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A new class of sugar analogues for use in the investigation of sugar transport

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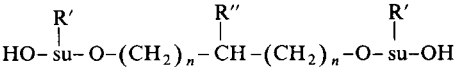
D-Mannose derivatives have been synthesised which are crosslinked through their C-4 hydroxyls to propyl-2-amine. Coupling to the amino group gave a fluorodinitrobenzene derivative, a nitroazidophenyl derivative and an azidosalicylamide derivative. Each of these derivatives was shown to have high affinity for the human erythrocyte sugar transport system. The affinity constant for the nitroazidophenyl derivative was not altered by temperature changes. In rat adipocytes treated with insulin, the affinity constants for the derivatives were up to 1000-fold lower than for the parent sugar. In the absence of insulin the affinity constants for the derivatives, but not for D-mannose, were 3-times higher than in insulin-treated cells. By preparation of radiolabelled derivatives we have shown that the compounds are not transported either by erythrocytes or by adipocytes. Thus the crosslinked sugars are good outside-specific analogues.

Introduction

We have designed a new class of sugar analogues for the study of sugar transport. The present work reports the effects of three of these new compounds on the human erythrocyte sugar transport system and on the insulin-dependent sugar transport system of rat adipocytes.

The general structure of this new class of sugar analogue is



where su–OH is a sugar moiety.

The sugars are crosslinked by a bridge, which can be of variable length. The position on the sugar chosen for crosslinking can be varied depending upon the specificity of the transport system being studied. In the case of the mammalian facilative transport system, specificity investigations of the spatial and bonding requirements of the transport site have indicated that the C-4 position would be most suitable [1–5].

Substitution of a reactive group can be made in the bridge at R″. For example, a photolabile group substituted at this position would give a potential photoaffinity labelling compound. Two of the compounds used in the present study are potential photoaffinity labels. The third compound contains a reactive FDNB group at R″. An advantage of introducing a bulky photoreactive group in the bridge rather than into the sugar moieties is that in such a position the bulky group should not interfere with the approach of groups on the transport system to binding positions on the sugar. Compounds of this type are thus called exoaffinity labels [17].

Substitution at R′, for example with isothio-

Abbreviations: FDNB, 5-fluoro-2,4-dinitrobenzene; DIDS, 4,4′-diisothiocyano-2,2′-disulphonic acid stilbene; BMPA, 1,3-bis(D-mannos-4′-yloxy)propyl-2-amine; NAP, 2-nitro-4-azidophenyl; ASA, 4-azidosalicylamide; DMSO, dimethylsulphoxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

cyanate groups, would give a bifunctional reagent similar to DIDS, the powerful inhibitor of the anion transport system [6].

The requirements that were sought in the design of this new class of sugar analogues were that they should be easily converted to radiolabelled compounds, that they should not be transported and should inhibit or inactivate the transport system from the outside of the cell. By synthesis of tritiated derivatives of the three compounds we have shown that they are not transported, even with large hydrophobic substitutions in the bridge. We have previously shown that noncrosslinked sugars with a large hydrophobic substitution, for example 6-*O*-benzyl-D-galactose, are transported rapidly through lipid regions of the membrane [3,5]. Thus the inclusion of the second sugar moiety must make the molecule as a whole very hydrophilic and unsuitable for transport through lipid regions of the membrane.

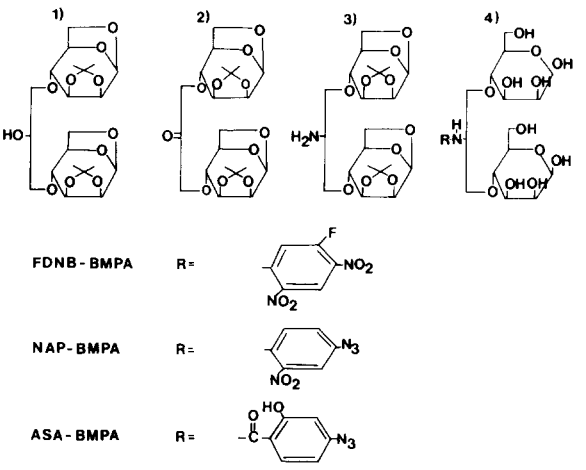
Materials and methods

Crude collagenase (type 1) was from Worthington enzymes. Bovine serum albumin, olive oil, D-mannose, D-galactose and 3-*O*-methyl-D-glucose were from Sigma. Porcine monocomponent insulin was from Novo Laboratories. Silicone oil was from Hopkins and Williams. Phloretin was from K&K laboratories. 3-*O*-[^{14}C]Methyl-D-glucose and D-[1-^{14}C]galactose were from Amersham International.

