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EFFECT OF TEMPERATURE ON KINETICS OF HEXOSE UPTAKE BY HUMAN PLACENTAL PLASMA MEMBRANE VESICLES

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Initial rates of passive and carrier-mediated D-galactose and D-glucose uptake were measured in membrane vesicles derived from the maternal surface of the human placental syncytiotrophoblast. Passive diffusion, as measured by L-glucose uptake, was slightly and continuously temperature-sensitive over a range 0–40°C ($Q_{10} = 1.1$). Below approx. 26°C, passive diffusion measured by D-galactose uptake in the presence of the inhibitor, cytochalasin B, was quantitatively similar to L-glucose uptake. Above this temperature, however, cytochalasin B appeared not to be as effective an inhibitor of carrier-mediated uptake. The initial rates of D-galactose carrier-mediated transport, generated at low concentration (10 μM) were very temperature-sensitive and yielded a non-linear Arrhenius plot. An Arrhenius plot of V_{max}, generated with higher concentrations, was linear. The linearity of the V_{max} Arrhenius plot, in conjunction with the high cholesterol content of this membrane preparation, suggests that a membrane lipid phase transition is not responsible for the non-linearity of the low concentration Arrhenius plot. A discontinuous temperature sensitivity of the interaction between D-galactose and the hexose transport system, as reflected by a marked sensitivity in K_m, appears responsible for the non-linearity in this Arrhenius plot.

Introduction

The syncytiotrophoblast of the human placenta is the first fetal cellular boundary between maternal and fetal circulations. It is through this transporting epithelium that the bulk of fetal nutrients must pass and among the important nutrients is glucose, the primary substrate for fetal oxidative metabolism. The microvillous surface of the syncytiotrophoblast has been shown to possess stereospecific, carrier-mediated D-glucose transport [1,2]. Like the glucose transport system of the erythrocyte and adipocyte and unlike that of the epithelia of the renal tubule and intestine, the glucose transport system of the microvillous surface of the syncytiotrophoblast is sodium-independent and sensitive to cytochalasin B. The syncytiotrophoblast is therefore a unique transporting epithelium with respect to glucose transport.

Since the glucose transport system undoubtedly spans the plasma membrane, transport activity is likely to be affected by the nature and physical state of the membrane lipids (see reviews, Refs. 3, 4). Such an influence of membrane lipids on the function of intrinsic proteins has been shown to be true in a variety of biological systems and tissues. This has been shown directly by manipulation of the type of lipid in the membrane as by culturing prokaryotes in defined media [5–7] or reconstitut-

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ing membrane proteins with specific phospolipids [8], or indirectly by monitoring intrinsic protein activity over a range of temperatures [9,10]. The latter approach has been used extensively with eukaryotic cells and cell membranes and is based on the finding that phospholipids undergo a phase transition from gel to liquid crystalline states, a fluidization, as the temperature is raised [11,12]. Such phase transitions occurring in biological membranes can have profound effects on membrane transport. Data on the effects of temperature on glucose transport kinetics, however, have been equivocal, from tissue to tissue, study to study (summarized in Ref. 13). Accordingly, we have examined the influence of temperature on the kinetics of the glucose transport system of the microvillous surface of the syncytiotrophoblast.

Materials and Methods

Plasma membrane vesicles from human placenta were prepared as described by Bissonnette et al. [2]. Uptake of labeled hexose by the vesicles was measured by the Millipore filtration technique of Carter et al. [14]. Uptake by simple diffusion was determined with L-glucose or with D-galactose and 10 μM cytochalasin B. When cytochalasin B was used, vesicles plus cytochalasin B were incubated for 30 min at room temperature prior to the transport study.

