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GLUCOSE TRANSPORT BY TRYPSIN-TREATED RED BLOOD CELL GHOSTS

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SUMMARY

1. Glucose transport has been studied in preparations of human erythrocyte ghosts which have been treated with trypsin (10μg/ml for 30 min). [^{3}H]D-Glucose uptake by this preparation has been measured using a Millipore filter technique, with [^{14}C]L-glucose serving as a marker for non-specific glucose uptake. Membrane vesicles prepared from erythrocyte ghosts did not preferentially take up D-glucose without prior treatment with trypsin.

2. This trypsin-treated erythrocyte ghost preparation demonstrates many of the characteristics of glucose transport: (1) the D-isomer is both taken up and released more rapidly than the L-isomer; (2) countertransport of D-glucose can be demonstrated; (3) competitive inhibition of D-glucose uptake is observed with certain sugars; (4) known inhibitors of glucose uptake, phlorizin and *N*-ethylmaleimide, inhibit D-glucose uptake.

3. Using very low concentrations of trypsin (1 μg/ml), the onset of glucose transport has been correlated with the pattern of cleavage of membrane peptides (assessed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate). The appearance of glucose transport is temporally associated with the hydrolysis of two major membrane peptides. Specific D-glucose uptake is not inhibited by extensive proteolysis (trypsin, 100 μg/ml) or selective elution of specific peptides (by manipulation of ionic strength).

INTRODUCTION

The study of sugar transport has generally involved the use of intact tissues or cells. Recently it has been shown that relatively pure preparations of membranes can retain intact transport systems. Kaback[1–3] has shown that bacterial spheroplast membranes essentially uncontaminated by cell wall or intracellular cytoplasm carry out the uptake of a number of amino acids and sugars against a cencentration gradient. We have shown[4,23] that plasma membrane vesicles prepared from siolated adipose cells of the rat demonstrate facilitated diffusion of glucose in a manner identical to sugar "transport" in the intact cell; this process does not involve either chemical modification (*i.e.* phosphorylation) of the sugar or its accumulation against a gradient.

Efforts to identify the glucose "carrier" using adipose cells were hampered by the small amounts of protein recoverable as pure plasma membranes.

In an attempt to extend our studies of glucose transport we turned to the red blood cell because: (1) large amounts of plasma membrane essentially free of intracellular contamination ("ghosts") are readily available, (2) the glucose transport system of the intact red blood cell has been ex ensively characterized[5,6], and (3) the properties of red cell membrane proteins have been intensively investigated in the last several years[7,8] and methods for "solubilizir.g" some of these proteins have been worked out.

In a companion article[9], we report that treatment of red cell ghosts with trypsin at low concentrations leads to a significant structural change, *i.e.* vesiculation of the membrane, without a significant change in the gross lipid composition of the membranes. This is associated with the selective hydrolysis of 2 major membrane peptides. Furthermore, the vesicles so formed differ in buoyant density from those formed by simple mechanical fragmentation of ghosts. The present report deals with the glucose transport observed in such trypsin-treated red cell ghosts.

MATERIALS AND METHODS

Methods for the preparation of lysed red cell ghosts[20], trypsin-treatment[9] and polyacrylamide gel electrophoresis of a sodium dodecyl sulfate solution of the membranes[8] have been reported. Except when specified, trypsin-treatment was done at a final enzyme concentration of 10μg/ml for 30 min at 20 °C and the reaction stopped with a 5-fold excess of soybean trypsin inhibitor. For uptake assays, trypsin-treated ghosts were suspended in 0.25 M sucrose–5 mM sodium phosphate buffer (pH 8.0) at a final concentration of 200–300 μg membrane protein per ml. Vesiculation was completed by rapid passage through a fine-bore needle (No. 27) just prior to assay.

The Millipore assay procedure, using D-[^{3}H]glucose for total sugar uptake and L-[^{14}C]glucose to correct for non-specific "leaking" into the vesicles, has been described in detail[4].

Chemicals were obtained as follows: D-[^{3}H]glucose and L-[^{14}C]glucose from New England Nuclear; D- and L-glucose (unlabeled), *N*-ethylmaleimide, crystalline trypsin, fructose, galactose and xylose were products of Sigma; phlorizin from Nutritional Biochemicals and 3-*o*-methylglucose and 2-deoxyglucose were from Calbiochem. Phlorizin was recrystallized 3 times from hot water prior to use. *N*-Ethylmaleimide concentrations were checked spectrophotometrically[11] just prior to use.

