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Translocation of oleic acid across the erythrocyte membrane. Evidence for a fast process

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To clarify divergent views concerning the mechanism of fatty acid translocation across biomembranes this issue was now investigated in human erythrocytes. Translocation rates of exogenously inserted radioactive oleic acid across the membrane of native cells were derived from the time-dependent increase of the fraction of radioactivity becoming non-extractable by albumin. No accumulation of non-extractable unesterified oleic acid occurred. The rate of transfer was markedly suppressed by SH-reagents and by ATP-depletion. The suppression, however, resulted from a mere decrease of incorporation of oleic acid into phospholipids and was not accompanied by an increase of non-extractable unesterified oleic acid. These findings were reconcilable with the concept of a slow, possibly carrier-mediated fatty acid transfer as well as a very fast presumably, diffusional process not resolvable by the albumin extraction procedure. This ambiguity was resolved by using resealed ghosts, which are unable to incorporate oleic acid into phospholipids. In such ghosts all of the oleic acid inserted into the membrane remains extractable by albumin even after prolonged incubation. On the other hand, ghosts containing albumin accumulated non-extractable oleic acid. The rate of accumulation was beyond the time resolution of the albumin extraction procedure at 4°C. Oleic acid uptake into albumin-containing ghosts became kinetically resolvable when the fatty acid was added as a complex with albumin. Correspondingly, time-resolvable release of oleic acid, originally complexed to internal albumin, into an albumin-containing medium was demonstrated at 4°C. Rate and extent of these redistributions of oleic acid were dependent on the concentrations of internal and external albumin. This indicates limitation by the dissociation of oleic acid from albumin and not its translocation across the membrane. Translocation of oleic acid, which is probably a simple diffusive flip-flop process, must therefore occur with a half-time of less than 15 s. These findings raise doubts on the physiological role of presently discussed concepts of a carrier-mediated translocation of fatty acids across plasma membranes.

Introduction

The mechanism of translocation of long chain fatty acids across biological membranes is not unambiguously clear. Simple diffusion and carrier-mediated processes have alternatively been proposed in myocytes [1–5], adipocytes [6–8] and hepatocytes [9–14]. This ambiguity may result in part from the necessity to introduce fatty acid into the systems to be analysed as a complex with albumin in order to obtain appropriate, physiologic extracellular levels of the fatty acids. In early studies on Ehrlich ascites tumor cells [15,16] and erythrocytes [17] it was postulated that unesterified fatty acids are first adsorbed to the membrane, then transported into the cell by an unspecific diffusion-like process and subsequently incorporated into phospholipids.

In an alternative concept translocation of fatty acid was supposed to involve a membrane receptor for albumin which enhances the dissociation of fatty acids from albumin. The observed saturation kinetics of the uptake of fatty acids was taken as evidence for a carrier-mediated transport [18,19]. Alternatively, saturation was proposed to result from non-linear changes of the membrane-bound fraction of unesterified fatty acid as a function of the molar fatty acid/albumin ratio [9,20].

Abbreviations: PCMBS, *p*-chloromercuriphenylsulfonic acid; NEM, *N*-ethylmaleimide; diamide, diazenedicarboxylic acid-*N*,*N*′-dimethylamide; DIDS, 2,2′-diisothiocyanatostilbene-4,4′-disulfonic acid; FABP, fatty acid binding protein; FITC, fluorescein isothiocyanate.

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As a final alternative, a saturating transfer system could also be pretended by subsequent metabolic processes [1,2]. Besides saturation a further piece of evidence for a protein-mediated fatty acid uptake stems from its inhibition by typical transport inhibitors such as phloretin and DIDS as well as by proteases [4,6–8,11–14,21]. Moreover, uptake of fatty acids has been reported to be stimulated by inward sodium gradients and by membrane potential [14,60]. Finally, the identification of fatty acid binding proteins (FABP) at the membrane of various cell types was taken as evidence for the existence of a transport protein for fatty acids [4,6–8,12,21,22]. A mediation of fatty acid uptake by cells via such proteins is supported by the inhibition of fatty acid uptake by a pretreatment of cells with a specific antibody against the FABP [4,8,12,21]. However, all the FABP analyzed in more details as yet proved to be extrinsic proteins [23,]. Moreover, the FABP in adipocytes, hepatocytes and cardiomyocytes with molecular weights between 33 and 85 kDa are heterogeneous [4,6–8,12,21,22] and are mechanistically different.

Transport rates of fatty acids derived from studies on biomembranes are low compared to rates derived on artificial lipid membranes which suggest half times for fatty acid translocation across the lipid bilayer of a few minutes or even less [9,24–27].

The present study aimed to clarify the obvious discrepancy between the interpretation of results obtained on artificial and on biological membranes. To this end a simple cell like the human erythrocyte was needed, since this cell does not have intracellular membranes and fatty acid metabolism is limited to the incorporation of fatty acids into phospholipids. Fatty acid uptake by whole cells was investigated using the time-dependent decrease of extractability of cell-bound fatty acids by albumin after primary insertion into the outer membrane leaflet. This procedure has successfully been used to measure the transbilayer translocation of lysophospholipids [28].

In an alternative approach that avoids complications by metabolic conversion of fatty acids translocation of fatty acids across the membrane was analyzed by following the equilibration of fatty acids between albumin in the medium and albumin trapped within resealed ghosts. It will be demonstrated that the 'true' translocation of fatty acid across the erythrocyte membrane is faster than the dissociation of fatty acids from albumin and occurs at a half-time of less than 15 s at 4° C.

Materials and Methods

Materials

Fresh human blood anticoagulated with heparin or citrate was obtained from the local blood bank and used within 5 days. Erythrocytes were isolated by centrifugation, the buffy coat removed and the cells washed three times with 154 mmol · l^{-1} NaCl.

