Metabolism of phospholipids in normal and spherocytic human erythrocytes

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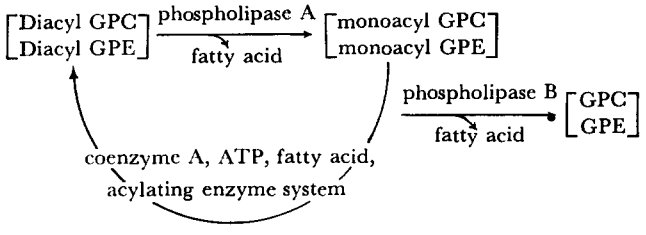
SUMMARY Human erythrocytes were incubated with oleic acid-l-C^{14} and monoacyl phosphatides to demonstrate the synthesis of diacyl glycerophosphoryl choline and diacyl glycerophosphoryl ethanolamine. These reactions proceeded at equal rates in normal and spherocytic cells. Phospholipase A activity was not demonstrable in erythrocytes. Normal and spherocytic cells appeared similar in the amount of acylating enzyme activity present. The amounts of endogenous monoacyl phosphatides available for acylation were similar in normal and spherocytic cells. In both types of cell, the predominant monoacyl phosphatide acylated was the α'-isomer.

PHOSPHOLIPIDS ARE present in human erythrocytes predominantly in the cell membrane and account for up to 24% of the dry weight of "ghost" preparations (2). Lecithin (diacyl GPC[1]), "ethanolamine phosphoglycerides" (diacyl GPE, and alkenyl-acyl GPE), and sphingomyelin are the most abundant phospholipids, with lysolecithin (monoacyl GPC) accounting for up to 3% of the phospholipid weight (3). Both monoacyl GPC and monoacyl GPE are hemolytic substances (4) and their presence in erythrocytes could possibly influence hemolytic anemia in humans. Kates et al. (5) reported an increase in monoacyl GPE in total blood cells from patients with congenital spherocytic anemia, but this finding has not been verified in two subsequent studies (6, 7). The involvement of monoacyl derivatives of GPC and GPE in phospholipid metabolism became more apparent after Lands showed (8) that preparations of rat liver microsomes contained an enzyme capable of transferring a fatty acid from its coenzyme A ester to the free hydroxyl group of monoacyl GPC. This type of acylation reaction has since been found to occur in rat brain (9), rabbit aorta (10), and human erythrocyte ghosts (11). The metabolism of the monoacylated phosphoglycerides appears to involve the following reactions:



An accumulation of monoacyl GPC or monoacyl GPE could therefore occur if phospholipase A were present in abnormal amounts or if either the acylating enzyme or the phospholipase B were absent.

The present paper describes the determination of some metabolic factors controlling monoacyl phosphoglyceride levels in normal and spherocytic erythrocytes of humans. No difference in the activity of the acylating enzyme system or phospholipase A was found in comparing spherocytic and normal cells.

EXPERIMENTAL PROCEDURES

Materials. Diacyl GPC was isolated from chicken egg yolks as described by Hanahan (12). This was treated with *Crotalus adamanteus* venom to yield α'-acyl GPC (13). α'-Acyl GPE was prepared by venom treatment of egg yolks (14). All of these derivatives were then purified by silicic acid chromatography before use in enzymatic studies. For chromatography, Mallinckrodt silicic acid (100 mesh) was used after removing the particles finer than 200 mesh. All solvents used were reagent grade. Oleic acid-l-C^{14} was purchased from the Volk Radiochemical Co. and the radioactivity of the

A preliminary report of this work has been presented (1).

