Acyl-CoA reductase and acyl-CoA: fatty alcohol acyl transferase in the microsomal preparation from the bovine meibomian gland

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Abstract Biosynthesis of wax esters, one of the two major products of the meibomian gland, was found to be catalyzed mainly by the microsomes of the bovine meibomian gland. The microsomal preparation catalyzed hexadecanoyl-CoA reduction to hexadecanol without any accumulation of the aldehyde intermediate. Maximal rates of reduction occurred at pH 6.5 and required both NADH and NADPH; the latter alone gave considerable rates whereas NADH alone was ineffective. Exogenous hexadecanal reduction catalyzed by the same preparation showed a preference for NADH. The hexadecanoyl-CoA saturation pattern was slightly sigmoidal and concentrations higher than 125 μM inhibited reduction. The fatty alcohol generated from hexadecanoyl-CoA was found as free alcohol and as wax esters. Esterification of hexadecanol to wax esters catalyzed by the meibomian gland microsomal preparation required exogenous acyl-CoA or ATP and CoA and was not affected by exogenous cholesterol. Maximal rates of esterification were observed at neutral pH. Hexadecanoyl-CoA concentrations higher than 125 μM inhibited esterification. Hexadecanol showed a typical substrate saturation pattern with an apparent K_m of 125 μM. Radio gas-liquid chromatography showed that, in the presence of exogenous hexadecanoyl-CoA, hexadecanol gave hexadecyl hexadecanoate whereas in the presence of ATP and CoA both C_{16} and C_{18} endogenous acids were used to esterify the alcohol. Consistent with the composition of the meibomian gland secretion, exogenous acyl-CoA longer than C_{14} and shorter than C_{20} gave maximal rates of esterification of hexadecanol. —**Kolattukudy, P. E. and L. Rogers.** Acyl-CoA reductase and acyl-CoA: fatty alcohol acyl transferase in the microsomal preparation from the bovine meibomian gland. *J. Lipid Res.* 1986. **27**: 404–411.

Supplementary key words wax esters • fatty alcohol • sebaceous gland • microsomal reductase

Meibomian glands, located on the eyelids along the line of the eyelash, secrete lipids to retard evaporation of moisture from the tear film and to perform other functions important to the eye (1, 2). The most abundant components of the human and bovine meibomian secretory lipids are wax esters and sterol esters (3–5). In spite of the importance of these sebaceous glands to the proper functioning of the eye, very little information is available on the biosynthesis of the meibomian secretion. Exogenous acetate was shown to be incorporated into the major secretory lipids by isolated bovine meibomian glands (6). A microsomal preparation from the meibomian gland was shown to catalyze chain elongation of fatty acyl-CoA to produce the very long chain compounds found in meibomian secretion (7). No other biochemical studies have been reported on the enzymology of synthesis of the unique lipids by the meibomian gland. In the present report we describe enzymatic reduction of acyl-CoA to fatty alcohol and its subsequent esterification to wax esters catalyzed by microsomal preparations from bovine meibomian glands.

MATERIALS AND METHODS

Intact steer eyelids were obtained from Colfax Meat Packing, Colfax, WA and kept at −80°C until used. NADH, NADPH, ATP, CoA, palmitoyl-CoA, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and dithioerythritol (DTE) were purchased from Sigma Chemical Company, St. Louis, MO. [1-^{14}C]Hexadecanoic acid (53–58 Ci/mol) and Omnifluor were purchased from New England Nuclear, Boston, MA. BF_3 (14%) in methanol was made by bubbling BF_3 gas into methanol until the weight increased by 14%.

Substrates

[1-^{14}C]Hexadecanoyl-CoA (53 Ci/mol) was prepared by reaction of the acid chloride of [1-^{14}C]hexadecanoic acid with CoA (8). [1-^{14}C]Hexadecanol (58 Ci/mol) was prepared by reduction of [1-^{14}C]hexadecanoic acid with $LiAlH_4$ in anhydrous tetrahydrofuran. [1-^{14}C]Hexa-

Abbreviation: DTE, dithioerythritol.

decanal (58 Ci/mol) was prepared from [1-^{14}C]hexadecanol by oxidation with pyridinium chlorochromate in CH_2Cl_2 (9). The labeled hexadecanol and hexadecanal were purified by thin-layer chromatography on silica gel G with hexane–ethyl ether–formic acid 40:10:1 (v/v) as the developing solvent. Substrate solutions (500 μM for hexadecanol and 150 μM for hexadecanal) were made in water by dispersing the labeled material by sonication (4 × 5 sec) with the needle probe of a Biosonik III sonicator. This method gave stable micellar solutions.