Test tubes containing 200 μl buffer A (100 mM D-mannitol/1 mM Tris-Hepes/1 mM $MgCl_2$ (pH 7.4) with 8 μCi [^{3}H]hexose (and sufficient unlabeled hexose to attain desired concentration) were incubated in a water-bath at specific temperature. Simultaneously, a vesicle-buffer A preparation containing 1–2 mg protein per ml was incubated in the same water-bath. The latter solution also contained ethanol as a cytochalasin B vehicle, 0.5% in the uptake experiment. A test-tube containing labeled hexose was then rapidly placed onto a vortex mixer positioned below a 1 ml Eppendorf pipette filled with ice-cold stop solution (10 mM Tris-Hepes/250 mM NaCl/0.2 mM phloretin (pH 7.4)). Immediately thereafter, and in the presence of vigorous swirling, the uptake was initiated by the addition of 60 μl of the vesicle solution and terminated by the addition of the cold stop solution. The reaction was timed with a Lab-Line Instruments, Inc., Kwik-set Labcron timer (No. 1405) with foot-pedal control. After mixing for an additional 2–3 s, the solution was filtered through a 0.45 μm Millipore filter (HAWP 02500, Millipore Corp., Bedford, MA) and washed with 10 ml ice-cold stop solution. Background filters were prepared by identical steps except that the vesicles were omitted. Filters containing trapped vesicles were placed in 15 ml liquid scintillation fluid (Biofluor, New England Nuclear, Boston, MA). The vials were stored overnight and counted for 10 min in a Packard model 3380 liquid scintillation counter. Uptake was normalized with respect to mg of protein determined by the method of Lowry et al. [15].

The following isotopes were obtained from New England Nuclear: D-[^{3}H]galactose, 10–25 Ci/mmol; L-[^{3}H]glucose, 10–20 Ci/mmol; D-[^{3}H]glucose, 25–50 Ci/mmol.

Methodology

The importance of an effective stopping solution for measurement of initial rates of transport into membrane vesicles was emphasized by Ludvigsen and Jarett [16]. To assess the effectiveness of our stop solution, the following experiments were conducted. First, 60 μl vesicle solution and 1.0 ml ice-cold stop solution were combined; subsequently, 200 μl of buffer A with [^{3}H]galactose at room temperature were added. At various times after the addition of isotope, the vesicles were filtered, washed and counted. The results showed that the cold stop solution was over 98% effective in preventing uptake over the first 2.0 s. Second, vesicles and labeled D-galactose were incubated at room temperature for 2 h then diluted with the 1 ml cold stop solution. At various times, the mix was filtered, washed and counted. A plot of label retained by the vesicles against time extrapolated to zero time indicated that over 93% of the vesicular label remained with the vesicles after 60 s, somewhat more time than required per normal separation of vesicles from the incubation medium. Finally, vesicles and labeled D-galactose were allowed to equilbrate for 2 h, then rapidly mixed with 1 ml cold stop solution, filtered and rinsed with 10, 20, 30 and 40 ml cold stop solution. Based on an extrapolation to zero rinse volume, the 10 ml rinse resulted in a retention of

over 95% labeled D-galactose. The stopping procedure was therefore very effective in inhibiting D-galactose entrance and exit.

Calculation of the rate of total vesicular uptake and uptake by simple diffusion assumes that no radioactive hexose remains bound to the vesicle exterior. This has been previously verified by Bissonnette et al. [2] using this vesicle preparation procedure and buffer A. Also, since the rate of carrier-mediated uptake is calculated as total uptake minus uptake by simple diffusion, any systematic error is minimized for interpretation of characteristics of the transporter.

