Factors Influencing Glucose Flux and the Effect of Insulin in Cultured Human Cells

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ABSTRACT Uptake of glucose-^{3}H into cultured HLM cells was measured. Equilibration of intracellular and extracellulaı pools occurred after 25 min. Glucose influx was determined subsequently by measuring the glucose-^{3}H entering in precisely 1 min. Although saturation kinetics were demonstrated these were not of the simple Michaelis-Menten type. The *Km* of the glucose carrier system is probably about 60 mM glucose. Galactose did not compete with glucose. Insulin stimulated glucose flux without increasing the value of V_{max}. The stimulation was fully demonstrable after 10 min, could be elicited at concentrations of 10^{-4} units/ml, and was absent 2-4 hr after removal. Increasing pH had little or no effect in stimulating glucose flux. Increasing osmotic pressure caused a marked increase and reduced the effect of insulin. Glucose influx was unaffected by anoxia. Glucose influx was increased and the effect of insulin abolished in the absence of K^+. Glucose influx was increased by mercuric chloride, iodoacetate, and fluoride which abolished the effect of insulin. Dinitrophenol decreased the rate of glucose uptake but did not alter the effect of insulin. Phlorizin reduced the rate of glucose uptake and abolished the effect of insulin. ATP and AMP enhanced the rate of glucose uptake. These findings are discussed in relation to the mode of action of insulin.

INTRODUCTION

Evidence for carrier-mediated transfer of monosaccharides has been obtained in many tissues and is reviewed by Le Fevre (1961); Randle and Morgan (1962); Hokin and Hokin (1963); and Wilbrandt (1961, 1963). Maio and Rickenberg (1962) have investigated monosaccharide transport in cultured cells. They have reported that the uptake of galactose by L cells displays saturation kinetics and is inhibited competitively by other sugars. Otherwise, little work has been done with this kind of material.

The effect of insulin in promoting an increase in the glycolytic metabolism of isolated tissues, such as rat diaphragm and the perfused rat heart, is well documented (Randle and Morgan, 1962). This effect has been attributed

both to an increase in the rate of transport of glucose into the cell and to an increased rate of conversion of glucose to lactic acid (Newsholme and Randle, 1961). Treatment with large amounts of insulin has also been shown to increase the rate of glycolysis of cell strains in culture (Paul and Pearson, 1960; Crockett and Leslie, 1963; and Vann, Nerenberg, and Lewin, 1963) but the nature of the effect has not been elucidated.

Cell monolayers would seem to offer unique advantages for the study of transport processes. In this communication a method is described for determining the rate of entry of glucose into the metabolic pools of cells cultured in this way. The effects of various factors and especially those of insulin on glucose uptake by HLM cells are detailed.

METHODS

Stock cultures of human fetal liver cells, strain HLM (Leslie et al., 1956), were used. The precise cellular origin of these cells has not been established. The "growth medium" used for maintaining the stock was a modified Waymouth's medium (Waymouth, 1959) containing 2% (v/v) human serum and 5% (v/v) calf serum, buffered with Tris-citrate–buffered balanced salt solution (Tris-citrate BSS, Paul, 1965). Standard cell culture techniques were used (Paul, 1965). In carrying out experiments two other media were used. Unless otherwise stated, "equilibration medium" consisted of Tris-citrate BSS, adjusted to pH 7.4, containing glucose at the concentrations required (if not stated this was 5.6 mM). "Test medium," unless otherwise stated, had the same basic composition but contained, in addition, labeled glucose (either uniformly labeled glucose-^{14}C or glucose-^{3}H, both obtained from the Radiochemical Centre, Amersham).

In preparation for an experiment, cells from stock cultures were harvested by trypsinization and suspended in growth medium. A cell suspension, diluted to contain 1-2 $\times$ 10^5 cells/ml, was dispensed into 25 ml conical flasks (5 ml/flask) using a Cornwall syringe. The mouths of the flasks were covered with aluminum foil and the cultures incubated at 37°C.