[^{14}C]- and [^{3}H]Oleic acid (spec. act. 56.75 mCi/mmol and 4.6 Ci/mmol, respectively) were obtained from Amersham Buchler (Braunschweig); PCMBS (*p*-chloromercuriphenylsulfonic acid sodium salt), wheat germ agglutinin, polylysine, FITC-albumin, ionophore A23187 and bovine serum albumin (essentially fatty-acid-free) were from Sigma (Munich); iodoacetate and inosine were purchased from Merck AG (Darmstadt); diamide (diazenedicarboxylic acid-*N*,*N*′-dimethylamide) from Calbiochem. (Frankfurt); NEM (*N*-ethylmaleimide) from Fluka (Neu-Ulm); phloretin from Carl Roth (Karlsruhe); DIDS (2,2′-diisothiocyanatostilbene-4,4′-disulfonate) was a kind gift of Professor K.F. Schnell, Regensburg).

The medium used for incubations contained (mmol · l^{-1}): KCl (90), NaCl (45), Na_2HPO_4/NaH_2PO_4 (12.5) and sucrose (44) (= medium A). The pH was 7.4 unless otherwise indicated.

Methods

Transfer of fatty acids. Variable amounts of a stock solution of ^{3}H- or ^{14}C-labelled oleic acid in ethanol mixed with non-labelled oleic acid were evaporated in a 1.5 ml vial under a stream of N_2 and 200 µl of a suspension of washed erythrocytes in isotonic saline (hematocrit 50%) were added. The sample was shaken at 22° C for 4 min to allow for incorporation of oleic acid into the membrane. Subsequently, 800 µl of medium A were added and the suspension transferred to a new snap cap vial. After centrifugation the cells were resuspended in 900 µl of medium A and incubated at 37° C. In order to analyze transfer of oleic acid from the outer to the inner membrane surface aliquots of this suspension were removed after various time intervals and the cells extracted with albumin (230 µmol · l^{-1}, three extractions, 2 min at 4° C) as described before for lysophospholipids [28].

Lipid analysis. After extraction of lipids from 50–200 µl samples of erythrocytes [29], lipids were separated by thin-layer chromatography on polygram P/SilG/UV (Macherey-Nagel, Düren, Art.No. 805022) using chloroform/ethanol/water/triethylamine (30 : 34 : 6 : 35, v/v) [30]. The radioactivity of the various lipid spots was quantified with a thin-layer chromatographic scanner (TLC-linear analyzer LB 282/LB 283; Berthold, Wildbad).

ATP-depletion. Three methods were used to deplete cells of ATP. One, an erythrocyte suspension in medium A (10% hct) was treated with iodoacetate (5 mmol · l^{-1}, 15 min, 37° C). After washing, the cells were incubated with either inosine (10 mmol · l^{-1}) or glucose (10 mmol · l^{-1}) for 45–90 min at 37° C [31]. Two, an erythrocyte suspension was incubated for 6–12 h at 37° C in medium A containing 2-deoxy-D-glucose (5–15 mmol · l^{-1} [32]).

Three, an erythrocyte suspension was incubated for 4 h at 37°C in medium A containing Hepes instead of phosphate and ionophore A23187 (5–10 $\mu mol \cdot l^{-1}$). After centrifugation cells were washed with medium A containing 1% albumin [33].

ATP-depleted cells were washed three times with medium A and 1 ml of packed erythrocytes was extracted with 2 ml of 1.2 M $HClO_4$ at 0°C. After centrifugation and neutralization of the supernatant with KOH [34], the ATP content was quantified by HPLC according to Ref. 35.

Treatments with SH-reagents. Washed erythrocytes were suspended in medium A (hematocrit 10%) and treated with iodoacetate (5 $mmol \cdot l^{-1}$, 15 min, 37°C). After three washings with medium A, cells were resuspended in medium A containing diamide (0.5–10 $mmol \cdot l^{-1}$) for 15–90 min at 37°C. Alternatively, cells were suspended in medium A and treated either with *N*-ethylmaleimide (0.1–5 $mmol \cdot l^{-1}$) for 5–30 min or with PCMBS (0.1–0.5 $mmol \cdot l^{-1}$) for 5–60 min at pH 8.0 and 37°C. The treatments were terminated by three washings of the cells with medium A.

Preparation of resealed ghosts. Erythrocytes were hemolysed according to the method of Bodemann and Passow [36]. To this end, 1 vol. of an erythrocyte suspension in isotonic KCl (hematocrit 50%) was mixed at 4°C for 15 min with 20 vols. of a solution containing 4 $mmol \cdot l^{-1}$ $MgSO_4$ and 3.5 $mmol \cdot l^{-1}$ acetic acid. The pH of the lysate was 6. After centrifugation of the lysate (10 min, 15000 × *g*) the supernatant was removed and portions of the sedimented unsealed ghosts were mixed (4°C, 1 h) with equal volumes of a solution containing 15 $mm \cdot l^{-1}$ $MgSO_4$ and variable concentrations of albumin. The pH during albumin trapping was 6.0–6.5. Isotonicity was reconstituted by addition of 0.2 vol. of a solution of 700 $mmol \cdot l^{-1}$ KCl and 10 $mmol \cdot l^{-1}$ Tris-HCl to 1 vol. of the ghost suspension. The suspension was shaken for 10 min (4°C, pH 7.4). Subsequently, ghosts were resealed by incubation at 37°C for 10 min. Resealed ghosts were sedimented by centrifugation (10 min, 4°C, 11000 × *g*) and washed 3 times with medium A.

Quantification of trapped albumin. The albumin concentration of the resealed ghosts was measured fluorimetrically using FITC-albumin, which was added to the non-labeled albumin during trapping. The concentration of trapped albumin was calculated per volume of packed ghosts after correction for the volume between the ghosts.

