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Research Article

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The labeled fatty acid then passed into a second "deeper" membrane pool of free fatty acids (F-2), which was not extractable with albumin. This process was energy-dependent and proceeded at a slower rate than the initial transfer from albumin to F-1.

Ultimately the labeled fatty acid was incorporated into phosphatides (PL). This process also was dependent upon cellular metabolism.

The kinetics of pulse label studies suggest that the processes observed were sequential and that precursor-product relationships exist between the F-1 and F-2 pools and the F-2 and PL pools. [Formula: see text] From the size and specific activities of these pools, calculations of the extent of phospholipid turnover were made. An approximate figure of 2% /hr or 30 nmoles/ml of packed red blood cells per hr was obtained. The figure was further calculated to represent an energy cost to the red blood cell of approximately 5% of [...]

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Stages in the Incorporation of Fatty Acids into Red Blood Cells

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ABSTRACT Mature human erythrocytes were incubated with ^{14}C -labeled palmitic acid bound to crystalline human albumin. Energy-dependent incorporation of the labeled palmitic acid into cell membrane phospholipids occurred, and various stages in this incorporation were defined.

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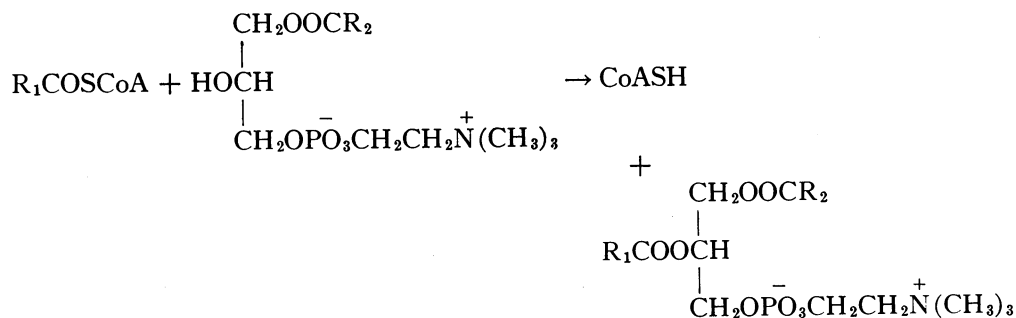
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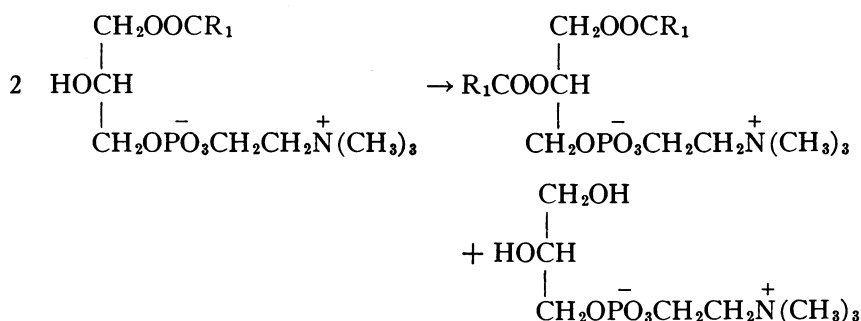
INTRODUCTION

The incorporation of unesterified fatty acids of the external medium into the phosphatides of erythrocyte membranes was first noted by Oliveira and Vaughan 5 yr ago (1). They found, using erythrocyte ghosts as experimental objects, that this incorporation was primarily into phosphatidylcholine and that ATP and coenzyme A were required. Subsequently Mulder, Van Den Berg and Van Deenen suggested that two biochemical pathways could be operative in the final stages of this incorporation (2). These pathways are: (a) Insertion of fatty acids (activated as acyl CoA) into preformed lysophosphatidylcholine by the pathway originally described by Lands (3).



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(b) Transesterification between two lysophosphatidylcholine molecules to form one phosphatidylcholine and one glycerophosphorylcholine, as described by Erbland and Marinetti (4).



Mulder and Van Deenen (5) later presented convincing evidence that in the presence of adequate CoA and ATP, the first reaction predominates, whereas without these cofactors the latter is the major pathway. Elsbach using white blood cells showed that at physiological pH the acylation pathway was dominant, whereas at lowered pH the transesterification pathway became important (6). Recently Donabedian and Karmen (7) have studied the relative rates of transfer of different fatty acids to red cells and their subsequent rates of incorporation into phospholipid. Because of the importance of phosphatides in membrane structure and because the metabolism of these compounds may well be important in membrane function, we believed that it was warranted to investigate in further detail the incorporation of fatty acids into red cell membranes, and particularly to examine the events immediately antecedent to this incorporation.

The purpose of this report is thus to describe experiments defining the stages in the transfer of fatty acids from extracellular albumin to human red cell phospholipids, and to estimate the energy required. The process will be shown to involve three metabolic pools of fatty acids. These pools have been designated as follows: (1) a pool of unesterified fatty acid, which can be removed from the cell by washing with defatted albumin (F-1 pool); (2) a pool of unesterified fatty acid which is *not* extractable by defatted albumin and which might be considered "deeper" in the membrane (F-2 pool); and (3) the fatty acids bound in ester linkages in membrane phosphatides (PL pool).

The results suggest that (a) transfer of fatty acids from albumin into the "superficial" red cell pool (F-1) is rapid and independent of cellular energy, (b) the subsequent transfer of fatty acids

from this pool to the "deeper" membrane pool (F-2) is less rapid and requires metabolic energy, and (c) uptake into the phospholipids (PL) proceeds from F-2 and is also energy dependent. The phosphatides involved in this process are phosphatidylcholine and phosphatidylethanolamine (ca. 95% of total incorporation into PL is concerned with these entities).

