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EFFECT OF PHLORETIN ON Na^+-DEPENDENT D-GLUCOSE UPTAKE BY INTESTINAL BRUSH BORDER MEMBRANE VESICLES*

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Abstract—In the present work we studied the effects of phloretin on Na^+-dependent D-glucose uptake by brush border membrane vesicles isolated from rabbit small intestine.

(1) Phloretin had no inhibitory effect on Na^+-dependent D-glucose uptake in the presence of equilibrated Na^+, although it inhibited the process in the presence of a Na^+ gradient. Phlorizin inhibited Na^+-dependent D-glucose uptake both in the presence and in the absence of a Na^+ gradient. Phloretin and phlorizin competed with each other for the inhibition of Na^+-energized D-glucose uptake. These results indicate that phloretin has no direct interaction with the Na^+-dependent D-glucose transporter *per se*, though phloretin and phlorizin may have a common step(s) in their inhibition mechanisms.

(2) Phloretin, but not phlorizin, was found to increase the equilibrium level of D-glucose uptake whether Na^+ was present or not. The increase in the equilibrium level was due to phloretin-induced binding of D-glucose to the brush border membrane, but not due to an increase in the intravesicular space. It seems to occur by a mechanism different from that by which phloretin inhibits Na^+-energized D-glucose uptake, because the equilibrium level of D-glucose uptake is increased by the ionized form of phloretin but Na^+-energized D-glucose uptake is inhibited by the unionized form.

It is generally accepted that small-intestinal epithelial cells have two distinct D-glucose transport systems, one being Na^+-dependent and located in the brush border membrane and the other Na^+-independent and located in the basolateral membrane. Phlorizin is a fully competitive inhibitor of the former [1–4], but phloretin, the aglycone of phlorizin, inhibits preferentially the latter [5–8]. From the results of absorption experiments with intact small-intestinal tissues, Alvarado [9] has suggested that the Na^+-dependent D-glucose transporter has two binding sites, i.e. the substrate (D-glucose) binding site and the phenol binding site and that the very potent inhibitory action of phlorizin is due to its simultaneous binding to these two sites. Subsequent observations, especially obtained with isolated brush border membrane vesicles, showed that phlorizin binds to the D-glucose binding site of the Na^+-dependent D-glucose transporter [4, 10, 11], but the question whether the suggested phenol binding site is located on the D-glucose transporter *per se* has not been answered.

To find some clues to this question we have investigated the effects of phloretin on Na^+-dependent D-glucose uptake by brush border membrane vesicles. We have obtained the results indicating that phloretin has no direct inhibitory effect on the Na^+-dependent D-glucose transporter. Another noteworthy finding is that a substantial amount of D-glucose is bound to the brush border membrane in the presence of phloretin, whether Na^+ is present or not.

MATERIALS AND METHODS

Preparation of brush border membrane vesicles. Brush border membrane vesicles were isolated from frozen rabbit small intestines by the method of Kessler *et al.* [12] with the following modifications: (1) the homogenizing solution was 0.3 M mannitol in 10 mM Tris-Hepes§, pH 7.4 (mannitol buffer) and was not diluted with water; and (2) after addition of 10 mM $CaCl_2$, non-brush border membranes in the homogenate were precipitated by centrifugation at 7000 *g* for 15 min. Brush border membrane vesicles were collected from the supernatant at 27,000 *g* for 30 min, washed once in buffer and finally resuspended in mannitol buffer containing 1 mM dithiothreitol at the concentrations of 8–15 mg protein/ml. A possibility of significant contamination with basolateral membrane vesicles could be excluded, because Na^+-independent D-glucose uptake was not appreciably inhibited by 0.4 mM phloretin (Fig. 1A).

D-Glucose uptake measurement. D-Glucose uptake was measured essentially as described by Kessler *et al.* [12] using the Millipore filtration method. For measurements of Na^+-dependent D-glucose uptake in the presence of a Na^+ gradient, 100 μl membrane vesicle suspension was rapidly mixed at 20° with 100 μl substrate solution (0.2 mM (^{3}H)-D-glucose, 0.2 M NaSCN and additions in mannitol buffer).