Oleic acid release from resealed ghosts. Before the resealing step ^{14}C-labelled oleic acid (20 nmol/ml ghosts) or labelled oleic acid complexed to albumin (15–740 $\mu mol \cdot l^{-1}$) were added to the ghost suspension in 15 $mmol \cdot l^{-1}$ $MgSO_4$ (see above). The molar ratio of fatty acid to albumin was constant, 1 : 20.

After resealing, 1 vol. of packed ghosts were suspended in 9 vols. of medium A containing albumin (9–230 $\mu mol \cdot l^{-1}$). The release of labelled oleic acid was quantified by two procedures.

(a) After increasing time periods 20 µl samples were mixed with 0.5 ml of medium A and centrifuged (5 min, 13000 × *g*, 4°C). The radioactivity in the packed ghosts was measured by liquid scintillation counting and related to the total radioactivity in the sample, which is equivalent to the radioactivity in the packed ghosts at $t = 0$.

(b) At 15 sec intervals 100 µl of the ghost suspension were mixed with polylysine (2 mg/ml of packed ghosts) or wheat germ agglutinin (1 mg/ml) in order to accelerate sedimentation, and were centrifuged for 15 s. The radioactivity in 10 µl supernatant was related to the total radioactivity of 10 µl ghost suspension.

Oleic acid uptake into resealed ghosts. 1 vol. of packed resealed ghosts with or without internal albumin was suspended in 1 vol. of medium A containing [^{14}C]oleic acid (0.5 nmol/ml ghosts) and varying amounts of non-labelled oleic acid complexed to albumin (15–150 $\mu mol \cdot l^{-1}$). The molar ratio of oleic acid to albumin was always 1 : 20. This suspension was incubated at 4°C under gentle agitation. At 15-s intervals samples were centrifuged and the radioactivity of supernatant related to the total radioactivity of ghost suspension.

Results

Transfer of oleic acid in intact erythrocytes

The present study aimed at a quantitative analysis of the kinetics of the process of fatty acid translocation from the outer to the inner layer of the erythrocyte membrane. Therefore oleic acid was inserted into the membrane in the absence of albumin. In this way essentially all of the added radioactivity (> 97%) was contained in the cells before starting transfer measurements. The transfer of oleic acid, which is defined here as the sum of the translocation of oleic acid across the membrane and its incorporation into phospholipids, was analyzed by measuring the time-dependent decrease of the fraction of the fatty acid extractable by albumin. This approach should be applicable to measure the rate of oleic acid transfer across the membrane provided that the rate of translocation between the two layers is sufficiently low relative to the rate of extraction of oleic acid from the outer membrane layer. Absolute amounts of fatty acid transferred were calculated from the non-extractable radioactivity and the specific activity of [^{14}C]oleic acid. Results obtained by this strategy are shown in Fig. 1. After a fast increase of the non-extractable fraction within the first minute (0.2 and 1.7 nmol of the 10 and 60 nmol/ml cells originally inserted; Fig. 1 inset) the non-extractable fraction increases more slowly with time without an indication of saturation even after incubation times up to 6 h. Even after this

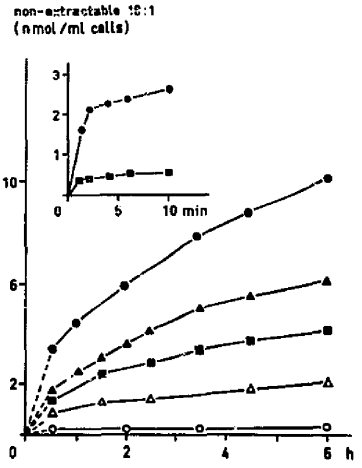

Fig. 1. Time-dependent increase of radioactivity non-extractable by albumin following insertion of 0.5 (○), 5 (△), 10 (■), 20 (▲) and 60 (●) nmol of [^{14}C]oleic acid (18:1) per ml of packed cells into the membrane. From the non-extractable radioactivity and the specific radioactivity of [^{14}C]oleic acid amounts of oleic acid per ml of packed cells were calculated. The inset shows the fast increase of non-extractable oleic acid during the initial time period.

extended period only 16–30% of the label originally inserted into the membrane has become non-extractable.

In erythrocytes fatty acids are not oxidatively degraded but will become incorporated into phospholipids following their activation by the ATP-dependent formation of acyl-CoA. These processes occur at the inner membrane surface [37]. An accumulation of unesterified fatty acid in the inner membrane layer will therefore only be detectable when the translocation is faster than the subsequent metabolic processes but too slow to allow reorientation during the extraction by albumin (see above). However, such an accumulation could not be demonstrated in the albumin extracted cells. Radiochromatograms of membrane lipid extracts prepared from intact cells either immediately after insertion (5 min, 22° C), or following incubations for up to 6 h with oleic acid and subsequent extraction with albumin, only contained label in the phospholipid fraction (Fig. 2). The essential lack of labelled, unesterified oleic acid in albumin-treated cells is not in line with results of others [38,39]. The discrepancy is probably due to differences in the lipid extraction procedure. In our study the lipid extracts were routinely prepared from intact, non-lysed cells. When cells were lysed before lipid extraction variable amounts of labelled unesterified fatty acids were in fact detectable.

Unesterified fatty acids as well as their hydroxamates were also found when lipids were extracted in the presence of hydroxylamine [40]. This procedure was elaborated for the detection of acyl-CoA as the apolar hydroxamate. In these procedures the occurrence of free fatty acids in the lipid extracts may be due to the hydrolysis of ^{14}C-labelled acyl-CoA during hemolysis of the cells or during derivatization of acyl-CoA by hydroxylamine, but could also result from significant hydrolysis of labelled phospholipids in the presence of hydroxylamine (data to be published elsewhere). In view of these considerations we do not think that significant amounts of labelled unesterified oleic acid in fact remain in the inner membrane layer after albumin extraction.

