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SPECIFIC D-GLUCOSE TRANSPORT IN SARCOLEMMA VESICLES

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Summary

The sarcolemmal fraction prepared from rat skeletal muscle consists of osmotically active vesicles that accumulate D-glucose in preference to L-glucose, apparently by facilitated diffusion into intravesicular space. Stereospecific D-glucose uptake by these vesicles is a saturable process, inhibited by phloridzin, by cytochalasin B, and by certain sugars, and enhanced by counterflow. An additional leak pathway permits entry of both D- and L-glucose into the vesicles.

Stereospecific D-glucose transport by sarcolemmal vesicles is enhanced to a small extent by insulin, provided the hormone is administered prior to cell disruption. In membranes prepared from insulin-pretreated muscle, Ca^{2+} produces a small further enhancement. Local anesthetics preferentially inhibit stereospecific D-glucose transport. Apparent uptake of both D- and L-glucose is greater when vesicles are suspended in salt solutions rather than sucrose, an effect attributed to increased functional vesicular volume.

Introduction

Carter et al. [1] demonstrated that vesicles prepared from plasma membranes of adipocytes retain a transport system specific for D-glucose. Such vesicles are able to accumulate D-glucose more rapidly than L-glucose, apparently by facilitated diffusion into an intravesicular space. We report here that the sarcolemmal fraction prepared from rat skeletal muscle by the method of Schapira et al. [2] also retains a D-glucose transport system, with properties similar to those demonstrated in adipocyte membrane vesicles.

Methods and Materials

Male rats (CD strain from Charles River Breeding Laboratories, Wilmington, Mass.), weighing 120—200 g were killed by cervical fracture. Skeletal muscle

was dissected from hind limbs. The membrane fraction enriched in sarcolemma was prepared by the method of Schapira et al. [2] with modifications described previously [3]. 10 g of muscle, from one rat, yielded approx. 0.8 mg of sarcolemma protein. Solutions were buffered with either Tris-HCl or K-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid).

Where indicated, membranes were sonicated for 10 s at 30—40 W with the microtip of a Branson sonifier, usually in KCl medium (135 mM KCl/10 mM NaCl/1 mM Tris, pH 7.4). They were then sedimented at 100 000 × *g* and resuspended in NaCl medium (145 mM NaCl/5 mM KCl/1 mM Tris, pH 7.4). All of these procedures were done at 4°C.

Uptake of labeled glucose by membrane vesicles was measured by the method of Carter et al. [1]. Unless otherwise indicated final concentrations in assay tubes were 145 mM NaCl, 5 mM KCl, 30 mM imidazole buffer, pH 7.4, and 5 mM each D-[6-^{3}H]glucose and L-[1-^{14}C]glucose (New England Nuclear Corp., Boston, Mass.). Aliquots of membrane suspensions, containing 60—100 μg protein, were added to assay tubes lacking glucose and kept at 20°C for 30 min. Transport was initiated by addition of an equimolar mixture of labeled D- and L-glucose and, after 1 min at 20°C, terminated by filtration of an aliquot. Blank tubes contained no membranes. D-[6-^{3}H]Glucose blanks gave 10—40 cpm above background compared to a minimum of 2000 cpm from the membrane suspensions. L-[1-^{14}C]Glucose blanks gave 6—10 cpm above background, compared to a minimum of 200 cpm for membrane suspensions.

For filtration, a glass flask with fritted glass support (Millipore Corp., Bedford, Mass.) was used with water suction. Millipore filters (type HAMK, 0.45 μm pore size) were presoaked in cold Ringer-bicarbonate buffer. After filtration of the sample, filters were washed with 5 ml cold Ringer-bicarbonate buffer, removed to scintillation vials, dried and dissolved in 2 ml 2-methoxyethanol (Eastman Organic Chemicals, Rochester, N.Y.). 10 ml Aquasol-2 (New England Nuclear) were added and samples counted in a Beckman LS-100C scintillation counter.

All determinations were made in triplicate. Standard deviation of triplicates was 2—5% of their mean. Each experiment was performed at least three times, with different membrane preparations. Uptake is expressed as quantity of glucose retained on the filter (minus blank) per mg membrane protein per min. The measurement we call uptake is net accumulation, not unidirectional flux and not descriptive of a single rate. The difference between uptake of D-glucose and L-glucose is termed specific D-glucose uptake.

Protein was determined by the method of Lowry et al. [4] with samples or bovine serum albumin standards in the same diluent, 250 mM sucrose in 1 mM Tris or HEPES. Ouabain-sensitive ($Na^+ + K^+$)-ATPase activity was measured as described earlier [3].

Monosaccharides, phloridzin, DL-propranolol, tetracaine-HCl and dibutyryl cyclic AMP (N^6,$O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphoric acid) were obtained from Sigma Chemical Co. St. Louis, Mo. Cytochalasin B was obtained from Calbiochem, LaJolla, Calif., and procaine-HCl from Mann Research Laboratories, New York, N.Y. [U-^{14}C]Sucrose was from New England Nuclear. Dr. R.J. Hosley, Eli Lilly Co., Indianapolis, Ind. generously supplied crystalline porcine insulin.