Although we lay particular stress on red blood cells and aspects of the maintenance of red cell membranes, phenomena such as those reported below have, of course, more general implications. For example, Spector and Steinberg have studied analogous situations in Ehrlich ascites cells (8-10).

METHODS

A. Materials. Crystallized human serum albumin was obtained from Dade Reagents Inc., Miami, Fla. Palmitic acid-1-¹⁴C, palmitic acid-9,10-³H and ¹²⁵I were obtained from New England Nuclear Corporation, Boston, Mass. All other reagents used were reagent grade. Polyvinylpyrrolidone (NP-K60) was obtained from the General Aniline and Film Corporation, New York. Silica Gel G for separation of neutral lipids and Silica Gel H for phospholipid separations were obtained from CAMAG Chemicals, Muttenz, Switzerland.

B. Cell collection and preparation. Peripheral venous blood was obtained with plastic syringes from normal human subjects. Heparin (3 U/ml) was added to prevent coagulation. Red cells were separated from plasma and white cells as follows. The freshly drawn blood was centrifuged at 300 *g* for 10 min in siliconized glass tubes; at the end of this time the white cell- and platelet-rich plasma was removed along with the top 10% of the red cell mass; subsequently, the bulk of the red cells was resuspended in 3 volumes of Krebs-Henseleit buffer (KHB) pH 7.4 (11) with 8 mM glucose, and centri-

fuged at 600 *g*; again the supernatant fluid and 10% of the residual red blood cells were removed. After this process was repeated once more, the residual white blood cell count on a red cell suspension reconstituted to an hematocrit of 50% was always less than 400 cells/mm³, and there were usually less than 200 white blood cells/mm³.

C. Preparation of defatted albumin and replacement of fatty acids on albumin. Crystalline human serum albumin was defatted by the method of Goodman (12). After lyophilization a chloroform-methanol extract was made (13, 14), and free fatty acids were assayed by the method of Dole and Meinertz (15). In our hands, 92–94% of the free fatty acid was removed by Goodman's procedure.

The fatty acids of albumin treated in this way could then be replaced with labeled fatty acids as follows.¹ 100 ml of KHB containing 800 mg of the defatted albumin preparation was placed in an 800 ml Erlenmeyer flask. 15 ml of heptane containing 4 mg of ¹⁴C- or ³H-labeled palmitic acid was gently layered upon this solution, and the air was displaced with nitrogen. The flask was closed and gently rocked for 36 hr at room temperature. The speed was controlled so that interfacial contact was maximal without production of emulsions or froth. After 36 hr the layers were separated, and the albumin mixture was filtered through No. 42 Whatman paper. Aliquots of both the albumin-containing and heptane layers were then taken for counting in a liquid scintillation counter in Buhler's toluene solution (16). At this point 94–96% of the added palmitic acid was in the aqueous layer, and FFA had been restored to the albumin to about 70% of the starting level.² The albumin-containing layer was dialyzed against fresh buffer. It was convenient to add small volumes of this component to the final incubation mixture; concentration was effected by ultrafiltration. The albumin with fatty acid attached is referred to as Alb-FA.

D. Incubation of red cells with fatty acid. The red cells prepared as above were resuspended to a final hematocrit of 50% in KHB with ca. 8 mM glucose and albumin-fatty acid complex (Alb-FA, 200 mg/100 ml). The fatty acid concentration was thus approximately 45 μEq/liter. Duplicate cell suspensions were incubated in small (25 ml) stoppered siliconized flasks at 37°C or at 4°C with gentle reciprocating agitation (40 cycles/min; excursion 2 inches).

Aliquots of these suspensions were taken for analysis

¹ The authors are grateful to Dr. Arthur Karmen who originally instructed us in this method. We used this method because we thought it might have the advantage, compared to the more usual method of adding the potassium salts of the fatty acids to the albumin, of reducing the possibility of formation of particulate entities. The method allowed the preparation of albumin-fatty acid complexes of very high specific activity and high saturation, with little waste of radioactive material.

² This corresponded to approximately 1.5 mEq of free fatty acid per mmole of crystalline albumin. In other studies we have found 1.5–3.2 mEq of free fatty acid per mmole protein on preparations of previously untreated human albumin from several different sources.

at various time intervals after the incubation was started. These were immediately pipetted into 10 volumes of normal saline at 4°C and spun in a refrigerated centrifuge at 500 *g* for 3 min. The sedimented cells were washed four times with 10 volumes of cold isotonic NaCl solution. No further radioactivity was removable by washing with isotonic saline.

For all determinations below, a small correction factor was introduced (5–10%) for losses of cells at the various stages. This correction was made by measuring total hemoglobin on aliquots.

E. Removal of unesterified fatty acids with albumin (F-1 pool). The washed red cells from D above were then usually subjected to a series of five washes at 20°C with defatted human albumin solution (1 g/100 ml) prepared as described above. Radioactivity was apparent in the first three washes, but no further activity was removed after the fourth wash. After lyophilization of the albumin-containing washings, the F-1 lipids were extracted with chloroform-methanol and analyzed by thin-layer chromatography (see below). In this albumin removable pool, over 96% of the activity was in unesterified fatty acids with only 2–3% in phosphatides and 1–2% unidentified.

