Biosynthesis of Alkyldiacylglycerols and Triacylglycerols in a Cell-Free System from the Liver of Dogfish (*Squalus acanthias*)*

Donald C. Malins† and John R. Sargent

ABSTRACT: A cell-free preparation of dogfish (*Squalus acanthias*) liver was incubated with [1-^{14}C]oleic acid and [9,10-^{3}H]-oleyl alcohol to study the relative rates of biosynthesis of triacylglycerols and alkyldiacylglycerols. The biosynthesis of triacylglycerols greatly exceeded that of alkyldiacylglycerols. Furthermore, radioactivity from both precursors was significantly higher in acyl chains of alkyldiacylglycerols than in alkyl chains. Fatty alochol is extensively oxidized to fatty acid in this system; whereas fatty acid is reduced to fatty alcohol to only a slight extent. Nevertheless, fatty alcohol is strongly favored over fatty acid as a precursor of the *O*-alkyl bond of alkyldiacylglycerols. The data may reflect regulation of alkyldiacylglycerol metabolism *via* enzymes controlling the equilibrium fatty acid $\rightleftarrows$ fatty alcohol.

The liver of dogfish (*Squalus acanthias*) offers certain advantages in studying the metabolism of glycerolipids. Notably, more than one-half of the wet weight of normal liver consists of oil composed largely of triacylglycerols and alkyldiacylglycerols (Malins *et al.*, 1965).

The mechanism of biosynthesis of the *O*-alkyl bond in lipids has recently been elucidated in cell-free systems (Snyder *et al.*, 1969a,b, 1970a,b; Hajra, 1969). So far, however, little is known about the relative rates of biosynthesis of ester and ether linkages, particularly with respect to the overall levels of triacylglycerols and alkyldiacylglycerols within cells.

The present work provides evidence that in a cell-free preparation from the liver of normal dogfish, the biosynthesis of ester linkages greatly exceeds the biosynthesis of ether linkages. Furthermore, fatty alcohol alone serves as a precursor for the small amounts of *O*-alkyl bonds formed. Also, enzymes controlling the equilibrium fatty acid $\rightleftarrows$ fatty alcohol may regulate the synthesis of alkyldiacylglycerols and triacylglycerols.

Experimental Section

Materials. A sample of [9,10-^{3}H]oleic acid (750 mCi/mmole), purchased from the Radiochemical Centre, Amersham, Bucks, England, was reduced with lithium aluminum hydride to [9,10-^{3}H]oleyl alcohol as described by Gauglitz and Malins (1960); [1-^{14}C]oleic acid (54 mCi/mmole) was purchased from New England Nuclear Corp., Boston, Mass. The following materials were obtained from the Boehringer Corp. Ltd. (London): Na_2ATP, Na_2NADP, Na_4NADPH, coenzyme A, and crystalline glucose 6-phosphate dehydrogenase. The Sigma Chemical Co., Ltd. (London), supplied the following materials: DL-α-glycerophosphate, sodium salt; D-glucose 6-phosphate, sodium salt; reduced glutathione; DL-glyceraldehyde 3-phosphate, diethyl acetal, monobarium salt; crystalline triose phosphate isomerase; lipid standards including oleic acid, oleyl alcohol, triolein, and selachyl alcohol.

Dogfish. Adult, male dogfish (*Squalus acanthias*) were captured from an indigenous population in a sea loch on the West coast of Scotland and were maintained in an aquarium at 14° for several days. The fish did not accept food during this period.

