Biochimica et Biophysica Acta, 770 (1984) 171–177
Elsevier

BBA 72021

SENSITIVITY OF Na^+-COUPLED D-GLUCOSE UPTAKE, Mg^{2+}-ATPase AND SUCRASE TO PERTURBATIONS OF THE FLUIDITY OF BRUSH-BORDER MEMBRANE VESICLES INDUCED BY *n*-ALIPHATIC ALCOHOLS

YVETTE J. FERNANDEZ *, ROSE-ANNE M. BOIGEGRAIN, CLAUDIE D. CAMBON-GROS and SALVADOR E. MITJAVILA

U-87-I.N.S.E.R.M., Institut de Physiologie, 2 rue François Magendie, 31400 Toulouse (France)

(Received August 8th, 1983)

Key words: n-Aliphatic alcohol; Membrane fluidity; Mg^{2+}-ATPase; Sucrase; Glucose transport; ESR; (Rat intestinal brush-border membrane)

The aim of our work is to show the importance of the role of hydrophobic bonds in maintaining Mg^{2+}-ATPase or sucrase activity and Na^+-coupled D-glucose uptake normal for the brush border of rat enterocytes. The activity of the two enzymes and the D-glucose uptake were therefore measured under the action of *n*-aliphatic alcohols and related to the fluidity determined by ESR. Three concentrations were used for the first eight alcohols, those of octanol being about 1500-times lower than those of methanol. For each alcohol the D-glucose uptake and the fluidity were linear functions of the logarithm of the concentration, the linear regressions being practically parallel and equidistant. The concentrations (C) of the eight alcohols inhibiting the D-glucose uptake by 80% were similar to those increasing the membrane fluidity by 3%. The linear relationship which existed in both cases between log $1/C$ and log P, P being octanol/water partition coefficients of the alcohols, was evidence of great sensitivity to the hydrophobic effect of the alcohols. Only the first alcohols, however, produced any notable inhibition of Mg^{2+}-ATPase and sucrase. Hydrophobic bonds are thus shown to have little influence in maintaining the activity of Mg^{2+}-ATPase and sucrase, but they modulate the Na^+-coupled D-glucose uptake.

Introduction

The lipid environment of membrane proteins, by giving them a defined orientation in space, plays an important role in maintaining numerous enzyme activities [1]. These activities can be modulated by the presence in the membrane of amphiphilic substances of exogenous origin which modify the motional parameters of the lipids [2]. Thus, for example, the activity of erythrocyte ($Na^+ + K^+$)-ATPase can be modified by various amphiphilic compounds (aliphatic alcohols, detergents, etc.) [3]. For the brush border of enterocytes it is now known that ethanol causes, to a greater or lesser degree, a decrease in the activity of certain hydrolases [4,5], a decrease in D-glucose transport [6,7] and an increase of fluidity [8]. These data, though obtained under different conditions, led us to think that there must be a relationship between the various effects of ethanol through hydrophobic interactions with membrane lipids.

The aim of our work is to bring out the importance of the role played by hydrophobic bonds in the normal maintenance of certain of these activities of the brush border. To do this we studied the action of various amphiphilic substances, the *n*-aliphatic alcohols, on the activity of sucrase, of

* To whom correspondence should be addressed.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

Mg^{2+}-ATPase, on Na^+-coupled D-glucose uptake and on membrane fluidity. This choice was made because of the different locations of these enzymes and the D-glucose carrier in the membrane [9–11] which suggest variable dependence of their activity relative to hydrophobic interactions of the protein and its lipid microenvironment. The changes in fluidity should permit an appreciation of the perturbations of the lipids due to the hydrophobic effect of the alcohols and allow the role of fluidity in the modulation of the various activities studied to be deduced.

