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EFFECTS OF INHIBITORS AND COMPETITOR ON THE TRANSPORT OF CARBOHYDRATE IN CRITHIDIA RILEYI

A THESIS

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TRANSPORT OF CARBOHYDRATE IN CRITHIDIA RILEYI

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SUMMARY

The entrance of carbohydrates into the cells of Crithidia rileyi has been studied with KCN as an inhibitor, using non-metabolizable carbohydrates: L-sorbose, L-xylose, D-xylose, L-arabinose, and D-ribose.

The competition between L-sorbose and D-glucose has also been studied.

At 0.5 mM external concentration the final intracellular concentration becomes equal to or exceeds the extracellular concentration, and the carbohydrate transport is dependent upon an active transport mechanism. At 20 mM extracellular concentration the maximum intracellular concentration does not exceed the extracellular concentration, and the carbohydrate transport is a facilitated diffusion.

The results of inhibition experiments with KCN show that the range of inhibition varies from 9 percent to 34.5 percent and show their maximum inhibition after 16 minutes of exposure. KCN inhibits the uptake of sorbose more than that of any other sugar. There was no sign of inhibition at 20 mM extracellular concentration.

The rate of sorbose transport indicates that the competition between sorbose and glucose is greater at higher external concentrations. The entrance of sorbose is sensitive to the presence of glucose and is dependent upon the concentration of glucose present. The results of the studies on the effect of the presence of glucose on the entrance of sorbose show that there is competition between monosaccharides presented simultaneously to the cells and the transport mechanism shows greater affinity for glucose than for sorbose.

CHAPTER I

INTRODUCTION

When a substance moves through a cell membrane against a concentration or electrochemical gradient, a mechanism other than diffusion is required. It has been found that molecules of substances are transported into the cell by a mechanism which involves the cell membrane in a special role of transport phenomena. It has been postulated that there are two types of transport systems in membrane transport, active and passive. It the transport is dependent on metabolic energy and occurs against a concentration difference it is called "active transport." Passive transport includes simple diffusion and facilitated diffusion. The energy for transport by both simple diffusion and facilitated diffusion is derived from thermal agitation. There are some differences between simple diffusion and facilitated diffusion. When the transport is independent of metabolism, and results in no accumulation against a concentration gradient, it is called "simple diffusion." While simple diffusion is a form of passive transport, not all passive transport is accomplished merely by simple diffusion. Facilitated diffusion has well known properties (e.g., cf., Cirillo, 1961): it may be influenced by monosaccharides with representative differences in structure and steric properties of the molecules, and may be inhibited competitively by penetrating substances (a carrier-mediated mechanism).

Pinocytosis is also an important transport mechanism. This process leads to invagination of parts of the cell membrane which are consequently separated from the larger advancing portion of the membrane.

The process of substance transport seems to be general to all types of cells. It is thought that probably more solutes enter into cells actively than passively.

In plant cells the root hair cells are capable of taking up, against a concentration gradient, the various necessary salt ions such as potassium, magnesium, calcium and nitrate (Hoagland, 1944; Stewart and Miller, 1954). In studies of fresh water protozoans it was shown that these organisms make use of active transport in voiding water (Kitching, 1954). From studies of sugar utilization by baker's yeast Rothstein (1954) found that the yeast cell membrane was impermeable to non-metabolizable sugars; however, Cirillo (1959, 1961a) showed that the cell membrane of baker's yeast has a special mechanism which permits the entrance of a number of nonfermentable monosaccharides. Carbohydrate transport into microorganisms has been studied by quite a number of investigators. Non-metabolizable sugars have been used for the studies of the transport system since glucose and the non-metabolizable sugars show competition for transport. It has been found that L-sorbose, L-galactose, D-xylose, D-arabinose, L-fucose, and D-ribose were transported by stereospecific carrier system. In this system transport was found to be independent of metabolic energy and there was no accumulation of sugars against a concentration difference. From the studies of carbohydrate transport in Crithidia luciliae Min (1965) found that the

mechanism of transport of sugars into this cell is different at low and at high external concentrations: (a) an active transport mechanism active at low external concentration and dependent upon a supply of metabolic energy; (b) facilitated diffusion, of importance only at high external concentrations. The carbohydrate transport into Escherichia coli has been studied and has been demonstrated to be a result of an active transport mechanism capable of accumulating sugar against great concentration differences (Doudoroff, et al., 1949; Pardec, 1957; Horecker and Monod, 1960; Osborn and McLellan, 1960; Hoffee, et al., 1962). Egan and Morse (1965) reported that carbohydrate transport into Staphylococcus aureus is mediated by specific permeases and a common membrane carrier for three reasons: (1) because the rate of entrance of sorbose did not saturate even at high concentrations; (2) by the ability of one carbohydrate to displace another accumulated within the cells (counterflow); and (3) lactose inhibits the entrance of other carbohydrates only in cells previously induced to transport lactose. They also suggested that the carbohydrate transport in general in Staphylococcus is necessary to invoke permeases specific for various carbohydrates.

