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INFLUENCE OF MEMBRANE LIPID FLUIDITY ON GLUCOSE AND URIDINE FACILITATED DIFFUSION IN HUMAN ERYTHROCYTES

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SUMMARY

A central question which must be resolved before acceptable molecular descriptions of facilitated diffusion systems can be provided is the nature of the spatial and functional relationships between the transport proteins and the membrane lipids In the work reported here, this question was addressed by investigating the dependence of the rates of glucose and uridine facilitated diffusion in human erythrocytes on membrane lipid fluidity Two approaches were used to alter the lipid fluidity treatment with ether, an anesthetic, and the exchange of a synthetic 3-ketosteroid, cholest-4-en-3-one, for membrane chloesterol Both of these treatments result in a significant increase in membrane lipid fluidity, as judged by the increase in the rates of passive diffusion of uridine through cell membranes and of glucose through membrane lipid bilayer vesicles Ether produces no change in the K_m of either transport process, a slight decrease in the V for glucose transport, and no significant change in the V for uridine transport Replacement of membrane cholesterol by cholest-4-en-3-one reduces the V for glucose transport slightly, without altering the K_m, and reduces both the K_m and V for uridine transport

The absence of the expected increase in the V of facilitated diffusion with increasing membrane lipid fluidity observed here with human erythrocytes is not consistent with models for the transport process which feature movement of transport proteins which are in direct contact with the bulk lipids of the membrane

INTRODUCTION

A dependence of the rate of active transport of β-galactosides and of β-glucosides on the physical state of the membrane lipids has been demonstrated in unsaturated fatty acid auxotrophs of *Escherichia coli* [1–3] This dependence was shown by the presence of as many as three discontinuities in the slopes of Arrhenius

Abbreviation HTG, 6-[(2[hydroxy-5-nitrobenzyl)thio]guanosine

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plots of transport, whose positions were determined by the fatty acid composition of the membrane lipids The lower and upper discontinuities could be correlated with the onset and completion, respectively, of the melting of the fatty acid chains of the membrane lipids

The influence of lipid physical properties on transport is compatible with some type of dynamic interaction between the transport protein and the lipid bilayer Another consequence that might be expected of such an interaction is a dependence of the rate of transport on membrane lipid fluidity above the phase transition boundaries Such a dependence has been shown in artificial mediated transport systems consisting of ion-transporting antibiotics added to lipid bilayer model membranes [4, 5] or to intact cells [6] Nonactin and valinomycin partition into lipid bilayers and stimulate cation fluxes by bidding the cation to form a complex which then diffuses through the lipid bilayer [4, 7, 8] The K^+ conductance induced in planar bilayer membranes by these antibiotics exhibits an increase with increasing temperature above T_c (the gel to liquid-crystalline lipid phase transition temperature) and a discontinuous decrease to essentially zero at the phase transition [5] Adding cholesterol to bilayer lipid membranes [5] or decreasing the fluidity of *Acholeplasma laidlawii* membrane lipids by adding cholesterol or by altering the fatty acid composition [6] also decreases the nonactin- and valinomycin-mediated monovalent cation fluxes Gramicidin, on the other hand, is a hollow, cylindrical molecule which acts by embedding itself within the membrane lipid to form a channel through the bilayer In contrast to the behaviour of "mobile carrier"-type antibiotics, gramicidin-mediated K^+ flux through a planar bilayer membrane decreases with decreasing temperature below T_c, but shows no temperature dependence (and therefore no dependence on lipid fluidity) above T_c [5] We have also recently shown that the maximum rate of mediated glucose transport by *A laidlawii* is dependent on the fluidity and physical state of the membrane lipids [9]

The effect of membrane lipid fluidity on a natural facilitated diffusion system has not previously been examined We have investigated this relationship in the human erythrocyte, which has well characterized facilitated diffusion systems for glucose [10–13] and for uridine [14–16] The fluidity of the membrane lipids was manipulated by adding ether or by exchanging the 3-ketosteroid cholest-4-en-3-one for membrane cholesterol The rates of passive permeation of liposomes (closed, spherical, multilamellar lipid bilayer vesicles) by glucose or of cells by uridine were determined to monitor alterations in membrane lipid fluidity

