Biochimica et Biophysica Acta, 413 (1975) 444–452
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

BBA 77149

THE BIGUANIDE INHIBITION OF D-GLUCOSE TRANSPORT IN MEMBRANE VESICLES FROM SMALL INTESTINAL BRUSH BORDERS

MARKUS KESSLER, WERNER MEIER[a], CARLO STORELLI[b] and GIORGIO SEMENZA

Laboratorium für Biochemie der ETH-Zürich, Universitätstrasse 16, CH-8006 Zürich (Switzerland)

(Received June 3rd, 1975)

SUMMARY

Biguanides inhibit D-glucose uptake in vesicles from small-intestinal brush border membranes. Evidence is presented that this inhibition is due to a reduced concentration of Na^+ in the microenvironment of the carrier(s) for D-glucose. Biguanides do not inhibit the uptake of either D-fructose or L-glucose.

INTRODUCTION

Among the effects of biguanides in mammals is the inhibition of glucose absorption in the small intestine [1–4]. Actually, this is one of the tissues in which these drugs are the most concentrated [5–6]. The mechanism(s) whereby biguanides produce this effect in the small intestine is (are), however, still unclear [7]. In vitro experiments, mainly with everted rings or sacs of hamster or rat small intestine [3, 4 8, 9], have shown that biguanides require either relatively long incubations or preincubations for their effect.

Not all transport systems are equally inhibited by biguanides. The uptake of D-glucose and of other glucalogues [3, 4, 8, 9] of *myo*-inositol [10], of amino acids [8–10] and of calcium [11] are all inhibited by biguanides, whereas that of D-fructose is not [4]. Since biguanides are known to become associated with mitochondrial membranes [12–14], thereby inhibiting oxidative phosphorylation [15–18], and since all transport systems known to be inhibited by biguanides ultimately require metabolic energy (they can accumulate the substrate against its electrochemical gradient), the hypothesis has been put forward that biguanides inhibit these intestinal transport systems by reducing the ATP at the disposal of the tissue, or by interfering with a possible second Na pump [8, 9].

However, in view of the fact that biguanides can be taken up by a variety of membranes, the following additional or alternative possibility should also be considered: that biguanides may exert an effect on the brush border membrane itself. Vesicles

[a] Present address: Chirurgische Forschungsabteilung des Kantonspitals, Basel (Switzerland).

[b] Present address: Istituto di Fisiologia Generale dell'Università, Via Amendola 165-A, 70126 Bari (Italy).

from brush-border membranes have proved of considerable value in differentiating between events occurring at the brush border membrane or elsewhere [19]. In the last few months, another preparation of membrane vesicles from small intestinal brush border has become available in which a higher transient "active" accumulation of glucose can be easily demonstrated (Storelli, C., Kessler, M., Müller, M., Murer, H. and Semenza, G., unpublished observations). In the present paper, we have investigated whether biguanides have any effect on the sugar uptake in these vesicles.

Our working hypothesis was suggested by the observation that all biguanide-sensitive transport systems are either sodium-dependent (Na^+ being in all likelihood a co-substrate) [20, 21] (D-glucose, *myo*-inositol, amino acids) or have a cation as substrate (calcium). Now, if biguanides dissolve into the lipid leaflet of the brush border membrane, as they do in mitochondrial and artificial membranes [12–14] and/or get adsorbed at the lipid/water interface, they introduce fixed positive charges into it. Net positive charges or neutralisation of negative fixed charges in the membrane will reduce the concentration of sodium and of other cations at the micro-environment of the membrane carriers as compared with the controls in the absence of biguanides. Thus, at non-saturating concentrations of the cationic substrate (Ca^{2+}) or of activator or co-substrate (Na^+), this decrease will result first of all in a reduced degree of occupancy of the carrier, and thus of its activity. Secondly, this inhibition should be compensated by increased bulk concentrations of Na^+, which presumably bring back the local Na^+ concentration to the original levels. Thirdly, the inhibition by biguanides should be, at identical Na^+ bulk concentrations, more evident at low rather than at high ionic strengths, since a high ionic strength should decrease the potential at the membrane surface [22]. Fourthly, biguanides at intermediate Na^+ concentrations should inhibit both concentrative "active" solute transport (i.e., in the presence of a trans-membrane gradient of $\tilde{\mu}_{Na^+}$) and equilibrative solute transport in the presence of Na^+ (but in the absence of trans-membrane gradient of $\tilde{\mu}_{Na^+}$). Fifthly, Na^+-independent transport processes, e.g., those of D-fructose and of L-glucose, should not be inhibited by biguanides.

