GEORGIA INSTITUTE OF TECHNOLOGY
ATLANTA, GEORGIA 30332

SCHOOL OF
BIOLOGY

January 29, 1968

National Institute of General Medical Sciences
National Institutes of Health
Public Health Service
Department of Health, Education and Welfare
Bethesda, Maryland 20014

Attention: Dr. William I. Gay, Chief
Research Grants Branch
932 Westwood Building

Subject: Grant No. 3 R01 GM12235-03
Transport of Carbohydrate in Crithidia luciliae

Dear Dr. Gay:

You will find enclosed ten (10) copies each of: a final progress report; a manuscript submitted to the Journal of Cellular Physiology; five reprints from American Zoologist; and forty (40) copies of a reprint from the Journal of Cellular Physiology.

A final invention statement was submitted December 13, 1967. I believe that our publications of work undertaken with support from the National Institutes of Health show real contributions to the subject of carbohydrates in Crithidia luciliae.

The support of the National Institutes of Health over these years has been greatly appreciated.

Sincerely yours,

Hong S. Min
Associate Professor
School of Biology

HSM:cm

Enclosures

GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF RESEARCH ADMINISTRATION

Genl Res 1960

Date: June 15, 1966

RESEARCH PROJECT INITIATION

Project Title: Transport of Carbohydrate in Crithidia Luciliae

Project No.: B-1406

Project Director: Dr. Hong S. Min

Sponsor: Public Health Service

Agreement Period: From 1 June 1966 until 31 May 1967

Type Agreement: Grant No. 5 R01 Gm 122235-03 TMP

Amount: $7,574 New Funds
526 Carry over from B-1404
$8,100

Grant Administrator

Research Grants Branch
National Institute of General Medical Science
National Institutes of Health
Bethesda, Maryland 20014

Reports Required

Interim progress - when application is made for continuation or renewal support (Form PHS-2590-1)

Terminal Progress - within 6 months after end of project

Assigned to: School of Applied Biology

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REPORTS 300-B-1406

RA-3 (4/65)

GEORGIA INSTITUTE OF TECHNOLOGY

OFFICE OF RESEARCH ADMINISTRATION

RESEARCH PROJECT TERMINATION

Date: 2 February 1968

Project Title: Transport of Carbohydrate in Crithidia luciliae

Project No: B-1406

Project Director: Dr. Hong S. Min

Sponsor: National Institutes of Health, Public Health Service

Termination Effective: Immediately

Charges Should Clear Accounting By: All acceptable charges have cleared.

Final Progress Report was mailed 2 February 1968.

Final Invention Statement was mailed 13 December 1967.

300-B-1406

COPIES TO:

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Other Mr. R. A. Martin
File B-1406

RA-4 (5/65)

Mailed 28 Apr 66

ADDENDUM A

PROGRESS REPORT

A. Summary Page

Title: Transport of Carbohydrate in Crithidia luciliae.

Grant No.: GM 12235

Principal Investigator: Hong S. Min

Sponsoring Institution: Georgia Institute of Technology

Period Covered: June 1, 1964 to April 10, 1966

Date of Preparation: April 15, 1966

Summary:

The entrance of carbohydrate into the cells of C. lucilliae has been studied, using nine non-utilizable monosaccharides. At 0.5 mM external concentration the rate of increase in intracellular concentration is constant for all sugars until the intracellular concentrations equal or exceed the extracellular concentration. At 20 mM external concentration, the rate of increase of intracellular concentration never exceeds the extracellular concentration. There is competition between monosaccharides presented simultaneously to the cells and transport mechanism shows enormously greater affinity for glucose than for other monosaccharides. The rate of carbohydrate entrance is inhibited 50% and 70% by KCN (10^{-4}M) and DNP (10^{-5}M) respectively at 0.5 mM external concentrations. However, these inhibitors do not affect transport from external concentrations of the order of 0.02 M. The transport of sorbose at 10 and 20 mM was studied in the presence of 5, 10, and 20 mM glucose. The transport of 20 mM sorbose was greatly inhibited by the simultaneous presence of 5 and 20 mM glucose as was the transport of 10 mM sorbose in the presence of 10 mM glucose.

The results indicate that glucose and sorbose utilize the same transport mechanism in C. lucilliae, but the system has a much higher affinity for glucose.

At the present studies are being made with other hexoses and pentoses in this laboratory. These data are interpreted as indicating two mechanisms for carbohydrate entrance: (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.

B. Detailed Report:

There has been much effort devoted to investigation of the entrance of carbohydrate into mammalian red blood cells, yeast, and various bacterial cells. However, there has not been any work done with protozoans until the entrance of carbohydrates into the cells of insect trypanosomid, Crithidia luciliae, was first studied (Min, '63). This microorganism is quite different from red blood cells or bacteria in its cellular organization and complexity. It is hoped that 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 environments and also provide information essential to understanding carbohydrate metabolism in these organisms.

Materials and Methods

Cultures of Crithidia luciliae were grown axenically in medium (Cosgrove, '63) containing 1.0% sucrose, 1.0% yeast extract (Difco), 0.05% liver extract (Nutritional Biochem.), 2.5 mg % hemin and 0.5% triethanolamine, pH 8.0. Stock cultures were grown in 125 ml. Erlenmeyer flasks containing approximately 50 ml. of medium per flask and experimental cultures were grown in 2.5 liter Corning no. 4422 "Low Form" culture flasks containing 1000 ml. of medium. Both cultures were kept at 25° C. The experimental cultures were harvested during the logarithmic phase; the organisms were then washed with carbohydrate-free amphibian Ringer (triethanolamine buffer, pH 7.6) by centrifugation. The final centrifugation, in Kolmer-Brown centrifuge tubes, separated the cells into several layers of which only the layer of motile unclustered organisms were saved. These 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% of the volume of suspension.

In all experiments, the "organic separator" technique of Ballentine and Burford ('60) was used 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 ref reached 12,000 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. The packed cells were resuspended in 2.0 ml. of distilled water and heated in boiling water for five minutes. After tubes were cooled to room temperature, 0.1 ml. each of protein precipitants, 5% $ZnSO_4$ and 0.3 N $Ba(OH)_2$, were added to each tube. Tubes were then centrifuged until all precipitate had been sedimented. The clear supernates were analyzed for carbohydrates using the following methods as required: total reducing sugar by Nelson's ('44) method; ketohexose by method of Dische and Devi ('60); pentose by the orcinol method of Hajdun (Umbreit et al., '59, p. 57). The analytical values so 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. The methods of determination of the extracellular and intracellular water content of the pellet of packed organisms and the packed cell volume were previously reported by Min ('63).

ADDENDUM A

Results:

The results of preliminary experiments were previously reported by Min ('65). The results of experiments, where the percent inhibition of carbohydrate entrance was determined after an exposure of 30 minutes to the mixtures of each carbohydrate and 10^{-4} M KCN, are shown in Table 1.

Table 1. Effect of KCN (10^{-4} M) on the entrance of carbohydrates into the cells of C. lucillae. Extracellular concentration 0.5 mM.

Carbohydrate	% Inhibition of Carbohydrate Entrance
L-sorbose	70
D-mannose	25
D-galactose	19
D-arabinose	44
L-arabinose	35
D-xylose	60
L-xylose	45

It appears from these results that the carbohydrate entrance is inhibited by KCN at 0.5 mM external concentrations, although the extent to which the entrance of these sugars inhibited vary considerably. However, this inhibitor does not affect transfer from external concentrations of carbohydrates of the order of 0.02 M.