After 2-3 days (by which time the cell population had increased to 2-4 $\times$ 10^6 per flask) the cultures were removed from the incubator and the overlying medium was discarded. 5 ml of equilibration medium were added, the flask was gently agitated, and the wash discarded. 5 ml of equilibration medium, which had been warmed to 37°C, were then added. After 30-45 min equilibration at 37°C (except when stated otherwise) a culture was removed from the water bath and the equilibration medium was discarded. The vessel was inverted and shaken rapidly to remove as much of the residual fluid as possible. It was placed immediately in a shaking water bath and 2 ml of test medium at 37°C were added for precisely 1 min (except in the experiments illustrated in Fig. 1 and Table VI, which were designed to study the time course of uptake and loss of glucose). The test medium was removed rapidly with a Pasteur pipette and the culture immediately washed four times in succession with 5-10 ml amounts of cooled (4°C) equilibration medium. This procedure removed the residual labeled glucose, which was presumed to be extracellular (Table I). After discarding

the final wash, 2 ml of 0.2 N perchloric acid at 4°C were added. The culture was then placed in crushed ice for 30-45 min. The cold acid extract was removed with a Pasteur pipette and added to a tube containing 0.5 ml of ice-cold N KOH to precipitate perchlorate. After centrifugation the extract was retained for determination of radioactivity. 2 ml of 2 N perchloric acid were then added to the culture flask and the vessel was placed in a shaking water bath at 70°C for 20 min to extract DNA nucleotides. Estimation of DNA was performed by the method of Ceriotti (1952).

Radioactivity in the neutralized extract was determined, in experiments using glucose-^{14}C, with a Nuclear-Chicago gas flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.) with a thin end window and an automatic changing device. The extract (0.2 ml) was plated on a stainless steel planchet and dried under infrared lamps. A 1:2 dilution (0.2 ml) of the extract was also plated out to determine self-absorption (which was invariably minimal).

TABLE I

REMOVAL OF EXTRACELLULAR RADIOACTIVITY FROM HLM CELL CULTURES AFTER EXPOSURE TO LABELED GLUCOSE (3.5 MC/MMOLE)

Replicate cultures were washed the number of times indicated and the residual activity in the cells determined by the method described in the text.

No. of washes	Residual activity in cells — Wash volume: 5 ml (experiment A)	Residual activity in cells — Wash volume: 10 ml (experiment B)
	counts/min/μg DNA phosphorus	
1	2440	610
2	935	95
3	636	82
4	570	50
5	554	97
6	400	70
7		73
8		56

When glucose-^{3}H was used, 1 ml of the neutralized extract was added to 8 ml of Scinstant scintillator NE572 (Nuclear Enterprises, Ltd., Edinburgh, Scotland), made up in dioxane. Samples were counted with a Packard Tricarb (Packard Instrument Co., Inc., Downer's Grove, Ill.) or Nuclear-Chicago liquid scintillation spectrometer. The efficiency was calculated from the ratio of counts obtained on two separate channels. Efficiency was approximately constant and no correction was necessary.

In experiments to study the effect of insulin, it was incorporated in the equilibration medium at the concentration required. Glucagon-free insulin was donated by Boots Pure Drug Co. Ltd., Nottingham, England and was received as a sterile preservative-free stock solution containing 200 units per ml. 1 unit per ml was used in the equilibration medium unless otherwise stated.

RESULTS

Uptake and Efflux of Glucose

Fig. 1 shows the uptake and loss of labeled material. In this experiment replicate cultures were maintained in glucose-^{3}H for 25 min and samples were taken during that time. After 25 min the remaining cultures were washed and returned to medium which did not contain glucose-^{3}H. The uptake curve, representing net entry (influx minus efflux), is distinctly less steep than the efflux curve. From the efflux curve two classes of labeled components were apparently present after 30 min of labeling; one passed out readily while the other did not leave the cell, even after 35 min incubation in medium devoid of glucose-^{3}H.

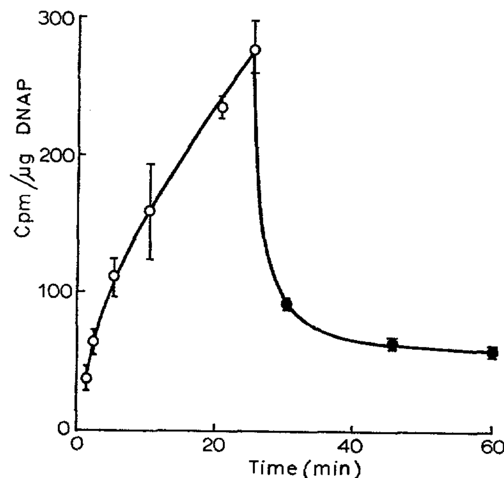

FIGURE 1. Net uptake and efflux of glucose-^{3}H. Cultures were maintained in glucose-^{3}H up to the point of inflexion; they were then washed and transferred to medium which did not contain glucose-^{3}H. Samples were taken at the points indicated. Means are shown; vertical lines indicate the range of the observations.