MATERIALS AND METHODS

Brush border vesicles were prepared from rabbit small intestines as described elsewhere (Storelli, C., Kessler, M., Müller, M., Murer, H. and Semenza, G., unpublished observations, see also refs 23, 24). In essence, the tissue was homogenized in hypotonic medium; after addition of Ca^{2+}, the brush-border vesicles were prepared by differential centrifugation. The vesicles had undetectable levels of cytochrome oxidase, $(Na^+ + K^+)$-ATPase, nucleic acids and had a sucrase specific activity of approx. 1.4 units/mg protein. Uptake of [1-^{3}H]-D-glucose, [1-^{14}C]-L-glucose, or [6-^{3}H]-D-fructose (all 1 mM) was measured at 20 °C in *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) Tris buffer, pH 7.5, as described by Hopfer et al. [19], with the additions or modifications given in the legends.

Radioactive compounds were obtained from Amersham, Bucks.; biguanides were a gift from Professors A. Pletscher, T. Hürlimann and F. Gey, Hoffmann-La Roche, Basel. All other compounds were obtained from commercial sources and were of the highest purity available.

RESULTS AND DISCUSSION

The preparation of brush border membrane vesicles (Storelli, C., Kessler, M., Müller, M., Murer, H. and Semenza, G., unpublished observations) used in the present study can produce a transient "active" accumulation of substrates which is higher and longer lasting than that produced by other kinds of brush border membrane vesicles. This property – which is probably due to a lower permeability for Na^+ – has allowed the unequivocal demonstration of the accumulation of amino acids in brush border vesicles [23] and was essential in the investigation of the mutual

