CHARACTERIZATION OF THE D-GLUCOSE TRANSPORT SYSTEM AND THE REGULATION OF METABOLITE TRANSPORT BY CYTOKININS AND CITRATE IN A WATER MOULD

by

SWEE HAN GOH

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TO MY PARENTS

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Abbreviations

BAP	benzylaminopurine
cAMP	adenosine 3':5' cyclic monophosphate
CCCP	m-chlorophenylcarbonylcyanidehydrazone
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol-bis-(aminoethyl ether)-N,N'-tetra-acetic acid
GA	gibberellic acid
HAP	hexylaminopurine
IAA	indole-3-acetic acid
6ipAde	N^6-(Δ^2-isopentenyl)adenine
6ipAdo	N^6-(Δ^2-isopentenyl)adenosine
K	kinetin
KR	kinetin riboside
NEM	N-ethylmaleimide
ONPG	o-nitrophenyl-β-D-galactoside
PHA	phytoheamagglutinin
PhenylAP	phenylaminopurine
Tris	trihydroxymethylaminomethane
Z	zeatin

ABSTRACT

D-glucose and 2-deoxy-D-glucose were transported by an active process into the cells of the water mould, Achlya. D-Fructose and glycerol failed to be transported by D-glucose grown cells. Metabolic inhibitors such as 2,4-dinitrophenol, cyanide, azide and CCCP (m-chlorophenylcarbonylcyanidehydrazone) inhibited transport markedly at micromolar concentrations, the most potent of these inhibitors being CCCP.

Competition studies revealed that D-galactose (K_i=1.55 x 10^{-3}M), D-mannose (K_i=5.8 x 10^{-4}M), 2-deoxy-D-glucose (K_i=1.7 x 10^{-4}M) and D-xylose (K_i=3.7 x 10^{-3}M) inhibited competitively D-glucose transport. Other sugars tested showed very slight to no inhibition, indicating the existence of a fairly specific D-glucose transport system. The uptake of D-glucose was pH and temperature dependent, each with a fairly well defined optimum. Sulfhydryl group reactive reagents such as I_2 (0.5 to 5.0 μM), Hg^{++} (1 to 200 μM) and NEM (1 to 500 μM) inhibited D-glucose uptake. All these results suggest that a protein carrier may mediate D-glucose

transport in Achlya.

Citrate, at concentrations of 1 mM or greater, inhibited D-glucose transport (K_i=8.2 x 10^{-3}M). Data are presented which indicate that citrate act by chelating Ca^{++} and so deprive the fungus of this cation which is absolutely essential for growth and active transport processes.

Indirect experimental observations support the idea that phosphorylation of D-glucose during transport may be essential for uptake. A similar phosphorylation process has been detected inyyeast.

Cytokinins (plant growth hormones), the most effective being 6ipAde (50 to 500 µM, K_i=1.88 x 10^{-4}M) and hexylaminopurine were found to inhibit D-glucose transport. These N^6-adenine derivatives appear to play important regulatory roles throughout the life cycle of Achlya in a variety of transport activities.

INTRODUCTION

Transport of metabolites through biological membranes and hormonal regulation of intracellular processes through hormone-membrane interaction are two major fields of research.

To a cell, the membrane is not only the window to the world but also acts as a protective barrier. Like intracellular enzymes, the activities of protein mediated transport systems located on cell membranes are regulated in very intricate fashions for the maximum economy to the cell. Tumour cells and certain viral transformed cell lines have been observed to take up D-glucose at faster rates when compared to normal cell lines. Understanding this anomaly may give some insight to the process of tumour formation. In the same light, differences in the topography of membranes between normal and tumour cells have been reported. It could well be, as suggested, that signals for regulating normal cell metabolism and proliferation cannot be transmitted to the cells owing to the absence or alteration of specific membrane located binding sites for such signals in tumour cells.

A better understanding of the regulation of sugar transport processes and the metabolism of such transported metabolites will undoubtedly contribute to the development of more efficient commercial fermentation processes. These are but some of the examples of membrane-hormone-transport related problems. Research into such fundamental processes may enable man to understand better the intricacies of cell growth.

The purpose of this study is two fold: first, to define some of the characteristics of the sugar transport system in the Oömycete, Achlya; and second, to examine some possible regulatory features of transport related to the growth of this fungus.

HISTORICAL

Plant Cytokinins

Cytokinins, a class of 'hormonal' compounds, exhibit a multitude of biological effects not only in plants but also in organisms as diverse as bacteria, fungi, algae and probably mammals as well. Some of these effects are summarized in a table (I). Since the isolation of kinetin by Miller et al in 1954 (67), similar purine compounds have been isolated or synthesized, the more significant ones being 6ipAde, zeatin [N^6-(trans-γ-hydroxymethyl-γ-methyl-allyl)adenine] and N^6-methyl and dimethyl-aminopurine. Structurally, an intact purine ring with an N^6-substituent of moderate size is necessary for a compound to exhibit high cytokinin activity (97). However certain exceptions do exist of which the urea derivatives like N,N' diphenylurea and N-3chlorophenyl-N'-phenylurea and 8-azakinetin are the more outstanding examples (29).

Cytokinins are intimately involved in nearly every facet of plant development. Their effects on metabolism vary from inducing cell division and cell enlargement to delaying senescence. Enzyme activities and their rates of biosynthesis and degradation have

been also shown to be affected by these hormones. RNA and DNA synthesis are also affected likewise. Table I summarizes some of the biological phenomena known to be influenced by cytokinins.

