Lysolecithin Metabolism of Thymus and Bursa Cells of the Chicken (34256)

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(Introduced by B. H. Waksman)
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Cytotoxic effects of lysophosphatides are well known (1). Thus, the investigation of lysophosphatidyl choline-metabolizing enzymes in lymphocytic cells seems to be of special interest with regard to molecular mechanisms of certain tissue-damaging immune reactions. Perlmann and co-workers (2, 3) found that peripheral lymphocytes became cytotoxic for tissue culture cells when they had been stimulated by phytohemagglutinin (PHA). Differences in histocompatibility between lymphocytes and target cells were not an absolute requirement for this cytotoxic reaction. Autologous and allogenic lymphocytes were equally effective. Therefore, it was suggested that PHA-stimulated lymphocytes may acquire surface properties typical for macrophages and that some steps in phagocytosis and cytotoxicity represent related phenomena. During phagocytosis various investigators (4) have noted changes in enzyme activities. Munder *et al.* (5) observed an increased activity of phospholipase A after rat peritoneal macrophages had engulfed silica particles. In the present investigation some aspects of the phospholipid metabolism of fowl thymus and bursa cells were compared. These cells were chosen because they were easily obtainable in sufficient quantities.

Materials and Methods. Eight to 10-week-old white leghorn chickens were used as cell donors. Thymic and bursa of Fabricius tissues were teased apart in phosphate buffered saline (PBS). The cells were filtered through gauze, spun at 150*g* for 10 min, and washed three times with PBS. Finally they were resuspended in medium 199. The cells were counted in a Coulter counter; the percentage of viable cells was determined by trypan blue exclusion. The viability of the cell preparations used for experiments was always about 90%. Homogenates were prepared by sonication of thymus and bursa cell suspensions in a Branson sonifier (3.5 A for 30 sec). Neutral lipids and phospholipids were extracted with chloroform–methanol by the method of Ways and Hanahan (6). They were separated by thin-layer chromatography (Merck silica gel H) using petroleum ether–diethyl ether–acetic acid (105:45:3 by volume) or chloroform-methanol–water (650:400:95), respectively, as developing solvents. The incorporation of radioactivity was scanned with a chromatogram scanner (Laboratory of Dr. Berthold, Wildbad, Germany). Analytical plates were sprayed with iodine and the spots were identified by comparing their positions with those of standards. The phospholipid bands were scraped off and their phosphorus content was determined by the method of Parker and Peterson (7). Lipid extracts from cell suspensions that were incubated with radioactive compounds of low specific activity were separated chromatographically on silica gel impregnated paper. The rhodamine 6G-stained zones were located on the basis of the positions of the marker compounds, cut from the paper, and their radioactivity was counted in a Packard model 3000 scintillation counter as described in detail elsewhere (8). Free fatty acids were assayed according to the method of Duncombe (9). Protein analysis was carried out by the biuret method.

Enzyme assays. 1. The enzyme activities were measured as described by Ferver *et al.*

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TABLE I. Percentage Distribution of Radioactivity among Bursa and Thymus Cells after Incubation with [1-^{14}C]-Oleic Acid.[a]

Cell population	Lecithin, sphingomyelin	Phosphatidyl ethanolamine	Free fatty acid	Diglycerides	Triglycerides	Cholesterol ester
Bursa	22.9	2.2	26.9	0.8	46.5	0.7
Thymus	26.6	1.4	31.6	2.9	36.5	1.0

[a] Cells (10^8) were incubated in 1 ml of medium 199 with 0.5 μCi = 11.6 mμmoles of oleic acid-^{14}C at 37° for 160 min. The total lipid was extracted and separated by thin-layer chromatography as described above and the radioactivity was determined by chromatogram scanning.

(8, 10) using radioactive labeled substrates: [1-^{14}C]-oleic acid was purchased from Radiochemical Center, Amersham, England; [1-acyl-^{14}C]-lysolecithin and [1,2-acyl-^{14}C]-lecithin were synthesized by the method of Hoelzl and Wagner (11). The water insoluble compounds were emulsified mechanically to improve the reproducibility of the enzyme tests.

2. The acyltransferase and the acyl–CoA–hydrolase activities were measured spectrophotometrically with the aid of Ellman reagent [5,5′-dithiobis(2-nitrobenzoic acid)] as first described by Lands and Hart (12). The CoA-ester of the oleic acid was synthesized from the mixed anhydride of the fatty acid according to the procedure of Goldman and Vagelos (13). The yield of oleyl–CoA was assayed by hydroxamic acid formation (14). Coenzyme A was obtained from Boehringer and Soehne, Mannheim, Germany.

