Jezyk, P., and Lands, W. E. M. (1968), *J. Lipids Res. 9*, 525.
Kanoh, H. (1969), *Biochim. Biophys. Acta 176*, 756.
Lands, W. E. M. (1965), *J. Amer. Oil Chem. Soc. 42*, 465.
Lands, W. E. M., Blank, M. L., Nutter, L. J., and Privett, O. (1966), *Lipids 1*, 224.
Lands, W. E. M., and Hart, P. (1965), *J. Biol. Chem. 240*, 1905.
Lands, W. E. M., and Merkl, I. (1963), *J. Biol. Chem. 238*, 898.
Lauderbaugh, T. (1968), Ph.D. Thesis, The University of Michigan.
Mabrouk, A. F., and Brown, J. B. (1956), *J. Amer. Oil Chem. Soc. 33*, 98.
Okuyama, H., Eibl, H., and Lands, W. E. M. (1971), *Biochim. Biophys. Acta 248*, 263.
Okuyama, H., and Lands, W. E. M. (1972), *J. Biol. Chem. 247*, 1414.
Okuyama, H., Lands, W. E. M., Christie, W. W., and Gunstone, F. D. (1969), *J. Biol. Chem. 244*, 6514.
Ono, K., and Fredrickson, D. S. (1964), *J. Biol. Chem. 239*, 2482.
Raulin, J., Lapous, D., Dauvillier, P., and Rérat, A. (1965), *C. R. Acad. Sci., Ser. A 260*, 344.
Raulin, J., Loriette, C. L., and Clément, G. (1963), *Biochim. Biophys. Acta 70*, 642.
Reitz, R. C., El-Sheikh, M., Lands, W. E. M., Ismail, I. A., and Gunstone, F. D. (1969), *Biochim. Biophys. Acta 176*, 480.
Scholfield, C. R., Davison, V. L., and Dutton, H. J. (1967), *J. Amer. Oil Chem. Soc. 44*, 648.
Selinger, Z., and Holman, R. T. (1965), *Biochim. Biophys. Acta 106*, 56.
Shorland, F. B., Weenink, R. O., and Johns, A. T. (1955), *Nature (London) 175*, 1129.
Sinclair, R. G. (1935), *J. Biol. Chem. 111*, 515.
vandenBosch, H., vanGolde, L. M. G., Eibl, H. and van Deenen, L. L. M. (1967), *Biochim. Biophys. Acta 144*, 613.
Waku, K., and Lands, W. E. M. (1968), *J. Lipid Res. 9*, 12.
Yang, S. F., Freer, S., and Benson, A. A. (1967), *J. Biol. Chem. 242*, 477.

Structure and Biosynthesis of Diesters of Alkane-2,3-diols of the Uropygial Glands of Ring-Necked Pheasants†

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ABSTRACT: The structure of the major components of the lipids excreted by the uropygial (preen) glands of chicken, quail, and ring-necked pheasants were determined by thin-layer chromatography and a combination of gas–liquid chromatography and mass spectrometry. Diacylalkane-2,3-diols constituted more than 90% of the lipids in all cases. In the pheasant C_{18} diol was the major (85%) component whereas C_{22}, C_{23}, and C_{24} diols predominated in chicken and quail. [1-^{14}C]Acetate injected under the gland was readily incorporated into preen oil in 24- to 72-hr experimental periods. The major part of the label was in the diol diesters and both acyl and diol portions were nearly equally labeled. The distribution of label among the C_{10}–C_{20} acyl moieties and C_{16}–C_{20} alkanediols, together with measurement of ^{14}C located in C-3 position of the individual diols, indicated *de novo* synthesis of carbon chains from acetate. Injection of [1-^{14}C]-palmitic acid under the gland gave rise to labeled diol diesters in which the acyl moieties contained 1.5 times as much ^{14}C as that in the diol portion. Distribution of the label among the diols and measurement of the ^{14}C located at C-3 of individual diols indicated that palmitic acid was incorporated intact into C_{18} diol and that the carboxyl carbon of the C_{16} acid became C-3 of the C_{18} diol. The data also indicated that exogenous C_{16} acid was elongated to C_{18} acid and subsequently incorporated into C_{20} diol. The major labeled product in the acyl portion was C_{18} acid, again suggesting elongation. Exogenous [1-^{14}C]stearic acid also labeled diol diesters but the acyl moiety was nearly four times as radioactive as the diol portion and incorporation into diol was much less than that observed with [1-^{14}C]palmitic acid. However, the major part of the label derived from the labeled C_{18} acid was found in C_{20} diol as expected from a condensation of the C_{18} with a C_2 unit. These results are consistent with the hypothesis that alkane-2,3-diols are synthesized by reduction of the acyloin formed by the condensation of a fatty aldehyde with active acetaldehyde. However, attempts to specifically label C-1 and C-2 of the diol with exogenous [2-^{14}C]pyruvate, [U-^{14}C]alanine, and [2-^{14}C]lactate failed probably because these substrates were readily incorporated into alkane chain of the diols *via* acetate.

A wide variety of alkane diols occur in microorganisms, animals and plants. Three major types are alkane-α,ω-diols (Murray and Schoenfield, 1955; Mazliak, 1962), alkane-1,2-diols (Horn and Hougen, 1953; Fieser *et al.*, 1957; Downing *et al.*, 1961; Karkkainen *et al.*, 1965; Nicolaides, 1965) and alkane-2,3-diols (Haahti and Fales, 1967; Hansen *et al.*, 1969; Saito and Gamo, 1970). No biosynthetic studies on these diols have been reported. However, the first two types are structurally closely related to ω-hydroxy acids and α-hydroxy acids, respectively, and were therefore suggested as arising by reduction of the corresponding acids (Kolattukudy, 1970a). On the other hand the mechanism of synthesis of the 2,3-diols is more intriguing because unlike the other diols no

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simple reaction can be postulated to account for their formation. One possible mechanism by which alkane-2,3-diols could be formed is by a condensation between a fatty aldehyde and acetaldehyde, most probably as the thiamine pyrophosphate derivative, to give an acyloin, which can then be reduced to give the 2,3-diol (Kolattukudy and Walton, 1972).

Uropygial glands of birds contain fairly large quantities of rather unusual lipids which are used to waterproof and condition the feathers. The major component of the preen lipids in turkey and chicken were identified to be diesters of alkane-2,3-diols of chain length C_{22-24}. It would be more convenient to perform experimental tests for the biosynthetic pathway proposed for the diols with an organism that synthesizes C_{18} or C_{20} diols because specifically labeled precursors in this range of chain length are available. It has been reported that the major diol in the preen lipids of green pheasants is octadecane-2,3-diol (Saito and Gamo, 1970). In this paper we describe structure determination of the diols of ring-necked pheasants by a combination of gas–liquid chromatography and mass spectrometry of the Me_3Si ethers and biosynthetic studies with labeled precursors. The results presented show that labeled acetate, palmitate, and stearate were incorporated into diols and that the carboxyl carbon of the palmitic acid became C-3 of octadecane-2,3-diol and that stearic acid was incorporated specifically into C_{20} diol in accordance with the above hypothesis.