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§ Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Aliquots of 20 μl were withdrawn at intervals and diluted in 2 ml ice-cold stop solution (0.25 M NaCl, 1 mM phlorizin and 1 mM Tris-Hepes, pH 7.4). The diluted sample was quickly filtered through a Sartorius filter (0.6 μm pore size) and washed with 8 ml stop solution. Radioactivity on the filters was measured by liquid scintilation spectrometry in ACS-II scintilation fluid (Amersham). For measurements of Na^+-dependent D-glucose uptake in the presence of equilibrated Na^+, vesicle suspensions were preincubated with 0.1 M NaSCN in mannitol buffer for 30 min at 25°. Uptake was initiated by the addition of the substrate solution containing 0.1 M NaSCN instead of 0.2 M NaSCN. For the Na^+-independent component of the D-glucose uptake, NaSCN was replaced with KSCN. Thus Na^+-dependent uptake refers to the difference between uptake in the presence of NaSCN and KSCN.

Phloretin was dissolved in ethanol, which was found to be without effect on Na^+-dependent D-glucose uptake by rabbit intestinal brush border membrane vesicles up to the final concentration of 5% (v/v).

Data from representative experiments are illustrated and each value presented is the mean of at least 3 determinations with the same vesicle preparation.

Protein determination. Protein was determined by the method of Lowry *et al.* [13] using bovine serum albumin as a standard.

Materials. (1-^{3}H)-D-Glucose was obtained from New England Nuclear. Phloretin and phlorizin were purchased from Sigma Chemical Co. Other chemicals were of reagent grade.

RESULTS

Time course of Na^+-dependent D-glucose uptake by brush border membrane vesicles. Figure 1 shows the time course profiles of Na^+-dependent and Na^+-independent D-glucose uptake by brush border membrane vesicles, under conditions of an initial gradient of 0.1 M NaSCN or KSCN across the membrane, in the absence and presence of 0.4 mM phloretin or phlorizin. In the absence of inhibitor was seen a typical Na^+-dependent transient accumulation of D-glucose, which reached a maximum between 15 and 60 sec, and then the accumulated D-glucose was released from the vesicles, the equilibrium level being attained after 60 min. Phlorizin drastically inhibited the transient accumulation of D-glucose. Its inhibitory effect seems instantaneous, because the degree of inhibition was almost the same between the vesicles preincubated with phlorizin for 60 min prior to addition of D-glucose and those to which D-glucose and phlorizin were added at the same time. Phloretin also inhibited the Na^+-dependent transient accumulation of D-glucose, though to a lesser extent. Unlike the case of phlorizin, however, its inhibition was time-dependent; more inhibition was seen when vesicles were preincubated with phloretin (Fig. 1 and see below). Preincubation without inhibitor had no significant effect on D-glucose uptake. In addition, it should be noted that the equilibrium level of D-glucose uptake was increased about two-fold by phloretin, but not by phlorizin.

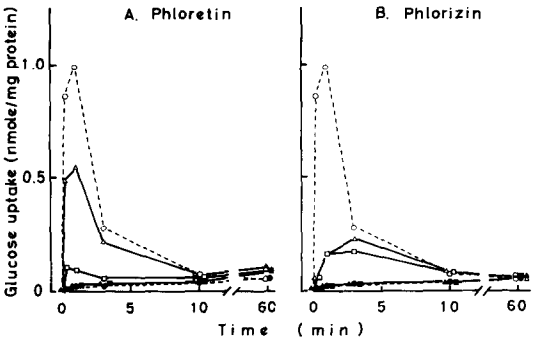

Fig. 1. Time course of D-glucose uptake by brush border membrane vesicles under different conditions. D-Glucose uptake by vesicles was measured in the presence of an initial gradient of NaSCN (open symbols) or KSCN (solid symbols) (100 mM outside, 0 inside) in the absence of presence of 0.4 mM phloretin (A) or phlorizin (B). ○, ●, Uptake by non-preincubated vesicles in the absence of inhibitor; △, ▲, uptake by non-preincubated vesicles in the presence of inhibitor; □, ■, uptake by the vesicles which, before adding D-glucose, had been preincubated with inhibitor for 60 min at 25°.

