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METABOLISM OF RED-CELL LIPIDS

I. INCORPORATION *IN VITRO* OF FATTY ACIDS INTO PHOSPHOLIPIDS FROM MATURE ERYTHROCYTES

SUMMARY

Erythrocytes freed from leucocytes and reticulocytes were demonstrated to incorporate fatty acids into their phosphoglycerides. This ability was decreased in the order rat, rabbit, man, ox and sheep. Lysis of the cells caused an increase of the rate of incorporation thereby abolishing the differences between erythrocytes of different species. Addition of coenzyme A and adenosine 5'-triphosphate promoted the fatty acid uptake, particularly in the lysates. In rabbit erythrocytes linoleic, oleic and palmitic acid were incorporated to a greater extent than stearic, myristic and lauric acid. The unsaturated fatty acids were esterified predominantly at the 2-ester position, whereas palmitic acid was directed to the 1-position of lecithin. By contrast to L-α-glycerophosphate, lysolecithin stimulated the incorporation of linoleic acid into lecithin of lysates of rabbit erythrocytes. The possible relation between the fatty acid uptake *in vitro* and the fatty acid renewal of phosphoglycerides from circulating erythrocytes is discussed.

INTRODUCTION

Studies on the lipids of red cells from various animal species have demonstrated that apart from differences in the phospholipid pattern, significant variations also exist in their fatty acid composition[1,2]. Some of these differences were shown to be attributable to variations in the nature of ingested lipids or the participation of ruminal processes[2,3]. Dietary experiments on animals[4] and man[5] have shown that notable quantitative alterations in the fatty acid pattern of red-cell phospholipids can be induced in a period far shorter than the average life span of the erythrocytes concerned. Such a renewal of lipids in circulating erythrocytes may be caused by an exchange of intact lipid molecules with those present in the serum[6], and by an enzymatic esterification of precursors of the red-cell phospholipids. Actually, studies performed by OLIVEIRA AND VAUGHAN[7,8] and in this laboratory[2,4] showed that red cells of various species are capable of incorporating various fatty acids into their phospho-

glycerides *in vitro*. The present paper deals with various aspects of this process, which is believed to be relevant to the dynamic character of phospholipids in many membranes.

MATERIALS AND METHODS

Labelled fatty acids ([1-^{14}C]lauric, [1-^{14}C]myristic, [1-^{14}C]palmitic, [1-^{14}C]stearic, [1-^{14}C]oleic, [1-^{14}C]linoleic, [1-^{14}C]linolenic acid; uniformly ^{14}C-labelled linoleic acid; sodium [1-^{14}C]acetate) were obtained from Radiochemical Centre, Amersham, Great Britain. The radiochemical purity of these compounds was tested by means of gas chromatography with scintillation counting as described below, and was found to be satisfactory, *e.g.*, for [1-^{14}C]linoleic acid, over 98% of the radioactivity was recovered in this acid.

α-Glycerophosphate and ATP were obtained from Fluka A.G.; CoA from Pabst Laboratories. 1-Oleoyl-2-stearoyllecithin was synthesized by Dr. G. H. DE HAAS. Lysolecithin (1-acylglycerol-3-phosphorylcholine) was prepared by treatment of lecithin with phospholipase A (*Crotalus adamanteus* venom (EC 3.1.1.4). and was purified by silicic acid chromatography with mixtures of chloroform and methanol.

Incubation experiments

Erythrocytes were obtained from fresh heparinized blood. The upper layer and buffy coat (containing the leucocytes) were separated from the red cells by centrifugation and cautiously removed. The cells were washed twice with Krebs–Ringer bicarbonate buffer[9] and subsequently subjected to ultracentrifugation[10] for 30 min at 100 000 × g. The upper third of the red-cell column in the centrifuge tube was discarded. The remaining packed cells were resuspended in Ringer solution and spun down again at moderate speed. The concentrated erythrocyte suspension was used as such in the assay mixtures. As verified by various methods[11], preparations of mature erythrocytes obtained by this procedure are entirely free of leucocytes. The radioactive substrates (about 4 μC), after removal of the organic solvent, were homogeneously dispersed by ultrasonication in 0.5 ml of Ringer solution (0.2% glucose) or in plasma, and then mixed with an equal volume of concentrated erythrocyte suspension. In a number of experiments the erythrocytes were disrupted by sonication as well. The addition of different cofactors to the incubation mixture is indicated in the relevant figures and tables. Incubation was carried out for 5 h at 37° under atmospheric conditions, and bacterial contamination was precluded.