Experimental Section

Materials. Ring-necked pheasants, *Phasianus colchicus*, were generously provided by the game farm of the Department of Wildlife of the State of Washington. Labeled fatty acids were purchased from Amersham/Searle Corp. About 100 μCi of [1-^{14}C]palmatic acid (specific activity 57.1 Ci/mole) or [1-^{14}C]stearic acid (specific activity 48.4 Ci/mole) was dissolved in 5–10 ml of ethyl ether and 1 drop (about 7 mg) of Tween-20 was added. After evaporating the ether with a stream of N_2 1 ml of 0.9% NaCl was added and the mixture was sonicated with the needle probe of Biosonik III for 1 min to give a stable, almost clear emulsion of the acid.

Sodium [1-^{14}C]acetate (specific activity 62 Ci/mole), sodium [2-^{14}C]pyruvate (specific activity 6.8 Ci/mole), sodium [2-^{14}C]propionate (specific activity 12 Ci/mole), DL-[U-^{14}C]-alanine (specific activity 10 Ci/mole), sodium [2-^{14}C]lactate (specific activity 29 Ci/mole), and [1-^{14}C]glucose (specific activity 58.8 Ci/mole), purchased from Amersham/Searle Corp., were dissolved in 0.9% NaCl.

Isolation and Identification of Diol Diesters. The oil was collected by gently squeezing the preen gland of live birds. It was dissolved in chloroform and subjected to analytical and preparative thin-layer chromatography on silica gel G (0.5 or 1 mm, respectively) with benzene as the developing solvent. The components were visualized with 2′,7′-dichlorofluorescein, and diol diesters were extracted from the silica gel with ethyl ether. The diol diesters were refluxed with 15 ml of a 14% solution of BF_3 in methanol and 5 ml of benzene for 12–16 hr. After addition of water the products were extracted with chloroform or ethyl ether. The extract was dried with anhydrous sodium sulfate and evaporated to dryness under reduced pressure. Diols and methyl esters were separated by thin-layer chromatography on silica gel G with hexane–ethyl ether–acetic acid–methanol (20:4:1:1, v/v) as solvent system. The methyl esters were subjected to gas–liquid chromatography on 4% OV-1 on 80–100 mesh gas chrom Q. The diols were subjected to gas–liquid chromatography on the same column after preparing their trimethylsilyl ethers by heating the diol fraction with *N*,O-bis(trimethylsilyl)acetamide for 15 min at 90° without any further purification. The details of chromatography are shown under the figures. The gas chromatograph was attached to a Perkin-Elmer-Hitachi RMU6D mass spectrometer with a Biemann separator interphase and mass spectra of individual components were recorded with 70-eV ionizing voltage. In order to test whether a single peak of the flame ionization detector represented an incompletely resolved mixture of components, spectra were recorded at the ascending and descending slopes of the gas chromatographic peak in addition to the top of the peak.

Incorporation of Labeled Substrates into Uropygial Lipids. The radioactive fatty acid solution (0.4 ml containing 40 μCi) was injected under each lobe of the preen gland of male pheasants. The birds were kept in a cage with usual food for varying periods of time (12–72 hr). At the end of the experimental period the birds were killed and the gland was excised. The two lobes of the gland were opened with a razor blade and then the oil was extracted with chloroform by immersing the open glands in chloroform for 1 min with stirring. This fraction is referred to as oil although this fraction did probably contain small amounts of tissue lipids. The tissue was then cut into thin slices and ground in a Ten-Broeck homogenizer with about 5 ml of water. Total lipids were extracted with an excess of a 2:1 mixture of chloroform and methanol according to the method of Folch *et al.* (1957). This fraction is called tissue lipids although there might be a little contamination from oil. The preen lipids already on the feathers by the end of the experiment were extracted by dipping the bird in a large volume of chloroform for about 1 min.

The various fractions of the lipids were separated by the same thin-layer chromatographic techniques, and diols were isolated as described in the previous section. The labeled diols purified by thin-layer chromatography were acetylated with a 2:1 mixture of acetic anhydride and pyridine at room temperature overnight. The diol diacetates were purified by thin-layer chromatography with benzene as the developing solvent. Radio gas–liquid chromatography of the labeled diol diacetates was done on 4% OV-1 on 80–100 mesh gas chrom Q. The experimental conditions are shown under the figure.

Chemical Degradation of Diols. Thin-layer chromatographically purified diol (10–20 mg) was treated with 100 mg of powdered sodium metaperiodate in about 5 ml of pyridine under nitrogen with vigorous stirring for 4 hr (Baumann *et al.*, 1969). About 5 ml of 0.005 M solution of $KMnO_4$ containing 0.002 M Na_2CO_3 was added and stirred for an additional 1–2 hr. The reaction mixture was decolorized with sodium bisulfite and diluted with two volumes of water and acidified. The lipids were extracted twice with chloroform and the extract was evaporated to dryness. The fatty acids were purified by thin-layer chromatography with hexane–ethyl ether–formic acid (40:10:1, v/v) as the developing solvent. The recovery of fatty acids was essentially quantitative. These fatty acids were analyzed by gas–liquid chromatography as their methyl esters which were prepared with BF_3–methanol reagent and purified by thin-layer chromatography with benzene as the solvent. In the labeling experiments individual methyl esters were collected from the effluent of the gas chromatography with Pasteur pipets packed with glass wool and hydrolyzed with 10% alcoholic KOH under nitrogen. The fatty acids recovered in the usual manner were purified by thin-layer chromatography as described above. These acids were subjected to micro-Schmidt degradation (Brady *et al.*, 1960).

