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THE INTERACTION OF PHENFORMIN AND PHLORIZIN WITH BRUSH BORDER MEMBRANE VESICLES, PHOSPHOLIPID LIPOSOMES, AND PHOSPHOLIPID LIPOSOMES CONTAINING BRUSH BORDER MEMBRANE PROTEIN

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Abstract—The actions of phenformin and phlorizin were compared using membrane preparations of three different types. Both drugs inhibited glucose transport *in vitro*, changed the fluorescence of the membrane bound probe ANS and altered membrane surface charge as measured by laser-Doppler anemometry. The implications of these findings are discussed in relation to the mechanism of action of the two inhibitors.

The biguanide compound phenformin inhibits the uptake of D-glucose from the small intestine [1], an effect which may contribute to its antidiabetic action. The molecular basis for this inhibition of hexose transport is unknown although an action invoking alteration in membrane surface charge has been proposed [2].

The availability of three different membrane preparations, namely phospholipid liposomes, phospholipid liposomes incorporating brush border proteins isolated from neonatal pig small intestine, and vesicles formed exclusively from pinched-off small intestinal brush border membranes from the same source has enabled us to explore more directly the mechanism by which phenformin interacts with constituent groups of the cell membrane mosaic. Under precisely similar conditions a comparison has been made with the corresponding action of phlorizin which is well established as an inhibitor of D-glucose transport by binding at its transport site in small intestine and kidney brush borders [3, 4].

In the experiments described here the interaction of phenformin and phlorizin with a given membrane type has been investigated in each of two ways: (i) by employing 8-anilino-1-naphthalene sulphonic acid (ANS) as a fluorescent probe of membrane structure, and (ii) by determining any alteration in membrane surface charge through measurement of the electrophoretic mobility and size of membrane vesicles using the dynamic light scattering technique of photon correlation spectroscopy and laser-Doppler anemometry [5, 6]. Finally, for a functional assessment of the relative potency of the two compounds in blocking membrane D-glucose transport the kinetics of labelled D-glucose uptake were determined for brush border vesicles in the presence or absence of each compound.

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MATERIALS AND METHODS

Materials. All the chemicals used were of ANALAR grade. D-[1-^{3}H]-glucose (8.3 Ci/mmol), D-[1-^{14}C]-mannitol (60 Ci/mmol), [U-^{14}C] sucrose (382 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, U.K.). 1-phenethylbiguanide hydrochloride (phenformin) was supplied by Aldrich Chemical Co. Ltd. (Gillingham, U.K.), Phloretin-2′-β-D-glucoside (phlorizin), 1-anilinonaphthalene-8-sulphonic acid (ANS) and L-α-phosphatidylcholine (from Soya bean) by Sigma Chemical Co. (Kingston-upon-Thames, U.K.). Liquid scintillation fluid ELS-294 came from Koch-Light Laboratories Ltd. (Colnbrook, U.K.). Unsuckled neonatal pigs were from the Babraham herd.

Membrane vesicle preparations. A distinction is made throughout this paper between liposomes which are composed solely of lipid, i.e. L-α-phosphatidylcholine, and those vesicles which contain proteins. The latter vesicles were of two types, (i) resealed pinched-off brush border membrane vesicles, and (ii) artificial vesicles made by incorporating brush border proteins into liposomal membranes.

Phosphatidylcholine liposomes were prepared by sonicating a mixture of 100 mg L-α-phosphatidylcholine in 5 ml of an aqueous buffer solution (composition of which is given for different experiments) under N_2 for 3 min; this produces a largely unilamellar liposome population. Sonication was carried out either with a Kerry Ultrasonic Generator (Type KG 80/1, frequency 80 kHz, 40 W, water bath sonicator), or an MSE 150 W Ultrasonic disintegrator using a titanium probe, frequency 20 kHz. In both cases the preparations were maintained at 4° in an ice-water bath, and sonicated for 3 min.

Resealed pinched-off brush border membrane vesicles were made by the method of Kessler *et al.* [7]; the prepared vesicles were suspended in 50 mM mannitol, 10 mM Tris-Hepes buffer at pH 7.5, and the protein content assayed by the method of Lowry *et al.* [8]. Purity of the vesicle preparations was rou-

tinely checked both by phase contrast microscopy and enzyme assay [9].