D-Glucose uptake in the presence of equilibrated Na^+. To determine whether the phloretin inhibition of Na^+-energized D-glucose transport occurred by direct effect on the D-glucose transporter *per se* or indirectly by uncoupling between transport of D-glucose and Na^+ and/or by a hastened dissipation of the Na^+ gradient, Na^+-dependent D-glucose transport was measured in the presence of Na^+ but in the absence of Na^+ gradient (Fig. 2). Phlorizin, which interacts directly with (the glucose binding site of) the D-glucose transporter [4, 10, 11], inhibited Na^+-dependent transport in this case as well, consistent with previous reports [14–16]. This result shows that the Na^+-dependent D-glucose transport

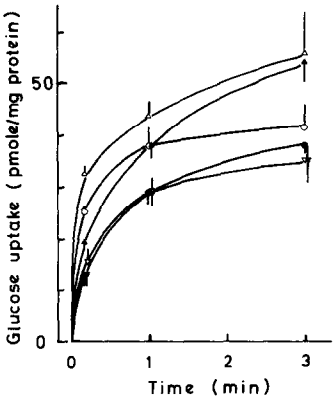

Fig. 2. D-Glucose uptake by brush border membrane vesicles in the presence of equilibrated Na^+. Brush border membrane vesicles were preincubated with 100 mM NaSCN (open symbols) or KSCN (closed symbols) for 30 min at 25°. At zero time 0.1 mM (final) (^{3}H)-D-glucose was added to the vesicle together with 100 mM NaSCN or KSCN and 0 (○, ●) or 0.5 (△, ▲) mM phloretin or 0.1 mM phlorizin (▽, ▼).

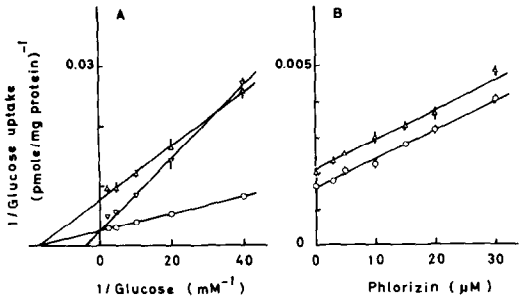

Fig. 3. Inhibition of Na^+-energized D-glucose uptake by phloretin and phlorizin. (A) Double reciprocal plots of Na^+-energized D-glucose uptake at 15 sec in the absence of inhibitor (○) and in the presence of 0.5 mM phloretin (△) or 15 μM phlorizin (▽). (B) Dixon plots of Na^+-energized D-glucose uptake at 10 sec in the presence of different phlorizin concentrations with (△) or without (○) 0.3 mM phloretin. The bars represent the S.D.; when not illustrated, the S.D. was smaller than the symbol.

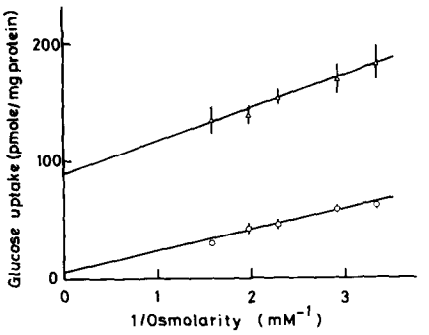

Fig. 4. Effect of medium osmolarity on D-glucose uptake by brush border membrane vesicles. D-Glucose uptake was measured at 90 min in the media of the indicated osmolarity given by different mannitol concentrations in the presence (△) or absence (○) of 0.3 mM phloretin. The osmolarity was calculated as the sum of contributions from all compounds, assuming ideal behavior. The bars represent the S.D.; when not illustrated, the S.D. was smaller than the symbol.