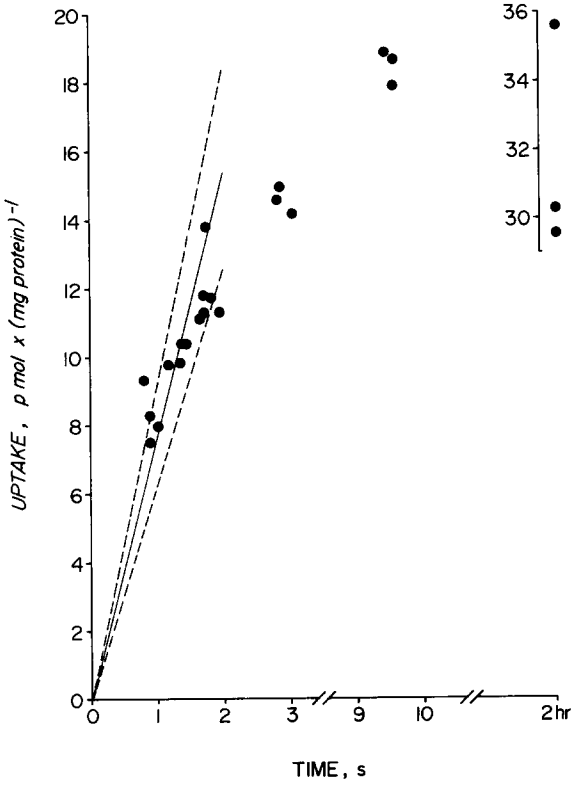

Fig. 1. D-Galactose uptake by membrane vesicles as a function of time. D-galactose concentration is 10 μM; temperature, 35°C. Shown is carrier-mediated uptake calculated as total D-galactose minus L-glucose uptake under identical conditions. Rate of initial uptake, J, was calculated as mean of individual uptake determinations for times no more than 2.0 s and is shown as solid line; ± 1. S.D. of the slope is shown by dashed lines.

Results

Low hexose concentration

Hexose concentrations at zero time were 0 and 10 μM for intra- and extravesicular spaces, respectively. The uptake of D-galactose in the presence and absence of 10 μM cytochalasin B and of L-glucose into plasma-membrane vesicles was determined over a temperature range of 0 to 40°C for uptake times of no more than 2.0 s. Uptake was corrected for background binding. Individual uptake values were divided by uptake time to give single flux values. Individual values ($n \geqslant 10$) were then averaged to determine initial velocity of uptake, J. Velocity of uptake by passive diffusion, determined as L-glucose uptake, was slightly temperature-sensitive with an approximate velocity in pmol/mg protein per s given by the equation for a straight line, $J = 4 \cdot 10^{-3}T + 0.4$ (T in °C), over a temperature range 0–40°C. Passive diffusion as determined by D-galactose uptake in the presence of 10 μM cytochalasin B was quantitatively similar, with $J = 7 \cdot 10^{-3}T + 0.5$ for $T \leqslant 26$°C. For $T > 26$°C, however, cytochalasin B-inhibited D-galactose uptake showed a marked increase in temperature sensitivity given by $J = 9 \cdot 10^{-1}T - 2$.

Velocity of carrier-mediated transport of D-galactose was calculated as the mean of $J_{\text{D-galactose}}$ minus $J_{\text{L-glucose}}$. Fig. 1 represents carrier-mediated D-galactose uptake at 35°C over an extended time. The results strongly suggest that for $t < 2.0$ s, J is a

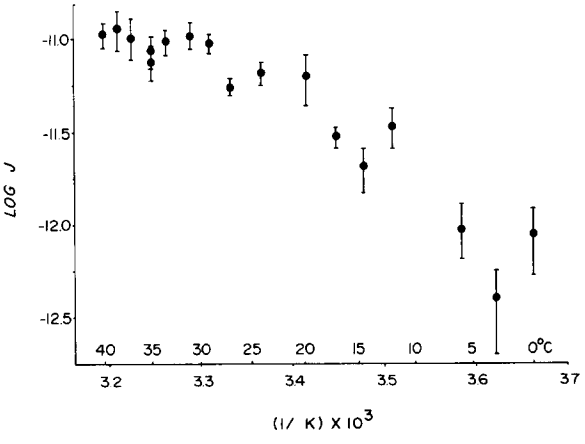

Fig. 2. Arrhenius plot of carrier-mediated D-galactose uptake. D-Galactose at time zero is 10 μM outside, zero inside. Datum points are means ($n \geqslant 10$), vertical lines represent $\pm$S.D.