RESULTS

In preliminary experiments, untreated preparations of red cell ghosts failed to show significant uptake of either D- or L-glucose by the Millipore filtration assay. Mechanical fragmentation of the ghosts was accomplished by passing them rapidly through a fine bore needle. The membrane vesicles produced by this procedure showed significant but essentially equal uptake of D- and L-glucose at all time points tested. Thus, "net" D-glucose uptake was not evident. We next looked at the effect

of proteolytic enzymes on glucose uptake by ghosts, since previous work on glucose transport in adipocyte plasma membranes[4,23] had utilized fat cells prepared by collagenase digestion of fat pads. After enzymatic digestion of red cell ghosts with crude bacterial collagenase (2 mg/ml), the membranes consistently showed a greater uptake of D- than L-glucose.

Trypsin treatment produced identical results (Table I). Neither intact nor mechanically disrupted ghosts showed any net uptake of D-glucose. Following trypsin digestion D-glucose was taken up in excess of L-glucose. Addition of mechanical vesiculation after trypsin-treatment led to a decrease in L-glucose uptake with a concomitant rise in "net" D-glucose; this was presumably due to the formation of tighter vesicles less "leaky" to the L-isomer. This trypsin-digested, mechanically disrupted preparation was used in subsequent experiments.

In the presence of equimolar D- and L-glucose, D-glucose uptake was rapid and usually complete by 15–20 min, whereas L-glucose was taken up more slowly. In incubations carried out over several hours at 20 °C, the final amount of L-glucose taken up in 2 h equaled or exceeded that of D-glucose (Table II); at this temperature uptake of D-glucose decreased slightly with time, presumably reflecting gradual lysis of some vesicles. Thus, at equilibrium D- and L-glucose "spaces" were identical.

In intact cells glucose transport is bidirectional[6]. To test for this in membrane vesicles, trypsin-treated ghosts were preloaded with labeled D- and L-glucose, then

TABLE I

EFFECT OF MECHANICAL DISRUPTION AND TRYPSIN ON GLUCOSE UPTAKE BY RED CELL GHOSTS

Ghosts and trypsinized ghosts were prepared as described previously[9]. Vesiculation was accomplished by 8–10 rapid passages through a fine-bore hypodermic needle (No. 27) of plain ghosts or trypsintreated ghosts. D-[^{3}H] and L-[^{14}C]Glucose uptake were measured by a Millipore filtration assay (4). Net D-glucose is the difference between D- and L-glucose uptake.

Preparation tested	*Time (min)*	*Glucose uptake (pmoles)*		
		L-*Glucose*	D-*Glucose*	*Net* D-*glucose*
Ghosts	0.5	52	38	−14
	1	91	63	−28
	2	62	46	−16
	3	68	51	−17
Vesiculated ghosts	0.5	229	204	−25
	1	179	148	−31
	2	182	169	−13
	3	200	189	−11
Trypsinized ghosts	0.5	574	767	193
	1	514	737	223
	2	501	748	247
	3	528	767	239
Trypsinized-vesiculated ghosts	0.5	329	529	200
	1	350	599	249
	2	361	670	309
	3	393	701	308

rapidly diluted with buffer containing no glucose and the rate of labeled glucose release was measured at appropriate time intervals. The rate of D-glucose release was in all cases much more rapid than that of L-glucose (Fig. 1). Using ghost vesicles prepared in 100 mM sucrose progressive increases in the osmolarity of the suspending medium above 200 mM led to a steady decrease in sugar uptake, (Fig. 2). This was true for both D- and L-glucose, so that at sucrose concentrations of 0.5 M or greater the uptake of both sugars was negligible. At 100 and 200 mM sucrose total D-glucose uptake was virtually the same, but L-glucose uptake was considerably less at the latter concentration, so that at 200 mM the highest "net" D-glucose uptake occurred. This indicated that the vesicles were more stable and thus less "leaky" to L-glucose at 200 mM as compared to 100 mM. At higher concentrations both total and net uptake fell progressively.

In intact red cells, "counterflow" of sugars[12] has been demonstrated, that is the accelerated passage of (usually labeled) sugar from one side of the membrane when the other side of the membrane is exposed to a high concentration of the same sugar or one known to be transported by the same system. We demonstrated this phenomenon in membrane vesicles in two ways (Fig. 3). Preloading the trypsin-

TABLE II

EQUILIBRATION OF D- AND L-GLUCOSE UPTAKE IN TRYPSINIZED RED CELL GHOSTS

Uptake assay was done at 37 °C.