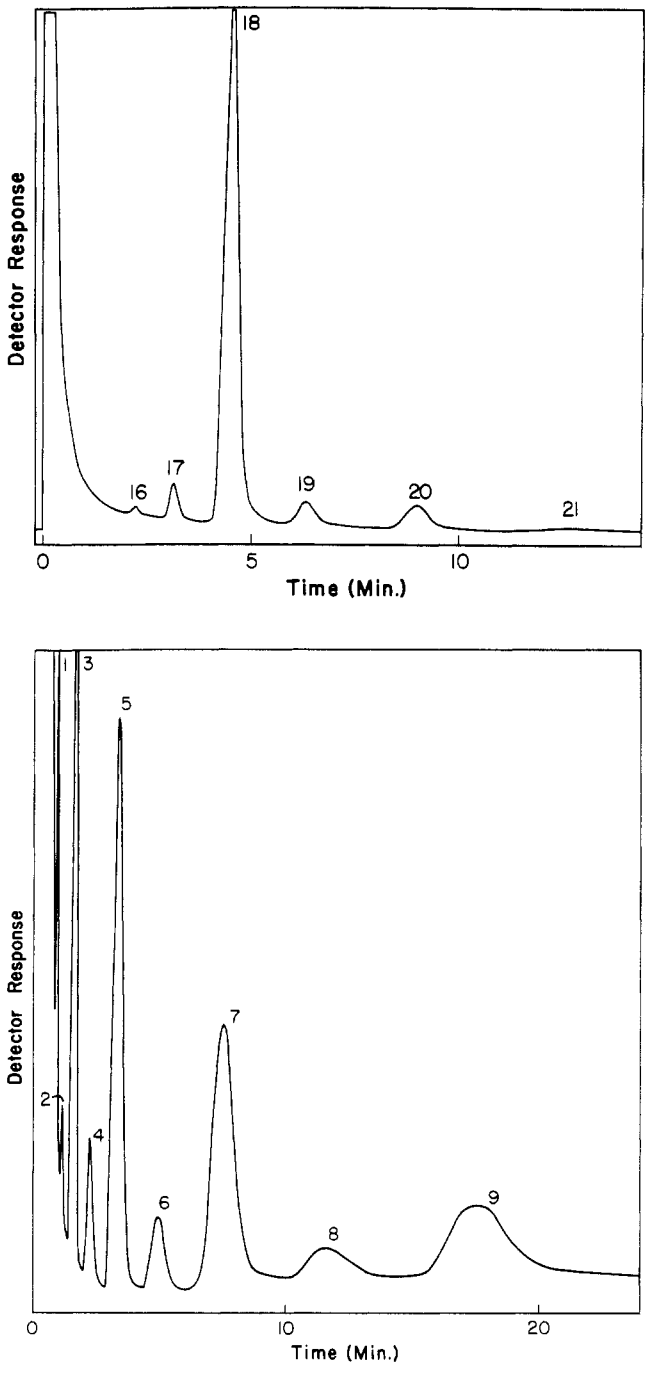

FIGURE 1: Gas–liquid chromatogram of diols (as trimethylsilyl ethers, top) and acyl moieties (as methyl esters, bottom) of the diol diesters isolated from preen oil of the ring-necked pheasant, *Phasianus colchicus*. The number on each peak of the top tracing represents the chain length. In the bottom tracing, 1, C_{10}; 2, C_{11}; 3, C_{12}; 4, C_{13}; 5, C_{14}; 6, C_{15}; 7, C_{16}; 8, C_{17}; 9, C_{18}; C_{19} and C_{20} were also detected but in much smaller quantities. The identification was by comparison of the retention time to authentic standards and by mass spectrometry. In each case coiled glass column (147 × 0.31 cm o.d.) packed with 3% OV-1 on 80–100 mesh gas chrom Q with 60 cm^3/min of carrier gas He was used. The column temperatures were 205° for the diol derivatives and 160° for the methyl esters.

Determination of Radioactivity. Radioactivity in liquid samples and in thin-layer fractions was measured directly as described before (Kolattukudy, 1965) except that a Packard liquid scintillation spectrometer was used. Internal standards were always used and usually ^{14}C was assayed with 70% efficiency. All counting was done with a standard deviation less than 3%. Radioactivity in the effluent of the gas chromatograph was continuously monitored with a Barber Colman radioactivity monitor.

Results and Discussion

Structure Determination of Diols and Acyl Moieties of the Diol Diester. Previous reports showed that chicken and turkey alkane-2,3-diols consisted of mainly C_{22}, C_{23}, and C_{24} (Haahti and Fales, 1967; Hansen *et al.*, 1969) while the diol of the preen gland of green pheasant was essentially a C_{18} alkane-2,3-diol. In order to attempt biosynthetic studies on alkane-2,-3-diols we searched for suitable experimental birds. We examined the preen lipids of chicken, quails, and ring-necked pheasants. The classes of preen lipids contained in the three species were very similar but the chain lengths were different. The chain lengths of pheasant diols were found to be particularly suitable for biosynthetic studies and therefore only the results obtained with the preen lipids of this bird are discussed below in detail. Thin-layer chromatography of preen oil of pheasants showed that the major component (over 90%) was a diol diester fraction. This fraction on treatment with BF_3–methanol gave rise to methyl esters and a diol fraction. Gas–liquid chromatography showed that the methyl ester fraction (Figure 1, bottom) contained C_{10}–C_{20} fatty acids, C_{18}, C_{16}, and C_{14} predominating in the order of decreasing amounts.

Gas–liquid chromatography of the trimethylsilyl ethers of the diols (Figure 1, top) showed that they contained one major component and five minor components. Mass spectrum of each component was used to determine their structures. The mass spectrum of the major peak (Figure 2) showed a very weak molecular ion at *m/e* 430 and a relatively intense ion at *m/e* 415 indicating a CH_3 loss. The weak ion at *m/e* 340 was assigned to loss of $(CH_3)_3SiOH$ from the molecular ion and a relatively weak ion at 325 was most probably formed by the loss of a CH_3 and $(CH_3)_3SiOH$ from the molecular ion. The most intense ion of the spectrum was at *m/e* 313, and is assigned to the major fragment ion produced by the cleavage of the carbon–carbon bond between the two carbons carrying trimethylsilyl ether functions. The other fragment ion produced from this cleavage was also found at *m/e* 117 but as expected, it was much weaker than that at *m/e* 313. The other possible α cleavages around the ether functions should give $M^+ - 15$ (*m/e* 415) and $M^+ - C_{17}H_{35}$ (at *m/e* 219). The mass spectrum showed a fairly strong ion at *m/e* 415 and weak ion at *m/e* 219. The only other significant ions in the spectrum were those at *m/e* 147 and 149, expected from molecules with polysilyl ether functions, and at *m/e* 73 and 75, characteristic of silyl ethers. Thus the fragmentation pattern clearly show that the major component is octadecane-2,3-diol. The mass spectra of all the other components showed patterns similar to the one discussed above and on the basis of their spectra (not shown) appropriate diol structures were assigned to them. For example, the component marked 20 (Figure 1, top) showed molecular ion at *m/e* 458 and a relatively intense $M^+ - 15$ ion at 443. The base peak at 341 represented α cleavage between the two carbons carrying the trimethylsilyl ether functions. The other fragment of this cleavage was also found at *m/e* 117. Thus this component was eicosane-2,3-diol. The other minor components were heptadecane-2,3-diol, nonadecane-2,3-diol and heneicosane-2,3-diol. Trace quantities of docosane-2,3-diol was also observed.