Extraction and characterisation of lipids

At the end of the incubation period the cells were separated from the medium by centrifugation and washed twice with Ringer solution. The lipids were extracted according to a modification of the method of FOLCH[12] introduced by BLIGH AND DYER[13]. In the experiments with hemolysates the solvents were added directly to the incubation medium. The combined chloroform layers containing the red-cell lipids were brought to dryness in a rotary evaporator and the residue was taken up in a small amount of a mixture of 50% methanol in chloroform for paper chromatography. Chromatograms were developed on silica-impregnated paper with diisobutylketone–acetic acid–water (40:25:4, v/v)[14] to separate and identify the labeled phospholipids.

The free ^{14}C-labelled fatty acids remaining after the incubation gave rise to trailing of radioactivity on the paper chromatograms, and were therefore removed by

washing the intact erythrocytes before the extraction with isotonic saline containing 6% bovine serum albumin. Alternatively paper chromatograms were developed in chloroform–acetic acid (98: 2, v/v) in advance of the chromatographic separation of the phospholipids in the Marinetti system. Phospholipids were detected by means of the Rhodamine 6G reagent and observation in ultraviolet light. Alternatively a tri-complex staining method[15] was used, which was modified to allow scanning of chromatograms for radioactivity. The paper strips were placed for 1 h in a solution containing (per l) 50 mg Ponceau red and 300 g $Al_2(SO_4)_3$, and the spots were outlined on the wet paper. The radioactivity of the paper chromatograms was scanned continuously with a thin-window Geiger-Müller counter tube coupled to a recorder. For more precise determinations of the radioactivity, the spots were cut out and counted in a liquid scintillation solution according to Bray[16]—containing dioxane, naphthalene, 2,5-diphenyloxazole and 1,4-bis-2-(5-phenyloxazole)-benzene—in a Packard liquid scintillation spectrometer Model 3203. It was not necessary to standardize the position of the paper in the counting vial[17] nor to correct for quenching[18]; the variation in measured activity of standard samples was found to be smaller than 3%.

Neutral lipids were separated on silica thin-layer plates with either hexane–diethylether (70: 30, v/v), or ethylene chloride–methanol (98:2, v/v) or CCl_4 (ref. 19). These systems permitted differentiation between phospholipids, free fatty acids, di- and triglycerides and cholesterol esters. The radioactivity was scanned with a chromatogram scanner modified for the counting of thin-layer plates. The active spots were then transferred into a counting vial and counted in the liquid scintillation counter.

Phospholipase A degradation of phospholipids

For the determination of the site of incorporation of the fatty acids in the phospholipid molecule, use was made of phospholipase A from *C. adamanteus*, which enzyme is known to attack only the β-fatty ester linkage of the phosphoglyceride molecule[20]. The radioactive phospholipid was emulsified by ultrasonic vibrations in 1 ml 0.10 M borate buffer (pH 7.0) containing 0.02 M $CaCl_2$, 1 mg of *C. adamanteus* venom and 2 mg of deoxycholate. After 3 h incubation lecithin and phosphatidylethanolamine appeared to be quantitatively converted in their respective lyso- analogs. The incubation mixture was brought to dryness and extracted repeatedly with methanol and, after chromatographic separation of the products of hydrolysis, the distribution of radioactive fatty acids among the two ester positions of the substrate was determined from the radioactivity present in the lyso- compounds and the diacyl-derivatives.

Gas-chromatographic identification of radioactive fatty acid constituents

After separation of the phospholipids, the lecithin spot was cut out and the fatty acids converted into their methyl esters[1]. All manipulations were performed in a CO_2 or N_2 atmosphere. A standard carrier mixture of fatty acid methyl esters was added and the mixture was separated over a polyethyleneglycol-adipate column, passed through a catharometer for detection and collected in the tubes of a gas-fraction collector filled with anthracene crystals, which were counted in the liquid scintillation counter.