[1] The abbreviations used are: GPC, glycerophosphoryl choline; GPE, glycerophosphoryl ethanolamine.

various samples was counted on a Nuclear-Chicago end-window, gas-flow counter. Labeled lecithin was synthesized by the previously reported method (15). In this method, alkenyl-acyl GPC is treated with iodine to form β-acyl-GPC. Oleic acid-l-C^{14} is then esterified enzymatically at the α'-position. However snake venom treatment of the labeled diacyl GPC used in these experiments released 84% of the radioactivity into the monoacyl GPC fraction and 15% into the fatty acid fraction. This indicates that there was migration of the acyl groups from the β to the α' position during the synthesis. The labeled lecithin is therefore a mixture of diacyl GPC (β-oleyl-l-C^{14}) and diacyl GPC (α'-oleyl-l-C^{14}). The product contained approximately 5,000 cpm/μmole.

Crotalus adamanteus venom was purchased from Ross Allen's Reptile Institute and a solution prepared as described previously (15) to contain 5 mg of dried venom per ml.

Venous blood was added to $^1/_2$ volume of a 3.5% sodium citrate solution. After centrifugation, the cells were washed twice with one volume of a 0.9% NaCl solution. Care was taken to remove the "buffy layer" of white blood cells. Cell counts on the packed erythrocyte preparations showed that 60–90% of the white blood cells had been removed. A better separation of white blood cells and erythrocytes was achieved by sedimenting the erythrocytes in a syringe with a 3% fibrinogen solution. Erythrocytes prepared in this manner gave experimental results identical with those given by the erythrocytes washed in NaCl. Microhematocrit readings on the packed cells before incubation gave values ranging from 78–92%. The packed erythrocytes were frozen by immersion in a $-60°$ bath for 5 min and then thawed in a 37° bath for 10 min. This suspension will be referred to as the hemolysate.

Methods. The following incubation mixture was used to determine the acylation of monoacyl GPC and monoacyl GPE. Oleic acid-l-C^{14} (90,000 cpm) was evaporated to dryness in a test tube. In some instances, 1 μmole of either monoacyl GPC or monoacyl GPE was also added before evaporating the solvents to dryness. One milliliter of phosphate buffer (0.1 M, pH 7.4) containing 1 μmole of sodium deoxycholate and 0.1 ml of 0.1 M $MgCl_2$ were added to the tube. This mixture was shaken at 37° in a water bath for 10 min to suspend the lipids and then 0.1 ml of ATP (10 μmoles) and 0.1 ml of coenzyme A (0.013 μmoles) were added. At zero time, 0.3 ml of the hemolysate was added, making a final volume of 1.6 ml. The incubations were stopped at 90 min by the addition of 10 ml of absolute ethanol. In later incubations, and in all experiments on the rate of acylation, the final mixture consisted of oleic acid-l-C^{14} (5.3 mμmoles, 90,000 cpm), 200 mμmoles of monoacyl GPC or monoacyl GPE, 0.1 ml $MgCl_2$ solution, 0.5 ml of phosphate buffer, 0.1 ml ATP (10 μmoles), and 0.1 ml of the hemolysate. The reaction was started by the addition of 0.2 ml of coenzyme A (0.026 μmoles). The final volume was 1.0 ml.

After stopping the reaction (by adding 10 ml of absolute alcohol to the hemolysate incubation), the mixture was homogenized in a glass homogenizing tube, the pestle washed with an additional 5 ml of absolute alcohol, and 5 ml of diethyl ether was added. The mixture was then shaken vigorously and centrifuged and the decanted supernatant solution was saved. The precipitate was resuspended in 5 ml of 33% diethyl ether in absolute ethanol[2], shaken, and centrifuged. The supernatant extract was combined with the earlier one. The lipid extract was evaporated to approximately 10 ml over a steam bath under a jet of nitrogen. The lipids were rinsed from the beaker into a large test tube with two 10-ml portions of chloroform. Six milliliters of water was added to the solution, the tubes were inverted and centrifuged, and the upper layer was discarded. The washing procedure was repeated and the chloroform layer evaporated to dryness. The lipids were then dissolved in 5 ml of 50% benzene in diethyl ether and put onto a silicic acid column.