Results and Discussion

Evidence for glucose entry into sarcolemma vesicles, in part by facilitated diffusion

Uptake of D-glucose by ghosts or vesicles prepared from plasma membranes of non-epithelial cells, including erythrocytes and adipocytes, is thought to occur by two pathways: the major physiological one, facilitated diffusion mediated by a carrier specific for D-glucose, and an additional leak pathway, minor in intact cells but exaggerated in vesicles, which allows penetration of L- as well as D-glucose [5,6]. Our results with sarcolemma vesicles prepared from rat skeletal muscle conform to this interpretation. In this section, we apply criteria discussed previously by Carter et al. [1], by which, in a membrane preparation, one can distinguish transmembrane transport of glucose into intravesicular space from binding of glucose to membrane surface. We show that uptake of D-glucose by sarcolemma appears to be into intravesicular space and at least in part by facilitated diffusion.

Fig. 1 shows that D-[^{3}H]glucose was both taken up and eluted more rapidly than was L-[^{14}C]glucose. High affinity binding to membrane surface could account for preferential uptake of D-glucose but not for its preferential release. Rather this result indicates that transport of D-glucose occurs, at least in part, by a stereospecific, bidirectional mechanism.

In the experiment illustrated in Fig. 1A, the quantity of L-glucose accumulated at 1 min was 0.6 the quantity of D-glucose accumulated at that time. This ratio is a crude index of the contribution of leak pathway to total D-glucose uptake. It varied widely in different preparations, from less than 0.5 to 0.9, with the lowest values found in preparations than had never been frozen. Mild

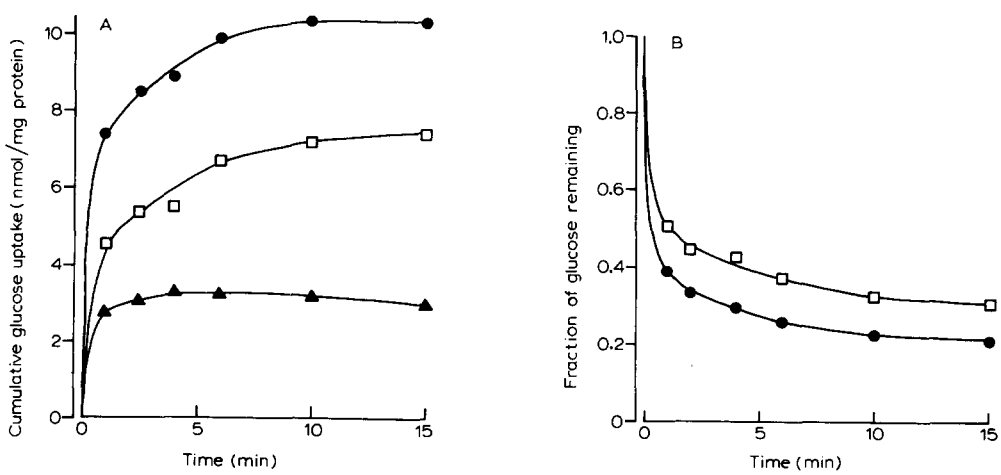

Fig. 1. (A) Time course of glucose uptake. Aliquots containing 90 μg membrane protein in NaCl medium were filtered at the indicated times after addition of labeled D- and L-glucose, each to a final concentration of 5 mM. Cumulative uptakes of D-glucose (●), L-glucose (□) and their difference (▲) are shown. (B) Time course of glucose release. After 2 h of uptake of labeled D- and L-glucose, samples were diluted 10-fold with NaCl medium lacking glucose. Aliquots containing 90 μg membrane protein were filtered at the indicated times after dilution. Glucose retained is expressed as fraction of glucose content of vesicles just before dilution. Symbols as in A.

sonication followed by centrifugation and resuspension of membranes did not reduce this ratio.

A key argument in support of facilitated diffusion is demonstration of counterflow. Exhibition of appropriate hexose to one side of the membrane should increase the rate of return of carrier to the second side and thus increase both availability of carrier and rate of uptake at the second side. That counterflow did occur in our preparation is illustrated in Fig. 2. There was an increase in uptake of D-[^{3}H]glucose by sarcolemma vesicles preloaded with unlabeled D-glucose, but not by those preloaded with unlabeled L-glucose. Uptake of L-[^{14}C]glucose was the same after either pretreatment. These data are compatible with counterflow but are incompatible with the notion that high affinity binding to membrane sites is responsible for D-glucose retention; in the latter case preexposure of membranes to unlabeled D-glucose would tend to saturate binding sites and thus reduce tracer accumulation.

Specific D-glucose uptake, but not total D-glucose uptake or L-glucose uptake, was a saturable function of glucose concentration (Fig. 3). This result supports the notion that two mechanisms are involved in uptake of D-glucose by

