Shinitzky, M., Dianoux, A. C., Gitler, C., & Weber, G. (1971) *Biochemistry 10,* 2106.
Smoluchowksi, M. (1917) *Z. Phys. Chem. 92,* 129.
Tanford, C., & Reynolds, J. A. (1976) *Biochim. Biophys. Acta 457,* 133.
Träuble, H., & Sackmann, E. (1972) *J. Am. Chem. Soc. 94,* 4499.
Wirtz, K. W. A., & Zilversmit, D. B. (1968) *J. Biol. Chem. 243,* 3596.
Woessner, D. E. (1962) *J. Chem. Phys. 36,* 1.
Yedgar, S., Hertz, R., & Gatt, S. (1974) *Chem. Phys. Lipids 13,* 404.

In Vitro Biosynthesis of Phospholipids by Chondrocytes and Matrix Vesicles of Epiphyseal Cartilage†

R. E. Wuthier,*,‡ F. H. Wians, Jr.,§ M. S. Giancola,¶ and S. S. Dragic#

ABSTRACT: Matrix vesicles are extracellular structures involved in endochondral calcification. They have a phospholipid composition distinct from that of chondrocytes from which they appear to be derived, but controversy exists concerning their origin. To elucidate the pathways involved in their formation, phospholipid biosynthesis by chondrocytes and matrix vesicles, either in tissue slices or as isolated fractions, was studied utilizing ^{14}C-labeled lipid precursors: acetate, palmitate, eicosatrienoate and L-serine. *Cartilage slices* incorporated L-serine into phospholipids of both chondrocytes and matrix vesicles; however, matrix vesicles were labeled more slowly than the cells. Serine was incorporated first into LPS (lyso form of phosphatidylserine) and then later into PS (phosphatidylserine), indicating a precursor–product relationship. This monoacyl base-exchange pathway may be unique to epiphyseal cartilage. *Isolated chondrocytes* actively incorporated all precursors into phospholipids, while *isolated matrix vesicles* did not. Chondrocytes incorporated significant amounts of L-serine into several nitrogenous phospholipids, whereas matrix vesicles incorporated only small amounts into PS and LPS. Since this was markedly inhibited by EDTA, it appears to have been by the non-energy-requiring base exchange. Chondrocytes incorporated significant amounts of acetate into all lipids, indicating a capacity for de novo fatty acid synthesis. Exogenous fatty acids, both saturated and unsaturated, were much more actively metabolized, but incorporation into specific phospholipids was selective. In particular, PS, diphosphatidylglycerol, and the lysophospholipids had significantly slower fatty acid turnover than the other phospholipids. Since isolated vesicles essentially lacked lipid synthetic capability, our findings indicate that matrix vesicles must be actively produced by chondrocytes. Although matrix vesicles were enriched in SPH, PS, and the lyso forms, none of these showed enhanced biosynthesis by either chondrocytes or matrix vesicles. This indicates that selective degradation of phospholipids and shedding of the modified membrane are involved in matrix vesicle formation.

Vesicles present in the extracellular matrix of epiphyseal cartilage have been shown to be associated with the initiation of endochondral calcification (Anderson, 1967, 1969; Bonucci, 1967, 1970; and others). They have been shown to be rich in alkaline phosphatase (Ali et al., 1970; Majeska & Wuthier, 1975) and phospholipids (Peress et al., 1974; Wuthier, 1975), both of which have been implicated in vesicle-mediated calcification (Fleisch et al., 1966; Eisenberg et al., 1970; Wuthier & Eanes, 1975). The phospholipid composition of matrix vesicles is distinctive, being enriched in PS,[1] SPH, and various lysophospholipids, and depleted in PC compared with chondrocytes (Wuthier, 1975). PS is known to have a strong affinity for Ca^{2+} (Nash & Tobias, 1964; Abramson et al., 1964; Cotmore et al., 1971; and others) and has been shown recently to be involved in phospholipid–calcium–phosphate complexes in matrix vesicles (Wuthier & Gore, 1977). Our recent studies have shown that matrix vesicle phospholipids are rapidly labeled in vivo with [^{32}P]orthophosphate (Wuthier et al., 1977). Curiously, however, most of the lipids specifically enriched in the vesicles (PS, SPH, LPC, and LPE) were not as rapidly labeled, relative to the cells, as PC which, in contrast, was depleted in the vesicles. We wished to further explore this paradoxical finding using a variety of other lipid precursors to elucidate the pathways of matrix vesicle phospholipid formation.

A further goal of this study was to unequivocally establish whether isolated matrix vesicles were capable of lipid biosyn-

† From the Departments of Orthopaedic Surgery and Biochemistry, University of Vermont, Burlington, Vermont 05401, and the Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208. *Received August 4, 1977.* This work was supported by funds from the United States Public Health Service, Grants no. AM 13523 and AM 18983, National Institute of Arthritis, Metabolism and Digestive Diseases.

