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THE EXCHANGE AND MAXIMAL NET FLUX OF GLUCOSE ACROSS THE HUMAN ERYTHROCYTE

I. THE EFFECT OF INSULIN, INSULIN DERIVATIVES AND SMALL PROTEINS

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SUMMARY

1. The exchange and maximal net fluxes of glucose across human red cells were determined. The results confirm those of Mawe and Hempling[17] in that the exchange flux is 2.5–3 times faster than the maximal net flux. When cells were incubated with insulin, the maximal net flux was elevated on the average by 47 %. There was no significant effect on the exchange flux. The maximal net flux was elevated by vasopressin, oxidized glutathione, and the individual A and B insulin chains. Oxytocin, reduced glutathione, cysteine and cysteic acid had no effect on the maximal net flux. The inhibition of the exchange flux and not of the maximal net flux by phospholipase C as reported by Odesser and Mawe[26] was also confirmed. This inhibition was prevented when red cells were treated with insulin prior to exposure to phospholipase C.

2. The results suggest that not only may insulin interact with membrane surfaces by means of disulfide–sulfhydryl interchanges but raise the possibility of interactions with amino acid side chains and fatty acids. The elevation of only the maximal net flux by insulin is interpreted in terms of a mobile carrier system and an interaction of insulin with glucose-free carrier, is suggested.

INTRODUCTION

The effect of insulin on the movement of glucose across biological membranes has been extensively investigated. Levine *et al.*[1] demonstrated the ability of insulin to increase the permeability of cells to hexoses. Insulin has been shown to facilitate the transport of glucose, galactose and other sugars across the membranes of cardiac muscle[2] and adipose cells[3,4]. In the presence of insulin, glucose incorporation into glycogen is markedly increased[5], and in a glucose-free medium[6], insulin increases both membrane potential and membrane resistance of frog skeletal muscle. As a result of these and other investigations[7,10], the cell membrane has been proposed as the primary site of insulin action.

The movement of glucose across the membrane of the human erythrocyte is

considered to be facilitated[11,12], *i.e.* a transfer believed to operate by means of a temporary stoichiometric association of the substrate molecule, glucose, with a reactive site (carrier) present in or on the cell surface. The effect of insulin on some of the parameters which indicate facilitation as a mechanism for glucose transport has been discussed by Henderson[13]. For example: Park *et al.*[14] demonstrated the existence of a definite degree of stereo-specificity in the glucose transport system in experiments on perfused heart and in addition the fact that insulin stimulates the stereospecific system only. However, no evidence has been shown for an insulin effect on glucose transport in the normal human erythrocyte. In those experiments in which insulin effects had been sought[15,16], only net fluxes of glucose were measured and the unidirectional fluxes were not examined. Since the work of Mawe and Hempling[17], it has been possible to analyze the transport of glucose on identical populations of cells in two ways; as a maximal unidirectional net efflux and as an exchange flux.

Using their technique, the effect of insulin, insulin derivatives and small proteins on these two components of glucose transport was investigated. The results which were obtained indicate that insulin elevated the maximal net flux of normal erythrocytes by 47 % and had no effect on the exchange flux. The individual insulin chains behaved in a similar fashion as did vasopressin and oxidized glutathione.

MATERIALS AND METHODS

Chemicals

Bovine insulin (25 units/mg), porcine insulin (23 units/mg), insulin A-chain oxidized, insulin B-chain oxidized, insulin S-sulfo A-chain and insulin S-sulfo B-chain were obtained from Mann Research Laboratories, New York, N.Y. Oxytocin, synthetic (10 units/mg), vasopressin, synthetic (150 units/mg), glutathione oxidized form, glutathione reduced form, phospholipase C, and β-D(+)-glucose were obtained from Sigma Chemical Co., St. Louis, Mo.; L-cysteine, and L-cysteic acid were from Calbiochem, Los Angeles, Calif.; bovine insulin, sterile (10 units/ml) was obtained from Squibb and Co., N.J., porcine ^{131}I-labeled insulin was from Abbott Laboratories, Chicago, Ill.; and D-[$^{14}C_6$]glucose was supplied by ICN Corp., City of Industry, Calif. All other materials were of reagent grade. Water was triply distilled, once from a tin-lined container, and twice from glass.

Analytical methods

Samples containing ^{14}C were added to a dioxane mixture and were counted in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 4322. Samples labeled with ^{131}I were counted in a Packard model 2001 Spectrometer well counter system. Osmolarities were determined by the freezing point depression method utilizing an Advanced Instrument Osmometer, Model 31-LAS. Hematocrits were determined with the use of a Spinco microcentrifuge (Microfuge) Beckman Instr. Model 15-2A. Tubes, 1 mm in diameter, were partially filled with a sample of the blood suspension. One end of the tube was sealed with clay and the tube centrifuged for 2 min at top speed in the microcentrifuge. The ratio of cells to the total sample volume was determined with the aid of an IEC microcapillary reader.