Phosphatidylcholine liposomes incorporating protein were made by first preparing brush border vesicles as above, the vesicles were then solubilised in Triton X-100 1% (v/v) (Crane *et al.* [10]). The vesicles were incubated with Triton X-100 for 60 min at 4°. The preparation was centrifuged at 30,000 *g* for 30 min. Protein analysis of supernatant and pellet indicated that about 70% of the membrane protein was solubilised. Triton X-100 was removed subsequently by means of a Bio Bead SM2 column (Kasahara and Hinkle [11]), and the extracted brush-border protein incorporated into phosphatidylcholine liposomes by sonicating the protein and phosphatidylcholine together in a ratio of 2:25 (w/w) for 30 min under N_2. These protein-supplemented liposomes were centrifuged at 50,000 *g* for 30 min, and the pellet resuspended in 1 ml of 10% sucrose solution.

Glucose uptake. D-glucose uptake measurements were made by the method of Murer and Hopfer [12]. Vesicle suspensions containing 1–2 mg/ml protein were incubated in 1 mM radio-labelled glucose at room temperature (in NaSCN 100 mM, mannitol 100 mM, Tris-Hepes 10 mM pH 7.5) aliquots were removed at intervals and filtered rapidly through a Millipore filter (PH 0.3 μm, 25 mm dia), and washed with 1 ml ice-cold stopping solution (198.5 mM NaSCN, 2 mM $MgSO_4$, 1 mM $HgCl_2$, 0.5 mM phlorizin, 10 mM Tris-Hepes buffer, pH 7.5). Extravesicular space was measured by addition to this solution of ^{14}C-sucrose or mannitol. The vesicles were washed a second time with 5 ml ice cold stopping solution without radiolabel. Radioactivity on the filters was measured by liquid scintillation spectrometry in ELS-294 scintillation fluid (Koch-Light). Binding of D-glucose, mannitol or sucrose to filters was less than 1%.

Fluorescence measurements. These experiments were performed in 1 mM sodium or potassium phosphate buffer, pH 7.4 containing 10 per cent sucrose, 400 μM ANS; vesicles or liposomes equivalent to 2 mg of lipid were added to each cuvette [13]. Phenformin or phlorizin solutions were prepared in the appropriate buffer and added in the concentrations given in each experiment. Fluorescence was excited at 375 nm and emission scanned from 420 to 500 nm; the peak fluorescence found at 480 nm was measured. All experiments were carried out at room temperature, i.e. 22°. The instrument was a Perkin–Elmer MPF-4. Phenformin increased ANS fluorescence so that fluorescence produced by ANS in its absence was subtracted from each value, phlorizin reduced ANS fluorescence so the difference between the initial fluorescence and that obtained in the presence of the drug is calculated as a positive value.

Photon correlation spectroscopy (PCS). Coherent light from a 15 mW He–Ne Laser (Spectra-Physics, 124A) at 632.8 nm was focused at the centre of the sample (2 ml) in a glass cuvette placed in a temperature controlled bath at 30 ± 0.05°. The light scattered at an angle of 90° was detected by a photomultiplier tube the photocurrent of which was amplified, digitized and analysed by a Malvern K7023 Autocorrelator. Measurement of the autocorrelation function $g^{(1)}(\tau) = \exp(-DK^2\tau)$ yields the diffusion coefficient (D) and hence particle diameter by the Stokes–Einstein relation. These experiments were carried out in 10 per cent sucrose, pH 5–6.

Laser-Doppler anemometry (LDA). For these determinations the He–Ne laser output passed successively through a beam splitter, filter array and lens to focus the two beams at a minimal crossover volume in the cuvette centre. Particle mobility at this point was controlled by a pulsed electric field applied through electrodes situated normal to the beam axis. The Doppler shift of the irradiation frequency due to particle movement is detected by the PM tube placed at a low forward angle. In this mode the autocorrelation function incorporates a cosine term characteristic of the electrophoretic mobility and hence particle surface charge density [5, 6].

In all experiments the standard error of the mean was calculated and the significance of differences between means determined using Student's *t*-test.