Wilbrandt (1961) pointed out that theoretically measurements of sugar efflux are preferable to those of influx as efflux can be determined under conditions where transport is virtually unidirectional, whereas influx measurements usually represent net inward transport under conditions in which movement out of and into the cell occurs simultaneously. With the technique used in this study, however, it is not practicable to take samples during the first few minutes of efflux. Moreover, efflux measurement is complicated by the likelihood that some of the radioactivity remaining within the cell represents products of glucose metabolism rather than residual glucose.

Rates of influx can be estimated from the amounts of labeled glucose entering cells during very brief exposures when the intracellular pool is labeled to a very small extent. In these experiments, the uptake of labeled glucose during a 1 min exposure period was taken to represent glucose influx. As

equilibrium was not reached in 25 min (Fig. 1) this is less than 5% of the equilibrium time; the error due to efflux could be assumed to be small. In all subsequent experiments (except those illustrated in Table VI) influx is therefore measured by the amount of glucose-^{3}H entering the cells in 1 min, as described in the Methods section.

Effect of Glucose Concentration on Glucose Influx

Glucose uptake by the perfused rat heart has been shown to conform to Michaelis-Menten kinetics (Park et al., 1959) and an approximate *Km* value of 157 mg per 100 ml (8.7 mM) has been calculated (Morgan et al., 1961). In erythrocytes similar *Km* values have been found (Widdas, 1954). Con-

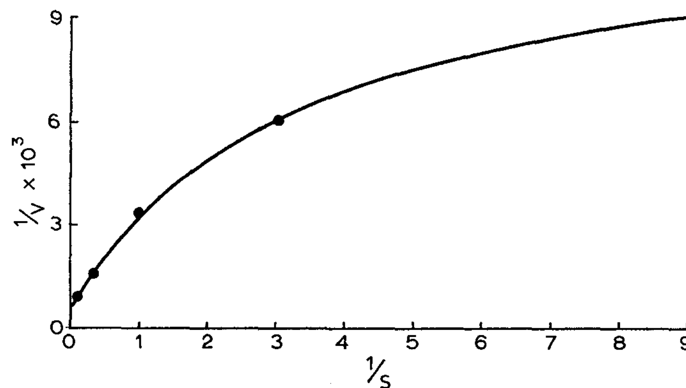

FIGURE 2. Lineweaver and Burk plot of the relationship between initial velocity of influx of glucose-^{3}H and glucose concentration in the medium, at low substrate concentrations (0.11–9 mM).

siderably lower *Km* values for glucose (1 mM) and galactose (0.5 mM) were reported for the L cell (Maio and Rickenberg, 1962).

We therefore performed experiments to determine the effect of glucose concentration on the initial rate of glucose influx in our system. When our findings are plotted in the reciprocal form of Lineweaver and Burk (Figs. 2-4) a linear relationship is not found consistently. At very low concentrations (which correspond to the conditions used by Maio and Rickenberg, 1962) the *Km* tends to a value of 1 mM glucose or less, whereas at high concentrations a value of the order of 50-100 mM glucose is approached. Hence, although saturation kinetics are approximated, our data cannot readily be reconciled with a simple carrier hypothesis. If two systems, one with a low *Km* and one with a high *Km*, were postulated, the findings could be better reconciled. The contribution of the component with the low *Km* varied from experiment to experiment (see Figs. 2-4). No reason for this could be discovered.

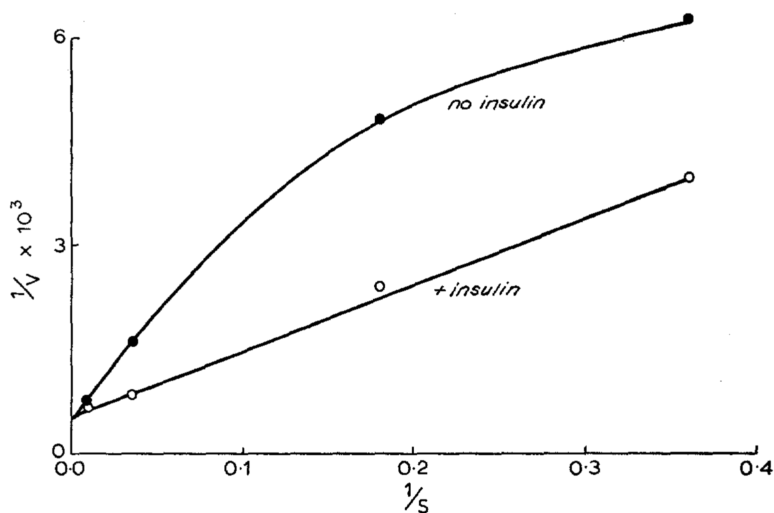

FIGURE 3. Lineweaver and Burk plot of the relationship between initial velocity of influx of glucose-^{3}H and glucose concentration in the medium, at high substrate concentrations (2.8–55.6 mM).