Red cells

A sample of 15–20 ml of blood from healthy male donors was obtained by

venipuncture and collected into a citrated tube (5 mg sodium citrate per ml blood). The blood was passed through three layers of gauze and washed 3 times at 4 °C in a glucose–saline solution adjusted to pH 7.3 with phosphate buffer. Whenever glucose was present in the medium it was at a concentration of 0.075 M. The osmolarity of this solution containing glucose was 380–390 mosmoles/l. An identical solution, lacking only glucose, was used in the maximal net measurements and had osmolarities of 295–310 mosmoles/l. Cells were obtained from several donors and were used either fresh or after 24 h storage. When cells were kept for 24, they were stored in the solution containing glucose and received an additional wash prior to use. Insulin and other agents tested were dissolved in glucose–phosphate–saline solution adjusted to pH 8.5–8.8 with 0.05 M NaOH. Solutions were freshly prepared just prior to use.

Flux measurements

An aliquot of 25 μl of the solution containing the agent to be tested was added to 0.5 ml of packed cells with hematocrits between 75 and 80 %, previously equilibrated with 25 μl [$^{14}C_6$]glucose (125 μCi/ml) for 30 min at 22–23 °C. A control sample without the agent was run concurrently under identical conditions.

Maximal net efflux and equilibrium fluxes were obtained on aliquots of blood from the same sample, using the method of Mawe and Hempling[17]. The usual procedure was to measure two maximal net effluxes followed by measurements of two equilibrium fluxes all under control conditions. Upon completion of these runs, the suspensions containing the agent were tested in the same manner. On the average, the total elapsed time required for a given set of control and experimental determinations was 15–20 min.

Theoretical considerations

The equilibrium or exchange flux is either the influx or the efflux of glucose when the concentrations of the sugar inside and outside the red cell are equal during the course of an experiment. By obvious necessity this is an isotopic measurement and is also termed the unidirectional flux of the tracer.

The conditions under which the equilibrium flux were measured were the same as that of a two-compartment closed system previously discussed by Solomon[19] and Sheppard[20]. Under these conditions the cell water forms one compartment while the medium water makes up the second compartment. Initially, almost the total amount of the tracer is present in the cell water compartment. The kinetics of the exchange have been described by Mawe and Hempling[17].

The net flux is a measurement of the change in glucose content of the red cell when there is a difference between the internal and external concentrations of the sugar. The maximal net flux is the net flux under ideal conditions, that is, when the concentration of the sugar on one side of the membrane remains well above the saturation level of the reactive site while on the other side the concentration of the sugar remains as close to zero as possible for the length of the measurement. Wilbrandt[15] has shown that true maximal net fluxes will be measured when precautions (such as the use of a large compartment into which the glucose can move) are taken to prevent back flux. In the maximal net flux experiments reported here, 10 μl of a red cell suspension which has been incubated with experimental agents is rapidly stirred into a beaker containing 10 ml of saline–phosphate, pH 7.3, 295–310 mosmoles/l. Under these conditions, the glucose concentration in the cell's environment

is reduced to $0.015 \cdot 10^{-3}$ M, a value which is considerably less than any of the suggested K_m values[21]. The conditions for the measurement of the maximal net flux are therefore met[22].

Widdas[23], LeFevre and McGinniss[24] and Wilbrandt[20] agree that the following equations describe the kinetics of the net glucose flux:

$$-\frac{d(G)_c}{dt} = \frac{d(G)_m}{dt} = V\left\{\frac{[G_c]}{K_m + [G_c]} - \frac{[G_m]}{K_m + [G_m]}\right\} \quad (1)$$

where V may be defined as the maximum rate of movement of which the carrier system is capable.

$(G)_c$, the amount of glucose in the cell compartment
$(G)_m$, the amount of glucose in the medium compartment
$[G_c]$, the concentration of glucose in the cell water
$[G_m]$, the concentration of glucose in the medium water
K_m, the dissociation constant of the glucose–carrier complex
(dR/dt) medium, cpm glucose entering medium from cell per second
R_c, the cpm glucose in the cell compartment
R_m, the cpm glucose in the medium compartment
A_c, the cpm glucose in the cell compartment/amount of glucose in the cell compartment

When $[G_m] \ll K_m$, $[G_c]$*, Eqn 1, simplifies to:

$$\frac{d(G)_m}{dt} = \frac{V[G_c]}{K_m + [G_c]} \quad (2)$$

If $[G_c] \gg K_m$ then Eqn 2 becomes:

$$\frac{d(G)_m}{dt} = V = -\frac{d(G)_c}{dt} \quad (3)$$

where Eqn 3 is a description of maximal net flux. We can write:

$$V = -\frac{(dR/dt)_{cell}}{A_c} = \frac{(dR/dt)_{medium}}{A_c} \quad (4)$$

where $(dR/dt)_{medium}$ is taken as cpm/s and is obtained from the slope of curves similar to those shown in Fig. 1.

Since all the radioactivity found in the medium at infinity, $cpm_{t=\infty}$, came from the cell with the exception of that amount which was present as an extracellular contaminant when the packed cells were mixed with the environment, we can write**:

$$(R_c)_{t=0} = (R_m)_{t=\infty} - (R_m)_{t=0} \quad (5)$$

* As noted previously the initial concentration of glucose in the cell is 0.075 M and the initial concentration of glucose in the environment is $0.015 \cdot 10^{-3}$ M. The K_m for glucose transfer has been reported at $1 \cdot 10^{-3}$–$5 \cdot 10^{-3}$ M. Therefore, the restrictions $[G_m] \ll K_m$, and $[G_c] \gg K_m$ are met and Eqn 3 may be applied to the experimental data.