RESULTS

Glucose uptake

The following experiments were undertaken to compare the actions of phenformin and phlorizin on sodium-dependent glucose uptake into pinched-off small intestinal brush-border membrane vesicles. These vesicles were made in sodium-free medium and incubated in sodium thiocyanate solution containing 1 mM D-[1-^{3}H]-glucose. There was a time dependent uptake of glucose reaching a peak between 24 and 77 sec; after that time the back-flux of glucose produced a reduction in vesicular levels. Phenformin produced a significant ($P=0.05$) inhibition of uptake (Fig. 1) at 18 sec. This inhibition was dose dependent. In separate experiments (not shown) the uptake of glucose at 60 sec in the presence of 1 mM phenformin was 2086 ± 640 pmole glucose/mg protein ($n = 7$), and at 2.5 mM phenformin, 714 ± 100 pmole glucose/mg protein ($n = 6$). Phlorizin 1 mM gave an even more pronounced inhibition of D-glucose uptake (Fig. 1).

Fluorescence measurements. ANS produces significant fluorescence only when bound to hydrophobic membrane components and changes in fluorescence are produced only when compounds become bound in close proximity to ANS molecules [13]. Fluorescence changes produced by phenformin or phlorizin were therefore examined using this method. Control experiments confirmed the fact that the addition of ANS and either phlorizin or phenformin produced, in the absence of liposomes or vesicles, no change in fluorescence from the basal level detected in buffer alone.

Phenformin is positively charged at pH 7.4 so ionic binding to the lipid membrane might be expected to change both surface charge and fluorescence. Addition of phenformin to phosphatidylcholine liposomes in sodium medium and in the presence of a fixed concentration of ANS (400 μM) produced a hyperbolic increase in fluorescence between 1 and 15 mM phenformin (Fig. 2). It was possible therefore, to calculate the phenformin concentration at which half-maximal stimulation of fluorescence occurred (K_m) and the maximum fluorescence $F_{(max)}$

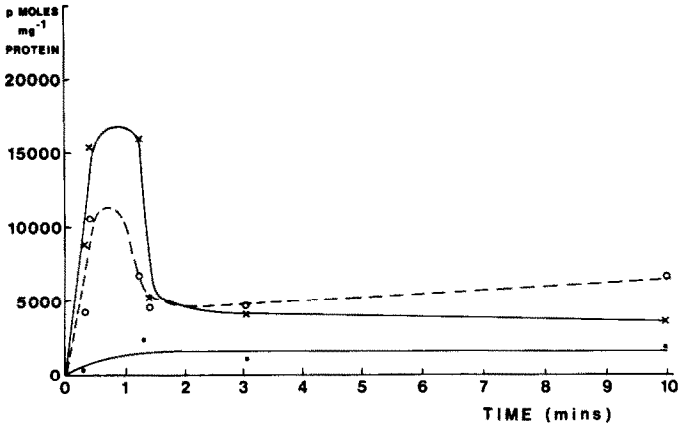

Fig. 1. Uptake of D-glucose into pinched-off brush border membrane vesicles (pmoles D-glucose. mg^{-1} protein) showing inhibition by phenformin and phlorizin, x = control, ○ = 1 mM phenformin, ● = 1 mM phlorizin. Standard error bars are omitted for clarity but in no case exceed ±4000 pmoles (n = 2–6).

produced by phenformin; these results are shown in Table 1 with the appropriate values calculated by Wilkinson's method i.e. S/V against S [14] from a minimum of 5 experiments. When the experiments were repeated in 1 mM potassium chloride solution the K_m was about half that in sodium, though $F_{(max)}$ was the same (Table 1). With pinched-off brush-border vesicles there was again a hyperbolic relationship between the increase in fluorescence and phenformin concentration, although there was no difference in $K_{(m)}$ or $F_{(max)}$ in sodium or potassium media. In the phosphatidylcholine liposomes containing brush-border protein on the other hand, there was a linear relationship between fluorescence and increasing phenformin concentrations, and no differences in fluorescence when these protein-supplemented liposomes were suspended in 1 mM sodium or potassium (Table 1). The effects of phenformin on fluorescence in phosphatidylcholine liposomes and those containing brush-border proteins indicates that the binding of the drug to these two membrane types is markedly different.

Addition of phlorizin to phosphatidylcholine liposomes, brush-border membrane vesicles or to phosphatidylcholine liposomes containing brush-border protein always produced a *reduction* in ANS fluorescence. This effect was also hyperbolic (see Fig. 3) and calculated $K_{(m)}$ and $F_{(min)}$ values are shown in Table 1. There were no significant differences between the effects of phlorizin on the brush-border membrane vesicles or protein-supplemented liposomes of Na or K media except that the $K_{(m)}$ for brush-border vesicles in sodium was significantly larger than for the other membrane types, and the maximum inhibition of fluorescence in sodium was significantly larger than in potassium medium. This is of interest because phlorizin inhibits sodium dependent glucose transport.

