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EFFECT OF ALBUMIN, LOW TEMPERATURE AND METABOLIC INHIBITORS ON TRANSPORT OF FATTY ACIDS INTO CULTURED HUMAN LEUKEMIC MYELOID CELLS

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Radioactively-labelled palmitic acid was used to study the effects of albumin, low temperature and several inhibitors of metabolism on transport of fatty acids into cultured human leukemic myeloid cells. When serum or albumin were present in the medium, uptake of fatty acid by cells as well as its further incorporation into phospholipids and neutral lipids were considerably reduced. Uptake and metabolic utilization of this fatty acid was reduced at low temperature, in the presence or absence of albumin in the incubation medium. In absence of albumin, addition of iodoacetate, sodium cyanide or sodium azide had but little effect on the total uptake of fatty acids while metabolic utilization was reduced. When albumin was present, these inhibitors reduced both total uptake and incorporation into lipids. The data suggest that incorporation of the fatty acid into the outer layer of the cell membrane is controlled by the concentration of free, uncomplexed molecules of fatty acid adjacent to the cell surface. In the absence of albumin this is a fast reaction which reaches nearly maximal uptake in three minutes. In the presence of albumin, this process is much slower and follows a nearly linear course between 3 and 60 minutes. Translocation into the inner layer of the membrane and subsequent utilization for metabolic processes is a much slower process, which seems to depend on the quantity of the fatty acid in the outer layer.

Introduction

Mammalian tissues utilize long chain fatty acids either as a source of energy (especially for heart and skeletal muscle), or for synthesis of membrane lipids; for this, free fatty acids are transported in blood as a complex with serum albumin. Many investigators have attempted to clarify the mode of transport of free fatty acids across the plasma membranes of organs or cultured cells and recently into liposomal dispersion of lipids. It has been suggested that free fatty acids incorporate into the outer leaflet of the bilayered membrane possibly by a passive process which is probably stabilized by binding between the methylene residues of the fatty acid and hydrophobic components of the membrane lipids [1–3]. Alternatively, this incorporation might be mediated by attachment of the fatty acid to a 'binding site' on the outer surface of the cell [4]. This binding site could be specific for the free fatty acid [5] or the albumin moiety of the complex of this protein with the fatty acid [6]. The mechanism of translocation from the outer to the inner leaflet of the membrane received most attention but is still essen-

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tially unresolved. The existence of a passive translocation ('flip-flop') has not been observed in cells [7] or liposomes [8]. 'Active', energy-dependent processes have therefore been sought [9,10]. Experimentally, it is difficult to distinguish between free fatty acids that are adsorbed onto the outer cell surface and those which are located within the membrane or inside the cell [11]. Also, should a carrier-mediated process exists [12], it is not clear whether fatty acids are first absorbed into and diffused within the outer layer and then bound to a carrier or whether the fatty acids in the medium are directly attached to the carrier. Translocation from the outer layer of the membrane has been related to formation of an acyl-coenzyme A intermediate in the membrane [12,13] or liposome [8], or to metabolic utilization of the fatty acid by membranous enzymes [14]. The presence of a specific receptor for fatty acids in bacterial cells has also been proposed [12].

In other studies, we tested the transport of several synthetic derivatives of fatty acids into cultured HL 60 myeloid cells [15], neuroblastoma and neuroglioma cells [16] or skin fibroblasts (Morand and Gatt, unpublished data). The derivatives could be classified into three groups: (A) those fatty acids which were transported across the cell membrane and incorporated into cellular lipids; (B) fatty acids which were taken up but not incorporated into neutral lipids or phospholipids; and (C) fatty acids which were neither transported at all across the cell membrane, nor associated with the cell membrane.

The present work was undertaken to study the effect of albumin and inhibitors of glycolysis or respiration on the transport and metabolic utilization of a natural fatty acid aiming to establish if the rate of association and subsequent translocation of the fatty acid across the cell membrane is an energy-dependent process and is related to its metabolic utilization.