	Time of incubation (min)					
	3	*5*	*15*	*30*	*60*	*120*
D-Glucose	869	841	824	819	738	646
L-Glucose	413	430	502	560	644	719
Net D-glucose	456	411	322	259	94	−73

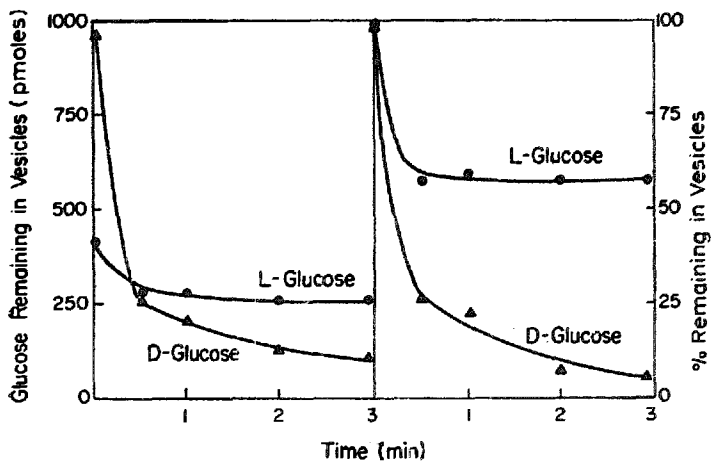

Fig. 1. Release of D- and L-glucose from red cell ghost vesicles. Trypsin-treated ghosts were preincubated with D-[^{3}H]glucose and L-[^{14}C]glucose (5 mM each) for 30 min at 20 °C in a total volume of 0.6 ml. Two 0.05-ml aliquots were filtered and washed, then the remaining 0.5 ml rapidly diluted with 4.5 ml of buffer containing no glucose. At the indicated times, 0.5-ml aliquots were filtered and washed. Since the two sugars had not equilibrated by 30 min (see Table II), results are expressed both as absolute rate of release of each isomer (left panel) and as a percent of the amount present prior to dilution (right panel).

treated ghosts with a high concentration of D-glucose, followed by rapid dilution in buffer containing D-[^{14}C]glucose at a low concentration, led to accelerated uptake of the labeled sugar as compared to control membranes preloaded with L-glucose.

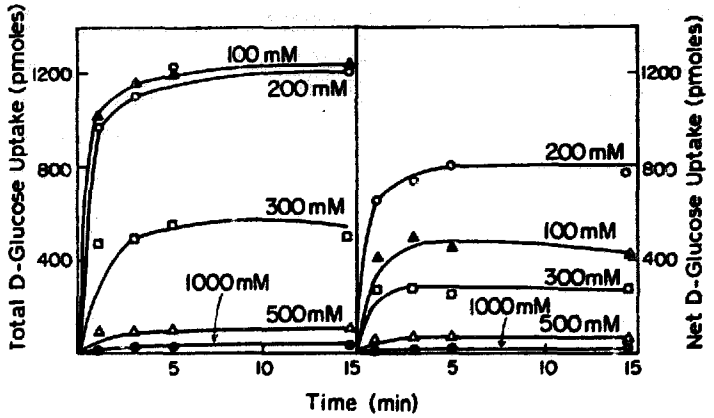

Fig. 2. Effect of osmolarity on glucose uptake by ghost vesicles. Trypsin-treated ghosts were suspended in 100 mM sucrose–5 mM sodium phosphate (pH 8.0) and equal aliquots placed in several tubes. An equal volume of the appropriate sucrose solution was added to each to give the final indicated concentrations, then D- and L-glucose added and uptake measured.

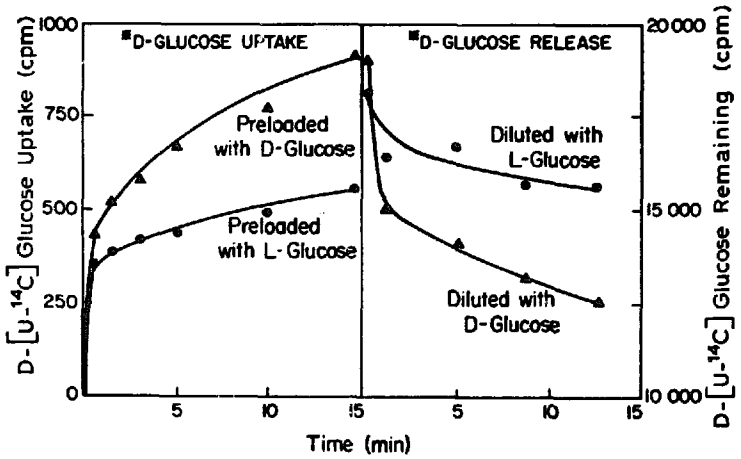

Fig. 3. Demonstration of counterflow in trypsinized red cell ghosts. Glucose uptake (left panel): vesicles were incubated with D- or L-glucose (50 mM) for 30 min at 20 °C, then 15 min at 0 °C. They were rapidly diluted with 19 vol. of buffer at 0 °C containing D-[U-^{14}C]glucose (2.5 mM) *plus* 2.5 mM L- of D-glucose, respectively, so that final concentrations of both sugars and specific activity D-[^{14}C]glucose were identical. Aliquots were filtered and washed at the indicated times. Glucose release (right panel): Vesicles were preincubated 30 min at 20 °C, then 15 min at 0 °C, with D-[U-^{14}C]glucose (5 mM) in a total volume of 0.7 ml. Two 0.1-ml aliquots were filtered and washed, then the remaining 0.5 ml diluted with 4.5 ml buffer at 0 °C containing either D- or L-glucose (50 mM). At the indicated times, 1.0-ml aliquots were filtered and washed.