Preparation of the Cell Fraction. Dogfish were killed by decapitation and their livers were immediately transferred to ice-cold 0.25 M sucrose containing 0.1 M potassium phosphate buffer, pH 7.0 (medium A). Portions of liver (8 g) were chopped finely and homogenized with 20 ml of medium A in a glass-Teflon homogenizer. The homogenate was centrifuged at $1.6 \times 10^2 g$, to yield a nuclear pellet, which was discarded. The supernatant was further centrifuged at $1.5 \times 10^5 g$ to sediment a combined mitochondrial–microsomal fraction. The latter was overlaid by a clear supernatant fraction which was in turn overlaid by a large oil fraction. The clear supernatant fraction was withdrawn by piercing a hole in the tube just above the pellet with a syringe needle. This method allowed the supernatant to be obtained almost quantitatively and free from oil. The pellet was then suspended with the supernatant and homogenized. The final suspension, containing 13.1 mg of protein/ml (Lowry *et al.*, 1951), represented essentially the complete homogenate less the nuclear fraction and oil. The entire process was carried out at 2–4°.

Incubation of the Cell-Free Fraction. The following cofactors were dissolved in medium A: $MgCl_2$, 14.0 mM; ATP, 14.0 mM; coenzyme A, 0.2 mM; reduced glutathione, 5.0 mM; DL-glyceraldehyde 3-phosphate, 30.0 mM; DL-α-glycerophosphate, 30.0 mM; and triose phosphate isomerase, 400 μg/ml. A portion (10 ml) of this solution was added to 10 ml of the cell-free fraction prepared above. Oleyl alcohol (50 μCi; 750 mCi/mmole) and oleic acid (10 μCi; 54 mCi/mmole) were added in 0.4 ml of 1% Triton X-100. Flasks were charged with nitrogen to minimize aerobic respiration, stoppered, and incubated at 14°. After 60 min each flask was supplemented with 2.0 ml of a solution containing NADP (13 mM), NADPH (16 mM), glucose 6-phosphate (100 mM), and glucose 6-phosphate dehydrogenase (2.0 mg/ml). Incubation was continued at 14° and the reaction was stopped at various times by rapidly chilling the flasks. The reaction media were finally centrifuged at $1.0 \times 10^5 g$ and the resulting pellets were retained.

Isolation and Characterization of Lipid Fractions. Total lipid was extracted from the pellets by the method of Hanson and Olley (1963). Using thin-layer chromatography as pre-

* From the NERC Fisheries Biochemical Research Unit, University of Aberdeen, Aberdeen, AB1 3RA, Scotland, U. K. *Received July 15, 1970.*

† Research Fellow, University of Aberdeen. Present address: Pioneer Research Laboratory, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Seattle, Wash. 98102.

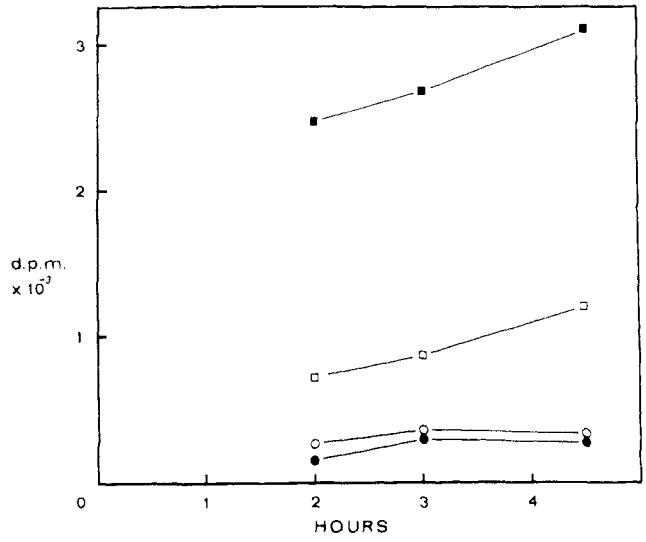

FIGURE 1: Incorporation of [9,10-^{3}H]oleyl alcohol and [1-^{14}C]oleic acid into alkyldiacylglycerols and triacylglycerols in a cell-free system from livers of *S. acanthias*. The curves depicted represent only 0.5% of radioactivity associated with each glycerolipid class present in the incubation medium. Alkyldiacylglycerols and triacylglycerols were obtained as described in the Experimental Section. (■—■) Disintegrations per minute of carbon-14 in triacylglycerols; (□—□) disintegrations per minute of tritium in triacylglycerols; (●—●) disintegrations per minute of carbon-14 alkyldiacylglycerols; (○—○) disintegrations per minute of tritium in alkyldiacylglycerols.