F. Extraction and assay of more tightly bound fatty acids and phosphatides (F-2 and PL pools). Lipids were now extracted from the red cells with aqueous isopropanol and chloroform by the method of Rose and Oklander (17). Two washes of the extract with 0.05 N KCl were performed to remove residual nonlipid contaminants. Samples were taken from these lipid extracts for counting, and then the solutions were evaporated to dryness under nitrogen at 50°C. The lipids were taken up in a small volume (ca. 0.1 ml) of chloroform-methanol (2:1). Thin-layer chromatography was performed on 16-inch long glass plates coated with Silica Gel H to separate the phospholipid classes. The developing solvent for phosphatides (PL pool) was the aqueous acetic acid-chloroform-methanol system described by Skipski, Peterson, and Barclay (18). Identification of phosphatide spots was achieved with ninhydrin (19) for phosphatidylethanolamine and Dragendorff's reagent for phosphatidylcholine (20). Identification of neutral lipid components was made by rechromatography of the neutral lipids. These lipids were eluted from the above plates with chloroform-methanol and rechromatographed on Silica Gel G with ether-hexane-acetic acid-chloroform as developing solvent (21). Radioactive spots corresponding to free fatty acids, triglycerides, and diglycerides were detected. In samples taken after short incubations, the vast majority (greater than 80%) of radioactivity was in free fatty acids. The unesterified fatty acid zone is referred to as the F-2 pool.

G. Determination of specific activity of pools and calculation of transfer between pools. The thin-layer plates were first counted in a Baird-Atomic gas flow strip scanner (model No. RSC-363). When specific activities of various fractions were to be determined, components were eluted (18). Aliquots were than taken for liquid scintillation counting in Buhler's solution and for chemi-

cal assay. For determination of phosphorus-containing compounds, a minor modification of the colorimetric method of Lowry, Roberts, Leiner, Wu, and Farr for phosphate was used (22). For free fatty acid, Dole's microtitration was employed (15). When the latter method was used, care was taken to filter any residual silica gel from the test solutions, and a blank was performed on a sample of the same amount of silica gel as that from which the lipid was eluted.

Fatty acid transfer between the pools (F-1, F-2, and PL) was estimated by dividing the number of counts detected in a given pool by the specific activity of the material in the preceding pool, e.g., transfer from F-1 into F-2 (in μmoles) = $\text{cpm in F-2}/\text{SA of F-1}$ (all specific activities [SA] as $\text{cpm per } \mu\text{mole FA}$). All calculations of fatty acid transfer with the exception of the initial transfer from albumin to F-1 were made after 1 hr of incubation with the standard incubation system and with a large excess of Alb-FA (ca. 25 times the F-1 pool). Calculation of fatty acid transfer from albumin to F-1 was made under the same conditions but after 5 min of incubation, since this phenomenon was only briefly linear (see below).

RESULTS

A. Effect of added white blood cells. On one occasion after the red cells had been obtained in the usual way, graded amounts of a white blood cell suspension obtained from the same donor were added to separate incubation systems. These white blood cells were obtained from the leukocyte-rich serum produced in the first centrifugation of the red blood cells. The white cells were washed twice in 20 volumes of Krebs-Henseleit buffer containing glucose, after gentle centrifugation (500 g for 8 min) in siliconized tubes. Smears taken after preparation showed a differential count of 45% polymorphonuclear leukocytes (PMN), 37% lymphocytes, 8% mononuclear cells and 10% swollen or smudge cells. Smears on the donors of the fresh blood showed 55% PMN, 45% lymphocytes, and 5% monocytes. Fig. 1 shows that the graded addition of up to 2000 white cells/ mm^3 in the final incubation mixture did not significantly change the character or extent of the incorporation noted. Above 3000 white blood cells/ mm^3 , a small increment in phospholipid activity could be detected. As noted in the Methods section, white cell counts were below 400 cells/ mm^3 in all subsequent incubations of red blood cells.

B. Gross uptake of fatty acid into lipid classes. The uptake of palmitic acid- ^{14}C into lipid classes extracted from cells incubated for 1 hr with

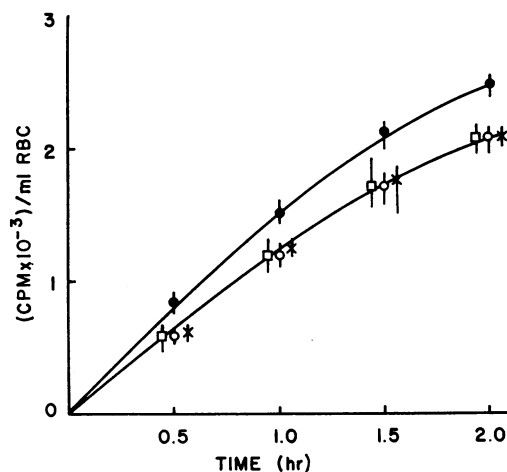


FIGURE 1 Effect of added white blood cells on incorporation of ^{14}C -labeled palmitic acid into phosphatides of red blood cell suspensions. All points are averages of four concurrent incubations. Ranges are indicated by the brackets. Points for various white blood cell concentrations were determined at the same times, but for clarity, they have been displaced slightly in the lower curve. \circ , no added white blood cells; \times , 500 white blood cells added per mm^3 ; \square , 2000 white blood cells added per mm^3 ; \bullet , 3000 white blood cells added per mm^3 . See Results, section A for further details.

Alb-FA- ^{14}C followed by saline washes, but without washes with defatted albumin, is shown in Table I, A. As shown in Fig 2a and b the rate of incorporation into phosphatides was almost constant for at least 3 hr, but incorporation into the total free fatty acids (F-1 and F-2) did not increase significantly after 90 min. Because of the large amount of activity present as free fatty acid in the nonphosphatide lipids (Table I, A), we wished to define the disposition of the free fatty acids of the erythrocyte. Thus, red cells incubated with Alb-FA- ^{14}C were washed not only with saline (as in this section) but also with defatted albumin as described in Methods section E. The results appear below.