Experimental procedures

Cells and culture conditions

The cultured cell line (HL 60), originally established from the peripheral blood of a patient with acute promyelocytic leukemia [17], retains many characteristics of the normal granulocytic progenitor cells (myeloblasts and promyelocytes). The cells were subcultured every 3–4 days at a density of $2.5 \cdot 10^5$ cells per ml of Alpha minimal essential medium (Gibco, Grand Island, NY) supplemented with 20% of fetal calf serum (heat inactivated for 30 min at 56°C, Gibco, Grand Island, NY), and incubated at 37°C in an incubator with humidified atmosphere of 5% CO_2 in air.

Incubation of cells with [1-^{14}C]palmitic acid

[1-^{14}C]Palmitic acid (50 mCi/mmol, New England Nuclear, Boston, MA) was diluted with non-radioactive palmitic acid to a final specific radioactivity of 1 mCi/mmol, dissolved in dimethylsulfoxide (DMSO) to a concentration of 20 nmol/μl and mixed with the medium of incubation. Various incubation media were used: medium free of serum or albumin, medium supplemented with fetal calf serum or with fatty acid-free albumin (Bovine, fraction V, Sigma, St Louis, MO). The medium containing the labelled fatty acid was preincubated at 37°C for 30 min and then mixed with a prewashed cell suspension and further incubated. The DMSO, whose final concentration did not exceed 0.25%, had no adverse effect on cell viability as determined by the multiplication potential of the cells and the Trypan blue exclusion test. Incubations were performed either at 37°C or 4°C with slow shaking and stopped by placing the tubes in an ice-water bath. Cells were sedimented by centrifugation, washed once with medium-10% fetal calf serum and twice with phosphate-buffered saline.

Lipid extraction and analysis

Lipids were extracted from washed cells with 2 ml chloroform/methanol (1 : 1; v/v) at 60°C for 10 min. After centrifugation, the pellet was discarded, 1 ml chloroform and 0.75 ml water were added to the supernatant to attain the solvent ratio of the procedure of Folch et al. [18]. After separating the phases, the lower phase was evaporated to dryness under nitrogen. Half of the lipid extract was used for counting radioactivity. The other half was chromatographed on a column of alumina (Aluminum oxide, Brockmann standardized, Merck) using a procedure adapted from Long and Staples [19]. Small columns, containing 500 mg of alumina in Pasteur pipets, were washed

with methanol and then with chloroform. Lipid extracts (in chloroform/methanol, 95 : 5, v/v) were applied to the alumina column, neutral lipids were first eluted with 2 ml of chloroform/methanol (95 : 5, v/v) and then phospholipids with 2 ml of chloroform/methanol (1 : 1, v/v). Radioactivity of each fraction (total lipids, neutral lipids and phospholipids) obtained after incubation of cells with [1-^{14}C]palmitic acid was measured in scintillator liquid (Insta-Gel II, Packard). Lipids were dried before dissolution in the scintillator liquid to avoid quenching by solvents. Absolute desintegrations and amounts of [1-^{14}C]palmitic acid taken up and incorporated into neutral lipids and phospholipids were calculated using standard curves. The part of the radioactivity of the total lipid extract which was retained on the alumina represented the free fatty acid pool. All values are expressed as nmol per one million cells and each is an average of at least two experiments: S.E. varied within a range of 10% of each value.

Results

Effect of albumin and serum on uptake and metabolic utilization of [1-^{14}C]palmitic acid by HL 60 cells

HL 60 cells were incubated in the presence of [1-^{14}C]palmitic acid in various media of incubation (Table I). The presence of serum or albumin inhibited considerably the uptake of palmitic acid by cells. Thus, when cells were incubated in medium free of serum or albumin, the total content of palmitic acid, free or lipid-bound, reached a value of 18 nmol/10^6 cells in 3 min and increased little after 60 min. When cells were incubated in the presence of serum or albumin, the total uptake was only about 0.3 nmol/10^6 cells after 3 min and increased up to 2.7 and 2.0 nmol/10^6 cells after 60 min, respectively. This diminution of the total uptake was also reflected in lesser incorporation into neutral lipids and phospholipids, which was reduced by 40–80% in the presence of serum or albumin.