Under this presupposition amounts of transferred oleic acid were calculated from the amount of non-extractable radioactivity and the specific activity of [^{14}C]oleic acid. From these amounts rates of transfer were determined for time intervals between 0.5 and 1.5 h, and between 1.5 and 2.5 h of incubation. The rates obtained for each of these time intervals were plotted against the respective concentration differences across the membrane. The resulting graph indicates a biphasic flux-gradient relationship (Fig. 3). Rates calculated for the initial period (10 min) are 20–50-fold higher than those for the later period. The observed nonlinear concentration dependence could arise from the contribution of several enzymes and substrates to the incorpo-

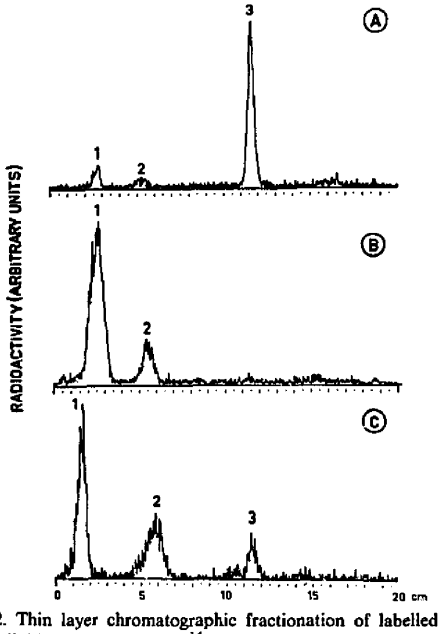

Fig. 2. Thin layer chromatographic fractionation of labelled membrane lipids extracted from [^{14}C]oleic acid incubated erythrocytes. Erythrocytes were incubated for 1 h (A, B) or 3 h (C) with [^{14}C]oleic acid and washed with medium in the absence (A) or presence of albumin (B, C) to remove oleic acid. After extraction lipids were separated by thin-layer chromatography and detected by radio-scanning. 1, Phosphatidylcholine; 2, phosphatidylethanolamine; 3, unesterified oleic acid.

ration of oleic acid into phospholipids. Alternatively it might reflect the characteristics of a transport system. In an attempt to distinguish between these two interpretations, the following studies were carried out.

Incorporation of fatty acids into phospholipids can be blocked by SH-reagents [41] and by ATP-depletion, which prevents formation of acyl-CoA. In case of a selective inhibition of acylation of fatty acids the metabolism of fatty acids might become slower than the preceeding translocation step. This should result in an accumulation of unesterified fatty acids in the inner membrane layer detectable by their inextractability by albumin. According to Fig. 4 pretreatment of erythrocytes with the alkylating SH-reagent NEM produces a marked concentration-dependent inhibition of oleic acid transfer. The same was observed for the SH-oxidizing agent diamide while the impermeable SH-reagent PCMBS had no effect (data not shown). ATP depletion of the cells also resulted in a gradual inhibition when the level was reduced below 10% of the normal value. At 7% of the original ATP level transfer was inhibited by at least 80%. The impermeable amino reagent DIDS and phloretin, both of which have been claimed to inhibit fatty acid uptake in other cell types [4,6–8,12–14,21], had no or only slight inhibitory effects (data not shown).

Despite this marked inhibition of transfer no significant increase of the absolute amount of unesterified oleic acid remaining in the cells after albumin extraction was observed (Fig. 4). Either the translocation rate is too fast to be measurable by the albumin extraction technique or translocation is slow and the inhibition of acylation is paralleled by an inhibition of translocation. In order to distinguish between these two possibilities, experiments were carried out on metabolically inactive resealed ghosts. We measured the time-dependent transfer of oleic acid from extracellular albumin to internally trapped albumin and the reverse process. This transfer comprises the dissociation of the fatty acid from the 'donor' albumin, its translocation through the membrane, and the association with the 'acceptor' albumin. If the rate of the dissociation of oleic acid from albumin and the rate of its association to albumin are faster than the rate of translocation of oleic acid, the latter rate should be measurable directly.

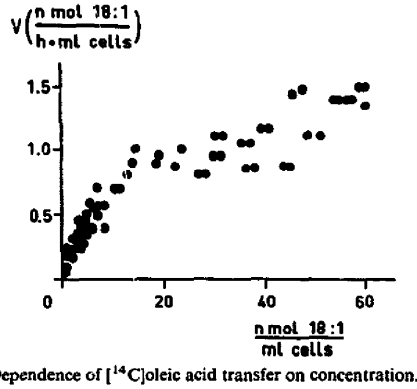

Fig. 3. Dependence of [^{14}C]oleic acid transfer on concentration. From the time-dependent increase of the non-extractable amounts of oleic acid (Fig. 1) transfer rates (V) were calculated for the intervals between 0.5 and 1.5 h as well as between 1.5 and 2.5 h. The rates were then plotted against the corresponding concentration in the outer membrane layer.