‡ Department of Chemistry, University of South Carolina, Columbia, South Carolina 29208.
§ F.H.W. is currently at the Armed Services Whole Blood Proccessing Laboratory, McGuire Air Force Base, New Jersey 08641.
¶ M.S.G. is currently in the Department of Physiology, School of Medicine, University of Vermont, Burlington, Vermont 05401.
# S.S.D. is currently in the Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40506.

[1] Abbreviations used: ATP, adenosine 5′-triphosphate; CoA, coenzyme A; CTP, cytidine 5′-triphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; DPG, diphosphatidylglycerol; SPH, sphingomyelin; LPC, LPE, and LPS, lyso forms of PC, PE, and PS, respectively; Tes, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]taurine.

0006-2960/78/0417-1431$01.00/0

TABLE I: Effect of ATP and Coenzyme A on the Incorporation of ^{14}C-Labeled Aliphatic Substrates into the Lipids of Isolated Epiphyseal Cartilage Fractions.[a]

Fraction	Lipid class	Substrate incorp (nmol per μCi per g of protein)					
		[2-^{14}C]Acetate		[1-^{14}C]Palmitate[d]		[1-^{14}C]Eicosatrienoate[d]	
		Control	ATP + CoA	Control	ATP + CoA	Control	ATP + CoA
Chondrocytes	Nonpolar[b]	139	139	1156	1373	312	366
	Polar[c]	12	11	436	249	191	198
	Total	151	150	1592	1622	503	558
Matrix vesicles	Nonpolar	1.8	1.9	6.1	10.0	323	169
	Polar	0.3	0.3	1.0	1.7	5	4
	Total	2.1	2.2	7.1	11.7	328	173

[a] Isolated chondrocytes and matrix vesicles were incubated at 37 °C for 4 h in autologous serum:synthetic cartilage lymph (1:1, v/v, pH 7.45) containing the indicated ^{14}C-labeled lipid precursors: [2-^{14}C]acetate, 10 μCi (58.8 Ci/mol); [1-^{14}C]palmitate, 10 μCi (55.1 Ci/mol); or [1-^{14}C]-$\Delta^{8,11,14}$-*all-cis*-eicosatrienoate, 2.5 μCi (57.5 Ci/mol). ATP and coenzyme A were added to give final concentrations of 3.2 mM and 0.032 mM, respectively. For further details, see Materials and Methods. [b] Nonpolar lipids were eluted from silicic acid columns with chloroform. They consisted primarily of triacylglycerols, free cholesterol, free fatty acids, cholesterol esters, and 1,2-diacylglycerols (Wuthier, 1975). [c] Polar lipids were eluted from silicic acid columns with methanol and consisted primarily of phospholipids. [d] The nonpolar fraction from these incubations would also contain any unincorporated ^{14}C-labeled fatty acids not removed during tissue washing.

thesis. Holtrop (1972) and some other workers believe vesicles to be the debris of dead cells, although both morphological (Bonucci, 1970) and biochemical data (Peress et al., 1974; Wuthier, 1975) suggest that they arise by budding from the plasma membrane of epiphyseal chondrocytes. While our earlier in vivo studies have indicated active biosynthesis from cells (Wuthier et al., 1977), it was possible that the vesicles themselves retained biosynthetic capability. Thus a negative finding in the present studies would indicate that the previously observed vesicle labeling was the result of active cellular synthesis.

Materials and Methods

Preparation of Cartilage Fractions. Tissues from 6- to 10-week old, male, hybrid chickens (Broiler Strain no. 663, Hubbard Hatcheries, Walpole, N.H.) were prepared as described previously (Wuthier, 1975). Epiphyseal cartilage shavings (0.1–0.3 mm thick) from both the proliferating and calcifying regions from 3 to 4 birds (1–2 g per zone per bird) were used for each experiment. In some studies chondrocyte and matrix vesicle fractions were isolated (Wuthier, 1975) before incubation with the lipid precursors, in others, whole tissue slices (Wuthier & Cummins, 1974) were used.

Incubation of Isolated Cartilage Fractions. For the isolated fractions the pellets were suspended in 2.0–2.5 mL of bicarbonate-buffered synthetic cartilage lymph, pH 7.45 (Majeska & Wuthier, 1975), aliquots being used for incubation and for protein assay (Lowry et al., 1951). In some studies autologous serum was added to the synthetic cartilage lymph (1:1, v/v) to enhance cell survival. Radioactive substrates ([2-^{14}C]acetate, [1-^{14}C]palmitate, or [1-^{14}C]-$\Delta^{8,11,14}$-eicosatrienoate) and cofactors (3.2 mM ATP and 0.032 mM CoA) were added. For studies on the incorporation of serine, L-[3-^{14}C]serine and either CTP or EDTA (2.0 mM each) were added. The flasks were gassed with 90% N_2–5% O_2–5% CO_2, a gas mixture designed to give O_2 and CO_2 tensions similar to that observed in vivo (Brighton & Heppenstall, 1971). The incubations were conducted for 4 h at 37 °C in a shaker bath (70 × 2 cm strokes/min). Final volumes of the incubation media were 2.0 and 4.0 mL for the matrix vesicles and cells, respectively. The incubations were terminated by chilling on ice and the contents of each centrifuged at 85 000*g* for 30 min to sediment the particulate matter. The supernate containing the bulk of the nonincorporated isotope was decanted and each pellet washed with 40 mL of the synthetic cartilage lymph and resedimented.