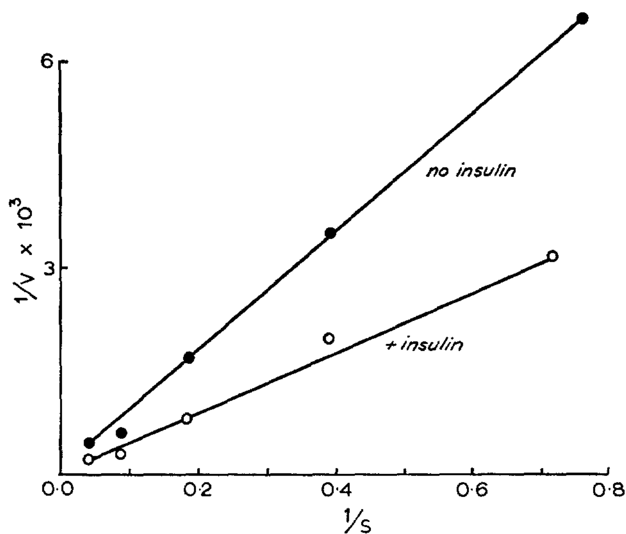

FIGURE 4. Lineweaver and Burk plot of the relationship between initial velocity of influx of glucose-^{3}H and glucose concentration in the medium (0.14–88 mM) showing linear relationship.

Competitive Inhibition of Glucose Uptake by Galactose

Competition among hexoses and pentoses for the glucose transport system of red blood cells (Le Fevre, 1961), muscle tissue (Randle and Young, 1960), Ehrlich ascites tumor cells (Crane et al., 1957), and L cells (Maio and Ricken-

berg, 1962) is well established. However, the results presented in Table II clearly indicate that galactose does not inhibit glucose influx in HLM cells even when the ratio of galactose: glucose is very high. The difference between the results obtained with 0.11 mM glucose (galactose: glucose ratio = 80.1) is not statistically significant.

Effect of Insulin on Glucose Influx

Table III shows that the addition of 1 unit of insulin per ml medium stimulated the rate of glucose influx by a factor of 1.5 to 2 after 10 min exposure and that increased exposure did not lead to greatly increased stimulation. The effect of insulin diminished after exposure periods greater than 4 hr.

TABLE II
GLUCOSE UPTAKE IN THE PRESENCE OF GALACTOSE

Glucose concentration	Glucose uptake	
	Control	+ 8.6 mM galactose
mM	*cpm/min/μg DNA phosphorus*	
0.11	11,200	6832
	±3871	±1585
0.33	15,700	16,660
	±3315	±4710
1.0	30,300	35,000
	±9626	±9625
3.0	67,200	46,100
	±15,180	±7350
9.0	104,000	98,500
	±17,200	±19,450

To determine how long this effect persisted cell cultures were equilibrated for the usual 30 min, exposed to insulin for 10 min, and washed three times with BSS. The cultures were then incubated in equilibration medium without insulin and influx of labeled glucose was determined after various intervals. The results (Table III, bottom) indicate that the insulin effect persisted for about 2 hr during which time it decreased progressively. These findings may be compared with those of Bleehen and Fisher (1954) who reported that maximum stimulation of glucose uptake by the perfused rat heart was achieved after 10 min exposure to insulin; however, this effect disappeared within 10 min of its removal.

Effect of Insulin Concentration on the Rate of Glucose Influx

When cells were exposed to insulin for 10 min before measuring glucose influx, stimulation of up to 45% could be measured with insulin concentra-

tions of 5 × 10^{-4} units per ml. If the exposure time were increased to 1 hr, stimulation of glucose influx of up to 30% could be measured with insulin concentrations down to 10^{-4} units per ml. The stimulation decreased as the concentration of insulin was reduced below 10^{-2} units per ml.