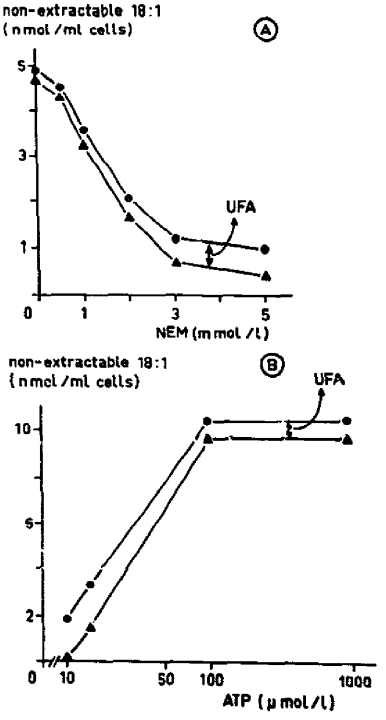

Fig. 4. Effects of *N*-ethylmaleimide (A) and of ATP-depletion (B) on the transfer of [^{14}C]oleic acid and its incorporation into phospholipids. Erythrocytes were either treated with 0.5–5 mmol·l^{-1} *N*-ethylmaleimide (pH 8.0, 15 min) or incubated by one of the three methods for ATP-depletion described in the Methods. Subsequently, cells were loaded with [^{14}C]oleic acid (12–20 nmol/ml SH-modified cells or 55–60 nmol/ml ATP-depleted cells) and were incubated at 37°C for 4.5 h followed by albumin extraction. The non-extractable amounts of total radioactive lipid (●) and of radioactive phospholipids (▲) are plotted against the NEM and ATP concentration. The small difference between the two curves represents unesterified fatty acid (UFA).

Translocation of oleic acid in resealed ghosts

To study oleic acid translocation, resealed ghosts containing albumin at different concentrations were exposed to external [^{14}C]oleic acid in the absence of external albumin. The amount of oleic acid not extractable by albumin was determined as described for whole cells. As expected, all of the inserted oleic acid is

TABLE I

Relative amounts of ^{14}C-labelled oleic acid remaining in resealed ghosts after albumin extraction

Albumin-free and albumin-containing ghosts were loaded from both sides (line 1) or from the outside (line 2–4) with [^{14}C]oleic acid (25 nmol/ml of packed ghosts). After washing with medium A resealed ghosts were incubated (hematocrit 10%, pH 7.4, 4°C) for 2 min and aliquots extracted 1–3 times with albumin. The percentages of oleic acid remaining bound after albumin extractions are related to fatty acid contents of ghosts washed in the absence of albumin. Data represent mean values from 3 to 10 experiments ± S.D.

Internal albumin concentration (μmol·l^{-1})	% remaining bound after albumin extractions, number of extractions		
	1	2	3
0	5	–	–
0	5 ±2.5	–	–
310	52	25.9±3.5	–
770	73.2±8.4	54.5±8.0	43.5±4.5

retained in the resealed ghosts after washing with saline medium A irrespective of the albumin content of the ghosts. On the other hand, oleic acid inserted from the outside or from both sides into the membrane of albumin-free ghosts is nearly completely removed from the ghosts by a single albumin extraction, not only immediately (Table I) but also after prolonged incubations in buffer (data not shown). This observation is reconcilable with two interpretations. Either, the absence of any translocation of oleic acid from the outer to the inner leaf of the bilayer or a very fast translocation leading to complete extraction of fatty from both layers of the membrane. On the other hand, when albumin-containing ghosts are exposed to [^{14}C]oleic acid, a considerable fraction of the inserted radioactive material is not extractable by a single albumin extraction (230 μmol·l^{-1}, 4°C, 2 min) even at the earliest possible measurements (Table I). This non-extractable oleic acid fraction does not increase when the ghosts are incubated in albumin free buffer for up to 6 h (data not shown).

Since according to Table I oleic acid bound to albumin-free ghosts remains fully extractable, its loss of extractability in albumin-containing ghosts must reflect its binding to the internal albumin. To get there, oleic acid has to be translocated rapidly across the membrane and has to become associated to internal albumin. In line with this notion the amount of oleic acid retained in albumin containing ghosts increases with the albumin concentration inside the ghosts, since albumin acts as a sink for oleic acid. On the other hand, the fraction retained decreases with increasing number of albumin extractions (Table I) confirming that oleic acid can leave the ghosts again. In this case oleic acid dissociates from internal albumin, migrates across the membrane and associates to external albumin.

These results demonstrate that information on the rate of oleic acid translocation across the membrane may in principle be obtained from transfer measurements on resealed ghosts. Consequently kinetics of fatty acid transfer between albumin trapped within resealed ghosts and albumin in the medium were analyzed in more detail.

Release studies

Attempts to follow the time course of oleic acid transfer from albumin-free resealed ghosts to external albumin were unsuccessful. In these experiments the labelled oleic acid was initially inserted into both (inner and outer) layers of the membrane by exposing open ghosts to oleic acid in the absence of albumin prior to resealing * followed by suspension of the resealed ghosts in albumin-containing medium. Already in the earliest sample, taken after 45 s, all of the oleic acid originally inserted into the ghosts was found in the extracellular medium (Fig. 5A (□)). When oleic acid was introduced into the ghosts as a complex with albumin (Fig. 5A (●), (○), (△)) the release of oleic acid became time-resolvable, proceeded more slowly and reached an equilibrium at higher residual levels. Interestingly, the release did not seem to follow a single exponential as would be expected for the equilibration of oleic acid between two compartments. We therefore evaluated the data according to a computerized curve fitting method [61,62] to determine the best fit to a sum of exponentials (plus a constant), i.e.

$$(c/c_0)t = A_1 \cdot e^{-k_1 \cdot t} + A_2 \cdot e^{-k_2 \cdot t} + \ldots + c$$

The programm used was made available by the Department of Medical Statistics, RWTH Aachen.

The best fit was obtained for a sum of two exponentials. The fractional sizes of the components (A_1, A_2), rate coefficients (k_1, k_2), and corresponding half-times are given in Table II for the different albumin concentrations. As becomes evident, the parameters for the fast component decrease with increasing size of the donor pool (albumin-oleic acid complex) while those of the slow component increase. The changes are much more pronounced for the pre-exponential terms, reflecting compartment sizes, than for the rates of release. After equilibration of oleic acid between internal and external albumin the molar ratios of oleic acid to al-

* To verify that oleic acid can in fact be inserted into the inner membrane layer of open ghosts a corresponding experiment was carried out using lysophosphatidylcholine as a probe. A considerable amount of the slowly translocating lysophosphatidylcholine was not accessible to extraction by external albumin after resealing of the albumin-free ghosts. This non-extractable lysophosphatidylcholine fraction must have been incorporated into the inner membrane layer.