C. Uptake into fatty acid pool not extractable with albumin and into the phospholipid pool (F-2 and PL). The uptake of palmitic acid- ^{14}C into lipid classes extracted from cells incubated for 1 hr with Alb-FA- ^{14}C , followed by washing with isotonic saline and with defatted albumin, is shown in Table I, B. Occasionally red cells were prepared in the usual fashion except that glucose was omitted from all washing and resuspension steps.

TABLE I
Radioactive Counts Obtained from Chromatograms of Extracts of Red Cell Lipids after Various Incubation and Washing Procedures

Component	Zone	A	B	C	D
		37°C incubation, after saline washes	37°C incubation, after saline and albumin washes	4°C incubation of ATP-depleted cells, after saline washes	4°C incubation of ATP-depleted cells, after saline and albumin washes
	R_f^*	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>
Nonphosphatide lipids‡	0.92-1.05	5920 (±410)	2200 (±160)	3000 (±280)	380 (±140)
Phosphatidylcholine	0.26-0.42	1880 (±320)	1700 (±400)	190 (± 40)	150 (± 40)
Phosphatidylethanolamine	0.60-0.77	1300 (±160)	1360 (±330)	160 (± 30)	160 (± 40)
Unidentified radioactivity§	—	240 (±100)	300 (± 70)	170 (±100)	60 (± 30)

All data are averages of results from four chromatographic plates derived from four separate incubations. Two donors supplied the cells for the experiments. Average deviations from the means are shown in parentheses.

* As range of R_f taken.

‡ 80% of activity in free fatty acids. Columns A and C represent essentially F-1 + F-2, and a small amount of esterified fatty acids. B and D are essentially F-2, plus a small amount of esterified fatty acids.

§ Remainder of strip.

After final resuspension but before incubation with fatty acid-albumin, the cell suspensions were left overnight at 20°C with 1% added polyvinylpyrrolidone (PVP). The cells were then washed once with isotonic saline before use. This procedure has been shown to reduce red cell ATP stores to less than 10% of normal.³ In studies done with ATP-depleted cells incubated at 4°C, uptake of palmitic acid-¹⁴C in phospholipid fractions was

barely detectable, and the amount of free fatty acid present depended on the type of washing procedure. The results of these studies are shown in Table I, C and D. It is notable that at 37°C a significant amount of labeled free fatty acid was present after washes with defatted albumin though less than after washing with saline alone (ca. $\frac{1}{3}$ remaining). Labeled free fatty acid was also present in the ATP-depleted cells incubated at 4°C after saline washes alone but no significant

³ Nathan, D. G. Unpublished experiments.

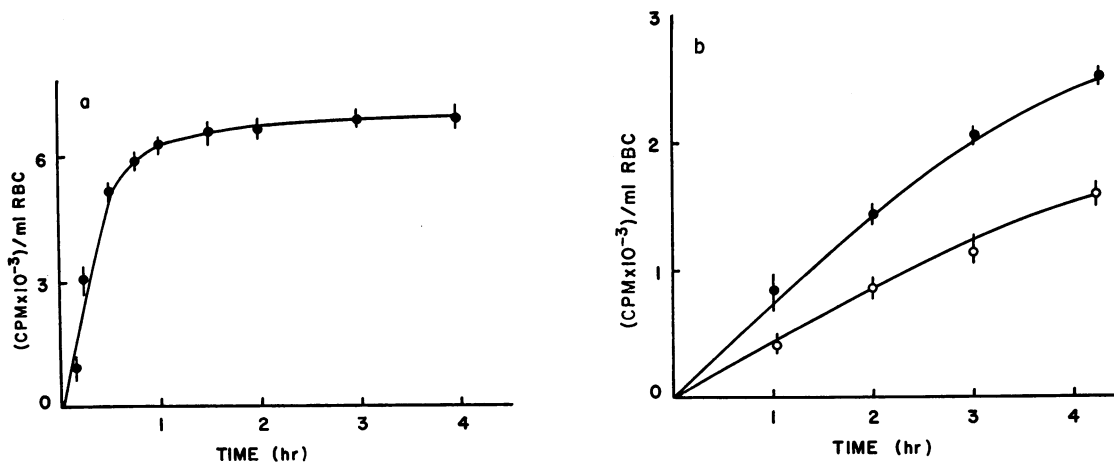


FIGURE 2 *a* and *b* Time course of incorporation of palmitic acid-¹⁴C into erythrocyte lipids. All points are averages of two concurrent incubations. The ranges are indicated by the brackets. In the left-hand panel (2 *a*) the total free fatty acids (F-1 + F-2) are shown. In the right hand panel (2 *b*), the phosphatides are shown. ●, phosphatidylcholine; ○, phosphatidylethanolamine.

label was found in this fraction after the cells were washed with defatted albumin. Combined with the observation that for cells depleted of ATP and incubated at 4°C almost no uptake of free fatty acids into phosphatides could be demonstrated with either washing procedure, these data indicate that the conversion of F-1 to F-2 and of F-2 to PL depends upon metabolic energy. In other experiments inhibition of new ATP synthesis with 25 mM sodium fluoride produced intermediate amounts of uptake which were limited to the 1st hr of incubation. Thereafter no new uptake of free fatty acid into phosphatide could be detected.