in the presence of equilibrated Na^+ is also mediated by the same D-glucose transporter as in the presence of a Na^+gradient. On the other hand, phloretin had no significant inhibitory effect on the Na^+-dependent D-glucose uptake. The total D-glucose uptake was increased, whether in the presence or absence of Na^+, by the addition of phloretin, because, as described later, it induces D-glucose binding to the brush border membrane. However, the Na^+-dependent uptake at 10 sec in the presence of phloretin was 13.0 pmole/mg protein, virtually the same as that obtained in the absence of phloretin (12.6 pmole/mg protein). These results indicate no direct interaction between phloretin and the Na^+-dependent D-glucose transporter *per se* in the brush border membrane.

Interaction between inhibition by phlorizin and phloretin. Na^+-energized D-glucose transport across the brush border membrane is inhibited competitively by phlorizin and non-competitively by phloretin (Fig. 3A; also see [1–3]). The Ki values for phlorizin and phloretin were 5 μM and 0.26 mM, respectively. As expected from the results in Fig. 1, the value for phloretin was time-dependent and a decreased value of 0.10 mM was obtained with the vesicles preincubated with phloretin for 30 min at 25°. Phlorizin is supposed to bind to both the D-glucose binding site of the transporter and the phenol binding site, but phloretin only to the latter site. This view implies that phlorizin and phloretin should compete with each other, in spite of their different kinetic types of inhibition. We tested this problem according to the kinetic analysis described by Colombo and Semenza [17] (Fig. 3B). The line obtained in the presence of both phlorizin and phloretin was parallel to that obtained in the presence of phlorizin alone. This result suggests mutual competition between phlorizin and phloretin for the inhibition of Na^+-energized D-glucose uptake by brush border membrane vesicles, as in the case of Na^+-dependent 6-deoxy-D-glucose uptake by hamster small-intestinal rings [17].

Effect of phloretin on the equilibrium level of D-glucose uptake. The phloretin-induced increase in the equilibrium level of D-glucose uptake can arise from changes in vesicle shape to yield a higher intravesicular space or from adsorption to the membrane of vesicles. To examine these possibilities the following two kinds of experiments were carried out.

If the phloretin effect is due to changes in vesicle shape, the equilibrium level of uptake of substrates other than D-glucose will also increase in the presence of phloretin. Na^+-dependent L-alanine uptake by brush border membrane vesicles in the presence of a Na^+ gradient showed a transient accumulation, which was inhibited by the addition of 0.4 mM phloretin. On the other hand, no increase in the equilibrium level of L-alanine uptake was induced by phloretin (data not shown).

The effect of medium osmolarity on uptake has been utilized to discriminate between binding to membrane and transport into an intravesicular space [18, 19]. When the equilibrium level of D-glucose uptake by brush border membrane vesicles was determined in the presence or absence of phloretin at different medium osmolarity and plotted against the inverse of medium osmolarity, a straight line was obtained whether phloretin was present or absent (Fig. 4). Being extrapolated to infinite medium osmolarity, the line obtained in the absence of phloretin passed near the origin, as in previous studies [18, 19], but the line in the presence of 0.3 mM phloretin intersected the y axis at the point of 89 pmole D-glucose taken up/mg protein, implying that this amount of D-glucose is bound to the membrane of brush border membrane vesicles under these conditions. The value of 114 pmole D-glucose bound/mg protein was obtained at 1 mM phloretin.