From the studies of Neurospora crassa Crocken and Tatum (1967) found that the transport of sorbose into these cells does not occur against a concentration gradient, but obeys saturation kinetics; is energy requiring; and is competitively inhibited by glucose. Thus, the sorbose transport into N. crassa is mediated by a carrier-mediated mechanism rather than by simple diffusion. Cirillo (1962) suggested that carbohydrate transport into yeast (Saccharomyces cerevisiae)

is also by a carrier-mediated transport mechanism because D-glucose was found to compete with L-sorbose for a common membrane transport system with an apparent affinity greater than twenty-five times that of sorbose, and the rate of glucose transport into fructose-loaded cells is greater than into unloaded cells.

In mammals, Alvarado (1967) suggests that xylose transport into the chicken small intestine occurs through a Na^+ dependent mobile carrier mechanism. From the study of the isolated small intestine of grass frogs (Rana pipiens) Csaky and Ho (1966) found that D-mannose transport into the intestines of those animals is by an active "pumping" transport mechanism. The active transport of pentose can be demonstrated only if the sugar is present in very low, preferably tracer, amounts on both sides of the intestinal epithelium. The transport of carbohydrates into red blood cells has also been studied by Rosenberg (1956), Lacko, et al. (1961), LeFevre (1962), and Miller (1965).

In this paper the effect of KCN as a metabolic inhibitor has been studied. The inhibitory effects of KCN were measured at appropriate time intervals after addition to the cell population; thus, it was possible to detect the intracellular carbohydrate concentrations after various time increments.

It has been found that when metabolizable and non-metabolizable sugars are present simultaneously the entrance of non-metabolizable sugar is inhibited competitively by the metabolizable sugar. This competition has been found in yeast, protozoa, and chicken intestine, and it has been suggested that the penetrating sugars into cells may share a common carrier.

Competition between sugars has been studied in yeast (Cirillo, Wilkins, and Joseph, 1963; Kotyk and Höffer, 1965; Egan and Morse, 1965) and in protozoans (Min, 1965, 1966). Competition between sorbose and glucose was also studied. After addition of the two sugars to the organism suspension, the intracellular concentration of sorbose was measured at the designated time intervals.

Cells of Crithidia rileyi have been used in this work for several reasons. This microorganism is quite different from red blood cells, yeast cells, or bacteria in its organization and complexity. It is also a convenient experimental organism. C. rileyi was isolated from a dipteran fly, Tabanus epistates, and is distinctly different from C. luciliae in morphology, but not in metabolism. Information on the entrance of sugars into these cells will broaden our understanding of the basic biological phenomenon of exchange of materials between cells and their environment, and also provide information essential to understanding carbohydrate metabolism in these organisms.

CHAPTER II

MATERIALS AND METHODS

Cells

Cultures of Crithidia rileyi were obtained from the laboratory of Dr. Hong S. Min, School of Applied Biology, Georgia Institute of Technology. Stock cultures were grown in 125 ml Erlenmeyer flasks containing approximately 50 ml of medium per flask and were kept in a refrigerator after sufficient growth at 25°C. Experimental cultures were grown in 2.5 liter Corning No. 4422 "Low Form" culture flasks containing 1000 ml of medium. Five ml of stock cultures were transferred aseptically into the experimental culture medium and the cultures were kept in 25°C incubator for three to four days.

Culture Medium (Cosgrove, 1963)

Ingredients:	sucrose	1.0%
	yeast extract	1.0%
	liver extract	0.8 ml
	hemin	2.5 mg %
	triethanolamine	0.5%

All the ingredients were dissolved by mixing on a magnetic stirrer at low speed. The medium was poured into 125 Erlenmeyer flasks and Corning No. 4422 "Low Form" culture flasks. After sterilization for 15 minutes at 15 pounds pressure in the autoclave, the flasks were cooled to room temperature and were inoculated.

General Procedure

The experimental cultures were harvested during the logarithmic growth phase by centrifugation in the Sorvall refrigerated centrifuge using the continuous flow system at a speed of 10,000 rpm (1200g). The organisms were then washed with carbohydrate-free amphibian Ringer (triethanolamine buffer, pH 7.6) three times by centrifugation. The final centrifugation, in Kolmer-Brown centrifuge tubes, separated the organisms into several layers of which only the layer of motile unclustered organisms were saved. The cells were resuspended in carbohydrate-free amphibian Ringer (phosphate, pH 7.6) for use. The concentration of cells was adjusted so that the packed cell volume was less than 5 percent of the volume of the suspension (Min, 1965).