Cytokinins are not exclusively found in plants. Such compounds have been isolated and characterised from certain bacteria and fungi (39, 50, 51, 65, 78, 101). The pathogenic symptoms exhibited by plants infected with such pathogens can be mimicked by singular addition of cytokinins. Cytokinins have also been shown to be constituents of tRNA from organisms as diverse as bacteria and mammals (7, 36). These purine derivatives are all found situated adjacent to the 3' end of the anticodon on the tRNA (35). Biochemical and genetical studies have shown that alterations or deletions of the substituted side chain(s) resulted in a reduction in the ability of such tRNAs to bind to ribosomes in the presence of the appropriate messenger. However, the charging process of these modified tRNAs with their specific amino acids is not affected (25, 31, 66). Whether the unique position of these cytokinins on tRNAs has any important relationship to their biological activities remains to be elucidated.

Cytokinins may also play an important role in plant tumourigenesis. Unlike normal cells in tissue culture, cells which are transformed are capable of autonomous growth in the absence of exogenously supplied cytokinins (112). This indicates that such tissues are capable of synthesizing their own cytokinin requirements in culture, a process which was repressed before, Braun et al have suggested that cytokinesins (glucose containing 3,7-dialkyl-2-alkylthio-6-purinone compounds) are the primary factors which induce cytokinesis (113). Also, cytokinesins are synthesized persistently in crown gall tumour cells. Normal plant cells require kinetin to induce cytokinesin synthesis. Wood and Braun have also demonstrated that cytokinesins are strong inhibitors of both plant and animal adenosine 3':5'-cyclic monophosphate phosphodiesterases (114, 115).

Despite the multitude of information concerning the biological activities of cytokinins, no satisfactory explanation has yet been given relating cytokinin activity to cytokinesis and cellular differentiation.

TABLE (I)

EFFECTS OF CYTOKININS ON PLANTS, MICRO-ORGANISMS AND ANIMALS

(A) IN PLANTS

SYSTEM	SPECIFIC EFFECT	CYTOKININ SPECIFICITY	LITERATURE REFERENCE
CELL GROWTH			
bean leaf	promoted leaf expansion	K,BAP	Scott, R.A. and Liverman, J. L. (1956) Plant Physiol. 31, 321-322. and Miller, C.O. (1956) Plant Physiol. 31, 318-319.
lettuce seeds	stimulation of germination related to cellular expansion	K	Haber, A.H., and Luippold, H.J. Plant Physiol. (1960) 35, 168-173.
sunflower hypocotyls	increased fresh and dry weight without elongation	K	de Ropp, R.S. (1956) Plant Physiol. 31, 253-254.
radish leaf discs	expansion promoted	general	Kuraishi, S. (1959) Sci. Papers Coll. Gen. Educ. Uni. Tokyo, 9, 67-104.
Lemna minor fronds	increase in frond area stimulated at low concentrations (10^{-6}M); inhibited at high concentrations	K	Tasser de Jong, J.G. and Veldstra, H. (1971) Plant Physiol. 24, 235-238.

Table (I) Continued

pea stem segments	elongation inhibited	Z	Witham, F.H. and Miller, C.O.,(1965) Plant Physiol. 18, 1007-1017.
MITOSIS AND CELL DIVISION			
tobacco pith tissue	stimulated mitosis and cytokinesis	K	Das, N.K., Patau, K., and Skoog, F. (1956) Plant Physiol. 9, 640-651.
unorganized tobacco tissue	stimulated cell division	K(auxin)	Skoog, F. and Miller, C.O. (1957) Symp. Soc. Exptl. Biol. II, 118-131.
mature pea root tissue	induces mitosis	K	Torrey, J.G. (1961) Exptl. Cell Res. 23, 281-299.
intact Allium cepa roots	mitosis inhibited	K	McManus, M.A. (1960) Nature 185, 44-45.
DIFFERENTIATION			
Shoot, Root and Bud Development and Initiation			
leaf squares of Peperomia sandersii	inhibits root and bud generation	K	Harris, G.P., and Ennid, M.H.H. (1964) Ann. Bot. N.S. 28, 509-526.
root segments of Isatis tinctoria and Convolvulus arvensis	initiated regeneration of shoots	K	Danckwardt-Lilliestrom, C. (1957) Physiol. Plant 10, 794-797 and Torrey, J.G. (1958) Plant Physiol. 33, 258-263.

Table (I) Continued

leaf cuttings of *Saintpaulia ionantha*	increased budding	K	Plummer, T.H. and Leopold, A.C. (1957) Proc. Am. Soc. Hort. Sci. 70, 442-444.
leaf discs of *Begonia rex*	induced shoot formation inhibited root growth	K	Schraudolf, H. and Reinert, J. (1959) Nature (Lond.) 184, 465-466.
Begonia leaf cuttings	root induction inhibited at high concentrations, promoted at low concentrations	K	Heide, O.M. (1965) Physiol. Plant 17, 789-804.
Neutralization of Apical Dominance			
Alaska pea stems (and other sources)	released axillary buds from either apical dominance or auxin treatment	K	Wickson, M. and Thimann, K.V. (1958) Physiol. Plant II, 62-74 and Sachs, T. and Thimann, K.V. (1964) Nature (Lond.) 201, 939-940.
Specialized Tissue Formation			
pea stem segments	functional xylem induced	K	Sorokin, H.P. and Thimann, K.V. (1958) Protoplasmia 59, 326-
N. tabacum	tracheid formation	K	Bergmann, L. (1964) Planta 62, 221-254.
pith parenchyma (Romaine lettuce)	induced to differentiate into tracheary elements	K(IAA)	Torrey, J.G., Fosket, D.E., Hepler, P.K. (1971) Am. Sci. 59, 338-352.
Abscission			
Phaseolus	retarded when applied to abscission zone; enhanced when applied elsewhere	K	Osborne, D.J., and Moss, S.E. (1963) Nature 200, 1299-1301.