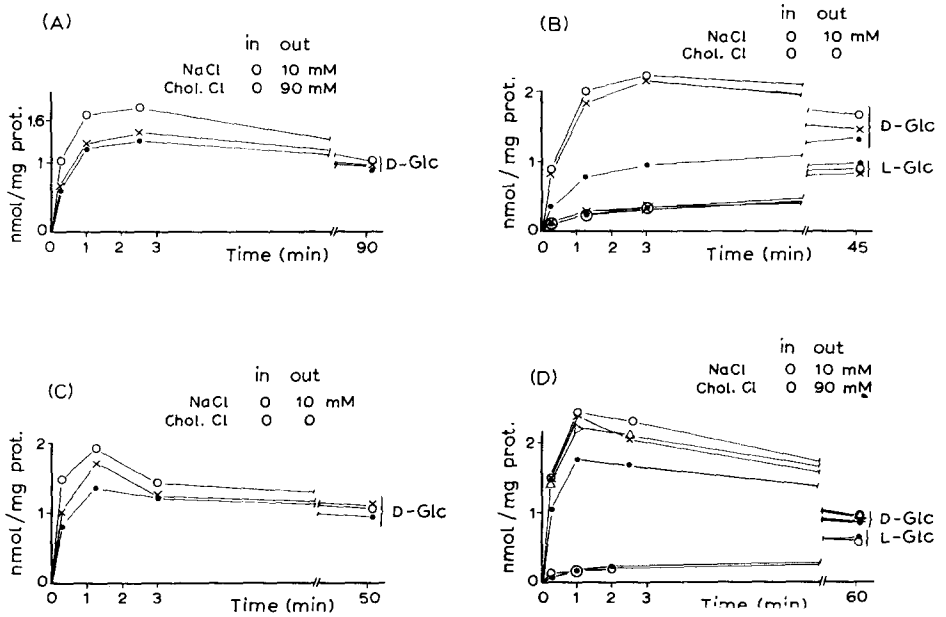

Fig. 1. Time course of D-glucose uptake in vesicles from brush border membranes, with or without biguanides, in the presence of transmembrane $\Delta\tilde{\mu}_{NA^+}$. (A), The vesicles were preincubated in 1 mM HEPES/Tris buffer, pH 7.5+2.5 mM phenethyl-biguanide+100 mM mannitol at approximately 0 °C for either 1 min (×) or 90 min (●). The incubation proper was carried out at 20 °C in a medium containing 10 mM HEPES/Tris, pH 7.5+2.5 mM phenethyl-biguanide+10 mM NaCl+90 mM choline chloride+1 mM D-glucose+1 mM L-glucose. (L-glucose uptake was measured routinely, but it is reported in some of the figures only). The controls (○) were treated similarly, except that no biguanide was present in either preincubation or incubation; they yielded the same uptake values whether preincubated or not. (B), Reversibility of biguanide inhibition. The vesicles were preincubated for 90 min at approximately 0 °C without biguanide (their uptake is indicated by ○), or in the presence of 2.5 mM phenethyl-biguanide as in Fig. 1A, and either incubated without washing (●) or after spinning them down for 20 min at 20 000 × *g* and resuspending them in biguanide-free medium (×). In the experiment reported, the incubation was carried out in 10 mM NaCl (without choline chloride), which would have revealed biguanide inhibition better (see Figs 2B and D). (C), D-glucose uptake in the presence of 1 mM (×) or 2.5 mM (●) of phenethyl-biguanide, or with no biguanide (○). Preincubation for 10 min as above; incubation in 10 mM NaCl without choline chloride at 37 °C. (D), Comparison among three biguanides. △, dimethyl biguanide; ×, butyl-biguanide; ●, phenethyl-biguanide; ○, no biguanide. During preincubation (at about 0 °C for 90 min) the biguanide concentration was 5 mM; during incubation (in 10 mM NaCl+90 mM choline chloride, as in Fig. 1A), it was 2.5 mM.

interaction phenomena between the Na-dependent D-glucose and L-amino acid transport system (24).

The efficiency of this preparation is also shown by the experiments reported in Figs 1(A–D) and 2(C): transient accumulation of D-glucose can be demonstrated with a transmembrane gradient of NaCl as low as from 10 mM (outer medium) to 0 (inner medium).

The experiments reported are all representative. Within the same vesicle preparation the results showed a very small scatter; however, it should be pointed out that quantitative variation was found among the various vesicle preparations, both in the absolute uptake values and in percent inhibition by biguanides. Thus, comparisons were always made among experiments carried out with the same vesicle preparation. Each point in the figures reported is the average of 2–3 determinations, never differing by more than a few percent.