Two series of test tubes (Sorvall's heavy wall centrifuge tubes, 12 ml capacity) were set up. At each of the designated time intervals 10 ml of cell suspension were transferred to centrifuge tubes containing 1 ml of organic separator (Di-n-butyl phthalate) (Ballantine and Burford, 1960) to separate cells and suspending fluid rapidly with a minimum of extracellular water in the pellet of packed cells. All such separations were made by centrifugation in the Sorvall refrigerated centrifuge (0°C), at maximum acceleration, until the rcf reached 10,000 g (45-55 seconds). After deceleration supernates were discarded and the layer of organic separator was removed. Any small droplets of aqueous solution adhering to the inner walls of the centrifuge tubes were removed by careful swabbing with absorbent paper. While the experiments were in progress the beaker of cell suspension was continually agitated on a shaker. The packed cells were resuspended in 2.0 ml of distilled water and heated in

boiling water for five minutes to disrupt the cells. After tubes were cooled to room temperature, 0.1 ml of 5 percent $ZnSO_4$ and 0.3 N $Ba(OH)_2$ were added to each tube to precipitate the proteins. Tubes were then centrifuged until all precipitate had been sedimented. The clear supernates were analyzed for free intracellular sugar.

Analysis (Nelson, 1944)

One ml of supernate (for 20 mM carbohydrates, 0.5 ml of clear supernate and 0.5 ml of distilled water were added) was pipetted into Folin-Wu blood sugar tubes. One ml of a mixture (prepared the day of use) of 25 parts of Copper Reagent A to one part of Copper Reagent B was added. One ml portions of appropriate standards and a blank were prepared the same way. The solutions were mixed well and heated in boiling water for 20 minutes. One ml of arsenomolybdate reagent was added to each. After being left a few minutes the mixtures were diluted to the mark of 25 ml, mixed well by inversion and read in Klett-Summerson Colorimeter with filter No. 54.

Reagents Utilized for Analysis

Copper Reagent A

Dissolve 25 gm of $Na_2(CO_3)$ anhydrous, 25 gm of Rochelle salt, 20 gm of $NaHCO_3$, and 200 gm of Na_2SO_4 (anhydrous) in about 800 ml of water and dilute to 1 liter, filter if necessary. This solution should be stored where the temperature will not fall below 20°C.

Copper Reagent B

Fifteen percent $CuSO_4 \cdot 5H_2O$ containing one or two drops of concentrated sulfuric acid per 100 ml.

Arsenomolybdate Color Reagent

Dissolve 25 gm of ammonium molybdate in 450 ml of distilled water, add 21 ml of concentrated H_2SO_4, mix, and add 3 gm of $Na_2HAsO_4 \cdot 7H_2O$ dissolved in 25 ml of H_2O, mix, and place in an incubator at 37°C for 24 to 48 hours.

Calculation

The extracellular water content of the pellet of packed organisms was determined by adding either of the non-penetrating, non-toxic substances, hemoglobin or potassium thiocyanate, to the suspension of organisms and measuring the amount of substance contained in the pellet.

Intracellular water content was calculated from wet weight and dry weight (100°C until the weight was constant) of pellets, taking into consideration the extracellular water determined previously.

The absolute values of these two quantities were then transformed to percentages using packed cell volumes determined on aliquots of the same suspension.

Mean values and standard errors are:

intracellular water : 79.0% $\pm$ 0.23

extracellular water : 9.67% $\pm$ 0.30

The analytical values obtained were converted to millimoles of carbohydrate per liter of cell water after correcting for the carbohydrate content of the extracellular water trapped in the pellet (Min, 1965).

CHAPTER III

RESULTS

The entrance of sugars into C. rileyi has been studied in this work with non-metabolizable sugars: D-xylose, D-ribose, L-sorbose, L-xylose, and L-arabinose. The presence of free intracellular carbohydrates is demonstrated in this study and the rate of entrance varies with the extracellular concentration of sugars. The intracellular concentrations of sugars were determined after 4, 8, 16, 32, and 64 minutes of exposure to two different external concentrations, 0.5 mM and 20 mM.