Several lines of evidence demonstrate that many of the lipid-soluble anesthetics have a fluidizing effect on membrane lipids They cause an increase in the area both of lipid films at the air-water interface and of biomembranes, which is 10-fold greater than could be explained by the volume occupied by the anesthetic molecules [17] A fluidization of the membrane by anesthetics, which can be reversed by increased atmospheric pressure, was seen in studies using fluorescent or spin-labeled probes [18, 19]

Cholesterol comprises about 40 mol % of the membrane lipid of human erythrocytes [20] and is a major component of many other biological membranes The presence of cholesterol has been shown to decrease the nonelectrolyte permeability of egg yolk lecithin liposomes [21], and of *A laidlawii* cells [22, 23] It was demonstrated, by determining the effects of several cholesterol analogs, that this reduction in permeability requires the presence of a planar sterol nucleus, a 3β-hydroxy group and

an intact side chain Sterols having these molecular structural features also cause a condensation of lecithin monolayers at the air-water interface [24] Fluorescence polarization measurements have revealed that cholesterol increases the apparent microviscosity and molecular order above the crystalline → liquid-crystalline phase transition temperature of phospholipid/water dispersions [25] These observations suggest that the substitution of another sterol for membrane cholesterol could alter the membrane lipid fluidity, and that this fluidity change should be detectable as a change in the passive nonelectrolyte permeability of the lipid region of the membrane Indeed, it has been shown that significant amounts of the 3-ketosteroid cholest-4-en-3-one, as well as other cholesterol analogs, could be exchanged for erythrocyte membrane cholesterol by incubating the cells with sonicated dispersions of the synthetic steroids with egg yolk lecithin [26] This replacement causes an increase in the swelling rate of erythrocytes in isotonic glycerol at 37 °C In the work presented here, we have exchanged cholest-4-en-3-one for erythrocyte membrane cholesterol, or have added ether to the cells, to fluidize the membrane lipids The rates of facilitated diffusion of glucose and uridine were determined to assess the effects of the changes in membrane lipid fluidity on these transport processes

METHODS

Lecithin (phosphatidylcholine) was purified from a lipid extract of egg yolks by column chromatography on silicic acid [27, 28] The yolks from three eggs were blended thoroughly with 110 ml of acetone, then filtered through Whatman No 1 paper and the filtrate discarded After blending the residue with 110 ml of chloroform/methanol (2 1, v/v) and filtering again, this second filtrate was taken to dryness and the deposited material redissolved under nitrogen in 125 ml of 5 % methanol in chloroform (v/v) with slight warming, cooled to room temperature, and filtered again. The filtrate was applied to a 6 cm-diameter column of 400 g of Unisil plus 50 g of celite, after packing the column as a slurry in methanol and washing with 1 l of chloroform The column was eluted stepwise with 1 l each of 7 5, 10, 12 5, 15, 17 5, 20, 22 5 and 35 % methanol/chloroform (v/v) and all these fractions were discarded Lecithin was then eluted with 35 l of 35 % methanol/chloroform, and the solvent from this fraction was evaporated The lipid was taken up in 10 % methanol/chloroform and a second column purification was carried out on a 3 cm-diameter, 15 cm column of Unisil After eluting with 1 5 l of 10 % methanol/chloroform and discarding this fraction, the lecithin was eluted with 13 5 l of 30 % methanol/chloroform The total yield was 2 96 g and the resulting lipid gave a single spot having the R_F of authentic egg yolk lecithin when analyzed by thin-layer chromatography