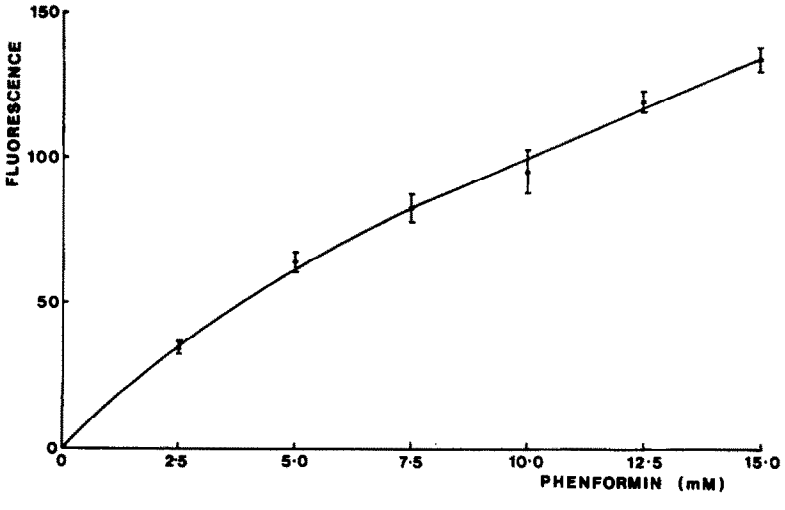

Fig. 2. Increase in ANS fluorescence of phosphatidylcholine liposomes produced by increasing concentrations of phenformin in medium containing 1 mM Na^+. Standard errors are indicated by the vertical bars (n = 6). Fluorescence in arbitrary units, measured at 480 nm.

Table 1. Effects of phenformin and phlorizin on ANS fluorescence of liposomes and vesicles*

Effects on ANS fluorescence of	Phosphatidylcholine liposomes only		Pinched off brush border membrane vesicles		Protein-supplemented liposomes Linear relationship		
	K_m (mM)	F_{max}	K_m (mM)	F_{max}	Slope	Intercept	Correlation Coefficient
Phenformin							
1 mM Na^+	18.16 ± 2.99	295.3 ± 19.6	53.31 ± 9.5	320.9 ± 68.4	4.44 ± 0.50	5.04 ± 4.8	0.883
1 mM K^+	7.08 ± 0.36	342.9 ± 13.3	41.18 ± 8.1	248.12 ± 49.5	4.37 ± 0.22	3.87 ± 2.1	0.981
	P < 0.005	n.s.	n.s.	n.s.	n.s.	n.s.	
	K_m (mM)	F_{min}	K_m (mM)	F_{min}	K_m (mM)	F_{min}	
Phlorizin							
1 mM Na^+	2.67 ± 0.41	77.03 ± 4.5	9.45 ± 1.1	100.1 ± 8.98	3.21 ± 0.30	63.19 ± 2.4	
1 mM K^+	4.00 ± 0.38	102.04 ± 6.9	3.93 ± 0.37	78.21 ± 3.9	3.35 ± 0.85	66.41 ± 5.8	
	P<0.05	P<0.025	P<0.001	P = 0.05	n.s.	n.s.	

* Significance values by Student's *t*-test for comparisons between Na^+ or K^+ media. Each mean value is derived from between 5 and 8 experiments.

Measurements of particle size and electrophoretic mobility by photon correlation spectroscopy and laser-Doppler anemometry

In view of the discrepancy between the effects of phenformin and phlorizin on ANS fluorescence demonstrated above and the previous assumption that ANS fluorescence reflects membrane surface charge [2] it was important to determine the action of these two substances on the surface charge density of each type of membrane preparation by a direct method. This was achieved by measuring liposome or vesicle electrophoretic mobility by the combined PCS-LDA system.

In these experiments membrane particles were subjected to an electric field under precisely controlled conditions as described above. Their mobilities and size were monitored continuously. Particle sizes are given in Table 2. It is clear that pinched-off brush-border membrane vesicles are largest, phosphatidylcholine liposomes smallest, and liposomes incorporating proteins intermediate, in size. Neither the presence of the two drugs nor substitution of sodium for potassium produced marked changes in any particle size, indicating the lack of complicating osmotic effects or aggregation.

From Table 2 it is also evident that vesicles formed from pinched-off brush-border membranes have a lesser mobility and hence surface charge density, than phosphatidylcholine liposomes [i.e. compare Table 2 (A, B, F and G), and (C and D)].