Studies on the intracellular accumulation of carbohydrates at 0.5 mM external concentrations show (Table 2) that there is an accumulation of free intracellular carbohydrates after the cells are exposed to sugars for 30 minutes. On the other hand, when the cells were exposed to the external concentration of 20 mM carbohydrates, there appeared no sign of accumulation of intracellular free sugar.

Table 2. Intracellular accumulation of carbohydrates at 0.5 mM external concentration. Incubation time 30 minutes.

Carbohydrate	$[c]_i / [c]_o$
L-sorbose	2.1
D-mannose	2.4
D-galactose	2.3
D-arabinose	5.0
L-arabinose	3.2
D-xylose	4.1
L-xylose	5.4
D-fucose	2.9
L-fucose	3.3
L-rhamnose	2.5

APPENDIX A

To study further the effect of simultaneous presence of glucose or galactose on the entrance of sorbose, the cells were exposed to various combinations of different external concentrations. The results of the studies on the competition between sugars are summarized in Table 3. It is apparent from the studies that the transport mechanism shows enormously greater affinity for glucose than for other monosaccharides. The results also indicate that the simultaneous presence of glucose suppresses the rate of entrance of sorbose, in varying degrees, as shown in Table 3.

Table 3. Effect of simultaneous presence of glucose or galactose on the entrance of sorbose, incubation time 30 minutes.

Extracellular Conc.	% Inhibition of Sorbose Entrance
0.02 M sorbose + 0.02 M glucose	65
0.02 M sorbose + 0.01 M glucose	45
0.02 M sorbose + 0.005 M glucose	35
0.02 M sorbose + 0.0025 M glucose	30
0.02 M sorbose + 0.01 M galactose	0

The data summarized in Table 4 show that, for the temperature interval 15°-25°C the Q_{10} for rate of entrance when the external concentration is 0.5 mM is 2.8 times larger than the Q_{10} when the external concentration is 20 mM. This large difference in Q_{10} parallels the differences in pattern of entrance and in specificity at these two external concentrations.

Table 4. Effect of temperature on the entrance of sorbose.

Extracellular Conc.	Rate (Δ-M/liter cell water/min)		Q_{10}
	15° C	25° C	
20 millimolar	0.65	1.8	2.8
0.5 millimolar	0.018	0.14	7.8

ADDENDUM A

DISCUSSION

The data indicate that there are two types of entrance of carbohydrates into the cells of C. lucilliae, as evidenced by the differences in kinetics of penetration, in specificity, in Q_{10} and in effects of metabolic inhibitions at low and high external concentrations of carbohydrate.

The occurrence of an active transport mechanism at low C is supported by the strong inhibition of the rate of carbohydrate entrance at 0.5 mM C when KCN is used as metabolic inhibitor. No apparent inhibition at 20 mM C indicates that the transport system at 20 mM C is unlike that at 0.5 mM C , not dependent upon metabolic energy. Also, intracellular accumulation of carbohydrates at 0.5 mM external concentration supports the occurrence of an active transport mechanism.

As previously reported (Min, '65), the kinetics of penetration resemble the Michaelis-Menton Law rather than the Fick's kinetics. The results of the studies on competition show (Table 3) that glucose greatly affects the rate of entrance of sorbose when both carbohydrate are present simultaneously. The mechanism predominating at 20 mM C is therefore facilitated diffusion.

The difference in the temperature coefficients of penetration at 0.5 mM and 20 mM C provide supporting evidence for a difference in mechanism, but no certain significance can be attached to the actual values.

The transport system functioning in C. lucilliae is determined by the external concentration of carbohydrate; an active transport system at lower concentrations and a facilitated diffusion at higher concentrations. This dual mechanism possessed by C. lucilliae has not been reported in any other cell. Carbohydrate entrance into yeast cells (Cirillo, '61) and red blood cells (Dowyer, '57) is by facilitated diffusion at all external concentrations. In contrast, carbohydrate transport in Escherichia coli is an active transport mechanism, capable of accumulating sugar against concentration gradients (Cohen and Monod, '57). As expected, the mechanism requires metabolic energy. It is very effective in accumulating the unchanged sugar against 100- to 10,000 fold differences. Except for these cases in bacteria, the accumulation of non-utilizable sugars, to twice the external concentration, demonstrated in C. lucilliae at a low external concentration has not been reported in other cells.

Recently, preliminary study has been made using another insect trypanosomid, Crithidia sp. from Arilus cristatus, which Hanson and McGhee ('63) isolated from a hemipteron. The data obtained so far indicate that this flagellated organism also utilizes both a facilitated diffusion and an active transport mechanism in transporting carbohydrates.

The occurrence of the active transport mechanism in cells of C. lucilliae and perhaps in other insect trypanosomids may be an adaptation to the conditions of their normal habitat, the gut of insects. The carbohydrate content of the gut would 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 flagellates would be competing with the host for the small amount of carbohydrate available. The effective active transport mechanism would permit survival of the flagellates under these conditions since apparently no metabolic reserves are accumulated by the flagellates and a continuous supply of carbohydrate is needed. When the concentration of carbohydrate in the gut is high, the facilitated diffusion mechanism is apparently sufficient.

ADDENDUM A

It is hoped that the current studies on the transport of carbohydrate in C. lucilise will provide a valuable tool for implementing the proposed study (See the application). Perhaps, these studies will enable us in the future to understand the phenomenon of exchange of materials between cells and their environments in general, and also provide information essential to understanding carbohydrate metabolism in these organisms.

Publications (Supported by NIH Grant):

Hong S. Min (1964) Further studies on the entrance of carbohydrates into cells of Crithidia lucilise. Amer. Zool., 4: 409.

Hong S. Min (1965) Studies on the transport of carbohydrate in Crithidia lucilise. J. Cell. and Comp. Physiol., 65: 243-248.

Hong S. Min (1965) Studies on the entrance of carbohydrates into cells of Crithidia lucilise; effects of extracellular concentrations and molecular configuration. Amer. Zool. 5: 201-202.

NOTE: Copies of reprints were previously submitted to NIH.

Manuscripts in preparation:

Hong S. Min (1966) Studies on the transport of carbohydrate in Crithidia lucilise: effects of inhibitors and the simultaneous presence of monosaccharides on the transport of carbohydrates.

Hong S. Min (1966) Studies on the transport of carbohydrate in Crithidia sp. from Arilus cristatus.

ADDENDUM A

LITERATURE CITED

Ballantine, R., and D. D. Burford 1960 Differtial density separations of cellular suspension. Anal. Biochem., 1: 263-268.

Bowyer, F. 1957 The kinetics of the penetration of nonelectrolytes into the mammalian erythrocyte. Intern. Rev. Cytol., 6: 469-511.

Cirillo, B. P. 1961 The transport of non-fermentable sugars across the yeast cell membrane. In: Symposium on Membrane Transport and Metabolism. A. Kleinzeller and S. Kotyk (eds.), Academic Press, Inc., New York, pp. 343-351.

Cohen, G. N., and J. Monod 1957 Bacterial permeases. Bacteriol. Rev., 22: 169-194.

Cosgrove, W. B. 1963 Carbohydrate utilization by trypanosomids from insects. Exp. Parasitol., 13: 173-177.

Dische, Z., and A. Devi 1960 A new colorimetric method for the determination of ketohexoses in the presence of aldoses, ketoheptoses and ketopentoses. Biochim. et Biophys. Acta, 39: 140-144.