When the cells were exposed to external concentration of 20 mM sorbose the rate of sorbose transport increased non-linearly with time and the intracellular concentration did not exceed the extracellular concentration (Table 1 and Figure 1a). From the studies of the carbohydrates at 20 mM extracellular concentration there were no signs of accumulation of intracellular free sugar. At 0.5 mM extracellular concentration intracellular accumulations reached their maximum after 16 minutes of exposure, then decreased. Intracellular concentration reached 3.3 mM at 16 minutes, but at 20 mM extracellular concentration intracellular concentration approached that of the external concentration after 32 minutes of exposure, and decreased gradually (Table 1 and Figure 1b).

The results of the studies on the effect of inhibitors on the rate of carbohydrate entrance are summarized in Table 2 and Figure 2.

Table 1. Intracellular Concentration of Sorbose

Extracellular Concentration $[C]_o$	Time (Min.)	Mean $[C]_i$	No. of Experiments	Standard Error ±
0.5 mM	4	2.57	6	0.130
	8	2.88		0.112
	16	3.31		0.125
	32	2.65		0.050
	64	1.85		0.148
20 mM	4	8.02	5	0.576
	8	10.65		0.458
	16	15.26		0.291
	32	18.83		0.277
	64	17.73		0.685

$[C]_i$ = Intracellular concentration in millimoles per liter of cell water.