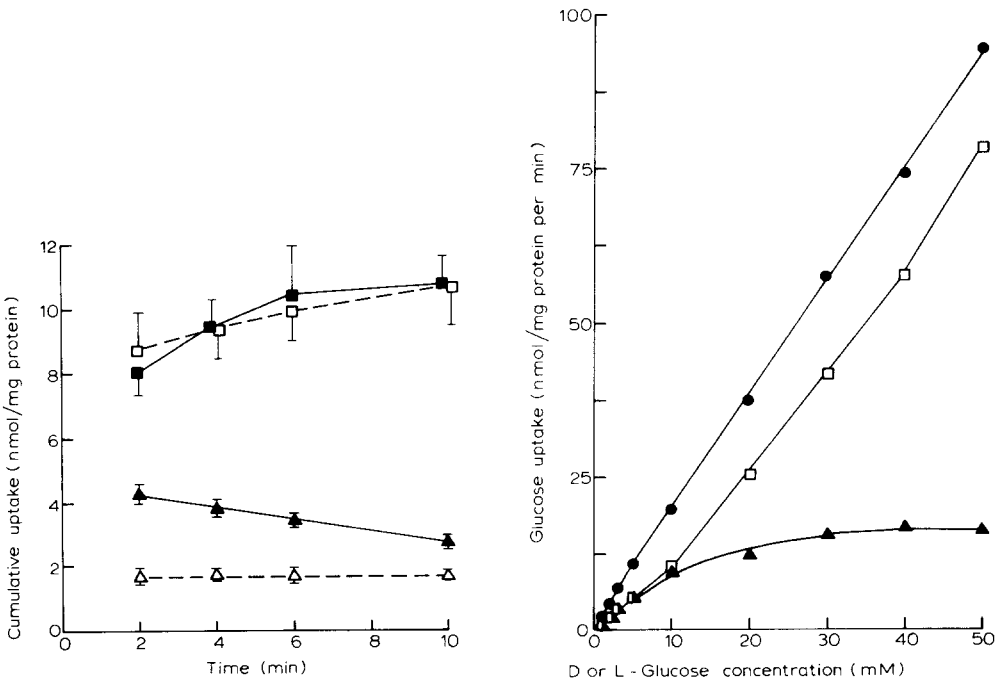

Fig. 2. Effect of preloading of vesicles with D- or L-glucose. Membranes were preincubated for 1 h at 20°C in NaCl medium containing 50 mM unlabeled D-glucose (closed symbols) or L-glucose (open symbols). At zero time, samples were diluted 11-fold with labeled and unlabeled D- and L-glucose to yield the same final concentration, 5 mM, of each isomer. Aliquots were filtered at the indicated times thereafter. Data are means ±S.E. of four preparations. Triangles show specific D-glucose uptake. Squares show L-glucose uptake.

Fig. 3. Effect of glucose concentration. Membranes suspended in NaCl medium were added last to tubes containing labeled D- and L-glucose, each at the final concentration indicated, and sucrose. The sum of glucose and sucrose concentration in each tube was constant. Aliquots were filtered after 1 min. Uptake of D-glucose (●), L-glucose (■) and their difference, specific L-glucose uptake (▲), are shown.

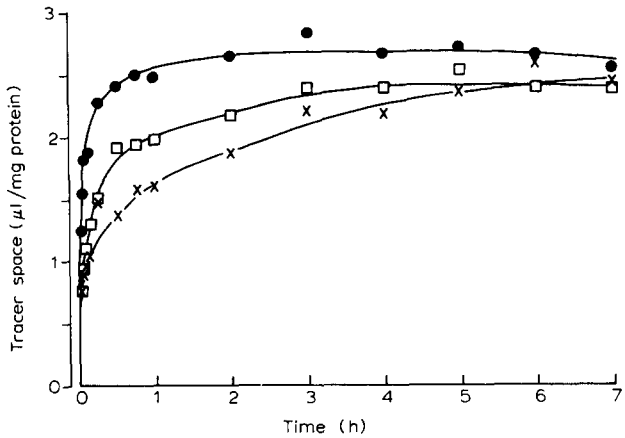

Fig. 4. Time course of accumulation of D-glucose (•), L-glucose (□) and sucrose (X). All tubes contained each of these three sugars at final concentrations of 5 mM. One set of triplicate tubes contained D-[^{3}H]-glucose and L-[^{14}C]glucose. Another set contained D-[^{3}H]glucose and [^{14}C]sucrose. Membranes, suspended in NaCl medium, were added at zero time and aliquots were filtered at the indicated times thereafter. Temperature, 21°C. Tracer spaces were calculated as (cpm/mg protein)/(cpm/μl medium).

sarcolemma vesicles: one carrier mediated and specific for the D-isomer, and the other a non-specific leak pathway.

If uptake of D-glucose is only by diffusion, including facilitated, then at equilibrium D-glucose is expected to have the same volume of distribution as other permeant molecules that are not concentrated or sequestered. In six of seven experiments of the type illustrated in Fig. 4, D-[^{3}H]glucose space reached an apparent plateau at some time during 7 h of uptake, but in only four of them was the plateau maintained. In the other two experiments ^{3}H space began to increase with a new slope at some time near the 4th hour of uptake and, when this occurred, so did the space of L-[^{14}C]glucose and [^{14}C]sucrose increase with approximately the same slope. Despite these unexplained inconsistencies seen after several hours of uptake, the data showed the following: (i) D-glucose uptake at 3 h was on a relatively flat part of the uptake curve, usually on an apparent plateau. (ii) L-Glucose space filled less rapidly than did D-glucose space but came within 11% of the latter in all seven experiments. (iii) Sucrose space filled at the slowest rate and did not reach a plateau, but, on the average, equalled L-glucose space at the 7th hour. Although these experiments do not demonstrate identity of volumes of distribution of D-glucose and the other sugars tested, they indicate that these sugar spaces, if measured at equilibrium, would differ by less than 11% and that no more than 11% of net D-glucose uptake can be ascribed to a process other than facilitated or simple diffusion.