The buffer used for washing and suspending the erythrocytes contained 140 mM NaCl, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 10 mM sodium pyruvate, 0 5 mM adenine and 20 mM Tris, adjusted with HCl to pH 7 4 D-Glucose was added for uridine transport experiments, and inosine for glucose transport or uridine passive flux experiments, to a level of 10 mM to serve as an energy source for the cells After washing the erythrocytes, they were routinely incubated at 37 °C for 1 h in buffer to produce high levels of ATP, then rewashed not more than 1 day before being used

The replacement of membrane cholesterol by exogenously supplied steroids was accomplished by incubating erythrocytes with sonicated dispersions of lecithin

plus the steroids in buffer [26] Steroids and egg yolk lecithin were combined at a ratio of 0 6 g cholesterol/g lecithin, or (0 3 g cholesterol+0 5 g cholest-4-en-3-one)/g lecithin, in 40 ml stainless steel Sorvall centrifuge tubes, and 0 5 ml buffer, mg of lecithin was added to the mixture A total volume of 10–20 ml (20–40 mg lecithin) was used per tube These mixtures were sonicated on ice under nitrogen for 20 min each in four 5-min bursts using a Biosonik III probetype sonicator at 180 W After sonication, the dispersions were centrifuged for 1 h at 48 000 × *g* to remove titanium powder and undispersed lipid The supernatants were then diluted 1-fold with buffer, and erythrocytes were added to a level of about 0 12 ml of packed cells/mg lecithin These mixtures were incubated for 2–8 h at 37 °C, mixing by continuous inversion at 50 rev /min, then cells were washed twice in buffer by centrigfuation before equilibration with permeant

The preloading of cells with labelled permeants and measurement of equilibrium exchange efflux of [^{14}C]glucose or uridine, and the determination of intracellular and interstitial volumes of cell pellets, were carried out exactly as recently described [29] The intracellular and interstitial spaces in the cell pellets were calculated from the extent of dilution of [^{14}C]sucrose and 3 0 methylglucose by cell suspensions 1 ml of packed cells corresponds to 0 59 ml of intracellular water The quench solution used to terminate glucose flux contained 1 25 mM KI 1 μM $HgCl_2$ and 0 1 mM phloretin in buffer [30], and that used for uridine flux measurements contained 0 05 mM 6-[(2-hydroxy-5-nitrobenzyl)thio]guanosine (HTG) in buffer [15] In order to test the effectiveness of these quench solutions, cells were preloaded with labelled permeant and the intracellular radioactivity present at various times after diluting cells with quench solution compared to that present in undiluted cells In all cases less than 3 % of the intracellular labelled glucose or uridine was lost within 5 min, the maximum time which elapsed in the assay procedure before separation of cells from the quench solution by centrifugation

Erythrocyte ghosts were prepared by hypotonic lysis of washed erythrocytes from fresh (less than 1 week old) human blood [31], and lipids were extracted from the washed ghosts by the method of Bligh and Dyer [32] The membranes were extracted twice with 190 ml of chloroform/methanol/water (1 2 0 8, v v v) centrifuging each time for 15 min at 13 000 × *g* and retaining the supernatants To the pooled supernatants were added 100 ml of chloroform, 100 ml of water, then 200 ml more of chloroform, shaking after each addition This mixture was centrifuged for 15 min at 13 000 × *g*, producing two liquid phases with denatured protein at the interface The upper phase and the protein material was aspirated and discarded, and the lower chloroform layer was evaporated to a small volume This was applied to a column of 5–10 g of Biosil-HA silicic acid in chloroform, and the column eluted with 100 ml of methanol This elution recovered all the lipid material, while non-lipid contaminants were retained on the column To avoid oxidation of the lipid, all operations were carried out under an atmosphere of N_2 or CO_2, and solvents were deoxygenated before use by vigorously bubbling N_2 through them