TABLE III

EFFECT OF TIME OF EXPOSURE TO INSULIN (1 UNIT/ML) ON THE RATE OF GLUCOSE INFLUX AND DURATION OF THE EFFECT AFTER REMOVAL OF INSULIN

Experiment No.	Time of exposure to insulin	Rate of glucose influx: No exposure to insulin (*C*)	Rate of glucose influx: With exposure to insulin (*T*)	Stimulation
	min	*cpm/min/μg DNA phosphorus*		%
1	1	1440	1343	−7
	30	1403	2517	79
	120	1867	2647	42
	240	1492	1930	29
	720	1310	1487	14
2	1	242	244	0
	10	198	297	50
	30	206	314	52
	90	246	261	6
	180	193	279	45
3	10	1132	2180	93
	120	1342	2375	77
	240	972	1845	90
	1020	1372	1555	13
	Time after 10 min exposure to insulin			
	min			
	15	1098	2180	99
	45	1200	1955	63
	75	1113	1833	65
	125	1240	1500	21
	270	820	940	15

The effect was, therefore, manifested at concentrations of insulin which could be expected in vivo (Randle and Taylor, 1960). The sensitivity was at least equal to that of the perfused heart preparation (Fisher and Lindsay, 1956) or the rat diaphragm (Randle and Taylor, 1960).

The Effect of Insulin at Different Glucose Concentrations

Results of an experiment to determine the effect of insulin on glucose influx at different glucose concentrations are plotted in the reciprocal form of

Lineweaver and Burk in Fig. 4. (In this instance the "low *Km*" component was virtually absent.) A similar trend was evident in those cases in which the kinetics were nonlinear (Fig. 3). The figures from both control and insulin-treated cultures gave curves which converged towards the same V_{max}. The effect of insulin on glucose influx could be demonstrated at very low glucose concentrations; for example, glucose influx was found to be increased by a factor of 2.5 by 1 unit per ml of insulin when the medium contained only 0.028 mM glucose.

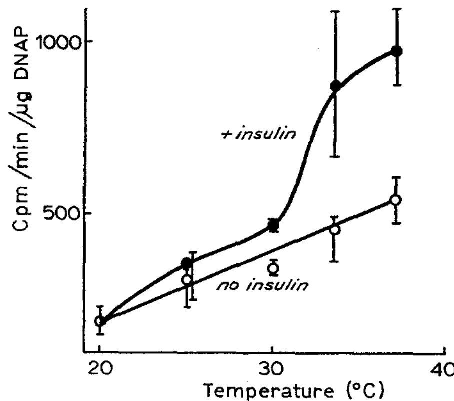

FIGURE 5. Relationship between rate of glucose influx and temperature.

Influence of pH, Temperature, and Osmotic Pressure on the Rate of Glucose Uptake and the Effect of Insulin

To study the effect of pH, cells were grown under standard conditions and transferred to media adjusted to different pH during the equilibration and test stages of the experiment. Tris-citrate buffers of constant ionic strength and otherwise similar to the equilibration medium were used.

No clear difference in the rate of glucose influx or in the effect of insulin over the pH range from 6.8 to 7.6 could be demonstrated.

Since it has been shown that the pH of the medium markedly influences glycolysis in cultured cells (Zwartouw and Westwood, 1958; Paul, 1959), glycolysis would not appear to be a limiting factor in regulating glucose flux in these cells.

To determine the effect of temperature, cultures were equilibrated at various temperatures and exposed to insulin for 10 min before measuring glucose-^{3}H flux. The results, shown in Fig. 5, indicated a Q_{10} for control cultures of about 1.7. Below 30°C there was virtually no effect of insulin on the rate of glucose influx but above this temperature insulin had an increasingly stimulatory effect.

Widely differing Q_{10} values for glucose transport and for the insulin effect

have been reported. Levine and Goldstein (1955) found a Q_{10} of 1.0-1.2 for the insulin effect in the perfused dog preparation. Narahara et al. (1960) reported a Q_{10} (27-37°C) of about 2 for pentose accumulation in rat skeletal muscles stimulated with insulin. Neglecting nonlinearity a Q_{10} (27-37°C) of 2.4 can be estimated from the results in Fig. 5.

Some workers have argued that low Q_{10} values provide evidence against the existence of a sugar transport mechanism (Kipnis and Cori, 1959). Randle (1961) points out, however, that it is doubtful whether the effect of temperature on the transport process per se was measured in these experiments. Since an interrelationship between energy metabolism and glucose transport is probable (Randle and Smith, 1958 *a*), the effect may be a result of the influence of temperature on both processes.

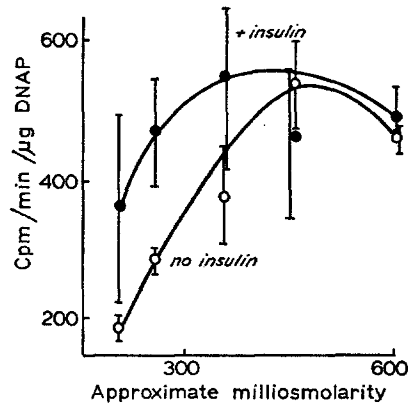

FIGURE 6. Relationship between rate of glucose influx and osmotic pressure of the medium.