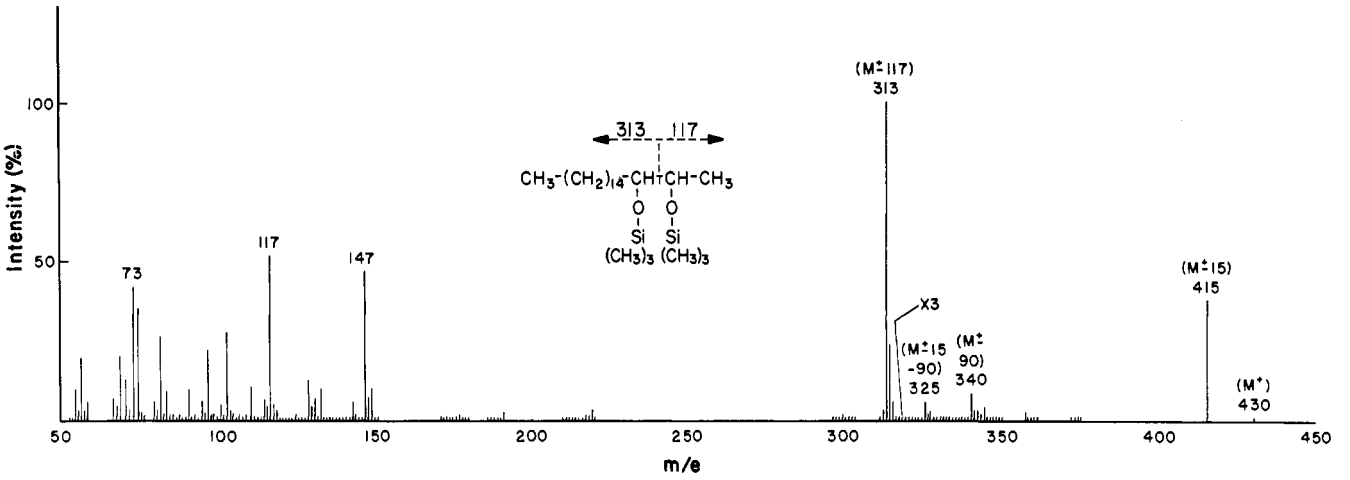

FIGURE 2: Mass spectrum of Me_3Si ethers of the major diol (18) shown in Figure 2. The spectrum was recorded at the top of the peak.

The Me_3Si ethers of diols appear to be the most convenient derivative for structure determination by gas–liquid chromatography and mass spectrometry. The simple fragmentation patterns observed (Figure 2) are readily interpreted, the preparation of the derivative is quick and simple and no purification is required prior to injection into the gas chromatograph.

In order to reconfirm the assigned structures of the diols and to aid in the analysis of products from the biosynthetic experiments a chemical degradation was used. Periodate oxidation followed by permanganate oxidation of the resulting aldehyde gave fatty acids which were analyzed as methyl esters by gas–liquid chromatography (the data can be seen in the figures from labeling experiments). The C_{16} acid was the major component followed by C_{18} acid with some C_{17} and C_{15} acids, and these results are completely in agreement with the mass spectral analysis.

Similar structural studies (structural data not shown) on the diols isolated from the preen glands of chicken showed that C_{22}, C_{23}, and C_{24} were the major diols confirming earlier reports (Haahti and Fales, 1967; Hansen *et al.*, 1969). In our attempts to find a suitable experimental system for biosynthetic studies we also examined the diols of Japanese quail (*Coturnix coturnix japonica*). The chain lengths of the diols of this bird resembled those of chicken in that C_{22}, C_{23}, and C_{24} were the major ones (Table I). However in quail C_{24} constituted only about 20% of the diol whereas in chicken this diol amounted to nearly one-half of the diol fraction. In any case these structural studies clearly show that the chain lengths of the diols exhibit great variation among the various species of birds. Since the predominant diol in ring-necked pheasant is C_{18}, this bird was chosen as the experimental organism.

TABLE I: Chain-Length Distribution of Alkane-2,3-diols in the Diol Diesters of the Preen Glands of Chicken, Quail, and Pheasants.

Chain Length	% Composition		
	Chicken	Quail	Pheasant
C_{16}	T	T	0.5
C_{17}	T	T	3.2
C_{18}	T	5.0	85.0
C_{19}	T	T	4.6
C_{20}	0.7	3.0	6.7
C_{21}	1.9	4.5	T
C_{22}	32.1	34.5	T
C_{23}	16.4	33.1	
C_{24}	48.9	19.8	

Incorporation of [1-^{14}C]Acetate into Diol Diesters. According to a hypothetical pathway (Kolattukudy and Walton, 1972) for biosynthesis of alkane-2,3-diols, a fatty aldehyde condenses with hydroxyethylthiamine pyrophosphate to give an acyloin which is reduced to a diol with subsequent acylation to give the diol diesters (Figure 3). In order to test such a hypothesis incorporation of labeled precursors into the diols had to be accomplished. Since acetate would be the precursor of the carbon chains that probably participate in the synthesis of diols, 40 μCi of [1-^{14}C]acetate was injected under the the glands of pheasants. The oil isolated after 3 days contained over 3×10^6 cpm. The gland tissue lipids contained 1.3×10^6 cpm. Thin-layer chromatography of the labeled oil showed that the major (90%) part of the label was in the diol diester fraction and that much smaller amounts of label were found in triglyceride, wax ester and polar lipid fractions (Figure 4). When the diol diester fraction was treated with BF_3–methanol reagent and the products analyzed by thin-layer chromatography, all the label was found in the methyl ester and diol fractions, in a ratio of 1.2:1. The diols were further analyzed by radio gas–liquid chromatography as their diacetates and the results (Figure 5, right) showed that the major part of the ^{14}C in the diol fraction was in C_{18} diol, followed by C_{20} diol and C_{19} diol. The amount of ^{14}C present in each diol was proportional to the chemical quantity of that diol. This labeling pattern is that expected from *de novo* synthesis of the diols from acetate. Treatment of the diols with periodate and permanganate followed by isolation of the products showed that virtually all the label was in the acids isolated. Radio gas–

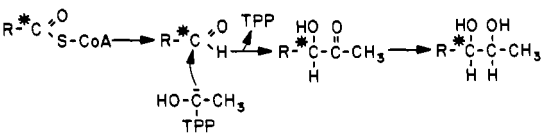

FIGURE 3: Hypothetical pathway for the biosynthesis of alkane-2,3-diols.