Table (I) Continued

Phaseolus leaves	promotion at low concentrations; inhibition at high concentration		Chatterjee, S.K. and Leopold, A.C. (1964) Plant Physiol. 39, 334-337.
Fruit Production			
figs	parthenocarpy induced	SD 8339 (synthetic)	Crane, J.C. and van Overbeek, J. (1965) Science 147, 1468-1469.
Vitis vinifera L.	increased fruit size and fruit set	BAP	Weaver, R.J., van Overbeek, J. and Pool, R.M. (1965) Nature (Lond.) 206, 952-953 and Weaver, R.J. and van Overbeek, J. (1963) California Agric. 17, 12-
plum and apple fruitlets	fruit and seed development	(endog)	Letham, D. (1963) New Zeal. J. Bot. I, 336-350.
Flowering			
Cichorium intybus L.	flower formation induced in cold requiring plant grown under non-inductive conditions	K(+Vit.E)	Michniewicz, M. and Kamienska, A. (1964) Naturwiss 51, 295-296.
Arabidopsis thaliana	overcomes long day requirements	K(+Vit.E)	Michniewicz, M. and Kamienska, A., (1965) Naturwiss, 52, 623-
tomatoe and pea (intact plants)	application to roots-inhibited flowering in tomato plants, enhanced in pear	K	Wittwer, S.H. and Dedolph, R.R. (1963) Amer. J. Bot. 50, 330-336.

Table (I) Continued

Special Organelles and Structures			
tobacco pith tissue	induced maturation of proplastids to plastids	K	Stetler, D.A. and Laetsch, W.M. (1965) Science 149, 1387-1388.
Solanum tuberosum	induction of tuber formation	K	Palmer, C.E. and Smith, O.E. (1969) Plant and Cell Physiol. 10, 657-664.
tobacco roots	induction of pseudonodule formation in the root cortex	K(IAA)	Arora, N., Skoog, F. and Allen, O.N. (1959) Amer. J. Bot. 46, 610-613.
SYNTHESIS			
Lemna minor	starch synthesis stimulated at high concentrations, inhibited at low concentrations	K,BAP	Hillman, W.S. (1957) Science 126, 165-166 and Tasser de Jong, J.G. and Veldstra, H. (1971) Plant Physiol. 24, 235-238.
detached Impatiens petals	anthocyanin production stimulated	K	Klein, A.O. and Hagen, C.W. Jr. (1961) Plant Physiol. 36, 1-9.
tobacco tissue	lignin synthesis activated	K	Bergmann, L. (1964) Planta 62, 221-254.
tobacco tissue	thiamine synthesis activated	K	Digby, J. and Skoog, F. (1966) Plant Physiol. 41, 647-652.
soybean tissue	deoxyisoflavone synthesis stimulated	general	Miller, C.O. (1969) Planta 87, 26-35.

Table (I) Continued

Amaranthus seedlings	betacyanin production increased	general	Kohler, K.H. and Conrad, K. (1966) Biologische Rundschen 4, 36-40.
cucumber cotyledons	chlorophyll content increased (in light)	BAP BAP	Fletcher, R.A. and McCullagh, D. (1971) Can. J. Bot. 51, 1347-1354.
leaves of intact plants	patterns of lipid fatty acids and lipid bound sugars altered	K,Z	Kull, U. and Büxenstein, R. (1974) Phytochem. 13, 39-44.

DORMANCY AND GERMINATION

Seed Germination

beech, hazel and rowan seeds	stimulation of germination of intact seeds	K(thiourea, GA)	Frankland, B. (1961) Nature 192, 678-679.
irradiated lettuce seeds	stimulation of recovery of germination	K	Haber, A.H. and Luippold, H.J. (1960) Plant Physiol. 35, 168-173.
Xanthium seeds	effects of naturally occurring inhibitors of seed germination such as coumarin and xanthetin are reversed	K(+red light)	Khan, A.A. and Tolbert, N.E. (1965) Physiol. Plant 18, 41-43.
bean seeds	enhanced germination	K,BAP, Phenyl AP, HAP	Miller, C.O. (1956) Plant Physiol. 31, 318-319.

Table (I) Continued

Seed Dormancy Release			
apple seedlings	induces dormancy break	K	Pieniazek, J. (1964) Acta. Agrobot. 26, 157-169.
Xanthium seeds	dormancy of 'upper' seed broken, with RNA synthesis involved	K	Khan, A.A. (1966) Physiol. Plant 19, 869-874.
pear embryos	dormancy release	K	Khan, A.A. Plant Growth Substances 1970 pp. 207-215, Carr, D.J. (ed), Springer-Verlag Berlin, Heidelberg, New York. 1972.
DELAY OF SENESCENCE			
barley seedlings (etiolated)	retarded loss of synthetic ability of proteins and chlorophyll with aging	K	Stobart, A.K., Shewry, P.R. and Thomas, D.R. (1972) Phytochem. 11, 571-577.
oat leaf sections	chlorophyll retained	K,BAP (Z and i^6Ade ineffective)	Varga, A. and Bruisma, J. (1973) Planta 111, 91-93.
isolated discs of *Xanthium* leaves	yellowing delayed, RNA and protein levels maintained	K	Osborne, D.J. (1962) Plant Physiol. 37, 595-602.
corn seedling leaves	chlorophyll and protein preserved (senescence enhanced at low concentrations)	BAP	Tavares, J. and Kende, H. (1970) Phytochem. 9, 1763-1770.

detached Xanthium leaves	chlorophyll and protein degradation retarded	K	Richmond, A. and Lang, A. (1957) Science 125, 650-651.
intact bean plants	leaf senescence retarded	BAP	Fletcher, R.A. (1969) Planta 89, 1-8.