RESULTS

Since feeding of corn oil causes a rapid and significant increase in the linoleic acid content of rabbit erythrocytes[2,4] these studies *in vitro* were primarily concerned with the uptake of this poly-unsaturated fatty acid into the erythrocyte lipids from this animal species. After incubation of intact red cells in plasma containing [14C]-linoleic acid, the labeled fatty acid was found to be incorporated mainly into the phospholipid fraction. Paper-chromatographic separation of the phospholipid species (Fig. 1), demonstrated that the major part of the radioactivity coincided with the
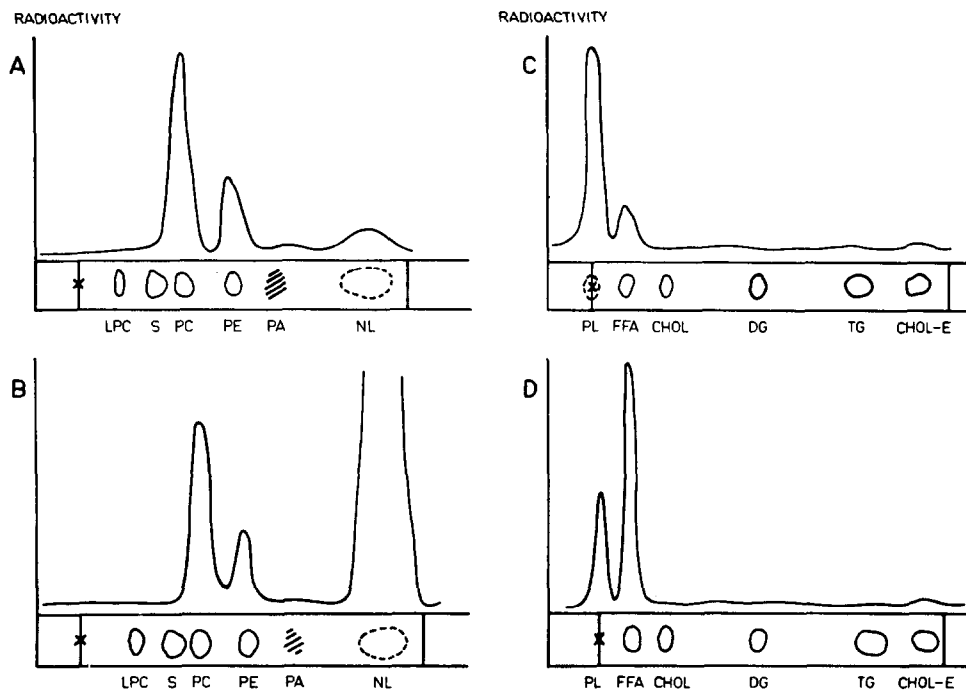

Fig. 1. Distribution of 14C in lipid fractions of rabbit erythrocytes after incubation with [1-14C] linoleic acid. A. Radioactivity of phospholipids after incubation in plasma. B. Radioactivity of phospholipids after incubation in Ringer solution. Distribution of radioactivity on a paper chromatogram of phospholipids extracted from rabbit erythrocytes incubated in plasma or Ringer. Abbreviations: LPC, lysolecithin; S, sphingomyelin; PC, phosphatidylcholine (lecithin) and other choline-containing phospholipids; PE, phosphatidylethanolamine and other ethanolamine-containing phospholipids; PA, phosphatidic acid; NL, neutral lipids. C. Radioactivity of neutral lipids after incubation in plasma. D. Radioactivity of neutral lipids after incubation in Ringer solution. Distribution of radioactivity on a thin-layer chromatogram of lipids extracted from hemolysed rabbit erythrocytes incubated in plasma or Ringer with 0.1 μmole CoA per ml and 5 μmoles ATP per ml. Abbreviations: PL, phospholipids; FFA, free fatty acid (linoleic acid); DG, diglyceride; TG, triglyceride; Chol, cholesterol; Chol-E, cholesterol ester.

lecithin spot, while a considerable activity was also recovered in the area of the cephalins (phosphatidylethanolamine). No significant activity coincided with the spots of phosphatidic acid and sphingomyelin. As regards the neutral lipids, their separation on chromatoplates allowed the conclusion that the major part of the radioactive linoleate in this fraction was present as unesterified fatty acid, while a very faint activity was detectable in the cholesterol esters (Fig. 1).

Controls carried out with plasma and serum alone did not reveal any incorporation of linoleic acid into the phospholipid fraction, thus precluding the possibility that the erythrocyte phospholipids labeled under the given conditions originated from the serum. This was substantiated by the observation that a similar incorporation of linoleate occurred when erythrocytes were incubated in a medium consisting of Ringer solution (Fig. 1). In this case, however, the radioactivity pattern of the chromatogram showed a much higher peak in the region of the unesterified fatty acids. The reason for this difference is probably to be attributed to the absence of serum albumin, which is known to bind fatty acids. The fatty acids adhering to the red cell for the greater part could be removed by adding bovine serum albumin to the incubation medium or by washing the red cells after their incubation in saline containing albumin.