Hanson, W. L., and R. B. McGhee 1963 Experimental infection of the hemipteron Oncopeltus fasciatus with Trypanosomatidae isolated from other hosts. J. Protozool., 10: 268-278.

Min, H. S., 1963 Studies on the transport of carbohydrate in Crithidia luciliae. Ph.D. Dissertation. University of Georgia.

Min, H. S., 1965 Studies on the transport of carbohydrate in Crithidia luciliae. J. Cell and Comp. Physiol., 65: 243-248.

Nelson, N. 1944 A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., 153: 375-380.

Umbreit, W. W., R. H. Burris and J. F. Stauffer 1959 Manometric Techniques. Burgess Publishing Co., Minneapolis, Minn., p. 338.

Terminal Progress Report: NIH Grant GM 12235

Title: Transport of Carbohydrate in Crithidia luciliae

Grant No.: GM 12235

Principal Investigator: Hong S. Min

Sponsoring Institution: Georgia Institute of Technology

Period Covered: June 1, 1964 to August 31, 1967

Summary:

The entrance of carbohydrate into the cells of C. luciliae has been extensively studied using nonmetabolizable monosaccharides. In addition, one other species of the same genus, C. sp. from Arilus cristatus has been studied to some extent. Also, the study on the transport of carbohydrate into human cancer cells grown in tissue culture (KB) was initiated and some preliminary results have been obtained.

The results of the studies on the entrance of carbohydrates in Crithidia luciliae and glucose transport in human cancer cells (KB) are submitted in the forms of reprints and manuscripts respectively.

Although there are some differences in values, the data obtained from the studies with C. sp. from Arilus cristatus indicate that this flagellated organism also utilizes both a facilitated diffusion and an active transport mechanism in transporting carbohydrates.

Radioactively labelled glucose was used to study the entrance of metabolizable sugar, glucose, into KB cells grown in monolayer culture. Entrance of glucose exhibits saturation kinetics and follows Michaelis-Menten kinetics. It appears that entrance is via a carrier-mediated mechanism.

Detailed Report:

During the past three years a great deal of effort has been devoted to investigation of the entrance of carbohydrate into Crithidia luciliae and other species of the same genus, Crithidia. The principal portion of the research carried out under the grant was published in the Journal of Cellular Physiology, 68: 237-240 (1966) and 40 reprints are substituted for that portion of the progress report. As the research on the carbohydrate transport in C. luciliae became nearly complete, studies on the transport of carbohydrate into cells of C. sp. from Arilus cristatus and human cancer cells grown in tissue culture (KB) have been started. These studies are by no means fully completed as yet, but there are sufficient amounts of data available from the studies made thus far to be included in this report.

Materials and Methods:

Cultures of Crithidia sp. from Arilus cristatus were grown axenically in a medium (Cosgrove, '63) containing 1.0% sucrose, 1.0% yeast extract (Difco), 0.05% liver extract (Nutritional Biochem.), 2.5% mg hemin and 0.5% triethanolamine, pH 7.9.

Stock cultures were grown in 125 ml Erlenmeyer flasks containing approximately 50 ml of medium per flask and experimental cultures were grown in 2.5 liter Corning No. 4422 "Low Form" culture flasks containing 1000 ml of medium. Both cultures were kept at 25°C. Harvesting and washing procedures were those of Min ('65). Washed organisms were resuspended in amphibian Ringer-phosphate buffered at pH 7.6 with triethanolamine.

In all experiments, the "organic separator" technique of Ballantine and Burford ('60) was used to separate cells and suspending fluid rapidly with a minimum of extracellular water in the pellet of packed cells. Procedures for such separations and obtainment of clear supernates for analyses were the same as those of Min ('65). The clear supernates were analyzed for carbohydrate using the following methods as required: total reducing sugar by Nelson's ('44) method; ketohexose by method of Dische and Devi ('60); pentose by the orcinal method of Mejbaum (Umbreit et al., '59, p. 274). The analytical values so 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. The methods of determination of the extracellular and intracellular water content of the pellet of packed organisms and the packed cell

volume were previously reported by Min ('65).

Results:

The results of preliminary experiments where the presence of free intracellular carbohydrate was determined after an exposure of 30 minutes to solutions of each carbohydrate (20 mM), show free intracellular carbohydrates when the cells are exposed to nonmetabolizable carbohydrates, but not when the cells are exposed to metabolizable carbohydrates as shown in Table 1.

TABLE 1

Entrance of carbohydrates into cells of C. sp. from Arilus cristatus. Extracellular concentration 20 mM, incubation time 30 minutes.

Carbohydrate	Intracellular Carbohydrate
Metabolized:	
Glucose	-
Fructose	-
Not metabolized:	
L-sorbose	+
D-xylose	+
L-xylose	+
D-arabinose	+
L-arabinose	+
L-rhamnose	+
D-fucose	+
D-mannose	+
D-lyxose	+
D-galactose	+
D-2-deoxyglucose	+
D-ribose	+

To study further the pattern of entrance of carbohydrates the cells were exposed to external concentrations of 0.5 mM sorbose and 20 mM sorbose. The results, presented in Figure 1, show that cells exposed to 0.5 mM sorbose,

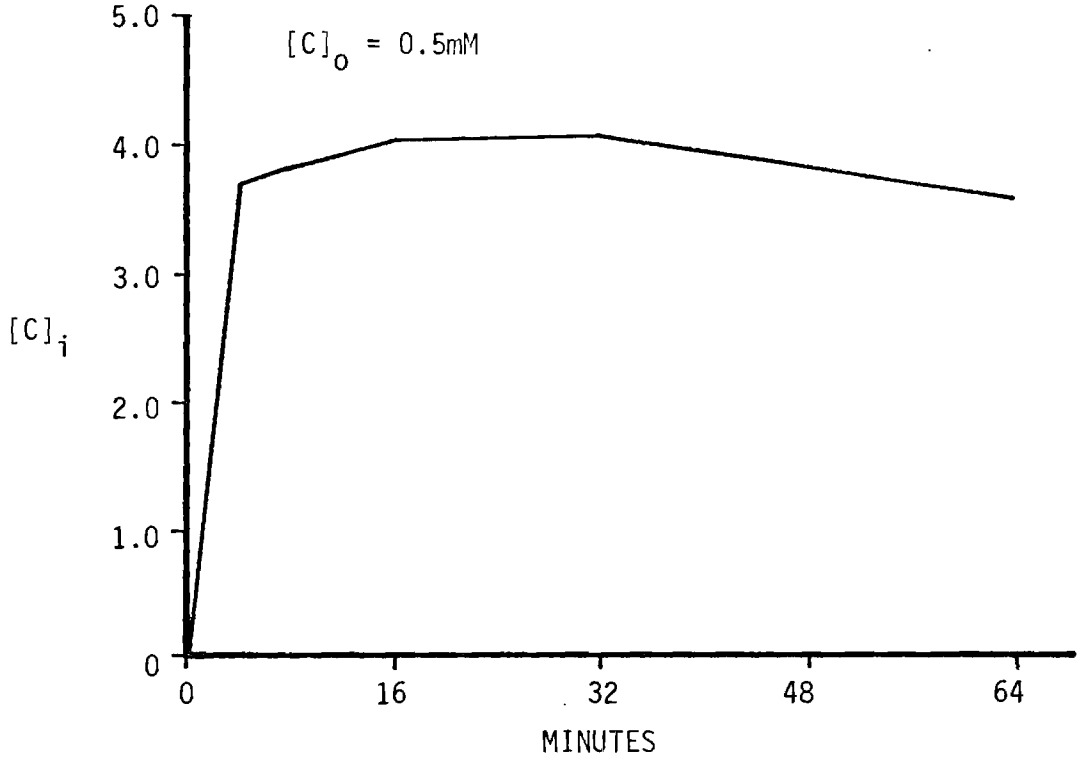

FIGURE 1

the concentration of free intracellular sorbose increased very sharply to 3.70 mM after 4 minutes of exposure, then increased gradually to 4.0 mM and decreased slowly to 3.65 mM after 64 minutes of exposure. On the other hand, when the cells were exposed to the external concentration of 20 mM sorbose, there appeared no sign of accumulation of intracellular free sugar and intracellular concentration approached at a declining rate the extracellular concentration (Figure 2). Because the mechanism of entrance appeared to be different at low and at high external concentrations, as evidenced by these data, the time courses of entrance of other carbohydrates were determined at external concentrations of approximately 0.5 mM and 20 mM. From the data, it was apparent that these carbohydrates fall into two distinct groups, differing in rate by varying factors at 0.5 mM external concentrations but not at 20 mM external concentrations.