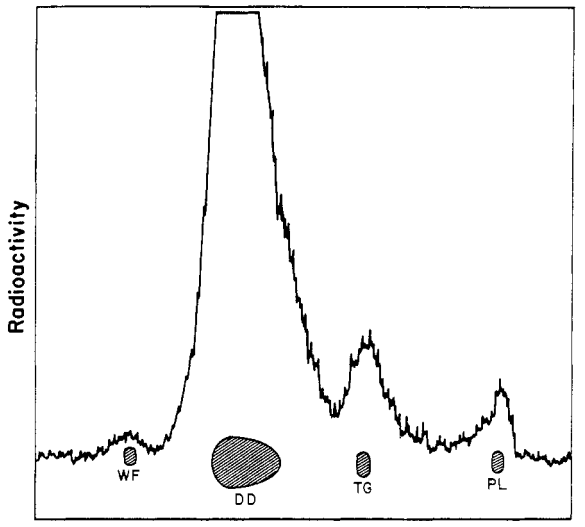

FIGURE 4: Radio thin-layer chromatogram of the preen oil isolated 3 days after injection of the pheasant with 40 μCi of [1-^{14}C]acetate. Chromatography was done on 0.50-mm silica gel G with benzene as the solvent system. PL, polar lipids; TG, triglycerides; DD, diol diesters; WF, wax ester fraction.

liquid chromatography of the methyl esters prepared from these acids showed that C_{16} acid contained the major part of ^{14}C followed by C_{18} and C_{17} confirming that the major labeled diol was the C_{18} diol (Figure 5, left). Gas–liquid chromatographic isolation of the individual methyl esters followed by hydrolysis and microSchmidt degradation showed that the carboxyl carbon of the C_{16} acid contained 13.8% of the radioactivity in this acid (Table II). This value is very close to the 12.5% expected from *de novo* synthesis of C_{16} acid. The carboxyl carbon of C_{18} acid derived from the diol contained 17.2% of the radioactivity of this acid. If this C_{18} acid were synthesized strictly by *de novo* synthesis from [1-^{14}C]acetate

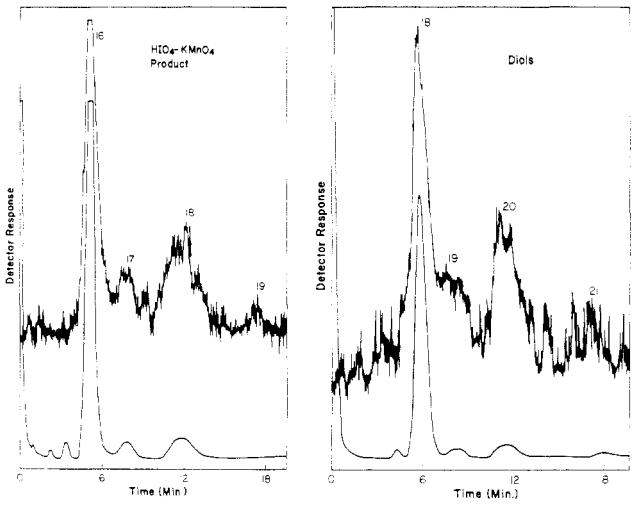

FIGURE 5: Radio gas–liquid chromatograms of the diols as diacetates (right) and methyl esters prepared from HIO_4–$KMnO_4$ products (left) of diols from the preen oil derived from [1-^{14}C]acetate. Chromatography was done with coiled stainless steel column (117 × 0.61 cm o.d.) packed with 3% OV-1 on 80–100 mesh gas chrom Q with 90 cm^3/min of argon. The column temperatures were 200° for diol diacetates and 170° for the methyl esters. The number on each peak represents chain length and in each case top tracing is radioactivity and bottom tracing is flame ionization detector response.

TABLE II: Radioactivity in the Carboxyl Carbon of the Fatty Acid Obtained by HIO_4–$KMnO_4$ Oxidation of Diols Derived from [1-^{14}C]Acetate and [1-^{14}C]Palmitate.

Chain Length of the Acid	Radioactivity (%)		
	[1-^{14}C]-Acetate (3 days)	[1-^{14}C]Palmitate	
		2 days	3 days
16	13.8	48.0	43.3
17	6.4	2.5	5.1
18	17.2	3.4	2.7
Post 18[a]		2.7	2.9

[a] This fraction represents ^{14}C collected from C_{19} and C_{20} regions.

the carboxyl carbon would contain 11% of the ^{14}C. The substantially higher experimental value indicates that labeled acetate was incorporated also by elongation of preformed C_{16} acid. Considering the fact that a relatively long experimental period was used it is highly probable that C_{16} acid formed by *de novo* synthesis from the injected labeled acetate also was elongated. Therefore the major source of C_{18} acid might have been elongation and the results of the Schmidt degradation only points out that some elongation must have occurred. Results discussed under a later section of this paper also point out the occurrence of elongation reactions leading to C_{18} acid formation. The carboxyl carbon of C_{17} acid derived by the periodate oxidation of the C_{19} diol contained 6.4% of the ^{14}C in this acid. If a propionic acid starter gave rise to the C_{17} acid 14.3% of the label would be expected to be at the carboxyl carbon. On the other hand if the C_{17} acid was derived by α oxidation of the C_{18} acid derived from [1-^{14}C]-acetate no radioactivity should be expected to be present at C-1 of the C_{17} acid. The experimental value indicates that both mechanisms may be operative in the formation of the C_{17} acid utilized in the C_{19} diol synthesis.

Incorporation of [1-^{14}C]Palmitic Acid and [1-^{14}C]Stearic Acid into the Diol Diesters. Fatty acids are known to undergo reduction to the aldehyde in other systems (Kolattukudy, 1970b, 1971; Day *et al.*, 1970: Snyder and Malone, 1970). Therefore incorporation of specifically labeled fatty acids of appropriate chain length may be used to test the proposed biosynthetic pathway for diol synthesis. Preliminary experiments with slices of glands showed that exogenous [^{14}C]fatty acids were incorporated into diol diesters but most of the ^{14}C was in the acyl moiety and therefore the mechanism of formation of diol could not be studied readily. Much longer periods of incubation of gland slices could not be done without bacterial contamination. On the other hand injection of labeled fatty acid under the two lobes of the gland of live pheasants could be used with longer experimental periods and under these conditions exogenous fatty acids were readily incorporated into the diols. Attempts to study the time course of incorporation of [1-^{14}C]palmitic acid into diols showed that the label in preen lipids was maximal 48–72 hr after injection and then decreased. Therefore in all experiments preen lipids were isolated within 72 hr.

