Isolation and identification of an alkyldiacylglycerol containing isovaleric acid

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Abstract We have isolated and identified a unique subclass of alkyldiacylglycerols from the pink portion of the harderian gland of the New Zealand white rabbit. Using chemical, enzymic, chromatographic, and physical procedures, the lipid class has been identified as 1-alkyldiacylglycerol containing 1 mole of isovaleric acid. More than 50% of the *O*-alkyl moieties consist of 16:0 and 18:0 carbon chains, whereas the other major *O*-alkyl moieties are 15:0 and 17:0 branched chains (≃30%). The long-chain acyl groups of the alkyldiacylglycerol subclass consist primarily of saturated fatty acids (60% 16:0 and 30% 18:0) and a small amount of branched-chain fatty acids (≃5%), whereas the 3-position appears to be occupied by isovaleric acid.

Supplementary key words pink harderian gland · rabbit · glycerol ethers · short-chain fatty acids

The *O*-alkyl moieties in glycerolipids exist primarily as alkyldiacylglycerols and as ether analogs of phosphatidylcholine and phosphatidylethanolamine. In mammalian cells, the *O*-alkyl chains consist of 14, 16, 18, and 20 carbon atoms and the *O*-acyl moieties range from C_{12} to C_{24} (1). Tumors of the harderian glands in mice have been shown to be a rich source of alkyl glycerolipids (2). This paper describes the isolation and identification of a subclass of alkyldiacylglycerols from the pink portion of the rabbit harderian gland (a secretory gland of the eye in mammals that possess a nictitating membrane) in which the 3-position of the glycerol moiety appears to be occupied by isovaleric acid.

EXPERIMENTAL PROCEDURES

Isolation of the alkyldiacylglycerols containing isovaleric acid

The pink portions of harderian glands were obtained from New Zealand white rabbits purchased from the Lab Animal Supply Co., Concord, Tenn. Total lipids, extracted from the glandular tissue by the method of Bligh and Dyer (3), were stored in chloroform at −23°C until used. Fig. 1 shows a TLC plate of the total lipid extract (lane *B*). The very small amount of material running at the same R_F value (0.60) as the standard long-chain alkyldiacylglycerol (lane *A*, *2*) is designated subclass II; the lipid class with an R_F value (0.46) just below the standard triacylglycerol (lane *A*, *3*) is designated subclass I; and the major, more polar lipid is designated as subclass III.

A glass column 2 cm in diameter, packed to a height of 30 cm with a chloroform slurry of Unisil (Clarkson Chemical Co., Williamsport, Pa.), was used to isolate the alkyldiacylglycerols from the lipid extract. After the column was washed with two-column volumes (≃150 ml) of hexane, 800 mg of the total lipid extract (dissolved in hexane) was applied to the column. Elution was carried out with 15% chloroform in hexane (v/v), and 75-ml fractions were collected.

The lipid class composition of each fraction from the column was qualitatively checked by TLC using appropriate reference compounds. Fractions 4 and 5 contained two subclasses (I and II) of alkyldiacylglycerols, whereas fraction 6 contained only a single subclass of alkyldiacylglycerols (I). Subclass III was not eluted from the column with this solvent. The alkyldiacylglycerols in subclass I (those containing a short-chain fatty acid) found in fractions 4 and 5 were isolated from subclass II (those containing only long-chain acyl moieties) by TLC on several chromatoplates coated with silica gel G (8 × 8 cm), using double development in benzene. The alkyldiacylglycerols (I) were eluted from the silica

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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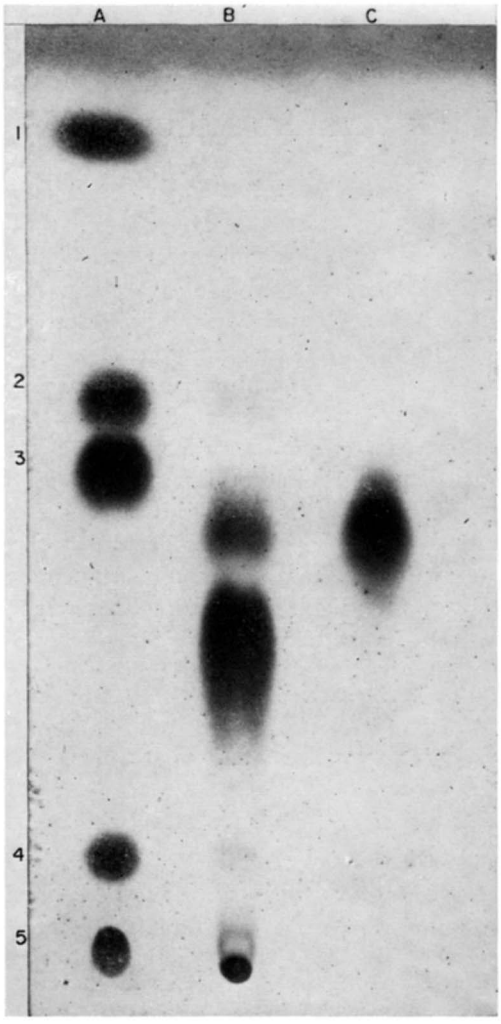