The fatty acid composition of the membrane lipids was analyzed by gas-liquid chromatography of the methyl esters on 10 % diethyleneglycol succinate on Anakrom 60/70 AS mesh To form the methyl esters, lipids were dissolved in a screw-cap tube in 10 ml of methanol with 5 drops of sulfuric acid and heated at 70 C for 2 h After cooling and adding 20 ml of water, the methyl esters were extracted twice with 10 ml

of hexane, and the extracts dried by passage through anhydrous sodium sulfate The steroid composition of the membrane lipids was determined by gas-liquid chromatography on 3 % QF-1 packed on Gas-Chrom Q (100–200 mesh) in a 210 cm × 3 mm glass column Prior to gas-liquid chromatographic analysis, the steroids were purified by column chromatography on Mallinckrodt No 2847 silicic acid and by thin-layer chromatography on Woelm Neutral Alumina [33]

The rate of passive diffusion of D-glucose from liposomes was measured by an enzyme-catalyzed assay system which couples oxidation of glucose 6-phosphate to NADP reduction [34] Liposomes were formed by vortexing extracted lipid in a glass tube under nitrogen with a 1 cm diameter glass bead in the presence of 50 μl of 300 mM D-glucose per mg of lipid The resulting suspensions were stored at 4 °C overnight, then most of the extravesicular glucose was removed by dialysis at 4 °C against 75 ml of isotonic salt solution (75 mM KCl, 62 mM NaCl, 2 mM magnesium acetate) for 5 consecutive 45 min periods, stirring by bubbling N_2 through the salt solution The reaction mixture used for glucose determination contained 8 8 units/ml of hexokinase (Sigma type F-300 sulfate-free), 0 9 units/ml of glucose-6-phosphate dehydrogenase (Sigma type XV), 1 mM ATP, 0 5 mM NADP, 75 mM KCl, 72 mM NaCl, 2 mM magnesium acetate and 28 mM Tris HCl, pH 8 0 All of these components except for NADP were present in the reference solution To carry out the assay, the double-beam spectrophotometer (Beckman model DB-GT) was zeroed at a wavelength of 340 nm with 2 0 ml of reaction mixture in the sample cell and reference solution in the reference cell The zero-time reading was taken 5 min after adding 10 μl of the liposome suspension to each cell The liposome suspension was then incubated at 50 °C and the absorbance of 10 μl aliquots, added to 2 ml of fresh reaction mixture, read 5 min after sampling at 20-min intervals After reading the 80-min sample, 0 2 ml of Triton X-100 was added to both cuvettes to release all the glucose remaining in the liposomes for the infinite time reading After correcting the final reading for dilution, the fraction of the total intravesicular glucose released was plotted vs time

To test for saturability of uridine flux in erythrocytes in the presence of 50 μM HTG, a potent inhibitor of facilitated diffusion of uridine [15], the effect of 4 5 mM unlabelled uridine on the efflux of 20 M [^{14}C]uridine was determined Erythrocytes in buffer, with or without added 4 5 mM uridine present, were preloaded by incubation for 30 min at room temperature with 20 μM [^{14}C]uridine, then 0 25 vols of 250 μM HTG in buffer were added to a final concentration of 50 μM Efflux was initiated by adding 2 ml of 50 μM HTG or 50 μM HTG plus 4 5 mM unlabelled uridine to 0 3 ml of cells, and the mixture was incubated at 37 °C with stirring Aliquots of 0 2 ml of the reaction mixture were taken at various times and added to 3 ml of ice-cold quench solution layered over 6 ml of dibutyl phthalate and centrifuged immediately This resulted in a layer of cell-free aqueous supernatant above the dibutyl phthalate [29], which was counted for radioactivity

The uptake of uridine by passive diffusion was measured by incubating 0 7 ml of a thick suspension of erythrocytes in buffer containing 50 μM HTG (quench solution) at 37 °C with an equal volume of [^{14}C]uridine in the same solution 0 2 ml samples were taken at 10-min intervals into 3 ml of ice-cold quench solution in 15 ml conical centrifuge tubes and immediately centrifuged for 1 min in the cold on the clinical centrifuge After aspirating the supernatants, the cells were washed three times

with ice-cold quench solution by resuspending and recentrifuging To extract radioactivity from the washed cells, they were lysed by adding 0 75 ml of distilled water, followed by vortexing Then 0 75 ml of trichloroacetic acid was added to each sample to precipitate the proteins and the tubes were spun for 10 min at top speed in the clinical centrifuge Aliquots of 1 ml of the supernatants were counted for radioactivity

