1970), *Escherichia* (Heath *et al.*, 1966; Schmidt *et al.*, 1969), and *Shigella* (Simmons, 1969). To isolate and identify these entities, we hydrolyzed the endotoxins with 1% acetic acid and separated the resulting partially degraded polysaccharide moieties ("degraded polysaccharides") by dialysis or by Sephadex gel filtration into two fractions. These corresponded to the O-specific side chain and oligosaccharide core, respectively. Further studies (Wang and Alaupovic, 1973) revealed that the O-specific side chain from the nonchromogenic strain *S. marcescens* Bizio is a linear polysaccharide which contains as a repeating unit a D-glucose–L-rhamnose disaccharide. This work represented the first structural elucidation of an O-specific side chain from the genus *Serratia* and provided evidence that even disaccharides may form repeating units.

The results presented in this article show that, in contrast to the disaccharide repeating unit of the nonchromogenic strain, the O-specific side chain of the chromogenic strain *S. marcescens* 08 consists of repeating units of a branched tetrasaccharide.

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