The three sugar analogues prepared for the present study were derivatives of 1,3-bis(D-mannos-4'-yloxy)propyl-2-amine (BMPA). Conjugation of BMPA with fluoronitrophenylazide (BDH) gave the nitrophenylazide derivative (NAP-BMPA). Conjugation with dinitrodifluorobenzene (Aldrich) gave the fluorodinitrobenzene derivative (FDNB-BMPA). Conjugation with *N'*-hydroxysuccinimidyl-4-azidosalicylate (Pierce) gave the azidosalicylamide (ASA-BMPA).

Preparation of 1,3-bis(D-mannos-4'-yloxy)propyl-2-amine and 1,3-bis(D-mannos-4'-yloxy)[2-^{3}H]propyl-2-amine

The preparation of 1,3-bis(D-mannos-4'-yloxy)propyl-2-amine will be described in detail elsewhere [18]. Essentially the route is from D-mannose and following Scheme I. D-Mannose was converted to 1,6-anhydro-D-mannose by pyrolysis. Reaction with acetone then gave 1,6-anhydro-2,3-*O*-isopropylidene-D-mannose. The free C-4 positions were then crosslinked with epichlorohydrin (1,2-epoxy-3-chloropropane) to give compound 1 in Scheme I. The alcohol group was oxidised with DMSO and phosphorus pentoxide to give compound 2. Reductive amination of the keto compound then gave compound 3. Acid hydrolysis of the amine then gave 1,3-bis(D-mannos-4'-yloxy)propyl-2-amine, which was crystallised as the hydrochloride salt. m.p. 77–78°C (decomp) m.s. ([+] ion F.A.B.), *m/z* 416 (*M* + 1). Anal. Calcd. for $C_{15}H_{30}O_{12}N_1Cl_1$: C, 39.87; H, 6.69; N, 3.10. Found: C, 39.75; H, 6.64; N, 2.96.



Scheme I. Structure and synthesis of 1,3-bis(D-mannos-4'-yloxy)propyl-2-amine derivatives. See text for details.

Incorporation of tritium was at the reductive amination state. 5.9 mg of oxidised compound 2 plus 19 mg of ammonium acetate were dissolved in 250 µl tetrahydrofuran and 250 µl methanol. 10 mCi cyano[^{3}H]borohydride (Amersham International) (7.65 Ci/mmol) in 750 µl methanol were then added. The mixture was then added to chloroform and water. The chloroform layer was separated and the water layer was extracted four more times with chloroform. The combined chloroform extracts were dried over sodium sulphate and the tritiated amine was then separated from nonradiolabelled impurities by preparative TLC in ethyl acetate/petroleum ether (4 : 1, v/v). The product was then hydrolysed with 4 ml of 1 M HCl for 3.5

h on a steam bath. The solution was neutralised with Amberlite resin IRA-93(OH). The product (1,3-bis(D-mannos-4′-yloxy)[2-^{3}H]propyl-2-amine) showed only a single radiolabelled spot on paper chromatography (butanol/ethanol/water, 40 : 11 : 19, v/v) which cochromatographed with non-labelled 1,3-bis(D-mannos-4′-yloxy)propyl-2-amine. Yield, 4 mCi.

Preparation of NAP-BMPA and NAP-[2-^{3}H]BMPA

All operations involving this compound were done in the dark or in dim light. 50 μmol (22.6 mg) of BMPA were dissolved in 21 μl of triethylamine plus 500 μl of DMSO. 150 μmol (27.3 mg) of fluoronitrophenylazide were then added and the reaction mixture was heated at 37°C for 48 h in the dark. Chromatography of the reaction mix showed only one substance which both contained the chromophore and gave a positive sugar stain. The solution was evaporated to dryness and the product was taken up in 3 × 1 ml of water. Insoluble material was filtered off. The combined filtrates were evaporated to dryness and the product was purified by preparative TLC in chloroform/methanol/water (65 : 25 : 4, v/v). The NAP-BMPA was identified as a red band which was well separated from minor impurities (mainly unreacted BMPA). The NAP-BMPA band was extracted into methanol. Hygroscopic crystals were obtained from isopropanol. Yield, 13 mg. m.p. 115–120°C (decomp). m.s. ([−] ion F.A.B.) m/z 576 ($M-1$). $\lambda_1 = 260$ nm, $E_1 = 1.19 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$; $\lambda_2 = 470$ nm, $E_2 = 2.6 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$.

