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Studies on the Nature and Formation of α-Glyceryl Ether Lipids in Bovine Bone Marrow*

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Received March 21, 1963

The distribution and biosynthesis of α-glyceryl ether phospholipids in bovine hematopoietic bone marrow have been studied. The two major phospholipid fractions, phosphatidyl ethanolamine and phosphatidyl choline, contain, in addition to the diacyl compounds, significant levels of vinyl ether derivatives (plasmalogens) and of glyceryl ether (chimyl, batyl, and selachyl alcohols) derivatives. In incubation experiments, radioactivity from glucose-6-C^{14}, sodium palmitate-1-C^{14}, and tritiated water were incorporated into glyceryl ether phospholipids by extracts of bone marrow. At the end of the incubation times studied, radioactivity of the glyceryl ether phospholipids was less than that of the nonglyceryl ether phospholipids. Possible relationships with plasmalogens are discussed.

Until recently most evidence on naturally occurring α-glyceryl ethers came from analyses of either the total lipid or neutral lipid fraction of the tissue examined (Bodman and Maisen, 1958). In 1958 a glyceryl ether–containing phospholipid, an analog of phosphatidyl ethanolamine, was reported for the first time (Carter *et al.*, 1958). Since that time glyceryl ethers have been found associated with the phospholipids from numerous sources (Ansell and Spanner, 1961; Pietruszko and Gray, 1962; Renkonen, 1962; Pietruszko, 1962). In at least two tissues, bovine erythrocytes (Hanahan and Watts, 1961) and red bone marrow (Thompson and Hanahan, 1962), glyceryl ether phospholipids account for over 10 mole per cent of the total.

Using an *in vitro* preparation of bovine hematopoietic marrow, Thompson and Hanahan (1962) have reported preliminary experiments showing that the glyceryl moiety of glyceryl ethers can be synthesized from glucose and that when glucose-6-C^{14} is employed, the glyceryl ethers isolated from the phosphatidyl ethanolamine fraction contain radioactivity almost exclusively in the 3-position (that bearing the primary alcohol substituent) of the glyceryl moiety. These results would suggest that the glucose-derived intermediate is α-glycerophosphate, as in the case of diacyl phospholipids.

In the present paper the details of these and further experiments using glucose-6-C^{14}, palmitic acid-1-C^{14}, and tritiated water are reported. In all cases the incorporation into the glyceryl ether phospholipids is much lower than that found for their diacyl counterparts.

Experimental

Materials

Bovine red sternal marrow and yellow marrow from femurs and tibiae were obtained through the cooperation of the Seattle Packing Company. The sternal bones were removed within 30 minutes after death of the animal and were preserved at 0° until the marrow could be removed and prepared for incubation. Incubation was initiated within 3 hours after the animal's death.

Calf marrow was obtained from the femurs and tibiae of 3- to 5-week-old calves under nitrous oxide and anectine chloride anesthesia.[1] These preparations

* This investigation was supported in part by grants from the National Science Foundation (G 22078), the United States Public Health Service (Rg-5519), and the American Cancer Society. G. A. Thompson holds a Public Health Service research career program award (AM-K3-9147) from the National Institute of Arthritis and Metabolic Diseases.

[1] We appreciate the donation of calf bone marrow by Dr. Patrick Goldsworthy, Department of Medicine, University of Washington.

were ready for incubation within 2 hours after their removal from the animal.

Samples of glucose-6-C^{14} (3.56 mc/mmole, and 25 mc/mmole) and of palmitic acid-1-C^{14} (29.7 mc/mmole) were purchased from the Nuclear-Chicago Corporation. Tritiated water (100 mc/mg) was obtained from the Volk Radiochemical Company.

Column chromatography was carried out using Mallinckrodt reagent grade silicic acid and Johns Manville Hyflo Super-Cel. Chloroform, methanol, and ethyl ether were reagent grade and were used without further purification. Hexane (Phillips technical grade) and ethyl acetate were redistilled, and fractions collected at 68° and 77°, respectively, were retained.

Procedures

Lipid Isolation.—Marrow preparations were extracted by the addition of 3–5 volumes of methanol, followed, in 10 minutes, by the addition of 3–5 volumes of chloroform. The mixture was then ground either in a mortar (small samples) or in a Waring Blendor (large samples) and filtered. The residue was reextracted with chloroform-methanol 1:1 (v/v). Purification of the crude extract was effected using the procedure of Dodge *et al.* (1963). For silicic-acid column chromatography of phospholipids the methods of Hanahan *et al.* (1960) were modified by the introduction of an additional elution step involving a 3:2 (v/v) mixture of ethyl acetate and methanol.