RESULTS

In the presence of 50 μM HTG, the rate of efflux of 20 μM [^{14}C]uridine from erythrocytes is identical in the presence and absence of added unlabelled 4 5 mM uridine Since the K_m for the equilibrium exchange flux of uridine in the absence of HTG is about 1 mM [35], this lack of saturability seen in the presence of the inhibitor indicates that uridine flux occurs by passive diffusion under these conditions

The presence of 100 mM ether caused a significant increase in the passive fluxes of glucose through liposomes or uridine through cell membranes At 50 C, the efflux of glucose from liposomes in the presence of ether was about 2 5 times the rate in its absence Similarly, the presence of this concentration of ether caused an acceleration of the rate of passive uptake of [^{14}C]uridine by erythrocytes to about 2 4 times the control level It can, therefore, be concluded that ether, present at 100 mM causes a significant increase in the fluidity of erythrocyte membrane lipids

Fig 1 is a Lineweaver-Burk plot showing the effect of 100 mM ether on the facilitated equilibrium exchange efflux of [^{14}C]glucose from erythrocytes at 15 °C In these cells, glucose efflux exhibited a K_m of 56 mM, which was not affected by the presence of ether This is evidence for the lack of any direct effect of this anesthetic on

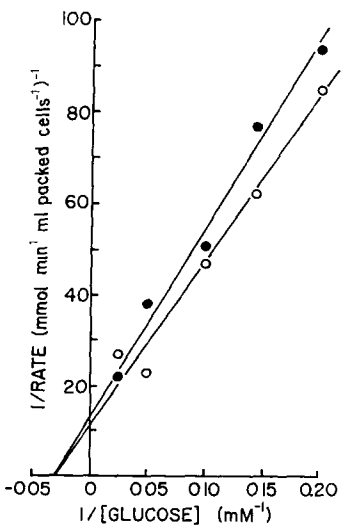

Fig 1 Effect of 100 mM ether on glucose facilitated diffusion The equilibrium exchange efflux of [^{14}C]glucose from human erythrocytes at 15 °C in the presence (●) or absence (○) of 100 mM ether is shown as a Lineweaver-Burk plot

the transport protein In contrast to the marked enhancement by ether of the rates of passive diffusion, V for the facilitated flux of glucose is slightly reduced, from 75 to 95 μmol min^{-1} ml packed $cells^{-1}$, in the presence of 100 mM ether Although the data obtained for uridine facilitated diffusion are rather scattered, no difference in rate caused by ether is apparent In the presence or absence of ether, a K_m of about 1 0 mM and a V of about 1 5 μmol $min^{-1} \cdot$ ml packed $cells^{-1}$ are obtained for uridine equilibrium exchange efflux

The effects of incubating erythrocytes with lecithin/steroid mixtures on their passive [^{14}C]uridine uptake is seen in Fig 2 This shows the uridine uptake in cells

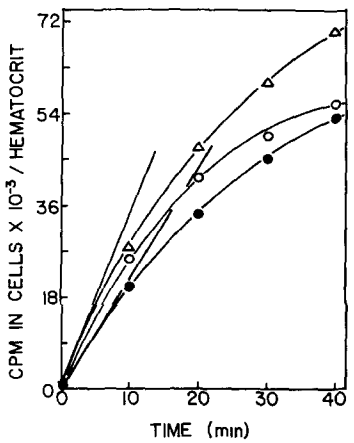

Fig 2 Effect of steroid exchange on passive uridine uptake After treatment with steroid/lecithin dispersions, erythrocytes were incubated at 37 °C with [^{14}C] uridine in the presence of 50 M HTG ○, control (no steroid exchanged), ●, cholesterol, △, cholesterol plus cholest-4-en-3-one