The incorporation *in vitro* of fatty acids into the intact erythrocyte is a rather slow process which continues, however, steadily with time (Fig. 2). In 5 h the uptake
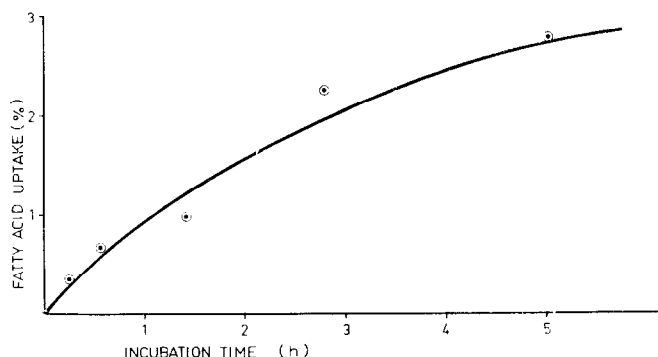

Fig. 2. Time course of fatty acid incorporation in rabbit erythrocytes. Incubation mixtures consisted of 0.5 ml of Ringer solution, 0.5 ml of intact erythrocytes and 4 μC of uniformly[14]C-labelled linoleic acid. The uptake is given in percent of fatty acid present in the medium.

of linoleate from the medium amounted to about 1.5 μg fatty acid per 0.5 ml of rabbit erythrocytes, which corresponds to about 0.6% of the linoleic acid present in the phospholipids from rabbit erythrocytes.

Several factors were found to enhance the incorporation of linoleate into lipids of rabbit erythrocytes (Table I). While addition of CoA and ATP to intact erythrocytes incubated in plasma stimulated incorporation, this effect was more pronounced when the cells were lysed before incubation. When lysed cells were incubated in the presence of ATP and CoA in a Ringer medium the fatty acid incorporation was stimulated as well. In general a somewhat higher uptake of linoleate in the red-cell lipid was observed when plasma was used as incubation medium. A possible explanation for this effect can perhaps be derived from the experiments compiled in Table II. Incubation of a hemolysate of red cells in Ringer solution supplemented with lysolecithin revealed a most significant increase of the incorporation of linoleic acid into the phospholipids. By contrast, addition of α-glycerophosphate had no effect. It is noteworthy that emulsification of the fatty acid together with a synthetic lecithin brought about a decrease of the labeling of the red-cell phospholipids during the incubation in Ringer solution.

TABLE I

INFLUENCE OF CO-FACTORS ON THE INCORPORATION OF [^{14}C]LINOLEIC ACID INTO INTACT AND HEMOLYSED RABBIT ERYTHROCYTES

Incubation mixtures contained 0.5 of plasma or Ringer solution, 0.5 ml of erythrocytes and 4 μC of [1-^{14}C]linoleic acid; 0.1 ml of a solution containing 50 μmoles ATP per ml and 1 μmole CoA per ml was added. Figures represent average of values from 3 or 4 experiments. The range of the observed individual values is given in parentheses.

Incubation conditions	% of fatty acid incorporated into phosphatides
A. Incubation in plasma	
Intact erythrocytes	2.4 (1.3–2.9)
Intact erythrocytes with CoA and ATP	7 (3.8–10.3)
Hemolysed erythrocytes with CoA and ATP	26 (13–47)
B. Incubation in Ringer solution	
Intact erythrocytes	2 (1–3)
Hemolysed erythrocytes	2 (1–3)
Hemolysed erythrocytes with CoA and ATP	17 (14–21)

TABLE II

INFLUENCE OF DIFFERENT SUBSTRATES ON FATTY ACID INCORPORATION IN RABBIT ERYTHROCYTE HEMOLYSATES

Incubation mixtures contained 0.5 ml of Ringer solution, 0.5 ml of hemolysed erythrocytes and 4 μC of [1-^{14}C]linoleic acid. The figures represent average values from 2–4 experiments. The range of the observed individual values is given in parentheses. Expts. 1–4 involved a 5-h incubation, while the incubation period in Expt. 5 was 20 h.

Expt. No.	Substrate added	Cofactors	% fatty acid incorporated into lecithin
1	No additions	50 μmoles ATP, 1 μmole CoA	17 (14–21)
2	1 mg of lysolecithin	50 μmoles ATP, 1 μmole CoA	40 (19–48)
3	1 mg of α-glycerophosphate	50 μmoles ATP, 1 μmole CoA	15 (10–21)
4	1 mg of γ-oleoyl-β-stearoyl-lecithin	50 μmoles ATP, 1 μmole CoA	5 (3–8)
5	1 mg of lysolecithin	250 μmoles ATP, 5 μmoles CoA	98 (92–99)

These results indicated that 1-acylglycero-3-phosphorylcholine may be intimately involved in the esterification process of linoleate.