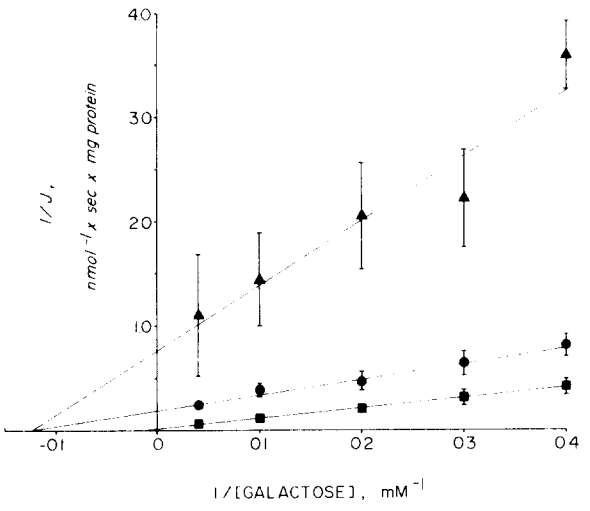

Fig. 3. Lineweaver-Burk plot of initial uptake rates over a concentration range of 2.5 to 25 mM D-galactose at 10 (▲), 22 (●) and 31°C (■). K_m and V_{max}, Table I.

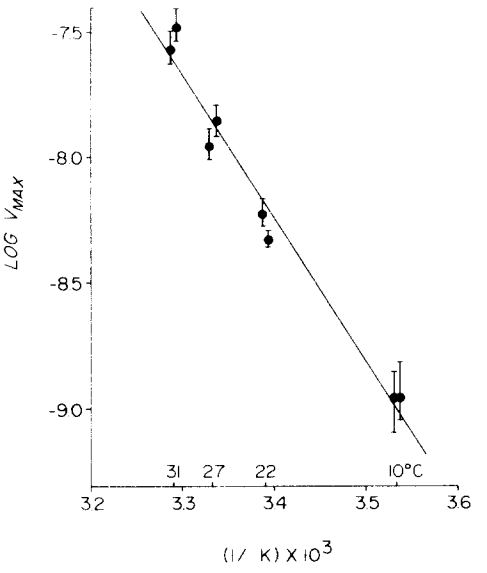

Fig. 4. Arrhenius plot of D-galactose V_{max} from Woolf transformation data, Table I.

good measure of carrier-mediated, initial uptake velocity of D-galactose into these membrane vesicles. Over the range 0–40°C, this velocity is highly sensitive to temperature. If these carrier-mediated uptake data are plotted as an Arrhenius plot (Fig. 2), they show a non-linear plot. Assuming the plot is biphasic, breaking at 24°C, the E_a values are about 18 kcal · mol^{-1} for $T \leqslant 24$°C and 6 for $T \geqslant 24$°C, corresponding to Q_{10} values of 2.9 and 1.4 (over 10–20 and 25–35°C), respectively.

High hexose concentration

D-Galactose and L-glucose initial uptake velocities were measured at extravesicular concentrations of 2.5 to 25 mM (intravesicular = 0) at 10, 22, 27 and 31°C. Initial uptake velocity of carrier-

TABLE I

K_m AND V_{max} FOR D-GALACTOSE AND D-GLUCOSE CARRIER-MEDIATED UPTAKE

K_m in mM; V_{max} in nmol/s per mg protein; K_m and V_{max} based on 44–61 individual uptake values; data calculated by Lineweaver-Burk (LB) and Woolf transformations; values in parentheses represent 95% confidence interval derived as described [34].

	Temp. (°C)	K_m		V_{max}	
		LB	Woolf	LB	Woolf
D-Galactose					
	10	9.1 (8.9– 9.3)	16 (14 –16)	0.8 (0.3– 1.3)	1.1 (0.8– 1.4)
		8.1 (8.0– 8.2)	6.6 (5.2– 8.0)	1.3 (0.5– 2.1)	1.1 (0.9– 1.5)
	22	8.2 (8.0– 8.3)	9.9 (8.6–11)	5.3 (2.7– 7.9)	5.9 (5.3– 6.8)
		6.8 (6.8– 6.9)	4.6 (3.7– 5.5)	5.6 (3.1– 8.1)	4.7 (4.5– 5.1)
	27	40 (38 –42)	33 (31 –35)	13 (5 –21)	11 (10 –13)
		38 (36 –40)	37 (35 –38)	14 (5 –23)	14 (12 –16)
	31	23 (19 –28)	31 (24 –39)	22 (8 –36)	27 (24 –32)
		41 (40 –42)	46 (45 –46)	30 (10 –50)	33 (29 –39)
D-Glucose					
	10	1.2 (1.2– 1.3)	3.1 (0.7– 5.6)	0.8 (0.5– 1.1)	1.4 (1.2– 1.6)
	22	4.3 (4.2– 4.3)	4.4 (4.0– 4.8)	5.6 (2.3– 7.0)	5.7 (5.2– 6.3)