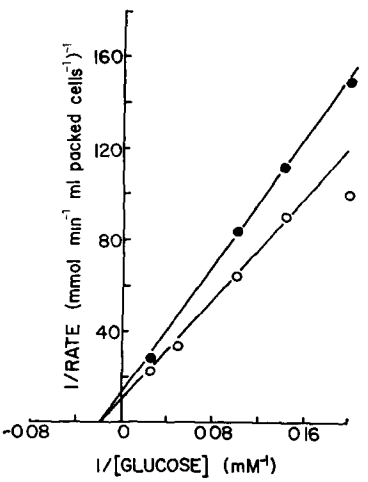

Fig 3 Effect of steroid exchange on glucose facilitated diffusion The equilibrium exchange efflux of [^{14}C]glucose from human erythrocytes at 15 °C is shown as a Lineweaver-Burk plot ●, cholesterol plus cholest-4-en-3-one, ○, cholesterol

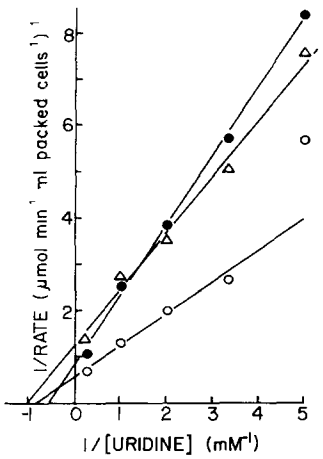

Fig 4 Effect of steroid exchange on uridine facilitated diffusion The equilibrium exchange efflux of [^{14}C]uridine from human erythrocytes at 15° C is shown as a Lineweaver-Burk plot ○, control (no steroid exchanged) ●, cholesterol, △, cholesterol plus cholest-4-en-3-one

TABLE I

EFFECTS OF INCUBATION WITH STEROIDS ON K_m AND V OF URIDINE EQUILIBRIUM EXCHANGE EFFLUX

Exogenous steroids	K_m (mM)	V (μmol min^{-1} ml^{-1})
Control	1 2	1 7
Cholesterol	1 8	1 2
Cholesterol cholest-4-en-3-one	1 0	0 8

incubated for 4 h at 37 ° C with either cholesterol or a mixture of 12 mg of cholesterol plus 20 mg of cholest-4-en-3-one bound to lecithin, as well as a control, using cells which were not incubated with steroids In the cells that were incubated in the presence of cholest-4-en-3-one, 13 % of the total membrane steroids consisted of this 3-keto-steroid Incubation with cholesterol decreased the rate of uridine uptake from that of the control, and incubation with cholesterol plus cholest-4-en-3-one increased it As determined from the slopes of tangents to the curves at t 0, the rate of uptake in cells incubated with both steroids is 1 6 times that in cells incubated with cholesterol only, indicating a significant difference in membrane lipid fluidity between them

Fig 3 is a Lineweaver-Burk plot of the facilitated efflux of glucose from erythrocytes incubated with cholesterol or with cholesterol plus cholest-4-en-3-one The value obtained for K_m was 34 mM in both cases, so it is not influenced by the nature of the exogenously supplied steroid Neither does the value of V seem to be directly related to the membrane lipid fluidity Cells incubated with cholesterol plus cholest-4-en-3-one, which would have a higher membrane lipid fluidity, has a V for glucose flux of 76 μmol min^{-1} ml packed cells^{-1}, while those incubated with cholesterol alone has a slightly higher V of 89 μmol min^{-1} ml packed cells^{-1}