Columns were prepared with 4 g of silicic acid, moistened with 50% benzene in diethyl ether. Fifteen fractions (20 ml each) were collected, as the lipids were eluted with 40 ml of 50% benzene in diethyl ether (fractions 1 and 2); 60 ml of 10% ethanol (95%) in diethyl ether (fractions 3–5); 80 ml of 50% ethanol (95%) in diethyl ether (fractions 6–9); 40 ml of 10% methanol in ethanol (95%) (fractions 10 and 11); and 80 ml of methanol (fractions 12–15). The elution of lipids by a similar method has been previously described (14). One milliliter of each fraction was dried on a planchette and the radioactivity counted. The counts from fractions 1 and 2 were added to give the activity of the fatty acid fraction; 6 through 8, the diacyl GPE fraction; and 12 through 14, the diacyl GPC fraction. The remaining fractions were counted: they contained less than 3% of the total radioactivity.

To determine the location of the oleic acid-C^{14} in the synthesized diacyl GPC, this lipid fraction was separated and concentrated to a 10-ml volume. A sample containing about 5,000 cpm was evaporated to dryness in a test tube and then dissolved in 3 ml of diethyl ether. To this, 0.2 ml of the *Crotalus adamanteus* solution was added and the mixture was shaken repeatedly for 6 hr. The reaction was stopped by adding 5 ml of methanol. The products of venom treatment were put on a silicic acid column

[2] All mixed solvents are described in terms of percentage by volume.

TABLE 1 ACYLATING ACTIVITY OF HUMAN ERYTHROCYTE HEMOLYSATES

Substrate Added to Incubation	Patient Source of Erythrocytes	Percentage Oleic Acid-l-C^{14} Recovered in: Diacyl GPE	Diacyl GPC
None	Normal	4 (3–6)	45 (37–59)
		t = 1.3	t = 1.4
	Spherocytosis	7 (2–14)	35 (17–54)
Monoacyl GPC (1 μmole)	Normal	1 (0–4)	76 (67–87)
		t = 0.5	t = 1.1
	Spherocytosis	2 (1–4)	64 (32–89)
Monoacyl GPE (1 μmole)	Normal	53 (38–69)	9 (7–12)
		t = 1.3	t = 3.9
	Spherocytosis	43 (36–54)	4 (2–6)

Figures are the average of triplicate incubations on samples from four patients with spherocytosis and from three normal subjects. The ranges are given in parentheses and values from the Student "t" test are given between the figures being compared.

moistened with 10% methanol in chloroform. Elution was carried out with stepwise increases in methanol concentration to separate fatty acids, diacyl phosphatides and monoacyl phosphatides (15).

To determine phospholipase A activity of the erythrocytes, 1 ml of C^{14}-labeled diacyl GPC (described under Materials) was evaporated to dryness in a test tube and 1 ml of the phosphate buffer containing sodium deoxycholate was added. This mixture was incubated for 10 min at 37° in a shaking water bath to suspend the lipids and then 0.3 ml of the hemolysate was added. In some cases, 0.3 ml of a 40% Pancreatin (Merck and Co., Inc., Rahway, New Jersey) solution was also added. In all cases, the final volume was 1.3 ml. The reaction was stopped at 90 min by the addition of 10 ml of absolute ethanol and the products were separated by silicic acid chromatography using stepwise increases in methanol (15).

TABLE 2 TIME COURSE OF SYNTHESIS OF DIACYL PHOSPHATIDES FROM MONOACYL PHOSPHATIDES BY ERYTHROCYTE HEMOLYSATES

Time of Incubation	Percentage Incorporation of Added Oleic Acid-1-C^{14} into Diacyl Phosphatides: Normal, Monoacyl GPC Added	Normal, Monoacyl GPE Added	Spherocytosis, Monoacyl GPC Added	Spherocytosis, Monoacyl GPE Added
min				
10	17	9	21	10
20	35	18	33	19
40	58	37	60	31

Incubation mixtures contained 200 mμmole added monoacyl GPC or monoacyl GPE, 5.3 mμmole oleic acid-1-C^{14}, 26 mμmole coenzyme A, 100 μmole ATP, 100 mμmole $MgCl_2$, 0.5 ml phosphate buffer, and 0.1 ml hemolysate. Figures represent average of values from three patients with spherocytosis and three normal controls.