** To be strictly true it would be necessary that $[G_c]_{t=\infty} = 0$. However, since the volume of the environment is so much greater than the volume of cells which are added, the $[G_c]_{t=\infty}$ is sufficiently close to zero as to make Eqn 5 virtually correct.

and substituting obtain

$$V = \frac{(dR/dt)_{medium}[G_c]}{[(R_m)_{t=\infty} - (R_m)_{t=0}]} \quad (6)$$

The amount of glucose present when $t = 0$, when normalized to an isomotic cell volume is a concentration denoted by $[G_c]$ in Eqn 6 and is equal to 0.075 M in these experiments.

A final correction* (S) for changes in cell volume is made and Eqn 7 is used to determine the net flux.

$$\text{Net flux} = \frac{(dR/d_t)_{medium}[G_c](S)}{[(R_m)_{t=\infty} - (R_m)_{t=0}]} \quad (7)$$

The exchange flux is determined by the use of Eqn 8.

$$\text{Exchange flux} = \frac{0.693\,[G_c](S)}{t_{1/2}} \quad (8)$$

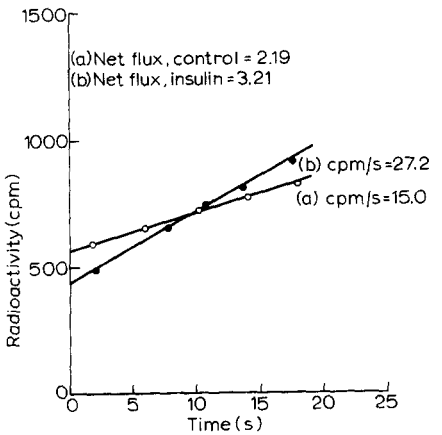
where $[G_c]$ and (S) have the same connotation as used for determining the net flux. The value $0.693/t_{1/2}$ represents in $2/t_{1/2}$ and in this context is the coefficient of exchange diffusion[17]. The value $t_{1/2}$ is read from the curve plotted semi-logarithmically as shown in Fig. 2.

All of the data obtained on flux measurements will be presented in tabular summary, but as it may be of some value, examples of the graphic display are shown in Figs 1 and 2. In Fig. 1 there are plotted two curves showing the measurement of the maximal net flux in (a) non-treated normal red cells and (b) insulin-treated normal human red cells. In each case the cpm obtained at sampling time t is plotted against the sampling time t. The value for the cpm/s is obtained from the slope and the cpm, medium, $t = 0$ is obtained from the intercept of the curve with the ordinate. Fig. 2 shows the measurement of the exchange flux in (a) non-treated normal human red cells and (b) insulin-treated normal human red cells. The values of one *minus* the ratio of the cpm at sampling time t to the cpm of the sample at infinity (30–60 min) are plotted against the sampling time t. The half time $(t_{1/2})$ is determined from the curve and this value is in turn utilized to determine the flux.

* As part of the calculations for determining fluxes under maximal net and exchange conditions, changes in the cell volume during the incubation period, due to the addition of small volumes containing the agents which were tested, must be taken into account. To determine the volume dilution correction (S), the following series of relationships are used. Approximate numerical values are used in these equations to give the reader an estimate of size of the correction normally made:

(a) ml cells = volume of suspension (0.5 ml) × hematocrit (80%) = 0.400
(b) ml plasma water = volume of suspension (0.5 ml) ml cells (0.4 ml) = 0.100
(c) Cell water = 0.70 × ml cells (0.4 ml) = 0.280
(d) Total water in initial volume of suspension
= plasma water (0.100) *plus* cell water (0.280) = 0.380
(e) Total water in the system = plasma water *plus* cell water (0.380)
plus ml additional water added (0.050) as test sample = 0.430
(f) $S = \frac{\text{total water in initial volume of cells}}{\text{total water in system}} = \frac{0.380}{0.430}$ = 0.884

The volume dilution correction is used in Eqns 7 and 8 for determining the flux.



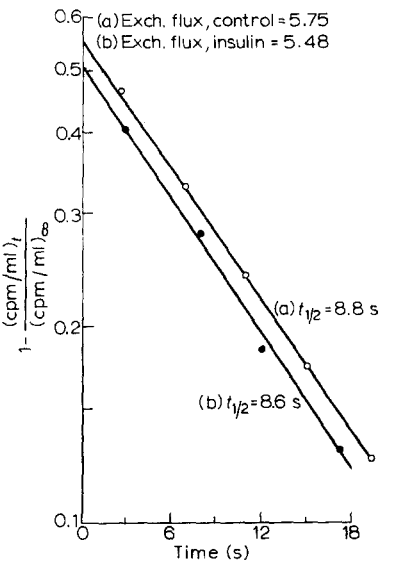


Fig. 1. The maximal net flux of [$^{14}C_6$]glucose in human erythrocytes. ○—○, net flux obtained in non-treated cells; ●—●, net flux obtained in insulin-treated cells. The ordinate represents the cpm [$^{14}C_6$]glucose appearing in the external medium.