In a typical experiment the preen oil isolated 72 hr after injection of 40 μCi of [1-^{14}C]palmitic acid contained 10 × 10^6 cpm and tissue lipids contained 2.6 × 10^6 cpm

TABLE III: Radioactivity Distribution among the Diols of the Oil Diol Diesters Derived from [1-^{14}C]Palmitate, [1-^{14}C]Stearate, and the Acids Derived from them by HIO_4–$KMnO_4$ Cleavage.[a]

Substrate	Diols				HIO_4–$KMnO_4$ Products			
	C_{18}	C_{19}	C_{20}	C_{21}	C_{16}	C_{17}	C_{18}	C_{19}
[1-^{14}C]Palmitate	45.9	12.5	31.4	10.2	44.6	16.3	36.0	3.2
[1-^{14}C]Stearate	19.6	9.27	71.1		20.6	14.8	64.6	

[a] The radioactivity distribution (%) was determined by radio gas–liquid chromatography as indicated under Figure 5 except that the column temperature for analysis of methyl esters was 160°. Other experimental details are described in the text.

while a wash of the outer surface of the bird gave 3.9 × 10^6 cpm. Thin-layer chromatography revealed that all three fractions contained a labeled material which cochromatographed with diol diesters. However the major part of this material was found to be in the preen oil fractions. Therefore in most cases we did further work only with the preen oil. All three sources of lipids contained small amounts of labeled triglycerides and varying amounts of ^{14}C were found in free fatty acids, polar lipids and wax esters which were generally ignored. Usually the lipids from the feather contained more labeled wax ester than the other sources of lipid.

The diol diester fraction was further purified by repeated thin-layer chromatography and then treated with BF_3–methanol reagent. All the radioactivity was contained in two thin-layer chromatographic fractions. The distribution of radioactivity between the two products showed that the methyl ester contained 1.5–2.5 times as much radioactivity as in the diol fraction. When the diol fraction was acetylated and the products subjected to thin-layer chromatography, it was found that all the radioactivity was in a component which cochromatographed with authentic alkane-2,3-diol diacetates. The radio gas–liquid chromatography of the diol diacetates showed that the major radioactive component was octadecane-2,3-diol (Table III). However eicosane-2,3-diol and nonadecane-2,3-diol also were labeled. In fact the amount of ^{14}C in the C_{20} diol was about two-thirds of that in C_{18} diol. The most obvious explanation for this observation is that the exogenous C_{16} acid underwent chain elongation prior to incorporation into the diol. Considering the relatively long periods of metabolism used in the present experiments such a suggestion appears reasonable. Experimental evidence for such a hypothesis was provided by the observation that the major part of the label in the acyl moiety of the diol diesters was in the C_{18} acid as discussed later in this paper. Since it was obvious that the carbon chain of the exogenous acid was modified prior to incorporation into the diol, it was not clear whether the C_{16} acid chain was incorporated intact into the C_{18} diol. According to the hypothesis previously proposed for the biosynthesis of the diol, the intact carbon chain of C_{16} acid should be incorporated and the carboxyl carbon of the acid should become C-3 of the C_{18} diol. In order to test this possibility the diols were cleaved with periodate and the resulting aldehydes were oxidized to the corresponding acids (Figure 6). All the radioactivity contained in the diol was recovered in the fatty acids showing that significant amounts of ^{14}C were not present in C-1 and C-2 of the diol. Radio gas–liquid chromatography of the fatty acids showed that the major acid was C_{16} acid as expected, with substantial amounts of C_{18} and C_{17} acids (Table III). If the intact 1-^{14}C-labeled C_{16} acid was incorporated into the C_{18} diol as shown in Figure 3 the carboxyl carbon of the C_{16} acid derived from the chemical cleavage of the diol should contain all the ^{14}C of the acid. If the incorporation of ^{14}C into C_{18} diol represents merely degradation into acetate and resynthesis, the carboxyl carbon of the C_{16} acid should contain only 12.5% of the ^{14}C in the C_{16} acid. The fact that radioactivity distribution among the various diols derived from 1-^{14}C-labeled C_{16} acid was different from that obtained in the experiments with [1-^{14}C]acetate argues against degradation and resynthesis. Furthermore the microSchmidt decarboxylation of C_{16} acid (Table II) showed that about one-half of the label in the C_{16} acid was located at the carboxyl carbon which was derived from C-3 of the C_{18} diol. This result clearly shows that intact C_{16} acid was incorporated into C_{18} diol and that the carboxyl carbon became C-3 of the diol. The other major diol, C_{20} diol, was presumably synthesized from C_{18} acid formed from the exogenous 1-^{14}C-labeled C_{16} acid by elongation. If this hypothesis is true the carboxyl carbon of the C_{18} acid chemically produced from the C_{20} diol should have no radioactivity. Experimental results (Table II) showed that the CO_2 released by Schmidt decarboxylation contained only 3.5% of the ^{14}C of the molecule. This quantity is much less than that expected from *de novo* synthesis of C_{18} acid from acetate derived from the exogenous 1-^{14}C-labeled C_{16} acid. This result also rules out the possibility that C_{18} acid involved in the formation of C_{20} diol was produced by elongation utilizing labeled acetate derived from exogenous C_{16} acid because if such was the case the carboxyl carbon of C_{18} acid derived from C_{20} diol should have all the ^{14}C in the carboxyl carbon.

If the proposed hypothesis for the biosynthesis of alkane-

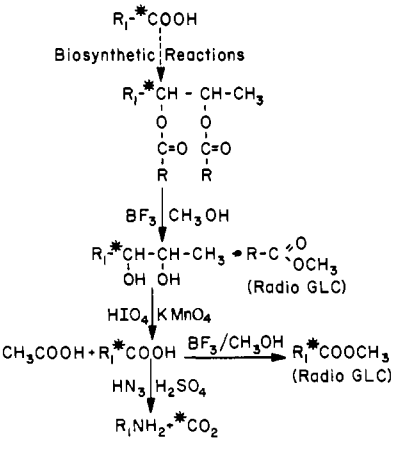

FIGURE 6: Hypothetical fate of C-1 (*) of a fatty acid, and the chemical degradation of the alkane-2,3-diols derived from this acid.

TABLE IV: Radioactivity Distribution (%) among the Diols of the Tissue Diol Diesters Derived from [1-^{14}C]Acetate, [1-^{14}C]Palmitate, and [1-^{14}C]Stearate.[a]

Substrate	C_{16}	C_{18}	C_{19}	C_{20}
[1-^{14}C]Acetate	4.28	82.0	3.91	9.84
[1-^{14}C]Palmitate	3.90	74.1	8.52	13.5
[1-^{14}C]Stearate		43.6	1.91	54.5

[a] A 3-day metabolic period was used in each case and the radio gas–liquid chromatographic conditions were the same as those under Figure 5. The other experimental details are described in the text.