Thin-layer chromatography on silica gel G was carried out using the solvent systems chloroform-methanol-water (95:35:4, v/v) for phospholipid separations and petroleum ether (60–70°)–diethyl ether–acetic acid (90:10:1 v/v) for neutral lipid separations. Location of lipid spots on the plates was determined by spraying with sulfuric acid and heating the plates at 300° for 10 minutes.

Phosphorus was determined by the method of King (1932), nitrogen by a micro-Kjeldahl procedure with selenium oxychloride as catalyst, and fatty acid ester content by measurement of the infrared absorption peak at 5.75μ. Plasmalogen was determined by the procedure of Gottfried and Rapport (1962). Glyceryl ether content was measured by the acetolysis and saponification procedure as used by Hanahan and Watts (1961).

The individual glyceryl ethers were identified by gas-liquid chromatography of the isopropylidine derivatives (Hanahan *et al.*, 1963), using a Barber Colman Model-10 argon chromatograph. Separations were made on 5-ft columns of 15% polyethylene glycol succinate on Anakrom AB, 60/70 mesh, under conditions of 175° column temperature and 16 psi inlet pressure of carrier argon. A typical retention time for the derivative of chimyl alcohol was 46 minutes. Hydrogenations of plasmalogens were carried out in a Parr Instrument Company apparatus using 1% pyridine in methanol as solvent, platinum oxide as catalyst, and a hydrogen pressure of approximately 40 psi.

Cleavage of the glyceryl ether molecule between the vicinal hydroxyl groups was brought about by oxidation with periodic acid. The procedure used is illustrated by this typical example: 50 mg of glyceryl ether mixture in 10 ml 95% ethanol was treated with 107.8 mg periodic acid (H_5IO_6) dissolved in 5 ml 95% ethanol. After remaining in the dark at room temperature for 1 hour the oxidation mixture was diluted with 15 ml water and extracted twice with 20-ml aliquots of chloroform. The chloroform extract, containing the ether-aldehyde product and traces of unreacted glyceryl ethers, was washed three times with water and dried over Na_2SO_4.

The aqueous portion remaining after chloroform extraction was adjusted to *p*H 5.5 with 6 N KOH and mixed with 316 mg dimedon in 7 ml 50% ethanol. After the material stood overnight at 4°, crystals were collected and recrystallized from aqueous ethanol; mp 188–189° (uncorr.), literature value 189° for formaldehyde derivative.

Radioactivity measurements were made using a Packard Tricarb Scintillation Spectrometer. Quenching in the diphenyloxazole-toluene type scintillation fluid was corrected by the use of internal standards.

Incubations.—Preparation of the marrow specimens for incubation was carried out at 4°. The marrow was pressed through cheesecloth to remove blood vessels and fragments of bone. This material was then treated by one of the procedures outlined below.

In experiments 1 and 2, aliquots of whole marrow cell suspensions were incubated in an equal volume of fresh citrated bovine plasma and of physiological saline (*p*H 7.4), respectively. Experiments 3–7 involved suspension of the cells in physiological saline at *p*H 7.4 and centrifugation at 1000 × *g* for 20 minutes at 4°. The floating pellet, containing mainly triglycerides, was discarded, and the phospholipid-enriched sediment was retained. In experiments 4–7 the sediment was treated further before incubation by rupturing all but a small percentage of the intact cells in a glass homogenizer. In addition to 0.03 M potassium phosphate buffer, *p*H 7.0, the incubation mixtures of experiments 4–7 were supplemented with the following factors: adenosine triphosphate, disodium salt, 2 μmoles; coenzyme A, 0.1 μmoles; reduced diphosphopyridine nucleotide, 1.5 μmoles; reduced triphosphopyridine nucleotide, 2.5 μmoles; glutathione, 20 μmoles; creatine phosphate, 7.5 μmoles; $MgCl_2$, 3 μmoles. Final volumes of the incubation mixtures were from 4–5 ml. Quantities of radioactivity used were from 2–10 μc per sample in the cases of glucose-6-C^{14} and palmitate-1-C^{14}, and 30 mc per sample in the case of tritiated water.