RESULTS

The acylation reaction was studied first in intact erythrocytes and then in erythrocyte hemolysates. In both cases, no reaction occurred unless coenzyme A was added. The reaction progressed slowly when no ATP was added and was accelerated by the addition of ATP. When the hemolysate was heated to 100° for 5 min no reaction occurred.

Table 1 presents the averages of incorporation of oleic acid-l-C^{14} into diacyl phosphatides in red cell hemolysates from four patients with spherocytosis and three control subjects without hemolytic disease. These incubations were done under three separate conditions. When no substrate was added to the incubation, the incorporation of the oleic acid-C^{14} into diacyl GPE was minimal, averaging 4%, whereas 45% of the oleic acid-C^{14} was incorporated into diacyl GPC. When 1 μmole of monoacyl GPC was added as substrate, the synthesis of diacyl GPC was increased to 76%. When 1 μmole of monoacyl GPE was added, an average of 53% of the C^{14} appeared in diacyl GPE and there was a reduction to 9% of the incorporation of C^{14} into diacyl GPC. The mean incorporations into lipids in cells from spherocytic patients were somewhat lower than those found in normal persons but not significantly so. The results were compared by using the Student "t" test. The t values are given in Table 1. These figures showed no significance at the $p = 0.05$ level with the exception of the incorporation into diacyl GPC when monoacyl GPE is added. In this circumstance, the hemolysates of spherocytic cells incorporated significantly lower amounts of isotope than the normal control samples.

To study the rate of synthesis of diacyl phosphatides, reactions were stopped at 10, 20, and 40 min. In these reactions, 200 mμmoles of monoacyl GPC or monoacyl GPE was added. Table 2 gives the percentage incorporation into diacyl phosphatides at these time intervals in hemolysates from three patients with spherocytosis and three control subjects. The synthesis of diacyl GPC occurred at approximately twice the rate of synthesis of diacyl GPE. If these figures are averaged and converted to mμmoles of C^{14}-diacyl phosphatides synthesized, it is found that in normal cells 0.084 mμmoles/min of diacyl GPC and 0.049 mμmoles/min of diacyl GPE were synthesized. The spherocytic cells formed 0.086 mμmoles/min of diacyl GPC and 0.046 mμmoles/min of diacyl GPE.

The results of incubations with sheep erythrocyte hemolysates are shown in Table 3. When no substrate

was added, 21% of the radioactivity was incorporated into the diacyl GPC fraction. When monoacyl GPC was added, this incorporation increased to 73%. When monoacyl GPE was added, the fraction containing diacyl GPE contained 70% of the incorporated C^{14}.

The endogenous monoacyl GPC that was acylated may be either α'- or β-acyl GPC. This can be determined by isolating the diacyl GPC synthesized from oleic acid-C^{14} when no exogenous monoacyl phosphatide is added to the incubation and treating it with *Crotalus adamanteus* venom, which contains a specific β-esterase (16). The results of such hydrolyses in studies on three patients with spherocytosis and two control subjects are shown in Table 4. When no enzyme was added to the isolated diacyl GPC fraction, 6% of the C^{14} appeared in the fatty acid fraction after 6 hr incubation. When enzyme was added, most of the radioactivity appeared in the fatty acid fraction. There was no increase in the C^{14}-monoacyl GPC fraction. The values for samples from patients with spherocytosis and controls are similar. In one experiment with spherocytic cells, sufficient radioactive C^{14}-diacyl GPE synthesized from oleic acid-l-C^{14} was isolated for venom treatment. In this case also, radioactive fatty acid was released but there was no increase in C^{14}-monoacyl GPE after incubation with the venom.