Incubation of Cartilage Slices. Tissue slices (2 g) from the zones of proliferation and calcification of the epiphyseal growth cartilage were incubated with L-[3-^{14}C]serine in 20 mL of the bicarbonate-buffered synthetic cartilage lymph under an atmosphere of the 90% N_2–5% CO_2–5% O_2 gas mixture. Incubations lasted for 1, 2, and 5 h at 40 °C as described above. The incubation fluid was decanted and the tissue slices were rinsed twice with 30 mL of the synthetic cartilage lymph. Chondrocyte and matrix vesicle fractions were released from the tissue slices by enzymatic digestion and differential centrifugation as previously described (Wuthier, 1975).

Lipid Extraction and Analysis. The cartilage fractions were extracted three times with chloroform–methanol (2:1, v/v) according to the method of Folch et al. (1957). Nonlipid contaminants were removed by Sephadex G-25 column chromatography (Wuthier, 1966) and nonpolar and polar lipid classes separated by silicic acid column chromatography (Wuthier, 1968). The purified polar lipid fractions were analyzed for class composition by two-dimensional paper chromatography (Wuthier, 1976). The radioactivity of the lipid-bearing areas from the chromatograms was measured by liquid scintillation counting (Wuthier, 1976). Corrections were made for self-absorption, counting efficiency and for background. The amount of each class of phospholipid was determined by P analysis of the lipid-bearing areas (Rouser et al., 1966) after digestion with 70% perchloric acid.

Chemicals. Omnifluor and all ^{14}C-labeled substrates were purchased from New England Nuclear, Boston, Mass. Tes (*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]taurine) and all organic solvents were purchased from Fisher Scientific Co. ATP, CoA (reduced), and CTP were purchased from Sigma Chemical Co. Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Inc.

Results

The incorporation of all labeled precursors into lipids was markedly greater in isolated chondrocytes than in isolated matrix vesicles (Tables I and III). Addition of ATP or CoA did not stimulate incorporation of either acetate or fatty acids into the lipids of either of these cartilage fractions. Addition of EDTA (Table III) resulted in a 64% inhibition of serine incorporation into cellular PS and over 90% inhibition in the isolated matrix vesicles. Added CTP had no stimulatory effect.

TABLE II: Specific Activity of Incorporated ^{14}C-Labeled Precursors in Phospholipids of Isolated Chondrocytes.[a]

	Substrate incorp (dpm/μg of lipid P)					
	[2-^{14}C]Acetate		[1-^{14}C]Palmitate		[1-^{14}C]Eicosatrienoate	
Phospholipid	Control	ATP + CoA	Control	ATP + CoA	Control	ATP + CoA
SPH	1680 ± 170	2160 ± 500	71 000 ± 2200	63 100 ± 2300	2 400 ± 220	2 440 ± 70
PC	1484 ± 20	1030 ± 144	69 100 ± 700	52 440 ± 270	7 090 ± 200	10 900 ± 2400
LPC	684 ± 18	884 ± 154	9 400 ± 550	3 360 ± 1000	*b*	*b*
PE	1580 ± 10	1310 ± 5	22 840 ± 230	13 890 ± 520	14 510 ± 990	11 060 ± 80
LPE	490 ± 10	376 ± 21	8 700 ± 2300	8 600 ± 60	*b*	*b*
PS	1220 ± 250	1170 ± 220	9 700 ± 700	7 360 ± 1400	6 500 ± 500	6 700 ± 500
LPS	2160 ± 290	2240 ± 220	2 450 ± 250	1 680 ± 300	*b*	*b*
PI	3020 ± 110	3270 ± 90	38 800 ± 2200	31 200 ± 2100	20 700 ± 1100	20 500 ± 2300
PA	1280 ± 165	1070 ± 280	44 700 ± 100	32 200 ± 300	8 200 ± 1700	4 900 ± 400
DPG	650 ± 200	390 ± 10	5 060 ± 440	2 940 ± 100	*b*	*b*
Total[f]	0.46[c]	0.43	10.4[d]	5.9	13.1[e]	13.5

[a] Isolated chondrocytes were incubated at 37 °C for 4 h with substrates indicated. For further details, see caption to Table I or Materials and Methods. Values represent the mean ± SEM of two to four analyses of duplicate incubations. [b] These lipids were detected on the chromatograms but were not analyzed quantitatively. [c] The percentage of added [^{14}C]acetate incorporated into *matrix vesicle* polar lipids was only 0.0018-0.0020%. [d] That of [^{14}C]palmitate was only 0.009-0.016%. [e] That of [^{14}C]eicosatrienoate was 0.046-0.065%. [f] % of added isotope incorporated.