Effect of medium pH on the phloretin effects. Figure 5 shows the effect of medium pH on the Na^+-dependent transient accumulation and the equilibrium uptake level of D-glucose in the presence and absence of phloretin. The control value of the transient D-glucose accumulation was lower at pH 5.8

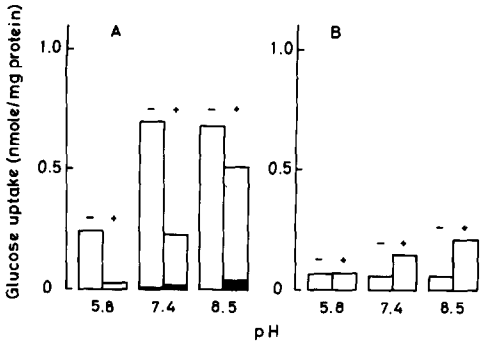

Fig. 5. pH effect on the Na^+-dependent transient accumulation (A) and the equilibrium level of D-glucose uptake (B) by brush border membrane vesicles. (^{3}H)-D-Glucose (0.1 mM) was added to the vesicles, together with 0.1 M NaSCN (□) or KSCN (■) and 0 (−) or 1 mM (+) phloretin, in the media of the indicated pH, and D-glucose uptake was measured at 15 sec (A) and 60 min (B). Buffers: pH 5.8, 10 mM Tris-Mes (2-(*N*-morpholino)ethanesulphonic acid); pH 7.4 and 8.5, 10 mM Tris-Hepes.

than at pH 7.4 and 8.5. This is attributed to the Km value for Na^+-energized D-glucose uptake being higher at pH 5.8 than at the other pHs (11); the concentration of D-glucose used was 100 μM, nearly equal to the Km for D-glucose at pH 7.4. The degree of inhibition by 1 mM phloretin of Na^+-dependent transient accumulation was greater at lower pH. The Ki value for phloretin of Na^+-energized D-glucose uptake at pH 8.5 was found to be 2.95 mM, much higher than that at pH 7.4. In contrast, the increase induced by phloretin in the equilibrium uptake level was larger at pH 8.5 than at pH 7.4 and almost zero at pH 5.8. These changes in the equilibrium level were virtually independent on Na^+. Since phloretin has a pKa of 7.4 [20, 21], these pH effects should be considered in terms of the respective concentrations of the ionized and unionized forms of phloretin (see Discussion).

DISCUSSION

The result of Fig. 3 suggests that there is a common step(s) in the mechanisms of inhibition by phlorizin and phloretin on Na^+-energized D-glucose uptake by brush border membrane vesicles. According to the hypothesis originally developed by Alvarado [9], this common step is probably one involving the binding to the phenol binding site on the Na^+-dependent D-glucose transporter or elsewhere in the brush border membrane. On the other hand, phloretin has no direct inhibitory effect on the Na^+-dependent D-glucose transporter (Fig. 2). Therefore, the Na^+-dependent D-glucose transporter *per se* is not thought to have the phenol binding site, implying that the transport system mediating Na^+-energized D-glucose transport (usually called the Na^+, D-glucose cotransporter) consists of different subunits. The subunit structure of the Na^+, D-glucose cotransporter has been suggested by the findings that it shows a molecular weight of about 160,000 under non-denaturating conditions [22] but of about 70,000 under denaturing conditions [23–25].

From labeling experiments with 4-azidophlorizin, in which the hydroxyl group of ring B of the phloretin moiety of phlorizin is replaced with an azido group, Hosang *et al.* [25] have obtained evidence indicating that a protein of Mr 72,000 is (a component of) the Na^+, D-glucose cotransporter in the intestinal brush border membrane. Since 4-azidophlorizin can be surmized to interact with the phenol binding site through the photoactive azido group [25, 26], the 4-azidophlorizin-labeled protein is thought to have the phenol binding site and thus not to be the D-glucose transporter *per se*, though it must be a component of the Na^+, D-glucose cotransporter.