EFFECTS ON TRANSPORT

detached tobacco	glycine transported, against concentration gradient, from cell to cell to site of application	K	Mothes, K., Engelbrecht, L. and Kulaeva, O. (1959) Flora, 147, 445-464.
detached oat leaves	glycine and phosphate transported to application site	K	Gunning, B.E.S. and Barkley, W.K. (1963) Nature (Lond.) 199, 262-265.
corn leaves	phosphate transported to application site	K	Muller, K. and Leopold, A.C. (1966) Planta 68, 167-185, 186-205.
bean leaves	phosphate movementtto application site enhanced	K	Seth, A.K. and Waring, P.F. (1967) J. Exptl. Bot. 18, 65-77.
bean leaves	no observed increase in phosphorus uptake and translocation	K	Resnick, M.E. and Montaldi, E.R. (1968) Biol. Prod. Veg. 5, 99-111.
whole bean plants, senescing	no mobilization of ^{14}C and ^{32}P compounds in the whole plant	BAP	Adedipe, N.O. and Fletcher, R.A. (1970) J. Exptl. Bot. 21, 968-974.

Table (I) Continued

tobacco chloroplast	increased permeability of chloroplast membrane to ^{14}C-leucine	K	Richmond, A.E., Sachs, B. and Osborne, D.J. (1971) Physiol. Plant 24, 176-180.
leaf discs and detached cotyledons of sunflowers	increased uptake of K^+, Rb^+, Li^+ but not Na^+.	K	Richmond, (1971) Physiol. Plant 25, 230-233 and Ilan, I., Gilad, T. and Reinhold, L. (1971) Physiol. Plant 24, 337-241.
bean primary leaves	Na^+ absorption	BAP	Jacoby, B. and Dagan, J. (1970) Physiol. Plant 23, 397-403.

DEGRADATION AND SYNTHESIS PROCESSES

RNA and DNA

onion root nuclei	RNA content increased	K	Guttman, R. (1957) J. Biophys. Biochem. Cytol. 3, 129-131.
onion root tip cells	doubling of RNA levels, DNA levels reduced	K	Jensen, W.A. Pollock, E. G., Healy, P. and Ashton, M. (1964) Exptl. Cell Res. 33, 523-530.
excised soybean hypocotyl	inhibition of RNA synthesis, especially rRNA	K	Vanderhoef, L.N. and Key, J.L. (1968) Plant and Cell Physiol. 9, 343-351.
peanut cotyledons	RNA levels increased; no DNA increase	BAP	Carpenter, N.B. and Cherry, J. (1966) Biochim. Biophys. Acta 114, 640-642.

Table (I) Continued

barley leaves, tobacco pith culture	mRNA synthesis increased or preserved	K	Srivastava, B.I.S. (1967) Ann. N.Y. Acad. Sci. 144, 260-278.
tobacco pith cells	rapid DNA increase	K(auxin)	Patau, K., Das, N.K. and Skoog, F. (1957) Physiol. Plant 10, 949-966.
Ribosome Levels			
detached wheat leaves (senescing)	levels maintained	K	Shaw, M. and Manocha, M. (1965) Can. J. Bot. 43, 747-755.
excised tobacco leaves	rRNA preserved	K	Srivastava, B.I.S. (1967) Ann. N.Y. Acad. Sci. 144, 260-278.
excised barley leaves	rRNA and ribosomes preserved	K	Srivastava, B.I.S. and Arglebe, C. (1968) Physiol. Plant 21, 851-857.
Chinese cabbage leaves	rRNA and ribosomes preserved	K	Berridge, M.V. and Ralph, R.K. (1969) Biochim. Biophys. Acta 182, 266-269.
Protein Levels			
tomato fruit locule plastids	increased amino acid incorporation	K(IAA)	Davis, J.N. and Cocking, E.C. (1967) Biochem. J. 104, 23-33.
isolated tobacco chloroplast	stimulation of synthesis (age dependent effect)	BAP	Kulaeva, O.N. and Romanko, E.G. (1967) Dokl. Akad. Nauk. SSSR (Bot. Sci. Sec.) 117, 464-467.

Table (I) Continued

mitochondria of *Vigna* seedlings	increase in protein specific activity	K	Bhattacharyya, J. and Roy, S.C. (1969) Biochem. Biophys. Res. Comm. 35, 606-610.
Tropaeolum majus (detached leaves)	decreased degradation	K	Mizrahi, Y., Amir, J. and Richmond, A.E. (1970) New Phytol. 69, 355-361.
darkened oat leaves	inhibition of proteolysis	K	Shibaoka, H. and Thimann, K.V. (1970) Plant Physiol. 46, 212-220.
corn seedling leaves	inhibition of proteolysis	BAP	Tavares, S. and Kende, H. (1970) Phytochem. 9, 1763-1770.
soybean chloroplast	increased amino acid incorporation in older leaf chloroplast; no response in those from younger leaves	K	Marchetti, S.E. and Baron, F.J. (1971) Adv. Frontiers of Plant Sciences 28, 397-404.
tobacco cultures	inhibition of uracil and leucine incorporation	K	Nudel, V. and Bamberger, E.S. (1971) Plant Physiol. 47, 400-403.
Lemna minor	alterations in rates of synthesis and degradation; influenced by medium.	BAP	Trewavas, A. (1972) Plant Physiol. 49, 47-51.
Cyclic AMP Levels			
soybean callus (cultured in liquid suspension)	increased levels of cyclic nucleotide with treatment	K	Brewin, N.J. and Northcote, D.H. (1973) J. Exptl. Bot. 24, 881-888.
tobacco tissue	addition results in cell division which is correlated to cAMP level changes	K	Lundeen, C.V., Wood, H.N. and Braun, A.C. (1973) Differentiation 1, 255-260.