Materials and Methods

Preparation of brush-border membrane vesicles. Male Wistar rats (200–230 g) were killed by decapitation. The small intestine was then removed, rinsed with cold NaCl (0.9%) and the mucosa was scraped. After preparation of the cellular homogenate, the brush border membrane vesicles were obtained by the calcium precipitation method of Schmitz et al. [12], modified by Kessler et al. [13]. After the last centrifugation, the pellet was resuspended in appropriated buffers. Activities are expressed relative to protein content measured by the method of Lowry et al. [14]. Sucrase was used as the marker of the brush border membrane vesicles. Sucrase activity of the homogenate and of the brush-border membrane vesicles was assayed routinely in each experiment. The brush border membrane vesicles were purified approximately 14-fold with respect to protein.

n-Aliphatic alcohols. Analytical grade *n*-aliphatic alcohols from methanol (C_1) to octanol (C_8) were purchased from Alltech Associates, 202 Campus Dr Armington, HTS., IL. Enzyme activities, D-glucose uptake and membrane fluidity were measured in the absence (control) or presence of alcohol. The final concentrations of these alcohols in the reactive medium are given in the various figures.

Determination of net Na^+-coupled D-glucose uptake. The experiments were carried out with shaking at 25°C after 10 min of preincubation. Preincubations (50 µl) were performed in a buffer containing 300 mM mannitol, 10 mM Hepes-Tris (pH 7.5) in the absence (control) or presence of alcohol and 25 µl of vesicles (0.2 mg protein) in suspension in the same buffer. After adding 50 µl of solution containing NaSCN, D-[6-^{3}H]glucose, D-[1-^{14}C]mannose and the alcohol at the same concentration, the uptake was started by shaking for 1.0 s. The incubation was stopped by adding 3 ml of an ice-cold stop solution (250 mM NaCl/1 mM Tris-HCl, pH 7.5). The diluted samples were immediately transferred onto a Millipore nitrocellulose filter (pore size: 0.45 µm) and washed twice with 2 ml of the same ice-cold stop solution. After the filters had been dried the ^{3}H and ^{14}C radioactivity was determined by liquid scintillation. The net Na^+-coupled D-glucose uptake was the difference between the amount of D-glucose uptake and D-mannose uptake (the D-mannose being used as a diffusional marker). Blanks were measured by adding, separately, 50 µl of incubation solution and 50 µl of preincubation solution to 3 ml of ice-cold stop solution and proceding as above. The results are expressed in nmol of net D-glucose uptake/mg protein.

Determination of enzyme activities. Sucrase (EC 3.2.1.48) and Mg^{2+}-ATPase (EC 3.6.1.3) activities were measured at 37°C after 10 min of preincubation and 15 min of incubation with constant shaking. The preincubation medium (total volume 0.8 ml) contained 0.5 ml of appropriate buffer (see Figs. 3 and 4), alcohols at the desired concentration and 0.1 ml of vesicles (0.1 mg protein) in suspension in a buffer of 300 mM mannitol and 2 mM Tris-HCl (pH 7.4). The incubation started by adding 0.2 ml of substrate. Controls were carried out by replacing the alcohol by distilled water in the preincubation medium. Sucrase activity was determined by the method of Hübscher et al. [15], in presence of saccharose as substrate. The reaction was stopped by boiling for 3 min. After the proteins had been eliminated by centrifugation, the amount of glucose liberated was determined [16]. The activity of the Mg^{2+}-ATPase was determined by the method of Quigley and Gotterer [17] in presence of Tris-ATP (Boehringer Mannheim GmbH) as substrate. After the reaction had been stopped by 0.25 ml of 2.5 M $HClO_4$ and proteins eliminated by centrifugation, the amount of P_i liberated was determined [18]. Enzyme activities are expressed in µmol of substrate hydrolysed per 15 min per mg protein.

Determination of membrane fluidity. The fluidity was determined by ESR at 22°C using *N*-oxyl-

4',4'-dimethyloxazolidine derivative of 5-keto-stearic acid as a spin label (Syva International, Palo Alto, CA 94304, U.S.A.). This spin-label probes and monitors the region of the headgroups of the phospholipids close to the surface of the lipid bilayer. 10 μl of a solution of 10 mM spin label in dimethylsulfoxide were added to 1 ml of vesicle suspension in 300 mM mannitol/2 mM Tris-HCl (pH 7.4) buffer and then vortexed for 5 min. 25 μl of labelled membrane suspension (0.25 mg protein) were shaken for 5 min in absence (control) or presence of alcohol (total volume 50 μl) and used to record ESR spectra (Brucker ER 200 D X band spectrometer). The freedom of movement of the nitroxide radical in the membrane was evaluated by measuring the $2T'_{\parallel}$ extreme hyperfine coupling on the ESR spectrum. The results are expressed in G.