At 0.5 mM external concentrations, sugars showed quite a range of intracellular accumulation as shown in Table 2. However, at 20 mM external concentration there was no accumulation of sugars at all.

TABLE 2

Intracellular accumulation of carbohydrates at 0.5 mM external concentration, incubation time 30 minutes.

Carbohydrate	$[c]_i/[c]_o$
L-rhamnose	25.4
L-fucose	21.0
D-arabinose	12.2
L-sorbose	9.6
D-fucose	8.8
D-xylose	7.8
D-mannose	5.6
D-lyxose	5.2
D-galactose	4.8
L-arabinose	4.8
D-ribose	4.4
L-xylose	4.4
2-deoxy-D-glucose	3.6

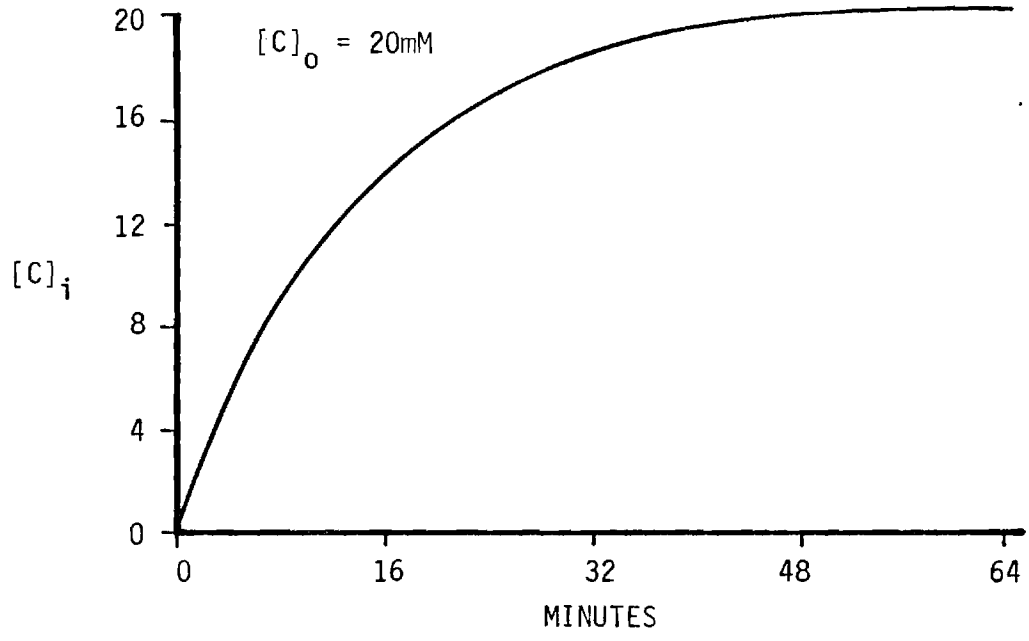

FIGURE 2

The results of the studies on the effect of inhibitors on the rate of carbohydrate entrance show that the rate of entrance is inhibited 30% and 50% by potassium cyanide (10^{-4}M) and dinitrophenol (10^{-5}M) respectively at 0.5 mM external concentrations. However, these inhibitors do not affect transport from external concentration of sugars of the order of 0.02 M.

Discussion:

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

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 and DNP are used as metabolic inhibitor. No apparent inhibition at 20 mM $[C]_o$ indicates that the transport system at 20 mM $[C]_o$ is unlike that at 0.5 mM $[C]_o$, not dependent upon metabolic energy. Also, intracellular accumulation of carbohydrates at 0.5 mM external concentration supports the occurrence of an active transport mechanism.

As previously reported (Min, '65, '66), the kinetics of penetration resemble the Michaelis-Menten Law rather than the Fick's kinetics. The mechanism predominating at 20 mM $[C]_o$ is therefore facilitated diffusion.

The transport system functioning in C. sp. from Arilus cristatus is also determined by the external concentration of carbohydrate; an active transport system at lower concentrations and a facilitated diffusion at higher concentrations. This dual mechanism possessed by C. sp. from Arilus cristatus was reported by Min ('65) in C. luciliae, a similar organism.

The occurrence of the active transport mechanism in cells of C. sp. from Arilus cristatus and C. luciliae, and perhaps in other insect trypanosomids may be an adaptation to the conditions of their normal habitat, the gut of insects. The carbohydrate content of the gut would 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 flagellates would be competing with the host for the small amount of carbohydrate available.

The effective active transport mechanism would permit survival of the flagellates under these conditions since apparently no metabolic reserves are

accumulated by the flagellates and a continuous supply of carbohydrate is needed. When the concentration of carbohydrate in the gut is high, the facilitated diffusion mechanism is apparently sufficient.

Publications (Supported by NIH Grant):

Hong S. Min (1964) Further studies on the entrance of carbohydrates into cells of Crithidia luciliae. Amer. Zool., 4: 409. (Abstract)

Hong S. Min (1965) Studies on the entrance of carbohydrates into cells of Crithidia luciliae; effects of extracellular concentrations and molecular configuration. Amer. Zool. 5: 201-202. (Abstract)

Hong S. Min (1966) Effects of Inhibitor, Competitors, temperature on transport of carbohydrate in Crithidia luciliae. J. Cell. Physiol., 68: 237-240.

Hong S. Min (1967) Entrance of carbohydrates into human cancer cells grown in tissue culture (KB cell line). Amer. Zool., 7: 203-204. (Abstract)

Manuscript Submitted to Journal of Cellular Physiology:

Michail A. Esterman and Hong S. Min (1968) Glucose transport into a human cancer cell (KB) grown in tissue culture.

Manuscript in Preparation:

Hong S. Min (1968) Studies on the transport of carbohydrate in Crithidia sp. from Arilus cristatus.

Publications in the Area of the Research:

Esterman, M. A. (1967) The kinetics of glucose transport into cancer cells (KB line), M.S. Thesis. Georgia Institute of Technology (Directed by H. S. Min).

Min, H. S. (1967) Entrance of carbohydrates into human cancer cells grown in tissue culture (KB cell line), Amer. Zool, 7: 203-204. (Abstract).

Min, H. S. and W. B. Cosgrove (1963) Entrance of carbohydrates into cells of Crithidia luciliae. J. Protozool., 10: 19.

Min, H. S. (1963) Studies on the transport of carbohydrate in Crithidia luciliae. Ph.D. Dissertation, University of Georgia.