FIG. 1. Thin-layer chromatogram, silica gel coated, developed twice in 100% benzene solvent, of: lane *A*, wax ester (*1*), long-chain (16:0) alkyldiacylglycerol (*2*), triacylglycerol (*3*), cholesterol (*4*), and free fatty acid (*5*); lane *B*, total lipids of the pink portion of rabbit harderian gland; lane *C*, purified subclass I of alkyldiacylglycerols containing isovaleric acid. (The major spot, subclass III, in the total lipids [lane *B*] is the subject of a separate report.)

gel on a sintered glass funnel with diethyl ether. The chemical and chromatographic analyses of subclass I are the subject of this paper; subclass II was discarded and subclass III is the subject of a separate study. Fig. 1, lane *C*, shows the final preparation (82 mg) which chromatographed as a single spot on silica gel G after double development in benzene. The sample was dissolved in hexane and stored at −23°C until used.

Analysis of *O*-acyl moieties

The preparation and the analysis of methyl esters (4) and 2-chloroethanol esters (5) of the acyl moieties of alkyldiacylglycerols (I) were based on a procedure used for the analysis of the wide range of chain lengths in fatty acids encountered in milk fats (6). The 2-chloroethanol, isovaleric acid, *n*-valeric acid, 2-methyl butyric acid, 2,2-dimethylpropanoic acid (pivalic acid), and butyric acid were purchased from Eastman Kodak Co., Rochester, N.Y., and palmitic, stearic, oleic, and linoleic acids were purchased from The Hormel Institute, Austin, Minn.

Methyl esters of fatty acids were analyzed by GLC using a Victoreen model 4000 chromatograph with a dual hydrogen flame detector fitted with 6 ft × 1/8 inch columns packed with 10% EGSS-X on 100–120 mesh Gas-Chrom P (Applied Science Laboratories, State College, Pa.). Temperature programming was carried out from 150 to 200°C at 2°C/min. Identifications of the various peaks were made by cochromatography with standards both before and after hydrogenation (over PtO) of the methyl esters. Quantitation was based on peak areas, calculated from peak height × width at one-half height measurements. Identification of all odd and branched-chain fatty acids was based on their relative retention times.

The 2-chloroethanol esters were analyzed with the same chromatograph equipped with 6 ft × 1/8 inch columns packed with either 6% SE-30 on 90–100 mesh Anakrom or the EGSS-X as above. Temperature programming was carried out from 75 to 200°C at 4°C/min on the EGSS-X column and from 75 to 230°C at 4°C/min for the first 15 min then at 15°C/min for the SE-30 column. Identifications of the various peaks were made by cochromatography with standards on both columns, and quantitation was performed as described for the methyl esters. By analyzing known mixtures of the 2-chloroethanol esters of isovaleric and palmitic acids, we found that weight to peak area correction factors calculated for the SE-30 column were lower than those calculated for the EGSS-X column. The molar percentage of short-chain acyl to long-chain acyl groups was therefore based on the results obtained with the SE-30 column.

Analysis of the *O*-alkyl moieties

To obtain an estimate of the molar percentage of the lipid class as alkylglycerol, a known weight of the purified lipid class was subjected to reductive cleavage by Vitride (70% $NaAlH_2[OCH_2CH_2OCH_3]_2$ in benzene, Eastman Kodak) (7). The recovered reduction products were diluted to a known volume, and the amount of alkylglycerols was determined by TLC photodensitometry (8). Based on TLC R_F value, the only other reduction products were fatty alcohols. The alkylglycerols were then isolated from the alcohols by TLC for subsequent analyses. The alkylglycerols were also isolated by TLC from the products of the HCl–methanol treatment

used for the preparation of methyl esters from this lipid class.