$$S.E. = \frac{\sqrt{\frac{\Sigma(x-\bar{x})^2}{N}}}{\sqrt{N}}$$

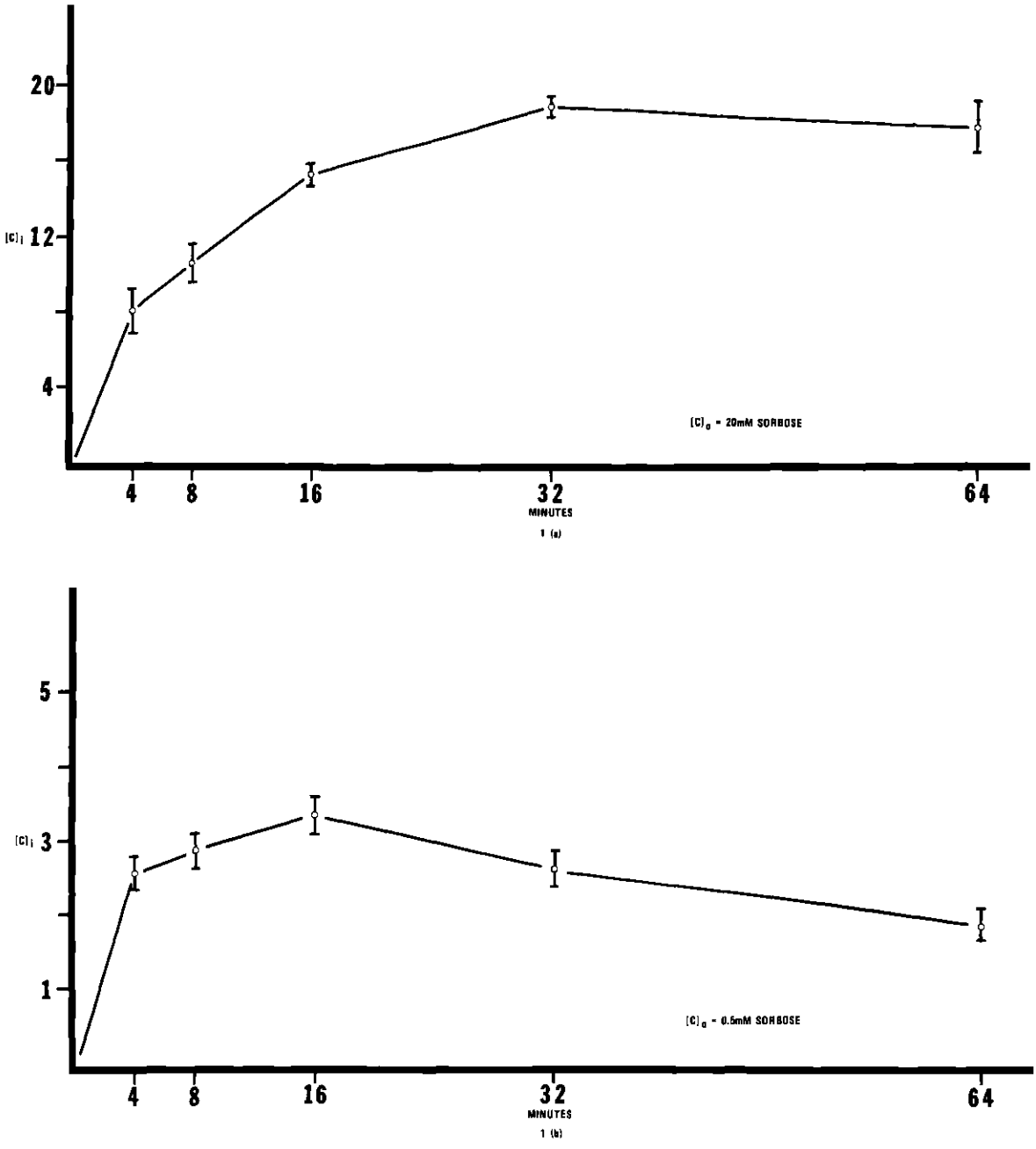

Figure 1. Intracellular Concentration of Sorbose as a Function of Exposure Time.
(a) $[C]_o$ = 20mM Sorbose: (b) $[C]_o$ = 0.5mM Sorbose
$[C]_i$ = Intracellular Concentration in Millimoles per Liter of Cell Water.

Table 2. Effect of KCN (10^{-4}M) on the Entrance of Carbohydrates into C. rileyi. Percent Inhibition of Carbohydrates After Exposure

Extracellular Concentration $[C]_o$	Time (Min.)	Mean Percent Inhibition	No. of Experiments	Standard Error ±
0.5 mM L-arabinose	4	20.6	5	0.900
	8	23.6		1.031
	16	27.4		0.921
	32	25.5		0.870
0.5 mM D-ribose	4	9.0	6	0.581
	8	18.2		0.826
	16	20.6		1.013
	32	22.4		0.797
0.5 mM L-sorbose	4	15.2	7	0.661
	8	33.7		0.618
	16	34.8		0.654
	32	18.0		0.535
0.5 mM D-xylose	4	7.6	7	0.351
	8	15.9		0.249
	16	18.3		0.413
	32	9.7		0.431
0.5 mM L-xylose	4	10.0	6	0.662
	8	15.9		0.881
	16	20.6		0.902
	32	19.4		0.789

$$\text{S.E.} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{N}} \Big/ \sqrt{N}$$

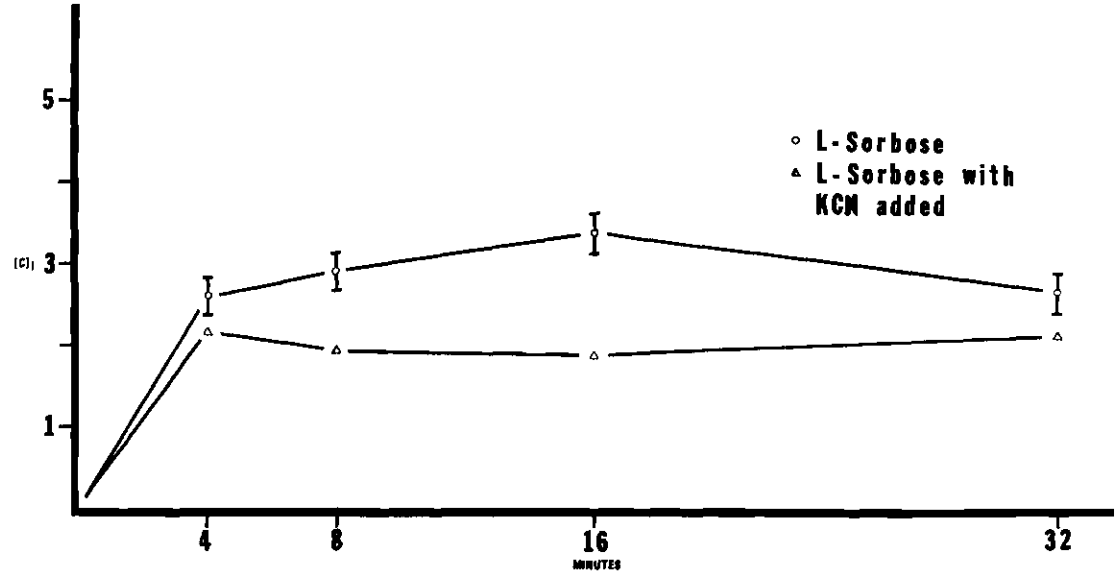

Figure 2. Intracellular Concentration as a Function of Exposure Time. $[C]_i$ = Intracellular Concentration in Millimoles per Liter of Cell Water. $[C]_o$ = 0.5mM. These Data Were Obtained from Table 1 and 2.