For the preparation of NAP-[2-^{3}H]BMPA, 679 μCi of [2-^{3}H]BMPA were treated with 13 mg fluoronitrophenylazide in 250 μl DMSO and 10 μl of triethylamine. Purification of the product was by TLC in chloroform/methanol/water (65 : 25 : 4, v/v). Yield, 277 μCi (stored in methanol).

Preparation of FDNB-BMPA and FDNB-[2-^{3}H]BMPA

All operations involving this compound were done in dim light. 50 μmol (22.6 mg) of BMPA were dissolved in 21 μl of triethylamine plus 500 μl of DMSO. 150 μmol (30.6 mg) dinitrofluorobenzene were added and the reaction mixture was heated at 37°C for 48 h. Chromatography of the reaction mixture showed only one substance which both contained the chromophore and gave a positive sugar stain. The solution was evaporated to dryness and the product was taken up into 2 × 1 ml of water. Insoluble material was removed by centrifugation. Purification of the product was by preparative paper chromatography (butanol/ethanol/water, 40 : 11 : 19, v/v). The preparative chromatography was carried out twice to ensure good separation from unreacted difluorodinitrobenzene. Hygroscopic crystals were obtained from isopropanol. Yield, 9.3 mg. m.p. = 138–141°C. ms. ([−] ion F.A.B.) m/z 598 ($M-1$). $\lambda_1 = 268$ nm, $E_1 = 6.11 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$; $\lambda_2 = 345$ nm, $E_2 = 1.29 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$.

For the preparation of FDNB-[2-^{3}H]BMPA, 679 μCi of [2-^{3}H]BMPA were treated with 6 mg difluorodinitrobenzene in 250 μl DMSO and 10 μl triethylamine. Purification of the product was by paper chromatography. Yield 238 μCi (stored in methanol).

Preparation of ASA-BMPA and ASA-[2-^{3}H]BMPA

All operations involving this compound were done in the dark or in dim light.

50 μmol (22.6 mg) of BMPA were heated at 40°C for 24 h with 55 μmol (15 mg) of N'-hydroxysuccinimidyl-4-azidosalicylate in 500 μl of N',N'-dimethylformamide and 20 μl of triethylamine. After removal of solvents, the product was taken up into water, filtered and then purified by preparative TLC in chloroform/methanol/water (65 : 25 : 4, v/v). Further purification was by paper chromatography in butanol/ethanol/water (40 : 11 : 19, v/v). Hygroscopic crystals were obtained from isopropanol. Yield 9.1 mg. m.p. = 143–145°C. m.s. ([−] ion F.A.B.) m/z 575 ($M-1$). $\lambda_1 = 272$ nm, $E_1 = 9.65 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$; $\lambda_2 = 305$ nm, $E_2 = 4.00 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$.

For the preparation of the radiolabelled compound, DMSO was found to be a better solvent. 340 μCi of [2-^{3}H]BMPA were treated with 10 mg of N'-hydroxysuccinimidyl-4-azidosalicylate in 250 μl of DMSO with 10 μl of triethylamine at 40°C for 24 h. Purification was by paper chromatography in butanol/ethanol/water (40 : 11 : 19, v/v). Yield 190 μCi (stored in methanol).

Preparation of erythrocytes

Human transfusion blood (1–3 weeks old) was separated from plasma and acid-citrate-dextrose by careful centrifugation below 20°C. Care was taken to remove white cells and platelets. The red cells were then washed a further four times in phosphate saline buffer at room temperature.

Preparation of adipocytes

These were prepared as previously described [1–3] following the recommendations detailed by Gliemann and Rees [7]. Tissue, chopped with scissors, was digested in Hepes buffer (pH 7.4 at 37°C) (140 mM Na^+; 4.7 mM K^+; 2.5 mM Ca^{2+}; 1.25 mM Mg^{2+}; 142 mM Cl^-; 2.5 mM $H_2PO_2^-/HPO_4^{2-}$; 1.25 mM SO_4^{2-}; 10 mM Hepes) containing 3.5% albumin, 0.5 mg/ml collagenase and 0.5 mM D-glucose. The digestion time was approx. 1 h. The digested tissue was filtered through nylon mesh and the isolated cells were carefully washed five times in Hepes/1% albumin buffer. The cells (at 30% cytocrit) were then divided into small batches, the number of which depended upon the experimental design, and were held in polystyrene tubes.