The rate of glucose influx into cells equilibrated in Tris-citrate buffer at various osmolarities was determined and the results are shown in Fig. 6. Osmolarity was adjusted by varying the concentrations of all salts in constant ratios.

The rate of uptake was found to increase with increasing osmolarity to a maximum at about 450 milliosmolar. The effect of insulin was least at the highest osmotic pressures employed.

Vann et al. (1963) found similar effects of osmolarity on glucose utilization by HeLa cells in the presence or absence of insulin. They reported that maximal stimulation by insulin occurred under slightly hypotonic conditions. In contrast, basal glucose utilization was maximal under somewhat hypertonic conditions. In view of the results presented here, it is possible that the effects observed by Vann et al. were secondary to effects of osmolarity on glucose flux.

Effect of Oxygen Tension

Since conditions of anoxia are known to accelerate glucose transport in muscle (Morgan et al., 1959; Randle and Smith, 1958 *b*) an experiment was

performed to determine whether similar effects could be observed with HLM cells. No difference in glucose influx could be detected between cultures equilibrated in the presence of N_2 and those equilibrated in the presence of air.

TABLE IV

INFLUENCE OF THE IONIC COMPOSITION OF EQUILIBRATION AND TEST MEDIA ON THE RATE OF GLUCOSE INFLUX AND THE RESPONSE TO INSULIN

		Rate of glucose uptake	
Experiment	Alteration to medium	Insulin not added Mean ± SD	Insulin added Mean ± SD
		cpm/min/μg DNA phosphorus	
	0	2353 (3)	3440 (3)
1	Mg^{++} omitted	2280 (3)	3977 (3)
	Ca^{++} omitted	2715 (3)	4048 (3)
2	Molar ratio $Na^{+}:K^{+}$		
	24:0	885±48 (6)	1028±93 (6)
	2:1	428±52 (5)	935±71 (6)
	1:2	602±62 (6)	1205±80 (6)
	0:24	695±52 (6)	1290±80 (6)
	24:0	1198±268 (6)	1147±317 (6)
	24:1	415±89 (6)	766±148 (6)
	1:2	333±45 (6)	475±79 (6)
	0:24	392±90 (6)	597±124 (6)

The figures in parentheses indicate the number of observations used in determining the mean and standard deviations.

Influence of Ions

The effects of omitting magnesium or calcium salts from equilibration and test media are shown in Table IV. Omission of either divalent cation had no marked effect. When a range of sodium to potassium ratios was studied, the striking fact became apparent that the omission of K^{+} induced increases in the rate of glucose uptake by control cultures to levels observed for insulin-treated ones, and, at the same time, abolished the effect of insulin.

Other alterations in the sodium to potassium ratio did not have any consistent effect.

Influence of Metabolic Inhibitors on the Rate of Glucose Uptake and the Effect of Insulin

Compounds which are known to react with sulphydryl, amino, and some other groups have been shown to inhibit glucose transport in erythrocytes

(Le Fevre, 1961). On the other hand many enzyme inhibitors such as fluoride, iodoacetate, azide, and cyanide do not inhibit transport in these cells.

Anoxia and inhibitors of oxidative phosphorylation, however, stimulate sugar transport in muscle, as does sodium fluoride (Morgan et al., 1959; Randle and Smith, 1958 *b*; Newsholme and Randle, 1961). This has been interpreted as indicating that products of oxidative phosphorylation somehow keep the glucose transport system inactive in muscle.

TABLE V

THE INFLUENCE OF VARIOUS COMPOUNDS ON THE RATE OF GLUCOSE UPTAKE BY HLM CELLS AND THE EFFECT OF INSULIN

Experiment No.	Concentration of compound added	Rate of glucose uptake	
		Insulin not added Mean ± SD	Insulin added Mean ± SD
	mM	*cpm/min/µg DNA phosphorus*	
59	0	1200 (2)	2310 (2)
	$HgCl_2$ (0.1)	2460 (2)	2435 (2)
	NaN_3 (10)	1497 (2)	2337 (2)
	NaF (10)	2248 (2)	2738 (2)
	NaCN (1)	1168 (2)	2437 (2)
	Sodium salicylate (5)	1595 (2)	2560 (2)
	2,4-Dinitrophenol (0.25)	882 (2)	1562 (2)
	Phlorizin (3)	672 (2)	675 (2)
60	0	623±136 (5)	1159±223 (5)
	Phlorizin (3)	319±42 (5)	384±75 (5)
	Sodium iodoacetate (1)	1974; 1773	1605; 2024; 1713
61	0	497±167 (5)	864±242 (5)
	Ouabain (1)	514±94 (5)	889±244 (5)
	p-Chloromercuribenzoate (0.005)	489±75 (5)	464±77 (5)
	N-Ethylmaleimide (0.01)	684±100 (5)	1100±360 (5)
	Stilbestrol (0.01)	527±136 (5)	634±49 (5)
	Iodoacetamide (0.025)	632±280 (5)	499±203 (5)

No. of observations indicated in parentheses.