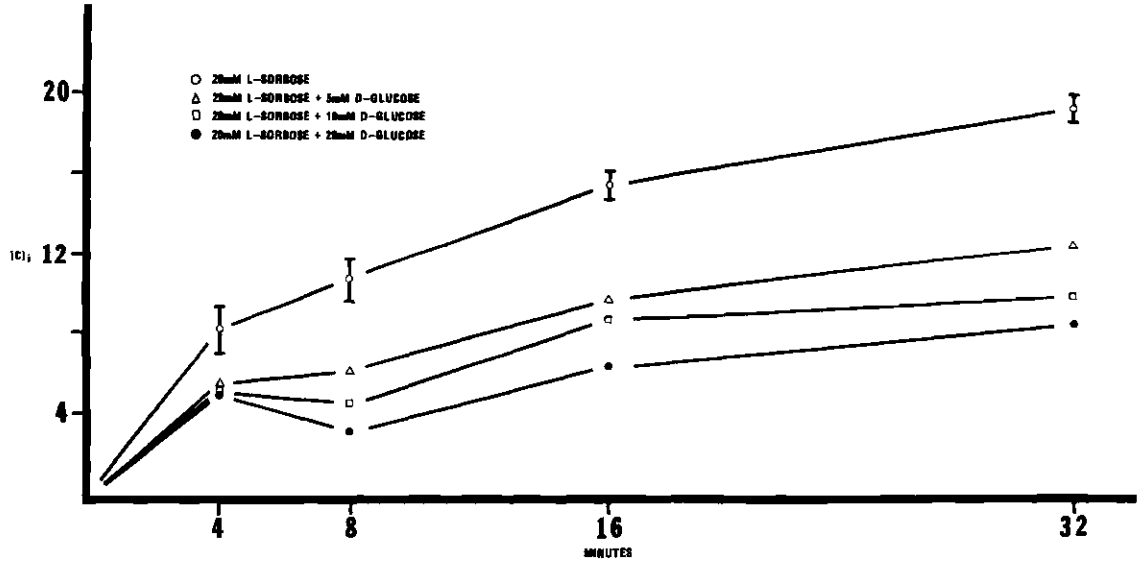

Figure 3. Pattern of L-Sorbose Entry into C · riley: Compared with Inhibited Pattern in Addition with Various Concentrations of D-Glucose. $[C]_i$ = Intracellular Concentration in Millimoles per Liter of Cell Water. These Data were Obtained from Table 1 and 3.

The data show that the rate of entrance of each carbohydrate studied is maximally inhibited after 16 minutes by KCN (10^{-4} M) at 0.5 mM external concentrations. The rate of inhibition is not linear with time, but the maximum inhibition occured after 16 minutes of exposure in all the experiments. The value of maximum inhibition is 34.5 percent after 16 minutes exposure at 0.5 mM sorbose, and the minimum value is 7.6 percent inhibition after four minutes of exposure at 0.5 mM D-xylose. It appears from the data that KCN inhibits the rate of sorbose entrance greater than any other sugar. However, this inhibitor does not affect transport from external concentrations of sorbose on the order of 0.02 M (20 mM).

To study the competition for entrance between sugars the cells were exposed to various combinations of different external concentrations. The entrance of sorbose was determined in the presence of various concentrations of glucose and compared to entrance in the absence of glucose. There is competition between sorbose and glucose presented simultaneously to the cells. The results of the competition between sorbose and glucose, as shown in Table 3 and Figure 3, indicate that at higher external concentrations of glucose the percent inhibition of sorbose was greater than that at lower external concentrations of glucose. The results show that the inhibition reached its maximum after 8 minutes of exposure and that the percent of inhibition decreases after 16 minutes of exposure. It apparently demonstrates that the mechanism responsible for the entrance of sorbose is sensitive to the presence of glucose and it is dependent upon the concentration of glucose present. The results of the studies on the effect of the simultaneous presence of

Table 3. Effect of Simultaneous Presence of D-glucose on the Entrance of L-sorbose Into C. rileyi. Percent Inhibition of L-sorbose Entrance After Exposure

Extracellular Concentration $[C]_o$	Time (Min.)	Mean Percent Inhibition	No. of Experiments	Standard Error ±
20 mM L-sorbose	4	37.5.	5	0.587
+ 20 mM D-glucose	8	70.8		1.198
	16	50.9		1.175
	32	55.9		0.922
20 mM L-sorbose	4	36.1	5	0.926
+ 10 mM D-glucose	8	57.7		1.311
	16	43.9		1.110
	32	48.5		1.211
20 mM L-sorbose	4	31.4	6	0.614
+ 5 mM D-glucose	8	42.4		1.107
	16	37.5		0.587
	32	34.9		0.843

$$S.E. = \sqrt{\frac{\Sigma(x-\bar{x})^2}{N}} \Big/ \sqrt{N}$$

glucose on the entrance of sorbose show that there is competition between monosaccharides presented simultaneously to the cells, and that the transport mechanism shows greater affinity for glucose than for sorbose.