Similarly, membranes preloaded with D-[^{14}C]glucose, at low concentration, then diluted into a medium containing a high concentration of D-glucose, showed accelerated release of the labeled sugar when compared to membranes similarly preloaded but diluted into a high concentration of L-glucose. Because of the rapid rate of D-glucose uptake and release, these differences could only be observed by carrying out the dilution step at 0 °C after preloading at 20 °C.

Many sugars have been shown to be competitive inhibitors of glucose transport in intact red cells[5]. Uptake into trypsin-treated ghosts showed the expected response;

it was markedly inhibited by 2-deoxyglucose and 3-*o*-methylglucose, partially by galactose, and not at all by L-glucose or fructose (Fig. 4) (for the particular case of phlorizin see below).

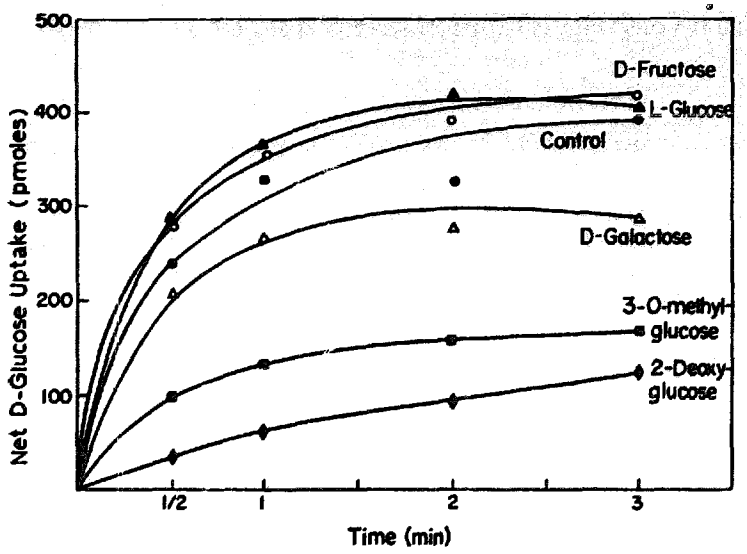

Fig. 4. Effect of various sugars on glucose uptake by trypsinized ghost vesicles. Uptake of D- and L-glucose (5 mM) were measured as usual. Other sugars were present at 50 mM concentration. Only net D-glucose uptake is shown.

Mechanism of trypsin activation of glucose transport

We have already shown[9] that trypsin treatment leads to vesiculation of red cell ghosts. This transformation appeared to be associated with the change in the permeability properties of the resultant particles. This was illustrated by observing the rate of washout of labeled D- and L-glucose from various types of ghost preparations (see ref. 9). Untreated ghosts preincubated with both sugars and collected on Millipore filters lost virtually all radioactivity when washed with minimal amounts of cold buffer, indicating the sugars were simply trapped in the pellet or contained within very "leaky" structures. Mechanical fragmentation led to only slightly longer retention of radioactivity with increasing washes. However, after trypsin treatment the labeled sugars were strikingly resistant to complete washout by cold buffer, indicating that they were contained in much more tightly resealed vesicles. Thus the sugar uptake was not obscured during the washing steps necessary for the Millipore filtration assay.

However, trypsin appeared to do more than simply fragment the ghosts and cause the formation of tightly resealed vesicles. Two separate types of experiments suggested that in some way limited proteolysis of the membrane proteins led to a markedly greater exposure of the glucose transport site. Sulfhydryl inhibitors have been shown to be inhibitors of glucose transport in red cells[13]. We examined the effect of *N*-ethylmaleimide on sugar uptake by trypsinized ghost vesicles and found a marked inhibition of D-glucose uptake (see Table III). However, exposing ghosts to the same concentration of the alkylating agent prior to trypsin treatment led to no significant inhibition of glucose uptake (Table III).

Similar results were obtained with the sugar analog phlorizin. As anticipated from prior studies of others[5], it proved to be a potent inhibitor of D-glucose uptake

when added to the trypsinized ghost preparation (Fig. 5) while having essentially no effect on L-glucose uptake. Less expected was the finding that such inhibition was largely irreversible. Phlorizin added to intact red cells or red cell ghosts was readily removed by repeated washings, but if the inhibitor was added to trypsin-treated ghosts repeated washing failed to reverse the inhibition seen (Table IV).

TABLE III

EFFECT OF *N*-ETHYLMALEIMIDE UPON GLUCOSE UPTAKE BY TRYPSINIZED-VESICULATED RED CELL GHOSTS

Red cell ghosts either before (2) or after (3) trypsin digestion were treated with *N*-ethylmaleimide (5 mM) for 30 min at 20 °C, the reaction stopped with β-mercaptoethanol (50 mM) and the membranes washed three times with buffer. With (2), trypsin treatment ane vesiculation was carried out after the washes. With (3), the membranes were revesiculated after the washes.