Fig. 1A shows the effect of incubation time on the uptake of palmitic acid, in the absence or presence of albumin. Without albumin, the uptake was very rapid during the first few minutes of incubation and then the total content of cell-associated radioactivity did not increase with time, the percent of uptake was about 18%. In the presence of 75 μM and 300 μM albumin, a rapid uptake occurred within the first minutes when the total content of [1-^{14}C]palmitic acid reached only 0.65 and 0.15 nmol/10^6 cells, respectively. This was followed by a nearly linear rate of uptake of about 2.6 and 1.6 nmol/10^6 cells per h (for about 1 h) respectively. Simultaneously, incorporation of palmitic acid into neutral lipids (Fig. 1B) and phospholipids (Fig. 1C) was higher in the absence of albumin that in its presence. For the first 30 min, the rate of incorporation of palmitic acid into neutral lipids was 1.1 nmol/10^6 cells per h in the absence of albumin but only 0.44 and 0.12 in the presence of 75 μM and 300 μM albumin, respectively.; respective values of incorporation into

TABLE I

UPTAKE AND METABOLIC UTILIZATION OF [1-^{14}C]PALMITIC ACID BY HL 60 CELLS IN VARIOUS MEDIA

One million cells were incubated for 3 min and 60 min with 100 nmol of [1-^{14}C]palmitic acid in medium free of serum or albumin, and in medium containing either serum or albumin. Incubation was performed at 37°C in a total volume of 2 ml. Lipids were extracted, separated into neutral lipids and phospholipids, and radioactivity measured as described in Experimental procedures.

	Incubation time (min)	Total uptake	Incorporation into	
			Neutral lipids	Phospholipids
Incubation in medium containing	3	0.3	0.05	0.06
10% fetal calf serum	60	2.7	0.6	0.7
Incubation in medium containing	3	0.3	0.05	0.07
150 μM albumin	60	2.0	0.4	0.8
Incubation in medium free of	3	18.0	0.25	0.15
serum or albumin	60	18.7	1.8	1.1

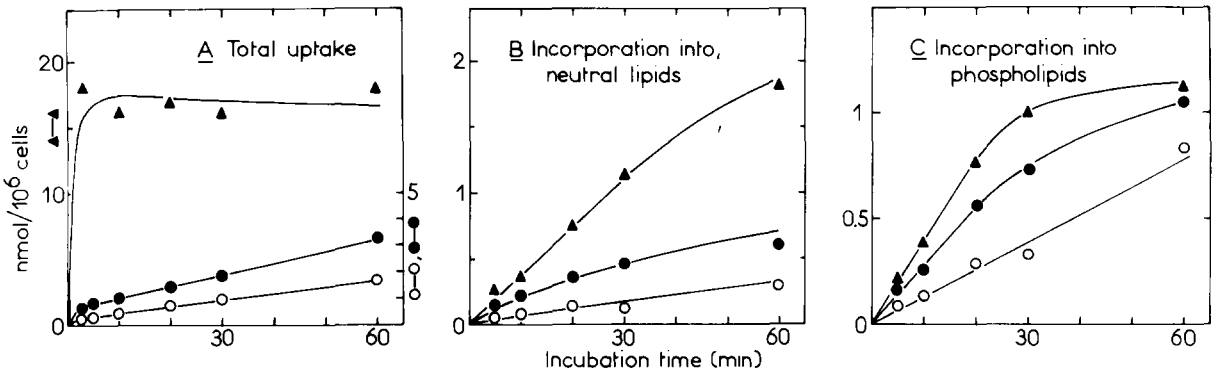

Fig. 1. Time-course of uptake and metabolic utilization of [1-^{14}C]palmitic acid by HL 60 cells in the presence of different concentrations of albumin. One million cells were incubated at 37°C with 100 nmol of [1-^{14}C]palmitic acid in medium free of albumin (▲——▲) or in medium containing either 75 μM (●——●) or 300 μM (○——○) albumin. At various times, the total uptake (A) and incorporation into neutral lipids (B) and phospholipids (C) were determined.

phospholipids were 1.0, 0.65 and 0.3 nmol/10^6 cells per h.