Results. [1-^{14}C]-Oleic acid incorporation by thymus and bursa cells. The incorporation of [1-^{14}C]-oleic acid into the lipids of thymus and bursa cells is shown in Table I. The majority of the uptake occurred in the triglycerides and phospholipids. The greatest incorporation in the phospholipid fraction was found in lecithin. Lecithin and sphingomyelin could not be separated well by chromatogram scanning and the radioactivity is therefore expressed as the sum of both fractions. Radioactivity was incorporated into phosphatidyl ethanolamine, diglyceride, and cholesterol ester to a much lesser extent. No incorporation at all could be detected in the lysolecithin fraction. Although both cell suspensions were slightly contaminated by red cells the contribution of radioactivity by red cell compounds is negligible. In comparison to leukocytes, red cells contain much smaller amounts of lipids (15).

Incorporation of oleyl–CoA-^{14}C lysolecithin-^{14}C, and lecithin-^{14}C into thymus and bursa cells. Because of the low specific activity of the labeled compounds the lipid extracts were separated on silica gel impregnated paper. The development of the chromatograms with diisobutylketone–acetic acid–water (240:150:30) did not allow the separation of free fatty acids, neutral fats, and cholesterol ester because their R_F values differed only slightly under the experimental conditions. The incubations were carried out for 160 min as follows: to 10^8 cells in 1 ml of medium 199 the labeled compounds were added in a concentration of 10–12 mμmoles. The incorporation of oleyl–CoA-^{14}C followed for both cell populations a percentage distribution of the radioactivity similar to that demonstrated for oleic acid in Table I. From this finding it cannot be concluded whether the CoA-ester is incorporated directly into the different constitutents simply by a transacylation reaction or whether it is hydrolyzed and reesterified.

The incubation with lysolecithin-^{14}C caused a marked increase of radioactivity into lecithin and into free fatty acid, neutral fat, and cholesterol ester, thus indicating that the free hydroxyl group of lysolecithin is esterified. Again, there were no differences between thymus and bursa cells.

When lecithin-^{14}C is added to the cell suspensions the total radioactivity is recovered only in the lecithin fraction in both cell populations. It may be that lecithin is incorporated into structural components, but it

TABLE II. Content of Phospholipid and Free Fatty Acid in Fowl Thymus and Bursa Cells.

	(mμmoles/mg of cell protein)				
	Lysolecithin	Sphingomyelin	Lecithin	Phosphatidyl-ethanolamine	Free fatty acid
Bursa cells	>0.1	1.144	5.397	2.081	>0.5
Thymus cells	>0.1	0.993	4.745	1.674	>0.5

seems more likely that lymphocytes cannot directly incorporate phospholipid into cellular constituents (16).

Phospholipid analysis of thymus and bursa cells. Table II shows average values of the different phospholipids determined in thymus and bursa cells. Free fatty acid and lysolecithin are present only in trace amounts in both cell types. However, bursa cells contain slightly more sphingomyelin, lecithin, and phosphatidylethanolamine. When the lipid concentrations are expressed as mμmoles per 10^8 cells (10^8 thymus cells contain 14.1 mg of protein, 10^8 bursa cells 20.9 mg of protein) bursa cells contain approximately 70% more phospholipid than thymocytes thus suggesting that the differences assayed correspond to differences in cell size.

Phospholipid-metabolizing enzymes in thymus and bursa cell homogenates. In both cell homogenates the activity of the following enzymes was determined: ligase, acyltransferase, acyl–CoA–hydrolyase, lysophospholipase, and phospholipase A. Table III shows that only the reaction rates of the acylation reaction are different. Though the activity of the acyltransferase in bursa cell homogenates is about three times higher than that of ligase it is assumed from the determination of the substrate affinities (see below) that the acylation reaction is not the limiting factor in the ligase test. The low activity of phospholipase A compared to the other enzymes studied is obvious; it is calculated by measuring the decrease of a known amount of lecithin added to the reaction mixture. As usual the values were corrected for endogenous substrate. Kinetic studies of phospholipase A could not be performed because of the inaccuracy of the method at that low range of its acitivity. Michaelis Menten kinetics of the other enzymes were studied at pH = 7.4. Figures 1 and 2 show the hyperbolic substrate concentration curve for acyltransferase and its double reciprocal plot according to Lineweater-Burk. The Michaelis constants are summarized in Table IV. An additional control experiment excluded the possibility that under the test conditions used lysolecithin is converted directly into lecithin and glycerylphosphoryl choline (GPC). The acyl–CoA–hydrolase, whose solubility is shown by a marked reduction in activity following multiple washings, was consistently assayed with acyltransferase because it competes with this enzyme for the same substrate. Its activity could not be influenced by

TABLE III. "Apparent" Enzyme Activities of Thymus and Bursa Cell Homogenates.