2,3-diol is correct, exogenous C_{18} acid would be expected to give rise to C_{20} diol. In order to test this possibility 1-^{14}C-labeled C_{18} acid was injected under the preen glands of pheasants and the products were processed as described for C_{16} acid experiments. Although stearic acid was readily incorporated into polar lipids, triglycerides and wax esters the amount incorporated into diol diester fraction was much less than that observed with [1-^{14}C]palmitic acid. For example, 3 days after injecting 40 μCi of [1-^{14}C]stearic acid into a bird only about 3.5×10^5 cpm could be found in the diol diester. About 21% of the radioactivity in the diol diester fraction was recovered in the diols; the rest of the ^{14}C was in the acyl moiety. Radio gas–liquid chromatography of diols (as diacetates) showed that the major labeled component was C_{20} diol (Table III) as expected from the proposed pathway. The C_{18} diol contained less than one-fifth of the radioactivity indicating some degradation. Cleavage of the diols by periodate permanganate gave rise to acids containing all the ^{14}C of the diol fraction. Radio gas–liquid chromatography showed that C_{18} acid contained the major part of the radioactivity (Table III) but C_{16} acid also contained significant amount of ^{14}C obviously suggesting that the exogenous [1-^{14}C]stearic acid did undergo some β oxidation to acetate followed by resynthesis of fatty acids which gave rise to the C_{18} diol.

The gland tissue lipids isolated from the experiments with [1-^{14}C]palmitic acid, [1-^{14}C]stearic acid, and [1-^{14}C]acetate contained labeled diol diesters. The radioactivity distribution in the diols of tissue lipids was markedly different from that of the diols of the oil. In the case of the experiments with C_{16} acid, the major part of the radioactivity in the tissue diols was in C_{18} diol whereas in the oil diols C_{20} and C_{19} contained nearly half of the total ^{14}C contained in the diol fraction (Table IV). Our interpretation of this observation is as follows. The exogenous substrates enter the gland tissue, where diol diesters are synthesized and then excreted into the gland oil. Therefore the diols found in the oil must have been synthesized earlier during the experimental period than those found in the tissue at the end of the experiment. It is probable that in the early phase of the experiment the intact chains of exogenous acids were incorporated into the diols directly and after elongation with unlabeled acetate, whereas during the later periods the labeled acetate derived from the substrate would be the major source of label for diol synthesis. If such is the case the diols in the tissue most probably came from the *de novo* synthesis rather than from the direct incorporation of the exogenous acids and under these conditions C_{18} diol would be expected to be the major labeled product as was the case when [1-^{14}C]acetate was the substrate. If this interpretation is correct the diols in the tissue from the experiments with C_{18} acid should contain substantial amounts of diol formed from the labeled acetate derived from β oxidation of C_{18} acid and therefore in this case C_{18} diol should contain proportionately more ^{14}C than in the corresponding fraction of oil diols. Experimental results in Table IV show that nearly one-half of the label in the tissue diol fraction was in fact in C_{18} diol whereas only about 20% of the label in the oil diol was in the C_{18}. Also in support of the interpretation discussed above is the observation that in the case of experiments with labeled acetate radioactivity distribution in the tissue diol was identical with that in the oil diols.

Incorporation of [1-^{14}C]Acetate, [1-^{14}C]Palmitate, and [1-^{14}C]Stearate into the Acyl Moieties of Diol Diesters. The acyl moieties of the diols were labeled by all three of the precursors used in this study. However, radioactivity distribution among the various acids depended on the precursor used. [1-^{14}C]Acetate was incorporated into C_{18}, C_{16}, and C_{14} acids of diol diesters with much less amounts in C_{15}, C_{17}, and C_{19} (Table V). The radioactivity in each component was proportional to the chemical quantity as expected from *de novo* synthesis of these acyl moieties from the labeled acetate. The major part of the radioactivity contained in the acyl moiety of the diol diesters isolated from the experiments with [1-^{14}C]palmitic acid was in C_{18} acid with much lower amounts of label in C_{16} and C_{17} acids. This labeling pattern clearly shows that the exogenous acid was elongated prior to incorporation into the acyl moieties of diol diesters. The fact that analysis of the diols from this experiment showed that substantial amounts of label was in C_{20} diol is in agreement with the conclusion that exogenous C_{16} acid underwent elongation prior to reaching the site of synthesis of diol diesters. Radio gas–liquid chromatographic analysis of the acyl moieties of diol diesters obtained from experiments with [1-^{14}C]stearic acid showed that this acid was incorporated intact into the diesters (Table V).

Incorporation of [2-^{14}C]Pyruvate, [2-^{14}C]Lactate, [U-^{14}C]-Alanine, [2-^{14}C]Propionate, and [1-^{14}C]Glucose into Diol Diesters. The results presented thus far in this paper show that C_{16} and C_{18} fatty acids are incorporated intact into the C_{18} and C_{20} alkanediols, respectively, and that the carboxyl carbon of the acid becomes C-3 of the diol. This finding is consistent with the proposed hypothesis for the biosynthesis of alkane-2,3-diols (Figure 3). We have observed that the fatty acids injected can in fact be reduced to the alcohol presumably involving an aldehyde intermediate. Such an aldehyde could be condensing with hydroxyethylthiamine pyrophosphate to give the acyloin which is subsequently reduced to the diol.

According to such a hypothesis C-1 and C-2 of the diol should originate from C-2 and C-3 of pyruvate. In order to test this possibility [2-^{14}C]pyruvate was injected under the preen glands of pheasants. Diol diesters were labeled in 24- to 36-hr experimental periods. Twenty-four hours after injection of about 20 μCi of [2-^{14}C]pyruvate the preen oil and preen tissue lipid fractions isolated contained 4×10^4 and 3.2×10^5 cpm, respectively. The diol diesters isolated from these fractions contained 2.5×10^4 and 2.0×10^5 cpm, respectively, and in both cases the diols and acyl moieties contained approximately equal amounts of radioactivity. The fatty acids obtained by HIO_4–$KMnO_4$ treatment of the diols contained virtually all the radioactivity of the diols showing that [2-^{14}C]pyruvic acid did not preferentially label the C-1 and C-2 of the diol under the present experimental conditions. In fact radio gas–liquid chromatography of acyl moieties

TABLE V: Radioactivity (%) Distribution among the Acyl Chains of the Oil Diol Diesters Derived from [1-^{14}C]Acetate, [1-^{14}C]-Palmitate, and [1-^{14}C]Stearate.[a]