CHAPTER IV

DISCUSSION

The data indicate that there are two types of entrance of carbohydrates into cells of C. rileyi, as evidenced by the difference in kinetics of penetration, in specificity, and in effects of metabolic inhibitions at low and high external concentrations of carbohydrates.

The pattern of increase of intracellular concentration in cells exposed to 20 mM extracellular concentration is very similar to the pattern predicted for diffusion of any solute into a cell initially free of the solute, when the major barrier to diffusion is the cell membrane (Table 4 and Figure 4). It appears that the kinetics of penetration of carbohydrates into C. rileyi follows the Michaelis-Menten Law rather than Fick's Law. At 0.5 mM external concentration it does not resemble the predicted pattern of entrance by diffusion (Table 4 and Figure 4).

From the studies of C. luciliae, Min (1965) suggested that the entrance of carbohydrates into C. luciliae is dependent on facilitated diffusion at 20 mM extracellular concentration, and at 0.5 mM extracellular concentration it is an active transport system as the accumulation of sugars inside the cell is energy dependent. The results obtained from this work agrees with the findings of Min (1965).

In C. rileyi there is competitive inhibition of sorbose by glucose when both are present simultaneously. When the concentration of glucose and sorbose are the same there is a severe inhibitory effect on the

Table 4. Comparison of Predicted Behavior Based on Fick's Law With Actual Behavior at 0.5 mM and 20 mM

Extracellular $[C]_o$	Time (Min.)	Mean $\frac{[C]_i}{[C]_o}$	No. of Experiments	Standard Error ±
0.5 mM	4	5.13	6	0.302
	8	6.47		0.385
	16	6.62		0.290
	32	5.28		0.091
20 mM	4	0.401	5	0.0156
	8	0.526		0.0224
	16	0.763		0.0159
	32	0.942		0.0439
20 mM theoretical	4	0.328		
	8	0.536		
	16	0.785		
	32	0.954		

$$S.E. = \frac{\sqrt{\frac{\Sigma(x-\bar{x})^2}{N}}}{\sqrt{N}}$$

The theoretical data were obtained by calculating $P\frac{A}{V}$ using the equation $e^{-P\frac{A}{V}t} = \frac{[C]_o - [C]_i}{[C]_o}$ (Fick's diffusion Law).