As shown in Fig 4 and in Table I, the effects of these steroids on uridine facilitated transport were somewhat more complex Incubation of erythrocytes with cholesterol caused a marked increase in the K_m, compared to the control, while incubation with the mixture of both steroids resulted in a much smaller decrease in the K_m Incubation with either lecithin/steroid mixture reduced the value of V, with cholesterol alone resulting in a higher V than cholesterol plus cholest-4-en-3-one No direct dependence of V of uridine flux on membrane lipid fluidity is apparent when the fluidity is manipulated by steroid exchange, but such a dependence could be obscured by the decrease in V caused by the incubation, whose physical basis was not determined

DISCUSSION

These results do not demonstrate any direct dependence of rates of facilitated diffusion on membrane lipid fluidity Ether, present at 100 mM, significantly increases the rate of passive diffusion of uridine through erythrocyte membranes and of glucose through membrane lipid bilayers, indicating that it fluidizes the membrane lipids However, this concentration of ether does not affect the K_m for facilitated diffusion of either glucose or uridine, and fails to increase the value of V for either permeant The slight decrease in V of glucose facilitated diffusion which is observed in the presence of ether may not be significant The failure of this anesthetic to affect the K_m values argues against an inhibition of these facilitated diffusion systems by ether, which is obscuring an increase in rate due to the fluidization of the membrane lipid

The results from the steroid exchange are more equivocal Although incubation with cholest-4-en-3-one plus cholesterol fluidizes the membrane lipid and cholesterol alone reduces the fluidity at 37 °C, as shown by the effects on passive uridine flux, this may not occur at 15 °C, the temperature at which facilitated transport was measured Cholesterol reduces lipid fluidity at temperatures above T_c, but increases fluidity below T_c [36], so the expectation of a rigidifying effect of cholesterol on the erythrocyte membrane lipids is contingent on the temperature being above the T_c of the lipids A fluidization caused by the replacement of cholesterol by cholest-4-en-3-one may also require this assumption A non-linearity occurring at 18–19 °C has been reported in plots of the viscosity of human erythrocyte membrane suspensions vs reciprocal temperature, which was attributed to a membrane lipid phase transition [37] However, this may be due to lipid cluster formation, which has been demonstrated in sarcoplasmic reticulum membranes and lipid extracts from them [38], rather than the main endothermic transition which consists of the melting of the hydrocarbon chains of the fatty acids

The fact that the incubation with either cholesterol alone or cholesterol plus cholest-4-en-3-one reduced V relative to the control for uridine facilitated diffusion raises the possibility that an inactivation of transport due to treatment with steroids obscured the superimposed effect of lipid fluidity Nevertheless, glucose facilitated diffusion demonstrated the same K_m and a very similar V after treatment with either steroid mixture

The absence of the expected correlation of the V values for uridine and glucose fluxes with membrane lipid fluidity observed here places some restrictions on the types of model which may be proposed for these transport systems The data imply either

that the portion of the transport systems undergoing movement during the permeation process is not in direct contact with the bulk lipid region, or else that no such movement occurs Several types of model having these characteristics can be envisioned

A fixed aqueous channel at the transport site, lined on the inside surface with permeant binding sites, such as had been proposed for glucose facilitated diffusion [39], would be compatible with this behavior This model, however, seems unlikely on the basis of other kinetic characteristics of this system [40] A carrier protein which translocates the permeant by a conformational change could show the characteristics demonstrated here if it had a rigid outer shell whose dimensions did not change significantly during transport, or if it was shielded from the bulk lipid phase by a surrounding aggregate of other proteins or of a specialized class of bound lipids The concept of a mobile carrier-type transport protein which shuttles from one side of the membrane to the other seems difficult to reconcile with the results reported here