Experiment 8 involved the use of a mitochondria-enriched fraction. This was obtained by centrifuging the 1000 × *g* (20 minutes) supernatant of the crude homogenate at 12,000 × *g* for 30 minutes in a Sorvall refrigerated centrifuge. The resulting pellet, considered to be composed principally of mitochondria, was resuspended in a portion of the supernatant which had been freed of microsomes by centrifugation at 105,000 × *g* for 60 minutes in a Spinco Model L refrigerated centrifuge. Cofactor additions were identical to those for experiments 4–7.

Incubations were carried out under air with shaking in a 37° bath. Intact cells were incubated for 3 hours, and cell homogenates for 1 hour. Incubations were terminated by beginning the usual lipid extraction procedures.

Results

Nature of Bovine Marrow Lipids.—Bovine marrow shows marked variations in lipid composition depending upon the age of the animal and the bone from which the marrow is obtained. The femurs and tibiae of calves 2–3 weeks of age contain hematopoietic marrow which was found to have a ratio of neutral lipid to phospholipid (by weight) of approximately 70. As the calf matures the phospholipid and presumably the blood-forming capacity disappear, leaving neutral lipid consisting almost exclusively of triglycerides but with traces of cholesterol. Analysis of this phospholipid-free marrow has confirmed the finding by Holmes *et al.* (1941) that glyceryl ethers are present. After silicic-acid chromatography of the lipid, the

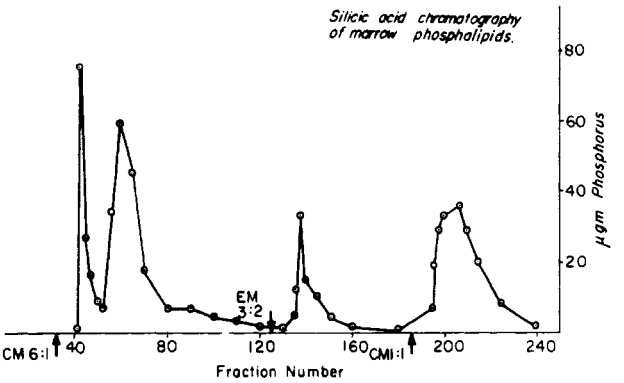

FIG. 1.—Elution diagram of bovine phospholipids. C = chloroform, M = methanol, E = ethyl acetate. All eluent mixtures are v/v. Fraction volume approximately 4 ml.

glyceryl ethers were found to be associated with the triglyceride components, indicating that they are present in the neutral lipid as the diacyl derivatives.

Adult sternal marrow used for some of the experiments reported here differed by having a higher proportion of phospholipid (neutral lipid-phospholipid = between 12 and 17).

The marrow phospholipid from all bones examined appears to have the same composition. Column chromatography of the phospholipid on silicic acid allowed a separation of the lipids as shown in the elution diagram (Figure 1). Thin-layer chromatography of aliquots from selected fractions provided qualitative data on the phospholipids present.[3] The first peak (fractions 41–50) consisted of compounds not positively identified but showing characteristics of phosphatidic acid and cardiolipin. The second peak (fractions 53–80) contained ethanolamine phospholipid, and the third (fractions 131–170) was composed of phosphatidyl serine and phosphatidyl inositol. The early portion of the last peak eluted from the column consisted solely of phosphatidyl choline. After the main body of phosphatidyl choline had been eluted, sphingomyelin also began to appear in the eluate. The peak was divided into a phosphatidyl choline fraction (fractions 194–220) and a sphingomyelin (+ some phosphatidyl choline) fraction (fractions 221–240, + chloroform-methanol 1:9 eluate).

Table I outlines the quantitative phospholipid distribution following silicic-acid chromatography. The isotope incorporation figures are the results of an experiment in which sternal marrow was incubated with glucose-6-C^{14} for 3 hours. Table II lists data concerning the properties of the various fractions.

The phosphatidyl ethanolamine and phosphatidyl choline fractions together accounted for about 70% of the total lipid phosphorus as well as 85% of the phospholipid C^{14}-incorporation. These two fractions were examined in considerable detail.

The fatty acid ester–phosphorus ratio for the phosphatidyl ethanolamine fraction was 1.42, thus suggesting a content of about 40% diacyl phospholipid and 60% monoacyl phospholipid. The plasmalogen content was found to be approximately 22 mole per cent, and glyceryl ethers equivalent to 28 mole per cent of the total were isolated from the preparation.