viously described (Malins *et al.*, 1965), alkyldiacylglycerols and triacylglycerols were isolated in 50–100-μg amounts from about one-half of the total lipid. To provide ample sample for analysis, the radioactive glycerolipids were diluted by adding a known amount of unlabelled lipid isolated from the liver (Malins *et al.*, 1965). The purity of the radioactive and unlabeled compounds was confirmed by previously described techniques (Malins *et al.*, 1965; Malins, 1968). Each fraction was further treated with pancreatic lipase by the method of Luddy *et al.* (1964). Thus, fatty acids were liberated from position 3 and positions 1, 3 of alkyldiacylglycerols and triacylglycerols, respectively. Using oleic acid and mono- and disubstituted glycerols as markers, fatty acids were isolated from the lipase digests on silicic acid plates with petroleum ether (bp 30–60°): diethyl ether–acetic acid (90:10:1, v/v). The absence of more than trace amounts of compounds, other than anticipated products of lipase digestion, confirmed the high purity of each glycerolipid class. *O*-Alkylglycerols were obtained from alkyldiacylglycerols *via* saponification and thin-layer chromatography (Malins, 1968). Free fatty acids and fatty alcohols were isolated by thin-layer chromatography as previously described (Mangold and Malins, 1960).

Radioassay and Calculations. Lipid fractions were dried and weighed directly in scintillation vials to which were added 10 ml of a solution of 2,5-diphenyloxazole (0.4%) and 1,4-bis-[2-(5-phenyloxazolyl)]benzene (0.01%) in toluene. Levels of tritium and carbon-14 in each sample were measured in a Packard Tri-Carb scintillation spectrometer. In the red window (50–1000, 50% gain) efficienceis for carbon-14 and tritium were 20.7 and 48.0%, respectively. In the green window (230–1000, 7.5% gain) the efficiencies for carbon-14 and tritium were 51.2 and 0.0%, respectively. The blue window (450–1000, 7.5% gain) was used for monitoring quenching, which was negligible in all cases.

All specific activities were expressed as disintegrations per minute of carbon-14 and tritium per micromole of lipid. The distribution of radioactivity between position 2 and positions 1, 3 of triacylglycerols was calculated from the specific activities of the triacylglycerols and fatty acids released by lipase digestion. The distribution of radioactivity among position 1 (alkyl), position 2, and position 3 of the alkyldiacylglycerols was calculated from the specific activities of alkyldiacylglycerols, *O*-alkylglycerols remaining after saponification, and fatty acids released by lipase digestion.

Results and Discussion

The cell-free system of Snyder *et al.* (1969a) for incorporation of fatty alcohol into *O*-alkylglycerols was employed in a modified form. The system used here contained the mitochondrial, microsomal, and high-speed supernatant fractions from a homogenate of dogfish liver. This preparation was essentially a complete homogenate without the cellular debris, nuclei, and residual oil. Removal of the high percentage of residual oil was necessary to minimize excessive dilution of radioactive products formed in the system.

Tritium-labeled oleyl alcohol was used to study *O*-alkyl-bond formation because fatty alcohol, rather than fatty aldehyde or fatty acid, is favorably incorporated into alkyl chains in cell-free systems (Snyder *et al.*, 1969b). [^{14}C]Oleic acid was employed to enable simultaneous comparison of the synthesis of ester linkages in glycerolipids. High percentages of 18:1 chains occur on each position of glycerol in the alkyldiacylglycerols and triacylglycerols of dogfish liver. For example, positions 1 (alkyl), 2, and 3 of alkyldiacylglycerols comprise 61, 18, and 39 mole %, and position 2 and positions 1, 3 of triacylglycerols contain 22 mole %, and 32 mole % of 18:1 chains, respectively (D. C. Malins and P. A. Robisch, unpublished results). Accordingly, radioactively labeled oleic acid and oleyl alcohol were logical choices for the present studies.