Preparation of enzyme

Meibomian glands were excised from freshly thawed frozen steer eyelids or freshly excised eyelids; both gave identical results. Glands were weighed and homogenized in 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM DTE and 0.25 M sucrose (10 ml/g of tissue). The homogenate was centrifuged for 5 min at 1,000 *g*. The supernatant was centrifuged for 15 min at 12,000 *g* to yield a "mitochondrial" fraction and the supernatant was centrifuged for 90 min at 105,000 *g*. The 105,000 *g* pellet was washed by resuspension in 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM DTE and 0.25 M sucrose and centrifugation for 60 min at 105,000 *g*. The washed 105,000 *g* pellet was resuspended in 20 mM sodium phosphate, pH 7.0, containing 1 mM DTE and 0.25 M sucrose (1 ml buffer/0.3 g original gland weight) and used as the enzyme source for both the esterification and reductase assays. For tentative subcellular localization, the same centrifugation protocol was followed. The pellets (12,000 *g* and 105,000 *g*) were washed by resuspension in buffer and recentrifugation at the appropriate speed (12,000 *g* or 105,000 *g*) and the final pellets were resuspended in a volume of buffer equal to the original volume. All procedures were done at 4°C.

Enzyme assays

Acyl-CoA reductase. Unless otherwise noted, each 0.5 ml of reaction mixture contained 120 μM [1-^{14}C]hexadecanoyl-CoA (sp act 20 Ci/mol), 5 mM NADPH, 5 mM NADH, enzyme (250–350 μg of protein), and 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM DTE and 0.25 M sucrose. The reaction mixture was incubated for 30 min at 30°C and the reaction was stopped by the addition of 100 μl of 6 N HCl. The reaction products were extracted by the method of Folch, Lees, and Sloane Stanley (10) and a small aliquot was assayed for ^{14}C to check for total recovery. The remainder of the sample was transesterified by refluxing it with 14% BF_3 in methanol for 2—3 hr; the recovered reaction products were chromatographed on 0.5-mm layers of silica gel G using hexane–ethyl ether–formic acid 65:35:1 (v/v) as the developing solvent. The silica gel from the primary alcohol region was scraped from the plate and either assayed directly for ^{14}C or the alcohol was eluted from the silica gel for further analysis.

Aldehyde reductase. Each 0.5 ml of reaction mixture contained 60 μM [1-^{14}C]hexadecanal (10–50 Ci/mol), NADPH and/or NADH, enzyme (60 μg of protein), and 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM DTE and 0.25 M sucrose. After incubating the reaction mixtures for 10 min at 30°C, 100 μl of 6 N HCl was added. After extraction of the products by the method of Folch et al. (10), an aliquot of the lipid extract was mixed with nonradioactive standards (hexadecanol, hexadecanoic acid, hexadecanal, and hexadecyl hexadecanoate) and subjected to thin-layer chromatography using hexane–ethyl ether–formic acid 65:35:2 (v/v) as the developing solvent. After spraying the plates with a 0.1% ethanolic solution of dichlorofluorescein, the components were visualized under UV light and the silica gel scraped from each region was either assayed for ^{14}C by liquid scintillation spectrometry or recovered for further study.

Esterification. Unless otherwise noted, each 0.5 ml of reaction mixture contained 100 μM [1-^{14}C]hexadecanol, 100 μM hexadecanoyl-CoA, enzyme (100 μg), and 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM DTE and 0.25 M sucrose. The mixture was incubated for 30 min at 30°C and the reaction was terminated by addition of 100 μl of 6 N HCl. Reaction products were extracted by the method of Folch et al. (10) and the wax ester fraction was isolated by thin-layer chromatography on 0.5 mM silica gel G plates using hexane–ethyl ether–formic acid 40:10:1 (v/v) as the developing solvent as indicated above for the reductase assay. The wax ester region was scraped from the plate and either assayed directly for ^{14}C or the wax esters were eluted for further analysis.