Results

Effects of alcohols on net Na^+-coupled D-glucose uptake

Fig. 1 shows the evolution of net D-glucose uptake with incubation time. Under our experimental conditions it had the classic characteristics of kinetics in presence of a gradient of sodium with an overshoot at about 15 s of incubation. For

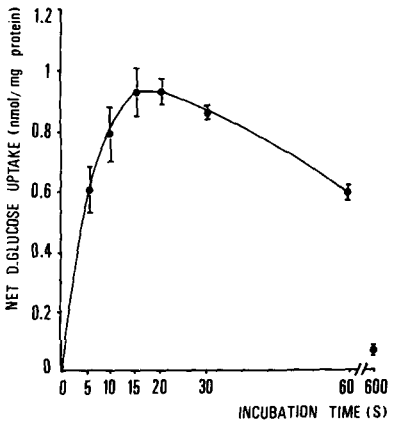

Fig. 1. Time-course of net Na^+-coupled D-glucose uptake. The reactive medium contains 0.2 mg of proteins, 1 μCi D-[6-^{3}H]glucose, 1 μCi D-[1-^{14}C]mannose, in 100 μl of 100 mM NaSCN, 0.1 mM D-glucose, 0.1 mM D-mannose, 300 mM mannitol and 10 mM Hepes-Tris (pH 7.5). Data represent means ± S.E. for four different brush-border vesicles preparations.

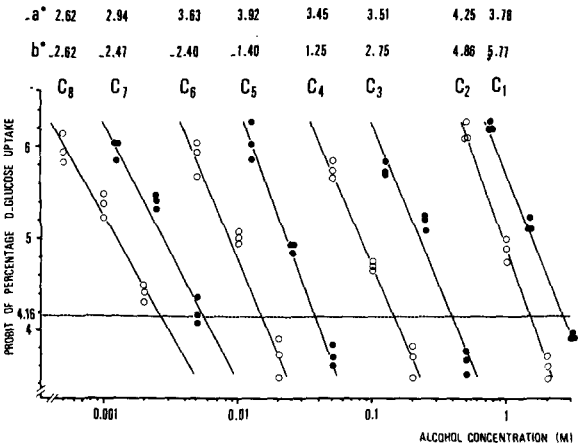

Fig. 2. Effects of *n*-aliphatic alcohols on Na^+-coupled D-glucose uptake. The reactive medium is that described in Fig. 1 with, in addition, the alcohols (C_1 to C_8) at the final concentrations indicated. * $y = ax + b$: linear regressions calculated for each alcohol taking account of the three experimental values obtained at each concentration (○ for even alcohols, ● for odd alcohols).

all the experiments carried out in presence of alcohols, the net D-glucose uptake was determined at 15 s of incubation. For the controls, the mean ± S.E. ($n = 6$) was 0.917 ± 0.085 nmol/mg protein. Fig. 2 gives the probit * of the percentage of net D-glucose uptake, relative to the control, against the logarithm of the alcohol concentration (from C_1 to C_8). The use of such coordinates produces 8 practically parallel and equidistant linear regressions and brings out the fact that the longer the aliphatic chain of an alcohol, the lower its active concentrations. Thus the active concentrations of methanol are about 1500 times higher than those of octanol.

Effects of alcohols on enzymic activities

For the controls, the activities (mean ± S.E.) ($n = 6$), expressed in μmol/15 min per mg protein, were 17.17 ± 0.54 for Mg^{2+}-ATPase and 20.24 ± 0.44 for sucrase. Figs. 3 and 4 show the variations of activity of Mg^{2+}-ATPase and sucrase, respectively, with alcohol concentration, practically in

* The 'probit' transformation allows an S-shaped curve to be represented as a straight line. This mathematical transformation of percentages to probits is performed using the appropriate tables [19].