In the system of Snyder *et al.* (1969b, 1970a,b), *O*-alkyl bonds are formed only in the absence of NADPH. Therefore, to promote the incorporation of both fatty alcohol and fatty acid into alkyldiacylglycerols it was imperative to add NADPH after the *O*-alkyl bonds had been synthesized. NADPH was added after 1 hr because maximum incorporation of fatty alcohol into *O*-alkyl bonds is reached at this time in dogfish liver homogenates (F. Snyder and P. A. Robisch, unpublished results).

Figure 1 shows the incroporation of radioactivity into both triacylglycerols and alkyldiacylglycerols using fractions representing 0.5% of the total radioactivity of each glycerolipid in the cell-free system. The finding that alkyldiacylglycerols and triacylglycerols were synthesized led to an examination of the distribution of radioactivities in hydrocarbon chains (Table I). The values quoted in Table I are corrected for dilution and represent the original specific activities. At each time period, incorporation of isotopes in the absence of ATP was less than 10% of the values shown in Figure 1. Furthermore, incorporation of radioactivity into triacylglycerols was dependent on added α-glycerophosphate.

In 2 hr there is a significant incorporation of tritium from oleyl alcohol into alkyl chains in comparison to carbon-14 ($^{14}C/^3H$ = 0.03) from oleic acid (Table I). Furthermore, calculations based on carbon to tritium ratios at 2 hr (Table II) and theoretical ratios in alcohol and acid pools if one were converted into the other instantaneously indicate that 15% of oleic acid was reduced to alcohol and 9% of oleyl alcohol

TABLE I: Specific Activities of Alkyldiacylglycerols and Triacylglycerols in a Cell-Free System from the Liver of *S. acanthias* after Incubation with [9,10-^{3}H]Oleyl Alcohol and [1-^{14}C]Oleic Acid.[a]

Lipid Class	2-hr Incubation (dpm/μmole)			4.5-hr Incubation (dpm/μmole)		
	^{14}C	^{3}H	^{14}C/^{3}H	^{14}C	^{3}H	^{14}C/^{3}H
Alkyldiacylglycerols						
O-alkylglycerols	5.0×10^2	1.4×10^4	0.03			
Fatty acids						
Position 2	4.0×10^4	8.8×10^4	0.50	1.9×10^5	6.0×10^4	3.2
Position 3	4.0×10^4	9.5×10^3	4.20	9.1×10^4	4.1×10^4	2.2
Triacylglycerols						
Fatty acids						
Position 2	6.0×10^5	2.1×10^5	2.8	2.6×10^6	9.0×10^5	3.0
Positions 1, 3[b]	9.5×10^5	9.8×10^4	9.5	1.3×10^6	2.3×10^5	5.7

[a] At the stated times, the lipid classes were isolated and assayed for radioactivity. Full details are given in the Experimental Section. [b] These data represent the molar radioactivities of positions 1 and 3 measured together, *i.e.*, the mean specific radioactivity.

was oxidized to acid. Thus, incorporation of oleyl alcohol into alkyl chains of alkyldiacylglycerols appears to have taken place under conditions favoring the reduction of acid to alcohol.