Infrared spectra of the alkylglycerols (in carbon disulfide) were obtained with a Perkin-Elmer model 337 spectrophotometer. Isopropylidene derivatives (9) of the alkylglycerols were analyzed by TLC and GLC. The GLC analyses were done on an EGSS-X column; temperature programming was carried out from 170 to 200°C at 4°C/min. Experimental samples were analyzed by GLC before and after hydrogenation over PtO. We identified the peaks by cochromatography of isopropylidene derivatives of synthetic 14:0, 16:0, and 18:0 alkylglycerols (purchased from Analabs, Inc., Hamden, Conn.) with the experimental samples. Identifications of odd and branched-chain alkylglycerols were based on their retention times.

Positional analysis of *O*-acyl moieties

Alkyldiacylglycerols (I) (16.4 mg) were hydrolyzed with pancreatic lipase (10) as modified for analysis of alkyldiacylglycerols (11). After 15 min at room temperature, the incubations were stopped by the addition of hydrochloric acid (final pH = 2) and the lipid products were extracted. Another identical sample was extracted directly with diethyl ether without acidification. The hydrolytic products were isolated by TLC on silica gel G layers developed in a solvent mixture of hexane–diethyl ether–glacial acetic acid 70:30:2 (v/v/v). A quantitative estimation of the amount of unreacted alkyldiacylglycerols, alkylacylglycerols, and alkylglycerols formed by the lipase hydrolysis was obtained through the use of standards (purchased from Analabs, Inc.) and TLC photodensitometry. The chromatographic bands representing the alkylacylglycerols, isolated by preparative TLC in the same solvent system, were eluted from the adsorbent with 20% methanol in diethyl ether. Portions of this fraction were esterified with 2-chloroethanol–HCl or methanol–HCl and analyzed as described earlier.

GLC of the intact alkyldiacylglycerols (I) containing a short-chain *O*-acyl moiety

High-temperature GLC was used to separate intac alkyldiacylglycerols (I) on the basis of carbon numbers. The same GLC instrument used for fatty acid analyses was fitted with a 1 ft × 1/8 inch stainless steel column packed with 2.5% SE-30 on 100–120 mesh Aeropak 30. The injection port was modified so that the sample could be injected directly on the column packing. The temperatures of injection port and detector were maintained at 320°C and 300°C, respectively, and the column temperature was programmed from 250 to 300°C at 5°C/min. The flow rate of the helium carrier gas was 20 ml/min. Cochromatography of the alkyldiacylglycerols (I) with trilaurin and trimyristin (purchased from Applied Science Labs) was done to assign carbon numbers.

RESULTS AND DISCUSSION

Short-chain *O*-acyl moieties in subclass I of alkyldiacylglycerols

GLC of the 2-chloroethanol esters derived from the alkyldiacylglycerols revealed that isovaleric acid was the only short-chain fatty acid in this lipid class. Identification was based on cochromatography with standard isovaleric acid on both the SE-30 and the EGSS-X columns. The 2-chloroethanol ester of isovaleric acid had a significantly shorter retention time than the ester of *n*-valeric acid on both liquid phases. Although the 2-chloroethanol esters of 2-methyl butyric and isovaleric acids were not separated on the SE-30 column, they were separated on the EGSS-X column as shown by their retention times in Table 1. The molar percentage of isovaleric acid in the alkyldiacylglycerols was 28%, an indication that each molecule of alkyldiacylglycerol (I) contained 1 mole of isovaleric acid.

Long-chain *O*-acyl moieties in subclass I of alkyldiacylglycerols

Palmitic and stearic acids were the two major long-chain fatty acids of the alkyldiacylglycerols (I); only a small degree of unsaturation was found (Table 2). On the basis of the results obtained for the 2-chloroethanol esters, the long-chain *O*-acyl moieties represented 35 mole % of the alkyldiacylglycerols in subclass I.

O-Alkyl moieties in subclass I of alkyldiacylglycerols

Table 2 shows that 15:0 (br), 16:0, 17:0 (br), and 18:0 aliphatic chains represented the major *O*-alkyl moieties in subclass I of the alkyldiacylglycerols. The same results were obtained for the alkylglycerols isolated after Vitride reduction or after acid methanolysis. Hydrogenation of the samples and subsequent GLC analysis revealed little change in the acyl distribution pattern (Table 2), proving that the *O*-alkyl moieties were not unsaturated.