The effects of these and other inhibitors, at concentrations comparable with those used by other authors, on the rate of glucose influx in HLM cells (Table V) were somewhat different from those on muscle and red blood cells.

Mercuric chloride, iodoacetate, and fluoride caused an increase in the rate of glucose influx in control cultures and masked or abolished the effect of insulin. Iodoacetate induced an increase in the rate of glucose influx to levels above those shown by cultures treated with insulin. The effects of iodoacetate and fluoride were shown to be dependent on pH. At pH 6.8 each

of these substances caused a fivefold increase in glucose flux, whereas at pH 7.8 it was doubled. This effect of fluoride on sugar uptake has also been observed with rat diaphragm (Newsholme and Randle, 1961).

Azide or salicylate had little or no effect but dinitrophenol caused a decrease in the rate of glucose uptake by both insulin-treated and control cultures without eliminating the effect of insulin. On the other hand, *p*-chloromercuribenzoate abolished the effect of insulin without altering the basal rate of glucose uptake. A similar trend is suggested in the presence of iodoacetamide and stilbestrol. Cyanide, *N*-ethylmaleimide, and ouabain had little apparent effect.

TABLE VI

EFFECT OF INSULIN AND FLUORIDE ON THE INFLUX AND EFFLUX OF GLUCOSE IN HLM CELLS

For experimental procedure see legend to Fig. 1.

		Intracellular radioactivity *cpm/µg DNA phosphorus*		
	Time	No addition	Insulin added	Fluoride added
	min		*1 U/ml*	*10 mM*
Influx experiment	1	35 (3)	180 (3)	191 (3)
	5	216 (3)	386 (3)	454 (3)
	15	427 (3)	608 (3)	689 (3)
	30	541 (3)	742 (3)	853 (3)
Efflux experiment	0	304 (3)	463 (3)	485 (3)
	5	163 (3)	168 (3)	218 (3)
	15	91 (2)	135 (3)	109 (3)
	30	77 (2)	100 (2)	80 (2)

No. of observations indicated in parentheses.

The effect of phlorizin on HLM cells appears to be similar to its effect on other cell types. Phlorizin has been shown to inhibit sugar transport in erythrocytes (Le Fevre, 1959), rat heart (Morgan and Park, 1957; Morgan et al. 1959; Park et al., 1959), Ehrlich ascites tumor cells (Crane et al., 1957), L cells (Maio and Rickenberg, 1962), and others. Keller and Lotspeich (1959) reported that phlorizin inhibited the insulin-activated transport of galactose into skeletal muscle, a finding in accord with that reported here.

It is notable that, in HLM cells, inhibitors of glycolysis generally stimulated glucose influx whereas the addition of substances known to interfere with oxidative phosphorylation did not accelerate uptake as they do in muscle (Morgan et al., 1959; Randle and Smith, 1958 *a*).

Comparison of the Effects of Insulin and Fluoride on the Influx and Efflux of Glucose

Since the addition of fluoride enhanced the rate of uptake of glucose by HLM cells, its effect on influx of glucose over a longer period and on the efflux of glucose was compared with the effect of insulin (Table VI). Fluoride caused an even greater increase in the rate of uptake than did insulin. The efflux of radioactivity was very rapid in both cases.

ATP and AMP also enhanced the rate of uptake of glucose by these cells (Table VII). The actions of fluoride and ATP appear to be additive.

TABLE VII
EFFECT OF FLUORIDE AND ADENINE NUCLEOTIDES ON THE RATE OF GLUCOSE INFLUX

	Rate of uptake
	cpm/min/μg DNA phosphorus
	Mean ± SD
Control	1094±165 (5)
+ NaF (10 mM)	2576±772 (5)
+ AMP (0.5 mM)	1958±134 (5)
+ ATP (1.5 mM)	2773±597 (5)
+ ATP (1.5 mM) and NaF (10 mM)	3780±519 (5)

DISCUSSION

The demonstration of saturation kinetics, competitive inhibition, and stereospecificity in the entry of sugars into various cell types has been taken as evidence for the participation of specific transport sites in this process (Le Fevre, 1961; Wilbrandt, 1961).