Because the vesicles are less permeable to sucrose than to glucose it is possible to elicit osmotic effects on D-glucose uptake in response to varied external sucrose concentrations, but only after relatively short periods of exposure to sucrose. Fig. 5 shows the dependence of D-[^{3}H]glucose space on reciprocal osmolarity of the medium in which uptake took place. Membranes, suspended either in NaCl medium (upper curve) or in sucrose (lower curve), were incu-

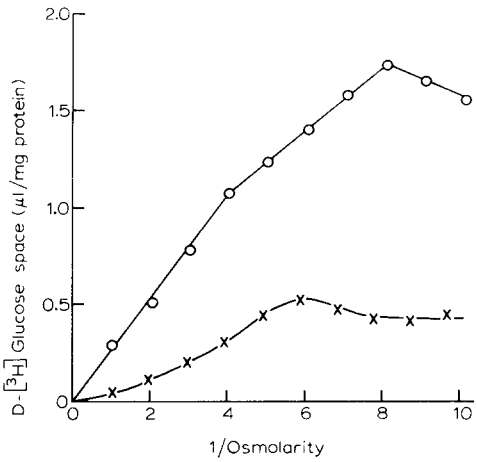

Fig. 5. Effect of osmolarity on glucose uptake in a representative experiment. The two curves represent portions of the same membrane preparation, one portion suspended in 145 mM NaCl/5 mM KCl (●———●) and the other in 250 mM sucrose (X———X). Membrane suspension sat at 4°C for several hours before the experiment. 25 μl of membrane suspension (100 μg protein) were added to tubes containing 75 μl sucrose, at concentrations ranging from 0 to 1.5 M, and allowed to sit at 20°C for 10 min. 25 μl of labeled D-glucose in 250 mM sucrose were added to yield a final glucose concentration of 5 mM. After an additional 10 min at 20°C, 100-μl aliquots were filtered. All solutions contained 1 mM Tris or HEPES, pH 7.4. D-[^{3}H]Glucose space was calculated as (cpm/mg protein)/(cpm/μl medium). Osmolarity is total osmolarity of the medium after all additions were made, calculated on the assumption that the activity coefficient of sucrose is 1 and that of NaCl or KCl is 0.75.

buted for 10 min at room temperature with various concentrations of sucrose. Labeled glucose was added and, after an additional 10 min, its uptake was determined.

Both curves in Fig. 5 show that D-[^{3}H]glucose space declines progressively as the medium is made more hypertonic, which indicates that glucose enters osmotically active space, presumably the interior of vesicles. Neither curve extrapolates to a positive intercept, which suggests that glucose is not bound to

TABLE I

EFFECT OF PHLORIDZIN AND CYTOCHALASIN B ON GLUCOSE UPTAKE

Membranes were sonicated and assayed as described in Methods and Materials. Preincubation with inhibitor for 30 min at 20°C preceded addition of labeled glucose. Specific D-glucose uptake is difference between uptakes of D- and L-glucose. Data are means ± S.E. of three or four preparations, expressed as percent of uptake in absence of inhibitor.

Addition	Uptake (percent of control)	
	Specific D-glucose	L-glucose
Phloridzin		
10^{-4} M	52 ± 1	95 ± 1
10^{-3} M	18 ± 4	98 ± 1
Cytochalasin B		
10^{-7} M	76 ± 4	102 ± 1
10^{-5} M	17 ± 2	99 ± 2

membrane surface. The distribution volume passes through a maximum, or even plateaus, as the medium is made more hypotonic, whether in saline or in sucrose, suggesting that progressively more vesicles rupture with increasing hypotonicity.

Inhibition of specific D-glucose transport

Phloridzin or cytochalasin B inhibits specific D-glucose uptake, but not L-glucose uptake, by sarcolemma vesicles (Table I). 50% inhibition occurred with 10^{-4} M phloridzin or with $8 \cdot 10^{-7}$ M cytochalasin B. These concentrations are not very different from those required to inhibit by 50% the transport of D-glucose analogs in whole muscle or cultured heart cells: about $2 \cdot 10^{-4}$ M phloridzin for 50% inhibition of insulin-stimulated 3-*O*-methylglucose release from rat soleus [7], or $8 \cdot 10^{-8}$ M cytochalasin B for 50% inhibition of 2-deoxyglucose uptake by chick embryo heart cells [8]. In addition to its effect on specific D-glucose uptake by sarcolemmal vesicles, 1 mM phloridzin abolished the selective release of D-glucose in washout experiments.

On the basis of competition studies, Battaglia and Randle [9] suggested that a number of other monosaccharides enter muscle by way of the same transport system that D-glucose uses. With this in mind we tested several other sugars for their ability to compete with D-glucose for entry into sarcolemmal vesicles. Results are shown in Table II.

2-Deoxyglucose and 3-*O*-methylglucose were most effective among the competitors tested and were more effective than D-glucose itself. D-Galactose, said not to utilize the same transport system as D-glucose in rat diaphragm [9,10], was as effective as D-glucose. D-Xylose, L-arabinose and D-mannose, all of which are competitors of D-glucose in diaphragm [9], produced smaller, but still significant, inhibition of specific D-glucose uptake. Only one of the competitors, D-mannose, had a significant effect on L-glucose uptake, and this was smaller than its effect on specific D-glucose uptake.

TABLE II

EFFECT OF OTHER SUGARS ON GLUCOSE UPTAKE

Membrane suspensions were added last to assay tubes containing the other constituents to yield final concentrations of 145 mM NaCl, 5 mM KCl, 1 mM Tris, pH 7.4, 0.1 mM each labeled D- and L-glucose and 10 mM of one of the unlabeled sugars listed. Aliquots were filtered after 1 min. Data are means ± S.E. of five preparations, expressed as percentage of uptake at 1 min in absence of additional sugar.