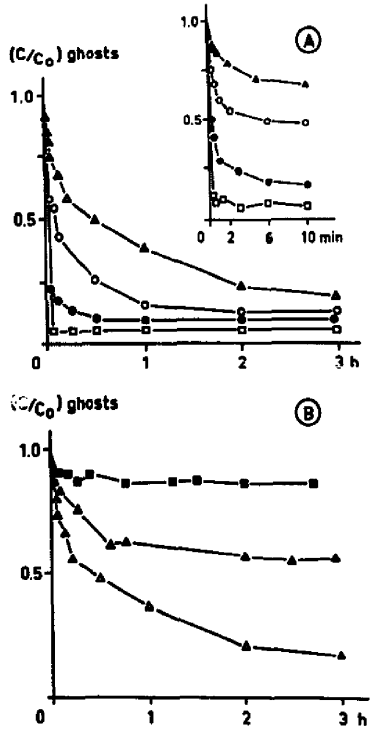

Fig. 5. Dependence of the release of oleic acid from resealed ghosts on the internal (A) and external (B) albumin concentrations. (A) Resealed ghosts prepared to contain [^{14}C]oleic acid and: 0 (□), 20 (●), 190 (○) and 470 (△) μmol·l^{-1} albumin (see Methods) were incubated in 220 μmol·l^{-1} albumin in medium A (hematocrit 7%, 4° C) and the time-dependent release of oleic acid from the ghosts determined. Data are given as fractional oleic acid content relative to the initial content in the ghost (C_0). Inset: Time course for the first 10 min. (B) [^{14}C]Oleic acid/albumin complex was trapped at a fixed albumin concentration of 470 μmol·l^{-1} within resealed ghosts and the time-dependent release of oleic acid into medium A containing 9 (■), 15 (▲) and 220 (△) μmol·l^{-1} albumin measured. The molar oleic acid/albumin ratio was 1:20 in all experiments. The curves are drawn by eye, for a precise evaluation see Table II.

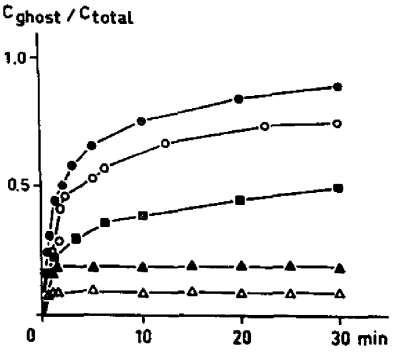

Fig. 6. Dependence of the oleic acid uptake by resealed ghosts on the external and internal albumin concentrations. Resealed ghosts free of albumin (△), (▲) and resealed ghosts containing 190 (○) and 470 (●), (■) μmol·l^{-1} albumin were incubated in media containing 15 (●), (○), (▲) and 150 (△), (■) μmol·l^{-1} albumin complexed with oleic acid. The molar oleic acid/albumin ratio was 1:20 in all experiments. The time-dependent uptake of oleic acid by the ghosts was calculated from the disappearance of the radioactivity from the medium. Data are given as fraction of radioactivity in the ghosts (C_{ghosts}) relative to that of total oleic acid present in the ghost suspension (C_{total}). The curves are drawn by eye, for a precise evaluation see Table II.

bumin inside and outside the ghosts should be equal. This proved to be the case (data not shown).

In the experiments described so far the internal albumin concentration was varied at constant external albumin concentration. Variation of the external albumin concentration at constant internal albumin concentration provided the same kinetic pattern of equilibration and of distribution of oleic acid between inside and outside the ghosts (Fig. 5B).

Uptake studies

In order to demonstrate the symmetry of fatty acid transfer in our ghost/albumin system the uptake of oleic acid was also measured, external albumin serving as the donor and internal albumin as the acceptor. Fig. 6 shows the same dependence of the kinetics of oleic

TABLE II

Rate constants (k_1, k_2) corresponding half-times ($t_{1/2}$), and fractional sizes of the components (A_1, A_2) for the biphasic release (Fig. 5) and uptake (Fig. 6) of oleic acid

See text for the evaluation procedure. Lines 1–3 release data, lines 4–6 uptake data.

Albumin		A_1	k_1	$t_{1/2}$	A_2	k_2	$t_{1/2}$
external (μmol/l)	internal (μmol/l)		(min^{-1})	(min)		(min^{-1})	(min)
220	20	0.89	1.28	0.50	0.11	0.014	50
220	190	0.51	1.11	0.63	0.49	0.022	32
220	470	0.28	0.68	1.02	0.72	0.026	27
15	470	0.53	0.86	0.81	0.47	0.099	7.0
15	190	0.50	0.83	0.83	0.50	0.101	6.9
150	470	0.36	1.21	0.57	0.64	0.035	19.8

acid uptake on the ratio between internal and external albumin concentration already demonstrated for the release measurements. When evaluated by the nonlinear least-square fitting procedure, the kinetics of uptake can again be described by two components (Table II). The half-times for the fast component of uptake (0.6–0.8 min) are not very different from those for the fast component of release (0.5–1.0 min). The half-times of the slow component of uptake, on the other hand, are considerable shorter than those for release. This is particularly true for the very low external albumin concentration (15 μmol/l). Adsorption of albumin to the ghosts may play a complicating role under these conditions. As in case of the release, the fractional size of the fast component decreases with increasing size of the donor pool.