Transport experiments

For the erythrocyte experiments the inhibition of the flux of 100 μM D-galactose was measured at 20°C. 50 μl of a 20% cell suspension were mixed with 50 μl of inhibitor plus D[1-^{14}C]galactose. Time-courses of $-\ln(1-f)$ (where f is the fractional filling) were linear up to about 50% filling. Higher fillings were not studied in detail. Generally a replicated single time point (e.g., 10 s) was used in the inhibition experiments where the inhibitor concentration was varied. The reaction was terminated with 2 ml of ice-cold stopping solution (10 μM $HgCl_2$, 0.3 mM phloretin in phosphate saline buffer). Cells were then spun briefly in a refrigerated bench centrifuge. The supernatants were removed with a microcapillary attached to a vacuum line. The pellets were resuspended and washed in a further 2 ml of stopping solution. The radioactivity associated with the cells at zero and at infinite time were estimated. Samples were processed no more than two at a time and the total time each sample was kept in stopping solution was kept to a minimum. Care was taken to ensure that the microcapillary removed all drops of solution adhering to the sides of the tubes. Using this technique the variation in duplicate samples was approx. 3% of the mean. From the radioactivity associated with the cells at equilibrium the sugar space was estimated as approx. 65 μl/100 μl of packed cells.

For the adipocyte experiments, 50 μM 3-*O*-[^{14}C]methyl-D-glucose was used as the substrate. Inhibition experiments were carried out at 37°C as previously described [3] except that 30% cell suspensions were used. In the presence of insulin (10 nM) the uptake time used was generally 2–3 s. This gave approx. 50% filling in the absence of the inhibitor. In the absence of insulin, the uptake time was 2–3 min. Uptake at these time points into cells treated with 50 μM cytochalasin B was also estimated in each experiment and was used to correct for trapped label and nonmediated uptake. This estimate of 'zero' uptake was slightly higher than the zero-time estimates made by directly adding phloretin stopping solution to the cells before the labelled 3-*O*-methyl-D-glucose only for noninsulin-treated samples. The extent of this nonmediated trapping of label is a variable which is dependent upon the state of the cells. In the presence of insulin nonmediated uptake over 3 s was undetectable (that is, the radioactivity taken up into cytochalasin-B-treated cells was equal to the radioactivity associated with the cells at zero time). The average variation in duplicate samples was approx. 3% of the mean in the presence of insulin and 4% of the mean in the absence of insulin. The average magnitude of the insulin response was a 60-fold increase in the uninhibited 3-*O*-methyl-D-glucose uptake rate.

For both the erythrocyte and adipocyte experiments the substrate concentration was kept low or was at equilibrium across the membrane and therefore the usual logarithmic expression for evaluating the rate constant was used [2,7]. In this equation, k (the rate constant) is equal to $-(\ln(1-f))/t$ where f is the fractional filling and t is the time. K_i values for erythrocyte and for adipocyte experiments were estimated from the equation $K_i = I/((v_0/v)-1)$ where I is the inhibitor concentration and where v_0 and v are the uninhibited and inhibited rate constants, respectively. Results are expressed throughout as mean ± S.E.

Results

Erythrocytes

The inhibition of D-galactose uptake in erythrocytes by FDNB-BMPA and by ASA-BMPA is shown in Fig. 1. The inhibitors were added with the substrate, so that the inhibitors are only available at the external surface of the transport system. The K_i for FDNB-BMPA is 312 ± 4 μM. Since the compound is only in contact with the cells for 10 s, it is unlikely that any of the inhibition is due to irreversible inactivation of the carrier. The K_i for ASA-BMPA is 146 ± 4 μM. This experiment was carried out in very dim light to prevent light-driven carrier inactivation. Thus both compounds have good affinity for the transport system. The FDNB derivative was chosen for study because of the well-known effects of nonderivatised 1-fluoro-2,4-dinitrobenzene on the transport system [8]. The azidosalicylamide was chosen for study because of the similarity of salicylate grouping to the structure of phloretin [16]. Rosenberg and Wilbrandt [9] suggested that the 2-hydroxyl in phloretin might be involved in a hydrogen bond to the keto group on the bridge of the molecule.

The radiolabelled FDNB-BMPA and ASA-BMPA showed virtually no uptake over a 90 min time-course (Table I). This is consistent with the high water/oil partition coefficients for these compounds. The rate constant in the presence and in the absence of 50 mM D-glucose was calculated for these compounds, but the apparent filling never reached much more than 1% in 90 min. Thus, these compounds could be useful potential affinity labels for the transport system.