The amount of added [^{14}C]linoleic acid which was incorporated into the red-cell phospholipids could be further elevated by increasing the amount of ATP and CoA. Thus, after 20 h of incubation of a hemolysate of rabbit red cells, under sterile conditions in Ringer solution containing appropriate amounts of lysolecithin, ATP and CoA, about 98% of the [^{14}C]linoleic acid added to the medium was recovered in the lecithin, and these experimental conditions have frequently been utilized in this laboratory to isolate a correspondingly labeled lecithin of high specific activity.

The incorporation of various other fatty acids into lecithin of rabbit erythrocytes was also studied under different conditions (Table III). None of the acids studied surpassed the extent of uptake of linoleic acid. The most active were oleic acid and palmitic acid, whereas stearic acid was less active. The short-chain saturated fatty acids lauric and myristic acid were also found to be incorporated to a lesser extent under the conditions of these experiments. Furthermore the position occupied by some of the fatty acids incorporated into the phosphoglyceride molecule was investigated (Table IV).

TABLE III

INCORPORATION OF ^{14}C-LABELLED FATTY ACIDS INTO THE LECITHIN FRACTION OF RABBIT-
ERYTHROCYTE MEMBRANES

The uptake of fatty acid in each series is given relative to that for linoleic acid. (Absolute figures
for linoleic acid can be derived from Tables I and II.) Incubation mixtures contained 0.5 ml
of erythrocytes, 0.5 ml of Ringer solution or plasma, 4 μC of [1-^{14}C]labelled fatty acid and
where mentioned 0.1 ml of a solution containing 50 μmoles of ATP per ml and 1 μmole of CoA per
ml. The figures are the average of two separate experiments. The individual measurements
varied up to 25% from the mean value.

Fatty acid added	Intact erythrocytes		Plasma with CoA and ATP	Hemolysed erythrocytes
	Ringer solution	Plasma		Plasma with CoA and ATP
Lauric acid	5	10	5	5
Myristic acid	10	10	-- --	. .
Palmitic acid	45	45	70	90
Stearic acid	10	20	20	10
Oleic acid	50	35	80	70
Linoleic acid	100	100	100	100
Linolenic	- -	25	. . .	---

TABLE IV

DISTRIBUTION OF LABELLED FATTY ACIDS IN LECITHIN SYNTHESIZED BY RABBIT ERYTHROCYTES

Radioactive lipids obtained during the incubation with one of the labeled fatty acids indicated
in Table III were hydrolysed with snake venom. The pattern of distribution of the fatty acids
between the 1- and 2-position was not affected by the differences in incubation procedure used in
these experiments (Table III). The figures for palmitic and linoleic acid represent average values
from 8 experiments; for oleic and myristic acid, from 4 experiments. The range of the individual
values is given in parentheses.

Radioactive fatty acid	% of the incorporated fatty acid located at the 2-position
Linoleic acid	92 (81–97)
Oleic acid	84 (80–88)
Palmitic acid	9 (1–22)
Myristic acid	68 (51–90)

By virtue of the positionally specific site of action of phospholipase A (C. ada-
manteus) it can be concluded that linoleic and oleic acid were incorporated predomi-
nantly at the 2-ester position of lecithin from rabbit erythrocytes. By contrast, pal-
mitic acid appeared to be directed mainly to the 1-ester position, while myristic acid
was distributed over both ester positions.

In a number of experiments, a small part of the radioactivity of the crude
lipid mixture from the erythrocytes incubated with uniformly-labeled linoleic acid
was found not to be extractable by chloroform but to remain in the water–methanol
phase. A search was made for the presence of short-chain degradation products of
fatty acids utilising the methods described by LYNEN et al.[21]. After treatment of this
fraction with KOH, chromatograms developed with ethanol–ammonia–water (20 : 1 : 4,
v/v) revealed several radioactive spots indicating the presence of small quantities of
acetate and other short-chain products. Although this finding suggested that incu-
bation of linoleic acid with mature erythrocytes gives rise to a limited breakdown of
the fatty acids, the production of ^{14}CO$_2$ measured over a period of 5 h was only 1%
of the fatty acids added to the incubation medium.