Fig. 2. The exchange flux of [$^{14}C_6$]glucose in human erythrocytes. ○—○, exchange flux obtained in non-treated cells; ●—●, exchange flux obtained in insulin-treated cells. The values of one *minus* the ratio of the cpm in the external medium at sampling time t to the cpm of the sample at infinity (30–60 min) are plotted logarithmically against the sampling time t.

Statistical analysis

Data obtained under a given set of similar conditions were grouped and averaged. Values exceeding the limit of $\pm$ 1.96 S.D. were discarded. The means from different groupings were compared for the presence or lack of significant difference by means of the student's t test. If $P > 0.05$ was found for a given comparison, it was considered that there was no significant difference between the two values in question.

RESULTS

The average net and exchange fluxes obtained with non-treated and insulin-treated red cells from nine different donors are shown in Table I. These values were obtained from both freshly drawn and stored cells. The values obtained for the net flux from the insulin-treated cells when compared to their respective controls show that the cells (fresh or stored) of the donors (with the one exception) all show a significant increase in the net flux after being exposed to insulin, and that the increase in the maximal net efflux ranged from 27 to 81 %. The net efflux, excluding the values with $P > 0.10$, showed an overall average increase of 47 %.

The values obtained for the exchange flux from the insulin-treated cells when compared to their respective controls show that in all cases except two, there is no significant difference between the control and insulin-treated cells. In the cases where there appear to be significant differences, the large fluctuation in values obtained for

THE AVERAGE NET AND EXCHANGE FLUXES OF [$^{14}C_6$]GLUCOSE OBTAINED FROM NON-TREATED AND INSULIN-TREATED RED CELLS

The averaged flux values for individual donors are expressed as mmoles/l cell water per s.

Donor	*Net flux ± S.E.*		*% change*	*P*	*Exchange flux ± S.E.*		*% change*	*P*	*Exchange flux/net flux*	
	Controls	*Treated cells*			*Controls*	*Treated cells*			*Controls*	*Treated cells*
1*	2.32 ± 0.10	3.27 ± 0.20	41	<0.01	7.05 ± 0.33	6.52 ± 0.29	(−8)	>0.10	3.04	1.99
1*	2.04 ± 0.08	3.02 ± 0.08	48	<0.01	4.56 ± 0.22	4.84 ± 0.34	6	>0.10	2.23	1.60
2*	2.36 ± 0.11	3.10 ± 0.21	32	<0.05	6.48 ± 0.27	5.19 ± 0.34	(−20)	>0.10	2.75	1.67
3*	2.41 ± 0.04	2.90 ± 0.28	20	>0.10	6.03 ± 0.11	6.50 ± 0.62	8	>0.10	2.50	2.24
3**	2.17 ± 0.05	2.01 ± 0.08	(−7)	>0.10	4.29 ± 0.09	5.06 ± 0.16	18	<0.01	1.98	2.52
4*	2.28 ± 0.07	3.04 ± 0.07	33	<0.01	—	—	—	—	—	—
5**	2.54 ± 0.08	3.23 ± 0.08	27	<0.01	5.94 ± 0.20	5.62 ± 0.27	(−5)	>0.10	2.34	1.74
6*	2.27 ± 0.07	4.11 ± 0.36	81	<0.01	6.43 ± 0.19	6.67 ± 0.11	4	>0.10	2.83	1.51
7*	2.42 ± 0.07	3.33 ± 0.25	38	<0.05	—	—	—	—	—	—
7**	1.98 ± 0.14	2.97 ± 0.16	50	<0.05	6.72 ± 1.09	6.48 ± 0.33	(−4)	>0.10	3.39	2.18
9*	2.45 ± 0.10	4.35 ± 0.22	78	<0.01	—	—	—	—	—	—
10*	2.29 ± 0.05	3.17 ± 0.08	38	<0.01	4.62 ± 0.25	6.01 ± 0.25	30	<0.05	2.02	1.90

* Day 1, freshly drawn cells.
** Day 2, 24-h-old cells.

TABLE II

THE AVERAGE NET AND EXCHANGE FLUXES OF [$^{14}C_6$]GLUCOSE OBTAINED FROM HUMAN RED CELLS EXPOSED TO DIFFERENT CONCENTRATIONS OF BOVINE INSULIN

The cells were incubated for 30 min at 23–25 °C. Fluxes are expressed as mmoles/l cell water per s.