Irrespective of no direct action on the Na^+-dependent D-glucose transporter, phloretin inhibits Na^+-energized D-glucose uptake by brush border membrane vesicles. This inhibitory effect of phloretin can occur by breaking coupling between the Na^+-dependent D-glucose transporter and the Na^+ gradient and/or by hastening dissipation of the Na^+ gradient. If the protein of Mr 72,000 is responsible for the above-mentioned coupling, interaction of phloretin with the phenol binding site of the protein possibly cause malfunction of the protein so that D-glucose becomes transported by the D-glucose transporter along its own chemical gradient in the presence of Na^+. In the case of phlorizin, functioning of the D-glucose transporter *per se* would be strongly blocked by binding of the glucose moiety of phlorizin to the glucose binding site of the D-glucose transporter.

4-Azidophlorizin in fact labels many proteins of brush border membrane vesicles besides the protein of Mr 72,000 [25], indicating that phlorizin and possibly phloretin can interact with many proteins. Furthermore, owing to its high hydrophobicity, phloretin (and the aglycone moiety of phlorizin) can interact with membrane lipids [27–29]. These interactions may perturb the membrane structure and change membrane permeability to Na^+, leading to a faster dissipation of the Na^+ gradient. Andersen *et al.* [27] have reported that phloretin dramatically increases the permeability of thin lipid membranes to cations. Like phloretin, *p*-chloromercuribenezene sulphonate [14], amphotericin B [15], ethanol [16] and phenolphthalein [8] have been found to inhibit Na^+-dependent D-glucose uptake by brush border membrane vesicles in the presence, but not in the absence, of Na^+ gradient. The inhibitory effect of these drugs on the concentrative D-glucose uptake has been also attributed to a hastened dissipation of the Na^+ gradient by increased membrane permeability to Na^+. It has not been investigated whether these compounds compete with phlorizin for the inhibition of Na^+-energized D-glucose uptake by brush border membrane vesicles.

The phloretin-induced increase in equilibrium level of D-glucose uptake is due to increased D-glucose binding to the brush border membrane (Fig. 4). This effect of phloretin probably represents a different effect from that causing an inhibition of the Na^+-energized D-glucose transport, as shown by pH effects on the phloretin actions (Fig. 5). Phloretin has a pKa of 7.4 [20, 21] and exists in the ionized and unionized forms in the range of physiological pH. The respective concentrations of the two forms can be calculated as described by Owen *et al.* [30].

Although the Ki values for phloretin of Na^+-energized D-glucose uptake at pH 7.4 and 8.5 are very different (0.26 and 2.95 mM, respectively), they are virtually the same in respect of the concentration of the unionized form of phloretin (0.13 and 0.22 mM, respectively); the small difference may reflect some changes in the binding site by pH change. Thus, only the unionized form of phloretin probably inhibits Na^+-energized D-glucose uptake by brush border membrane vesicles. Phlorizin also has been suggested to bind the Na^+, D-glucose cotransporter only in the unionized form [11]. On the other hand, the concentrations of the ionized form of phloretin at a total concentration of 1 mM, used in the experiment of Fig. 5, is calculated to be 0.02, 0.50 and 0.93 mM at pH 5.8, 7.4 and 8.5, respectively, the values being parallel to the extent of increase in the equilibrium uptake level (Fig. 5). Therefore, the ionized form of phloretin is thought to cause D-glucose binding to the brush border membrane. Ferguson [31] studied the effect of phenformin, a biguanide compound, on Na^+-dependent D-glucose uptake by intestinal brush border membrane vesicles. Although the authors did not refer to, their result (Fig. 1 of [31]) shows that phenformin increased the equilibrium level of D-glucose uptake. Since the drug did not change significantly the vesicle size [31], it is likely that the increase in the equilibrium level of D-glucose uptake was due to adsorption of D-glucose to the membrane. Phenformin is also thought to be active in the ionized form, because at pH 7.5, where the experiments were carried out, phenformin with a pKa of 11.8 is almost exclusively in the ionized form. At present we have no experimental data concerning the mechanism of phloretin-induced binding of D-glucose to the brush border membrane.