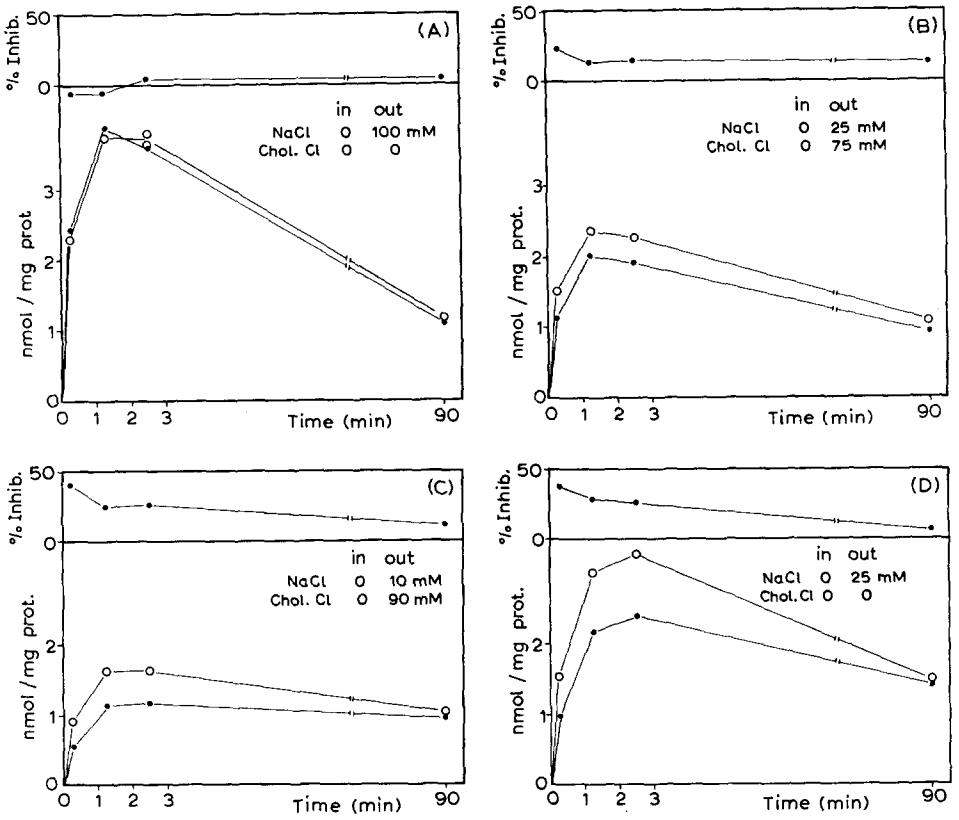

Fig. 2. Effect of 2.5 mM phenethyl-biguanide on concentrative D-glucose uptake at various concentrations and $\Delta\tilde{\mu}$-values of NaCl and at different ionic strengths. The vesicles were preincubated for 90 min at approx. 0 °C in 1 mM HEPES/Tris buffer, pH 7.5+100 mM mannitol. Incubation was at 20 °C carried out in 10 mM HEPES/Tris buffer, pH 7.5+100 mM mannitol+substrates, with the following additions: 100 mM NaCl (A); or 25 mM NaCl+75 mM choline chloride (B); or 10 mM NaCl+90 mM choline chloride (C); or 25 mM NaCl without choline chloride (D). Full circles: in 2.5 mM phenethyl-biguanide in both preincubation and incubation; empty circles: no biguanide present. The top panels indicate the extent of inhibition as percent of the controls.