It is worthwhile mentioning that we used fatty acid-free albumin and that, when analyzed by gas chromatography, the fatty acid content of the albumin lipid extract was extremely small as compared to the amount of radioactive palmitic acid added. Thus, the 'inhibitory' effect of albumin on the uptake of [1-^{14}C]palmitic acid is not related to dilution by non-radioactive fatty acids.

Fig. 2A shows the effect of increasing concentration of albumin on the decrease of the total cellular uptake of [1-^{14}C]palmitic acid. A drastic drop of the uptake was observed when the concentration of albumin increased from 0 to 50 μM. No significant differences were observed when further raising the concentration to 300 μM albumin. This decrease of total uptake was also reflected by a decrease of incorporation of palmitic acid into neutral lipids and phospholipids. The inhibition of incorporation of palmitic acid into neutral lipids was more pronounced than into phospholipids (Figs. 2B and 2C). To determine if some of the fatty acid was passively adsorbed and not internalized, cells, incubated for 20 min with [1-^{14}C]palmitic acid either in medium or in medium supplemented with 300 μM albumin, were washed repeatedly with medium-10% serum and then with phosphate-buffered saline. Irrespective of the conditions of incubation, the washings had but little

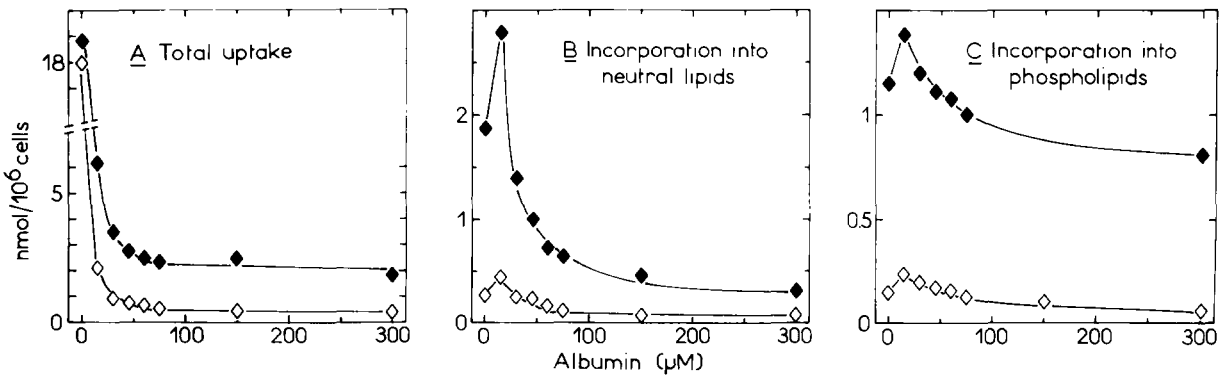

Fig. 2. Effect of increasing concentration of albumin on the total uptake and metabolic utilization [1-^{14}C]palmitic acid by HL 60 cells. One million cells were incubated at 37°C for 3 min (◇——◇) and 60 min (◆——◆) with 100 nmol of [1-^{14}C]palmitic acid in 2 ml of medium containing increasing concentration of albumin. Total uptake (A) and incorporation into neutral lipids (B) and phospholipids (C) were determined.

effect on the values of total cell-bound radioactivity.

The effect of increasing concentration of [1-^{14}C]palmitic acid on its uptake and utilization by cells incubated in the absence of serum or albumin was determined next. Fig. 3A shows that, in the absence of albumin, the rate of total uptake of palmitic acid by cells increased linearly or even in a slightly parabolic manner with increasing concentration of fatty acid while the simultaneous incorporation of [1-^{14}C]palmitic acid into neutral lipids and phospholipids was saturable (Figs. 3B and 3C).