Min, H. S. (1964) Further studies on the entrance of carbohydrates into cells of Crithidia luciliae. Amer. Zool. 4: 409.

Min, H. S. (1965) Studies on the transport of carbohydrate in Crithidia luciliae. Jour. Cell. and Comp. Physiol., 65: 243-248.

Min, H. S. (1965) Studies on the entrance of carbohydrates into cells of Crithidia luciliae; effects of extracellular concentrations and molecular configuration. Amer. Zool., 5: 319-320.

Min, H. S. (1966) Studies on the transport of carbohydrates in Crithidia sp. from Arilus cristatus. Amer. Zool., 6: 319-320.

Min, H. S. and M. A. Esterman (1966) Effect of the presence of glucose on the transport of carbohydrate in Crithidia luciliae. Amer. Zool., 6: 350.

Min, H. S. (1966) Effects of inhibitor, competitors, and temperature on transport of carbohydrate in Crithidia luciliae. Jour. Cell. Physiol., 68: 237-240.

Literature Survey

Ballantine R., and D. D. Burford (1960) Differential density separations of cellular suspension. Anal. Biochem., 1: 263-268.

Cohen, G. N., and J. Monod (1957) Bacterial permeases. Bacteriol. Rev., 22: 169-194.

Cosgrove, W. B. (1963) Carbohydrate utilization by trypanosomids from insects. Exp. Parasitol., 13: 173-177.

Dische, Z., and A. Devi (1960) A new colorimetric method for the determination of ketohexoses in the presence of aldoses, ketoheptoses and ketopentoses. Biochim. et Biophys. Acta, 39: 140-144.

Hanson, W. L., and R. B. McGhee (1963) Experimental infection of the hemipteron Oncopeltus fasciatus with Trypanosomatidae isolated from other hosts. J. Protozool., 10: 268-278.

Min, H. S., (1965) Studies on the transport of carbohydrate in Crithidia luciliae. J. Cell and Comp. Physiol., 65: 243-248.

Min, H. S., (1966) Effects of inhibitor, competitors, and temperature on transport of carbohydrate in Crithidia luciliae. J. Cell. Physiol., 68: 237,240.

Nelson, N. (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem., 153: 375-380.

Umbreit, W. W., R. H. Burris and J. F. Stauffer (1959) Manometric Techniques. Burgess Publishing Co., Minneapolis, Minn., p. 338.

GLUCOSE TRANSPORT INTO A HUMAN CANCER CELL (KB) GROWN IN TISSUE CULTURE[1]

(Title for running page)
GLUCOSE TRANSPORT INTO KB CELLS

By

Michail A. Esterman[2] and Hong S. Min
School of Biology
Georgia Institute of Technology
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ABSTRACT

Radioactively labelled glucose was used to study the entrance of a metabolizeable sugar, glucose, into KB cells grown in monolayer culture. Entrance of glucose exhibits saturation kinetics and follows Michaelis-Menten kinetics. These observations lead to the conclusion that entrance is via a carrier-mediated mechanism similar to that observed by several workers in studies using Ehrlich ascites, HeLa, and L-cell lines.

An affinity constant (K_m) of 10 mM was calculated for glucose transport by the KB cells, which indicates a relatively low affinity for glucose.

At low external sugar concentrations (5 mM) glucose appears to be accumulated by the KB cells.

INTRODUCTION

Carbohydrate transport has been studied in single celled populations of bacteria (Horecker et al., '60) protozoa (Min, '65) yeast (Cirillo, '62), erythrocytes (LeFevre, '62) and tissue cultured cell lines (Rickenberg and Maio, '61). Much has been learned about amino acid transport in cancer cells grown in tissue culture (Johnstone and Scholefield, '57); however, only relatively few results have been reported concerning sugar transport into tissue cultured cancer cells. The studies that have been reported have been limited to the Ehrlich ascites (Crane et al., '57; Cirillo and Young, '64; Kolber '63; Nierenberg and Hogg, '58) and the HeLa cell lines (Vann et al,, '63). It is hoped that the information obtained from the study of glucose transport into KB cells will increase the understanding of the physiology of the cancer cell grown in cultures.

MATERIALS AND METHODS

Cells

Tissue cultured human strain KB cells derived from an oral carcinoma were obtained from the laboratory of Dr. R. H. Fetner of Georgia Institute of Technology and the cells were maintained in continuous culture on Eagle's minimum essential medium (Eagle, '59) at 35°C supplemented with nonessential amino acids, penicillin (0.5 mg/l), streptomycin (0.5 mg/l), sodium bicarbonate (0.35 g/l), and 10 percent pooled human serum from fasted patients. Hanks' salt solution (Hanks and Wallace, '49) was substituted for Earle's. The cells were grown in monolayer culture in 8 ounce prescription bottles until the maximum 10 g phase was reached. The growth medium was then decanted and the cells were washed three times with equal volumes of Hanks' salt solution.

Packed-cell volume was determined on two different bottles in sedimentation tubes, Kimas #46815, by centrifugation for 5 minutes at 3,300 g.

Chemicals

Uniformly labeled D-glucose with a specific activity of 1 mc/mM was obtained from Cal Biochem. Nonradioactive reagent grade glucose was obtained from Fisher Scientific and used without further purification.

General Procedure

To each bottle of washed cells 5 ml of twice the desired concentration of glucose and 5 ml of labeled solution (0.1 μc/ml) were added. The bottles

were incubated at 35°C and at each of the designated time intervals one bottle was removed. The cells were scraped off the glass surface with a rubber policeman and transferred to 15 ml centrifuge tubes that had previously been coated with GE SC-87 Dri-Film and contained 0.1 ml of organic separator (Octoil and Di-n-butyl pthalate, 6:5) (Ballentine and Buford, '60). The cells were centrifuged at 3,300 g for 1-2 minutes. The supernatant was removed and all traces of liquid were removed by swabbing with absorbent paper. The cells were then resuspended in ice cold Hanks ' solution and immediately centrifuged for 1 minute at 3,300 g. This step removed carbohydrate trapped in the intercellular spaces so that no correction was necessary for intercellular sugar. The supernatant was discarded and any adhering moisture was removed by swabbing. The cell pellets were resuspended in 0.8 ml of 0.1 N lithium hydroxide to disrupt the cell membrane. Then 0.1 ml each of 5 percent $ZnSO_4$ and 0.3 N $Ba(OH)_2$ was added to precipitate the proteins. The sample was centrifuged until all the precipitate had sedimented. The clear supernatant was transferred to 25 ml liquid scintillator vials, neutralized with HCl and then 15 ml of scintillation fluid were added. The intracellular water was analyzed for radioactivity with a Packard Tri Carb liquid scintillator. With each experiment a blank was made to determine background. The blank was treated as above except that no labeled solution was added. The average background was 54 cpm for the 28 experiments. A standard was prepared for each experiment by transferring 0.5 ml of twice the desired concentration

of glucose and 0.5 ml of labeled solution to a scintillation vial and adding 15 ml of scintillation fluid.

Results

Time Course of Glucose Uptake

KB cells in monolayer were exposed to a series of four concentrations of glucose. Samples were taken at appropriate time intervals to determine the pattern of glucose uptake. Figure 1 shows that uptake is initially linear with time at all concentrations studied, and then rapidly attains a steady-state. Entrance from a 10 mM glucose solution reaches the steady-state before the first sample is taken, thus it can only be assumed that the entrance is initially linear.

Saturation Kinetics

The effect of the external glucose concentration on the rate of entrance into the KB cells is shown in Figure 2. As the external concentration of glucose is increased, a saturation effect is observed.