After silicic-acid chromatography the first few tubes containing eluted phosphatidyl ethanolamine were heavily contaminated with an unknown nitrogen-containing compound, which had an R_f value on thin-layer plates very slightly higher than that for phosphatidyl ethanolamine. A tube-by-tube assay of radioactivity indicated that this compound was also radioactive and therefore introduced a certain error in calculating specific activities. The unknown compound, by its chromatographic behavior and products of hydrolysis (hexose and, tentatively, lignoceric acid), is suspected to be cerebroside.

By all the criteria employed, namely, thin-layer chromatography, infrared spectroscopy, nitrogen and phosphorus analyses, and by paper electrophoresis and chromatography of the hydrolysis products (Thompson and Hanahan, 1963), the phosphatidyl choline fraction appeared almost pure, with slight amounts of sphingomyelin present. The mole percentages of plasmalogen and glyceryl ether in the fraction are 3 and 12, respectively. This is lower than the expected 25% monoacyl compounds called for by the fatty acid–phosphorus ratio of 1.75.

It might be well to note at this point that although the glyceryl ether isolation has yielded quite reproducible results in several analyses of the phosphatidyl ethanolamine and the phosphatidyl choline components, a test of the recovery by submitting a lipid sample containing a small, known quantity of pure chimyl alcohol to the complete isolation scheme showed that only 85% was recovered. Likewise the plasmalogen content may be somewhat higher than that measured due to the possibility (Rapport and Norton, 1962) that some degradation of these compounds takes place during chromatography. Indeed, the sum of the parts, as judged by the plasmalogen assay after chromatography, did not equal the whole plasmalogen content found for the total lipid.

An analysis of the glyceryl ethers of each fraction (Table III) demonstrated some interesting differences in the distributions. However, in both cases chimyl alcohol (1-*O*-hexadecyl glycerol), batyl alcohol (1-*O*-octadecyl glycerol), and selachyl alcohol (1-*O*-octadecenyl glycerol) constitute the bulk of the material. Corresponding data for the bovine erythrocyte are included for comparison.

Isotope Incorporation Studies.—In two cases, one using leg marrow (exps. 1 and 2, Table IV) and one sternal marrow (exp. 3, Table IV), the glyceryl ethers were

TABLE I

DISTRIBUTION OF PHOSPHOLIPIDS IN BOVINE MARROW

As recovered after silicic acid chromatography. See text for details.

Fraction	Principal Lipid	Per Cent Total Phosphorus (average of 5 experiments)	Specific Radioactivity (cpm/mmole phosphorus)
41–50	Unidentified	6	371,000
53–80	Phosphatidyl ethanolamine	28	235,000
131–170	Phosphatidyl serine phosphatidyl inositol	11	667,000
194–220	Phosphatidyl choline	43	1,020,000
220–240 + Chloroform-methanol 1:9 eluate	Sphingomyelin[a]	12	110,000

[a] Considerable phosphatidyl choline contamination.

[3] Throughout this paper the individual phospholipid fractions will be referred to by the name of the parent diacyl compound despite the presence of plasmalogen and glyceryl ether derivatives.

TABLE II

ANALYSES OF PHOSPHOLIPID FRACTIONS ELUTED FROM SILICIC ACID

Recovery was 98% of phosphorus applied.

Fraction	Total Wt. (mg)	Phosphorus (μmoles)	Nitrogen/ Phosphorus[a]	Plasmalogen/ Phosphorus[a]	Glyceryl Ether/ Phosphorus[a]
Unidentified	8.7	3.8	7.08	0.13	—
Phosphatidyl ethanolamine	45.0	38.7	1.43	0.22	0.28
Phosphatidyl inositol, phosphatidyl serine	24.7	15.4	0.84	0.04	—
Phosphatidyl choline	49.4	57.7	1.08	0.03	0.12
Sphingomyelin	—	6.6	—	0.04	—

[a] Molar ratio.

TABLE III

DISTRIBUTION OF GLYCERYL ETHERS IN PHOSPHOLIPID FRACTIONS

Determined by gas-liquid chromatography of the compounds as the isopropylidine derivatives. Results given as mole per cent of total.