TABLE III: Incorporation of L-[3-^{14}C]Serine into Phospholipids of Isolated Chondrocytes and Matrix Vesicles.[a]

	Amount incorp (dpm/μg of lipid P)					
	Control		EDTA		CTP	
Phospholipid	Chondrocytes	Matrix vesicles	Chondrocytes	Matrix vesicles	Chondrocytes	Matrix vesicles
SPH	5 530 ± 1110	50 ± 25	1 990 ± 470	130 ± 45	4 450 ± 360	80 ± 40
PC	90 ± 20	40 ± 15	60 ± 10	90 ± 40	100 ± 10	35 ± 15
LPC	2 810 ± 790	420 ± 85	240 ± 100	275 ± 85	720 ± 40	425 ± 100
PE	1 670 ± 350	155 ± 60	1 090 ± 350	185 ± 85	1 050 ± 310	210 ± 45
LPE	840 ± 320	140 ± 80	450 ± 130	485 ± 185	330 ± 80	250 ± 75
PS	66 200 ± 7700	7570 ± 1350	24 000 ± 4700	650 ± 280	60 500 ± 5600	7290 ± 1790
LPS	27 500 ± 3700	2650 ± 380	11 500 ± 2400	548 ± 214	20 350 ± 1600	1990 ± 360
Total[b]	2.9 ± 1.7	0.09 ± 0.02	1.5 ± 0.9	0.02 ± 0.00	2.1 ± 1.2	0.11 ± 0.03

[a] Values are the mean ±SEM of duplicate samples from each of three separate experiments. Control samples were incubated with 2.5 μCi of L-[3-^{14}C]serine (56.5 Ci/mol) in synthetic cartilage lymph for 4 h. As indicated, EDTA or CTP (2 mM each) was added to the incubation medium. For further details, see Materials and Methods. [b] % of added isotope incorporated.

TABLE IV: Comparison of the Incorporation of Various Lipid Precursors into the Phospholipids of Isolated Chondrocytes.[a]

		% of dpm incorp into phospholipids			
Phospholipids	% of lipid P	Acetate	Palmitate	Eicosatrienoate	Serine
SPH	6.8 ± 0.5	5.5 ± 0.2	6.5 ± 0.2	1.4 ± 0.2	13.9 ± 2.9
PC	59.4 ± 2.1	54.5 ± 0.5	79.6 ± 0.4	55.6 ± 0.8	2.7 ± 0.3
LPC	3.1 ± 0.8	1.1 ± 0.3	0.6 ± 0.0	*b*	1.6 ± 0.7
PE	12.0 ± 1.9	17.3 ± 0.2	6.6 ± 0.2	28.6 ± 1.0	11.1 ± 4.0
LPE	4.7 ± 0.3	1.7 ± 0.2	1.1 ± 0.4	*b*	1.0 ± 0.2
PS	2.2 ± 0.2	1.6 ± 0.1	0.5 ± 0.0	1.5 ± 0.1	56.0 ± 3.5
LPS	0.9 ± 0.2	1.0 ± 0.1	0.1 ± 0.0	*b*	7.5 ± 1.6
PI	7.0 ± 0.4	14.6 ± 0.9	4.1 ± 0.1	12.4 ± 0.4	1.6 ± 0.6
PA	0.9 ± 0.3	1.0 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	1.1 ± 0.3
DPG	2.0 ± 0.1	1.0 ± 0.2	0.2 ± 0.0	*b*	0.0 ± 0.0
X[c]	0.6 ± 0.2	0.8 ± 0.2	0.3 ± 0.0	*b*	4.3 ± 0.5

[a] Isolated chondrocytes were incubated at 37 °C for 4 h with the substrates indicated. For further details see caption to Tables I and III, or Materials and Methods. Values are the means ±SEM of duplicate analyses of two to three samples from the control incubations. [b] These lipids were detected on chromatograms but were not analyzed quantitatively. [c] An unidentified phospholipid migrating near to SPH.

Acetate was incorporated into all classes of cellular phospholipids, generally in proportion to the amount of each class present (Table IV). PC, PE, and PI contained the bulk of the radioactivity, but PI had the highest specific activity (Table II). Palmitate was incorporated heavily into PC, with smaller but significant incorporation into SPH, PE, and PI. SPH, PC, PA, PI, and PE, in that order, had the highest specific activity, with PS and the lysophospholipids being markedly lower (Table II). In contrast, the polyunsaturated fatty acid, eicosatrienoate, was incorporated proportionally more into PE and PI (Table IV). The order of decreasing specific activities was: PI > PE > PA > PC > PS > SPH (Table II). Serine was in-