Effect of low temperature and metabolic inhibitors on uptake and metabolic utilization of [1-^{14}C]palmitic acid by HL 60 cells

Fig. 4B shows the effects of low temperature and metabolic inhibitors on the transport of fatty acids into cells incubated in medium containing albumin. At 37°C, the amount of free as well as lipid-associated [1-^{14}C]palmitic acid increased from 3 min to 60 min. At 4°C, the uptake of fatty acids was very much reduced; the total content of cell-associated [1-^{14}C]palmitic acid was very low after 3 min and did not increase after 60 min of incubation. Also, at this low temperature, there was no incorporation into neutral lipids nor phospholipids. The time- and temperature-dependent increase of cell-associated free or lipid-bound palmitic acid was considerably reduced in the presence of either sodium cyanide (inhibitor of respiration) or iodoacetate (inhibitor of glycolysis). Thus, the increase of the total uptake of [1-^{14}C]palmitic acid between 3 min and 60 min of incubation was reduced by 70%, while incorporation into neutral lipids and phospholipids was reduced by about 10% and 70%, respectively. Sodium azide decreased the incorporation into neutral lipids and phospholipids notably while the increase of cell-associated free palmitic acid was relatively less affected.

Fig. 4A shows results of an experiment, similar to that of Fig. 4B except that the cells were incubated in medium free of serum or albumin. As already shown in Fig. 1, the content of cell-associated palmitic acid did not change between 3 min and 60 min of incubation, while the incorporation into neutral lipids and phospholipids increased. Incubation at 4°C reduced the free fatty acid content by 30% and there was no incorporation whatsoever into neutral lipids and phospholipids. Sodium cyanide, iodoacetate and sodium azide inhibited the incorporation into neutral lipids and phospholipids by 70, 75 and 55%, respectively. However, in this case, the metabolic inhibitors had only little effect on cell-associated free palmitic acid. This contrasts with the data observed using medium containing albumin where inhibitors decreased considerably the accumulation of free palmitic acid in cells after 60 min of incubation (Fig. 4B).

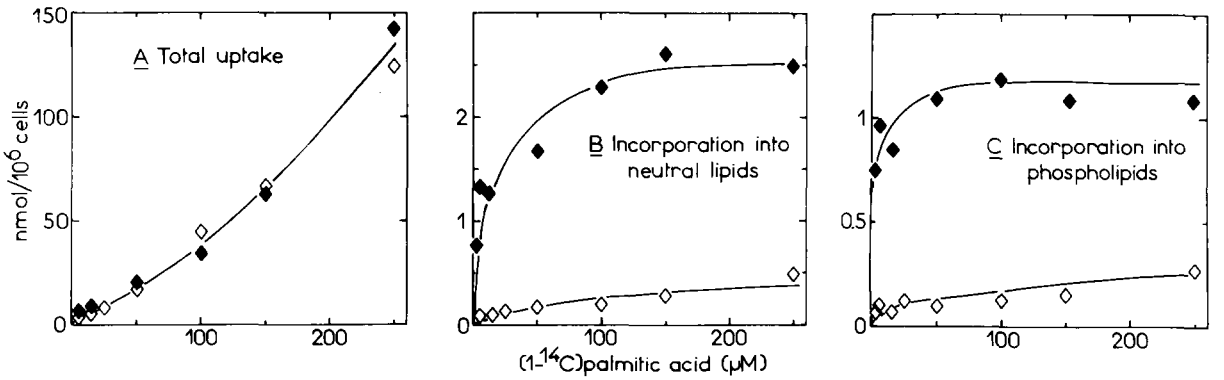

Fig. 3. Effect of [1-^{14}C]palmitic acid concentration on its uptake and metabolic utilization by HL 60 cells in medium free of serum or albumin. One million cells were incubated at 37°C for 3 min (◇——◇) and 60 min (◆——◆) with increasing concentration of [1-^{14}C]palmitic acid in 2 ml of medium free of serum or albumin. Total uptake (A) and incorporation into neutral lipids (B) and phospholipids (C) were determined.