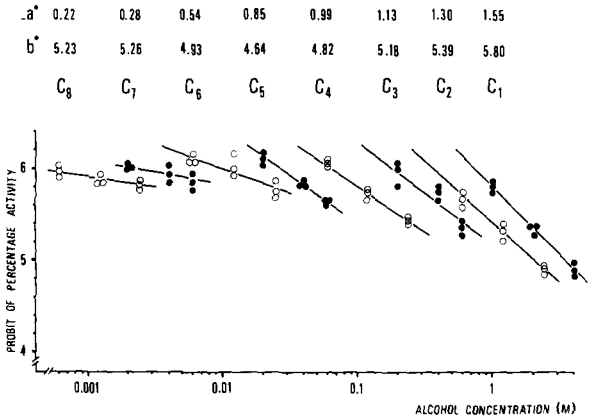

Fig. 3. Effects of *n*-aliphatic alcohols on Mg^{2+}-ATPase activity. The reactive medium contains 0.1 ml of brush-border vesicles (0.1 mg protein) in 300 mM mannitol, 2 mM Tris-HCl (pH 7.4), 0.5 ml of 2 mM ouabain, 15 mM $MgCl_2$, 240 mM NaCl, 40 mM KCl, 60 mM Tris-HCl (pH 7.4) and 0.2 ml of 25 mM Tris-ATP. The volume is made up to 1 ml with diluted alcohols (C_1 to C_8; final concentrations indicated in abscissa). * $y = ax + b$: linear regressions calculated for each alcohol taking account of the three experimental values obtained at each concentration (○ for even alcohols, ● for odd alcohols).

the same range of concentrations used for D-glucose uptake. In the case of Mg^{2+}-ATPase there is a linear relationship between the probit of the percentage activity relative to the control and the logarithm of the alcohol concentration, for all eight alcohols. Unlike the results obtained for D-glucose uptake (Fig. 2), the slopes of the linear regressions are smaller and decrease progressively from C_1 to C_8. Sucrase activity (Fig. 4) is even less sensitive to the effects of alcohol; from propanol on, the higher concentrations of alcohols acting on Mg^{2+}-ATPase have practically no action on sucrase. To check that the zones of active concentrations of alcohols are not higher for sucrase, three extra concentrations of propanol (0.8 M, 1 M, 1.6 M), butanol (0.3 M, 0.6 M, 1.2 M), pentanol (0.12 M, 0.3 M, 0.5 M) and hexanol (0.1 M, 0.2 M, 0.5 M) were used. In these conditions, for the first four alcohols, it became clear that sucrase activity diminished considerably with increasing concentration. On the other hand, this enzyme is hardly sensitive to the action of the other alcohols, even at the strongest concentrations used.

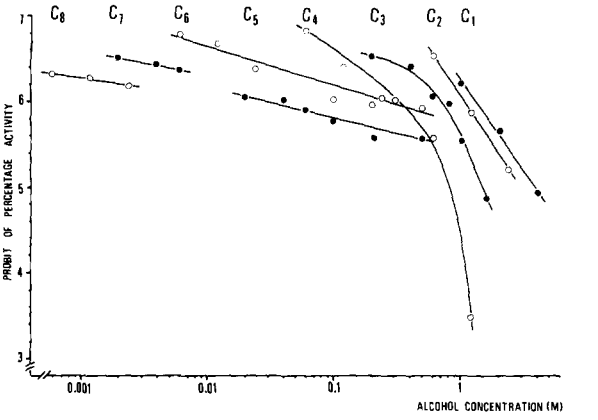

Fig. 4. Effects of *n*-aliphatic alcohols on sucrase activity. The reactive medium contains 0.1 ml of brush-border vesicles (0.1 mg protein) in 300 mM mannitol, 2 mM Tris-HCl (pH 7.4), 0.5 ml of 80 mM sodium phosphate buffer (pH 6.5) and 0.2 ml of 200 mM saccharose. The volume is made up to 1 ml with diluted alcohols (C_1 to C_8; final concentrations indicated in abscissa). For the sake of clarity, the mean of the three experimental values only is shown for each concentration (○ for even alcohols, ● for odd alcohols).