To determine phospholipase activity, C^{14}-labeled lecithin was incubated with erythrocyte hemolysates. Phospholipase activity would then be shown by an increase of radioactivity in the fatty acid and monoacyl GPC fractions (15). In the absence of enzyme, 2% of the C^{14} appeared in the fatty acid fraction (Table 5). When the erythrocyte hemolysate was added, there was no significant increase in radioactivity in the fatty acid or monoacyl GPC fraction. When Pancreatin, a known source of phospholipase A, was added, hydrolysis occurred causing an increase in the fatty acid-C^{14} fraction to 20% and an increase in the monoacyl GPC fraction to 52%.

DISCUSSION

Monoacyl GPC and monoacyl GPE are capable of altering erythrocyte shape (17) and causing hemolysis (4). Since monoacyl GPC occurs normally in plasma (18) and in erythrocytes (3), it is reasonable to suspect an abnormality of phospholipid metabolism as a possible cause of some of the previously unexplained hereditary hemolytic anemias, especially those characterized by changes in the cell shape, such as spherocytosis. Kates et al. studied the incorporation of P^{32} into the phospholipids of spherocytic cells and found the specific activities of phosphatidic acid, diacyl GPC, and diacyl GPE, to be similar to those in normal cells. They concluded that the rates of synthesis of these phospholipids,

TABLE 3 ACYLATING ACTIVITY OF SHEEP ERYTHROCYTE HEMOLYSATES

Substrate Added (0.2 μmoles)	Percentage of Added Oleic Acid-l-C^{14} Recovered in:	
	Diacyl GPE	Diacyl GPC
None	10 (9–13)	21 (17–26)
Monoacyl GPC	1 (1–2)	73 (65–76)
Monoacyl GPE	70 (67–72)	4 (3–4)

Figures represent the average results in three incubations; ranges are given in parentheses.

as described by Kennedy (19), was normal. In other tissues, the synthesized diacyl phosphatides may be hydrolyzed by phospholipase A to monoacyl phosphatides (15). These monoacyl phosphatides may be further hydrolyzed to GPC or GPE or may be resynthesized to diacyl phosphatides by the acylating enzyme system (see diagram in the Introduction). Kates et al. postulated (5) an absence of acylating enzyme activity in spherocytic cells to explain the accumulation of monoacyl GPE. Also an abnormally high activity of phospholipase A or an absence of phospholipase B could explain an excess of monoacyl GPE.

Our results demonstrate that normal cells contain enzyme systems capable of acylating α'-acyl GPC and α'-acyl GPE. The acylation of monoacyl GPC by erythrocyte ghosts has been previously demonstrated (11). In the hemolysates of spherocytic cells, these enzyme systems are also present. In human erythrocytes, the rate of synthesis of diacyl GPC is approximately twice the rate of synthesis of diacyl GPE. This difference in enzyme activity toward the two substrates occurs in normal and spherocytic cells. The absolute rates for each substrate (Table 2) show no significant differences between normal and spherocytic cells, indicating no relative deficiency of the enzymes that acylate the β-position.

Other studies have not confirmed the finding of Kates et al. that blood cells of patients with spherocytosis

TABLE 4 DISTRIBUTION OF C^{14} IN LIPID FRACTIONS AFTER HYDROLYSIS OF SYNTHESIZED DIACYL GPC BY CROTALUS ADAMANTEUS VENOM

Lipid Fraction	% C^{14} Recovered in Lipid Fractions		
	No Enzyme Added	Spherocyte Hemolysate Added	Normal Erythrocyte Hemolysate Added
Fatty acids	6	89	92
Diacyl GPC	86	5	5
Monoacyl GPC	8	7	4

Figures represent triplicate incubations on samples from three patients with spherocytosis and two normal subjects.