Table (I) Continued

EFFECTS ON SPECIFIC ENZYMES

(Activities and Synthesis)

RNase			
excised barley leaves	reduced activity	K	Srivastava, B.I.S. and Ware, G. (1965) Plant Physiol. 40, 62-64.
tobacco leaves	reduced activity	K	Bagi, G. and Farkas, G.L. (1968) Experientia 24, 397-398.
detached oat leaves	inhibition of RNase I activity	K(protein synthesis inhibitors or auxins)	Udvardy, J. Farkas, G.L. and Marre, E. (1969) Plant Cell Physiol. 10, 375-386.
barley leaf discs	counteraction of increase in RNase and proteinase activities	K,BAP	Atkin, R.K. and Srivastava, B.I.S. (1969) Physiol. Plant. 22, 742-750.
darkened oat leaves	inhibits rise in RNase level	K	Shibaoka, H. and Thimann, K.V. (1970) Plant Physiol. 46, 212-220.
Pisum sativum (apical regions of epicotyls)	suppresses IAA stimulated RNase activity	BAP	Birmingham, B.C. and Maclachlan, G.A. (1972) Plant Physiol. 49, 371-375.
Other Enzymes			
barley roots	Tyramine methylpherase, specific increase in this but not four other enzymes	K	Steinhart, C., Mann, J.D. and Mudd, S.H. (1964) Plant Physiol. 39, 1030-1038.

Table (1) continued

Phaseolus hypocotyl discs	α-amylase, increased synthesis	K	Clum, H.H. (1967) Plant Physiol. 42, 568-572.
peanut and squash seeds	isocitrate lyase, hormonal stimulus produced by embryonic axis replaced	BAP	Penner, D. and Ashton, F.M. (1967) Biochim. Biophys. Acta 148, 481-485.
squash seedlings	proteinase, hormonal stimulus produced by embryonic axis replaced	BAP,K phenylAP	Penner, D. and Ashton, F.M. (1967) Plant Physiol. 42, 791-796.
Agrostemma embryos	nitrate reductase, increased activity, additive response in presence of nitrate	BAP	Kende, H., Hahn, H. and Kays, S.E. (1971) Plant Physiol. 48, 702-706. and Kende, H. and Shen, T.C. (1972) Biochim. Biophys. Acta 286, 118-125.
Agrostemma embryos	nitrate reductase, increased activity due to increased de novo synthesis	BAP	Hirchberg, K. Hubner, G. and Borriss, H. (1972) Planta 108, 333-336.
excised pea roots	nitrate reductase, decrease in synthesis	K	Sahulka, J. (1972) Biol. Plant. 14, 330-336.
excised pea roots	glutamate dehydrogenase, antagonism of IAA induced synthesis	K	Ibid
carrot callus	glutamate dehydrogenase, concentration effect	K	Werner, D. and Goglin, D. (1970) Planta 91, 155-164.
general	respiratory kinases, activities inhibited	BAP	Tuli, V., Dilley, D.R. and Wittwer, S.H. (1964) Science 146, 1477-1479.
tobacco callus	hexose monophosphate shunt enzymes, specific activities decrease with decrease in growth rates	K	Scott, K.J., Daley, J. and Smith, H.H. (1964) Plant Physiol. 39, 709-711.

Table (2) continued

soybean callus, tobacco leaves	adenine phosphoribosyl transferase, inhibited	K	Nicholls, P.B. and Murray, A.W. (1968) Plant Physiol. 43, 645-648.
detached wheat leaves	acid phosphatase, lipase, esterase, levels altered	K	Sodek, L. and Wright, S.T.C. (1969) Phytochem. 8, 1629-1640.
tobacco leaves	aminoacyl tRNA synthetase, increased activity	K	Anderson, J.W. and Rowan, K.S. (1966) Biochem. J. 101, 15-18.
soybean cotyledons	altered leu-tRNA and synthetase function	BAP	Anderson, M.B. and Cherry, J.H. (1969) Proc. Natl. Acad. Sci. U.S. 62, 202-209.
Chinese cabbage nuclei and chloroplast extracts; tobacco and carrot nuclei	protein kinases, activities inhibited	K	Ralph, R.K., McCombs, P.J.A., Tener, G. and Wojcik, S.J. (1972) Biochem. J. 130, 901-911.
lentil embryonic axis	peroxidase isozymes; altered distribution patterns	K(IAA)	Gasper, T., Khan, A.A. and Fries, D. (1973) Plant Physiol. 51, 146-149.

RESPIRATION AND TRANSPIRATION

Stomatal Opening

barley leaves	transpiration and opening enhanced in detached mature leaves, but not young leaves	K(GA)	Livné, A. and Vaadia, Y. (1965) Physiol. Plant 18, 658-664.
mature primary barley leaves	increased rate of opening and CO_2 intake	K	Meidner, H. (1967) J. Exptl. Bot. 18, 556-561.