Substrate	C_{12}	C_{14}	C_{15}	C_{17}	C_{17}	C_{19}	C_{19}	C_{20}
[1-^{14}C]Acetate	8.80	16.4	3.30	19.8	5.67	39.0	7.04	
[1-^{14}C]Palmitate				11.8	11.5	63.7	8.71	4.37
[1-^{14}C]Stearate		6.61		2.30	2.30	74.6	4.00	10.2

[a] A 3-day metabolic period was used in each case and the methyl esters obtained from BF_3–CH_3OH treatment of radiochemically pure diol diesters isolated from the oil was analyzed by radio gas–liquid chromatography under the conditions indicated in Figure 5.

of the diol diesters and the fatty acids obtained by HIO_4–$KMnO_4$ degradation of the diol showed a pattern of radioactivity distribution very similar to that obtained with acetate injection experiments. These results indicate that the label from the exogenous pyruvate was incorporated into the diol diesters *via* acetate derived from the pyruvate. Chemical degradation of the labeled diols derived from [U-^{14}C]alanine and [2-^{14}C]lactic acid also showed that virtually all the ^{14}C of the diol was contained in the long chain side of the diol. Since our degradation studies involved analysis of the purified degradation products, it is highly unlikely that these results are due to some labeled non-diol material contained in the diol fraction. Thus exogenous labeled pyruvate, alanine, and lactate, three possible precursors of the C-1 and C-2, did not preferentially label these two carbons of the diol. Therefore we conclude that either the hypothesis in Figure 3 is untenable or the exogenous substrates are so readily converted into acetate that they do not provide labeled hydroxyethylthiamine pyrophosphate at the site of diol synthesis under the present experimental conditions. We also incubated preen gland homogenates with [1-^{14}C]palmitaldehyde or [1-^{14}C]palmitoyl-CoA and hydroxyethylthiamine pyrophosphate under many different conditions but very little diol formation could be detected with the preparations thus far examined.

Since our attempts to test whether C-1 and C-2 of the diol originate from hydroxyethylthiamine pyrophosphate failed, we considered other possible routes for diol biosynthesis. Since even carbon chains predominate in the diols a usual fatty acid synthesis-type mechanism involving a decarboxylation near the terminal stage was considered unlikely. However, if a methylmalonyl-CoA substituted for the final malonyl-CoA in a fatty synthesis type reaction sequence an α-methyl α,β-unsaturated acid could be formed which could be converted into an alkane-2,3-diol by decarboxylation and epoxidation of the double bond followed by hydration of the epoxide. Since propionic acid is the usual precursor of methylmalonyl-CoA, [2-^{14}C]propionic acid would be expected to label the C-2 of the diol. However a detailed analysis of the HIO_4–$KMnO_4$ oxidation products of the pure labeled diol derived from [2-^{14}C]propionate showed that virtually all the ^{14}C in the diol was in the long alkane chain. The radioactivity distributions among the acyl moieties of the diol diester and the fatty chains derived from the diol was remarkably similar to those observed with [1-^{14}C]acetate except that the odd chains appeared to have slightly more radioactivity than with acetate. Therefore it is concluded that the C-1 and C-2 of the diol do not originate from propionate and that label from propionate was incorporated into the diol presumably *via* acetate indirectly derived from propionate.

We have also made attempts to test whether the thiamine pyrophosphate derivative of glycoaldehyde such as that involved in *trans*-ketolase reactions might be involved in diol synthesis but with no success. Injection of [1-^{14}C]glucose followed by isolation of the preen products and detailed analysis of the diols and their HIO_4–$KMnO_4$ degradation products showed that this substrate also labeled the alkane chain but did not preferentially label the C-1 and C-2 of the diol.

The results described in this paper constitute the first report of biosynthetic studies on alkane diols and they clearly show that a variety of labeled materials injected under the preen gland can give rise to labeled diols. Regarding the biosynthetic pathway for diols it is clear that the carboxyl carbon of palmitic acid becomes the C-3 of the C_{18} diol and that C_{18} acid gives rise to C_{20} diol in a similar manner. This finding rules out several possible routes for diol synthesis and it is consistent with the acyloin pathway shown in Figure 3. However, until the origin of the C-1 and C-2 of the diol can be elucidated the acyloin pathway must remain only a working hypothesis. At present, attempts to determine the nature of the precursor of the C-1 and C-2 of the diol are under way in this laboratory.

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References

Baumann, W. J., Schmid, H. H. O, and Mangold, H. K. (1969), *J. Lipid Res. 10*, 132.

Brady, R. O., Bradley, R. M., and Trams, E. G. (1960), *J. Biol. Chem. 235*, 3093.

Day, J. I. E., Goldfine, H., and Hagen, P. O. (1970), *Biochim. Biophys. Acta 218*, 179.

Downing, D. T., Kranz, Z. H., and Murray, K. E. (1961), *Aust. J. Chem. 14*, 619.

Fieser, L. F., Huang, W., and Bhattacharyya, B. K. (1957), *J. Org. Chem. 22*, 1380.

Folch, J., Lees, M., and Sloane Stanely, G. H. (1957), *J. Biol. Chem. 226*, 479.

Haahti, E. O. A., and Fales, H. M. (1967), *J. Lipid Res. 8*, 131.

Hansen, I. A., Jang, B. K., and Edkins, E. (1969), *J. Lipid Res. 10*, 267.
Horn, D. H. S., and Hougen, F. W. (1953), *J. Chem. Soc.*, 3533.
Karkkainen, J., Nikkari, T., Ruponen, S., and Haahti, E. (1965), *J. Invest. Dermatol. 44*, 333.
Kolattukudy, P. E. (1965), *Biochemistry 4*, 1844.
Kolattukudy, P. E. (1970a), *Annu. Rev. Plant Physiol. 21*, 163.
Kolattukudy, P. E. (1970b), *Biochemistry 9*, 1095.
Kolattukudy, P. E. (1971), *Arch. Biochem. Biophys. 142*, 701.
Kolattukudy, P. E., and Walton, T. J. (1972), *Progr. Chem. Fats Other Lipids 13*, 119.
Malziak, P. (1962), *Phytochemistry 1*, 79.
Murray, K. E., and Schoenfield, R. (1955), *Aust. J. Chem. 8*, 424.
Nicolaides, N. (1965), *J. Amer. Oil Chem. Soc. 42*, 691.
Saito, K., and Gamo, M. (1970), *J. Biochem. (Tokyo) 67*, 841.
Snyder, F., and Malone, B. (1970), *Biochem. Biophys. Res. Commun. 41*, 1382.