Effect of temperature

The time courses of oleic acid release and uptake could only be resolved at 4°C. At higher temperatures the equilibration of oleic acid between internal and external albumin was too fast to be time-resolvable with our method (data not shown).

Effect of SH-reagents

As shown above, oleic acid uptake into intact, metabolizing erythrocytes is strongly inhibited by SH reagents. This is not the case for oleic acid release from albumin-containing released ghost treated with *N*-ethylmaleimide prior to lysis (data not shown). This insensitivity of the oleic acid equilibration kinetics in ghosts to SH reagents demonstrates that the inhibition observed in intact cells is the result of an inhibition of reactions following the translocation step.

Discussion

Membrane translocation of oleic acid is a fast process

The experiments performed on intact erythrocytes did not provide evidence that the slow incorporation of exogenously added oleic acid into phospholipids, which occurs at the inner membrane surface [37], is limited by a correspondingly slow translocation of oleic acid across the membrane. As a major point of evidence against this view no accumulation of free fatty acid could be demonstrated in cells in which acylation was prevented by ATP depletion or by SH blockage. Such an accumulation would have been expected if the translocation of oleic acid occurred at a rate comparable to or slower than that of the acylation reaction. Unless one assumes that ATP depletion and SH-reagents inactivate a putative carrier protein for the fatty acid, the only reasonable interpretation for this result consists in the assumption, that the translocation of fatty acid is a process too fast to be measurable by the albumin extraction procedure. Studies on resealed ghosts proved this interpretation to be true.

In the ghost system a very high rate of translocation of oleic acid could be demonstrated by measuring the equilibration of oleic acid between albumin trapped within ghosts and albumin in the medium. The kinetics of this equilibration are probably not limited by the translocation across the membrane. This follows from the following arguments:

(1) Accumulation of oleic acid can be time-resolved in albumin containing resealed ghosts but not in albumin-free ghosts, which have only membrane binding sites. Moreover, oleic acid inserted into both layers of albumin-free ghosts prior to resealing can be removed completely and instantaneously (in less than 15 s at 4°C!) by a single extraction with albumin (Table I, Fig. 5). Transbilayer reorientation must therefore have a half-time < 15 s.

(2) The rates of oleic acid equilibration vary with the ratio of the albumin concentrations inside and outside the ghosts. Observations of this type might be taken to indicate that membrane translocation of oleic acid limits the rate of its redistribution. The argument would be that under the conditions of a fixed oleic acid/albumin ratio the transmembrane gradient of unbound oleic acid varies linearly with the transmembrane albumin gradient to which it is linked by the albumin concentrations and the thermodynamic equilibrium constant of the complex (K_{eq}). The rates of equilibration should then vary in the same proportion according to the basic laws of diffusion.

While this is true in principle the transbilayer redistribution rate of oleic acid should also vary with the albumin gradient when either the dissociation of the fatty acid from the 'donor' albumin or its association to the 'acceptor' albumin is rate-limiting. Since it is well established (see, for example, Ref. 43) that the dissociation is much slower than the association, the former step would be more likely to be rate-limiting. The argument goes as follows. If the dissociation rate of oleic acid is slower than its transmembrane reorientation rate, the transmembrane gradient for the unbound fatty acid will be lower than that predicted by the albumin concentration gradient and K_{eq} down to the limiting case where the system behaves as if there were no membrane barrier. Even under these limiting conditions, to be expected at high rates of transmembrane reorientation, the rate of redistribution of fatty acid between the two pools of albumin will still be a function of the pool sizes since these determine the steady-state concentration of unbound fatty acid. This follows from the steady-state condition

$$FA_{unbound} = k_{off} \cdot C_{FA/ALB} - k_{on} \cdot FA_{unbound} \cdot C_{ALB}$$

(with k_{off} the first-order rate constant of dissociation of

the fatty acid albumin complex, k_{on} the second-order rate constant for association, and $C_{FA/ALB}$ and C_{ALB} the concentrations of the donor and the acceptor pool, respectively). It can also be demonstrated experimentally by using suitable probes, as will be reported elsewhere (Federwisch, M. and Bröring, K., unpublished data).

The question of the rate-limiting step can thus not be answered by arguments involving the influence of the albumin gradients. It may therefore be helpful to compare our measured rate coefficients with the rate coefficients of dissociation for the fatty acid-albumin complex reported in the literature.

Among the available data [20,42–44] we have chosen those obtained at temperatures closest to our experimental temperature of 4°C (Scheider [43] 1°C, Svenson et al. [44] 9°C), and extrapolated them to 4°C using the activation energy of 14.3 kcal/mol [43] and 15 kcal/mol [44]. The comparison of the dissociation rate constant obtained in this way (0.25 and 0.37 min^{-1}, respectively) with our data is complicated by the biphasic kinetics of uptake and release of fatty acids in our ghost/albumin system. The literature data refer to fatty acid/albumin ratios higher than those in our experiments (0.05 : 1). However, in preliminary experiments we have already demonstrated similar biphasic transfer kinetics and comparable rate constants at a fatty acid/albumin ratio of 1 : 1. The extrapolated literature data for the dissociation rate constants of fatty acid-albumin complexes range between the high k_1 values in the early phase of oleic acid uptake and release by the ghosts, and the lower k_2 values (Table II). Regardless of this ambiguity it can be concluded that the rate constant of translocation must be higher than the highest measured k_1 values (1.1–1.3 min^{-1}). This means that oleic acid transfer across the erythrocyte membrane must be a process occurring at 4°C with a half time of less than 30 s. The basis of the biphasic kinetics is presently unclear.