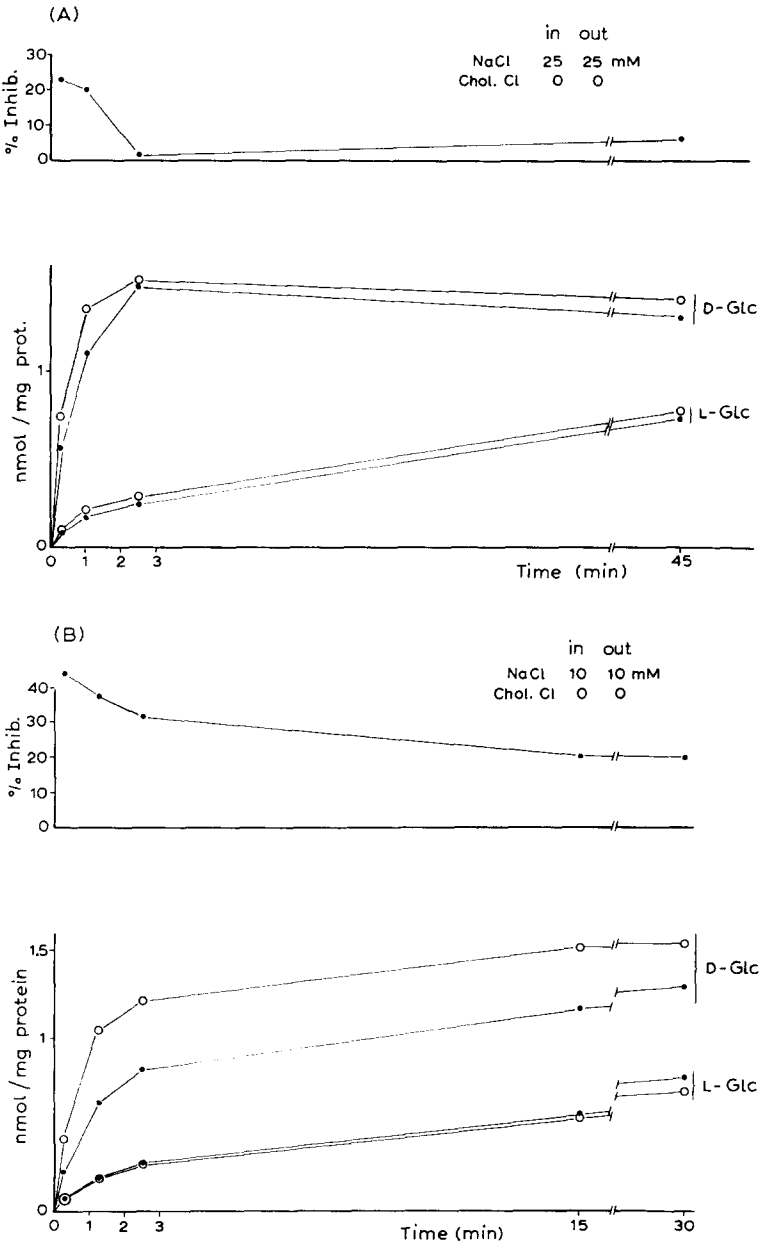

Fig. 3. Inhibition of equilibrative D-glucose uptake (i.e., in the absence of $\Delta\tilde{\mu}_{Na^+}$) by phenethyl-biguanide. The vesicles were preincubated for 90 min at approx. 0 °C in 2 mM HEPES/Tris buffer, pH 7.5+100 mM mannitol+10 or 25 mM NaCl. Incubation at 20 °C was carried out in media of the same composition, except for the addition of substrates and the increase in buffer concentration to 10 mM. Full circles: in 2.5 mM phenethyl-biguanide in both preincubation and incubation; empty circles: no biguanide present. A: in 25 mM NaCl. B: in 10 mM NaCl. No choline chloride. The uptake of D-glucose has reached the equilibrium value at 45 min, as a number of separate experiments have shown.

Preincubation, even if short, was found to be necessary for biguanide inhibition, in agreement with previous reports on everted sacs or rings [3, 4, 8, 9]. Preincubation for 90 min at room temperature was used routinely (Fig. 1A), since it did not affect our vesicle preparation adversely. The inhibition by biguanide was reversible upon washing (Fig. 1B) and dose-dependent (Fig. 1C). Not all biguanides were equally effective: among those tested at 2.5 mM concentration, the inhibition of concentrative D-glucose uptake is the largest with phenethyl-biguanide (Fig. 1D). Hereafter, most of our work was carried out with this compound.

Our results on sugar uptake in vesicles from brush-border membranes are in excellent agreement with the predictions of the working hypothesis (see Introduction), namely: (i) concentrative D-glucose uptake (in the presence of a transmembrane gradient of $\tilde{\mu}_{Na^+}$) is inhibited by biguanides more at low than at high external bulk concentrations of NaCl (Figs 2A, B and C); (ii) increased bulk concentrations of NaCl counteract the inhibition brought about by the biguanides: the D-glucose uptake in 25 mM NaCl+2.5 mM phenethyl-biguanide approaches that in 10 mM NaCl with no biguanide (Figs 2B and C). (iii) At constant Na^+ and biguanide concentrations, increasing the ionic strength reduces inhibition by biguanides (Figs 2B and D). (iv) Equilibrative D-glucose uptake (i.e., in the absence of $\tilde{\mu}_{Na^+}$ gradient) is also inhibited by biguanides and the inhibition is again dependent on the concentration of NaCl (Fig. 3). (v) D-Fructose and L-glucose uptakes, which are Na-independent, are not affected by biguanides (Fig. 4) We have found also that biguanides at low Na^+ concentrations inhibit both binding and uptake of this cation in these vesicles (data not shown). In addition, our results rule out a number of possible alternative mechanisms: (i) A first trivial possibility, namely that the inhibition may