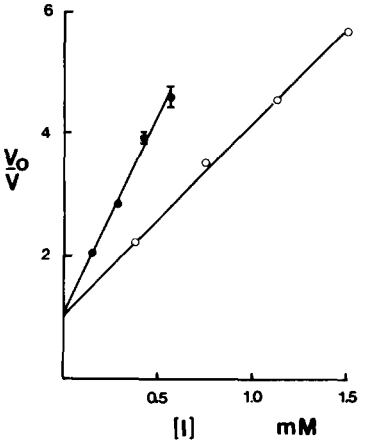


Fig. 1. The effect of FDNB-BMPA (○) and of ASA-BMPA (●) on the uptake of 100 μM D-galactose in erythrocytes at 20°C. The K_i for FDNB-BMPA is 0.312 ± 0.004 mM ($n = 8$). The K_i for ASA-BMPA is 0.146 ± 0.004 mM ($n = 8$). The average rate constant (v_0) for uninhibited D-galactose uptake was 0.091 s^{-1}. Where not shown S.E. values are smaller than the symbol.

The same considerations also apply to the NAP derivative. The inhibition constant was measured at 2°C ($K_i = 312 \pm 15$ μM) at 20°C ($K_i = 348 \pm 18$ μM) and at 37°C ($K_i = 260 \pm 8$ μM) (Fig. 2). The inhibition constant was measured over a range of temperatures in order to determine whether the high affinity for the analogue was related in any way to membrane fluidity. There is virtually no change in affinity as the temperature is increased. Over the same range of temperature D-galactose uptake changes by 300-fold (the rate constants for D-galactose uptake are $1.17 \cdot 10^{-3}$ s^{-1} at 2°C, $6.91 \cdot 10^{-2}$ s^{-1} at 20°C and 0.34 s^{-1} at 37°C). This evidence thus suggests that there is little, if

TABLE I

UPTAKE OF BIS(MANNOSYLOXY)PROPYLAMINE DERIVATIVES

The uptake of tritiated 1,3-bis(D-mannos-4-yloxy)propyl-2-amine derivatives (50 μM) by erythrocytes and adipocytes in the presence and in the absence of 50 mM transported sugar. Rate constants were estimated for the fractional equilibration with the D-galactose space (erythrocytes) and with the 3-*O*-methyl-D-glucose (MG) space (adipocytes) (linear regression). All estimates are approximations and inaccuracies are due to the low fillings and poor penetration into olive oil.

	Erythrocyte uptake (min^{-1})		Adipocyte uptake (min^{-1})		Water/olive oil partition ratio (approx.)
	− 50 mM glucose	+ 50 mM glucose	− 50 mM MG	+ 50 mM MG	
FDNB-[2-^{3}H]BMPA	$2.17 \cdot 10^{-5}$	$-7.5 \cdot 10^{-6}$	$1.04 \cdot 10^{-3}$	$6.42 \cdot 10^{-4}$	1900
ASA-[2-^{3}H]BMPA	$-9.11 \cdot 10^{-5}$	$6.33 \cdot 10^{-5}$	$2.37 \cdot 10^{-4}$	$1.33 \cdot 10^{-4}$	900
NAP-[2-^{3}H]BMPA	$1.25 \cdot 10^{-5}$	$4.17 \cdot 10^{-6}$	$6.35 \cdot 10^{-4}$	$5.00 \cdot 10^{-4}$	1500

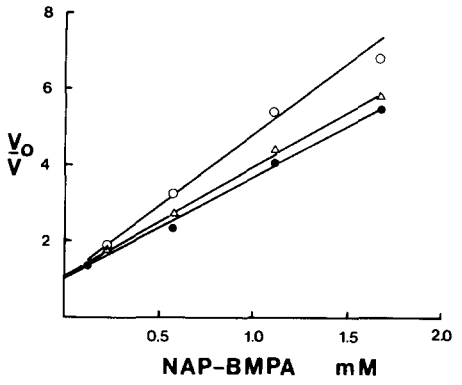


Fig. 2. The effect of NAP-BMPA on the uptake of 100 μM D-galactose in erythrocytes at 2°C (△) $K_i = 0.312 \pm 0.015$ mM ($n = 8$) at 20°C (●) $K_i = 0.348 \pm 0.018$ mM ($n = 8$) and at 37°C (○) $K_i = 0.260 \pm 0.008$ mM ($n = 8$). The rate constants for uninhibited D-galactose uptake were $1.17 \cdot 10^{-3}$ s^{-1} at 2°C, $6.91 \cdot 10^{-2}$ s^{-1} at 20°C and 0.336 s^{-1} at 37°C.