N-Ethylmaleimide (5 mM) added to:	*Net D-glucose uptake (nmoles/mg protein)*			
Time (min):	*0.5*	*1*	*2*	*3*
(1) None	23.3	28.3	34.2	33.0
(2) Ghosts before trypsin	22.0	27.8	31.4	33.3
(3) Ghosts after trypsin	7.8	11.3	13.9	20.6

TABLE IV

EFFECT OF TRYPSIN TREATMENT OF RED CELL GHOSTS ON REVERSIBILITY OF PHLORIZIN INHIBITION OF GLUCOSE TRANSPORT

Irreversibility of phlorizin inhibition of glucose uptake. Ghosts or trypsin-treated ghosts were incubated with phlorizin (1 mM) for 15 min at 20 °C, then washed four times. The ghosts (but not the trypsin-treated ghosts) were then treated with trypsin before assaying glucose uptake

Phlorizin(1 mM) added to:	*Net D-glucose uptake (nmoles/mg protein)*			
Time (min):	*0.15*	*1*	*2*	*3*
None	24.3	34.6	30.2	27.9
Ghosts	25.3	36.4	34.9	38.5
Trypsin-treated ghosts	3.6	16.7	15.3	20.3

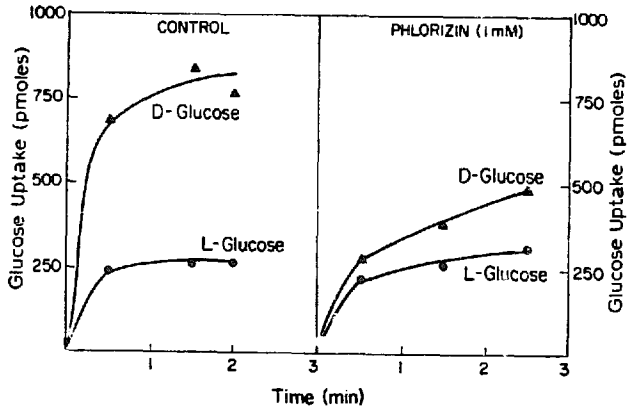

Fig. 5. Effect of phlorizin (1 mM) on D- and L-glucose uptake by trypsin-treated red cell ghosts.

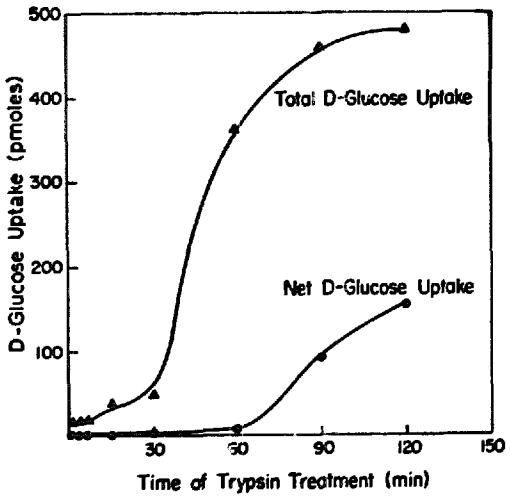

Fig. 6. Effect of trypsin on glucose uptake by red cell ghosts. Ghosts were incubated with trypsin (1 μg/ml) at 20 °C for the indicated time, then the reaction stopped with an excess of soybean trypsin inhibition. Total (D-glucose) and net (D-glucose *minus* L-glucose) uptakes were measured over 2 min at 20 °C.