D. Uptake of free fatty acids into the F-1 pool. In addition to confirming the experiments of Oliveira and Vaughn (sections B and C above), the data suggested that two distinct pools of red cell free fatty acid exist. Accordingly, the uptake of free fatty acid into the albumin-extractable F-1 pool was studied in more detail. As shown in Fig. 3, uptake into this pool was rapid and reached an almost constant level after 15 min. The uptake occurred to the same extent and at roughly the same rate at different temperatures and in the absence of ATP stores.

In order to rule out the possibility that incorporation into F-1 was simply passive adsorption

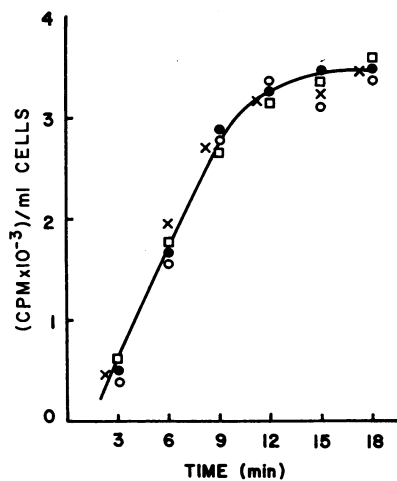


FIGURE 3 Incorporation of palmitic acid-¹⁴C into F-1 (albumin-extractable fatty acid) pool under various conditions. All points represent single samples from concurrent incubations. Points were taken at the same times and are slightly displaced where necessary for clarity. □, 20°C; ●, 37°C; ○, 40°C; ×, ATP-depleted cells at 37°C. See Methods, section E, for further details.

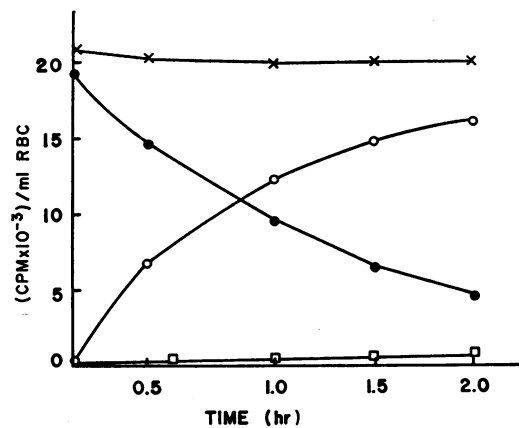


FIGURE 4 Incorporation of ³H-labeled palmitic acid into total erythrocyte lipids and attachment of ¹²⁵I-labeled albumin onto erythrocytes. The starting ratio of ¹²⁵I to ³H counts in the windows selected was 6:5. ○, lipid ³H counts; ●, medium ³H counts; □, cell ¹²⁵I counts; ×, medium ¹²⁵I counts. See Results, section D, for further details.

of the whole Alb-FA complex to the cell surface, a doubly labeled experiment was performed by incubating red cells prepared in the usual way in the presence of ³H- and ¹²⁵I-labeled albumin preparation.

Doubly labeled Alb-FA was obtained as follows. Defatted albumin prepared as usual was iodinated with ¹²⁵I by the method of Reif (23) and was subsequently loaded with tritiated palmitic acid. The uptake of palmitic acid-³H onto such albumin was identical with uptake onto a noniodinated control sample handled simultaneously. The ratio of activity of ¹²⁵I to ³H in the final preparation was 6:5. Red cells prepared in the usual way were then incubated in the presence of this preparation. Frequent aliquots of both the cells and the incubation media were taken for analyses. After four saline washes, total red cell radioactivity was assayed by the combustion procedure of Nathan, Gabuzda, and Gardner (24). Radioactivity in aliquots of the incubation medium was determined directly in Buhler's solution with a correction for quenching due to small amounts of albumin present. The contributions of ³H and ¹²⁵I to the total radioactivity were established by counting through two widely separated "windows" on an Ansitron model No. 2 scintillation counter. The results of this experiment are summarized in Fig. 4. Clearly, fatty acid was incorporated into the cell lipids, but

albumin remained in the medium. Thus, no significant net adsorption of Alb-FA complex to the cell membrane occurred.

E. Relationships between pools. Passage of free fatty acid from the "surface" pool (F-1) to later pools was evaluated as follows. After a brief initial incubation with labeled fatty acid-albumin complex (12 min), the cells were washed in saline four times and then reincubated in Krebs-Henseleit buffer containing 1% PVP and 8 mM glucose. Aliquots were taken at various times and analyzed as indicated above. The uptake from F-1 to (F-2 + PL) under various conditions is shown in Fig. 5. At 37°C, approximately 80% of the "pulse"-labeled F-1 fraction was converted to (F-2 + PL) in 3 hr. This transfer was markedly reduced at 20°C and virtually absent in ATP-exhausted cells.

Further dissection of the passage of fatty acid to PL is shown in Fig. 6. The design of this incubation was the same as that for Fig. 5. Here the

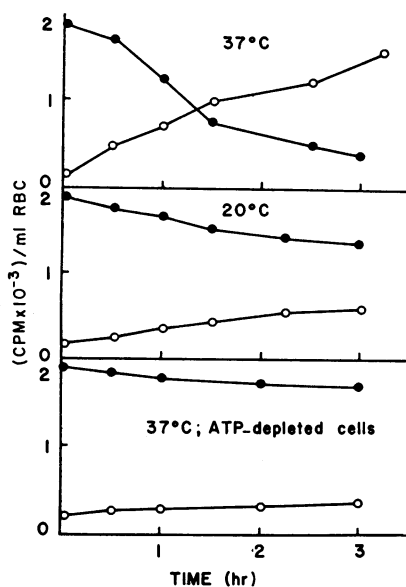


FIGURE 5 Incorporation of ¹⁴C-labeled palmitic acid from "pulse-labeled" F-1 into F-2 + PL under varying conditions. The cells were saline washed after brief initial incubation with Alb-FA and then reincubated in saline with 1% polyvinylpyrrolidone and glucose. After this reincubation, aliquots were washed with saline and extracted (washed) with albumin solution at the times indicated on the abscissa. The albumin extracts and the total residual cellular lipids were counted. ●, F-1; ○, F-2 + PL. See Results, section E, for further details.