Addition	Uptake (percent of control)	
	Specific D-glucose	L-Glucose
2-Deoxyglucose	48 ± 2	98 ± 2
3-*O*-Methylglucose	56 ± 1	95 ± 2
D-Glucose	77 ± 2	97 ± 1
D-Galactose	78 ± 3	97 ± 2
D-Xylose	83 ± 2	97 ± 3
L-Arabinose	85 ± 2	97 ± 2
D-Mannose	85 ± 2	94 ± 1
α-Methylmannoside	95 ± 3	102 ± 3
D-Fructose	95 ± 2	96 ± 2
L-Fucose	96 ± 1	96 ± 2
L-Rhamnose	97 ± 1	98 ± 3
L-Glucose	101 ± 2	96 ± 2

Localization of D-glucose transport system

We compared glucose uptake by the subcellular fraction enriched in sarcolemma, P_1, with uptake by its nearest neighbor on a discontinuous sucrose gradient, P_2. P_1 is collected from the 8.5—30% sucrose interface and has the highest specific activities of the plasma membrane markers $(Na^+ + K^+)$-ATPase, 5′-nucleotidase and adenylate cyclase. P_2 is collected from the 30—35% sucrose interface, contains all of the above enzymes at a lower specific activity than found in P_1, has the highest specific activity of $(Mg^{2+} + Ca^{2+})$-ATPase and is thought to be enriched in transverse tubules but contaminated with sarcolemma and perhaps sarcoplasmic reticulum. Denser fractions collected from the gradient show no preference for D-glucose over L-glucose.

In four pairs of preparations, specific D-glucose uptake was 10 times as high in P_1 as in P_2 (2.2 ± 0.2 vs. 0.2 ± 0.01, mean ±S.E., nmol/mg protein per min). This shows that the transport system is a property of sarcolemma.

Effect of insulin

In an effort to increase specific D-glucose uptake by membrane vesicles, we incubated excised muscle with insulin, 1 munit/ml, for 30 min at 23°C prior to homogenization and isolation of sarcolemma. In 5 of 13 experiments insulin was exhibited only during preincubation of the tissue; in the remaining 8 experiments the same concentration of insulin was also present in all solutions used during membrane isolation. Since there was no difference between the two groups, results were pooled for analysis. In each of the 13 experiments membranes prepared from contralateral hind limb muscles, preincubated without insulin, served as paired controls.

Pretreatment with insulin increased specific D-glucose uptake in each of 13 paired experiments (range 5—50%; mean ±S.E., 26 ± 5%). L-Glucose uptake also increased significantly but to a lesser extent, 10.8 ± 0.4%.

Because the effect of insulin was relatively small we wondered whether there may have been no true increment in specific D-glucose uptake but rather that insulin may have caused a redistribution on sucrose density gradients so that fraction P_1 was further enriched in sarcolemma. To test this possibility we re-expressed glucose uptake in terms of $(Na^+ + K^+)$-ATPase activity, instead of in terms of membrane protein concentration. On this basis, specific D-glucose uptake was higher, by 18 ± 5%, as a result of pretreatment of muscle with insulin. L-Glucose uptake, per unit $(Na^+ + K^+)$-ATPase, was not affected by insulin pretreatment (3 ± 3%).

In contrast to the effect of pretreatment with insulin, insulin added to assay tubes at final concentrations up to 1 munit/ml had no effect.

The insulin effect we observed is small compared to that on specific D-glucose uptake by adipocyte membrane vesicles reported first by Martin and Carter [11] and subsequently by Ip et al. [12]. Among the possible reasons for the small size of the effect in muscle are that fibers were damaged during dissection, that insulin may not have reached deep fibers due to slow diffusion through extracellular space, and that insulin-induced alterations in sarcolemma may not persist through the preparative procedure (see next section).

Effect of Ca^{2+}

Holloszy and Narahara [13] noted increased sugar transport in muscle stimulated to contract. They suggested that redistribution of intracellular Ca^{2+} might be responsible. Since then Bihler [14] and Clausen et al. [15] have suggested that certain other activators of the glucose transport system in skeletal muscle, including ouabain, hyperosmolarity and perhaps insulin, may act indirectly through elevation or redistribution of cell Ca^{2+}. Therefore we looked for an effect of Ca^{2+} on glucose uptake by sarcolemma vesicles.

Two kinds of experiments were performed using membranes prepared from untreated muscles. In the first, membranes were sonicated in the presence of 1 mM $CaCl_2$ and then centrifuged overnight on discontinuous gradients of sucrose solutions containing 1 mM $CaCl_2$. Portions of membranes from the same preparations were treated similarly, but without $CaCl_2$, to serve as controls. Sonication and centrifugation in the presence of 1 mM $CaCl_2$ led to a significant increase in specific D-glucose uptake when that measurement was expressed per mg protein but not when it was expressed per unit $(Na^+ + K^+)$-ATPase activity (six experiments). In three experiments in which gradient centrifugation was omitted, there was no effect of Ca^{2+} on glucose uptake per mg protein. In three experiments with membrane preparations that had never been frozen, Ca^{2+} had no effect.

In the second kind, Ca^{2+} was added to membrane suspensions 30 min prior to assay. In 10 such experiments with preparations not pretreated with insulin, specific D-glucose uptake was not significantly altered in the presence of 1 mM $CaCl_2$ (103.6 ± 2.0% of control without added Ca^{2+}). In vesicles prepared from insulin-pretreated muscle, however, addition of Ca^{2+} to assay tubes produced a small but highly significant increase in specific D-glucose uptake, 108 ± 0.7% of control without added Ca^{2+} ($P << 0.01$). This increase cannot be attributed to a non-specific effect of Ca^{2+} on the vesicles themselves, such as on their sealing properties, because there was no corresponding increase in uptake of L-glucose by these vesicles, nor was there increased uptake of either isomer by vesicles from untreated muscle. Rather, it appears that insulin pretreatment potentiates an effect of Ca^{2+} on the D-glucose transport system. These observations are consistent with the following reports.