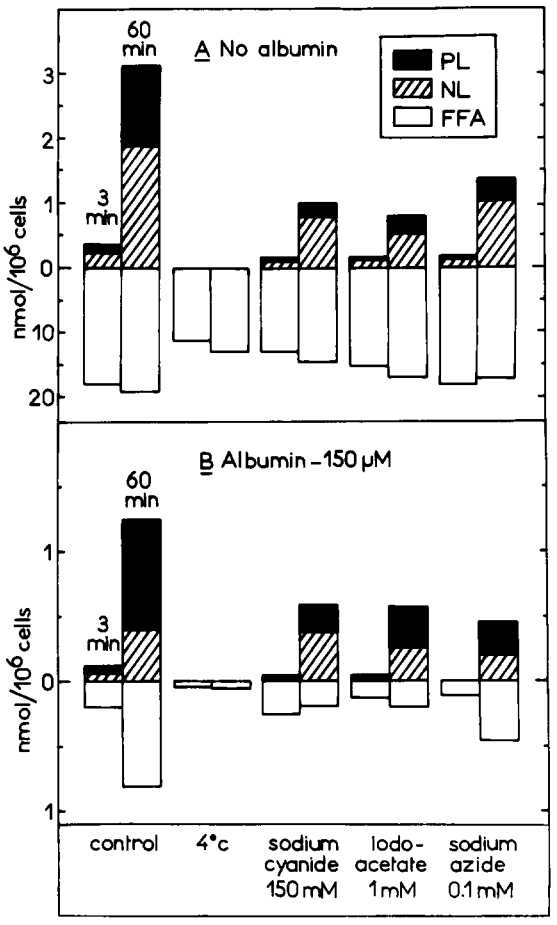

Fig. 4. Effect of low temperature and metabolic inhibitors on uptake and metabolic utilization of [1-^{14}C]palmitic acid by HL 60 cells incubated in the absence or presence of albumin. One million cells were incubated at 37°C or 4°C, and in the presence of either sodium cyanide, iodoacetate or sodium azide. Incubations were performed in a volume of 2 ml of medium free of serum or albumin (A), or in medium containing 150 µM albumin (B). Open bars represent the amount of cell-associated free palmitic acid (FFA), dark bars and hatched bars the amount of cell-associated palmitic acid incorporated into phospholipids (PL) and neutral lipids (NL), respectively.

Discussion

This study is part of a research effort aimed at elucidating the mechanisms of transport of fatty acids across the membrane of eukaryotic cells. In previous investigations we showed that certain synthetic derivatives of fatty acids containing fluorescent polycyclic rings are transported and further utilized metabolically, while others are excluded entirely from the transport process altogether [15]. While working with these derivatives we noticed that addition of serum or albumin to the culture medium reduced considerably the rate of transport as well as the subsequent metabolic utilization of the fatty acid. This prompted us to pay closer attention to the mode of transfer of the synthetic, as well as natural, fatty acids. In this paper we used radioactively-labelled palmitic acid to study the effect of albumin on fatty acid association with the cellular membrane. A very pronounced inhibition was observed, down to a residual value of only 5% of the parallel uptake in the absence of albumin. The amount of fatty acid associated with the cell affects the rate of its incorporation into neutral lipids and phospholipids (Figs. 1 and 2). Thus, within 1 h, in the absence of albumin the total uptake was 18.7 nmol per million cells, of which 15% only (2.9 nmol) was present in the lipids of the cell. In the presence of 150 µM albumin, only 2.0 nmol palmitic acid was transported however, out of this 60% (1.2 nmol) were localized in the neutral lipids and phospholipids. In the absence of albumin, the rate of uptake of fatty acid is high during the first 3 min but the quantity associated with the cells remains practically constant afterwards. This is paralleled by a slow, nearly linear incorporation into cellular lipids. In contrast, when albumin is present, the rates of uptake and metabolic utilization are very similar. These data suggest that, in the absence of albumin, the initial association of the fatty acid with the cell membrane is independent of and most probably not linked to subsequent activation or metabolic utilization. It is likely that, in the absence of albumin, much of the free fatty acid associated with the cell resides in the outer layer of the membrane and has not been translocated into the inner leaflet or the cytosol. The fact that the degree of fatty acid association with the cells could not be reduced by washing with serum, suggests that the fatty acid has indeed been absorbed into the membrane and was not just adsorbed onto the cell surface. Another possible explanation for the rapid uptake of palmitic acid in the first 3 min without further change in the following 60 min, when albumin was absent, might be that the fatty acid indeed is transported rapidly into the cell but,