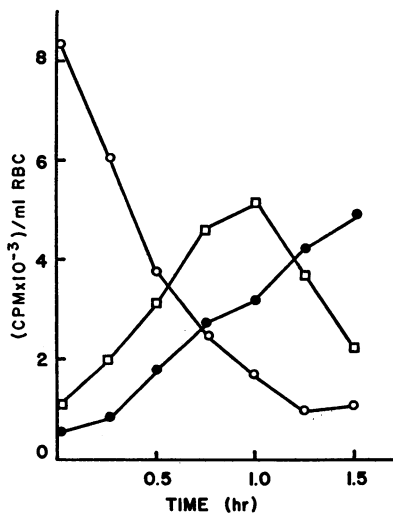


FIGURE 6 Incorporation of ¹⁴C-labeled palmitic acid from "pulse-labeled" F-1 into F-2 and into PL, separately determined. The cells were saline washed after brief initial incubation with Alb-FA and then reincubated in saline with 1% polyvinylpyrrolidone + glucose. After this reincubation, aliquots were washed with saline and extracted with albumin at the times indicated on the abscissa. The extracts were counted (F-1), and the residual cellular lipids (F-2 and PL) were separated by thin-layer chromatography and then counted. ○, F-1; □, F-2; ●, PL. See Methods, sections E, F, and Results, section A, for further details.

separate conversion of F-1 to F-2 and PL pools is shown. It can be seen that counts in the F-1 pool progressively decrease whereas those in the F-2 pool progressively increase. After approximately 1 hr of incubation, the F-2 pool reaches its maximum activity, and then its activity begins to decrease while that of the phospholipid pool (PL) continually increases.

In order to study "backflow" out of pool F-2 and "forward" flow from F-2 to PL, an experiment establishing a "pulse" label in F-2, then removing the radioactive F-1 fraction and replacing it with unlabeled free fatty acid, was performed as follows. After a brief initial incubation (17 min) with extremely active ³H-labeled Alb-FA, the red blood cells were washed in saline as usual. They were then washed in five washes of defatted albumin to remove F-1. After this, they were reincubated for 8 min at 37°C in donor plasma saved during the original preparation of the red cells. After the reestablishment of a nonradioactive F-1 pool, the plasma was removed, and the cells were

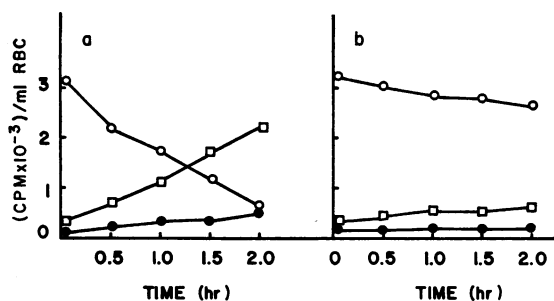


FIGURE 7 *a* and *b* Incorporation of palmitic acid-³H into F-1 and PL pools from "pulse-labeled" F-2 pool. The cells were albumin-washed after a brief initial incubation with highly radioactive palmitic acid-³H albumin. A non-radioactive F-1 pool was reestablished with normal donor serum, and the cells were reincubated for various periods as indicated on the abscissa; aliquots were taken and extracted again with albumin. These extracts (F-1) were counted, and the residual cellular lipids (F-2 and PL) were separated and counted. *a*, the left-hand panel, shows the results of reincubation at 37°C. *b*, the right-hand panel, shows the results of reincubation at 4°C. ○, F-2; ●, F-1; □, PL. See Results, section E, for further details.

again washed once with saline and incubated in buffer. One-half of the cells were incubated in duplicate at 37°C with 8 mM glucose present and one-half were incubated in duplicate at 4°C without glucose. Aliquots were removed at various times, and the cells were washed, extracted, and analyzed as usual. The results of this experiment are seen in Fig. 7 *a* and *b*. At 37°C the fatty acids in F-2 were steadily transferred to PL, and there was also a slow transfer back to F-1. At 4°C these rates were very markedly diminished.

F. Pool size and extent of fatty acid transfer between pools. The size of the various pools and the approximate transfer rates between those pools are indicated in Tables II and III.

TABLE II
Pool Sizes

F-1	0.19 ± 0.05 μmole fatty acid/ml packed cells
F-2	0.16 ± 0.06 μmole fatty acid/ml packed cells
PL*	1.3 ± 0.4 μmoles phosphatide/ml packed cells

All data are averages of six determinations on cells from three separate donors.

* It should be noted that, if expressed as moles total fatty acid, the value would be double. This would imply equal participation of fatty acids at positions 1 and 2 of the glycerol backbone, which at this stage is not warranted.

TABLE III
Transfer Rates

	nmoles fatty acid/ml cells per hr
Alb-FA to F-1*	700 ± 80
F-1 to F-2	42 ± 12
F-2 to F-1	5
F-2 to PL	30 ± 9

All data are averages of six determinations on cells of three separate donors except for F-2 → F-1 which is the average of one pair of determinations on one donor.

* All rates were determined after 1 hr of incubation except for Alb-FA → F-1 which was determined after 5 min of incubation.