Michaelis-Menten Kinetics

In Fugure 3 the internal glucose concentration at steady-state is plotted against the external concentration in the reciprocal form of the Michaelis-Menten equation derived by Lineweaver and Burke (Patton, '65). A least mean square line was calculated from the means of the uptake data and excellent agreement to Michaelis-Menten kinetics is shown. The K_m calculated for glucose entrance into KB cells is 10 mM and Vmax is 20 mM/min.. Figure 3 also indicates that the transport of glucose

follows a Langmuir adsorption isotherm (Langmuir, '18) which suggests that uptake may involve a reversible adsorption process.

DISCUSSION

The results of these experiments indicate that the entrance of glucose into KB cells is not by a simple diffusive process. The kinetics of glucose entrance, (a) saturation kinetics, (b) Michaelis-Menten kinetics) and (c) Langmuir adsorption isotherm suggests that the transport is of the carrier mediated type. Similar observations have been made on transport in Ehrlich ascites cells grown in tissue culture by Cirillo and Young ('64) and Kolber ('63). Also, Earle's L-cells were shown by Rickenberg and Maio ('61) to possess the carrier mechanism for carbohydrate transport.

Experiments by Eagle et al. ('58) show that the optimum glucose concentration in the media for maximum growth of KB cells is 2-5 mM. Figure 1 suggests possible accumulation of glucose below a concentration of 5 mM in the external media. However, no further experiments were done to ascertain if the cells were actually actively transporting glucose at low external sugar concentrations. However, this result lends support to the statement by Min ('65) that cells exposed to low concentrations of metabolites build up reserves to permit survival when the carbohydrate is present in insufficient quantities for optimum conditions.

The Michaelis constant (10 mM) obtained from Figure 3 indicates the transport system of the KB cells has a low affinity for glucose compared

to the Ehrlich ascites, 0.7 mM, (Crane et al., '57) and Earle's L-cells, 1.0 mM (Rickenberg and Maio, '61). Although these three cell lines are quite similar in their metabolic activity, the sugar transport system appears to be different. Therefore, it is proposed that entrance of sugars into KB cells is not rate limiting for glycolysis, and appears in agreement with the earlier findings of Eagle ('55).

LITERATURE CITED

Ballentine, R. and D. D. Burford 1960 Differential density separation of cellular suspension. Anal. Biochem., 1:263-268.

Cirillo, V. P. 1962 Sugar transport by S. cerevisiae protoplast. J. Bacteriol., 1251-1253.

_____ and D. K. M. Young 1964 Uphill sorbose transport induced by counterflow in Ehrlich ascites cells. Arch. Biochem. and Biophys. 105:86-88.

Crane, R. K., R. A. Field, and C. F. Cori 1957 Studies of tissue permeability, I. The penetration of sugars into the Ehrlich ascites tumor cells. J. Biol. Chem., 224:649-662 .

Eagle, H. 1955 Nutrition needs of mammalian cells in tissue culture. Science, 122:501.

_____ , Barban, S., Levy, M. and H. O. Schalze 1958 Carbohydrate utilization by cell cultures. J. Biol. Chem. 233:551-558.

_____ 1959 Amino acid metabolism in mammalian cell culture. Science 130:432-437.

Hanks, J. H. and R. E. Wallace 1949 Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exp. Biol. Med., 71:196.

Horecker, B. L., J. Thomas and J. Monod 1960 Galactose transport in E. coli. I. General properties as studied in a glactokinaseless mutant. J. Biol. Chem. 235:1580-1585.

Johnstone, R. M. and P. G. Scholefield 1964 Amino acid transport in tumor cells. Adv. in Cancer Res., 9:143-226.

Kolber, A. R. 1963 Evidence for mediated transport of monosaccharides in ascites tumor cells. Fed. Proc., 22:167.

Langmuir, I. 1918 The adsorption of gases on plane surfaces of glass, mica and platinum. J. Am. Chem. Soc., 40:1361-1402.

LeFevre, P. G. 1962 Rate and affinity in human red blood cell sugar transport. Am. J. Physiol., 203:286-2 90.

Min, H. S. 1965 Studies on the transport of carbohydrate in Crithidia luciliae. J. Cell. Comp. Physiol., 65:243-248.

Nierenberg, M. W. and J. F. Hogg 1958 Hexose transport in ascites tumor cells. J. Am. Chem. Soc., 80:4407-4412.

Patton, A. R. 1965 Biochemical Energetics and Kinetics. W. B. Saunders Co., Philadelphia.

Rickenberg, H. V. and J. J. Maio 1961 The transport of galactose by mammalian culture cells. In: Symposium on Membrane Transport and Metabolism. A. Kleinzeller and S. Kotyk (eds.), Academic Press, Inc., New York, pp. 343-351.

Vann, L. S., S. T. Nerenberg, and C. J. Lewis 1956 The kinetics of glucose transport through the human red cell membrane. Exp. Cell Res., 11:59-66.

FOOTNOTES

1. Submitted to the Graduate Division, Georgia Institute of Technology, as a thesis in partial fulfillment of the requirements of the degree of Master of Science. Supported in part by USPHS Grant GM-12235 and NASA Grant NsG-657 to Hong S. Min.

2. Present address: Eli Lilly and Company, Indianapolis, Indiana.

3. $[C]_i$ = intracellular concentrations in micromoles per milliliter of packed cell volume.

   $[C]_o$ = extracellular concentration in micromoles per milliliter.

LEGENDS FOR FIGURES

Fig. 1. Relationship between intracellular concentration and time of exposure.

Fig. 2. Effect of external glucose concentration on the rate of transport.

Fig. 3. Effect of external glucose concentration on glucose uptake.