The effect produced by both phenformin 1 mM and phlorizin 1 mM is a decrease in vesicle mobility but in all experiments phenformin is much more potent than phlorizin. Furthermore, whereas phenformin is fully effective in the presence of either K^+ or Na^+, phlorizin has little or no effect in the presence of Na^+; nor does it prevent the phenformin-induced decrease in mobility [see Table 2 (B and D)].

The phenformin effect is not due simply to a change in ionic strength or the addition of mono-

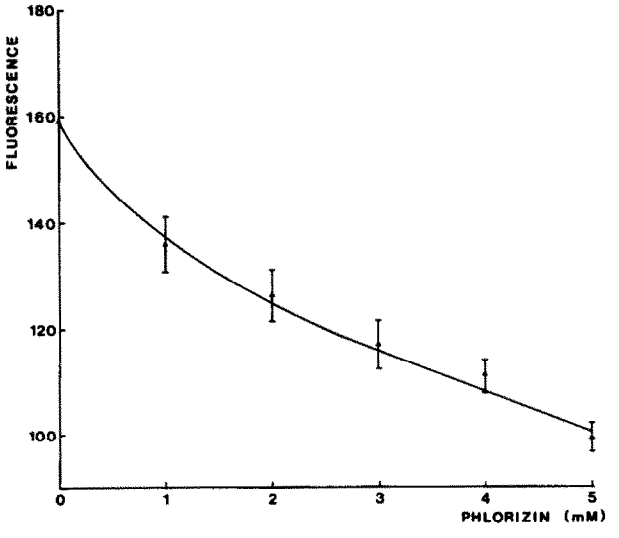

Fig. 3. Reduction in ANS fluorescence produced by increasing phlorizin concentrations in medium containing 1 mM Na^+. Standard errors are indicated by the vertical bars (n = 4). Fluorescence in arbitrary units, measured at 480 nm.

Table 2. Effects of phenformin and phlorizin on the electrophoretic mobility of liposomes and vesicles

Membrane preparation	Experimental condition	Electrophoretic mobility (μm/sec)/(V/cm) ± S.E.	P	Decrease in mobility (%)	Vesicle size (mμ ± S.E.)
Phosphatidylcholine liposomes	A. 1. KCl 1 mM	8.02 ± 0.16 (6)	–	–	173 ± 3.4 (6)
	2. KCl 1 mM + Phenformin 1 mM	6.22 ± 0.19 (6)	<0.0005	22	204 ± 4.3 (6)
	3. KCl 1 mM + Phlorizin 1 mM	7.00 ± 0.12 (6)	<0.0005	13	258 ± 3.7 (6)
	B. 1. NaCl 1 mM	8.21 ± 0.11 (6)	–	–	264 ± 8.8 (6)
	2. NaCl 1 mM + Phenformin 1 mM	5.99 ± 0.15 (5)	<0.0005	27	254 ± 7.1 (5)
	3. NaCl 1 mM + Phlorizin 1 mM	8.56 ± 0.06 (5)		0	250 ± 1.4 (6)
	4. NaCl 1 mM + Phenformin 1 mM + Phlorizin 1 mM	5.61 ± 0.10 (6)	<0.0005	32	174 ± 6.8 (6)
Brush-border membrane vesicles	C. 1. KCl 1 mM	3.63 ± 0.12 (5)	–	–	407 ± 6.0 (5)
	2. KCl 1 mM + Phenformin 1 mM	3.17 ± 0.00 (3)	<0.025	13	386 ± 5.4 (6)
	3. KCl 1 mM + Phlorizin 1 mM	3.27 ± 0.14 (6)	n.s.	10	408 ± 22.0 (6)
	D. 1. NaCl 1 mM	3.53 ± 0.06 (6)	–	–	435 ± 12.8 (6)
	2. NaCl 1 mM + Phenformin 1 mM	2.91 ± 0.08 (6)	<0.0005	18	410 ± 14.5 (7)
	3. NaCl 1 mM + Phlorizin 1 mM	3.29 ± 0.11 (6)	n.s.	7	491 ± 11.0 (5)
	4. NaCl 1 mM + Phenformin 1 mM + Phlorizin 1 mM	2.84 ± 0.07 (6)	<0.0005	20	423 ± 19.0 (5)
Phosphatidylcholine liposomes sonicated with bovine serum albumin*	E. 1. KCl 1 mM	8.04 ± 0.08 (6)	–	–	397 ± 5.0 (6)
	2. KCl 1 mM + Phenformin 1 mM	6.92 ± 0.19 (6)	<0.0005	14	343 ± 9.8 (6)
	3. KCl 1 mM + Phlorizin	7.60 ± 0.13 (6)	<0.025	6	313 ± 9.9 (6)
Phosphatidylcholine liposomes incorporating brush-border membrane protein	F. 1. KCl 1 mM	7.99 ± 0.09 (6)	–	–	331 ± 9.6 (6)
	2. KCl 1 mM + Phenformin 1 mM	6.12 ± 0.00 (6)	<0.0005	23	312 ± 9.4 (6)
	3. KCl 1 mM + Phlorizin 1 mM	7.64 ± 0.12 (6)	<0.05	4	276 ± 9.0 (6)
	4.† KCl 1 mM	8.93 ± 0.09 (6)	–	–	310 ± 7.4 (6)
	5.† KCl 1 mM + Phenformin 1 mM	5.50 ± 0.11 (5)	<0.0005	38	287 ± 7.9 (6)
	6.† KCl 1 mM + Phlorizin 1 mM	7.21 ± 0.10 (6)	<0.0005	19	277 ± 5.9 (6)
	G. 1. NaCl 1 mM	7.68 ± 0.24 (5)	–	–	160 ± 4.3 (6)
	2. NaCl 1 mM + Phenformin 1 mM	5.31 ± 0.10 (6)	<0.0005	31	167 ± 5.2 (6)
	3. NaCl 1 mM + Phlorizin 1 mM	7.70 ± 0.16 (6)	n.s.	0	173 ± 7.4 (6)