Glucose entry into HLM cell cultures exhibits saturation kinetics but these are not strictly of the Michaelis-Menten type. One possibility is that two mechanisms (or more) are involved in glucose flux. On the other hand, in experiments with whole cells kinetic studies of this kind may have little meaning especially when glucose, the main energy substrate for cell metabolism, is being varied over a wide range. For example, at very low concentrations of glucose, pyruvate production is much reduced; hence, the nonlinearity at low substrate concentrations may merely reflect inhibition of total energy metabolism at these levels. This explanation gains some support from the effect of insulin in restoring a linear relationship between $1/V$ and $1/S$ (Fig. 3). This could be due to increased glucose influx in the presence of insulin, which would enable total energy metabolism to proceed more effectively at low glucose concentrations. If this hypothesis is correct then the

true kinetics of glucose flux are more likely to be represented by the "high *Km* component." In this event the value of *Km* for glucose influx in HLM cells is of the order of 60 mM glucose, higher than values reported previously.

No inhibition of glucose entry by galactose could be discerned. Similar observations have been made in other systems. Battaglia and Randle (1960) reported that galactose and other sugars do not compete with glucose for transport into rat diaphragm and they suggested that a separate carrier exists for each group of sugars. Also, Nirenberg and Hogg (1958) found that in the Gardener lymphosarcoma ascites tumor cell a 200-fold excess of galactose was required to demonstrate inhibition of glucose uptake.

Most of the metabolic inhibitors which stimulated glucose influx inhibit glycolysis from the glyceraldehyde-3-phosphate dehydrogenase step onwards. Since ATP is degraded to ADP in the hexokinase and phosphofructokinase reactions, an accumulation of ADP, and possibly AMP, at the expense of ATP would be expected. Hence the effect of glycolytic inhibitors can be interpreted in at least two ways. On the one hand, if the pool of labeled glucose is metabolized to pyruvate in significant amounts during a 1 min exposure, the addition of a glycolytic inhibitor might cause some accumulation of labeled intermediates. On the other hand, the increase of intracellular radioactivity in the presence of glycolytic inhibitors could represent an increase in movement of glucose across the cell membrane, probably by a mechanism similar to that postulated for muscle by Randle and Smith (1957, 1958 *a*, 1960). They suggested that the glucose carrier mechanism is normally kept in an inhibited state in muscle. This is presumed to involve an ATP-dependent phosphorylation reaction. Conditions which decrease the supply of ATP decrease the rate of carrier inactivation and hence increase the transport rate. Thus, sugar transport in muscle is accelerated by fluoride, 2,4-dinitrophenol, salicylate, cyanide, and anoxia (Randle and Smith, 1958 *a*; Morgan et al., 1959; Newsholme and Randle, 1961). In HLM cells only substances which could be expected to inhibit glycolysis had a stimulatory effect on glucose transport. Inhibitors of respiratory chain phosphorylation, such as cyanide, 2,4-dinitrophenol, and salicylate, were ineffective. This discrepancy may be related to the fact that most tissue culture cell lines have a high rate of aerobic glycolysis and use "glycolytic ATP" as a major source of energy.

Many of our findings are compatible with a mechanism in which ATP, AMP, or their derivatives regulate the glucose flux. One could either postulate that ATP actively inhibits the mechanism (Randle and Smith, 1957, 1960; Haynes et al., 1960) or, alternatively, that AMP and/or ADP activates it. Wu's finding (1959) that the amount of AMP in HeLa cells is markedly increased by fluoride (although the total concentration of adenine nucleotides is decreased) would fit in with either hypothesis since fluoride has a

marked effect on glucose flux. Our finding that the addition of AMP or ATP to the medium increases net glucose influx might be held to argue for activation by AMP and/or ADP since ATP is almost certainly degraded at the cell surface by the ATPases there. It is somewhat surprising that these substances are directly effective since they are not thought to enter cells; they may act directly at the cell surface.

One can only speculate about the way in which insulin influences glucose flux. However, it is worth pointing out that several workers have adduced evidence for a primary action of insulin which involves increased turnover of ATP (Kaplan and Greenberg, 1944; Sacks, 1945; Haugaard et al., 1951; Paul and Pearson, 1960). It may be relevant that most of the factors we have found to increase glycolysis, and which may do so by altering the relative amounts of adenosine phosphates, also abolish the effect of insulin.

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