TABLE 1. GLC retention times of 2-chloroethanol esters of various short-chain fatty acids[a]

Fatty Acid	SE-30	EGSS-X
Butyric	6.2	6.0
2,2-Dimethylpropanoic	7.1	5.1
2-Methyl butyric	9.1	8.0
Isovaleric	9.1	8.8
n-Valeric	10.8	10.7
Short-chain acyl moiety from subclass I	9.1	8.8

[a] Retention times are given in minutes under GLC conditions described in the text.

TABLE 2. Long-chain acyl and alkyl compositions of the alkyldiacylglycerol lipid class

Chain Designation	Alkyldiacylglycerols (I)				1-Alkyl-2-acylglycerols[a]	
	Acyl		Alkyl			
		H_2[b]		H_2	Acyl	Alkyl
			wt %			
14:0	0.8	0.9	7.6	7.4	tr[c]	7.8
Br[d] 15:0			17	17		15
15:0			2.3	2.6		2.3
Br 16:0			2.3	2.0		1.9
16:0	57	60	45	43	61	47
Br 17:0 + 16:1	7.5	4.7	14	16	4.0	14
17:0	2.2	2.4	0.7	tr	2.0	1.1
18:0	27	32	11	11	33	12
Br 19:0 + 18:1	4		tr	tr	tr	tr
18:2	1					
Others	tr	tr				

[a] Derived from hydrolysis of alkyldiacylglycerols (I) by pancreatic lipase.
[b] H_2 indicates results obtained after hydrogenation.
[c] Tr, trace, less than 1%.
[d] Br, branched chain.

The substantial amount of branched chain *O*-alkyl moieties (≃30%) differs from the pattern (16:0, 18:0, and 18:1) normally found in most mammalian cells (1). This might be related to the utilization of isovaleric acid in fatty acid synthesis followed by the reduction of these longer odd-chain acids to the corresponding alcohols which serve as the precursors of the *O*-alkyl moieties. The molar percentage of alkylglycerols found for the alkyldiacylglycerols (I) was 37%, which is close to the theoretical value for this molecular species.

The infrared spectra were essentially the same as those reported previously for alkylglycerols (12); a broad OH absorption band occurred in the 3500 cm^{-1} area, and a strong ether absorption band occurred at 1120 cm^{-1}. Periodate oxidation produced alkyl glycolic aldehyde (based on TLC migration and GLC retention of standards), which demonstrates that the *O*-alkyl moiety was located at the 1-position of glycerol. The isopropylidene derivatives of the alkylglycerols had the same TLC R_F value as the isopropylidene derivative of hexadecylglycerol, which rules out the presence of any 1,2-alkane diols (13) in the samples.

GLC analysis of the intact alkyldiacylglycerols (I)

The gas–liquid chromatogram in Fig. 2 shows that the alkyldiacylglycerols separated into three major peaks (carbon numbers 34–40). The shoulders associated with the peaks are probably due to the large amount of odd or branched-chain *O*-alkyl groups, or both, in this lipid subclass. The main peak of alkyldiacylglycerols (I) had a retention time essentially the same as that of trilaurin, whereas trimyristin had a retention time beyond the last peak of alkyldiacylglycerols (I). On the basis of the GLC separations of diacylglycerols and dialkylglycerol acetates (14), the carbon number of the main alkyldiacylglycerol (I) peak would correspond to 37 (the carbon of glycerol being excluded). The *O*-alkyl and *O*-acyl chain length compositions described in the previous sections predict that this carbon number would represent the major component, assuming a random distribution of the *O*-alkyl and long-chain *O*-acyl groups.