Product identification

A portion of the recovered reaction products was mixed with the mixture of authentic standards indicated above and subjected to thin-layer chromatography on 0.5 mm silica gel G using hexane–ethyl ether–formic acid 75:25:2 (v/v) as the developing solvent. After spraying the plates with dichlorofluorescein and locating the components under UV light, the product distribution was determined by assaying the silica gel scraped from the various regions directly for ^{14}C. The plates were also scanned for radioactivity using a Berthhold scanner. Primary alcohol fractions obtained from acyl-CoA and aldehyde reduction were acetylated using pyridine–acetic anhydride 1:2. The acetylated products were purified by thin-layer chromatography using hexane–ethyl ether 70:30 (v/v) as the developing solvent and analyzed by radio gas–liquid chromatography at 200°C. The wax ester fraction isolated from the esterification reaction mixture by thin-layer chromatography was eluted with ethyl ether and subjected to radio gas–liquid chromatography at a column

temperature of 280°C. Radio gas–liquid chromatography of alcohol acetates and wax esters was done using a Perkin-Elmer Model 801 gas chromatograph equipped with a flame ionization detector and an effluent splitter, attached to a Barber-Coleman radioactivity monitor. A coiled stainless steel column (0.3 cm OD × 198 cm) packed with 5% OV-1 on Gas Chrom Q (80–100 mesh) was used in both cases with a carrier flow rate of 90 ml/min.

Determination of protein

The protein precipitated by trichloroacetate was redissolved in 0.1 M NaOH and analyzed for protein by the method of Lowry et al. (11) using bovine serum albumin as the standard protein.

Determination of radioactivity

Aliquots of products or silica gel containing labeled products were assayed for radioactivity by liquid scintillation spectrophotometry using a Packard Tri-Carb liquid scintillation counter, Model 3255. A solution of 4 g/l of Omnifluor dissolved in 30% ethanol in toluene was used as the scintillation mixture. The counting efficiency of ^{14}C was 75%; the efficiency was not decreased by the inclusion of silica gel and the amounts of dichlorofluorescein contained in the thin-layer chromatographic fractions.

RESULTS

When a cell-free preparation from bovine meibomian gland was incubated with [1-^{14}C]hexadecanoyl-CoA, NADH, and NADPH, and the products were analyzed by thin-layer chromatography after transesterification with BF_3/methanol, a significant portion of the ^{14}C was found to co-migrate with authentic hexadecanol. To determine the subcellular fraction that catalyzes the apparent acyl-CoA reduction, mitochondrial pellets, a microsomal preparation, and the high speed supernatant were separately incubated with radioactive hexadecanoyl-CoA, NADH, and NADPH, and the products were analyzed, after transesterification, by thin-layer chromatography. Based on the amount of fatty alcohol generated, the microsomal fraction contained about three-quarters of the total acyl-CoA reductase activity with about 18% in the mitochondrial pellet and 8% in the high speed supernatant. Since a major part of the acyl-CoA reductase was in the microsomal fraction, this fraction was routinely used for further studies.

Product identification

When the microsomal preparation from the bovine meibomian gland was incubated with [1-^{14}C]hexadecanoyl-CoA, NADH, and NADPH for 30 min and the lipid products were extracted and subjected to thin-layer chromatography, four major radioactive products were found (**Fig. 1**). About 18% of the radioactivity recovered was found in wax esters and 4% in fatty alcohols. To test whether the radioactivity found in the wax ester fraction was in the alcohol portion, this material was subjected to transesterification and the products were analyzed by thin-layer chromatography. More than one-third of the total radioactivity was found to be in the fatty alcohol portion, the remaining being in the methyl ester fraction. Thus, between the free fatty alcohol fraction and the alcohol portion of the wax esters, 10.3% of the total radioactivity was recovered. Thus, the microsomal preparations catalyzed acyl-CoA reduction to alcohol and an acyl-CoA alcohol transacylase catalyzed esterification of a large portion of the alcohols generated. As might be expected, hexadecanoyl-CoA was also incorporated into polar lipids which remained in the origin accounting for about 16% of the total radioactivity. That this radioactivity represented acyl lipids was shown by the fact that more than 95% of the radioactivity in the origin was recovered in the fatty acid methyl ester fraction when the material was subjected to transesterification, followed by thin-layer chromatography. A little over one-half of the total radioactivity was recovered in free fatty acids, indicating that hexadecanoyl-CoA also underwent hydrolysis. Since the alcohol generated by the acyl-CoA reductase was distributed between free alcohols and wax esters, for routine assays, the total products were subjected to transesterification and the total alcohols were recovered by thin-layer chromatography and assayed for radioactivity.