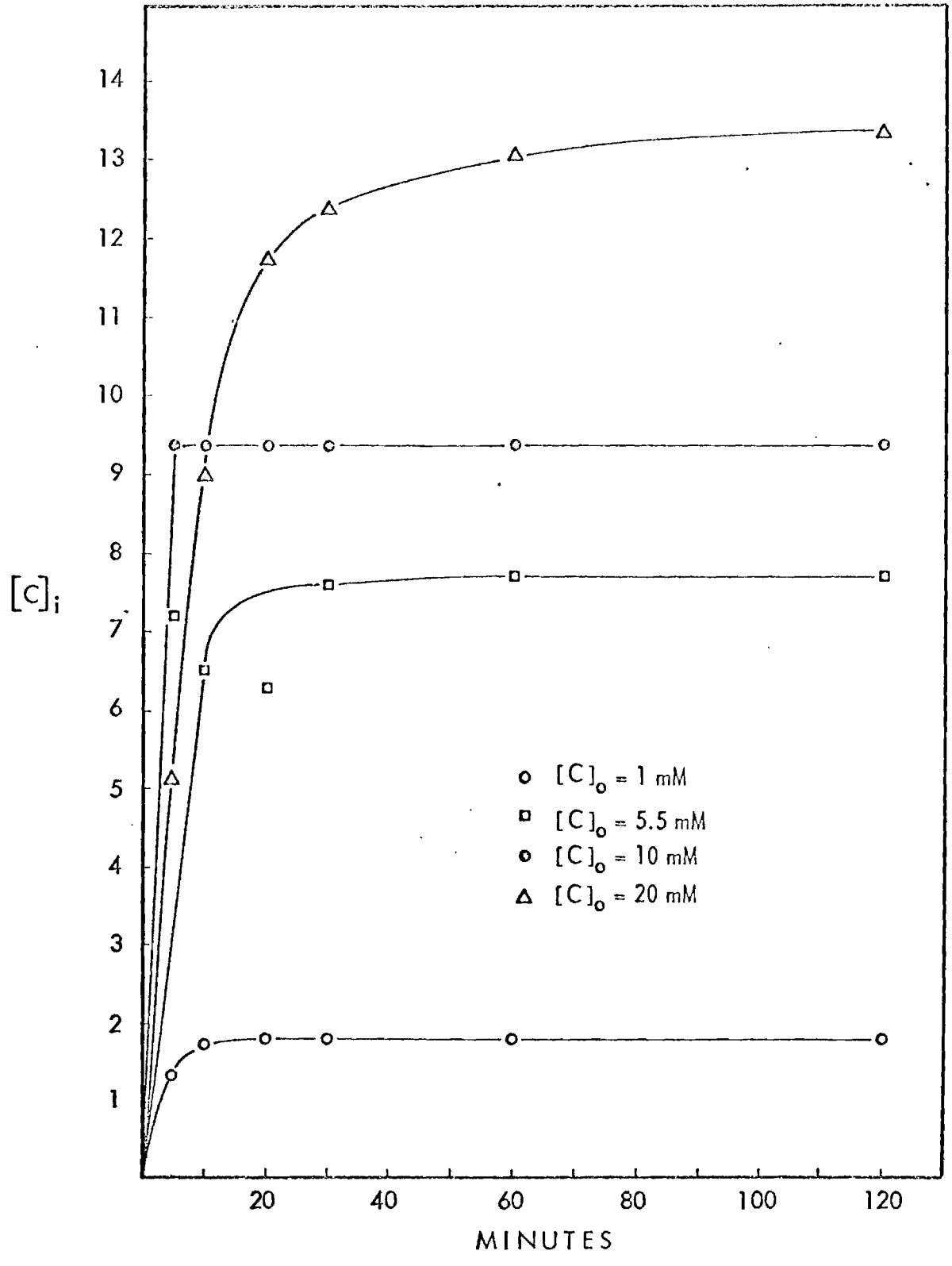
14
13
12
11
10
9
8
7
6
5
4
3
2
1
[C]i
20
40
60
80
100
120
MINUTES
[C]o = 1 mM
[C]o = 5.5 mM
[C]o = 10 mM
[C]o = 20 mM

FIGURE NO. 1

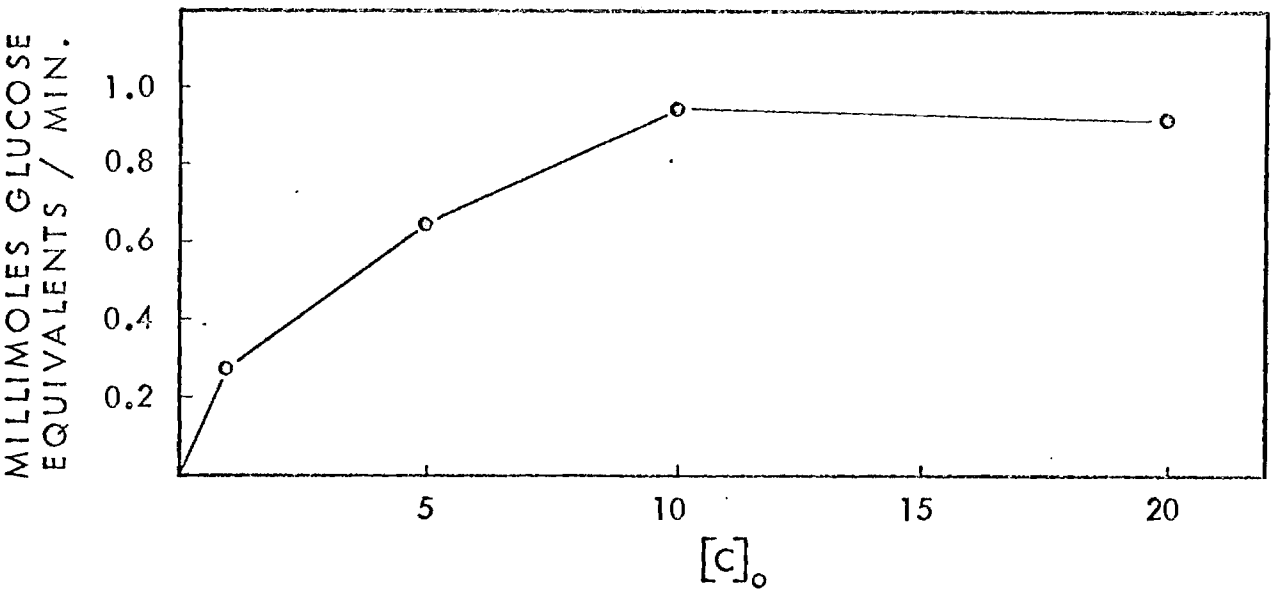
MILLIMOLES GLUCOSE EQUIVALENTS / MIN.
1.0
0.8
0.6
0.4
0.2
5
10
15
20
[C]o

FIGURE NO. 2

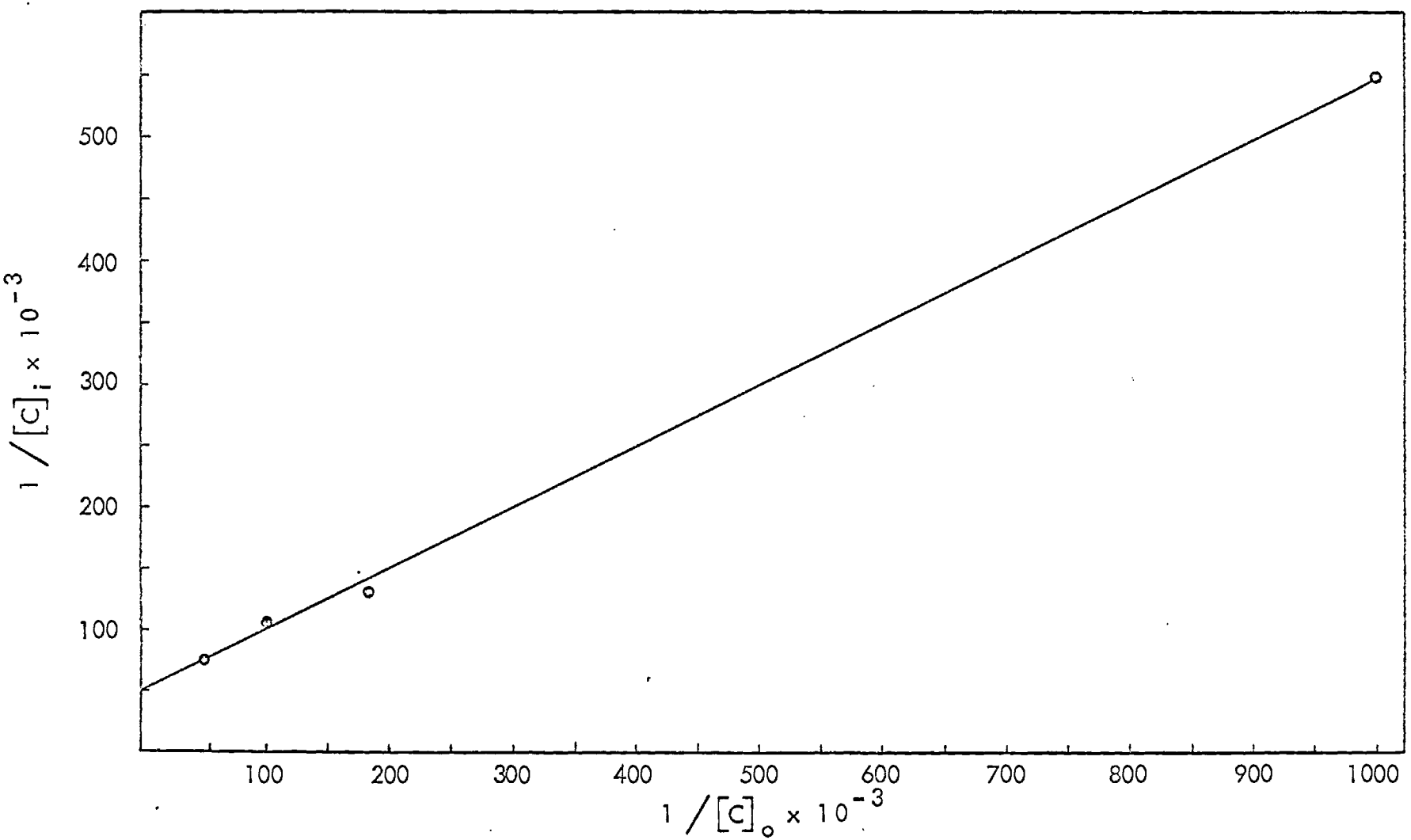
500
400
300
200
100
$1/[C]_i \times 10^{-3}$
100
200
300
400
500
600
700
800
900
1000
$1/[C]_o \times 10^{-3}$