TABLE 5 ABSENCE OF PHOSPHOLIPASE A ACTIVITY IN HUMAN ERYTHROCYTE HEMOLYSATES

	% C^{14} Recovered		
	No Enzyme Added	Hemolysate Added	Hemolysate and Pancreatin Added
Fatty Acids	2	4	20
Diacyl GPC	87	82	32
Monoacyl GPC	11	12	52

Distribution of C^{14} in lipid fractions after incubation with lecithin labeled with oleic acid-1-C^{14}. Each figure represents the average of duplicate incubations on cells from three patients with spherocytosis.

contain increased amounts of monoacyl GPE (6, 7). Hanahan et al. have emphasized (20) the possibility of monoacyl GPE being formed on storage or under the influence of heparin. A number of relatively mild procedures can lead to partially degraded products as artifacts (21), and there has been some controversy regarding the natural occurrence of "lysophosphatides." The synthesis of C^{14}-diacyl phosphatides in erythrocytes with no added phospholipid indicates that endogenous monoacyl phosphatides are present in the hemolysate and are available for acylation. It is possible, however, that additional monoacyl phosphatides are present but not available for reaction owing to their close association with the lipoproteins of the cellular membranes. It is also possible that a different enzyme is required to acylate β-acyl phosphatides and this may not be present in these cells. In that case, no endogenous β-acyl GPC or β-acyl GPE would be detected by this method.

Snake venom treatment of the diacyl phosphatides synthesized from endogenous monoacyl phosphatides (when no substrate is added to the incubation mixture) allows one to determine whether the oleic acid-l-C^{14} was incorporated at the α'- or β-position. This indicates whether the endogenous monoacyl phosphatide fraction that was acylated was the α'-acyl or β-acyl isomer. The predominant monoacyl GPC isomer acylated was α'-acyl GPC in both normal and spherocytic cells. This was also the predominant isomer of monoacyl GPE that was acylated in spherocytic cells. α'-Acyl GPC is also the predominant isomer acylated in serum (22) and in aortic tissue (10), but this may not indicate the enzymatically produced isomer. The thermodynamically stable form appears to be the α'-isomer with an equilibrium value of $\alpha':\beta$ of approximately 9:1 (22). Furthermore, the results reported for monoglycerides at pH 8.0 by Mattson and Volpenhein (23) suggest that spontaneous acyl migration could rapidly occur in vivo. Thus β-acyl GPC may not exist for a very long time even if it is produced enzymatically.

We were unable to demonstrate phospholipase A activity in normal or spherocytic cells. A very low phospholipase activity in serum has been described (24). Monoacyl GPC is present in plasma (18) and the phospholipids of cells and plasma are apparently exchangeable to some extent (25). This interchange may explain the origin of the monoacyl phosphatides that are present in the cells and acylated by the erythrocyte membrane enzyme system.

There may be no net synthesis of phospholipids in mature erythrocytes (26). However, the fatty acid composition of the erythrocyte phospholipids is influenced by dietary changes within 10 days (27). This incorporation of fatty acids could be explained by the activity of the acylating enzyme system and the recent note of Mulder et al. (27) considers this possibility. If the acylating enzymes controlled the level of diacyl phosphatides in these cells, we might expect to find variations in the amount of the acylating activity in cells from species with different phospholipid contents. In sheep erythrocytes, there is little diacyl GPC (28), although our results show a very active acylation of monoacyl GPC by sheep erythrocytes. This indicates that the level of diacyl phosphatides in erythrocytes is not apparently controlled solely by the acylating enzyme. It is possible that in these cells the level of diacyl phosphatides is governed by the activity of phospholipase A in the cell or in plasma or that there is another metabolic pathway to the formation of acyl phosphatides. However, a more significant factor controlling erythrocyte phosphatide composition may prove to be differences in the protein portion of the cellular lipoproteins that lead to more stable binding of one phosphatide over another. This may also be a critical factor in spherocytic cells that may have relatively normal metabolic activities, but still appear to have altered lipoprotein membranes.

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