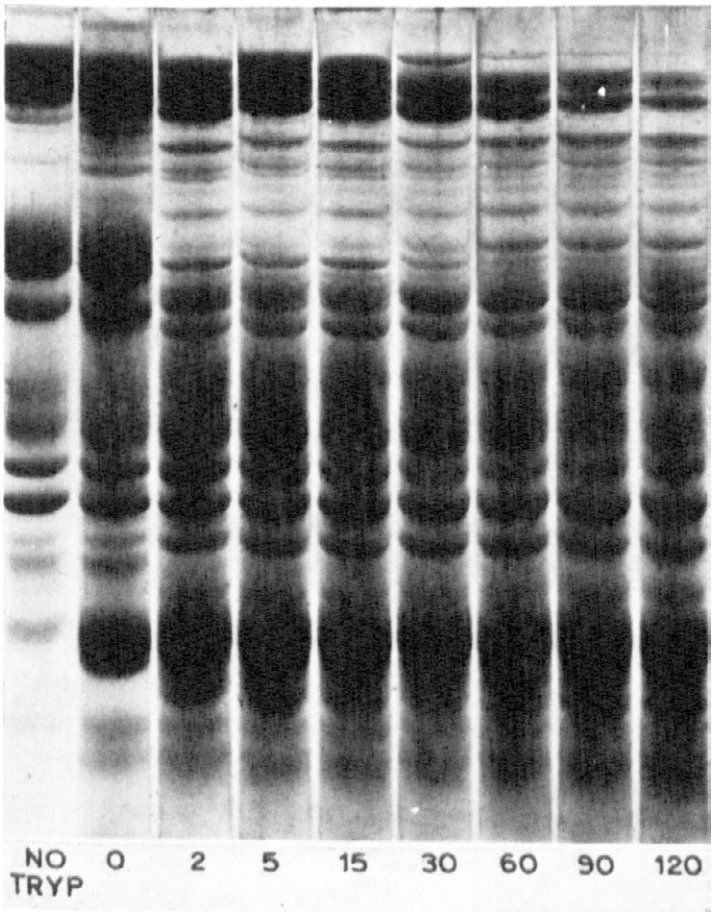

Fig. 7. Effect of high trypsin concentration on red cell ghost membrane proteins. Red cell ghosts were incubated with trypsin (100 μg/ml) for various times and the reaction stopped by the addition of soybean trypsin inhibitor; they were solubilized with sodium dodecyl sulfate and analyzed by polyacrylamide gel electrophoresis. From left to right: no trypsin, zero time (trypsin inhibitor before trypsin), 2, 5, 15, 30, 60, 90 and 120 min incubation with trypsin.

Membrane protein changes and glucose transport

By the use of selective proteolysis and extraction procedures, as monitored by polyacrylamide gel electrophoresis, it was possible to approach the question of the minimal requirements for glucose transport in red cell vesicles. We have previously shown[9] that exposure of membranes to low (1 μg/ml) concentrations of trypsin resulted in selective hydrolysis of two of the six major bands present with a coincident spontaneous vesiculation of the membrane and a decrease in buoyant density. By measuring glucose uptake after various times of exposure of the membranes to trypsin, two distinct phases were observed to occur (Fig. 6). First there was a marked but equal increase in both D- and L-glucose uptake (60 min) followed by a progressive increase in D-glucose uptake (90 min on) with no further change in L-glucose uptake. In three separate experiments this exact sequence occurred, although the length of the trypsin incubation required for the initial increase in total glucose uptake to occur varied from 5 to 30 min for the three preparations. At higher trypsin concentrations (10 μg/ml and above) both total and "net" D-glucose uptake increased at the earliest time points tested (2 min) and could not be separated. Coincident with the structural and glucose transport changes noted, there occurred hydrolysis of protein Bands III and IV as monitored by gel electrophoresis[9].

At high trypsin concentrations (100 μg/ml), there was extensive hydrolysis of all the major proteins (Fig. 7) in the red cell membranes except for Band VI.

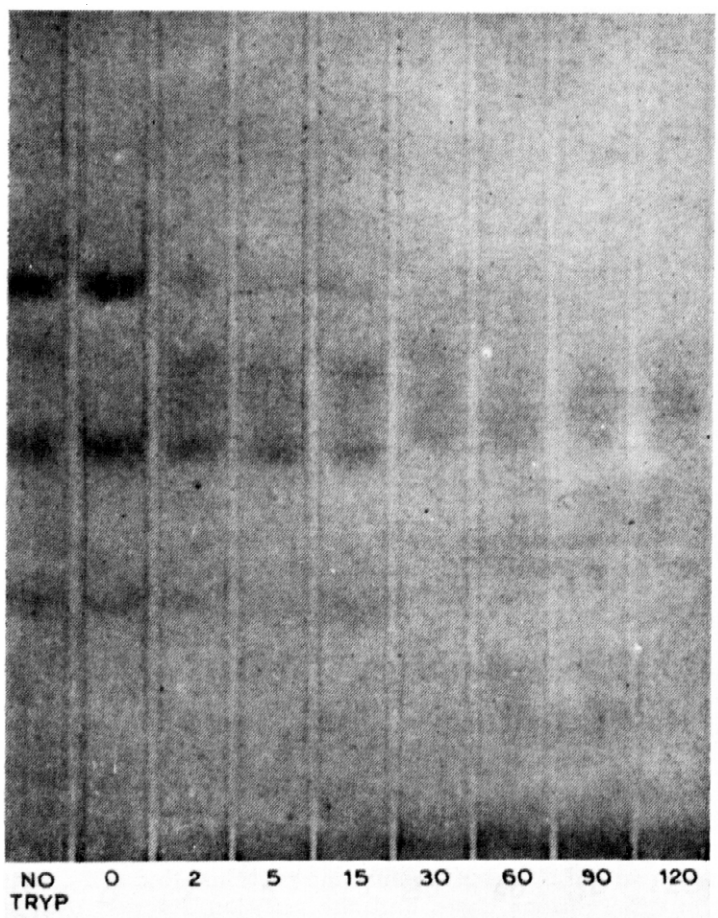

Fig. 8. Effect of high trypsin concentration on red cell ghost membrane glycoproteins. Experiment as in Fig. 7 except gels stained with periodic acid–Schiff reagent instead of Coomassie Blue.