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REFERENCES

1 Linden, C D , Wright, K L , McConnell, H M and Fox, C F (1973) Proc Natl Acad Sci U S 70, 2270–2275
2 Linden, C D , Keith, A D and Fox, C F (1973) J Supramol Struct 1, 523–543
3 Linden C D and Fox, C F (1973) J Supramol Struct 1, 535–534
4 Szabo, G , Eisenman, G and Ciani, S (1969) J Membrane Biol 1, 346–382
5 Krasne, S , Eisenman, G and Szabo, G (1971) Science 174, 412–415
6 Van Der Neut-Kok, E C M , DeGier, J , Middelbeck, E J and Van Deenen, L L M (1974) Biochim Biophys Acta 322, 97–103
7 Ciani S , Eisenman, G and Szabo, G (1969) J Membrane Biol 1, 1–36
8 Eisenman, G , Ciani, S and Szabo, G (1969) J Membrane Biol 1, 294–345
9 Read B D and McElhaney, R N (1975) J Bacteriol 123, 47–55
10 Sen, A K and Widdas, W F (1962) J Physiol 160, 392–403
11 Mawe, R C and Hempling, H G (1965) J Cell Comp Physiol 66, 95–104
12 Miller D M (1971) Biophys J 11, 915–923
13 Stein, W D (1972) Ann N Y Acad Sci 195, 412–428
14 Oliver, J M and Paterson, A R P (1971) Can J Biochem 49, 262–270
15 Cass, C E and Paterson, A R P (1972) J Biol Chem 247, 3314–3320
16 Cass, C E and Paterson, A R P (1973) Biochim Biophys Acta 291, 734–746
17 Seeman P (1972) Pharmacol Rev 24, 583–655
18 Spero, L and Roth, S (1970) Fed Proc 29, 474
19 Hubbell, W L and McConnell, H M (1969) Proc Natl Acad Sci U S 63 16–22
20 Weed, R I , LaCelle, P L and Udkow, M (1974) in The Human Red Cell in vitro (Greenwalt, T J and Jamieson, G A , eds), Chapt 4, Grune and Stratton, New York

21 Demel, R A , Bruckdorfer, K R and Van Deenen, L L M (1972) Biochim Biophys Acta 255, 321–330
22 DeKruyff, B , Demel, R A and Van Deenen, L L M (1972) Biochim Biophys Acta 255, 331–347
23 DeKruyff, B , De Greef, W J , Van Eyk, R V W , Demel, R A and Van Deenen, L L M (1973) Biochim Biophys Acta 298, 479–499
24 Demel, R A , Bruckdorfer, K R and Van Deenen, L L M (1972) Biochim Biophys Acta 255, 311–320
25 Cogan, V , Shinitzky, M , Weber, G and Nishida, T (1973) Biochemistry 12, 521–528
26 Bruckdorfer, K R , Demel, R A , De Gier, J and Van Deenen, L L M (1969) Biochim Biophys Acta 183, 334–345
27 Lea, C H , Rhodes, D N and Stoll, R D (1955) Biochem J 60, 353–363
28 White, B J , Tipton, C L and Dressel, M (1974) J Chem Educ 51, 533–536
29 Read, B D , Bell, N and McElhaney, R N (1975) Anal Biochem 67, 22–29
30 Levine, M and Stein, W D (1966) Biochim Biophys Acta 127, 179–193
31 Dodge, J T , Mitchell, C and Hanahan, D J (1963) Arch Biochem Biophys 100, 119–130
32 Bligh, E G and Dyer, W J (1959) Can J Biochem Physiol 37, 911–930
33 Bruchovsky, N (1971) Endocrinology 89, 1212–1221
34 Demel, R A , Kinsky, S C , Kinsky, C B and Van Deenen, L L M (1968) Biochim Biophys Acta 150, 655–665
35 Cass, C E , Gaudette, L A and Paterson, A R P (1974) Biochim Biophys Acta 345, 1–10
36 Oldfield, E and Chapman, D (1972) FEBS Lett 23, 285–297
37 Zimmer, G and Schirmer, H (1974) Biochim Biophys Acta 345, 314–320
38 Lee, A G , Birdsall, N J M , Metcalfe, J C , Toon, P A and Warren, G B (1974) Biochemistry 13, 3699–3705
39 Naftalin, R J (1971) Biochim Biophys Acta 233, 635–643
40 Lieb, W R and Stein, W D (1972) Biochim Biophys Acta 265, 187–207