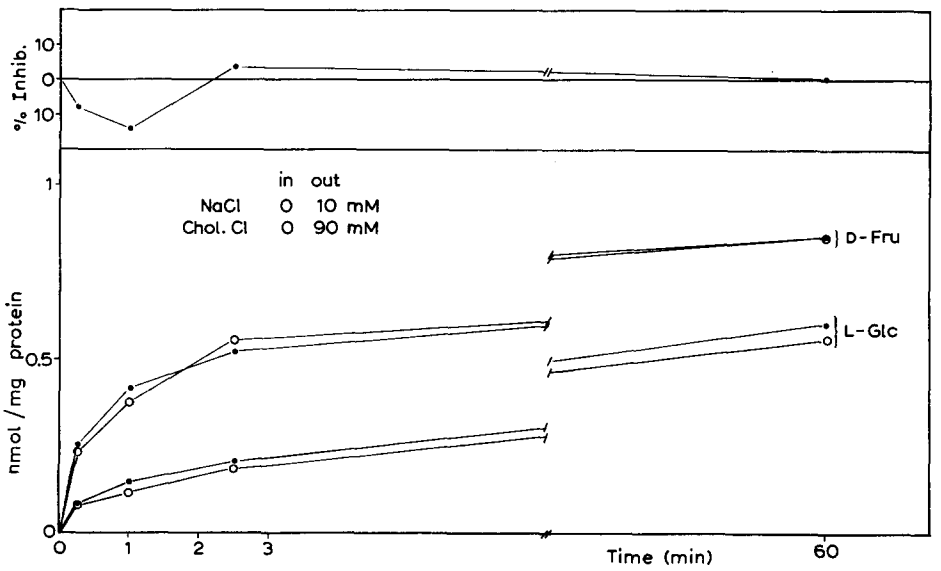

Fig. 4. Lack of inhibition of D-fructose uptake by phenethyl-biguanide. Preincubation and incubation (the latter in 10 mM NaCl+90 mM choline chloride) as in Fig. 2C, except that the substrates were D-fructose and L-glucose. Full circles: in 2.5 mM phenethyl-biguanides in both preincubation and incubation; empty circles: no biguanide.