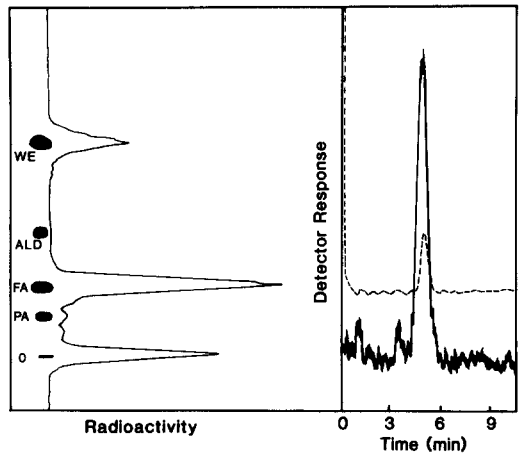

Fig. 1. Left: thin-layer chromatogram of the products generated from [1-^{14}C]hexadecanoyl-CoA, NADH, and NADPH by the microsomal preparation from the bovine meibomian gland. Right: radio gas–liquid chromatogram of fatty alcohols (as acetates) generated by BF_3/methanol treatment of the total products shown on the left. The dashed line represents the flame ionization detector response from the co-injected hexadecanol acetate. Location of authentic wax ester (WE), hexadecanal (ALD), hexadecanoic acid (FA), hexadecanol (PA) and origin (O) are indicated. Chromatographic conditions are described in the text.

That the incorporation of hexadecanoyl-CoA into the fatty alcohol was entirely a direct conversion, not involving degradation, was demonstrated by the fact that radio gas–liquid chromatographic analysis of the products showed that all of the radioactivity in the alcohol fraction was in hexadecanol (Fig. 1). Thin-layer chromatographic analysis revealed barely detectable amounts of aldehydes and therefore these aldehydes were not further analyzed. Presumably, the aldehyde represented the intermediate in the conversion of the acyl-CoA to the alcohol.

Effect of protein concentration, incubation time, and pH on acyl-CoA reduction

Protein concentration-dependence of acyl-CoA reduction showed a sigmoidal pattern (**Fig. 2A**), most probably because the detergent effect of hexadecanoyl-CoA inhibits the enzyme activity when low levels of microsomal protein are used, as previously observed with other particulate acyl-CoA reductase systems (12–14). Acyl-CoA reduction was rectilinear up to 45 min of incubation (Fig. 2B). The acyl-CoA reductase showed a pH optimum near 6.5 (Fig. 2C). This optimum is similar to that observed for several other acyl-CoA reductases (14–18), but more acidic (13) and more basic (19, 20) pH optima have been reported for some cases.

Cofactor and substrate concentration dependence of acyl-CoA reduction

NADPH was an essential cofactor, with NADH being completely ineffective (**Fig. 3A**). However, addition of NADH together with NADPH gave rates higher than that obtained with NADPH alone. Addition of ATP and CoA in addition to the reducing agents tended to increase the rates obtained, presumably because of regeneration of hexadecanoyl-CoA from the free hexadecanoic acid generated by the thioesterase activity found in the microsomal preparation (data not shown). The addition

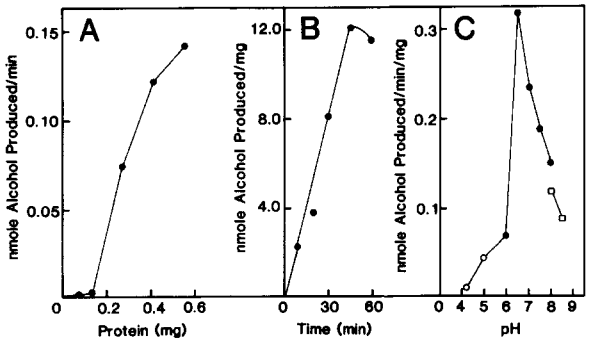

Fig. 2. Effect of protein concentration (A), incubation time (B), and pH (C) on the formation of hexadecanol from hexadecanoyl-CoA in the presence of NADH and NADPH by the microsomal preparation from bovine meibomian gland. Experimental details are described in the text. Citrate-phosphate (○), phosphate (●), and Tris-HCl (□) buffers were used as indicated for the pH experiment.