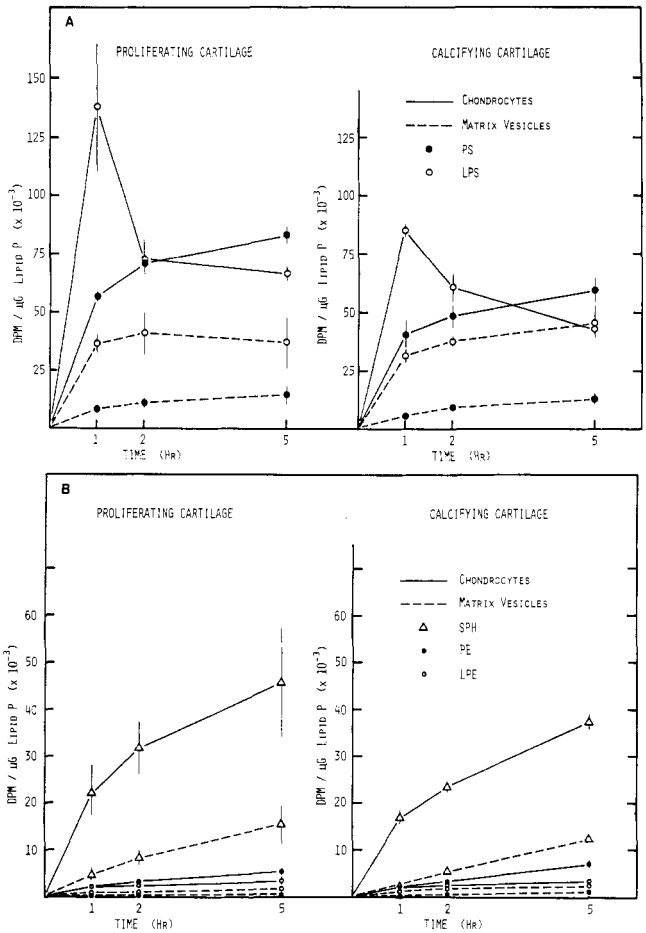

FIGURE 1: Time course of incorporation of L-[3-^{14}C]serine into phospholipids of chondrocytes and matrix vesicles. Chicken epiphyseal cartilage slices were incubated with 4.2 μCi of L-[3-^{14}C]serine (56.6 Ci/mol) for the times indicated. After incubation, chondrocytes and matrix vesicles were isolated and analyzed for isotope incorporation into phospholipids (for further details, see Materials and Methods). (A) Incorporation into PS and its monoacyl form, LPS; (B) incorporation into SPH, PE, and its monoacyl form, LPE. Note the more rapid rates of serine incorporation into the various phospholipids of the cells than the matrix vesicles, and of proliferating than calcifying cartilage. Particularly note the primary labeling of LPS by both chondrocytes and matrix vesicles, followed later by the labeling of the diacyl form, PS (from A).

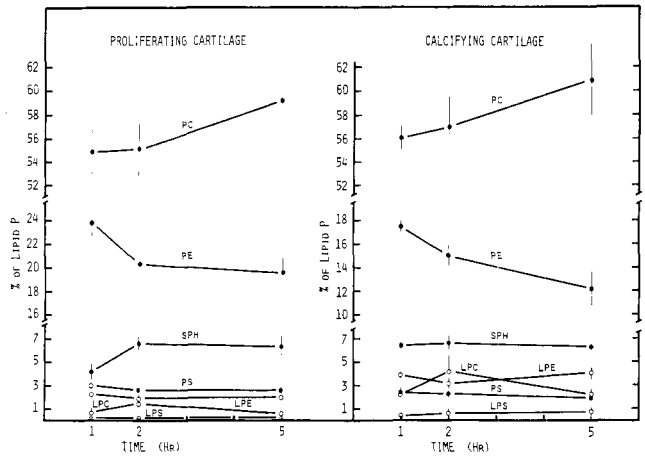

FIGURE 2: Change in phospholipid composition of chondrocytes during in vitro incubation of cartilage slices with L-[3-^{14}C]serine. Data points are means of duplicate samples from three separate experiments, the vertical bar indicating the SEM. Note the progressive increase in the percentage of PC and the decrease in PE during the incubation. No significant changes in percentage composition of phospholipids of matrix vesicles occurred.

corporated predominantly into the nitrogen-containing phospholipids, PS containing 56% of the total incorporated radioactivity. PS and LPS had the highest specific activities, followed by SPH, and then PE and LPC at much lower levels (Table III).