* These liposomes were prepared with 25 mg phosphatidylcholine and 2 mg of BSA or solubilized brush border membrane protein suspended in 1 ml of buffer solution.

† The solubilized brush border membrane protein was prepared in these experiments in buffer containing dithiothreitol rather than in 10 per cent sucrose solution.

valent cation. This is demonstrated by the fact that when the KCl or NaCl concentration is increased to the same extent (i.e. from 1 to 2 mM) there is no reduction whatsoever in electrophoretic mobility (for brush-border membrane vesicles 3.30 ± 0.00, 3.73 ± 0.14 (μm/sec)/(V/cm) in KCl 1 and 2 mM, respectively; for phosphatidylcholine liposomes incorporating brush-border membrane proteins, 8.20 ± 0.24, 8.59 ± 0.32 in KCl and 7.68 ± 0.24, 7.86 ± 0.15 (μm/sec)/(V/cm) in NaCl, 1 and 2 mM, respectively). Furthermore, neither the presence of a model amphoteric protein (BSA) or incorporation of solubilized brush-border membrane protein markedly decreased the mobility of the phosphatidylcholine liposomes *per se*. On the other hand only the brush border membrane proteins enhanced the effect of phenformin in decreasing the mobility, and hence surface charge, of the phosphatidylcholine liposomes [Table 2 (F and G)].

Theoretically, the electrophoretic mobility of a particle is dependent primarily upon its surface charge density and, within certain limits, relatively independent of particle size. There should therefore be little effect of particle size from one preparation to another (of the same type) upon surface charge density and electrophoretic mobility. Our observations are in accord with this prediction [e.g. compare Table 2 (F and G)].

DISCUSSION

Phenformin and phlorizin both inhibited glucose uptake into pinched-off brush-border vesicles, although it is clear that phlorizin is the more effective of the two drugs. In experiments with either compound an overshoot was seen, i.e. after about 60 sec, the amount of glucose taken up into the vesicles rapidly decreased. Of the possible explanations for the overshoot, which has previously been detected [7, 12], the most likely is that movement into vesicles of sodium thiocyanate is more rapid initially than efflux of potassium chloride, therefore the vesicles first swell and then shrink as they equilibrate osmotically. Despite the difference in potency these results demonstrate conclusively that in the concentrations used in our experiments both phenformin and phlorizin will inhibit glucose transport *in vitro*.

Interactions of phenformin and other biguanides with liposomes and mitochondrial membranes have been extensively investigated by Schäfer and Bojanowski [15], Schäfer and Reiger [16] and Schäfer [17]. They have found that phenformin partitions preferentially into a hydrophobic medium and is therefore likely to become concentrated in the membrane phospholipid phase. Indeed their experimental results show a good correlation between the binding to various membrane types of different biguanide analogues and their octanol/water partition coefficients. Since phenformin is positively charged this hydrophobic binding to the membrane may be expected to increase correspondingly the positive charge on the membrane surface, or at least to decrease its net negativity.