Table (I) Continued

Respiration			
tobacco leaves	inhibition	K	Sugiura, M. (1963) Bot. Mag. Tokyo 76, 359-362.
soybean callus	inhibition; concentration dependent effect; adenine reversal	Z,K	Moore, T.S. Jr. and Miller, C.O. (1972) Plant Physiol. 50, 594-598.
(B) PLANT DISEASES			
Bacterial			
Agrobacterium tumefaciens infecting Vinca rosea	crown gall tumour; implied in tumour induction	endog.	Wood, H.N. and Braun, A.C. (1967) Ann. N.Y. Acad. Sci. 144, 244-250.
Corynebacterium fascians	fasciation disease (witches broom) symptoms duplicated by treatment	K,BAP, phenylAP	Samuels, R.M. (1961) Ph.D. thesis, Indiana University; Klambt, D. Thies, G. and Skoog, F. (1966) Proc. Natl. Acad. Sci. U.S. 56, 52-59; Thimann, K.V. and Sachs, T. (1966) Am. J. Bot. 53, 731-739 and Helgeson, J.P. and Leonard, N.J. (1966) Proc. Natl. Acad. Sci. U.S. 56, 60-63.
Pseudomonas tabaci	antagonizes toxic effect of bacterium which causes 'wildfire' disease in tobacco	K	Lovrekovich, L. and Farkes, G.L. (1963) Nature (Lond.) 198, 710-

Table (1) continued

Viral			
tobacco mosaic in tobacco leaves	lesions reduced, virus production stimulated	K,KR,BAP 6ipAdo	Milo, G.E.Jr. and Srivastava, B.I.S. (1969) Virology 38, 26-31.
tobacco mosaic in bean leaves	inhibition of lesions and viral production	"	Ibid
turnip yellow mosaic (Chinese cabbage)	protein synthesis maintained in leaves, formation of intact viral particles reduced	K	Berridge, M.V. and Ralph, R.K. (1969) Biochim. Biophys. Acta 182, 266-269.
tomato spotted wilt (petunia leaves)	number and size of lesions reduced	K	Selman, I.W. (1964) Ann. Appl. Biol. 53, 67-76.
tobacco mosaic, (tobacco leaf discs)	viral production inhibited	K	Kiraly, Z. and Szirmai, J. (1964) Virol. 23, 286-288.
tobacco aucuba mosaic	viral production stimulated in detached leaves, in attached leaves either stimulated or inhibited; dependent on age and concentration	K	Daft, M.J. (1965) Ann. Appl. Biol. 55, 51-56.
Fungal			
Taphrina deformans (peach leaves)	production of IAA and kinetin like substances implicated in neoplastic growth induction	endog.	Sommer, N.F. (1961) Physiol Plant. 14, 460-469.
Erysiphae (powdery mildew)	development on cucumber leaves checked completely	K	Dekker, J. (1963) Nature 197, 1027-1028.

Table (I) Continued

Uromyces and Botrytis	no effect	K	Ibid
Helminthosporium (In excised oat leaves)	synergistic enhancement of toxic effect of vitorin toxin	K	Luke, H.H. and Wheeler, H. (1966) Phytopath. 56, 138-139.
Verticillium alboatrum (potato roots)	decrease in pectolytic enzyme of the fungus	K,BAP	Patil, S.S. and Dimond, A.E. (1968) 58, 868-869.

(C) IN LOWER PLANTS, FUNGI AND MICRO-ORGANISMS

Mosses			
Tortella caespitosa	budding induced	general	Gorton, B.S., Skinner, C.G. and Eakin, R.E. (1957) Arch. Biochem. Biophys. 66, 493-
Funaria hygrometrica	budding induced, target cell interaction	BAP	Brandes, H. and Kende, H. (1968) Plant Physiol. 43, 827-837.
Ferns			
Marsilea drummondii (water Fern)	induction of copious root growth in aseptic culture	K	Allsop, A. and Szweykowska, A. (1960) Nature (Lond.) 186, 813-814.
Algae			
Spirogyra longata	promoted cell division, increased elongation	K	Olszewska, M.J. (1958) Bull. Soc. Sci. Lettres Lodz. Classe III 9, 11

Table (I) Continued

marine algae (mixed)	both stimulation and inhibition of growth in culture	endog.	Bentley-Mowat, J.A. and Reid, S.M. (1968) Ann. Bot. 32, 23-32.
Volvox	possible role in growth and spheroid development	dneog.	Van Staden, J. and Breen, C.M. (1973) Pl. Sci. Lett. 1, 325-330.
Pylaiella litoralis, Ectocarpus fasciculatus (marine brown algae)	required for normal morphology when grown in completely defined culture medium	K	Pedersen, M. (1968) Nature 218, 776-
Fungi			
Melampsora lini (rust)	required in medium for aerial mycelial development in culture of rust infected flax leaves	coconut milk	Turel, F.L.M. and Ledingham, G.A. (1957) Can. J. Micro. 3, 813-819.
Saccharomyces	with IAA, greatly slowed growth rate	K	Kennell, D. (1960) Exptl. Cell Res. 21, 19-33.
Neurospora crassa (infertile strains)	increased perithecial production in crosses	K	Lee, B.O. (1961) Nature 192, 288-
Acetabularia mediterranea	regeneration of fragments inhibited	K	Brachet, J. (1958) Exptl. Cell Res. 14, 650-651.
Achlya sp.	regulation of metabolite and ion transport	6ipAde	LéJohn, H.B. and Stevenson, R.M. (1973) Biochem. Biophys. Res. Comm. 54, 1061-1066 and LéJohn, H.B. and Cameron, L.E. (1973) Biochem. Biophys. Res. Comm. 54, 1053-1061.