*Insulin concn**	*Net flux ± S.E.*		*P*	*Exchange flux ± S.E.*		*P*	*Exchange flux/net flux*	
	Controls	*Treated cells*		*Controls*	*Treated cells*		*Controls*	*Treated cells*
0.03	2.00 ± 0.11	2.26 ± 0.31	>0.10	—	—	—	—	—
0.30	2.07 ± 0.11	2.76 ± 0.11	<0.01	5.99 ± 0.26	6.55 ± 0.71	>0.10	2.89	2.37
2.7	1.96 ± 0.09	2.78 ± 0.09	<0.01	6.24 ± 0.30	6.35 ± 0.33	>0.10	3.18	2.28
6.7	2.20 ± 0.10	2.84 ± 0.06	<0.01	5.43 ± 0.24	6.12 ± 0.32	>0.10	2.47	2.15
10.0	1.67 ± 0.16	2.29 ± 0.16	<0.05	5.35 ± 0.67	4.83 ± 0.34	>0.10	3.20	2.11
13.3	2.37 ± 0.07	3.35 ± 0.16	<0.01	6.28 ± 0.36	5.62 ± 0.15	>0.10	2.65	1.68
26.7	2.28 ± 0.09	3.17 ± 0.15	<0.01	6.02 ± 0.16	6.79 ± 0.23	<0.05	2.64	2.14

* The insulin concentration is in μmoles ($\times 10^{-2}$) per ml packed red cells. These values are based on: (1) an average hematocrit of 75 %, and (2) a molecular weight of 5800 for insulin.

the exchange flux and the unusually low control values raise doubts as to the significance of these differences.

In a related series of experiments (Table II) the insulin concentration was extended to a range of from 0.25 to 40 units/ml of packed cells. This corresponds to an approximate range of $0.3 \cdot 10^{-2}$ to 0.26 μmole insulin (M_r of insulin, 5800) per ml packed red cells (average hematocrit of 75 %). Examination of the exchange fluxes shows that only with the highest concentration of insulin used in this series (0.26 μmole/ml cells) was there a significant increase in the exchange flux. Examination of the net fluxes show that, with the exception of the value obtained at the lowest concentration of insulin which was utilized, the net flux is significantly elevated at every level of insulin. The threshold level appears to be located between 0.3 and 3.0 nmoles insulin per ml packed cells. There does not appear to be any linear relationship between the increase of the net flux and the level of insulin in the incubation mixture. It should be noted, however, that a 30–40 % increase is attained almost immediately and that an average increase of about 37 % is maintained over the entire concentration range of $0.30 \cdot 10^{-2}$ to $26.7 \cdot 10^{-2}$ μmole insulin per ml packed cells.

Experiments utilizing insulin which had been heated in boiling water for 30 min resulted in an 80 % decrease in the elevation of the maximal net flux normally observed with unheated insulin. Insulin was not heated for longer periods of time since after 30 min it tended to become an insoluble gel which could not be quantitatively transferred to the red cell suspensions.

In the measurement of the maximal net flux, the environment into which the glucose-loaded cells are placed is of a lower osmolarity than the environment in which the red cells are equilibrated with glucose initially. Consequently a number of experiments were performed in which insulin-treated red cells were introduced into a medium devoid of glucose but having an osmolarity adjusted with NaCl equal to that in which the red cells were equilibrated. An elevation in the maximal net flux of the order of 40 % in the insulin-treated red cells was still observed.

In view of evidence[7,25] which has suggested that the physiological action of insulin is on the membrane and that the membrane interaction occurs by means of an interchange between the –S–S bonds of the insulin molecule and the sulfhydryl groups of the target membrane, a number of substances such as oxytocin, vasopressin and oxidized glutathione which contain a disulfide linkage within the molecule were tested. As controls for these substances, two compounds lacking disulfide linkages but possessing sulfhydryl groups, reduced glutathione and L-cysteine, were included for testing. The results (Table III) show that there is no significant difference between the exchange flux values of non-treated cells and treated cells. There are, however, significant increases in the net efflux of glucose with those cells which were incubated with either vasopressin or oxidized glutathione while the net flux is not significantly altered in the cells exposed to either oxytocin, reduced glutathione or L-cysteine.

In addition, the effects on glucose transport by insulin A-chain, insulin B-chain, insulin S-sulfo A-chain, insulin S-sulfo B-chain, and cysteic acid were investigated. The results obtained (Table IV) indicate that: (1) The net flux is markedly enhanced by the A-chain, B-chain, and to a lesser though still substantial extent by the S-sulfo A-chain and the S-sulfo B-chain. (2) The exchange flux is not significantly increased by any of these agents with the exception of the S-sulfo B-chain. (3) Cysteic acid,

TABLE III

THE AVERAGE NET AND EXCHANGE FLUXES OF [$^{14}C_6$]GLUCOSE IN HUMAN RED CELLS TREATED WITH AGENTS CONTAINING DISULFIDE OR SULFHYDRYL GROUPS

Flux values are expressed as mmoles/l cell water per s.