Source	Fraction	Length of Side Chain[a]						
		15:0	15:1	16:0	16:1	17:1	18:0	18:1
Marrow	Phosphatidyl ethanolamine			33.6	tr		29.0	37.0
Marrow	Phosphatidyl choline			40.3	1.6		15.2	42.9
Erythrocyte	Phosphatidyl ethanolamine[b]	tr	tr	31	7	4	31	26

[a] The number preceding the colon indicates the number of carbon atoms; that following the colon indicates the number of double bonds. [b] See Hanahan *et al.* (1963).

isolated from the phosphatidyl ethanolamine fraction and from the phosphatidyl choline fraction after incubation of the tissue with glucose-6-C^{14}. A comparison of the molar specific activities of the nonglyceryl ether phospholipids with those of the glyceryl ether–containing ones revealed that in the phosphatidyl ethanolamine fraction the nonglyceryl ether compounds incorporate about two to three times as much radioactivity as did the glyceryl ether type. In the phosphatidyl choline fraction the difference is even more pronounced with the ratio being between 12 and 14 in favor of the nonglyceryl ether lipid.

Over 95% of the radioactivity incorporated into phospholipids and triglycerides from glucose-6-C^{14} resided in the glyceryl moiety. For triglycerides and diacyl-phosphatides this was determined by alkaline hydrolysis followed by assay of the ether-soluble and water-soluble products for radioactivity. The isolated glyceryl ethers were oxidized with periodic acid, removing the 3-carbon of the glyceryl moiety and, as previously reported for phosphatidyl ethanolamine (Thompson and Hanahan, 1962), providing proof that this carbon atom contained almost all the radioactivity. In Figure 2 evidence is presented to show that glyceryl ethers from the phosphatidyl choline fraction too are labeled almost exclusively in this position. Recoveries of the 3-carbon atom as the dimedon derivative of formaldehyde ranged from 40–65% of theory. The remainder of the molecule was recovered in approximately theoretical yields.

A number of isotope experiments were carried out in which the total phospholipid mixture was analyzed for glyceryl ethers rather than first separating the various phospholipids. The yield of glyceryl ethers makes it appear unlikely that these compounds could be present in more than trace amounts in any phospholipid other than phosphatidyl choline and phosphatidyl ethanolamine.

A summary of the results from several isotope incubations is given in Table IV. There were some differences in the preliminary treatment of the marrow samples (see Procedures). For example the tissue used in experiments 1 and 2 was obtained from the whole-cell preparation, whereas the tissue in experiments 3–7 was the sediment after centrifugation of the preparation in saline. In the latter experiments a large floating pellet was discarded after centrifugation. It seems likely that cells having functions similar to those of adipose tissue may be present in marrow alongside hematopoietic cells. Experiments 5–8 were carried out using a cofactor-enriched medium similar to that of Margolis and Vaughan (1962).

All the methods used for preparing tissue for incubation yielded preparations in which the percentage of total phospholipid radioactivity recovered in glyceryl ethers showed little variation. The incorporation of tritium followed a pattern resembling that of glucose-6-C^{14}, in that approximately 98% of the radioactivity

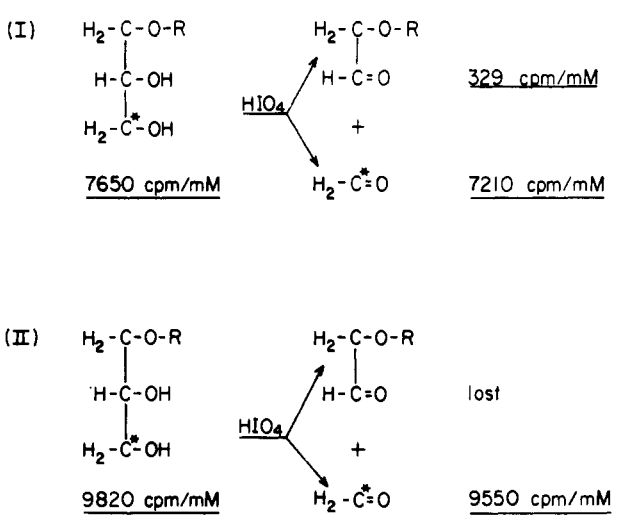

FIG. 2.—Results from degradation of radioactive glyceryl ethers isolated (I) from marrow phosphatidyl ethanolamine fraction and (II) from marrow phosphatidyl choline fraction. See under Experimental for details.

TABLE IV

INCORPORATION OF ISOTOPIC TRACERS INTO BOVINE MARROW LIPIDS

All samples were incubated at 37° with shaking under air. Details of the individual experiments are outlined under Experimental.