Table (1) continued

Acetabularia mediterranea	concentration dependent effect on stem formation	K	Zetsche, K. (1963) Planta 59, 624-634.
Acetabularia crenulata	differentiation affected; shorter stalks; cap formation stimulated	K	Spencer, T. (1968) Nature 217, 62-64.
Bacteria			
Brucellus abortus	selective effect in change in population from non-virulent to virulent	K(DNA products)	Braun, W., Firshein, W. and Whallen, J. (1957) Science 125, 445-447.
E. coli	cell division rate increases	K	Kennell, D. (1960) Exptl. Cell Res. 21, 19-33.
B. megaterium, Agrobacterium tumefaciens	growth stimulated; no change in morphology	K	Maruzzella, J.C. and Garner, J. G. (1963) Nature (Lond.) 200, 385-
E. coli S. aureus Erwinia carotovora	some growth stimulation	K	Ibid
C. michiganense	inhibition of growth	K	Ibid
Cl. thermocellum	cellulose digestion occurs even in the absence of yeast extract	K	Quinn, L.Y., Oates, R.P. and Beers, T.S. (1963) J. Bact. 86, 1359-
E. coli	inhibition of tRNA methylases	Ribosides of K,BAP, Z	Wainfan, E. and Landesberg (1971) FEBS. Lett. 19, 144-148.

Table (I) Continued

(D) ANIMALS

Lower Animals			
Paramecium caudatum	generation time shortened especially at sub-optimal conditions	K	Guttman, R. and Back, A. (1960) Science 131, 986-987.
Planaria	irreversible alteration in differentiated tissues via xanthine oxidase effect	K and analogues	Henderson, T.H., Skinner, C.G. and Fakin, R.E. (1962) Plant Physiol. 37, 552-555.
Hydra	inhibition of regeneration	6-sub-purines	Skinner, C.G., Shive, W., Ham, R.G., Fitzgerald, D.C. Jr. and Eakin, R.E. (1965) J.Amer. Chem. Soc. 78, 5097-5110.
Higher Animals (whole animals)			
rats with Yoshida tumour cell transplant	high rate of cell division induced on injection	K	Ogawa, Y., Abe, Y. and Fujioka, K. (1957) Nature (Lond.) 180, 985-986.
mice	radioprotective effect for survival seen on injection	6ipAdo	Prasad, N., Bushong, S.C. and Barton, H.L. (1971) Experientia 27, 1332-1333.
mice immunized with sheep red blood cells	inhibition of immune response	6ipAdo	Diamanstein, T., Wagner, B. and Bhargavo, A.S. (1971) FEBS. Lett. 15, 225-228.
Cell Cultures and Organ Tissues			
calf spleen	inhibition of tRNA methylases	ribosides of K,BAP, Z	Wainfan, E. and Landesberg (1971) FEBS. Lett. 19, 144-148.

Table (I) Continued

skin cultures	retarded growth of epithelium; no effect on cells of carcinoma and cystic disease	K	Orr, M.F. and McSwain, B. (1960) Cancer,Res. 20, 1362-1364.
Triturus viridescens	stimulated mitosis in mucosa of alimentary tract	K	Buckley, W.B., Witkus, E.R. and Berger, C.A. (1962) Nature 194, 1200-1201.
Sarcoma 180 cells	cytotoxic, growth inhibition	6ipAdo	Grace, J.T., Hakala, M.T., Hall, R.H. and Blakeslee, J. (1967) Proc. Amer. Assoc. Cancer Res. 8, 23-
human and rat cells (PHA stimulated)	mitosis inhibited at 10^{-5}M; stimulated at 10^{-7}M	6ipAdo	Gallo, R.C., Whang-Peng, J. and Perry, S. (1969) Science 165, 400-402.
gastro intestinal mucosa; lymphoid tissue	inhibition of growth in vitro and in vivo	6ipAdo	Suk, D., Simpson, C.L. and Mihich, E. (1970) Cancer Res. 30, 1429-1436.
rat spleen cells (PHA stimulated)	inhibition via effect on tRNA methylase	6ipAdo	Hacker, B. and Feldbush, T.L. (1971) Cancer 27, 1384-1387.
human lymphocytes (PHA stimulated)	inhibition of DNA and RNA synthesis (immunosuppresion)	6ipAdo	Ibid
human myelogenous leukemic cell line (Roswell Park 6410)	growth inhibition at 3 x 10^{-6}M, not at 2 x 10^{-6}M	6ipAdo	Rathbone, M.P. and Hall, R.H. (1972) Cancer Res. 32, 1647-1650.

Metabolic Transport

Living cells have for a long time been recognized as possessing selectively permeable plasma membranes. It was not until the late nineteen forties that Doudoroff et al (22) suggested that the transport of sugars into bacteria is not due to a mere diffusion process through a semipermeable membrane, but rather one in which stereospecific transport systems function. It was the work of Jacob, Monod and coworkers (17) and Fox, Carter and Kennedy (27,28) that finally provided the evidence for the existence of such transport systems for sugars. Using the powerful tool of bacterial genetics, significant advances have been made in the better understanding of such a phenomenon.