In the 4.5-hr sample alkyl chains contained negligible levels of both isotopes. Therefore, these data were not considered sufficiently reliable to include. Nevertheless, the very low ratio of carbon-14 to tritium 1 hr after the addition of NADPH indicates that fatty alcohol is the favored precursor of the alkyl chain. The negligible incorporation of fatty acid into alkyl chain is reminiscent of previous findings in which [1-^{14}C]-palmitic acid was weakly incorporated into alkyl chains in the liver of live *S. acanthias* (Malins, 1968). Accordingly, previous evidence also did not support a significant role for fatty acid in the formation of *O*-alkyl bonds. The present results generally favor the pathway for *O*-alkylglycerol biosynthesis proposed by Snyder *et al.* (1969a,b, 1970a,b) which requires fatty alcohol for formation of the *O*-alkyl structure.

Generally, fatty acids of alkyldiacylglycerols contain considerably higher levels of tritium and carbon-14 in relation to the alkyl chains (Table I). This finding suggests that, for this lipid class, transacylation is extensive in comparison to *O*-alkyl-bond formation. It is noteworthy that conditions for the oxidation of fatty alcohol to fatty acid are favorable during the first hour of the incubation (Snyder *et al.*, 1969a) in the absence of added NADPH. Thus, oxidation of fatty alcohol undoubtedly contributed significantly to the relatively low specific activities of alkyl chains. A lack of enzyme, or inactivity of enzyme, are additional factors that may favor a reduced synthesis of *O*-alkyl bonds in this system. Furthermore, cleavage of *O*-alkyl bonds is extensive *in vivo* (Malins, 1968), and such a reaction could contribute substantially to the negligible level of radioactivity in the alkyl chains observed in the 4.5-hr sample.

Previous work demonstrated that the levels of glycerolipids in liver are readily altered by changes in the environment (Malins and Barone, 1970). In the present experiment the much higher specific activities of fatty acids in the triacylglycerols suggest that these glycerolipids were synthesized at a much higher rate than the alkyldiacylglycerols. It is possible that certain environmental stimuli promote the biosynthesis of fatty alcohol from fatty acid in the live animal. An increase in the rate of formation of fatty alcohol would stimulate a net synthesis of the alkyl chain. Such conditions would ultimately result in production of alkyldiacylglycerols at the expense of triacylglycerols. Indeed, the present results may indicate that the biosynthesis of alkyldiacylglycerols is governed by enzymes controlling the equilibirum fatty acid $\rightleftarrows$ fatty alcohol.

In previous experiments, little attention has been focused on the metabolic interrelations existing between alkyldiacylglycerols and the analogous triacylglycerols. The double-label technique, in comparison to the use of a single isotope, provides an added dimension to a study of these classes of lipids. For example, ^{14}C/^{3}H ratios are highest in those positions of glycerol that are incorporated before the equilibrium of alcohol to acid is complete.

There are significant alterations with time in the ^{14}C/^{3}H ratios on positions 2 and 3 of the alkyldiacylglycerols. A dramatic increase in these ratios associated with fatty acyl groups on position 2 and a significant decrease for position 3 is evident. These changes are not readily interpreted. However, 1 hr after the addition of NADPH there is a distinct preference for tritium on position 2 of alkyldiacylglycerols as suggested by the ^{14}C/^{3}H ratio of 0.50. This preference is not nearly as significant after 4.5 hr. These results may indicate that either synthesis of fatty acyl groups on

TABLE II: Radioactivities of Free Fatty Acids and Fatty Alcohols Isolated from a Cell-Free System from *S. acanthias* Liver after Incubation with [9,10-^{3}H]Oleyl Alcohol and [1-^{14}C]Oleic Acid.[a]

Lipid Class	2 hr ^{14}C/^{3}H	3 hr ^{14}C/^{3}H	4 hr ^{14}C/^{3}H	4.5 hr ^{14}C/^{3}H
Fatty alcohols	0.03		0.01	
Fatty acids	2.23	1.94	1.27	1.44

[a] The conditions, the same as those employed in Table I, are described in the Experimental Section.

position 2 does not proceed *via* ROH → RCOOH → RCOS-CoA → RCOOR′ pathways or that the turnover rate of these groups is very great and that, prior to the addition of NADPH, acyl chains on position 2 are synthesized primarily from fatty acid derived from the oxidation of fatty alcohol. Furthermore, because [1-^{14}C]chimyl alcohol is converted rapidly into free fatty acid in the liver of live dogfish (Malins, 1968), tritiated *O*-alkylglycerols must be considered as a possible source of tritiated acyl groups on position 2 of the alkyldiacylglycerols.