Effects of alcohols on membrane fluidity

For the controls, the mean ± S.E. ($n = 5$) of 2 $T'_{//}$ was 56.0 ± 0.2 G. A typical ESR spectrum of 5-doxyl stearic acid is shown in Fig. 5 for the label incorporated into the membrane. A decrease in 2 $T'_{//}$ (parameter showed on Fig. 5) indicates increased fluidity. Fig. 6 shows that the probit of the percentage of 2 $T'_{//}$ relative to the control is a linear function of the logarithm of the concentration for each of the alcohols. The results are comparable to those obtained for the D-glucose uptake (Fig. 2) but, in the case of fluidity, higher concentrations were used to make the decrease of 2 $T'_{//}$ clearer.

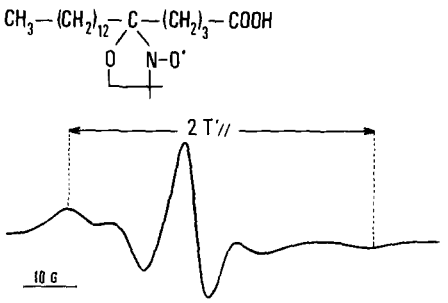

Fig. 5. First derivative ESR spectrum of 5-doxyl stearic acid incorporated in brush-border vesicles. The reactive medium contains 0.25 mg of proteins, 0.05 mM spin label in 50 µl of 300 mM mannitol and 2 mM Tris-HCl (pH 7.4).

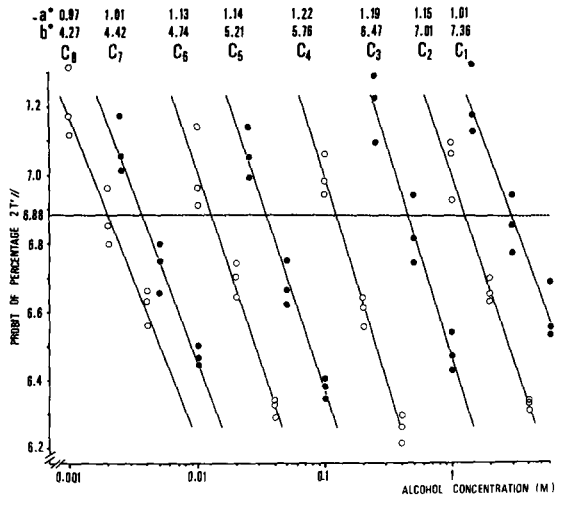

Fig. 6. Effects of *n*-aliphatic alcohols on membrane fluidity. The reactive medium is that described in Fig. 5 with, in addition, the alcohols (C_1 to C_8) at the final concentrations indicated. * $y = ax + b$: linear regressions calculated for each alcohol taking account of the three experimental values obtained at each concentration (○ for even alcohols, ● for odd alcohols).

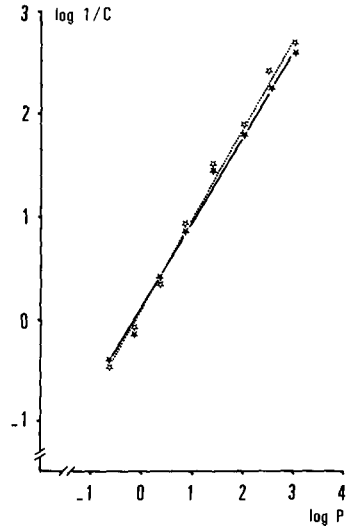

Fig. 7. Relationship between log $1/C$ and log P. The values of C represent the alcohol concentrations in the medium reducing D-glucose uptake by 80% or the parameter 2 $T'_{\parallel}$ by 3%. The values of P represent the octanol/water partition coefficients of each alcohol [20]. Linear regressions for D-glucose uptake (☆———☆, $y = 0.845x + 0.098$) and for 2 $T'_{\parallel}$ parameter (☆· · · · · ☆, $y = 0.890x + 0.097$).