*Agent**	*Net flux ± S.E.*		*% change*	*P*	*Exchange flux ± S.E.*		*% change*	*P*	*Exchange flux/net flux*	
	Controls	*Treated cells*			*Controls*	*Treated cells*			*Controls*	*Treated cells*
Oxytocin	2.40 ± 0.03	2.61 ± 0.10	8	>0.05	5.87 ± 0.18	5.27 ± 0.24	(−10)	>0.05	2.44	2.02
Vasopressin	2.36 ± 0.13	2.89 ± 0.13	22	<0.01	5.31 ± 0.18	5.86 ± 0.42	10	>0.10	2.25	2.03
GSSG	2.19 ± 0.03	2.62 ± 0.07	20	<0.01	5.23 ± 0.27	5.87 ± 0.52	12	>0.10	2.39	2.24
GSH	1.80 ± 0.21	2.21 ± 0.08	18	>0.10	7.53 ± 0.68	6.54 ± 0.98	(−13)	>0.10	4.18	3.08
Cysteine	1.96 ± 0.16	2.24 ± 0.16	14	>0.10	6.62 ± 1.1	6.25 ± 0.77	(− 6)	>0.10	3.38	2.79
Insulin	2.28 ± 0.09	3.17 ± 0.15	39	<0.01	6.02 ± 0.16	6.79 ± 0.16	13	>0.10	2.64	2.14

* The concentrations of the compounds shown, in μmoles/ml packed cells, are as follows: oxytocin 0.56; vasopressin 0.54; GSSG 4.08; GSH 7.90; L-cysteine 7.40; insulin 0.28.

TABLE IV

THE AVERAGE NET AND EXCHANGE FLUXES OF [$^{14}C_6$]GLUCOSE IN HUMAN RED CELLS TREATED WITH INSULIN DERIVATIVES

The incubation temperature was 23–25 °C. Flux values are expressed as mmoles/l cell water per s.

*Agent**	*Net flux ± S.E.*		*% change*	*P*	*Exchange flux ± S.E.*		*% change*	*P*	*Exchange flux/net flux*	
	Controls	*Treated cells*			*Controls*	*Treated cells*			*Controls*	*Treated cells*
A-chain	2.24 ± 0.07	3.82 ± 0.15	70	<0.01	6.51 ± 0.24	6.82 ± 0.51	5	>0.10	2.91	1.79
B-chain	2.26 ± 0.13	3.51 ± 0.10	55	<0.01	6.52 ± 0.25	6.70 ± 0.36	4	>0.10	2.84	1.91
S-sulfo A-chain	2.23 ± 0.10	2.92 ± 0.09	31	<0.01	6.61 ± 0.51	6.94 ± 0.60	13	>0.10	2.96	2.38
S-sulfo B-chain	2.17 ± 0.08	2.85 ± 0.13	31	<0.01	5.70 ± 0.48	7.10 ± 0.26	25	<0.05	2.63	2.49
Cysteic acid	2.21 ± 0.22	2.24 ± 0.05	2	>0.10	5.72 ± 0.34	4.41 ± 0.33	(−23)	>0.10	2.59	1.97
Insulin	2.28 ± 0.09	3.17 ± 0.15	39	<0.01	6.02 ± 0.16	6.79 ± 0.23	13	>0.10	2.64	2.14

* The concentrations of the various agents shown in the above table are expressed in μmoles/ml packed red cells and are as follows: A-chain, 0.66; B-chain, 0.49; S-sulfo A-chain, 0.66; S-sulfo B-chain, 0.48; cysteic acid, 8.88; insulin, 0.28.

used as a representative control for molecules possessing $-SO_3$ groups, gave no significant change in either the net or exchange fluxes.

When red cells are exposed to phospholipase C[26] for short periods of time, no effect on the maximal net flux is observed, however, a marked inhibition of the exchange flux is evident. When red cells are pretreated with insulin, the inhibition normally obtained with phospholipase C is prevented (Table V).

The degree of binding of insulin to the red cell was measured under conditions which simulate those which exist during both flux measurements by washing the cells (after incubation with ^{131}I-labeled insulin) with either glucose–saline–phosphate or saline–phosphate solution. The data obtained indicated that at most only 1–2 % of the added insulin remained bound to the cells and that the extent of the binding was in no way affected by the choice of wash solution.

TABLE V

THE AVERAGE NET AND EXCHANGE FLUXES OF [$^{14}C_6$]GLUCOSE IN HUMAN RED CELLS

Red cells were exposed to: (1) phospholipase C, (2) insulin followed by phospholipase C, (3) phospholipase C followed by insulin. Fluxes are expressed as mmoles/l cell water per s.

	Net flux ± *S.E.*	% *change*	P^*	*Exchange flux* ± *S.E.*	% *change*	P^*	*Exchange flux* / *net flux*
Untreated cells	2.38	—	—	6.16 ± 0.34	—	—	2.59
Phospholipase C-treated cells	2.38 ± 0.08	0	—	4.24 ± 0.26	−31	<0.01	1.78
Insulin-treated cells exposed to phospholipase C	2.97 ± 0.14	+25	<0.01	5.70 ± 0.17	− 7	>0.10	1.92
Phospholipase C-treated cells exposed to insulin	2.67 ± 0.10	+12	<0.05	4.73 ± 0.14	−23	<0.01	1.77

* The values for P are for comparisons between the treated samples and their respective controls. There is no significant difference ($P>0.10$) between the exchange flux values of 4.24 and 4.73.