FIGURE NO. 3

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HONG S. MIN, Georgia Institute of Technology.

Further studies on the entrance of carbohydrates into cells of *Crithidia luciliae*.

The entrance of carbohydrate into the cells of *C. luciliae* has been studied, using nine non-utilizable monosaccharides. At 0.5 mM external concentration the rate of increase in intracellular concentration is constant for all sugars until the intracellular concentrations equal or exceed the extracellular concentration. At 20 mM external concentration, the rate of increase of intracellular concentration never exceeds the extracellular concentration. There is competition between monosaccharides presented simultaneously to the cells and transport mechanism shows enormously greater affinity for glucose than for other monosaccharides. The rate of carbohydrate entrance is inhibited 50% and 70% by KCN (10^{-4}M) and DNP (10^{-4}) respectively at 0.5 mM external concentration. However, these inhibitors do not affect transport [illegible] external concentrations of the order of 0.02 M. [illegible]se data are interpreted as indicating two mec[illegible]ms for carbohydrate entrance: (a) an active [illegible]nsport mechanism, active at low external co[illegible]ation and dependent upon a supply of metabo[illegible]rgy; (b) facilitated diffusion, of importance [illegible]gh external concentrations. (Supported by gr[illegible]M 12235-01 from the U.S.P.H.S.)

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HONG S. MIN, Georgia Institute of Technology.

Studies on the entrance of carbohydrates into cells of *Crithidia luciliae*; effects of extracellular concentrations and molecular configuration.

Previously it was reported that at 0.5 mM external concentration the rate of increase in intracellular concentration is constant for all sugars until the intracellular concentration equals or exceeds the extracellular concentration, and at 20 mM external concentration, the rate of increase of intracellular concentration never exceeds the extracellular concentration (Cell. & Comp. Physiol., Vol. 65, 1965). The entrance of carbohydrate has been further studied, using external concentrations greater than 20 mM. The results of the studies using 40, 80, and 160 mM sorbose also show that the maximum intracellular concentration approached the extracellular concentration after 32 minutes. Similar studies using various monosaccharides exhibited generally the similar pattern of entrance. However, the results clearly indicate that transport of various monosaccharides from the external concentrations of the order of 0.02 M exhibit a specificity of mechanism related to configurational properties of the carbohydrate molecules.

Detailed analysis of these data reaffirms that the transport system functioning in *C. luciliae* is determined by the external concentration of carbohydrate; an active transport system at lower concentrations and a facilitated diffusion at higher concentrations. (Supported by Grant GM 12235 from the U.S.P.H.S.)

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HONG S. MIN, Georgia Institute of Technology.
Studies on the entrance of carbohydrates into cells of *Crithidia sp.* from *Arilus cristatus*.

The entrance of carbohydrate into the cells of *C. sp.* from *Arilus cristatus* has been studied, using non-utilizable monosaccharides. At 0.5 mM external concentration the rate of increase in intracellular concentration is constant for all sugars until the intracellular concentrations equal or exceed the extracellular concentration. At 200 mM external concentration, the rate of increase of intracellular concentration decreases continuously and the maximum intracellular concentration never exceeds the extracellular concentration. There is competition between monosaccharides presented simultaneously to the cells and the transport mechanism shows enormously greater affinity for glucose than for other monosaccharides. The rate of carbohydrate entrance is inhibited by KCN and DNP at 0.5 mM external concentrations. However, these inhibitors do not affect transport from concentrations of the order of 0.02 M. These data are interpreted as indicating two mechanisms for carbohydrate entrance: (a) an active transport mechanism, active at low external concentration and dependent upon a supply of metabolic energy; (b) facilitated diffusion, of importance at high external concentrations. It appears that despite the differences in metabolic patterns, the cells investigated in this study have similar transport mechanisms to the previously reported *Crithidia luciliae* (J. Cell. and Comp. Physiol., *65*:243-248). (Supported by Grant GM 12235 from the U.S.P.H.S.)

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HONG S. MIN and MICHAEL A. ESTERMAN, Georgia Institute of Technology.

Effect of the presence of glucose on the transport of carbohydrates in *Crithidia luciliae*. (By title only.)

The treatment of sorbose at 10 and 20 mM was studied in the presence of 5, 10, and 20 mM glucose. The transport of 20 mM sorbose was greatly inhibited (more than 90%) by the simultaneous presence of 5 and 20 mM glucose as was the transport of 10 mM sorbose in the presence of 10 mM glucose.

The results indicate that glucose and sorbose utilize the same transport mechanism in *C. luciliae*, but the system has a much higher affinity for glucose.

At the present studies are being made with other hexoses and pentoses in this laboratory. (Supported by grant GM 12235 from the U.S.P.H.S.)

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HONG S. MIN, Georgia Institute of Technology.

Entrance of carbohydrates into human cancer cells grown in tissue culture (KB cell line).

The entrance of carbohydrates into the cells of KB cancer cell line has been studied, using labeled monosaccharides. At 10 mM external concentration the rate of increase of intracellular concentration decreases continuously and the maximum intracellular concentration never exceeds the extracellular concentration. It appears that the kinetics of penetration resemble the Michaelis-Menton Law rather than the Fick's kinetics. On the other hand, the pattern of entry of sugars between 1.0 and 5.0 mM external concentrations does not resemble the predicted pattern of entrance by diffusion, but instead the rate of entrance is constant up to $[C]_i/[C]_o$ of 1.80.

The data indicate that the transport system has a high affinity for sugars and the saturation of the system occurs very rapidly at 10 mM external concentration. The enzyme-like kinetics observed in these studies suggest that a carrier mechanism may be involved in the transport of sugars into cancer cells (KB). The large value for V_{max} (18 mM/min) and the rapid attainment of the steady state intracellular concentration by the KB cells suggests that the cells have a large demand for metabolizable sugars.

It appears that the mode of carbohydrate entrance into KB cells is dependent upon the external concentration of sugars and a carrier system involved. (Supported by Grants GM-12235 from the U.S.P.H.S. and NsG-657 from NASA.)