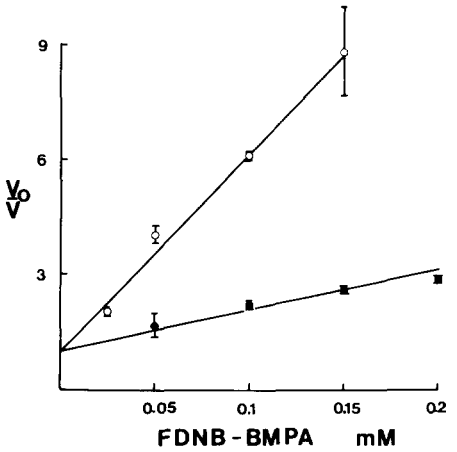


Fig. 3. The effect of FDNB-BMPA on 50 μM 3-*O*-methyl-D-glucose uptake in adipocytes at 37°C. In the presence of 10 nM insulin (○) $K_i = 21.2 \pm 1.2$ μM ($n = 8$). In the absence of insulin (●) $K_i = 99.8 \pm 13$ μM ($n = 8$). The rate constants (v_0) for uninhibited uptake are 0.345 s^{-1} in the presence and $5.95 \cdot 10^{-3}$ s^{-1} in the absence of insulin.

any, effect of membrane fluidity on the affinity for this crosslinked analogue, but more analogues would have to be synthesised and tested to confirm this. As with FDNB-BMPA and ASA-BMPA the NAP-BMPA shows no detectable uptake into red cells over 90 min.

Adipocytes

The inhibition of 3-*O*-methyl-D-glucose uptake into fat cells by FDNB-BMPA is shown in Fig. 3. In Fig. 3 the uptake $t_{1/2}$ of the substrate in the absence of insulin is 116 s, which is 60-fold slower than the uptake $t_{1/2}$ in the presence of insulin ($t_{1/2} = 2.00$ s). This large difference is presumably due to an increase in the number of active plasma membrane transport sites in the presence of insulin which modifies just V_{max} for 3-*O*-methyl-D-glucose transport [12–14]. However, perhaps surprisingly, the affinity constant for FDNB-BMPA is lower in the presence of insulin ($K_i = 21.2 \pm 1.2$ μM) than in the absence of insulin ($K_i = 99.8 \pm 13.0$ μM).

The difference in half-saturation constant between D-mannose (Fig. 7) and FDNB-BMPA is very large for insulin treated adipocytes. There is an approx. 1000-fold enhancement in affinity due to derivatisation (Table II).

The high affinity binding of FDNB-BMPA is displaceable by 3-*O*-methyl-D-glucose as shown by the data in Fig. 4. The plots of inhibition at 50 μM and at 10 mM 3-*O*-methyl-D-glucose are virtually parallel, which is consistent with competitive displacement of the inhibitor by the 3-*O*-methyl-D-glucose [3].

For the erythrocyte, ASA-BMPA was the best inhibitor. This is not true for the fat cell, where it is the worst (Fig. 5). In the presence of insulin, the K_i was 400 ± 13 μM (the $t_{1/2}$ for the substrate was 2.44 s). In the absence of insulin, the K_i was 1400 ± 120 μM (the $t_{1/2}$ for the substrate was 174 s). We have previously found [1–5] that for simple sugar substitutions the K_i values are very similar for erythrocytes and adipocytes. Thus these new

TABLE II

K_i VALUES FOR BIS(D-MANNOSE) DERIVATIVES

K_i values (half-saturation constants), in μM, were measured in human erythrocytes at 20°C and in rat adipocytes in the presence and in the absence of 10 nM insulin at 37°C.

	Erythrocytes	Adipocytes −insulin	Adipocytes +insulin
D-Mannose	26810	18980	24280
FDNB-BMPA	312	100	21
ASA-BMPA	146	1400	400
NAP-BMPA	348	115	46

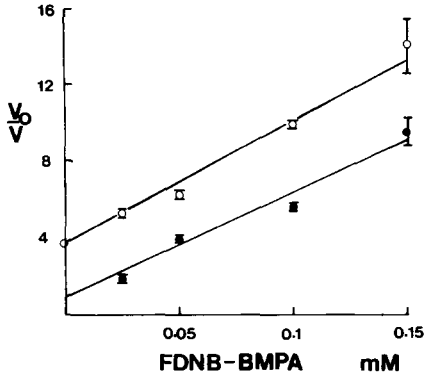


Fig. 4. The competitive displacement of FDNB-BMPA from insulin-treated adipocytes at 37°C by 3-*O*-methyl-D-glucose at 50 μM (●) and at 10 mM (○). The rate constant (v_0) for uptake of 50 μM 3-*O*-methyl-D-glucose is 0.331 s^{-1}.

compounds may be more sensitive to differences between the erythrocyte and adipocyte transport systems.

