The Enzymatic Synthesis of Wax in Liver*

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SUMMARY

An enzyme system present in both mammalian and dogfish liver was found to catalyze the conversion of cetyl alcohol to wax.

The product was isolated by column chromatography and repeated thin layer chromatography. It was identified by thin layer chromatography, by analysis of the hydrolysis products, and by gas chromatography.

Microsomal and supernatant fractions of liver were active. Synthesis was stimulated by detergents such as Triton X-100, Tween 20, and bile salts. Wax formation occurred without activation, i.e. formation of acyl coenzyme A. With an enzyme preparation from which lipids had been substantially removed wax synthesis required both long chain fatty acids and cetyl alcohol.

It is postulated that the synthesis of waxes from long chain alcohols and fatty acids without activation is possible in an environment from which water is essentially excluded and can be envisioned as resulting from the interaction of two nonpolar compounds and an enzyme in a micellar state.

In the course of a study in this laboratory of the metabolism of cetyl alcohol in the liver of the dogfish, Squalus acanthias, it was observed that this compound is converted extensively to another material. On investigation, the latter was found to be an ester of cetyl alcohol and long chain fatty acids (wax). The enzymatic formation of waxes has been previously described with extracts of porcine pancreas (1, 2). The present paper describes enzymatic formation of waxes by an enzyme found in mammalian and dogfish liver.

METHODS

Live dogfish were obtained through the facilities of the Duke University Marine Laboratories at Beaufort, North Carolina.

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Cetyl alcohol-14C was obtained from Nuclear-Chicago, and was purified by thin layer chromatography on silica gel. For this purpose the plates were developed in hexane-ether-acetic acid, 80:20:1, and the cetyl alcohol-14C was eluted from the silica gel with ethyl ether. Palmitic acid-14C, carboxyl-labeled triolein-14C, and ethanol-14C were obtained from New England Nuclear and were more than 99% pure by thin layer and gas chromatography. Diisopropyl fluorophosphate was obtained from Merck. Standard fatty acid methyl esters, alcohols, and fatty acids were obtained from Applied Science Laboratories.

Homogenates of dogfish liver were prepared in 2 volumes of 0.1 M Tris buffer, pH 7.8, containing 2% urea, or in 0.1 M phosphate buffer, pH 7.8. The homogenates were centrifuged at 8000 x g for 20 min at 3° to remove cell debris, mitochondria, and supernatant fat. This preparation contained 40 mg of protein per ml. Similar homogenates were prepared from pig and rat liver without urea. Dogfish liver microsomes were obtained by the method of Hogeboom, Schneider, and Pallade (4).

An enzyme from which lipids had been partially removed was prepared as follows: the preparation from dogfish liver homogenate described above was centrifuged again to remove as much lipid material as possible and fractionated by the addition of solid ammonium sulfate. The material obtained at 40% saturation was centrifuged at 15,000 x g for 3 hours at 3° and reprecipitated with 40% ammonium sulfate. The precipitate was resuspended in a volume of 0.1 M phosphate buffer, pH 7.8, equal to the volume of the homogenate (34 mg of protein per ml). This fraction contained a considerable amount of insoluble enzymatically active material. (Fractions obtained with higher concentrations of ammonium sulfate contained little enzyme activity.) A portion of the suspension was extracted for 30 min at 2° with an equal volume of ethyl ether. The mixture was centrifuged to separate the phases, and the supernatant ether containing the extracted lipid was discarded. The lower phase, containing soluble and insoluble enzymatic activity, was resuspended with a glass homogenizer. The ether extraction was repeated three more times, once overnight. Ether in solution was removed at reduced pressures at 2°.

Incubation media were extracted by the method of Dole (5). The lipid extracts were dried under nitrogen and chromatographed on thin layers of Silica Gel H (Brinkmann Instruments, Inc., Westbury, New York). Plates were developed in benzene. (RF of standard cetyl palmitate and the product of the enzymatic reaction was approximately 0.6.) Thin layer chromatography was also performed on aluminum oxide with 1% benzene in hexane (RF of the above substances, 0.5) (6). Both of these methods
are capable of separating waxes from cholesterol esters, squalene, and vitamin A esters. Spots were visualized by iodine vapor or by fluorescein spray. The relationship of radioactivity to visualized spots was determined as follows: the plates were sprayed with Neatan plastic spray (Brinkmann Instruments, Inc.) and the vertical lanes containing the separated compounds were cut into 4-mm wide horizontal strips. The plasticized strips were placed in scintillation vials, phosphor solution was added, and the radioactivity was determined in a scintillation counter. Radioactivity is plotted as a function of distance from the origin and the positions of unknowns and reference compounds are noted as horizontal bars (7).