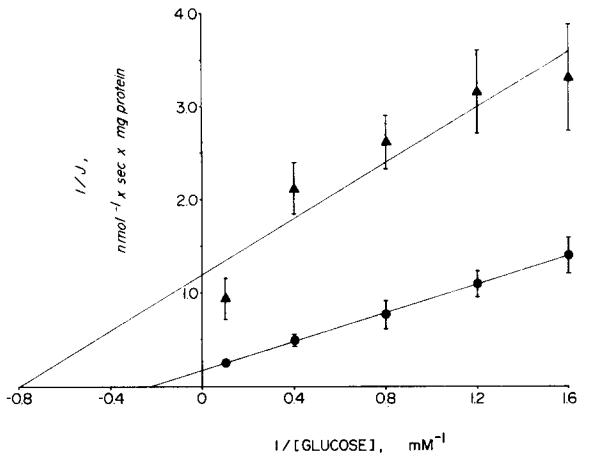

Fig. 5. Lineweaver-Burk plot of carrier-mediated D-glucose initial rates at 10 (▲) and 22°C (●). K_m and V_{max}, Table I.

mediated D-galactose transport was analyzed with Lineweaver-Burk and Woolf transformations of Michaelis-Menten kinetics. Best estimates of K_m and V_{max} are obtained when K_m lies within the concentration range used to generate the kinetic data. Extrapolation of the Lineweaver-Burk plot to the X and Y intercepts ($-K_m^{-1}$ and V_{max}^{-1}, respectively) resulted in K_m values within the concentration range of 2.5 to 25 mM at 10 and 22°C, as shown in Fig. 3. However, at 27 and 31°C, data points extrapolated to near the origin, indicating a K_m outside that concentration range. The extravesicular concentration range was thus changed to 5–50 mM. Table I summarizes the K_m and V_{max} data. The change in K_m with temperature is clearly discontinuous between 22 and 27°C; in contrast, an Arrhenius plot of V_{max}, Fig. 4, is linear with an E_a of about 26 kcal · mol^{-1}.

To circumvent the problems inherent in extrapolating from D-galactose transport to the more physiologically important D-glucose transport in this vesicle system, we attempted to determine K_m and V_{max} for those temperatures examined with D-galactose. Fig. 5 and Table I show data obtained at 10 and 22°C utilizing D-glucose concentrations of 0.625 to 10 mM. In these experiments with D-glucose, uptake was determined during the first 1.5 s. At 10 and 22°C, uptake generally exceeded 50% of the equilibrium value (for equilibrium, $t = 2$ h) for uptake times greater than 1.5 s. Experiments at higher temperatures were consequently not attempted.

Discussion

We have measured initial rates of passive diffusion and carrier-mediated hexose transfer into plasma membrane vesicles. Although these rates represent net inward flux rather than absolute unidirectinal flux, the use of very short uptake times and D-galactose as the transported hexose allows qualitative interpretation of the temperature sensitivities of the uptake velocities.

The rate of passive diffusion as determined by L-glucose uptake appears slightly temperature-sensitive, with a Q_{10} of approx. 1.1 over the range 0–40°C. Such a rate measured by D-galactose uptake in the presence of 10 μM cytochalasin B also appears slightly temperature-sensitive below 26°C; more sensitive above. Consequently, cytochalasin B inhibition of transport may not be as effective at warm as at low temperatures. This is consistent with the findings of Taverna and Langdon [17] for human erythrocytes. In separate experiments, no difference in rates of transport was found for D-galactose in the presence of cytochalasin B at 15°C between control vesicles and vesicles which had been incubated at 37°C for up to 4 h and then returned to 15°C. This suggests that the apparent decrease in effectiveness of cytochalasin B inhibition above 26°C was not due to irreversible changes of the cytochalasin B binding site(s).