At these higher trypsin levels, there was also extensive hydrolysis of the three major glycoproteins of the ghost membrane (Fig. 8). Aliquots of membranes at each time point were assayed for D- and L-glucose uptake and there was no loss of "net" D-glucose uptake even after 120 min of tryptic digestion. Since the properties of the glucose transport system once exposed were unaltered by more extensive proteolysis, ghosts were routinely prepared for transport studies using 10 μg/ml trypsin for 30 min at 20 °C.

Extraction of ghost membranes with dilute EDTA containing buffers led to selective loss of 3 of the 6 major protein bands[8] (numbers I, II, V) which were recovered apparently unchanged in the eluate (Fig. 9). Despite this, glucose transport as measured by "net" D-glucose uptake remained unchanged.

High salt extraction of red cell ghosts led to selective solubilization of protein Band VI[8] which was recovered in the extracting solution. Again, specific glucose uptake was unaffected by this procedure.

It should be pointed out that after each of the above extraction procedures, as with untreated ghosts, trypsin treatment was required to demonstrate specific uptake of D-glucose.

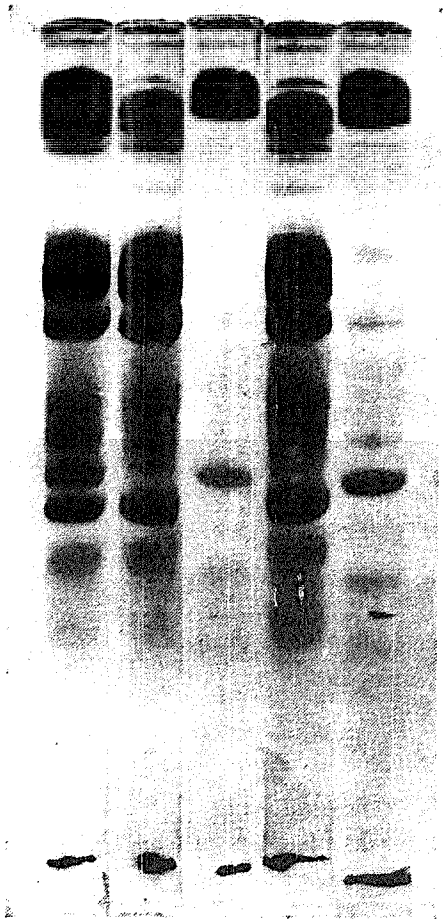

Fig. 9. Extraction of ghost membranes at low ionic strength. Ghost membranes were extracted with EDTA (1 mM) in sodium phosphate (0.5 mM, pH 8.0) for 30 min at 37 °C without or with 0.25 M sucrose, and the pellets recovered by centrifugation. Acrylamide gel electrophoresis was carried out on aliquots of the supernate and of sodium dodecyl sulfate solubilized membranes. From left to right: unextracted ghost membranes, membranes after extraction without sucrose, supernate, membranes after extraction in 0.25 M sucrose and the supernatant of the sucrose extraction.

DISCUSSION

Intact red blood cells have been used extensively for the study of glucose transport, and many details of the kinetics and conformational requirements of the glucose "carrier" system described[5,6,12].

In addition, $(NH_4)_2SO_4$-treated erythrocyte ghosts exhibit sterospecific binding of D-glucose by a process that does not appear to involve transmembrane transport[14].

A number of laboratories have reported "solubilization" of a glucose-binding protein, but it remains likely that uptake into vesicles rather than binding in the usual sense was being measured[15,16].

In the present experiments it appears likely that the "specific" D-glucose uptake represents transport ("facilitated diffusion") for the following reasons: (a) The D-glucose is not only taken up more rapidly than the L-isomer, but released more rapidly (Figs 1 and 3); (b) at equilibrium uptake of equal amounts of the two isomers are observed (Fig. 2); (c) increasing the *trans* concentration of glucose accelerates both entrance and exit of [^{14}C]D-glucose (Fig. 5), suggesting a "counterflow" phenomenon; (d) known inhibitors of glucose transport in other systems, both of competitive (sugar analogs) and non-competitive (*N*-ethylmaleimide) types inhibit glucose uptake in the system described (Figs 6 and 7, Table II).

These findings are compatible with a system of facilitated diffusion into and out of membrane vesicles. Most important is the finding of a "counterflow" phenomenon, for which the presence of a bi-directional system for sugar transport is a prerequisite[17].