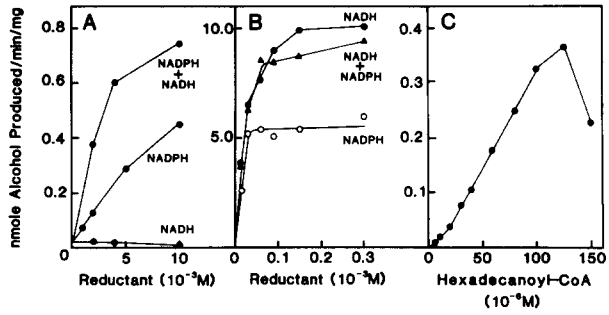

Fig. 3. Effect of reduced pyridine nucleotide concentration on the rate of reduction of hexadecanoyl-CoA to hexadecanol (A), hexadecanal to hexadecanol (B), and the effect of hexadecanoyl-CoA concentration on its reduction to the alcohol in the presence of NADH and NADPH (C) catalyzed by the microsomal preparation from bovine meibomian glands. Experimental conditions are described in the text.

of glucose-6-phosphate and glucose-6-phosphate dehydrogenase also tended to increase the yield of the alcohol as might be expected of regeneration of NADPH.

Since NADH enhanced the rates obtained with NADPH alone, we determined the effects of various concentrations of the two reductants on the conversion of hexadecanoyl-CoA to hexadecanol (Fig. 3A). As the concentration of NADPH increased, the rate of conversion of hexadecanoyl-CoA to the alcohol increased, whereas NADH alone at such concentrations gave no detectable rates of formation of the alcohol. At each concentration tested, up to 10 mM, addition of NADH stimulated acyl-CoA reduction obtained by NADPH alone. That the apparent synergistic effect of NADH was not due to its supplementation of reducing power, which might have been exhausted by a NADPH oxidase, was shown by the observations that there was little NADPH oxidase activity that could be detected in the microsomal preparations that were used.

To test the possibility that NADPH was absolutely necessary for acyl-CoA reduction to the aldehyde stage and that NADH was the preferred reductant to convert the aldehyde to the alcohol, [1-^{14}C]hexadecanal was tested as a substrate. Incubation of the microsomal preparation with labeled hexadecanal followed by analysis of the products, as indicated above for acyl-CoA reduction, showed that the fatty aldehyde was readily converted to fatty alcohol (data not shown). This aldehyde reductase activity showed a preference for NADH, although NADPH alone also gave about half the rate obtained with NADH alone (Fig. 3B). In this case, a mixture of NADH and NADPH gave the same rate as that obtained with NADH alone. In any case, it is clear that the aldehyde reductase showed a preference for NADH as the reductant.

As the concentration of hexadecanoyl-CoA increased up to about 125 μM, the rate of alcohol formation increased and subsequent increases in hexadecanoyl-CoA concentration caused a marked inhibition of the enzyme

activity (Fig. 3C). This pattern was unaffected by the pyridine nucleotide concentration up to 10 mM. The substrate saturation pattern did show a slight sigmoidal character, presumably because at the low hexadecanoyl-CoA concentrations the substrate binds as a detergent to the microsomal membrane. Thus, the effective concentration of the available substrate would be less than that actually added. In any case, determination of the classical K_m was not done because of this sigmoidal nature.

Subcellular location of fatty alcohol esterifying enzyme

To study fatty alcohol esterifying activity which would give rise to wax esters, one of the major unique products of meibomian glands, [1-^{14}C]hexadecanol, was used as a substrate. After incubation with the enzyme preparation, the products were subjected to thin-layer chromatography and the radioactivity in the wax ester fraction was assayed. Differential centrifugation of meibomian gland extracts followed by assays with the resulting subcellular fractions revealed that about two-thirds of the esterifying activity was in the microsomal fraction; the remaining activity was equally distributed between the mitochondrial fraction and the high speed supernatant. Therefore, for further experiments microsomal fractions were routinely used as the source of esterifying enzyme.