In contrast to the slight sensitivity of L-glucose uptake, carrier-mediated D-galactose transport into these vesicles is much more temperature-sensitive. Also, as indicated by the 10 μM D-galactose uptake Arrhenius plot, Fig. 3, this carrier-mediated D-galactose transport is not a simple function of temperature. For $T \leqslant 24$°C, E_a is 18 kcal · mol^{-1}, corresponding to a Q_{10} of about 2.9 over 10–20°C; for $T \geqslant 24$°C, E_a is 6, corresonding to a Q_{10} of 1.4 over 25—35°C. Such non-linearity in an Arrhenius plot for the activity of a membrane protein, either enzyme or transporter, is often interpreted as being due to a lipid phase transition [18–21]. In their reconstituted glucose transporter system, Melchior and Czech [8] report a similar non-linear Arrhenius plot. They interpret their results as the transporter functioning at near maximal rate when imbedded

in a fluid or liquid-crystalline membrane but relatively inoperative in a gel lipid matrix. They suggest that as temperature increases, ever larger areas of gel membrane fluidize with a concomitant increase in total membrane transport activity. Although this is a plausible explanation for the temperature sensitivity of a transport system reconstituted with a simple phospholipid composition, it is unlikely to be the explanation for the vesicle preparation used in this study. The microvillous membrane of the human placental syncytiotrophoblast is rich in cholesterol; the molar ratio of cholesterol to phospholipid is about 1 : 1.4, i.e., the membrane lipid is about 40% cholesterol [22]. Membrane lipid phase transitions, however, are absent in membranes containing more than about 30% cholestrol [23–26]. Consequently, it is highly unlikely that the placental microvillous membrane would undergo a lipid phase transition; therefore, it is also highly unlikely that the observed non-linearity in the 10 μM D-galactose Arrhenius plot, Fig. 2, is due to such a phase transition of membrane lipid surrounding the transporter. An Arrhenius plot of V_{max} yields a linear plot, Fig. 4. This further suggests that a membrane lipid phase transition does not occur in this membraneous preparation over the temperature range studied. The transporter itself may, however, be discontinuously temperature-sensitive.

A non-linearity in the Arrhenius plot has been attributed to the temperature sensitivity of the membrane protein rather than, or in addition to, such a sensitivity in the protein-lipid interaction in several membrane enzyme or transport systems. This includes the β-galactosidase transport system of *E. coli.* [27], the Na^+, Mg^{2+}-ATPase of *Acholeplasma laidlawii* B [28] and possibly alkaline phosphatase of dog kidney [29]. Further, Silvius and McElhaney [30] have shown by theoretical models that in addition to changes in membrane lipid phase state, discontinuous enzyme or transporter temperature sensitivities can lead to multiphasic Arrhenius plot.

The striking effect of temperature on K_m in this study (Table I) strongly suggests a discontinuous temperature sensitivity in the interaction of D-galactose with the transporter. This sensitivity of the D-galactose-transporter interaction appears sufficient to account for the non-linear Arrhenius plot generated from the 10 μM D-galactose initial rate data. The elucidation of the molecular basis for this unusual sensitivity requires further investigation. However, it may be involved in a temperature-dependent ionization of functional groups of the transporter. Such a mechanism appears involved in the temperature sensitivity of phosphofructokinase [31].

K_m values obtained for D-glucose at 10 and 22°C are lower than those obtained for D-galactose under identical conditions, yet are consistent with those in other tissues reported by numerous authors and summarized by Stein [32]. Johnson and Smith [1] have reported a K_m of 31 mM for 3-*O*-methylglucose at 22°C utilizing a preparation similar to that used in this study, however, their rates were obtained with equilibrium exchange transfer while ours were obtained using zero-trans uptake. The apparent discrepancy in these K_m values may be due to differences in glucose analog used or in mode of study; kinetic parameters appear to be strongly dependent upon experimental protocols [13,33].

D-Galactose and D-glucose compete for the same transporter in the placental microvillous preparation [1,2]. Consequently, the discontinuous temperature sensitivity of carrier-mediated D-galactose transport reported in this study is likely to exist for placental D-glucose transport as well.