Results of lipase hydrolysis

We had previously shown that the three products formed during the hydrolysis of alkyldiacylglycerols with pancreatic lipase were free fatty acids, 1-alkyl-2-acylglycerols, and 1-alkylglycerols (11). The amounts of these products produced by lipase hydrolysis of the alkyldiacylglycerols in this study are shown in Table 3. The alkylglycerols are formed as a result of acyl migration in the 1-alkyl-2-acylglycerol. GLC of the 2-chloroethanol

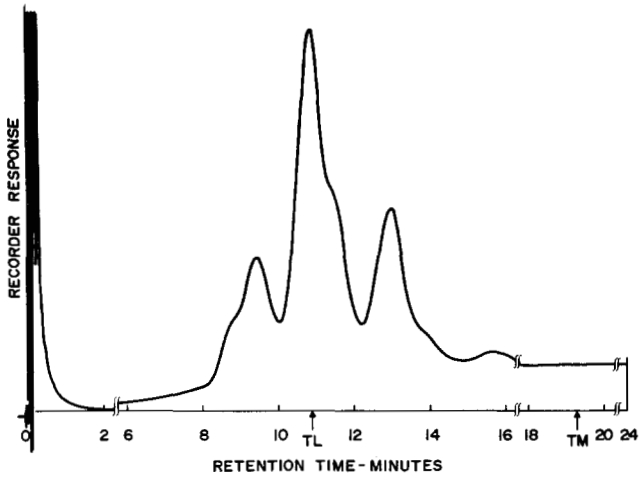


FIG. 2. Gas–liquid chromatographic separation of the intact alkyldiacylglycerols (subclass I). *TL* and *TM* correspond to the retention times of trilaurin and trimyristin, respectively. (See text for chromatographic conditions used.)

TABLE 3. Products other than free fatty acids produced by lipase hydrolysis of alkyldiacylglycerols (I)

Compound	TLC R_F[a]	Mole %	Mole % of Acyl Groups as Isovaleric Acid
Alkyldiacylglycerols (I)	0.64	45.5	40
Alkylacylglycerol[b]	0.16	50.0	3.6
Alkylacylglycerol[c]	0.10	2.5	
Alkylglycerol	0.01	2.0	

[a] On silica gel G, using hexane–diethyl ether–glacial acetic acid 70:30:2 (v/v/v).

[b] R_F value corresponds to the standard 1-alkyl-2-hexadecanoylglycerol.

[c] R_F value corresponds to 1,2-diacylglycerol but is presumably 1-alkyl-2-isovaleroylglycerol.

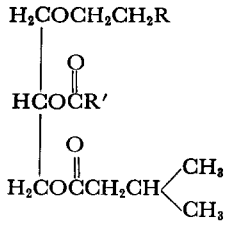
esters of the fatty acids in the alkylacylglycerol fraction produced from alkyldiacylglycerols (I) showed only trace amounts (< 4 mole %) of isovaleric acid.

The free fatty acids produced by the lipase hydrolysis contained 80 mole % isovaleric acid. The major hydrolysis product, alkylacylglycerol, had an R_F (TLC) which corresponded to that of the standard 1-alkyl-2-acylglycerol and not to the 1-alkyl-3-acylglycerol which migrates at a higher TLC R_F value. The same hydrolysis products, except for a lower amount of free fatty acids, were found when the extraction was done in the absence of added HCl. Since only a very small amount of alkylglycerol was found after lipase hydrolysis of the alkyldiacylglycerols, the trace amounts of isovaleric acid in the alkylacylglycerols cannot be explained by acyl migration of the isovaleric acid. Therefore, the conclusion can be drawn that the alkyldiacylglycerols (I) contain essentially all of the isovaleric acid in the 3-position unless pancreatic lipase exhibits intermolecular selectivity for these lipid classes.

The fatty acid composition at the 2-position and the *O*-alkyl composition at the 1-position of the alkylacylglycerols isolated from the lipase incubations are given in Table 2. Both compositions agree reasonably well with those determined for the products isolated after Vitride reduction and acid methanolysis of the alkyldiacylglycerols (I). The fact that the alkylglycerol composition of this partial glyceride is the same as in the original substrate also argues against the presence of any fatty acid esters of alkane diols being present in the alkyldiacylglycerol (I) preparation from the pink portion of the harderian gland of rabbits.

CONCLUSION

The data reported in this paper document the existence of a unique subclass of *O*-alkyl lipids that contains isovaleric acid (formula I).



(R = primarily 14:0, 16:0, 13:0 br, and 15:0 br)
(R′ = primarily 15:0 and 17:0)

(I)

Although isovaleric acid has previously been detected in triacylglycerols from the mandibular canal of a porpoise (15), our work is the first to describe its presence in alkyldiacylglycerols. Furthermore, the significant proportion of branched *O*-alkyl moieties in this lipid class indicates that isovaleric acid might also be selectively incorporated into long-chain fatty alcohols (precursor of the *O*-alkyl moiety) in the pink portion of the rabbit harderian gland.

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