In confirmation of previous reports[22,23] erythrocyte preparations used in the present study did not incorporate acetate into the lipid fractions. It was therefore likely that the ^{14}C-labeled fatty acids were incorporated into the red-cell lipids without any previous conversion into other long-chain homologues. This view was supported by the results of the gas-chromatographic analysis of the labeled fatty acid constituents of the red-cell phospholipids. As demonstrated in Fig. 3, after incu-
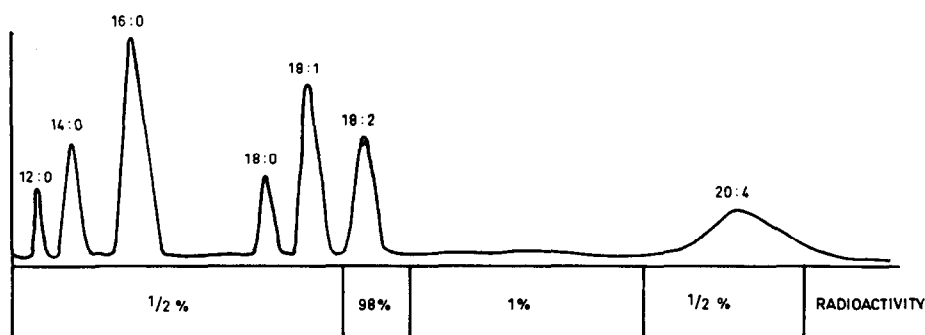

Fig. 3. Gas-chromatographic identification of the radioactive fatty acid incorporated in rabbit-erythrocyte lecithin. The methyl esters of fatty acids hydrolysed from radioactive lecithin—obtained after incubation of erythrocytes with [1-^{14}C]linoleic acid—were separated by gas chromatography after dilution with a carrier mixture of fatty acid methyl esters. Abbreviations: 12:0, lauric acid; 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid.

bation of rabbit erythrocytes with [^{14}C]linoleic acid, over 98% of the radioactivity was recovered in the corresponding fatty acid methyl ester fraction. Experiments carried out with erythrocytes from man and rat, known to contain a high level of arachidonate[24], gave similar results. Our observations support the conclusion of LEUPOLD AND KREMER[25] that a conversion of linoleate into arachidonate by blood cells is not to be attributed to the mature erythrocytes.

Since the erythrocytes of various animal species differ greatly with respect to the phospholipid distribution and composition of fatty acid constituents[2], comparative experiments on the incorporation of fatty acid into erythrocytes of some mammals have been made.

The uptake of linoleic acid was found to be of the same magnitude in erythrocytes from rabbit and rat, while human erythrocytes appeared to be somewhat less active. However, the capability of intact erythrocytes to incorporate fatty acid into the phospholipids was most significantly decreased for sheep and ox (Table V). It may be recalled that the erythrocytes from both ruminants have a remarkable low lecithin content, this being compensated for by a high level of sphingomyelin. In addition the phospholipids of red cells from these ruminants contain a very low content of poly-unsaturated fatty acids, but it has been demonstrated recently by DE GIER AND VAN DEENEN[3], that a high level of linoleic acid can be induced in sheep erythrocytes by avoiding the action of the rumen. Although the extent of incorporation in vitro of linoleic acid into the lipids of erythrocytes from sheep and ox was decreased compared with rabbit, about the same distribution pattern of the radioactive fatty acids was observed. After incubation of intact ox erythrocytes, the linoleic acid incor-

TABLE V

INCORPORATION OF [^{14}C]LINOLEIC ACID INTO PHOSPHOLIPIDS OF ERYTHROCYTES OF DIFFERENT ANIMAL SPECIES

Incubation mixtures contained 0.5 ml of plasma, 0.5 ml of intact or hemolysed erythrocytes and 4 μC of [1-^{14}C]linoleic acid. To the hemolysates was added 0.5 ml of a solution containing 50 μmoles ATP per ml and 1 μmole CoA per ml. The figures represent averages from at least 3 experiments. The range of the observed individual values is given in parentheses.