DISCUSSION

The flux data obtained with insulin-treated cells show that in general insulin elevates the net flux and has no significant effect on the exchange flux of glucose in human erythrocytes. Previous reports[15,16] of net influx measurements in human erythrocytes indicated that insulin had no effect on red cell permeability to glucose. However, under the experimental conditions of the previous investigations, a maximal net flux was not measured because during influx experiments the internal concentration of glucose in the cells rapidly exceeds the K_m for glucose transport[27]. In the present experiments this difficulty was specifically eliminated by measuring the maximal net efflux into a medium in which the concentration of glucose never approached the K_m for glucose transport, thus providing more sensitive conditions for detection of an insulin effect.

Rieser and Rieser[28] reported that insulin stimulates aldose–hexose transport into the human erythrocyte. Unlike the experiments reported here, this effect was obtained only after the red cells had been exposed to chymotrypsin for one hour at 38 °C. They suggested that the proteolytic enzyme removed some substance from the cell surface, allowing the red cell to become responsive to insulin with respect to the transport of aldose–hexoses. In the experiments reported here, a response of the maximal net flux to insulin without any other prior treatment of the cell has been observed. The levels of insulin utilized both in their experiments and those reported here are comparable when expressed as units/ml of cell water[29]. On such a basis, an insulin level of 0.9 unit/ml of cell suspension (hematocrit 12.5–15 %) used by Rieser and Rieser[28] would be equivalent to 8.6–10.2 units/ml of cell water. The insulin levels used here, when similarly expressed, would range from 0.1 to 152 units/ml of cell water.

There are, however, several differences in experimental techniques which may be responsible for the difference in observations reported in these two investigations: (1) In the experiments reported here the period of incubation with insulin was 30 min at 23 °C. Rieser and Rieser[28] incubated the cells for 15 min at 38 °C. (2) In the experiments reported here red cells were preloaded with glucose prior to insulin treatment. In the efflux experiments reported by Rieser and Rieser[28] cells were treated with chymotrypsin or chymotrypsin *plus* insulin before incubation with glucose. (3) Strikingly, different methods for the analysis of glucose movement were used in the two investigations. The methods utilized in the present experiments, involving the measurement of the rapid movement of an isotope of glucose and the calculation of fluxes from these initial glucose movements, may present a system more sensitive to discrete changes in the carrier mechanism.

Although several workers[17,30,31] have shown that differences up to twice the tonicity between cell and external media (differences not encountered in the experiments reported here) have no effect on the glucose flux in human erythrocytes, speculation may arise that the effect of insulin on maximal net efflux is related to the differences in tonicity between the glucose-equilibrated cell (385 mosmoles/l) and the glucose-free media (310 mosmoles/l). In several experiments the tonicity of the external media was adjusted with NaCl prior to measurement of the maximal net flux of untreated and insulin-treated cells. Under experimental conditions which were essentially isosmotic, insulin still elevated the maximal net efflux to the same degree as noted previously. The failure of heating to block the insulin effect completely does not strongly indicate a contaminant as to be the agent responsible for the elevation of the glucose flux. The usual elevation of the flux by insulin treatment is reduced by 80 % by heating and it is quite possible that several moieties on the insulin molecule may be responsible for the flux enhancement and that some of these are not necessarily heat denaturable. The elevation of the maximal net efflux in contrast to the absence of an effect on the exchange flux cannot be accounted for on the basis of insulin binding inasmuch as the number of molecules of insulin bound to the red cell is exceptionally low and is the same both in the presence or absence of glucose in the external medium.

Unlike the results obtained here for the red cell, it has been reported[32] that adrenocorticotropic hormone, oxytocin, vasopressin and the separated chains of oxidized insulin and of insulin *S*-sulfonate did not alter the permeability of frog muscles to 3-*O*-methyl-D-glucose. The concentration levels utilized in the experiments

reported here are several hundredfold greater than the quantities utilized by Wohltman *et al.*[32], and this may account for the difference. However, there are reports that these compounds do affect glucose permeability in rat hemidiaphragm[33], isolated hearts[34] and adipose tissue[35].

To effect an elevation of the net flux and at the same time not influence the exchange flux implies an effect directly on the free carrier. An effect on the binding capacity of the carrier for glucose would be evidenced by an alteration in the exchange flux. The same can be said for a proposed effect on the movement or capacity of loaded carrier. Since the exchange flux is unaffected and the overall pattern of insulin is to raise the net flux value towards the levels of the exchange flux, only the possibility that insulin interacts with free carrier appears capable of explaining the elevation of the maximal net flux. The movement of the carrier–hexose complex across the cell membrane is considered to be the rate-limiting step in the passage of sugar across the red cell membrane. The difference in rates between the exchange and net fluxes obtained with normal non-treated cells has been attributed to the different mobilities of the complexed carrier and the free carrier. It has been suggested[18] that free carrier moves some four times slower than complexed carrier. It is suggested here that the insulin elevation of the net flux, has the appearance on the whole, of an increase in the mobility of the free carrier. If the flux values obtained with insulin are inserted into a series of expressions utilized by Britton[36] to demonstrate differences in carrier mobility, it can be shown that an insulin-elevated net flux is equivalent to a 2-fold increase in the mobility of the free carrier.