DISCUSSION

In red cells, where all modes of cell destruction finally involve alterations in membrane structure (25) and where cell aging appears to be related to a gradual reduction in membrane mass (26-28), the importance of a membrane renewal system is clear.

Efforts to correlate defects of red cell survival or shape (29, 30) with the composition of red cell membranes have been relatively unsuccessful (31). Recently metabolic studies with ³²P designed to uncover possible differences between normal red cells and those with defective membrane function have been reported (32, 33). The further possibility that similar defects may be correlated with anomalies in the renewal of the fatty acids of membrane phosphatides prompted us to these studies. In these experiments with normal cells, a system has been developed in vitro to allow exploration of the dynamics of red cell fatty acid turnover in disease states.

In examining the data in Fig. 3, it appears that the initial stage of free fatty acid incorporation into erythrocyte membrane is a rapid, energy-independent transfer from binding sites on albumin to those on the erythrocyte membrane. This process is complete within a few minutes and apparently is not limiting for the subsequent reactions (Table III). In addition, it appears to be a true transfer of free fatty acid and not the adhesion of albumin-fatty acid complex to the red cell membrane, as shown by the data with labeled albumin-labeled fatty acid (Fig. 4). Goodman (34) compared the affinity of red cell binding

sites for fatty acids with the various fatty acid binding sites of albumin. In his experiments, commercial ^{131}I -labeled albumin was introduced into the system consisting of red cells and fatty acid-loaded albumin. Our reason for reexamining the possibility that the fatty acid-albumin complex per se might be significantly bound to erythrocytes stemmed from the question of possible differences between the labeled albumin used by Goodman and that to which his fatty acids were bound. The present results confirm and extend Goodman's initial observation.

The free fatty acid remaining in albumin-extracted labeled red cells (Table I, B) suggests that there is a second pool of cellular fatty acid that is not accessible to albumin. We operationally describe this as a pool "deeper" in the membrane. It should be noted that while this may be indeed anatomically farther from the cell surface, the data do not prove this point; these fatty acids may simply be bound more avidly than they are in F-1 or on albumin.

The data in Fig. 5 suggest that the transfer of free fatty acid into the F-2 pool proceeds from the F-1 pool and is dependent upon cellular energy. Additionally, a precursor-product relationship is strongly suggested between these two pools although the continual removal of fatty acid from F-2 to PL makes definitive interpretation of a precursor-product relationship difficult. Nevertheless, in "pulse-labeled experiments"⁴ typified by the data in Fig. 6, nearly 90% of the activity in the F-1 fraction appears to pass into or through the F-2 fraction during the time of the experiment.

A similar relationship between fractions F-2 and PL is demonstrated in Fig. 7 *a* and *b*. Here it is also shown that there is a slight backflow from the "deeper" F-2 pool in addition to a much larger forward flow of fatty acid from F-2 into the phospholipid pool. Energy is apparently necessary for flow from F-2 to both F-1 and PL.

When one employs the data on pool sizes and transfer rates in Tables II and III, some approximate calculations of the cost of the transfer process in terms of ATP available from red cell glycolysis can be made. 1 ml of red blood cells utilizes 2 μmoles of glucose per hr (35). This produces 4

⁴ This refers to experiments in which the period of initial labeling was 12 min ($\frac{1}{3}$ of the total incubation time).

μmoles of lactate per hr and 4 μmoles of ATP per hr, assuming that the pathway involving 2,3-diphosphoglycerate is not significantly operative (36). Various authors estimate that 7–30% of this energy is used in maintaining the sodium-potassium ion pump (37, 38). This leaves a minimum net unassigned energy production of approximately 3 μmoles of ATP per ml of cells per hr. The figure we have obtained for the final rate of palmitic acid transfer from F-2 into phospholipid is 30 nmoles/hr. This corresponds to a turnover of the fatty acids of phosphatidylcholine and phosphatidylethanolamine of approximately 2%/hr or 50%/day. One may assume that the acylation of lysolecithin requires 2 moles of $\sim\text{P}$ to activate 1 mole of fatty acid since AMP is produced in the formation of acyl CoA (39). The net cost of the reaction $\text{F-2} \rightarrow \text{PL}$ is a total of 60 nmoles of $\sim\text{P}$ per hr per ml of cells. According to Oliveira and Vaughn (1) and Mulder, de Gier, and Van Deenen (40), palmitic acid which is used in these experiments is approximately six times less active in the acylation of lysolecithin than linoleic acid, but approximately two times more active than stearic and oleic acids. From the distribution of the fatty acids of red cell lecithin (41), it may be calculated that a further factor of approximately 2.5-fold in the over-all rate of this reaction may be invoked. This factor gives an estimated cost of the last stage of phosphatide fatty acid renewal in the mature red cell of 150 nmoles of $\sim\text{P}$ per hr, or approximately 5% of the cellular energy. It should be reiterated that this figure represents only the final stage of incorporation and that energy requirements of antecedent stages of fatty acid transfer (i.e. $\text{F-1} \rightarrow \text{F-2}$) have not yet been evaluated. We have shown that $\text{F-1} \rightarrow \text{F-2}$ is indeed dependent upon metabolism. If the assumption is made that this process operates at about the same energy cost as does $\text{F-2} \rightarrow \text{PL}$, then we may speculate that the over-all requirement for incorporation of plasma fatty acids into membrane lipids is about 10% of the metabolic energy.

We have recently noted a 4-fold increase in the rate of the incorporation of fatty acid into PL in red cell suspensions moderately rich (12%) in reticulocytes. This finding suggests that the process of acylation may account for a very significant amount of metabolic activity in these young and active cells. It may also imply a difference in fatty

acid permeability in the membranes of these cells or a difference in the rate of the F-1 → F-2 reactions.