Column silicic acid chromatography was performed by the method of Hirsch and Ahrens (8).

Free alcohols and fatty acid methyl esters were separated by gas-liquid chromatography with the use of a Warner-Chilcott model 1600 apparatus with dual hydrogen flame ionization or a strontium 90 ionization detector. The column packed with 15% ethylene glycol succinate on Anakrom AB (90 to 70 mesh) was 30 inches long with 0.1 inch internal diameter. Column temperature was 160° and the carrier flow was 40 ml per min. It was not necessary to chromatograph the alcohols as acetyl derivatives provided the columns were conditioned for a sufficiently long period of time (1 week).

Authentic cetyl palmitate was prepared by the method of Haati (9), isolated by chromatography on Silica Gel H with benzene as solvent, and eluted from the Silica gel with diethyl ether. The product gave a single gas-liquid chromatographic peak with a retention time of 24 min. A portion of the cetyl palmitate was transmethylated with boron trifluoride-methanol by the method of Morrison and Smith (10) and the mixture was analyzed by gas-liquid chromatography. It contained equimolar amounts of cetyl alcohol and methyl palmitate.

For identification purposes, larger quantities of cetyl esters were obtained from the enzymatic reaction as follows: 100 ml of dogfish liver homogenate were incubated with 50 mg of cetyl alcohol-14C (5,000,000 cpm) and 500 mg of Triton X-100. The mixture was incubated overnight at 25° and extracted by the method of Dole (5). The extracted lipids were applied as a band to four 0.75-mm silica gel plates and developed with benzene. The areas corresponding to standard cetyl palmitate were scraped off the plates and eluted with ethyl ether. The crude lipid mixture obtained was rechromatographed on two 0.5-mm aluminum oxide plates together with standard cetyl palmitate, cholesterol palmitate, and vitamin A palmitate, and developed in 1% benzene in hexane. This procedure separated the unknown from residual contaminating cholesterol esters and vitamin A esters. The wax was recovered and a final purification was made on silica gel eluted with benzene. A single band was detected with the same RF as synthetic cetyl palmitate.

RESULTS

Synthesis of Wax by Dogfish Liver Homogenate

One milliliter of dogfish liver homogenate, free of debris, mitochondria, and excess lipid, was incubated at 20° for 8 hours with 1 μmole of cetyl alcohol-14C (500,000 cpm) and 6 mg of Triton X-100. Thin layer chromatography of the lipid extracted from the incubation mixture revealed a large radioactive peak of nonpolar material which migrated just below cholesterol esters on silica gel developed in benzene (Fig. 1). After 8 hours of incubation, active preparations yielded about 0.2 μmole of the unknown product calculated from the specific activity of cetyl alcohol-14C. Enzyme immersed in boiling water for 15 sec showed no activity. Fig. 2 shows formation of the compound with time with the use of heated and active enzyme. The product was separable from palmitic acid, palmitaldehyde, and glyceryl ether on silica plates developed with hexane-ethyl ether-acetic acid, 85:15:1. The compound had the same RF as standard cetyl palmitate on silica gel eluted with benzene, hexane, toluene, or...
Identification of Product

A portion of the cetyl esters isolated as described under "Methods" was hydrolyzed at 70°C in a sealed tube for 4 hours with 2 N KOH in 50% ethanol. A fraction of this material was applied to a silica gel plate and developed in hexane–ether–acetic acid, 80:20:1, together with reference cholesterol, cetyl alcohol, and palmitic acid. The results indicated that the unknown compound was partially hydrolyzed to cetyl alcohol and fatty acid. All of the radioactivity remained in the unhydrolyzed compound and in the spot corresponding to cetyl alcohol. Of the radioactivity 27% remained in the unhydrolyzed compound and 73% was recovered in the area corresponding to cetyl alcohol. Another portion of the wax was transmethylated with boron trifluoride-methanol. A small fraction of the methylated mixture was chromatographed by thin layer chromatography with hexane–ethyl ether–acetic acid, 90:10:1. Two spots were observed which corresponded to standard cetyl alcohol and methyl oleate. No other spots were detected.

The mixture containing free alcohol and fatty acid methyl esters was examined by gas chromatography on an ethylene glycol succinate column at a temperature of 100°C. The principal peaks obtained were the methyl esters of palmitic, palmitoleic, stearic, oleic, two unidentified acids (probably 20- and 22-carbon unsaturates), and free cetyl alcohol. With reference to quantitative standards, the total amount of fatty acid in millimicromoles was very close to the quantity of cetyl alcohol. In one experiment 36 μmole of cetyl alcohol and 33.6 μmole of total fatty acid methyl esters were measured and in another 37.7 μmole of alcohol and 38.3 μmole of methyl esters (Fig. 3).