Esterification of labeled hexadecanol required the addition of either hexadecanoyl-CoA or ATP and CoA and was not affected by exogenous cholesterol. The protein concentration-dependence of the rate of esterification was sigmoidal when palmitoyl-CoA was used as the substrate (**Fig. 4A**). On the other hand, when ATP and CoA were provided, the rate of esterification of hexadecanol increased rectilinearly with increasing protein concentrations (Fig. 4A). Probably endogenous fatty acids available for activation were limiting when ATP and CoA were used. The detergent effect of hexadecanoyl-CoA probably inhibits the esterification reaction when the protein concentration is very low, and when the protein concentration is very high hexadecanoyl-CoA binds to the microsomal membranes as a detergent and thus is prevented from participating in the reaction; thus the rate does not show any further increase. Although ATP and CoA gave linear increases with protein concentration, the esterification obtained with these substrates would involve both the activation of endogenous fatty acids and the esterification of the fatty alcohol using the activated fatty acids as the substrate. To avoid this complication and to study the esterification reaction per se, we chose to use hexadecanoyl-CoA as the substrate and used protein concentrations that gave increasing rates with increasing amounts of protein.

Esterification of hexadecanol showed a rectilinear increase with increasing incubation time up to 1 hr, either with ATP and CoA or with hexadecanoyl-CoA as the substrate. Optimal rates of esterification were obtained at near neutral pH, and this pH profile (Fig. 4B) was similar to that observed with a particulate preparation from avian uropygial gland (13).

The effect on the rate of hexadecanol esterification of hexadecanoyl-CoA concentration is shown in Fig. 4C; the severe inhibition of esterification was observed at the high concentrations because of the detergent effect of the substrate (Fig. 4C). From the typical substrate saturation pattern obtained with hexadecanol (Fig. 4D), double reciprocal plots were constructed and a K_m of 125 μM was calculated for hexadecanol.

Incubation of hexadecanol with the meibomian microsomal preparation followed by thin-layer chromatographic analysis of the products revealed only one major product that had an R_f identical to that of wax esters; no other products could be detected (**Fig. 5, left**). Hexadecanoyl-CoA gave a much higher amount of wax ester

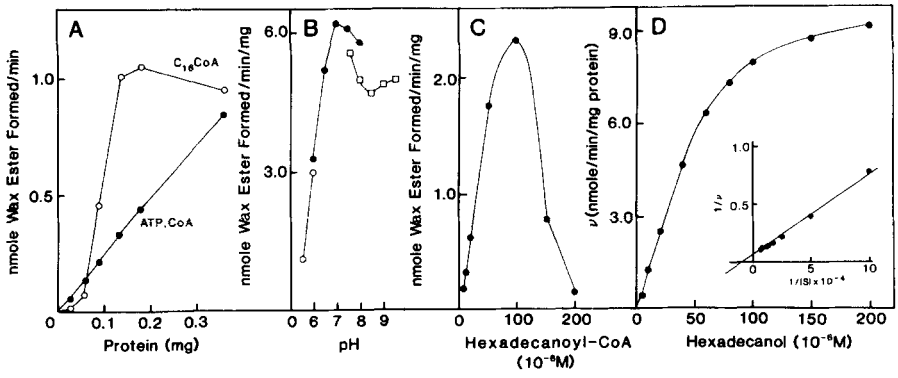

Fig. 4. Effect of bovine microsomal protein concentration (A) on the rate of incorporation of hexadecanol to wax esters in the presence of hexadecanoyl-CoA or ATP and CoA; effect of pH (B), concentration of hexadecanoyl-CoA (C), and hexadecanol (D) on the esterification of hexadecanol with hexadecanoyl-CoA catalyzed by the bovine meibomian gland microsomes. Experimental details are described in the text. (○) Citrate-phosphate; (●) Na phosphate; (□) Tris-HCl.