In cartilage slices cellular LPS had the most rapid serine incorporation and turnover of any of the phospholipids. The proliferating zone showed more rapid labeling than the calcifying (Figure 1A). The label appeared progressively, but more slowly in cellular PS. In the matrix vesicles LPS was also labeled more rapidly than PS, but both were labeled more slowly than in the cells. Cellular SPH also progressively accumulated activity (Figure 1B), again with the matrix vesicles being labeled more slowly. PE and LPE incorporated radioactivity even more slowly. Progressive changes in the phospholipid composition of the cells occurred during the incubation period (Figure 2). The most obvious changes were the progressive decline in the percentage of PE, and the proportional increase in PC. Matrix vesicles showed no significant changes with time. Differences between zones of the growth plate in phospholipid composition of the cells and matrix vesicles were also evident. Cells from the calcifying zone were enriched in SPH and the lyso forms of PS, PE, and PC compared with those of the proliferating region. Matrix vesicles also displayed zonal differences in phospholipid composition. Those from the calcifying region were reduced in PS, PE, and PA, and enriched in LPS and LPC. As emphasized before (Wuthier, 1975), matrix vesicles were notably richer in PS, SPH, and the lysophospholipids than the cells.

Discussion

In these studies two approaches were taken to elucidate the mechanism of matrix vesicle phospholipid biosynthesis. In the first the incorporation of various aliphatic precursors into phospholipids of isolated chondrocytes and matrix vesicles was studied. This provided a comparison of the lipid biosynthetic capability of the two tissue entities. In the second, a time study of the incorporation of L-[3-^{14}C]serine into cartilage tissue slices was conducted. This permitted us to follow the metabolism of key phospholipids under conditions in which the anatomical relationship between the cells and the matrix remained essentially intact.

The data obtained with isolated chondrocytes and matrix vesicles revealed that *isolated* cells actively incorporated acetate, fatty acids and serine into phospholipids, whereas the *isolated* matrix vesicles did not. The small, but measurable incorporation of [^{14}C]acetate (0.46% of the total isotope added) shows that the isolated chondrocytes were capable of de novo fatty acid synthesis. On the other hand, the very substantial incorporation of the ^{14}C-labeled fatty acids (10.4% and 13.5% for palmitate and eicosatrienoate, respectively) indicates that rapid turnover of exogenous fatty acids occurs in chondrocyte phospholipids. This in agreement with earlier studies by Wolinsky & Guggenheim (1970) which showed rapid uptake of fatty acids into the total phospholipid fraction of chick cartilage. In contrast, the isolated vesicles not only did not incorporate acetate, but barely utilized fatty acids, even with added cofactors. Further, they incorporated L-[^{14}C]serine into phospholipids at only about 10% of the specific activity of the cells. This appeared to be primarily by the non-energy-requiring Ca^{2+}-dependent base-exchange pathway of Hübscher (1962), since it was not stimulated by CTP, and was almost totally blocked by 2 mM EDTA (Table III). The minor incorporation of fatty acids into phospholipids probably was through reverse action of phospholipase A, an enzyme which

TABLE V: Relative Turnover of Fatty Acids of Chondrocyte Phospholipids.[a]

Phospholipid	A (% of total fatty acid)[b] 16:0[c]	A (% of total fatty acid)[b] PUFA[d]	B (dpm per g of lipid P per μCi)[f] 16:0[c]	B (dpm per g of lipid P per μCi)[f] 20:3[e]	B/A[a] 16:0	B/A[a] 20:3
SPH	37.2 ± 4.3	2.2 ± 0.0	7100 ± 220	976 ± 88	191	444
PC	27.1 ± 1.2	6.4 ± 1.2	6910 ± 70	2836 ± 80	255	444
LPC	25.7 ± 0.0	13.2 ± 3.2	940 ± 55		36.5	
PE	7.8 ± 1.2	21.3 ± 4.4	2284 ± 23	5804 ± 376	293	272
LPE	12.7 ± 1.6	13.7 ± 3.6	870 ± 230		68.5	
PS	16.8 ± 2.5	19.9 ± 8.0	970 ± 70	2600 ± 200	57.5	130
LPS	12.8 ± 4.2	17.1 ± 3.9	245 ± 25		19.0	
PI	9.2 ± 0.5	15.5 ± 3.8	3880 ± 220	8280 ± 440	422	434
PA	14.5	20.7	4470 ± 10	3280 ± 680	308	158
DPG	10.2 ± 1.9	12.7 ± 3.0	506 ± 44		49.5	

[a] Ratio of the specific activity (dpm/μg of lipid P) to the abundance of the fatty acid present in the phospholipid (% of the total). In the case of 20:3 fatty acid, the ratio is that of the specific activity of incorporated 20:3 to the total polyunsaturated fatty acid. [b] Data (Wuthier, 1975) represent the percentage of the total fatty acid present in each phospholipid. [c] 16:0, palmitate. [d] PUFA is the total polyunsaturated fatty acid present in the phospholipid. [e] 20:3, $\Delta^{8,11,14}$-*all-cis*-eicosatrienoate. [f] Data in column B, derived from Table II, are normalized to the same amount of labeled fatty acid per incubation. They were derived from the control values.

appears to be present in high levels in epiphyseal cartilage (Wuthier, 1973). In any case, the weak biosynthetic capability of isolated matrix vesicles, taken with the rapid incorporation in vivo of [^{32}P]orthophosphate into phospholipids of both cells and matrix vesicles (Wuthier et al., 1977), demonstrates that matrix vesicles must arise by an active, cell-mediated process.