REFERENCES

1. F. Alvarado and R. K. Crane, *Biochim. biophys. Acta* **56**, 170 (1962).
2. F. Alvarado, *Biochim. biophys. Acta* **112**, 292 (1966).
3. D. F. Diedrich, *Arch. biochem. Biophys.* **117**, 248 (1966).
4. G. Toggenburger, M. Kessler and G. Semenza, *Biochim. biophys. Acta* **688**, 557 (1982).
5. G. A. Kimmich and J. Randles, *J. membrane biol.* **23**, 57 (1975).
6. U. Hopfer, K. Sigrist-Nelson, E. Ammann and H. Murer, *J. cell. Physiol.* **89**, 805 (1976).
7. E. M. Wright, C. H. Van Os and A. K. Mircheff, *Biochim. biophys. Acta* **597**, 112 (1980).
8. W. B. Im, D. W. Misch, D. W. Powell and R. G. Faust, *Biochem. Pharmacol.* **29**, 2307 (1980).
9. F. Alvarado, *Biochim. biophys. Acta* **135**, 483 (1967).
10. C. Tannenbaum, G. Toggenburger, M. Kessler, A. Rothstein and G. Semenza, *J. supramol. Struct.* **6**, 519 (1977).
11. G. Toggenburger, M. Kessler, A. Rothstein, G. Semenza and C. Tannenbaum, *J. membrane Biol.* **40**, 269 (1978).
12. M. Kessler, O. Acuto, C. Storelli, H. Murer, M. Muller and G. Semenza, *Biochim. biophys. Acta* **506**, 136 (1978).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. P. C. Will and U. Hopfer, *J. biol. Chem.* **254**, 3806 (1979).
15. P. S. Aronson, J. P. Hayslett and M. Kashgarian, *Am. J. Physiol.* **236**, F392 (1979).
16. L. G. Tillotson, E. A. Carter, K. Inui and K. J. Isselbacher, *Archs biochem. Biophys.* **207**, 360 (1981).
17. V. E. Colombo and G. Semenza, *Biochim. biophys. Acta* **288**, 145 (1972).
18. U. Hopfer, K. Nelson, J. Perrotto and K. J. Isselbacher, *J. biol. Chem.* **248**, 25 (1973).
19. U. Hopfer, K. Sigrist-Nelson and H. Murer, *Ann. N.Y. Acad. Sci.* **264**, 414 (1975).
20. J. D. Owen, *J. membrane biol.* **16**, 65 (1974).
21. M. L. Jennings and A. K. Solomon, *J. gen. Physiol.* **67**, 381 (1976).
22. P. Malathi, H. Preiser and R. K. Crane, *Ann. N.Y. Acad. Sci.* **358**, 253 (1980).
23. J. Lemaire and D. Maestracci, *Can. J. physiol. Pharmacol.* **56**, 760 (1978).
24. A. Klip, S. Grinstein, J. Bieber and G. Semenza, *Biochim. biophys. Acta* **598**, 100 (1980).
25. M. Hosang, E. M. Gibbs, D. F. Diedrich and G. Semenza, *FEBS Lett.* **130**, 244 (1981).
26. E. M. Gibbs, M. Hosang, B. F. X. Reber, G. Semenza and D. F. Diedrich, *Biochim. biophys. Acta* **688**, 547 (1982).
27. O. S. Andersen, A. Finkelstein, I. Katz and A. Cass, *J. Gen. Physiol.* **67**, 749 (1976).
28. J. L. Cousin and R. Motais, *Biochim. biophys. Acta* **507**, 531 (1978).
29. J. W. L. Robinson, *Gen. Pharmac.* **12**, 273 (1981).
30. J. D. Owen, M. Steggall and E. M. Eyring, *J. membrane biol.* **19**, 79 (1974).
31. D. R. Ferguson, E. K. Matthews, M. D. L. O'Connor and A. D. Schuz, *Biochem. Pharmacol.* **30**, 1613 (1981).