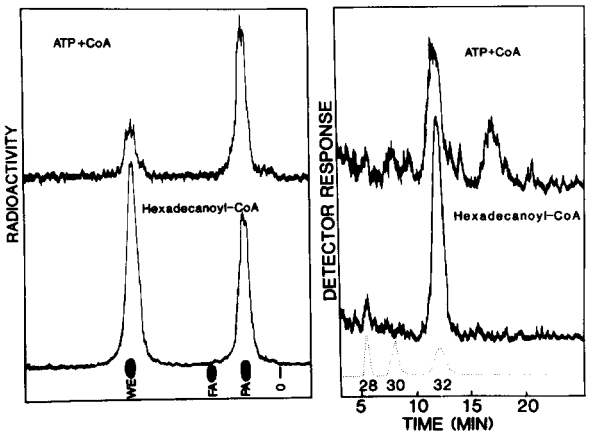

Fig. 5. Radio thin-layer chromatogram of the products generated from [1-^{14}C]hexadecanol and ATP with CoA or hexadecanoyl-CoA by the bovine meibomian gland microsomes (left) and radio gas-liquid chromatogram of the wax esters obtained from this thin-layer chromatography (right). The location of authentic wax esters (WE), hexadecanoic acid (FA), hexadecanol (PA), and origin (O) are indicated. The dashed line represents the flame ionization detector response from the co-injected hexadecyl esters of C_{12}, C_{14}, and C_{16}.

when compared to that obtained with ATP and CoA. Radio gas-liquid chromatographic analysis of the product showed that the major radioactive wax ester had a retention time identical to that of hexadecyl hexadecanoate when hexadecanoyl-CoA was used as a substrate (Fig. 5, right). Small amounts of radioactivity were also found in two components that showed much lower retention time (not shown in the figure because a different chromatographic condition had to be used to detect them). When ATP and CoA were used as the substrates, the endogenous fatty acids must have been activated and used as the acyl donor for wax ester synthesis. Under these conditions, the wax esters formed were composed of hexadecyl hexadecanoate and another component that showed a retention time equivalent to that of hexadecyl octodecanoate. Small amounts of the more volatile components, similar to those observed when hexadecanoyl-CoA was used as a substrate, were obtained also when ATP and CoA were used. These must be short chain acyl esters of the exogenous hexadecanol but were not identified because they are not significant components of the meibomian lipids. In any case, it appears that exogenous hexadecanol was esterified to C_{16} and C_{18} fatty acids from an endogenous source. When different exogenous acyl-CoAs were tested, $C_{15:0}$, $C_{16:0}$, and $C_{18:1}$ acyl-CoA gave maximal rates of hexadecanol esterification (**Fig. 6**).

DISCUSSION

Since the first demonstration of acyl-CoA reductase activity in the *Euglena* cell-free preparations (15), such reductase activities have been found in a variety of organisms in both the plant and animal kingdoms (13–21). The first acyl-CoA reductase that was obtained in soluble form was from the higher plant *Brassica oleracea* (16) and, in this case, two separable protein fractions catalyzed the conversion of acyl-CoA to fatty alcohol. One fraction catalyzed acyl-CoA reduction to the aldehyde and another converted the aldehyde to the alcohol. A similar pair of reductases was subsequently obtained from bovine cardiac muscle (19, 22), and the acyl-CoA reductase found in *Clostridium butyricum* (20) was quite similar to that obtained from both the plant and animal sources. In the *Euglena,* on the other hand, the acyl-CoA reductase is localized in the microsomal membranes (23) and in this case evidence for the formation of an aldehyde was obtained only indirectly by trapping the presumed aldehyde intermediate as phenylhydrazones (15). The mammalian acyl-CoA reductases (14, 18, 24) and the avian acyl-CoA reductase (13) appear to be quite similar to that described for *Euglena.* In these cases no aldehyde intermediate accumulated. The acyl-CoA reductase in the meibomian gland is also located mainly in the microsomal membranes and no aldehyde was found to accumulate. The microsomal location of acyl-CoA reductase is beginning to appear as the usual occurrence and in such cases the conversion of acyl-CoA to the alcohol occurs without accumulation of any free aldehyde intermediate.