because of the much smaller rate of metabolic utilization, is subsequently exocytosed. In such a case, between 3 min and 60 min a steady state is set up as a consequence of the bidirectional flow across the cell membrane. But, should it eventually turn out that in the absence of albumin most of the fatty acid has been incorporated only into the outer leaflet, the steady state is probably rapidly established for the fatty acid between the medium and that portion of the membrane. In this case, it would be anticipated that only a small portion of the membrane-associated fatty acid is translocated into the inner leaflet and the cytosol, most or all of the latter being immediately 'activated' and incorporated into cellular lipids. In the presence of albumin, all components of the above process (i.e. association, translocation, activation and transfer to lipids) occur at a considerable reduced rate and seem to be linked to each other. Thus, the data of this paper, which show a reduction of the fatty acid uptake in the presence of albumin, do not support, for the HL 60 cells (as well as for skin fibroblasts; Morand and Gatt, unpublished data), the presence of receptors for albumin-fatty acid complex as proposed by Weisiger et al using hepatic cells [6].

To further test if a relationship exists between transport and metabolic utilization, the effects of low temperature and different inhibitors of metabolism (such as sodium azide and sodium cyanide) were investigated. As anaerobic glycolysis probably occurs in the HL 60 cells, iodoacetate was also used to inhibit this process. In the absence of albumin, the inhibitors had but little effect on the total uptake of the fatty acid, although the metabolic utilization was reduced by 55–70%. In the presence of albumin, sodium cyanide, iodoacetate and sodium azide reduced notably the total uptake as well as the incorporation into cellular lipids. When cells were incubated at 4°C there was no incorporation at all into the cellular lipids. In the absence of albumin, the uptake of palmitic acid was reduced to only 60% of the value at 37°C, while in the presence of albumin the effect of low temperature on the uptake of fatty acid was much more pronounced. The finding that, in the presence of albumin, metabolic inhibitors cause only a partial reduction of the quantity of cell-associated fatty acid supports the notion of two stages of fatty acid uptake or, as it has been previously proposed, of two pools of free fatty acids [11,20]: a passive entry into the outer layer of the membrane and an energy-dependent translocation.

Based on the present results, we suggest that the concentration of free, unbound fatty acid in the water layer adjacent to the surface of the cell might be the factor determining the degree of absorption into the outer layer; this subsequently affects the overall process. In the presence of albumin, free unbound fatty acids would be in equilibrium with the molecules complexed to albumin and those bound to the cell [21]. This, as well as the interrelationship between the rates of association and metabolic utilization [22], permits the following formulation of the transport process: in the absence of albumin, the high concentration of free fatty acid molecules permits rapid penetration into the outer leaflet, probably without mediation of specific receptors. This process, which reaches saturation in 3 min, is followed by a slower translocation process which might be energy-dependent. When albumin is present, only a small portion of the fatty acid molecules is in the form of free, uncomplexed molecules, and the rate of association with the outer leaflet is much slower. Furthermore, we assume that no specific receptors are involved in the initial step of fatty acid influx into the outer layer of the membrane and that the subsequent translocation might occur with the mediation of a transport protein accompanied by 'activation' with the aid of a membranous thiokinase [12,13] or by other, as yet unexplained, mechanisms. Elucidation of these possibilities is the subject of further research on fatty acid transport to be done with natural fatty acids, as well as synthetic derivatives.

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