Acknowledgements

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References

1 Johnson, L.W. and Smith, C.H. (1980) Am. J. Physiol. 238, C160–C168
2 Bissonnette, J.M., Black, J.A., Wickham, W.K. and Acott, K.M. (1981) J. Membrane Biol. 58, 75–80
3 Lenaz, G., Curatola, G. and Massoti, L. (1975) Bioenergetics 7, 223–299

4 Sandermann, H., Jr. (1978) Biochim. Biophys. Acta 515, 209–237
5 De Kruyff, B., Van Dijck, P.W.M., Goldbach, R.W., Demel, R.A. and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 330, 269–282
6 Rottem, S., Cirillo, V.P., De Kruyff, B., Shinitzky, M. and Razin, S. (1973) Biochim. Biophys. Acta 323, 509–519
7 Morrisett, J.D., Pownall, H.J., Plumlee, R.T., Smith, L.C., Zehner, Z.E., Esfahani, M. and Wakil, S.J. (1975) J. Biol. Chem. 250, 6969–6976
8 Melchior, D.L. and Czech, M.P. (1979) J. Biol. Chem. 254, 8744–8747
9 Brasitus, T.A., Schachter, D. and Mamouneas, T.G. (1979) Biochemistry 18, 4136–4143
10 De Smedt, H. and Kinne, R. (1981) Biochim. Biophys. Acta 648, 247–253
11 Chapman, D., Bryne, P. and Shipley, G.G. (1966) Proc. R. Soc. (London) Ser. A 290, 115–142
12 Chapman, D., Williams, R.M. and Ladbrooke, B.D. (1967) Chem. Phys. Lipids 1, 445–475
13 Plagemann, P.G.W., Wohlhueter, R.M., Erbe, J. and Wilkie, P. (1981) Biochemistry 20, 3366–3370
14 Carter, J.R., Avruch, J. and Martin, D.B. (1972) J. Biol. Chem. 247, 2682–2688
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
16 Ludvigsen, C. and Jarett, L. (1979) J. Biol. Chem. 254, 1444–1446
17 Taverna, R.D. and Langdon, R.G. (1973) Biochim. Biophys. Acta 323, 207–219
18 Kumamoto, J., Raison, J.K. and Lyons, J.M. (1971) J. Theor. Biol. 31, 47–51
19 Linden, C., Wright, K.L., McConnell, H.M. and Fox, C.F. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2271–2275
20 Raison, J.K. (1973) Bioenergetics 4, 285–309
21 Linden, C.D. and Fox, C.F. (1975) Acc. Chem. Res. 8, 321–327
22 Smith, N.C. and Brush, M.G. (1978) Medical Biol. 56, 272–276
23 Ladbrooke, B.D., Williams, R.M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333–340
24 Hinz, H.J. and Sturtevant, J.M. (1972) J. Biol. Chem. 247, 3697–3701
25 Rothman, J.E. and Engleman, D.M. (1972) Nature New Biol. 237, 42–44
26 Engelman, D.M. and Rothman, J.E. (1972) J. Biol. Chem. 247, 3694–3697
27 Sullivan, K.H., Jain, M.K. and Koch, A.L. (1974) Biochim. Biophys. Acta 352, 287–297
28 Silvius, J.R., Read, B.D. and McElhaney, R.N. (1978) Science 199, 902–904
29 Le Grimellec, C., Giocondi, M., Carriere, B., Carriere, S. and Cardinal, J. (1982) Am. J. Physiol. 242, F-246–F253
30 Silvius, J.R. and McElhaney, R.N. (1981) J. Theor. Biol. 88, 135–152
31 Bock, P.E. and Frieden, C. (1976) J. Biol. Chem. 251, 5630–5636
32 Stein, W.D. (1967) The Movement of Molecules across Cell Membranes, Academic Press, New York
33 Kessler, M., Tannenbaum, V. and Tannenbaum, C. (1978) Biochim. Biophys. Acta 509, 348–359
34 Snedecor, G.W. and Cochran, W.G. (1967) Statistical Methods, pp. 155–157, Iowa State University Press, Ames, IA