Erythrocytes obtained from	% of fatty acid incorporated into phospholipids
Intact cells	
Rat	2.8 (2.0–3.1)
Rabbit	3.0 (2.4–3.9)
Human	1.9 (1.8–2.3)
Ox	0.7 (0.5 0.8)
Sheep	0.4 (0.3 0.4)
Hemolysates	
Rabbit	26 (13–47)
Ox	31 (11–50)

porated was about equally distributed among lecithin and phosphatidylethanolamine (Fig. 4). When compared with rabbit erythrocytes a relatively higher incorporation into the ethanolamine-containing phosphoglycerides of the ox erythrocytes occurred, but in the latter case the specific activity is much higher for lecithin. The sphin-
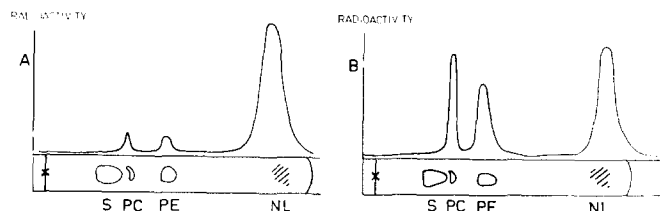

Fig. 4. Distribution of ^{14}C in lipid fractions of ox erythrocytes after incubation with [1-^{14}C]linoleic acid. A. Radioactivity of phospholipids after incubation of intact erythrocytes. B. Radioactivity of phospholipids after incubation of erythrocyte hemolysates with ATP and CoA. Abbreviations: S, sphingomyelin; PC, phosphatidylcholine (lecithin) and other choline-containing phospholipids; PE, phosphatidylethanolamine and other ethanolamine-containing phospholipids; NL, neutral lipids.

gomyelin content of ox erythrocytes is much higher than that of red cells from the non-ruminants studied, but in both cases no incorporation of fatty acid into this sphingolipid was observed. Apparently the differences in the velocity of uptake of fatty acids from the medium by erythrocytes from different species are only partly or indirectly related to the well-established differences in phospholipid composition between these cells. This interpretation was supported by the fact that red-cell hemolysates from ox and rabbit, when incubated with linoleic acid and ATP and CoA, in contrast to intact erythrocytes, did not give any significant differences in the amount and distribution of the linoleic acid incorporated (Table V).

DISCUSSION

In confirmation of our previous investigations and the work of OLIVEIRA AND VAUGHAN[7,8] on erythrocyte ghosts, the present study supports the view that mature

erythrocytes are capable of incorporating fatty acids into the phosphoglycerides. Erythrocytes of various animal species appear to exhibit this feature, but intact red cells of ox and sheep are less active *in vitro* than those of rat, rabbit and man. Various observations suggest that this distribution is related only indirectly to the known differences in phospholipid composition between the ruminants and non-ruminants studied. On the other hand these erythrocytes have been demonstrated to differ in permeability behaviour[26]; in binding of phospholipids[27] and susceptibility to phospholipase A (ref. 28), thus indicating that certain differences in the fine structure of the erythrocyte membrane may exist between the species concerned. Therefore it is not unlikely that the differences in the rate of fatty acid incorporation are brought about by a different penetrability of these red-cell membranes, either for the fatty acid derivative or another reaction partner. Actually with lysed cells in the presence of ATP and CoA the distinction between the uptake of linoleic acid by the phosphoglycerides from rabbit and ox erythrocytes was abolished. In general the rate of fatty acid incorporation was greatly increased in hemolysates, as compared with intact cells. CoA and ATP had a marked stimulating effect on the incorporation of fatty acids, particularly when the experiments were carried out with hemolysates. Accordingly, OLIVEIRA AND VAUGHAN[7,8] and ROBERTSON AND LANDS[29] demonstrated that in human red-cell ghost the fatty acid uptake is much smaller though not completely eliminated in the absence of CoA, and also dependent on the presence of ATP.

Significant differences were observed in the rate of esterification between various fatty acids. As regards rabbit erythrocytes, a dominant activity was observed for linoleic acid, but oleic and palmitic acid also revealed a much better incorporation than stearic, myristic and lauric acid. Furthermore the fatty acids turned out to be directed to the specific ester sites frequently demonstrated to be reserved primarily for saturated and (poly)unsaturated fatty acids[30]. In rabbit erythrocytes, linoleic and oleic acid were esterified at the 2-position of lecithin, whereas palmitic acid was preferentially attached to the 1-ester position. OLIVEIRA AND VAUGHAN[8], however, reported that in human red-cell ghost, palmitic acid was incorporated like linoleic acid mainly at the 2-position. It will be of interest to classify the factors responsible for this difference between the experiments of both laboratories carried out under somewhat different conditions.