be simulated by the vesicles being made grossly leaky by the biguanides, can be ruled out safely because biguanides clearly affected the velocity of glucose uptake rather than the equilibrium uptake (see top panels in Figs 2 and 3); these equilibrium levels are a measure of intra-vesicular space, not of substrate binding [19, Storelli, C., Kessler, M., Müller, M., Murer, H. and Semenza, G., unpublished observations) and because both L-glucose uptake (Figs 1 and 3) and D-fructose uptake were not inhibited by these drugs (Fig. 4). (ii) The inhibition of concentrative D-glucose transport (in the presence of $\Delta\tilde{\mu}_{Na^+}$) could be attributed, at least in principle, to an increased Na^+-conductance of the membrane: i.e., the primary effect of biguanides might be that of making the $\Delta\tilde{\mu}_{Na^+}$ dissipate faster. This mechanism is very unlikely on a priori grounds, the cation conductance of a membrane being, if anything, decreased by the introduction of positive fixed charges [25, 26]. Actually, it has been shown experimentally that biguanides do decrease the conductance for a positively charged permeant and increase that for a negatively charged permeant [14]. Furthermore, the experiment of Fig. 3 directly rules out an increased Na^+-conductance of the membrane vesicles (and thus a faster dissipation of the $\tilde{\mu}_{Na^+}$ gradient) as the major mechanism of biguanide effect: 2.5 mM phenethyl-biguanide inhibits both concentrative D-glucose uptake in the presence of $\tilde{\mu}_{Na^+}$ gradient (Fig. 2) and non-concentrative D-glucose equilibration in the absence of $\tilde{\mu}_{Na^+}$ gradient (Fig. 3), i.e. under both conditions by some 30–45 % in 10 mM NaCl and by some 10–40 % in 25 mM NaCl, depending on the vesicle preparation. (iii) In view of the suggested direct role of the $(Na^+ + K^+)$-ATPase in active sugar transport in the small intestine ("direct coupling", ref. 27) it may be suggested that the reduced local cation concentrations at the membrane surface may inhibit this cation-stimulated ATPase and thus inhibit D-glucose uptake in this vesicle preparation. However, a role of $(Na^+ + K^+)$-ATPase in our vesicles and thus an effect of biguanides on it can be ruled out for the following reasons: the cation-stimulated ATPase activity of these vesicles is minimal or nil (Storelli, C., Kessler, M., Müller, M., Murer, H. and Semenza, G., unpublished observations); K^+ was absent from the incubation media; the vesicles were not supplemented by ATP or any other intermediate; and biguanides (at least butyl-biguanide) do not inhibit small intestinal $(Na^+ + K^+)$-ATPase (in guinea-pig) (28). (iv) The concentrations of all cations (H^+ included) in the local microenvironment are likely to be decreased by biguanides. Thus, the possible effect of a local alkalinisation in the domain of the glucose carrier should also be considered. (Since D-fructose is transported by a system other than that of D-glucose [Refs 29, 30 and references therein], the lack of inhibition of fructose uptake by biguanides does not by itself rule out an effect on D-glucose carrier via alkalinisation).

D-Glucose uptake in 25 mM NaCl + 2.5 mM phenethyl-biguanide (Fig. 2, B) is somewhat larger than that in 10 mM NaCl in the absence of biguanides (Fig. 2, C) i.e., the inhibition by 2.5 mM phenethyl-biguanide is smaller than that brought about by a decrease of NaCl bulk concentrations from 25 mM to 10 mM. Now, assuming that the distribution profile of H^+ is the same as that of Na^+ – whatever function they follow – we should now compare this biguanide inhibition with that (if any) brought about by a corresponding decrease in the bulk concentration of H^+ (i.e., by increasing the medium pH from 7.5 to not more than 7.9). In actual fact, the uptake of D-glucose in these vesicles is found to be pH-insensitive from pH 6 to at least 8 (Storelli, C., Kessler, M., Müller, M., Murer, H. and Semenza, G., unpublished observations).

Thus, the inhibition brought about by 2.5 mM phenethyl-biguanide cannot be explained by local alkalinisation.

We conclude, therefore, that the effect of biguanides on D-glucose uptake in this preparation of brush border vesicles is fully accounted for by the working hypothesis described at the beginning of this paper, which is within the framework of the co-transport theory of Na-activation of glucose/galactose transport across the brush border pole of small intestinal cells [20, 21].

An alternative or additional, albeit less probable, mechanism could also explain our data: biguanides may interact directly with some negatively charged groups in the carrier proteins. Such hypothetical interactions, though, would have not to be possible in the D-fructose carrier.

Finally, we want to point out that the proposed mechanism for biguanide inhibition of Na-dependent transport systems in isolated vesicles from brush border membranes need not be the sole, or even the overriding mechanism in the effect of biguanides in the small intestine, let alone in the whole organism [2, 31–34]. It is quite likely, rather, in view of the many effects of these drugs, that other mechanisms are operative as well. An assessment of the contribution of the individual factors was outside the scope of the present investigation.

ACKNOWLEDGEMENTS

This work was supported in part by the Swiss National Science Foundation, Berne, and by Hoffmann-LaRoche, Basel.