A fast translocation of oleic acid is in line with indirect evidence obtained on phospholipid vesicles. Fatty acid transfer across such pure lipid membranes has been claimed to be a process occurring with half-times in the range of minutes or less [9,24,27]. Such fast translocation is theoretically well-founded in spite of the negative charge of the anion, since it has been shown that the pK_a value of the carboxylgroup is increased in a hydrophobic environment relative to that in water [17,45–51]. This results in an incomplete dissociation of the carboxyl group in the membrane at physiological pH. The uncharged species will have high translocation rates comparable to those of short-chain fatty acids, cholesterol, nonionic detergents, diacylglycerols and bile acids [52–56]. Moreover, even evidence for a fast translocation of the anionic form of long-chain fatty acids was recently presented [57].

The problem of carrier-mediated fatty acid transfer

The finding of a very rapid translocation of a long-chain fatty acid across the erythrocyte membrane raises critical questions as to the physiological relevance of transmembrane carriers for such fatty acids claimed to exist in a number of cells [3–8,10–14,21]. An involvement of proteins in the translocation of fatty acids across biomembranes has been postulated, based on the following observations.

(1) Saturation kinetics, sodium dependence and electrogenicity of fatty acid uptake [3,5–7,13,14,22,23,60]. These findings may indeed indicate carrier mediation. The complexity of the conditions, however, under which they are obtained, leaves room for alternative interpretations. Saturation phenomena have been shown to occur in the process of partitioning of fatty acids between albumin and membranes under certain conditions [19,20]. Sodium and membrane potential dependence may reflect changes of the metabolic integrity of the cell rather than involvement of energy-linked cotransport. Quite generally, secondary metabolic reactions are sometimes hard to separate from the primary process of translocation. Our observations and earlier findings [38] of a very slow SH- and ATP-dependent increase of the fatty acid fraction non-extractable by albumin in whole erythrocytes may be interpreted as a typical feature of enzyme reactions providing for the incorporation of translocated fatty acid into phospholipids at the inner membrane surface. The concept that the time-dependent increase of the non-extractable fatty acid is not limited by translocation but by acylation is in line with a positive correlation between the sequence of transfer rates of oleic acid in erythrocytes of various species (data not shown) and the order of their acylation capacities [58].

(2) Inhibition of fatty acid uptake by protein-modifiers and transport inhibitors [4,6–8,11–14,21]. In the case of inhibition of fatty acid uptake by permeable SH-reagents and by phloretin [4,7,8,12–14,21] the effects could be explained by effects of these treatments on fatty acid metabolism. The inhibition of fatty acid uptake by the impermeable amino reagent DIDS [6,7] and by trypsin [13], however, may be considered as good evidence for an involvement of a carrier in translocation.

(3) Identification of fatty acid binding proteins (FABP) in cell membranes [4,6–8,12]. The identification of FABP in membranes is not questioned. However, their role in the translocation of fatty acids across the membrane has not been demonstrated. Experiments on the extractability of FABP from membranes indicate that they are extrinsic proteins [23]. According to current ideas on membrane transport mechanisms such proteins are no candidates for carriers.

The role of intracellular FABP's

Besides the complications arising from fatty acid

metabolism and from their binding to membrane bound FABP, effects of intracellular FABP on fatty acid uptake have to be considered. The uptake of fatty acids into cells with intracellular binding sites for fatty acids on proteins is comparable to the albumin-ghost system used in the present study. In either case binding proteins act as intracellular acceptors for fatty acids. In our albumin-ghost system a very fast translocation ($t_{1/2} < 15$ s at 4°C) of oleic acid across the membrane was derived from its fast equilibration between internal and external albumin. A similar fast uptake of exogenously added oleic acid may be expected in FABP containing cells. Indications that intracellular FABP indeed influences the uptake kinetics as measured by the albumin extraction procedure can be found in the literature. Several authors [6,13] postulate that the initial (≤ 30 s) rate of fatty acid uptake from albumin/fatty acid complexes by hepatocytes, cardiomyocytes and adipocytes is limited by the translocation rate of the fatty acid across the membrane, since during this initial period fatty acids accumulate in a pool not extractable with albumin in spite of the absence of metabolic conversion of the fatty acid. Moreover, following such a short period of fatty acid uptake a slow release of the initially cell-bound fatty acid could be demonstrated upon addition of extracellular albumin [4,6–8,13,14]. In our study such a release of fatty acids from ghosts containing fatty acids bound to albumin was time resolvable, whereas removal of fatty acids from the cell membrane was not. The internal albumin concentrations required to obtain a fast uptake of oleic acid into ghosts from albumin containing media correspond to FABP concentrations in heart intestine and liver tissue (200–400 $\mu mol \cdot l^{-1}$, Ref. 59).

Comparative considerations on fatty acid uptake

On the basis of our results it can be speculated that in cells other than erythrocytes, too, fatty acids will equilibrate rapidly between extracellular albumin and intracellular FABP. It seems possible that saturation kinetics of the initial rate of uptake of fatty acids by adipocytes, hepatocytes and cardiomyocytes do not represent the properties of a transport protein, but are the result of simple diffusive equilibration of fatty acids between extracellular albumin and intracellular FABP. Since membranes of tissue cells do certainly not differ from the erythrocyte membrane and artificial phospholipid membranes in their basic bilayer properties they should also be highly permeable to fatty acids. Such cells should be able to meet their fatty acid demands by simple diffusion. Indeed, the v_{max} values for the (initial) fatty acid uptake into myocytes (at 37°C) or adipocytes (at 23°C) are comparable, when related to unit surface area, to fatty acid influx into ghosts at 4°C when calculated for the physiological oleic acid/albumin ratio of 1:1 from our measured rate constants (myocyte $1.5 \cdot 10^{-19}$, adipocyte $5 \cdot 10^{-19}$, ghost $1.2 \cdot 10^{-19}$ $mol/\mu m^2$ per min). Even allowing only for a moderate activation energy the values for the ghost membrane will at least be quite similar to those in other cells at identical temperatures.

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