McDonald et al. [16] have shown in adipocytes that exhibition of insulin to intact cells increases the binding of $^{45}Ca^{2+}$ to subsequently isolated plasma membranes, an observation we have confirmed in skeletal muscle (unpublished). Grinstein and Erlij [17] similarly found that merely increasing availaibility of intracellular Ca^{2+} by means of a Ca^{2+} ionophore did not mimic the effect of insulin on 3-*O*-methylglucose efflux from rat soleus muscle.

Effect of dibutyryl cyclic AMP

It was first shown in 1969 that dibutyryl cyclic AMP inhibits glucose uptake by rat diaphragm [18]. Subsequently, it has been pointed out that agents which stimulate glucose transport in adipocytes, such as insulin and clofibrate, tend to lower cellular cyclic AMP levels whereas agents which inhibit glucose transport, such as isoproterenol and procaine, elevate cyclic AMP levels. On the basis of this inverse relationship, Taylor et al. [19] suggested that cyclic AMP may inhibit glucose transport into adipocytes, perhaps through phosphoryla-

tion of the glucose transporter.

To test this hypothesis we looked for an effect of dibutyryl cyclic AMP on glucose uptake by sarcolemma vesicles. In the absence of ATP, exposure to dibutyryl cyclic AMP reduced both specific D-glucose uptake and L-glucose uptake by 8—10% (six preparations, 2 mM dibutyryl cyclic AMP), a result more compatible with altered leakiness of vesicles than with inhibition of stereospecific transport. In 12 experiments with membranes that had been sonicated and resuspended in media containing MgATP, addition of dibutyryl cyclic AMP, 0.02—1 mM, produced no effect.

One might argue that the latter negative results present no challenge to the hypothesis under examination because the conditions used do not guarantee that membrane proteins were phosphorylated.

Effect of local anesthetics

Local anesthetics have been shown to inhibit glucose transport in intact cells. In human erythrocytes, for example, 1 mM tetracaine or 12 mM procaine inhibits [^{14}C]glucose uptake by about 40% [20]. When the glucose transport system of rat muscle or adipose tissue is stimulated, as by insulin, 0.5 mM tetracaine reduces 3-*O*-methylglucose release by half. In unstimulated tissue, however, up to 2 mM tetracaine has no effect, and at higher concentrations it appears to make the cells leaky to 3-*O*-methylglucose and also to ions [21]. Procaine, at sufficiently high concentrations, inhibits glucose uptake by adipocytes [19,22] although at relatively low concentrations it stimulates both transport and metabolism of glucose [22]. In view of these reports it was of interest to examine effects of local anesthetics on glucose uptake by membrane vesicles.

Results are shown in Table III. Tetracaine, procaine, and propranolol all induced preferential inhibition of specific D-glucose uptake compared to L-glucose uptake. At higher concentrations (not shown) L-glucose uptake was also progressively decreased as if the vesicles had become leaky to both isomers.

TABLE III

EFFECT OF TETRACAINE, PROCAINE AND DL-PROPRANOLOL

Membranes, suspended in NaCl medium, were added last to tubes containing the other constituents to yield final concentrations of 135 mM NaCl, 10 mM KCl, 1 mM Tris, pH 7.4, 5 mM each labeled D- and L-glucose and additions as indicated. Aliquots were filtered after 1 min at 20°C. Data are means ± S.E. of three preparations, expressed as percentage of uptake wihout addition.

Addition		Uptake (percent of control)	
		Specific D-glucose	L-Glucose
Tetracaine	1 mM	54 ± 6	97 ± 4
	2 mM	32 ± 4	94 ± 1
Procaine	1 mM	92 ± 4	100 ± 2
	2 mM	82 ± 2	101 ± 3
	10 mM	47 ± 7	95 ± 4
DL-Propranolol	1 mM	75 ± 9	93 ± 2
	2 mM	59 ± 8	94 ± 1

The half-inhibitory concentrations of tetracaine and procaine were similar to those effective on erythrocytes [20].

Effect of salts

Our initial experiments on glucose uptake were conduced with membranes suspended and assayed in isotonic sucrose. Later, in an effort to impose transmembrane ionic gradient similar to those maintained across cell membranes, we sonicated the sarcolemmal fraction in 135 mM KCl/10 mM NaCl medium, centrifuged, and then resuspended and assayed the pelleted vesicles in 145 mM NaCl/5 mM KCl medium. Under these conditions accumulation of both D- and L-glucose was increased 2–4-fold compared to vesicles exposed only to sucrose (Table IV). Subsequently we found that the sonication step could be omitted; isosmotic substitution of NaCl for sucrose in the suspension or assay medium was sufficient to increase accumulation of glucose by the vesicles. Other preliminary experiments showed that KCl was as effective as NaCl and that longer periods of preincubation in salt led to increased effect. This response to salts is very different from the one reported by Aronson and Sacktor [23] for vesicles prepared from kidney brush border, in which greater than 40-fold stimulation of glucose uptake is produced by Na^+, but not by K^+, and the stimulation diminishes with time of exposure to Na^+ due to dissipation of the transmembrane Na^+ gradient.