The constant ^{14}C/^{3}H ratio on position 2 of triacylglycerols at both times suggests that acylation has occurred essentially after equilibrium of the conversion of fatty alcohol into fatty acid is reached. In contrast, the decrease in the ^{14}C/^{3}H ratios suggests that fatty acid on positions 1, 3 is incorporated before the conversion of alcohol to acid is maximum. This could well reflect early esterification of positions 1 and 3.

The importance of diet in lipid metabolism of the liver remains to be discussed. It has been postulated that fish may synthesize large amounts of triacylglycerols from a 2-acylglycerol structure that is carried essentially unchanged through the food chain (Brockerhoff *et al.*, 1964a). This stable structure is thought to originate in the phytoplankton (Brockerhoff *et al.*, 1964b). The present data clearly show that considerable [1-^{14}C]oleic acid is incorporated into position 2 of triacylglycerols. Comparable results were obtained *in vivo* after the administration of [1-^{14}C]palmitic acid (D. C. Malins and P. A. Robisch, unpublished results). Furthermore, recent evidence suggests that the cell-free system employed in the present work exhibits a strong dependence on both α-glycerophosphate and ATP for the formation of triacylglycerols (J. R. Sargent, unpublished results). These findings are not compatible with a significant role for either acyl migration or transacylation reactions in the biosynthesis of triacylglycerols from an exogenous 2-acylglycerol structure, but are consistent with the well-known pathway for triacylglycerol biosynthesis established by Kennedy and Weiss (1956). Nevertheless, the quantitative contribution of the latter pathway to the biosynthesis of triacylglycerols in the liver of *S. acanthias* remains to be established.

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References

Brokerhoff, H., Hoyle, R. J., and Ronald, K. (1964a), *J. Biol. Chem. 239*, 735.

Brokerhoff, H., Yurkowski, H., Hoyle, R. J., and Ackman, R. G. (1964b), *J. Fish. Res. Bd. Can. 21*, 1379.

Gauglitz, E. J., Jr., and Malins, D. C. (1960), *J. Amer. Oil Chem. Soc. 37*, 425.

Hajra, A. K. (1969), *Biochem. Biophys. Res. Commun. 37*, 486.

Hanson, S. W. F., and Olley, J. (1963), *Biochem. J. 89*, 101P.

Kennedy, E. P., and Weiss, S. B. (1956), *J. Biol. Chem. 222*, 193.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Luddy, F. E., Barford, R. A., Herb, S. E., Magidman, P., and Reimenschneider, R. W. (1964), *J. Amer. Oil Chem. Soc. 41*, 693.

Malins, D. C. (1968), *J. Lipid Res. 9*, 687.

Malins, D. C., and Barone, A. (1970), *Science 167*, 79.

Malins, D. C., Wekell, J. C., and Houle, C. R. (1965), *J. Lipid Res. 6*, 100.

Mangold, G. K., and Malins, D. C. (1960), *J. Amer. Oil Chem. Soc. 37*, 383.

Snyder, F., Blank, M. L., Malone, B., and Wykle, R. L. (1970b), *J. Biol. Chem. 245*, 1800.

Snyder, F., Malone, B., and Blank, M. L. (1970a), *J. Biol. Chem. 245*, 1790.

Snyder, F., Malone, B., and Wykle, R. L. (1969a), *Biochem. Biophys. Res. Commun. 34*, 40.

Snyder, F., Malone, B., and Wykle, R. L. (1969b), *Biochem. Biophys. Res. Commun. 34*, 315.