Experiment	Tracer	Per Cent Incorp. into Total Lipid	Per Cent of Total Incorp.		Per Cent Phospholipid Radioactivity in Glyceryl Ethers
			Neutral Lipid	Phospholipid	
1	Glucose-6-C^{14}	0.43	55.5	44.5	0.38
2	Glucose-6-C^{14}	1.45	62	38	
3	Glucose-6-C^{14}	1.7	69	31	0.6
4	Palmitic acid-1-C^{14}	32.6	85.8	14.2	0.32
5	Palmitic acid-1-C^{14}	29.7	87.8	12.2	—
6	T_2O		73.4	26.6	0.5
7	Glucose-6-C^{14}	0.65	86.5	13.5	0.7
8 (mitochondria)	Glucose-6-C^{14}		22.7	77.3	0.2

found in the phosphatides resided in the glyceryl portion.

The mitochondrial fraction, unwashed and probably contaminated with microsomes, incorporated less radioactivity from glucose-6-C^{14} than did the complete homogenate. The glyceryl ethers, isolated in a yield amounting to approximately 8 mole per cent of the mitochondrial phospholipid, showed a distribution similar to that of phosphatidyl ethanolamine. The pattern, in mole per cent, was as follows: 16:0, 33.9; 16:1, tr.; 17:1, tr.; 18:0, 30.3; 18:1, 35.8.

Preliminary experiments have been performed to investigate the possible relationship between plasmalogens and glyceryl ether phospholipids. Aliquots of lipid extracted from a marrow cell suspension incubated with glucose-6-C^{14} were analyzed for glyceryl ethers both before and after catalytic hydrogenation. Hydrogenation has been shown (Marinetti *et al.*, 1958) to saturate the vinyl ether double bond of plasmalogens, converting them to glyceryl ether derivatives. The specific activities of glyceryl ethers isolated from the nonhydrogenated and the hydrogenated samples were 26.2 cpm/mg and 33.4 cpm/mg, respectively. A difference in the side-chain distribution was noted, with a higher percentage of C_{16}-ether in the hydrogenated sample. These experiments must be repeated to confirm the results, but indications are that the vinyl ether compounds have a specific activity almost twice that of the glyceryl ethers. These results are consistent with the concept that plasmalogens might serve as precursors but could not be products of glyceryl ethers.

DISCUSSION

The experimental findings show that glyceryl ethers are present in the neutral lipids and the phospholipids of bovine bone marrow. In the latter lipid class each of the two major phospholipid fractions contains significant concentrations of glyceryl ether derivatives. The glyceryl ethers of the ethanolamine-containing lipids may be distinguished from those of the choline phosphoglycerides by virtue of the quantitative distribution of their hydrocarbon side chains.

Marrow tissue is the origin of circulating erythrocytes, which, in the case of the ox, have been shown (Hanahan and Watts, 1961) to contain a high percentage of glyceryl ethers in the cell membrane ethanolamine phospholipids. As might be expected, the side-chain distribution patterns for the glyceryl ethers of this phospholipid fraction in both mature red cells and marrow cells are very similar. Phosphatidyl choline is virtually absent from the bovine erythrocyte (Hanahan *et al.*, 1960). It was therefore rather surprising to discover that phosphatidyl choline is the major phospholipid of marrow. Even more unexpected was the high incorporation of radioactivity detected in this lipid fraction. The explanation of these data may be forthcoming when we learn more about the lipids of those marrow products formed *in situ*, i.e., reticulocytes and white cells.

In the human erythrocyte, all phospholipids appear to be localized in the cell membrane (Dodge *et al.*, 1963). All intracellular organelles have atrophied in this cell. Erythrocytes of the cow appear to have a similar construction. In bovine marrow cells, which still possess a full complement of nuclei, mitochondria, and microsomes, glyceryl ethers are not restricted to the cell membrane, as evidenced by their isolation from the mitochondrial fraction. This finding makes it rather unlikely that the high levels of glyceryl ether derivatives in the erythrocyte have evolved to participate specifically in some specialized process of the cell membrane. The likelihood of some distinctive role for glyceryl ether lipids in marrow and red cells remains great, however, and is further strengthened by the finding in our laboratory that rat marrow lipids contain glyceryl ethers in amounts equivalent to those found in bovine marrow.[3] In addition, Pietruszko (1962) has recently shown that 20% of pig marrow phosphatidyl choline exists as the glyceryl ether analog.