Since, in our preparation, uptake of both D- and L-glucose and their difference are all increased by addition of salts, the effect appears to be on some property of the vesicles and not on specific D-glucose transport. Among the possible explanations we considered were that binding of glucose to membranes might be increased in the presence of salts or, since membranes suspended in salt solutions tend to aggregate, that trapping in interstices between vesicles might account for increased retention of both isomers. These possibilities were rejected because the criteria taken as evidence for transport into intravesicular

TABLE IV

EFFECT OF MONOVALENT IONS

One portion of membranes was sonicated, centrifuged, resuspended and assayed in sucrose. An equal portion was sonicated and centrifuged in KCl medium, then resuspended and assayed in NaCl medium. Aliquots were filtered 1 min after addition of labeled D- and L-glucose, each to a final concentration of 5 mM. Three separate preparations are shown. Total D-glucose uptake (D), L-glucose uptake (L) and specific D-glucose uptake (Δ) are shown for the two conditions with the fold increase due to salt treatment in parentheses.

Experiment	Uptake (nmol/mg protein per min)					
	D	L	Δ	D	L	Δ
Sonicated in	250 mM sucrose			135 mM KCl/10 mM NaCl		
Assayed in	250 mM sucrose			145 mM NaCl/5 mM KCl		
1	2.1	1.3	0.8	9.9 (4.7)	6.4 (4.8)	3.5 (4.4)
2	2.8	1.6	1.2	10.3 (3.7)	5.8 (3.7)	4.5 (3.8)
3	4.1	2.7	1.4	9.5 (2.3)	6.5 (2.4)	3.0 (2.2)

space, discussed in an earlier section, were satisfied whether membranes had been exposed only to sucrose or to NaCl. The possibility that salt treatment makes the vesicles more permeable to molecules such as glucose cannot be itself explain the data of Table IV because in that case in increase in specific D-glu-