Relationship between D-glucose uptake and membrane fluidity

To bring out the relationship between the effects of alcohols on the D-glucose uptake and on the 2 $T'_{\parallel}$ parameter, we chose to compare the respective percentage decreases obtained in zones of concentration very close together; which is the case for the concentrations corresponding to the ordinate 4.16 (Fig. 2) and the ordinate 6.88 (Fig. 6). They were responsable for the inhibition of 80% of the D-glucose uptake and for 3% of fluidization, respectively. In both cases a linear relationship exists between log $1/C$ and log P (Fig. 7), C being the active concentrations of alcohols in the medium and P the octanol/water partition coefficients [20]. The equations of the linear regressions obtained are practically the same.

Discussion

The gradual changes of certain physicochemical properties of *n*-aliphatic alcohols, in particular their liposolubility, is the origin of their action on various biological systems. For the *n*-aliphatic alcohol series, as for other anaesthetic drugs, there exists, in certain cases, a linear relationship between the logarithm of equiactive concentrations (C) and the number of carbon atoms in the aliphatic chain or the logarithm of the partition coefficient, P (octanol/water, membrane/water) [21–23]. Hansch and Dunn [20] have listed the equations obtained by taking log $1/C$ as a function of log P for various sets of congeners and for very different biological activities. The slope of the linear regression indicates the sensitivity of the biological system studied to the perturbations produced by the hydrophobic effect of the compounds. The use of the *n*-aliphatic series of alcohols is thus suitable for defining to what extent the membrane activities studied depend on the lipid environment.

The results obtained show that hydrophobic bonding does not play a fundamental role in maintaining Mg^{2+}-ATPase and sucrase activity. Similar results had already been obtained for the alkaline phosphatase of the brush border [24]. As far as Mg^{2+}-ATPase is concerned (Fig. 3), the inhibitions observed are less sensitive to the alcohol concentration if the alcohol is liposoluble. Previous results [24] had led to expectations of

stronger inhibitions, but the membrane fraction used was less pure. For sucrase (Fig. 4) the first four alcohols have, in the range of concentrations used, a non-negligible effect on its activity. However, the ineffectiveness of the higher alcohols in inhibiting it implies mechanisms other than the hydrophobic effect. Given their partition coefficients [20], a large fraction of the first four alcohols is present at equilibrium in the aqueous phase. This could partially explain the inhibition of the enzyme through the setting up of hydrogen bonds between the -OH groups of these alcohols and polar groups of the enzyme situated in the hydrophilic zone of the membrane. In this case it could be expected that identical concentrations of these alcohols would exert identical actions, which is not the case since the methanol concentrations are much higher than the butanol. Taking account of structural data for numerous hydrolases, notably sucrase [9,25,26], it is possible to explain these results by supposing that all the inhibitions observed are due to the simultaneous intervention of the alcohols in the hydrophilic part of the enzyme bearing the enzyme site and in the hydrophobic part inserted in the lipids. In relation with the hydrophobic effect, a modulation of sucrase activity by phospholipids has already been observed [27]. This hydrophobic interaction would probably be located in a zone, near the headgroups of the phospholipids, where the higher alcohols, by their apolar character, should interfere little.

The D-glucose uptake, like the 2 $T'_{\parallel}$ parameter, is strongly modified by the perturbations imposed on certain hydrophobic zones of the membrane. The slope of the linear regressions between log $1/C$ and log P, of the order of 0.8 in both cases, indicates, according to Hansch and Dunn [20], that the zones concerned are very sensitive to the hydrophobic effect of the alcohols. In the case of fluidity, given the position of the spin label in the membrane, these zones are well defined. But the problem is more complex for the D-glucose uptake. The alcohols could act either directly on the carrier, given the transmembrane position of this protein [10,11], or indirectly by a faster disappearance of the electrochemical gradient of sodium due to an increase in membrane permeability. Using 340 mM ethanol, Tillotson et al. [7] have shown that this alcohol inhibits D-glucose uptake indirectly by dissipating the gradient of sodium. Our experimental procedure does not allow the two hypotheses to be differentiated: by their ability to displace hydrophobic bonds, the alcohols can either perturb the lipids-carrier interaction zone or facilitate the formation of channels favorising the passage of sodium [28].