The three isotopically labeled tracers used in our studies were all incorporated to some extent. The ratios of glyceryl ether phospholipid specific activities to those of diacyl phospholipids were always similar and less than one. Thus the possibility that diacyl phospholipids serve as intermediates cannot be dismissed.

There is much indirect evidence that makes a metabolic relationship between glyceryl ethers and plasmalogens seem likely. Available for comparison are pertinent data concerning the human erythrocyte. This cell contains no detectable glyceryl ethers.[4] However, Farquhar (1962) has found plasmalogens in the phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl choline fractions in 67, 8, and 12 mole per cent, respectively. Analyses of the length and unsaturation of the vinyl ether side chains present in the different fractions produced results similar to those found for glyceryl ethers in the present study.

One possible route by which glyceryl ethers may be synthesized lies in the formation of a plasmalogen-type molecule followed by the enzymatic hydrogenation

[3] Thompson, G. A., Jr., unpublished data.

[4] Hanahan, D. J., unpublished data.

of the vinyl ether double bond. The experiment involving tritiated water was designed to test the possibility of hydrogenation involving one or more hydrogen ions from water. When compared to a glucose-6-C^{14} control sample, no increased incorporation of tritium into glyceryl ethers due to tritium uptake by the side chain was noted. However, since the over-all amount of radioactivity incorporated was small and since it was not determined into which position or positions of the glyceryl moiety tritium was incorporated, the validity of these results must be confirmed by further experimentation. Catalytic hydrogenation experiments of the type reported here promise to be more useful in studying the role of plasmalogens as possible glyceryl ether precursors.

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The Biochemistry of Plasmalogens. III. Concentrations in Tissues of the Rat as a Function of Age*

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Received March 7, 1963

The plasmalogen concentrations of heart, lung, liver, spleen, and skeletal muscle were determined by two independent methods in rats 1, 2, 3, 4, and 8 weeks of age. In contrast to the marked increase noted in brain during this period of rapid growth, no consistent change in plasmalogen concentration with age was observed in any of these tissues when comparisons were made on the basis of fresh weight of tissue, total lipid content, or total phosphatide content. A simple ultramicro spectrophotometric method for specific iodination has been found to be satisfactory for determination of the plasmalogen content of total lipid extracts.

It was established by Minder and Abelin (1954) and confirmed by Korey and Orchen (1959), Erickson and Lands (1959), and Bieth *et al.* (1961) that the aldehydogenic phosphatide content of the brain of the rat increases severalfold as the animal increases in age. In contrast, it was found (Minder and Abelin, 1954) that in other organs (heart, kidney, muscle, and liver) the plasmalogen content decreases with age, a sharp drop occurring between 3 and 4 weeks. These changes were determined in relation to fresh weight of tissue. Only three groups of animals were compared, namely, 3-week-old, 4-week-old, and "adult" rats. We have restudied this problem in order to develop a more complete picture with respect both to age and to other bases of reference, e.g., total lipid and total phosphatide. Five organs (heart, lung, liver, spleen, and muscle) were studied in five groups of rats (1, 2, 3, 4, and 8 weeks old). A more reliable method than that available to Minder and Abelin (Schiff reaction) was used to determine the aldehydogenic phosphatide content, namely, *p*-nitrophenylhydrazone formation (Wittenberg *et al.*, 1956). Plasmalogens were also measured by the more specific determination of α,β-unsaturated ethers (Rapport and Franzl, 1957). The findings of Minder and Abelin regarding change with age in heart, muscle, and liver could not be confirmed.

* This work was supported by research grants B-1570, A-2965, H-3838, and M-2562, National Institutes of Health, U. S. Public Health Service.

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Experimental Procedure

Sprague-Dawley albino rats (Holtzman strain) used in this study were kept with their mothers, with free access to a stock Rockland rat diet. Groups of five rats each were sacrificed at ages 1, 2, 3, 4, and 8 weeks. Animals in the first group were sacrificed by decapitation; for the others, ether anesthesia was used, followed by immediate dissection. The selected organs were trimmed, washed in 0.9% saline solution, blotted quickly on a paper towel, and weighed. The specimens were then stored in ice until processed (within 2 hours). Pools of tissues were ground in a glass homogenizer or a Waring Blendor with 20 volumes of chloroform-methanol (2:1, v/v) at room temperature. The chloroform-methanol extracts were then filtered and washed by the method of Folch *et al.* (1951). Lipids