Data on serine incorporation by tissue slices indicated that an unusual pathway of PS biosynthesis occurs in epiphyseal cartilage. LPS was the most rapidly and transiently labeled phospholipid (Figure 1A). The specific activity curves indicate that LPS must be the precursor of PS in this pathway. As mentioned before, serine incorporation appeared to be by a base-exchange mechanism. To our knowledge, this is the first reported biosynthesis of PS by a monoacyl version of this pathway. LPE appears to have been the serine acceptor in the base-exchange mechanism, based on the fact that the levels of PE progressively declined during the incubation period (Figure 2). Serine incorporation into PS and SPH was slower in the matrix vesicles than in the cells. This is in agreement with our previous in vivo studies with [^{32}P]orthophosphate in which both PS and SPH were labeled more slowly in the vesicles than in the cells. Since these are the two phospholipids which are especially enriched in the matrix vesicles, these findings indicate that the increase does not result from enhanced synthesis. Rather, considering the elevated amounts of lysophospholipids also present in the vesicles, the data indicate that it must be the result of selective degradation of the *other* phospholipids. This interpretation is supported by the fact that the fatty acid turnover of LPS and PS (Table V) was very slow, being less than half that of the other monoacyl and diacyl lipids.

In fact, the monoacyl lipids all showed very slow turnover rates with the saturated fatty acid, palmitate. These were only one-third to one-sixth that of the diacyl forms. This finding indicates that [^{14}C]palmitate containing lysophospholipids are not the direct precursors of labeled diacyl forms, but rather are degradation products. Further, in incubations with the polyunsaturated fatty acid, eicosatrienoate, diacyl phospholipids were rapidly labeled, whereas the monoacyl forms almost completely disappeared. These findings, coupled with our earlier demonstration of active phospholipase A activity in epiphyseal cartilage (Wuthier, 1973), strongly support a degradative origin of the lysophospholipids. The rapid incorporation of both saturated and polyunsaturated fatty acids, coupled with the disappearance of the lysophospholipids during tissue incubation with the latter, suggests that the high levels of lyso forms seen in vivo must result from disruption of a deacylation–reacylation cycle, perhaps one similar to that described by Hill & Lands (1970) in other tissues. It is probable, in the final stages of epiphyseal differentiation in which chondrocytes appear to be exfoliating matrix vesicles and undergoing disintegration of intracellular organelles, that the balance shifts toward the monoacylated forms. It is of interest here that coupled with this phenomenon, we have recently found that prostaglandin biosynthesis is also elevated in the calcifying region of epiphyseal cartilage (Wong et al., 1977).

Further comment should be made on the incorporation of fatty acids into the phospholipids. Palmitate was selectively incorporated into SPH and PC (Table II), which was a reflection of the high levels of this fatty acid in these cartilage lipids (Wuthier, 1975). However, relating the specific activity of the incorporated palmitate to the percentage of this fatty acid present in the individual phospholipid classes (Table V) showed that palmitate actually turned over more rapidly in PI, PA, and PE than in PC or SPH. In contrast, PS, DPG, and the lysophospholipids (LPC, LPE, and LPS) all showed very slow turnover of palmitate. Eicosatrienoate, on the other hand, showed preferential incorporation into PI and PE. Again while the specific activity tended to reflect the polyunsaturated fatty acid content of these lipids (Table V), in PS and PA it did not. In both these lipids eicosatrienoate turned over much more slowly than in the other phospholipids. The data thus show that the relative turnover of saturated and polyunsaturated fatty acids in the various phospholipids was quite selective. In particular they indicate that in PS the turnover of both saturated and unsaturated fatty acids is very slow. This finding concurs with our previous observation that PS was degraded more slowly by phospholipase A in epiphyseal cartilage than the other phospholipids (Wuthier, 1973) and helps explain the enrichment of this lipid in matrix vesicles.

Acknowledgments

We thank Dr. Robert J. Majeska, Mr. Peter Harrison and Mr. John Weija for their generous assistance in these studies.

References

Abramson, M. B., Katzman, R., & Gregor, H. P. (1964) *J. Biol. Chem. 239*, 70.