	Cell homogenate [mμmoles min^{-1} mg^{-1}]	
Enzyme	Bursa	Thymus
Lysophospholipase	12.6	14.4
Ligase	1.5	1.5
Acyltransferase	4.5	19.5
Acyl–CoA–hydrolase	2.3	2.1
Phospholipase A	0.023	0.032

TABLE IV. Michaelis Constants of Lysophospholipase for Lysolecithin, Ligase for Oleic Acid, and Acyl–CoA–Hydrolase and Acyltransferase for Oleyl–CoA in Bursa and Thymus Cell Homogenates.

	K_m	
Enzyme	Bursa cell homogenate (M)	Thymus cell homogenate (M)
Lysophospholipase	2.2×10^{-4}	1.7×10^{-4}
Ligase	1.6×10^{-5}	1.6×10^{-5}
Acyl–CoA–hydrolase	7.7×10^{-6}	7.3×10^{-6}
Acyltransferase	7.3×10^{-6}	7.3×10^{-6}

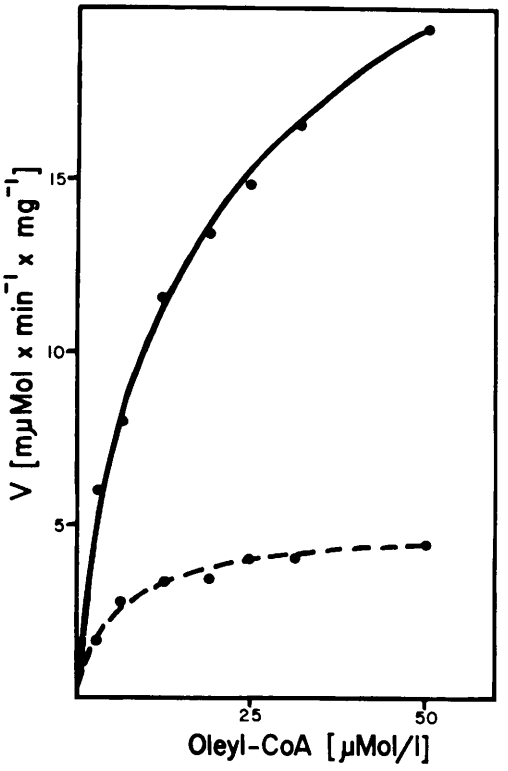

FIG. 1. Substrate concentration curve of acyltransferase from bursa (--); and thymus cell homogenates (—). Test conditions: sodium phosphate buffer (0.1 *M*); oleyl-CoA (6–50 μM); lysolecithin (60 μM); Ellman reagent (1 m*M*); 0.5 mg of homogenate protein, total volume, 2 ml; pH of the reaction mixture = 7.4.

diisopropylfluorophosphate, which Lands and Hart (12) showed to be an effective inhibitor of liver microsome acyl-CoA-hydrolase.

Discussion. Since the enzymes studied are structurally bound and some of their substrates are insoluble in water the reaction rates and Michaelis constants must be considered as "apparent" values. The experiments demonstrate the presence of ligase, acyltransferase, acyl-CoA-hydrolase, lysophospholipase, and phospholipase A in fowl thymus and bursa cell homogenates. The only significant difference between the enzyme patterns is seen in acyltransferase activity. The lecithin formation seems to be due to the acylation of the monoacyl precursor because no direct conversion of lysolecithin into lecithin and GPC could be found. This is not necessarily in contradiction to Elsbach's findings (17, 18) in phagocytic cells because in his system the reaction was measured at acid pH and in the presence of extremely high lysolecithin concentrations (4 m*M*). The reaction rates of both lysolecithin catabolizing enzymes studied are about 500 times higher than that of phospholipase A. The lecithinase may be associated with mitochondrial fragments and provide the substrate of the fatty acid oxidation. Its low activity may reflect the limited number and small size of mitochondria in lymphocytic cells (19). Nevertheless, the results do not strongly support Lands's concept of a complete "fatty acid exchange cycle" (20). It rather seems that the monoacylphosphatide must have been brought to these cells from other sources (*e.g.*, blood or macrophages).

That the incorporation experiments reflect the same enzyme activity (with the exception of phospholipase A) in homogenates and in whole cell preparations may indicate that these enzymes are active in the cell membrane or probably at its surface. The lower reaction rates in intact cells may have been produced by suboptimal concentrations of the labeled substrates (more than 10 mμmoles of lysolecithin/10^8 cells lead to a significant increase in the number of trypan blue stained cells). Furthermore, the intact surface membrane may only allow a limited access of the substrates.