Schäfer and Bojanowski [15] also demonstrated an increase in fluorescence of ANS produced by the binding of phenformin to mitochondrial membranes and interpret this as an increase in photon emission, i.e. quantum yield, by ANS rather than an increase in its binding. They also found a close correspondence between the affinity constants calculated by the change in ANS fluorescence and the binding of labelled phenformin to mitochondrial membranes. Schäfer and Rowohl-Quisthoudt [18] have demonstrated an increase in ANS fluorescence due to addition of phenformin to liposomes and attribute this primarily to a change in membrane surface charge. This finding is in line with our observations since phenformin increased ANS fluorescence in a saturable fashion. We have also shown by measurement of electrophoretic mobility that the binding of phenformin alters membrane surface charge. We believe however than ANS cannot be taken as an accurate reporter of surface charge as Schäfer assumes because phenformin and phlorizin both change the surface charge in the same direction yet phenformin increases ANS fluorescence whereas phlorizin markedly decreases it.

Phlorizin is a phenylglucoside, blocking glucose transport by liganding to both a sugar and phenolic binding site located some 15 Å apart [19]. Hydrogen bonding via hydroxyl groups is believed to maintain the phlorizin-transport site interaction, and ionic charge–charge, i.e. electrostatic, interactions appear minimal [20].

In contrast, the biguanide phenformin has a strong basic primary dissociation constant and at physiological pH values will exist almost exclusively as a singly charged cation [21], a pK of 11.8 corresponding to >99.9 per cent charged species at pH 7.0 [22]. Not unexpectedly therefore phenformin decreases the net negative surface charge of the membrane vesicles. However the effect cannot be accounted for simply by a reduction of the surface membrane net negativity through coulombic cationic–anionic group interaction or screening, nor by a change in ionic strength, for whereas phenformin 1 mM decreased mobility by >30 per cent (see Table 2), the addition of up to twice this concentration, i.e. to a total of 2 mM, of the monovalent counter-ions K^+ or Na^+ caused no such decrease. Membrane partitioning of phenformin is the most probable explanation for the extensive change in the electrostatic surface potential. Making the not unreasonable assumption that the amount of drug bound to membrane preparations closely parallels the organic/aqueous phase partition coefficient for phenformin *in vitro* (approx. 6.7: see [15]), there would be, assuming minimal dielectric field effects in the membrane environment, an effective phenformin counter-ion concentration of up to 6.7 mM in the proximity of the membrane-bulk phase interface and ionic double layer. The combined effect of this binding-concentration phenomenon and its influence on electrostatic surface potential will have far reaching consequences for the control of cation-dependent transfer processes such as amino-acid and glucose uptake [2, 7].

Phlorizin blocks sodium dependent, saturable glucose transport across brush border membranes in renal proximal tubule and small intestine [3, 4]. Our results compare the membrane effects of this inhibitor with those of phenformin, an agent which from

Schäfer's results appears to have relatively non-specific effects related mainly to its capacity to partition into lipid membranes. On the other hand our ANS experiments show that the presence of protein in vesicles changes the binding of phenformin to a marked degree, transforming the characteristic hyperbolic relationship between fluorescence and increasing phenformin concentrations in liposomes to a linear increase in fluorescence with increasing amounts of phenformin in liposomes containing brush-border proteins.

Moreover the incorporation of protein into phosphatidylcholine liposomes did not decrease the liposome mobility and hence membrane electrostatic surface potential, *per se* [see Table 2 (E and F)] nor did it decrease the effect of phenformin [see Table 2 (F)]. This again highlights a lack of congruity between ANS fluorescence and the direct surface charge measurements presented here.

Finally, whatever the molecular basis for the membrane action of phenformin, these experiments are consistent with the idea that biguanides may act as antidiabetic agents, at least in part, by inhibiting glucose absorption from the small intestine and blocking the sodium dependent uptake process through which glucose enters the small intestinal epithelial cells from the gut lumen. Their systemic effects are generally less well understood, and although they evidently do partition into phospholipid membranes and change surface charge, as we have demonstrated here, more specific antidiabetic actions at concentrations used clinically are not ruled out.

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