NAP-BMPA (Fig. 6) also shows higher affinity in insulin treated cells. The K_i in the presence of insulin is 46.1 ± 2.0 μM (the substrate $t_{1/2}$ is 2.32 s), while in the absence of insulin the K_i is 115 ± 10.0 μM (the substrate $t_{1/2}$ is 146 s).

The uptake rates for all three labelled derivatives are shown in Table I. The apparent fractional fillings in adipocytes are larger than those found

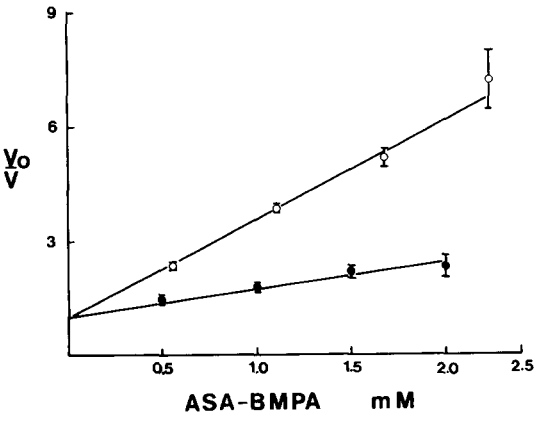


Fig. 5. The effect of ASA-BMPA on 50 μM 3-*O*-methyl-D-glucose uptake in adipocytes at 37°C. In the presence of 10 nM insulin (○) $K_i = 400 \pm 13$ μM ($n = 8$). In the absence of insulin (●) $K_i = 1400 \pm 120$ μM ($n = 8$). The rate constants (v_0) for uninhibited uptake are 0.282 s^{-1} in the presence and $3.97 \cdot 10^{-3}$ s^{-1} in the absence of insulin.

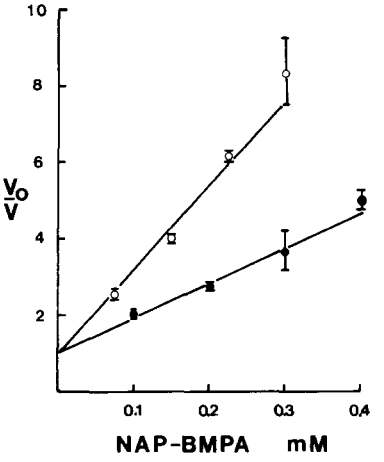


Fig. 6. The effect of NAP-BMPA on 50 μM 3-*O*-methyl-D-glucose uptake in adipocytes at 37°C. In the presence of 10 nM insulin (○) $K_i = 46.1 \pm 2$ μM ($n = 8$). In the absence of insulin (●) $K_i = 115 \pm 10$ μM ($n = 14$). The rate constants (v_0) for uninhibited uptake are 0.208 s^{-1} in the presence and $4.21 \cdot 10^{-3}$ s^{-1} in the absence of insulin.

in erythrocytes, but this probably represents more leakage or nonspecific trapping in the former type of cell. In any case, the uptakes never exceed an apparent 12% filling in 90 min, which is comparable with our previous estimate of methyl-β-D-glucoside uptake [3] and is in marked contrast to the uptake of noncrosslinked sugars substituted with hydrophobic substituents [3], where uptake through the lipid was found to be quite rapid in adipocytes.

Discussion

In a previous paper [3] we reported that certain sugars were specific for either the inside or the outside of the transport system. Thus 4,6-*O*-ethylidene-D-glucose was found to be a good external inhibitor, while propyl-β-D-glucoside was found to be a good internal inhibitor. The inhibition by these sugars was competitive in nature and the sugars could be confined to a particular membrane surface for only short periods. These sugars were quite lipophilic and penetrated the membrane lipid. Although the affinity of these compounds was good and very close to the affinity for the parent sugar D-glucose, no enhancement in affinity due to substitution was found. Thus it seemed to us that

it would be useful to have a side-specific irreversible inhibitor of transport with high affinity, and this is why the new class of crosslinked analogues was developed.