Lieb and Stein[37] have recently proposed that the transport system be considered to be an oligomeric protein embedded in the membrane. They suggest that transport is the movement of substrate within such a protein and that there are two classes (high affinity and low affinity) of binding sites for glucose, each binding site represented by a different subunit of the oligomeric protein. In addition, they propose that the transition frequency of substrate is directly proportional to the number of substrate molecules bound (*i.e.* the occupancy of the other sites). With this proposition as a frame of reference it can be seen that the exchange flux and maximal net flux as determined in the experiments reported here appear to be analogous respectively to the situation where all the sites are occupied and where only some of the sites are occupied. In addition our results indicate that the transfer of glucose across the membrane under the experimental conditions designed to give a maximal net flux is slower by a factor of 2 than that obtained under conditions designed to obtain an exchange flux. When the presence of a carrier entity, whether of unknown make up or of the nature of an oligomeric protein is assumed, one also assumes that at least one face of this entity must be exposed to the external media. The size of an insulin molecule and the size of the red cell pores (8–10 Å diameter[38]) precludes the possibility of even a portion of the insulin molecule from passing through the membrane. Therefore, the effect obtained with insulin could be expected to be a surface phenomenon and in particular an interaction with the face of the carrier entity which is assumed to be exposed to the external media.

The general consensus as to a mechanism concerning insulin action is more or less summed up by Levine[8,39] who believes that the primary site of insulin action is at the cell membrane. The manner in which insulin interacts with the cell membrane is as yet uncertain. However, a number of interesting observations have been made

in this regard. Insulin has been shown[7] to bind to rat skeletal muscle cell membranes by means of electrovalent and covalent (disulfide) linkages. Those results indicated an involvement of the cyclic disulfide linkage of the insulin A-chain and the sulfhydryl groups on receptor protein. Similar conclusions of a thiol–disulfide exchange reaction have come from others studying the binding of insulin to rat epididymal fat pads and hemidiaphragms[25].

The similarity of action (namely an increase in permeability) by phospholipase C, phospholipase A and insulin on the cell membranes of free adipose cells with respect to glucose entry has been demonstrated[9,40,41]. It is suggested that the results of those experiments offer evidence that plasma membrane phospholipids play a role in the facilitated transport of glucose. It was suggested[9] that limited hydrolysis of membrane phospholipids might transform membrane lipoproteins from laminar to globular configurations with a resultant increased permeability to small molecules. No mention is made[41], however, as to whether insulin might be involved in a manner similar to the phospholipases. It has been suggested that an alteration of the configuration of plasma membrane lipoproteins could occur by formation of complexes between phospholipases, Ca^{2+} and their phospholipid substrates[41]. A similar interaction between insulin, Zn^{2+} and membrane lipoprotein has been proposed[39]. Several reports[35,43] have indicated that pretreatment of the tissue with *N*-ethylmaleimide and the resultant blocking of membrane sulfhydryl groups has had no effect on the subsequent binding of ^{131}I-labelled insulin. These results suggest that the thiol–disulfide interaction is not the primary mechanism in all cases of insulin–tissue interactions.

Another proposal for a mechanism of insulin and other disulfide–hormone action is presented by Robinson[44]. The author suggests that there may be an interaction between the sulfhydryl groups of the hormone and the *cis* double bonds in lipid fatty acids. He proposes that such an interaction could affect the packing of the hydrocarbon chains, such that there would be an alteration in the surface charge properties as well as in protein conformation. Such changes, if they occurred, could in turn affect membrane permeability.

Phospholipase treatment of the red cell in the experiments reported here caused a marked decrease in the exchange diffusion of glucose but had no effect on the maximal net flux. This sort of result suggests that the lipid moieties of the carrier entity are more readily accessible to phospholipase C when carrier is combined with glucose than when free of glucose. It further suggests that the carrier when combined with glucose is in a more open configuration (with regard to lipid moieties) than when free of glucose. The ability of insulin to protect the exchange flux inhibition by phospholipase C, may be merely the result of non specific binding resulting in steric hindrance between phospholipase C and membrane substrate or on the other hand it may suggest the ability of insulin to interact with *cis* double bond in fatty acids as proposed by Robinson[44].

The elevation of the maximal net flux, in a manner similar to that of insulin, by vasopressin and GSSG, which contain disulfide bonds, while GSH and cysteine were without any effect (Table III) lends support to the concept of thiol–disulfide interactions as a mechanism for insulin action on the membrane. However, the disulfide groups may be only one of several interaction sites. That this is possible as far as glucose transport in human red cells is concerned is evident from the effect

on the net flux obtained with insulin derivatives (Table IV). These molecules with the exception of the S-sulfo A-chain contain neither disulfide bridges nor sulfhydryl groups.

In addition, the decrease in the inhibition of the exchange flux brought about by phospholipase C, by pretreatment of the red cells with insulin, lends support to a possible interaction of insulin with lipid moieties. Our results suggest a varied and complex insulin–membrane interaction—an interaction of the insulin molecule with membrane sulfhydryl groups, protein side chains and fatty acid chains. The evidence that such interactions are possible may shed light on many an anomalous result obtained in insulin–membrane studies, and may also represent the varied possibilities of interaction operable under normal, abnormal or pathological conditions.

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