Ali, S. Y., Sajdera, S. W., & Anderson, H. C. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67,* 1513.
Anderson, H. C. (1967) *J. Cell Biol. 35,* 81.
Anderson, H. C. (1969) *J. Cell Biol. 41,* 59.
Bonucci, E. (1967) *J. Ultrastruct. Res. 20,* 33.
Bonucci, E. (1970) *Z. Zellforsch. Mikrosk. Anat. 103,* 192.
Brighton, C. T., & Heppenstall, R. B. (1971) *J. Bone Jt. Surg., Am. Vol. 53,* 719.
Cotmore, J. M., Nichols, G., Jr., & Wuthier, R. E. (1971) *Science 172,* 1339.
Eisenberg, E., Wuthier, R. E., Frank, R. B., & Irving, J. T. (1970) *Calcif. Tissue Res. 6,* 32.
Fleisch, H., Russell, R. G. G., & Straumann, F. (1966) *Nature (London) 212,* 901.
Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226,* 497.
Hill, E. E., & Lands, W. E. M. (1970) in *Lipid Metabolism* (Wakil, S. J., Ed.) p 613, Academic Press, New York, N.Y.
Holtrop, M. E. (1972) *Calcif. Tissue Res. 9,* 140.
Hübscher, G. (1962) *Biochim. Biophys. Acta 57,* 555.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265.
Majeska, R. J., & Wuthier, R. E. (1975) *Biochim. Biophys. Acta 391,* 51.
Nash, H. A., & Tobias, J. M. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51,* 476.
Peress, N. S., Anderson, H. C., & Sajdera, S. W. (1974), *Calcif. Tissue Res. 14,* 275.
Rouser, G., Siakotos, A. N., & Fleischer, S. (1966) *Lipids 1,* 85.
Wolinsky, I., & Guggenheim, K. (1970) *Calcif. Tissue Res. 6,* 113.
Wong, P. Y.-K., Majeska, R. J., & Wuthier, R. E. (1977) *Prostaglandins 14,* 839.
Wuthier, R. E. (1966) *J. Lipid Res. 7,* 558.
Wuthier, R. E. (1968) *J. Lipid Res. 9,* 68.
Wuthier, R. E. (1973) *Clin. Orthop. Relat. Res. 90,* 191.
Wuthier, R. E. (1975) *Biochim. Biophys. Acta 409,* 128.
Wuthier, R. E. (1976) in *Lipid Chromatographic Analysis* (Marinetti, G. V., Ed.) p 59, Marcel Dekker, New York, N.Y.
Wuthier, R. E. & Cummins, J. W. (1974) *Biochim. Biophys. Acta 337,* 50.
Wuthier, R. E., & Eanes, E. D. (1975) *Calcif. Tissue Res. 19,* 197.
Wuthier, R. E., & Gore, S. T. (1977) *Calcif. Tissue Res. 24,* 163.
Wuthier, R. E., Majeska, R. J., & Collins, G. M. (1977) *Calcif. Tissue Res. 23,* 135.

Small-Angle X-Ray Scattering and Differential Scanning Calorimetry Studies on Reversibly Modified Human-Serum Low Density Lipoproteins†

L. Mateu,* T. Kirchhausen, and G. Camejo

ABSTRACT: Small-angle x-ray scattering diagrams of human serum low density lipoprotein (LDL) were recorded at several temperatures in solutions of different freezing points. It was found that modifications of the x-ray patterns observed on cooling the lipoprotein samples below 0 °C are due to reversible alterations of the LDL surface structure induced by the freezing process (independent of temperature). With both intact and partially dehydrated LDL, differential scanning calorimetry (DSC) carried out in the body temperature range revealed a heat absorption characteristic of the transition from a liquid crystal to an isotropic liquid phase of cholesteryl esters within the lipoproteins (Deckelbaum, R. J., Shipley, R. J., Small, P. M., Lees, R. S., & George, P. K. (1975) *Science 190,* 392). However, small-angle x-ray scattering diagrams recorded with the same LDL sample before and after the partial removal of water were found to be very different: the scattering curve for intact LDL showed a strong band centered at $(36\ \text{Å})^{-1}$ which disappeared upon drying and reappeared upon restoring the water. Our results suggest that the presence of this signal strongly depends on the molecular structure of the lipoprotein surface.

The precise location and organization of the molecular constituents within the low density lipoprotein (LDL) is not yet definitely established. The current concept is that the proteins and polar groups of phospholipids are exposed to the aqueous environment, covering the cholesteryl esters and triglycerides which are located toward the center of the particle (Pollard et al., 1969; Mateu et al., 1972; Luzzati et al., 1976; Tardieu et al., 1976).

The organization of the apolar constituents still remains one of the incompletely resolved problems. Deckelbaum et al. (1975) first observed a close resemblance between the thermal behavior of LDL solutions and that of cholesteryl esters derived from these particles. In the same angular region ($(36\ \text{Å})^{-1}$) of the small-angle x-ray diagrams recorded at 10 °C, both systems display a diffraction signal whose intensity vanishes near body temperature. In addition, by differential scanning calorimetry (DSC) techniques, they also detected a thermal transition in the same temperature range of the disappearance

† From the Laboratorios de Estructura Molecular y Lipoproteinas, Centro de Biofisica y Bioquimica Instituto Venezolano de Investigaciones Cientificas (IVIC), Apartado 1827, Caracas 101, Venezuela. *Received August 10, 1977.* This investigation was supported in part by Research Grant S1-0353 from CONICIT (Venezuela).

0006-2960/78/0417-1436$01.00/0