REFERENCES

1 Biró, L., Bányász, T., Kovács, M. B., Bajor, M. (1961) Klin. Wochenschr. 39, 760–762
2 Czyzyk, A., Tawecki, J., Sadowski, J., Ponikowska, I. and Szczepanik, Z. (1968) Diabetes 17, 492–498
3 Lorch, E. (1971) Diabetologia 7, 195–203
4 Caspary, W. F. and Creutzfeld, W. (1971) Diabetologia 7, 379–385
5 Wick, A. N., Mobley, P. W. and Stewart, C. J. (1970) in Diabetes, 154–155, Excerpta Medica, Amsterdam
6 Yoh, Y.-J. (1967) Jap. J. Pharmacol. 17, 439–449
7 Love, A. H. G. (1969) Diabetologia 5, 422
8 Robinson, J. W. L. and Luisier, A. L. (1972) Naunyn-Schmiedeberg's Arch. Pharmacol. 274, R 93
9 Luisier, A. L. and Robinson, J. W. L. (1972) Experientia 28, 729
10 Caspary, W. F. and Creutzfeld, W. (1973) Diabetologia 9, 6–12
11 Caspary, W. F. and Creutzfeld, W. (1971) Verh. Dtsch. Ges. Inn. Med. 77, 194–197
12 Schäfer, G. and Bojanowski, D. (1972) Eur. J. Biochem. 27, 364–375
13 Schäfer, G. (1974) Eur. J. Biochem. 45, 57–66
14 Schäfer, G. and Rieger, K. (1974) Eur. J. Biochem. 46, 613–623
15 Pressman, B. C. (1962) Fed. Proc. 21, 55
16 Pressman, B. C. (1963) J. Biol. Chem. 238, 401–409
17 Chappell, J. B. (1963) J. Biol. Chem. 238, 410–417
18 Chance, B. and Hollunger, G. (1963) J. Biol. Chem. 238, 432–438
19 Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K. J. (1973) J. Biol. Chem. 248, 25-32
20 Crane, R. K. C. (1965) Fed. Proc. 24, 1000–1005
21 Schultz, S. G. and Curran, P. C. (1970) Physiol. Rev. 50, 637–718
22 Davies, J. T. and Rideal, E. K. (1963) in Interfacial Phenomena, 2nd edn., pp. 75–94, Academic Press, New York

23 Sigrist-Nelson, K., Murer, H. and Hopfer, U. (1975) J. Biol. Chem. 250, 5674–5680
24 Murer, H. Sigrist-Nelson, K., and Hopfer, U. (1975) J. Biol. Chem., in press
25 McLaughlin, S. G. A., Szabo, G., Eisenman, G. and Ciani, S. M. (1970) Proc. Natl. Acad. Sci. U.S. 67, 1268–1275
26 Neumcke, B. (1970) Biophysik 6, 231–240
27 Kimmich, G. A. (1973) Biochim. Biophys. Acta 300, 31–78
28 Luisier, A. L. and Robinson, J. W. L. (1973) in "Compar. Physiol." (Bolis, L., Schmidt-Nielsen, K. and Maddrell, S. H. P. eds) pp. 465–475, North Holland Publ. Co.
29 Honegger, P. and Semenza, G. (1973) Biochim. Biophys. Acta 318, 390–410
30 Sigrist-Nelson, K. and Hopfer, H. (1974) Biochim. Biophys. Acta 367, 247–254
31 Hollobangh, S. L., Rao, B. and Kruger, F. A. (1970) Diabetes 19, 45–49
32 Kruger, F. A., Altschuld, R. A. and Hollobangh, S. L. (1970) Diabetes 19, 50–52
33 Berger, W. (1968) Diabetologie 4, 382
34 Creutzfeld, W., Willms, B. and Caspary, W. F. (1971) 7th Congr. of the Int. Diabetes Fed., pp. 95–106, Excerpta Medica, Amsterdam