The C-4 position was considered to be the most suitable position for crosslinking, since this position can accomodate quite bulky substitutions [1–5], while bulky substitutions at other positions in the sugar are not well tolerated. Unfortunately the carbohydrate chemistry of the C-4 position in D-glucose is the most difficult because a suitably protected D-glucose is difficult to prepare. Thus at the outset of the study we chose D-mannose because, although it had lower affinity than D-glucose, the epimerisation of the C-2 position would not result in loss of a crucial hydrogen bonding position [4,2]. However, we now have reason to believe that the problem of preparing a range of C-4 crosslinked D-glucose analogues is probably not insurmountable, and we are re-exploring this possibility. Such a range would then be an interesting contrast to the D-mannose range of this new class of derivatives.

The high affinity of the red cell transport system for the crosslinked analogues can be contrasted with the K_i for D-mannose (Fig. 7). The K_i was measured under equilibrium conditions with only low concentrations of D-mannose. The K_i value of 26.81 ± 2.3 mM which we have obtained (Fig. 7) is similar to the value obtained for 10 mM D-mannose inhibition of L-sorbose transport (K_i = 20 mM) by Barnett, Holman and Munday [4]. In our hands, higher D-mannose concentrations show a higher apparent half-saturation constant for inhibition of 100 μM D-galactose uptake. We accept that this finding may be an artefact but we have been unable to determine its cause. The K_i value for inhibition by low concentrations of D-mannose is 2–3-fold higher than the K_i for inhibition of transport by low concentrations of equilibrated D-glucose, where the K_i value is approx. 7–12 mM [4,10,11]. In the red blood cell the enhancements in affinity for our D-mannose derivatives are therefore approximately 70-fold for FDNB-BMPA and for NAP-BMPA and 150-fold for ASA-BMPA.

There are several possible explanations for the enhancement in affinity for the three crosslinked D-mannose derivatives studied here. A large part of the enhancement may be due to a specific hydrophobic interaction between the aromatic grouping and a hydrophobic site on the membrane protein. A very large enhancement in affinity is found for the nitrobenzene derivatives of nucleosides [15]. There may be hydrophobic interaction between the aromatic grouping and the membrane lipid. The latter interpretation is not, however, supported by the lack of temperature dependence of the affinity constant or by the very low hydrophobicity of the molecule as a whole.

The reason why the half saturation constants for the new compounds are so different in erythrocytes and adipocytes is not clear. Also, an explanation of the observation that the inhibition constants for this new class of compounds is insulin-dependent is not available with the present limited range of the crosslinked class of analogue. This insulin effect is not due to either the slightly more significant nonmediated uptake rate found in basal cells, or to the slightly greater variability between replicate samples found in basal cells. It is our view that the difference in affinity is related to some aspect of transport site topography or hydrophobicity that is not revealed by the simpler sugar substitutions. A large number of simple sugar sub-

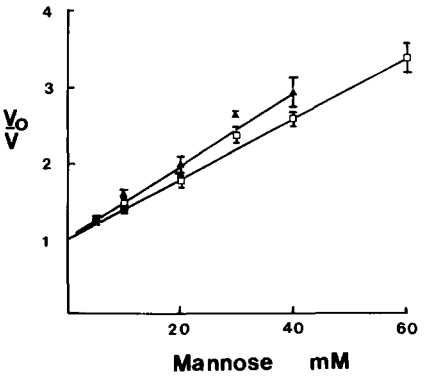


Fig. 7. Inhibition of 100 μM D-galactose uptake by equilibrated D-mannose in erythrocytes at 20° (●). $K_i = 26.81 \pm 2.3$ mM ($n = 8$). In adipocytes at 37°C the K_i values for equilibrated D-mannose inhibition of 3-*O*-methyl-D-glucose uptake are 24.28 ± 1.28 mM ($n = 24$) in the presence of 10 nM insulin (□) and 18.98 ± 1.0 in the absence of insulin ($n = 8$) (▲). The rate constants (v_0) are 0.12 s^{-1} for erythrocyte D-galactose uptake and 0.314 s^{-1} and $3.97 \cdot 10^{-3}$ s^{-1} for adipocyte 3-*O*-methyl-D-glucose uptake in the presence and absence of insulin, respectively.

stitutions have been previously studied [1] and these do not show an insulin-sensitive half-saturation constant. D-Mannose (Fig. 7) and several other noncrosslinked sugars we have been studying recently have exactly the same half-saturation constant in the presence and in the absence of insulin. It would be of interest to determine which portions of the crosslinked sugars are responsible for the extra inhibition of 3-*O*-methyl-D-glucose flux found in insulin-treated cells. It may be that the hydrophobic portion is responsible for the effect.

An explanation of the virtually undetectable uptake and poor lipid solubility of these compounds must be that the second sugar makes the molecule as a whole very hydrophilic.

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