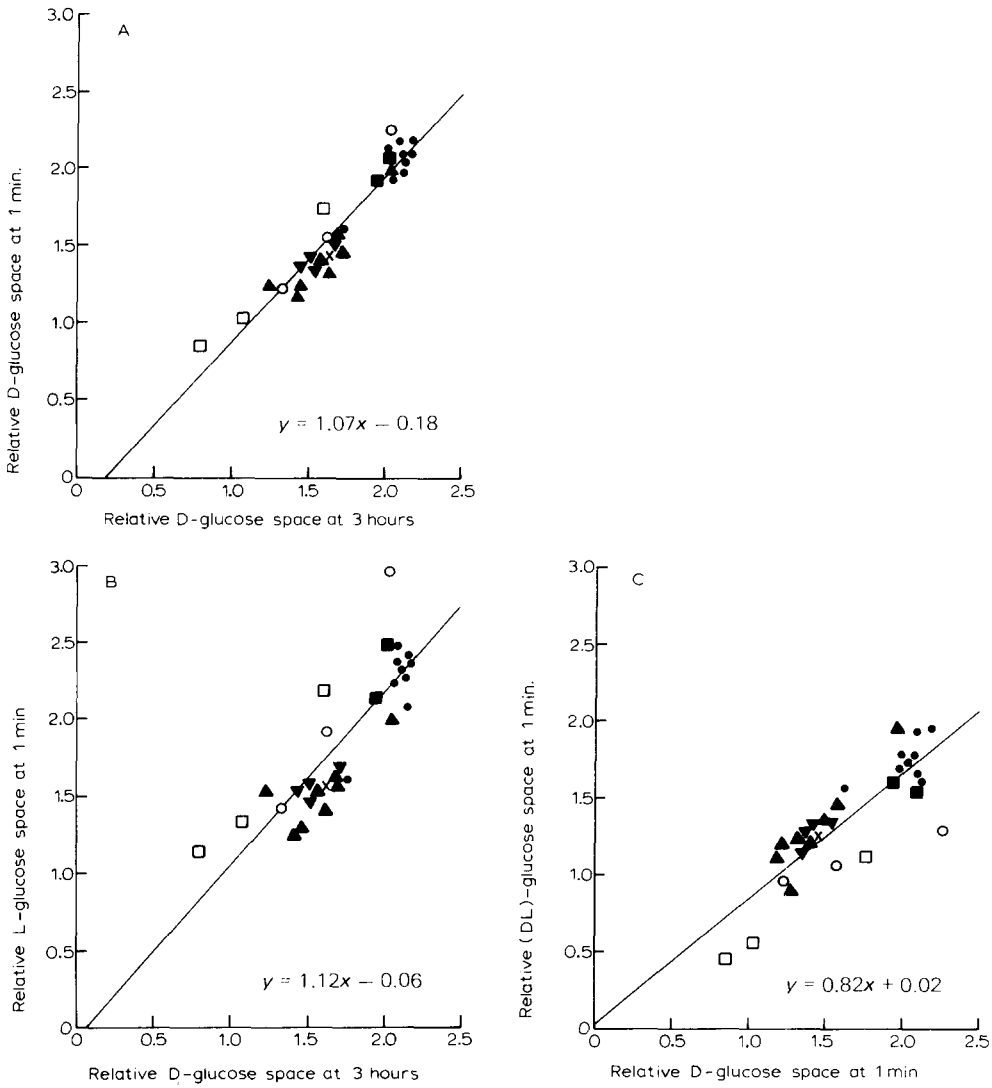

Fig. 6. Effect of salts on accumulation of glucose by sarcolemma vesicles. One volume of membranes, suspended in 250 mM sucrose, was added to tubes containing seven volumes of a 308 mosM salt solution. After 30 min, one volume of a mixture of D-[^{3}H]glucose and L-[^{14}C]glucose in 177 mM imidazole buffer, pH 7.4, was added to yield final glucose concentrations of 5 mM each. Aliquots from each tube were filtered after 1 min and after 3 h. All incubations were at 20°C. Sucrose and salt solutions were buffered to pH 7.4 with Tris. Symbols represent classes of salts. ●: NaCl, NaF, KI, KBr, KCl, LiCl, LiBr, NH_4CL, and CsCl. ○: $MgCl_2$, $MnCl_2$, and $CaCl_2$. X: Choline chloride. ■: NaSCN and NaN_3. ▲: sodium pyruvate, isethionate, citrate, tartrate and acetate; potassium gluconate and isethionate; ammonium acetate. ▲: Na_2SO_4, $(NH_4)_2SO_4$, Li_2SO_4 and $MgSO_4$. □: $NaHCO_3$, $KHCO_3$ and NH_4HCO_3. Each point is the mean from three experiments done on different preparations. Data are expressed as tracer spaces of membranes incubated in salt solutions relative to those of membranes incubated in sucrose only. Linear regressions for all 30 points are shown.

cose uptake would not be expected. A more likely explanation seemed to be that vesicles in salt have a greater effective intravesicular volume than do vesicles in sucrose, either because they have a larger average radius or because more of them are sealed.

To investigate the possibility that salts increase effective intravesicular volume, and to examine effects of a variety of salts, we did the experiments summarized in Fig. 6. Vesicles, suspended in sucrose, were preincubated at 20°C for 30 min with 7 volumes of one of 30 different salts, or for controls, with 7 volumes of sucrose. Transport was initiated by addition of 1 volume of solution containing D-[^{3}H]glucose and L-[^{14}C]glucose and aliquots were filtered 1 min or 3 h later. Fig. 6A is a plot of D-glucose space at 1 min against D-glucose space at 3 h, each space relative to control D-glucose space at the corresponding time. In Fig. 6B the abscissa is the same as in Fig. 6A and the ordinate is L-glucose space after 1 min of uptake relative to control L-glucose space at that time. Fig. 6C is described below.

Two points concerning vesicle volume can be made from the figure. First, if we take D-glucose space at 3 h as an index of total intravesicular volume, which seems reasonable on the basis of data shown in Fig. 4, then the spread of points along the horizontal axis indicates that vesicles in salts do have a larger effective volume than vesicles in sucrose. In some cases the volume is more than doubled compared to controls.

For the second point let us note that, owing to the difference in surface/volume ratio, diffusion into large spheres is slower than diffusion into small spheres, and let us make additional assumption that L-glucose enters vesicles solely by diffusion. If increases in vesicle volume were due entirely to increase in average radius, then early uptake of L-glucose would be a non-linear function of volume and points in Fig. 6B would depart from the line of identity on a curve that is concave downward. They do not. In particular, at the largest volumes, where an effect of increased radius would be most pronounced, points lie close to the line of identity.

To summarize, the data in Figs. 6A and 6B suggest that, in general, vesicles in salt have a larger functional volume than do vesicles in sucrose without having a larger average radius. The volume measured in the intravesicular volume in which the labeled glucose remains after the vesicles have been washed on the Millipore filter prior to counting. If vesicles are very leaky, some labeled glucose can be washed out of vesicles during this step, giving a falsely low estimate of intravesicular volume. However, if the vesicles are well-sealed, labeled glucose, once inside the vesicle, is less likely to be washed out during preparations for counting. Therefore we propose that most of the salts tested promote sealing of vesicles and thereby increase functional intravesicular volume.

We found no evidence for salt-induced increase in stereospecific D-glucose transport beyond that attributable to increased vesicular volume. Indeed, the contribution of stereospecific D-glucose uptake to total D-glucose uptake at 1 min was reduced in salts compared to sucrose. This is shown in Fig. 6C where the difference between D-glucose space and L-glucose space at 1 min relative to control (an index of D-glucose uptake by the specific transport system) is plotted against D-glucose space at 1 min relative to control (an index of D-glucose uptake by specific transport and diffusion). The regression line for

the 24 solid symbols has a slope of 0.76 ± 0.07, significantly less than unity. That is, the relative contribution of specific D-glucose transport to total D-glucose transport is, in general, smaller in vesicles exposed to monovalent ions than vesicles exposed only to sucrose.

It is worth noting that most of the 30 salts tested fall into two groups with respect to the increases in glucose uptake which we attribute to increase in functional volume. One group, which approximately doubled glucose space compared to sucrose, includes halides of monovalent cations, sodium azide and thiocyanate, and ammonium acetate. The other, which increased glucose space by about 50% compared to sucrose, includes salts of organic acids, sulfates, choline chloride, and NaF. The remaining salts tested, represented by open symbols, are chlorides of divalent ions and bicarbonates of monovalent ions. With the exception of $MgCl_2$, these appeared to make the vesicles leakier in that they increased the L-glucose uptake to a greater extent than D-glucose uptake.

There is no obvious relationship between the effect of these salts on the functional volume of sarcolemma vesicles and any single physical property of the salts. Their relative effects on functional volume do not correlate throughout the list with their relative activity coefficients or with their relative equivalent conductances, although there are partial correlations. We had anticipated that the effect might be due to changes produced in surface charges on the membrane, but, if that is an explanation, it does not cover all cases.

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