The requirement for tryptic digestion of ghost membranes in order to demonstrate transport must be explained. Erythrocyte ghosts prepared by the method of Dodge *et al.*[10] are exceedingly "leaky" and appear to offer no measurable barrier to the entry (or exit) of L-glucose; minimal washing of the pellet removes virtually all radioactivity[9]. In addition to permeability characteristics, the membrane ghosts prepared by the method of Dodge differ from that of the intact erythrocytes in other respects. For example, ghost membrane peptides are extensively iodinated in the presence of lactoperoxidase, whereas with the intact cell only a single component is iodinated[18]. While this may partly reflect the increased permeability of the ghosts, a more compelling observation is that trypsin, even when bound to a macromolecular carrier, can extensively degrade the membrane peptides of the ghost prepared by the method of Dodge, whereas the intact erythrocyte is highly resistant to proteolytic attack[19]. Furthermore, whereas the membrane lipids of intact erythrocytes are not susceptible to attack by phospholipase A, or phospholipase C, these enzymes lead to extensive breakdown of membrane phospholipids when incubated with erythrocyte ghosts prepared by the method of Dodge *et al.*[20]. These findings suggest that some rearrangement of membrane structure occurs during ghost preparation by the method of Dodge *et al.* With respect to glucose transport, red blood cells lysed under other conditions retain their selective uptake of sugars[21,22]. Using ghosts prepared by successive washes in a hypotonic medium containing Mg^+ and Ca^{2+}, glucose transport can be demonstrated without further membrane modification[22]. Whether the discrepancy between this latter ghost preparation and the ghost prepared by the method of Dodge is due solely to differences in their non-specific permeability

characteristics, or whether these particles differ in membrane configuration as well is uncertain.

By the use of very low trypsin concentrations, we have shown that there are at least two separate aspects to the trypsin effect. The first, involving a structural change in which spontaneous vesiculation occurs coincident with a distinct change in the buoyant density of the membranes, has been described in a companion paper[9]. At least some of the vesicles so formed are much more tightly sealed structures than the parent ghost, as demonstrated by the sugar washout studies[9]. Secondly, it is clear that in some manner the glucose "carrier" becomes more readily accessible to the surrounding medium following limited proteolysis. This was shown in several ways. At low trypsin concentrations, equimolar uptake of D- and L-glucose appears concurrent with the transformation to vesicles; preferential D-glucose uptake is seen only with more prolonged incubation. This suggests that a further change in the membrane, other than vesiculation, is required for the expression of D-glucose transport (Fig. 6). Furthermore, concentrations of *N*-ethylmaleimide which do not inhibit transport when added to ghosts prior to trypsin, induce profound inhibition when added to trypsinized membranes (Table III). And finally, phlorizin has been shown to produce an inhibition of glucose transport which cannot be reversed by routine washing procedures, although it can be readily washed out in intact red cells or untreated ghosts (Table IV).

There are several possible explanations for the increased availability of the glucose carrier site after trypsin treatment. This could simply reflect a major rearrangement of membrane structure coincident with hydrolysis of structurally important proteins. Since transport appears under conditions in which only two major proteins are significantly hydrolyzed[9], this would indicate that one or both of these proteins are important for the structural integrity of the membrane.

Other explanations are possible. Trypsin may be hydrolyzing a protein that is directly blocking the glucose transport site and thus could make this site more accessible to substrate without significant change in the overall conformation of the membrane. Since the transport site is available in the intact erythrocyte, this would imply that the preparation of the ghost is accompanied by changes in the disposition of membrane proteins. Other evidence suggests that this may occur[19,20]. An even more attractive idea, but no more compelling, is the possibility that limited tryptic digestion is acting directly on the carrier protein itself, causing a conformational change that increases availability of the glucose binding site.

The selective extraction and high trypsin experiments raise several interesting points. The selective extraction of protein Bands, I, II and V by dilute buffers and of Band VI by hypertonic saline leaves a morphologically intact vesicle still capable of transporting glucose after proteolysis. Presumably none of these four peptides could by the glucose carrier. Bands III and IV and the three major glycopeptides, as well as a large number of minor peptides components, are not removed by these procedures. In the high trypsin experiments, Bands III, IV and glycopeptides are extensively hydrolyzed. However, the ability of the membrane to transport glucose is largely unaltered. (The fate of many of the minor peptides is obscured by the superimposition of proteolysis products.) The majority of the peptide residues present after such treatment remain associated with the membrane, and are not released or "solubilized" (ref. 9). This indicates that the glucose carrier in this situation is either

a minor peptide species resistant to proteolysis, or an active proteolysis fragment of the original glucose transport protein, with its glucose binding site intact and perhaps with enhanced mobility in the membrane. If a proteolysis fragment is performing the transport function, Components III and IV become prime suspects for the original carrier moiety, as their hydrolysis is closely associated with the activation of the transport function.

NOTE ADDED IN PROOF

Kahlenberg *et al.* (*J. Biol. Chem.* 247 (1972) 4572) have recently reported findings similar to those above concerning the uncovering of glucose transport in erythrocyte membranes by proteolysis.

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