P = Permeability

A = The surface area of the cell

V = Volume of the cell

t = Time

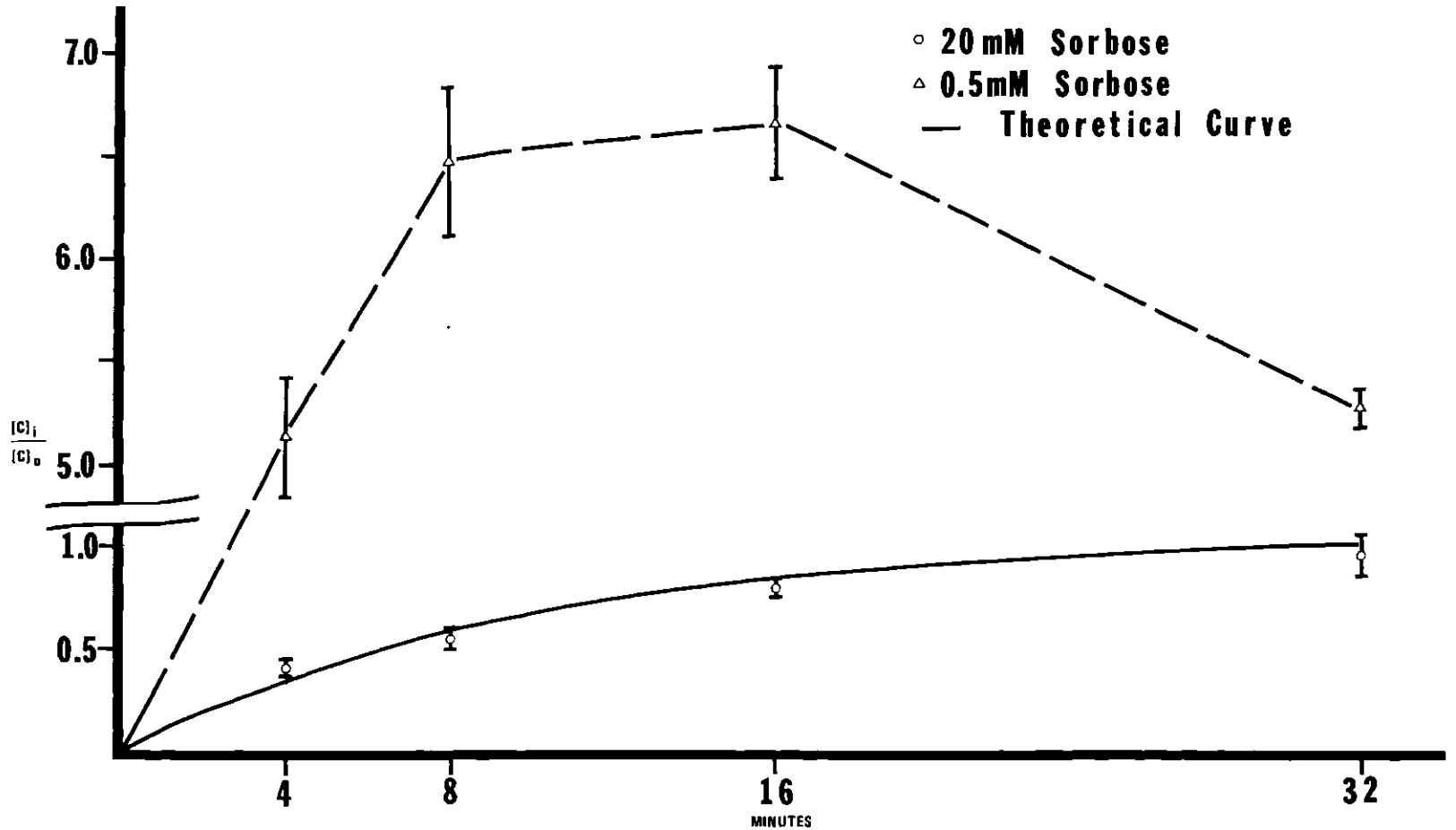

Figure 4. Comparison of Predicted Behavior, Based on Fick's Law, with Actual Behavior at 0.5mM and 20mM.

entrance of sorbose by glucose competitively (70.8 percent at 8 minutes). With a decrease in the concentration of glucose, the competition between sorbose and glucose also decreases and at 0.5 mM extracellular concentration there is no suppression of the entrance of sorbose by the simultaneous presence of glucose. These findings suggest that sorbose and glucose share the same carrier, and that the carrier for facilitated diffusion is different from that of the active transport mechanism. The results of the studies on competition show that glucose greatly affects the rate of entrance of sorbose when both carbohydrates are present simultaneously. The mechanism predominating at 20 mM $[C]_o$ is, therefore, facilitated diffusion.

On the other hand, the pattern of entry of 0.5 mM carbohydrates does not resemble that of 20 mM $[C]_i/[C]_o$ of 1.0 (Figure 4). This accumulation of sugar inside the cell indicates that active transport is occurring. The gradual decline in the intracellular concentration which occurs after reaching the maximum value can be attributed to a gradual decrease in the availability of the energy required to sustain the concentration difference. The occurrence of an active transport mechanism at low $[C]_o$ is supported by the strong inhibition of the rate of carbohydrate entrance at 0.5 mM $[C]_o$ when KCN is used as metabolic inhibitor. The lack of apparent inhibition at 20 mM $[C]_o$ indicates that the transport system at 20 mM $[C]_o$ is unlike that at 0.5 mM $[C]_o$, i.e., not dependent upon metabolic energy.

A carrier hypothesis has been proposed in sugar transport in yeast cells (Cirillo, 1961), in E. coli (Kepes, 1967), in Neurospora (Croken and Tatum, 1967). In these studies that sugar is transported

in by facilitated diffusion and in no case has there been any sign of intracellular accumulation of sugars. From the studies of C. luciliae (Min, 1965) and C. rileyi the sugar concentration inside the cell is far greater than the extracellular concentration when the cells are exposed to 0.5 mM $[C]_o$. On the other hand, at 20 mM $[C]_o$ the intracellular concentration never exceeds $[C]_o$.

It has been demonstrated that the transport of carbohydrates is inhibited in yeast (Höfer, 1962), C. luciliae (Min, 1966), and in C. rileyi by the metabolic inhibitor KCN. It has also been proposed that there is competition between sorbose and glucose in yeast, Neurospora, C. luciliae, and C. rileyi.

Further comparison of the results with C. rileyi and the results with C. luciliae shows many similar points. The transport system functioning in C. rileyi is determined by the external concentration of carbohydrate, an active transport system at lower concentrations, and a facilitated diffusion at higher concentrations. The occurrence of the active transport mechanism in cells of C. rileyi may be an adaptation to the conditions of their normal habitat, the gut of flies. The carbohydrate content of the gut could be expected to vary greatly and it could be expected that for considerable periods of time the carbohydrate content of the gut would be low. During this period of low carbohydrate concentration the flagellate would be competing with the host for the available carbohydrate. The effective active transport mechanism would permit survival of the flagellates under these conditions.

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