The question can be raised whether the fatty acid incorporation occurring during incubation of red cells or ghosts *in vitro*, represents a process which is also involved in the fatty acid renewal of the membranous phosphoglycerides *in vivo*. In this context it is interesting to note that in rabbit erythrocytes there is some degree of parallelism between the rate of incorporation *in vitro* and the occurrence of the different fatty acids in the phosphoglycerides from this cell membrane. Also suggestive are some coincidences between the results of the studies *in vitro* and dietary-induced alterations in fatty acid composition of circulating red cells. So far, the most significant effects were observed *in vivo* in those fatty acid constituents which in the present investigation also revealed the highest rate of incorporation into the red-cell phosphoglycerides. Administration of a regimen high in linoleate to rabbit[4] and lamb[3] for a period too short to give a maximal contribution of newly formed erythrocyte membranes, gave a striking augmentation of the linoleic acid level in the red-cell membranes, often mainly at the expense of the oleic acid content. The short-term dependence of linoleic and oleic acid content of red cells on the nature of ingested

lipids was observed by FARGUHAR AND AHRENS[5] and by THOMASSON *et al.*[31] to apply to the human erythrocyte as well. Attempting to relate the *in vivo* and *in vitro* phenomena it is of primary interest to make a comparison of the extent of uptake of fatty acids by the erythrocytes under both conditions. Although a red-cell hemolysate when supplemented with various cofactors very actively incorporates the added lino-leate into lecithin, the esterification by intact erythrocytes incubated in plasma with-out added ATP and CoA is a rather slow process. On the other hand, the alteration of the linoleic acid content of erythrocytes by dietary means is also a time-consuming process. Under given experimental conditions an augmentation of 15–20% in the linoleic acid level in rabbit erythrocytes was obtained after a dietary period of 10–14 days[2]. In man and sheep[31,3] such alterations were reached after about 4–6 weeks. It is worth noting that *in vitro* also the incorporation of linoleic acid into erythrocytes from rabbit exceeds that of erythrocytes from man and sheep. Though circumstantial evidence strongly suggests that the incorporation of fatty acids into red-cell phos-pholipids as revealed by studies *in vitro* may play an important role in the circulating erythrocytes, this does not imply that this process is solely responsible for the obser-vations made *in vivo*. Although at present a fair quantitative comparison between the incorporation *in vitro* of fatty acids, being highly dependent on experimental con-ditions and the events *in vivo*, cannot be made, our tentative calculations indicate that the extent of this capability is probably not sufficient to account for the dietary-induced alterations in fatty acid composition. The observations of REED[6] on the exchange of phospholipid molecules between erythrocytes and serum, which have been confirmed in this laboratory, make it very likely that, in addition to the enzy-matically-regulated fatty acid incorporation, other processes have a part in the rene-wal of red-cell phospholipids.

As regards the mechanism of the fatty acid esterification into erythrocyte phos-phoglycerides *in vitro*, the observed positionally specific location of saturated and unsaturated long-chain constituents already suggests a relationship with the path-ways studied by LANDS *et al.*[32,33]. These investigations showed that the re-acylation of 1-acyl and 2-acyl lyso- derivatives by microsomal enzymes preferentially involves the utilisation of (poly-)unsaturated and saturated fatty acids respectively. This process probably accounts for the well-known dissimilar distribution of fatty acid constituents in natural lecithins[30]. In this context it is of interest that LLOVERAS *et al.*[34] and VAN DEN BOSCH AND VAN DEENEN[35] reported that in rat tissues both isomeric lysolecithins can be produced by an enzymatic hydrolysis of the diacyl analogue. The observed positionally specific esterification of saturated and unsatur-ated fatty acids into lecithin of rabbit erythrocytes *in vitro*, may theoretically be brought about, therefore, by an enzymatic acylation of the corresponding lyso-compounds with fatty acid CoA, a view recently also supported by OLIVEIRA AND VAUGHAN[8] and ROBERTSON AND LANDS[29].

In agreement with this explanation is the observation that L-α-glycerophos-phate, known to be the major fatty acid acceptor for the synthesis of phosphatides and glycerides in many tissues[36] and also in leucocytes[37], did not enhance the fatty acid incorporation in red blood-cell lysates, whereas lysolecithin greatly promoted this process. Accordingly, the ability of red-cell ghosts to acylate lysophosphogly-cerides, to be dealt with in detail in an accompanying paper, supports this concept. However, at this stage it would be premature to conclude that this conversion is

exclusively responsible for the fatty acid incorporation into phospholipids from erythrocytes. Further exploration of the various problems left may be very rewarding, since the red cell with its limited metabolic capacities may render fruitful information about the dynamic events concerning lipids from animal membranes in general.

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