In conclusion, our results show clearly that the fluidity modified by perturbation of the hydrophobic bonds of the membrane does not play a very important part in maintaining the activity of Mg^{2+}-ATPase and sucrase but modulates the Na^{+}-coupled D-glucose uptake. These data allow one to predict the possible importance of other factors capable of inducing a change in fluidity (variation of lipid composition, of temperature, etc...) on the Na^{+}-coupled D-glucose uptake.

Acknowledgements

This work was partly supported by grant from the Institut National de la Santé et de la Recherche Médicale (C.R.L. 81 70 24). The authors wish to thank G. Reynes and A. Belliure for their technical assistance.

References

1 Sandermann, H., Jr. (1978) Biochim. Biophys. Acta 247, 209–237
2 Gordon, L.M., Sauerheber, R.D., Esgate, J.A., Dipple, I., Marchmont, R.J. and Houslay, M.D. (1980) J. Biol. Chem. 255, 4519–4527
3 Roelofsen, B., Zwaal, R.F.A. and Van Deenen, L.L.M. (1971) in Membrane-Bound Enzymes (Porcelatti, G. and Di Jeso, F., eds.), Vol. 14, pp. 209–228, Plenum Press, New York, London
4 Dinda, P.K., Hurst, R.O. and Beck, I.T. (1979) Am. J. Physiol. 237, E68–E76
5 Dinda, P.K. and Beck, I.T. (1982) Am. J. Physiol. 242, G442–G447
6 Dinda, P.K. and Beck, I.T. (1981) Am. J. Dig. Dis. 26, 23–32
7 Tillotson, L.G., Carter, E.A., Inui, K.I. and Isselbacher, K.J. (1981) Arch. Biochem. Biophys. 207, 360–370
8 Gray, J., Hoyumpa, A., Dunn, G., Henderson, G., Wilson, F. and Swift, L. (1979) Clin. Res. 27, 683A
9 Maroux, S. and Louvard, D. (1976) Biochim. Biophys. Acta 419, 189–195
10 Toggenburger, G., Kessler, M. and Semenza, G. (1982) Biochim. Biophys. Acta 688, 557–571
11 Klip, A., Grinstein, S. and Semenza, G. (1979) FEBS Lett. 99, 91–96

12 Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J. and Crane, R.K. (1973) Biochim. Biophys. Acta 323, 98–112
13 Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M. and Semenza, G. (1978) Biochim. Biophys. Acta 506, 136–154
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
15 Hübscher, G., West, G.R. and Brindley, D.N. (1965) Biochem. J. 97, 629–642
16 Hyvärinen, A. and Nikkilä, E.A. (1962) Clin. Chim. Acta 7, 140–143
17 Quigley, J.P. and Gotterer, G.S. (1969) Biochim. Biophys. Acta 173, 469–476
18 Fiske, C. and SubbaRow, J. (1926) J. Biol. Chem. 66, 375–400
19 Fisher, R.A. and Yates, P. (1963) Statistical Tables for Biological, Agricultural and Medical Research, p. 68, Oliver and Boyd, Edinburgh
20 Hansch, C. and Dunn, W.J. (1972) J. Pharm. Sci. 61, 1–19
21 Rang, H.P. (1960) Br. J. Pharmacol. 15, 185–200
22 Seeman, P. (1972) Pharmacol. Rev. 24, 583–655
23 Schneider, H. (1968) Biochim. Biophys. Acta 163, 451–458
24 Mitjavila, S., Lacombe, C. and Carrera, G. (1976) Biochem. Pharmacol. 25, 625–630
25 Sigrist, H., Ronner, P. and Semenza, G. (1975) Biochim. Biophys. Acta 406, 433–446
26 Takesue, Y. and Nishi, Y. (1978) J. Membrane Biol. 39, 285–296
27 Brunner, J., Hauser, H., Semenza, G. and Wacker, H. (1977) in Biochemistry of Membrane Transport (Semenza, G. and Carafoli, E., eds.), pp. 105–113, Springer Verlag, Berlin
28 Tanford, C. (1980) The Hydrophobic Effect, 2nd Edn., pp. 211–212, Wiley-Interscience, New York