Our data confirm some of the observations made in the recent work of Donabedian and Karmen (7) in which they studied the relative rates of transfer and incorporation of various fatty acids into red blood cells. These authors also found that the initial transfer of fatty acids to the membrane surface was rapid and reversible. In addition, they found that this first step was faster at lower concentrations of albumin. They observed that the extent of final incorporation into phospholipids of specific fatty acids was a function of the concentration of each of those fatty acids in the total cellular free fatty acid pool (F-1 plus F-2 in our scheme). This observation would fit our proposed precursor-product relationship between the F-2 and PL pools. Additionally, Donabedian and Karmen noted significant incorporation into triglycerides after 3 hr of incubation. Our findings of modest (less than 20%) incorporation after 1 hr are in accord with this observation.

A consideration of how closely the system studied *in vitro* represents the normal situation *in vivo* is pertinent. Tarlov (42), using rat red cells and entirely different methods, has demonstrated the renewal of red cell membrane phospholipids *in vivo*. In Tarlov's experiments half of the renewal occurred through a *passive* exchange of lecithin between plasma and cells. He has also suggested that approximately one-half of the renewal occurs through acylation of lysolecithin. Both he and Elsbach (43) (who used phagocytizing white blood cells *in vitro*) have presented evidence that the acylation process is considerably augmented by the presence of plasma lysolecithin which, being highly membrane-soluble, diffuses freely into the cell and is rapidly converted into lecithin. In one recent experiment we have noted a 2- to 3-fold increase in fatty acid incorporation into PL with sublytic concentrations of lysolecithin. Recent studies (44, 45) suggest that, under the conditions we used, 1 nmole of lysolecithin per mg of albumin (or approximately 2 nmoles of lysolecithin per ml of packed cells) might have been added to our system. We were, however, unable to detect lysolecithin in the cell extracts which we studied by thin-layer chromatography.

The lecithin formed from lysolecithin is in

much slower equilibrium with plasma than the substrate lysolecithin, which exhibits rapid rates of equilibration (42). One could hypothesize that the red blood cell had developed a "trapping mechanism" both for the lecithin backbone and for plasma free fatty acids, by the acylation system described. The advantages of such a trapping mechanism to a cell that lacks the ability to synthesize either free fatty acids or phosphatides *de novo* (46, 47), and that progressively loses membrane lipid as it ages, are clear. In addition to the advantages to the cell of trapping essential building blocks, this series of reactions would appear to serve as a method of limiting the intramembrane concentration of lysolecithin which is dangerous at high levels (48).

Comparison of the F-2 → PL fatty acid incorporation rates which we have found with those given in previous studies is of little value because pool size corrections were not made previously (1, 2). The data of Tarlov (42) are amenable to such comparison, however, since he measured incorporation of preformed lysolecithin labeled in the choline moiety. Such a measurement of incorporation does not depend on knowledge of the size of fatty acid pools antecedent to final acylation. He found a turnover dependent upon acylation of approximately 1%/hr using rat erythrocytes and measuring phosphatidylcholine synthesis alone. Our results of approximately 2%/hr for phosphatidylcholine plus phosphatidylethanolamine in humans with somewhat lower amounts of total red blood cell phospholipid appear to be quite comparable.

In the absence of a known chemical pathway, the energy requirements for the F-1 → F-2 step are beyond precise calculation. It is interesting to consider, however, from the work of Wittels and Hochstein (49) that carnitine fatty acyltransferase or a similar transferase might be operative at this stage. In preliminary experiments, however, we have found no stimulation of incorporation of free fatty acids into phospholipids in the presence of added carnitine.

Although significant phospholipase A activity has not been demonstrated in erythrocytes, one should consider a possible alternative pathway, i.e., one in which fatty acids could be transferred from F-1 to F-2 by a cycle involving sequential acylation and deacylation of the 1-position of

lecithin. Waite and Van Deenen (50) have recently found phospholipase A₁ in liver homogenates. This enzyme hydrolyzes the acyl ester link at the 1-position of lecithin. On subcellular fractionation of the tissue, the enzyme showed a distribution similar to that previously reported for the fragmented plasma membrane (51). Isolated plasma membranes from liver cells incorporate bound unesterified fatty acids into phospholipids more actively than any other cell fraction of this tissue (52). An acylation/deacylation cycle involving the enzyme mentioned above would require the presence of a small quantity of phospholipid which would become labeled even before F-2.

It is possible that the F-1 → F-2 step may normally limit the extent of over-all phosphatide synthesis. This possibility is suggested by the repeated observation (1, 5) that homogenates and ghosts are more active in over-all incorporation of fatty acid than intact cells. One could suppose that the usual membrane limits have been broken and high concentrations of free fatty acid can be directly presented to the final synthetic pathway. In addition, the data of Wittels and Hochstein (53) and of Jacob and Lux (54) suggest that in prelytic states due to either primaquine treatment or vitamin E deficiency, the over-all process of fatty acid incorporation into phosphatide is considerably increased. Again this could be due to an "opening up" of the membrane barrier which might effectively circumvent the F-1 → F-2 step and allow the F-2 → PL step to proceed maximally.

It will be of considerable interest to see if this system of reactions is different in the young red cell and reticulocyte which might well have more active membranes than the population of cells studied here. In this regard it should be recalled that in the isolation of cells a definite bias to older cells was made by removing the top 10% of red blood cells during repeated washes. It will also be interesting to see if perturbations of this series of reactions exist in congenital hemolytic anemias, thalassemias, and other hemolytic disorders in which cell membrane maintenance appears to be abnormal.

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