The question arises whether changes in the reaction rates of phospholipases in lymphocytes can be brought about by nonspecific or

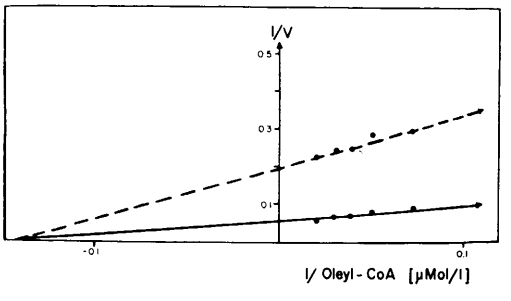

FIG. 2. Double reciprocal plot of the substrate concentration curve of Fig. 1 according to Lineweaver-Burk.

immunologically specific stimulation. The induction of phospholipase A especially would be of great interest. Preliminary experiments have been carried out in fowl thymus and bursa cell cultures stimulated with PHA; in comparison to nonstimulated cells no difference in phospholipase A activity could be demonstrated. These results are not surprising in view of Perlmann's suggestion (2) that the cells responsible for cytotoxicity in his system are the peripheral immunologically competent and PHA-responsive lymphocytes. Further experiments with such cells might settle the question whether or not lysophosphatides are involved in certain cytotoxic immune reactions.

Summary. The purpose of the present investigation was to determine whether lysophosphatide-metabolizing enzymes in lymphocytic cells might be an important component of certain tissue-damaging immune reactions. Thymus and bursa cells were used for technical reasons. The two cell populations differed in total lipid content according to differences in cell size but there was no difference with regard to phospholipid composition. In the cell homogenates the "apparent" reaction rates and Michaelis constants of the following enzymes were determined: ligase (acid:CoA ligase, EC 6.2.1.3), acyltransferase (acyl-CoA:lysolecithin-*O*-acyltransferase, EC 2.3.1 ?), acyl-CoA-hydrolase, lysophospholipase (lysolecithin acylhydrolase, EC 3.1.1.5), and phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4); the only difference between both cell homogenates was shown for the transferase activity. The investigation of the incorporation of radiolabeled substrates used in the enzyme assays indicated that the enzymes determined in the cell homogenates (with the exception of phospholipase A) are also active in the intact cells, but to a much lesser extent.

1. Munder, P. G., Ferber, E., and Fischer, H., Z. Naturforsch. **20b**, 1048 (1965).

2. Perlmann, P., Perlmann, H., and Holm, G., Science **160**, 306 (1968).

3. Holm, G. and Perlmann, P., J. Exptl. Med. **125**, 721 (1967).

4. Karnovsky, M. L., Physiol. Rev. **42**, 143 (1962).

5. Munder, P. G., Modolell, M., Ferber, E., and Fischer, H., Biochem. Z. **344**, 310 (1966).

6. Ways, P. and Hanahan, D. J., J. Lipid Res. **5**, 318 (1964).

7. Parker, F. and Peterson, N. F., J. Lipid Res. **6**, 455 (1966).

8. Ferber, E., Munder, P. G., and Fischer, H., Z. Klin. Chem. **4**, 199 (1966).

9. Duncombe, W. G., Biochem. J. **88**, 7 (1963).

10. Ferber, E., Munder, P. G., Kohlschütter, A., and Fischer, H., European J. Biochem. **5**, 395 (1968).

11. Hoelzl, J. and Wagner, H., J. Lipid Res. **7**, 569 (1966).

12. Lands, W. E. M. and Hart, P., J. Biol. Chem. **240**, 1905 (1961).

13. Goldman, P. and Vagelos, P. R., J. Biol. Chem. **236**, 2620 (1961).

14. Snyder, F. and Stephens, N., Biochim. Biophys. Acta **34**, 244 (1959).

15. Winterbourn, C. C. and Batt, R. D., Biochim. Biophys. Acta **152**, 255 (1968).

16. Cline, M. J., Physiol. Rev. **45**, 674 (1965).

17. Elsbach, P., Biochim. Biophys. Acta **125**, 510 (1966).

18. Elsbach, P., Van den Berg, J. W. O., Van den Bosch, H., and Van Deenen, L. L. M., Biochim. Biophys. Acta **106**, 338 (1965).

19. Schneider, W. C. and Hageboom, G. H., Cancer Res. **11**, 1 (1951).

20. Lands, W. E. M., J. Biol. Chem. **235**, 2233 (1960).

Received June 9, 1969. P.S.E.B.M., 1969, Vol. 132.