The cofactor requirement for the present acyl-CoA reductase appears to be rather unusual. The soluble reductases seem to generally use NADH for the acyl-CoA reduction and NADPH for the aldehyde reduction (16, 19, 20, 22). The avian and mammalian microsomal acyl-CoA reductases use NADPH as the preferred reductant and generate fatty alcohol (13, 14, 18). On the other hand, a mixture of NADH and NADPH gave the optimal rate of reduction in the present case. It is possible that the present reductase system uses NADPH for the first step to generate the aldehyde, which might then require

Fig. 6. Rates of esterification of hexadecanol with different exogenous acyl-CoAs. Assays were done as described in the text with 80 μM CoA esters.

NADH for more effective conversion to the alcohol. The results obtained with hexadecanal support this hypothesis, even though a comparison of products generated in the presence of NADPH alone with those generated in the presence of NADPH and NADH revealed no accumulation of aldehyde in the presence of NADPH alone. This result may suggest that the aldehyde intermediate might be enzyme-bound, possibly as a thiohemiacetal, and NADH might more effectively remove this aldehyde from the enzyme resulting in a more rapid overall rate of reduction in the presence of both NADH and NADPH. Such an enzyme-bound intermediate could also explain why the aldehyde does not accumulate in the present system just as in the other animal systems previously examined.

Three types of acyl donors have been demonstrated to be involved in wax ester synthesis (25). Under physiological conditions, acyl-CoA or acyl lipids probably provide the acyl group for wax ester biosynthesis, although under certain experimental conditions, free fatty acids can also be incorporated into wax esters without the involvement of an acyl intermediate (25, 26). In the present case acyl-CoA appears to be a good substrate for esterification, and the esterification observed in the presence of ATP and CoA most probably involves acyl-CoA generated by activation of endogenous fatty acids. However, the possibility of involvement of an acyl lipid intermediate cannot be completely ruled out. Radio gas-liquid chromatography of the products demonstrated that when hexadecanoyl-CoA was used as the substrate, the major product obtained was hexadecyl hexadecanoate, whereas when ATP and CoA were added to activate endogenous fatty acids, C_{18} fatty acids also were esterified. However, much longer fatty acids did not participate in the esterification process. The specificity of the esterifying enzyme observed with exogenous acyl-CoA in the present study is clearly consistent with the observation that the naturally occurring wax esters in the meibomian gland contain C_{14} to C_{18} and normal and methyl branched acids, whereas the sterol esters found in the same gland are types of chains composed of much longer fatty acids (5, 6).

The subcellular localization of the esterifying activity appears to be the same in most biological systems so far examined. As was observed in the present study with meibomian glands, the microsomal membranes catalyzed wax ester synthesis in *Euglena* (23), in mammalian (27–29) and avian tissues (such as the uropygial glands) (13), insects (30), and marine organisms (31). The occurrence of acyl-CoA reductase and fatty alcohol acyl-CoA transacylase in the same membrane could allow a functional coupling between the two enzyme systems. Thus, in the bovine meibomian glands, fatty acids generated by the cytoplasmic synthase, which was already shown to be immunologically identical to the fatty acid synthase in other organs (7), can provide the fatty acids to the microsomal membranes. In the microsomal membranes, these fatty acids are elongated using malonyl-CoA and NADPH as the substrates as previously demonstrated (7) and these fatty acids are then reduced by the acyl-CoA reductase to the alcohol and the alcohols are esterified to the wax esters. Such a functional coupling between the production of the acyl chains, reduction, and esterification has been previously demonstrated with microsomal membranes from *Euglena* (23). The was esters could be transported into the lumen of the microsomal membranes by this process and, as the wax esters accumulate, these organelles could become fat bodies and these fat bodies would subsequently fuse to form larger fat bodies as observed for the avian uropygial gland (32). Ultimately, the cells would lyse and empty their contents into the lumen through which the secretion would reach the eye. The observation that in sebaceous glands the onset of lipogenesis is preceded by proliferation of membranes is consistent with such a hypothesis (33, 34). The wax esters, together with the other components found in the meibomian secretion, protect the eye mainly by preventing the evaporation of the preocular tear film. They also protect the eye lid surface by coating it and prevent drying of the corneal surface during sleep. How the composition of the complex mixture of the meibomian secretion is specifically suited for these and possibly other functions is not known.

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