Gas chromatography of the unhydrolyzed wax at 200°C yielded several broad, poorly separated peaks, one of which had the retention time of cetyl palmitate. Free cetyl alcohol was not present.

Detection of Naturally Occurring Fatty Alcohol in Dogfish Liver Lipids

When the alcohol fraction obtained from enzymatically synthesized wax was examined by gas chromatography with a 35-fold increase in electrometer sensitivity a number of peaks in addition to cetyl alcohol were found, two of which had exactly the same retention time as stearyl and oleyl alcohols. The alcohol fraction was then acetylated by the method of Farquhar (11). The two peaks tentatively identified as stearyl and oleyl alcohol now had the same retention time as standard acetylated derivatives of stearyl and oleyl alcohols. The amount of these other alcohols was less than 1% of the cetyl alcohol present and was small enough so that the peaks could not be detected at electrometer sensitivities which gave full scale deflection of the cetyl alcohol. These results suggest that very small quantities of wax might be present in dogfish liver itself. Under the same conditions, the commercial cetyl alcohol-14C did not contain other peaks.

Long chain alcohols were detected in dogfish liver lipids as follows: 50 ml of the untreated enzyme preparation (i.e., from which lipids had not been removed) and 5 ml of oil (supernatant lipid of dogfish liver homogenate) were extracted separately by the method of Dole (5), saponified, and re-extracted. Alcohols in the nonsaponifiable lipids were separated by thin layer chromatography on silica gel with hexane–ethyl ether–acetic acid, 70:30:1. Each fraction was examined by gas chromatography before and after acetylation. Trace quantities of cetyl, stearyl, and oleyl alcohols were identified. The total amount of alcohol was estimated to be about 1 μg per ml of homogenate and 20 μg per g of shark liver oil.

Formation of Wax from Fatty Acids and Cetyl Alcohol

Although the results of the isolation and hydrolysis of the enzymatically formed compound indicated that it was composed of cetyl alcohol and long chain fatty acid esters, incorporation of palmitate-14C into the same compound was relatively small, and initially a fatty acid requirement could not be satisfactorily established. This was thought to be due to the large amount of free fatty acid present in the enzyme preparation. In addition, the quantity of fatty acid was increased during the course of incubation by the presence of lipase activity. However, by the use of
the enzyme preparation from which the lipids had been partially removed it was possible to show a large increase in the incorporation of palmitate-14C into wax (Table I). Thin layer chromatography revealed that the compound labeled with cetyl alcohol-14C and the compound labeled with palmitic acid-14C migrated in exactly the same location. It was also shown that incorporation of cetyl alcohol was increased by the addition of unlabeled oleic acid or palmitic acid and that the incorporation of palmitic acid-14C did not occur in the absence of added unlabeled cetyl alcohol (Table I).

Effect of Added Cofactors and Dipropyl Fluorophosphate

Neither the rate nor the extent of the reaction was influenced by ATP, CoA, Mg++, or CTP, so that no requirement for activation or cofactors could be shown either in the homogenate or the enzyme preparation treated for lipid removal. With the use of dogfish liver microsomes it was possible to show triglyceride synthesis requiring added CoA, ATP, and Mg++. These microsomes were able to catalyze wax formation equally well in the presence or absence of the cofactors needed for the formation of fatty acid acyl-CoA.

Enzyme with lipid removed was incubated with cetyl alcohol, Triton, and carboxyl-labeled triolein-14C. Triolein-14C fatty acid was incorporated less than half as well as palmitate-14C although about 75% hydrolysis of triolein to fatty acid and lower glycerides took place. Carboxyl-labelled tripalmitin was also a very poor substrate, and its fatty acid was incorporated only 10% as well as palmitate on a molar basis. Addition of calcium ion did not stimulate the reaction as might be expected for a lytic reaction as are all reactions which do not involve activated intermediates. Bergström and Borgrström (20) have discussed the conditions that are necessary for the reversal of a hydrolytic reaction to proceed at a significant rate. The conditions involve formation of micelles which are closely associated with the catalytic site of the enzyme. These micelles have a lower water content than the bulk of the medium and contain a high concentration of reactants (with the fatty acid predominantly nonionized). When one of the reactants is polar (such as glycerol) it must be present in high concentrations in the aqueous phase. Since the relative rates of the synthetic and hydrolytic reactions will depend on the exact composition of the phase surrounding the catalytic site of the enzyme it may be possible to favor one reaction or the other by changing experimental conditions. Inside the cell where many enzymes are localized in lipid matrices the water content may be at a low enough level that activation reactions would not be required for formation of ester linkages between sufficiently nonpolar reactants.

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REFERENCES

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