Generally there are three transport processes which are operative in micro-organisms: (a) facilitated diffusion (b) active transport (c) group translocation. Facilitated diffusion is similar to passive diffusion, but differs in several important aspects. The process is a stereospecific one; i.e., it is able to selectively exclude certain compounds from entering the cell. Also the rates of uptake of metabolites are faster than by passive diffusion. Facilitated

diffusion also exhibits 'saturability', a property which resembles that of a simple enzyme reaction.

Active transport is similar to facilitated diffusion, but with one key difference. This process enables the transport of solutes against a concentration gradient. Metabolic energy is necessary and is coupled in some unknown fashion to such a process.

Group translocation involves the conversion of the metabolite into another derivative during the transport process. The membrane components involved in this transport process exhibit enzymatic properties. Unlike active transport, uptake of metabolites by group translocation may or may not require metabolic energy. This depends on whether the uptake process at the membrane level involves an endergonic or exergonic reaction (87).

The transport of sugars by mammalian cells differs, in some important aspects, from that observed in micro-organisms. First, the hormone insulin is necessary for activating the transport of sugars in numerous tissues e.g. skeletal and cardiac muscles and adipose and optical tissues. However, erythrocytes, liver, placental and tumour cells are independent of insulin action. Second, of the tissues examined, only

that of the intestinal epithelium is capable of active transport. Facilitated diffusion is the predominating process of sugar transport (56). Also a Na^+ containing medium is essential for sugar transport as was first shown in 1958 by Riklis and Quastel for excised guinea pig gut (83).

Available evidence shows that specific transport systems are operative in very diverse living systems. Most of the work done pertaining to sugar transport has been confined mainly to bacterial systems, owing somewhat to a greater understanding of bacterial genetics. However an increasing knowledge of such a phenomenon has been rapidly accumulating among the fungi and yeast.

The Phosphoenolpyruvate-phosphotransferase System (PEP-PTS)

The phosphoenolpyruvate-phosphotransferase system first detected by Roseman and coworkers concerning sugar transport by group translocation (53) has been well documented in studies with *E. coli*, *Salmonella typhimurium* and *Staphylococcus aureus* (88). Unlike *E. coli* and *Salmonella typhimurium*, *Staphylococcus aureus* may transport all sugars via the

phosphoenolpyruvate phosphotransferase system as suggested by Roseman (88). Recently Dutton et al demonstrated that maltose accumulation and metabolism in S. aureus does not involve the phosphoenolpyruvate phosphotransferase system (23). A preliminary report presented by Van Demark and Plackett showed that sugar transport in a strain of Mycoplasma is through a phosphoenolpyruvate phosphotransferase system (103). Further characterization by Cirillo and Razin (15) indicated that M. gallisepticum, M. mycoides var. mycoides and M. mycoides var. capri but neither fermentative Acholeplasma strains nor the non-fermentative Mycoplasma species utilize the phosphoenolpyruvate phosphotransferase system for sugar transport. A phosphoenolpyruvate phosphotransferase system dependent sugar transport process was detected in a vertebrate intestine (108). Negative results have been reported from studies involving transport of specific sugars in N. crassa (72,92), Pseudomonas aeruginosa (77) and the obligate aerobe Brucella abortus (81). Lin has cautioned that when comparison of results are made, one has to bear in mind not only the specific species but also the strains of organisms used in the studies (61).

D-glucose Transport

Hoffee et al characterized the D-glucose 'transport systems' of S. typhimurium and E. coli (41). The carriers were specific for D-glucose and the accumulation of the sugar, in each case, involved an active process. Chromatography of cell extracts indicated that 90% of the accumulated sugar was unchanged. Eagon found that 2-deoxy-D-glucose was transported by facilitated diffusion in Ps. aeruginosa (24). In a strict aerobe, Azotobacter vinelandii, D-glucose uptake is mediated by a carrier specific system (3) and multiple sites exist for coupling D-glucose transport to the respiratory chain of membrane vesicles of such organisms (4).

Neville et al (72) and Scarborough (92) demonstrated the presence of a derepressible D-glucose transport system in N. crassa. The high affinity uptake system, which was inhibited by azide, 2,4-dinitrophenol and sulfhydryl reagents, was repressed when spores were germinated in a D-glucose containing growth medium. The low affinity system appeared to function by facilitated diffusion. A transport system involving a membrane 'carrier' and common to D-glucose and L-sorbose uptake in Saccharomyces

cerevisiae was reported by Cirillo (12).

Active transport of D-glucose in mammalian tissues was first reported by Clark in 1922 using frog kidneys (16) and later by Barany and Sperber in 1939 using rabbit intestine (2) in their studies. Since then, D-glucose uptake by intestinal epithelium isolated from painted turtles (26), toads (20), bull-frogs (55) and fishes (69, 70) has been reported. Using isolated brush border membrane from rat small intestine, Ulrich et al showed that D-glucose uptake is by facilitated diffusion and dependent on Na^+. D-Galactose competed with D-glucose transport, indicating possibly a transport system common to both sugars (103). Stereospecific carrier mediated uptake of D-glucose and 2-deoxy-D-glucose by facilitated diffusion have been reported for chick embryo fibroblast (52), erythrocytes, brain slices and skeletal and cardiac muscles (57).

Hexose-6-Phosphate Transport

Hagihira et al in 1963 isolated an E. coli K12 mutant which possessed a glucose-6-phosphate transport system distinct from that for D-glucose (33). Further characterization by Winkler showed that mannose-6-phosphate, fructose-6-phosphate and glucose-6-phos-

phate share the same uptake system. Also a hexose-6-phosphate mutant grew on the hexoses, mannose, fructose and glucose but not when supplied with the corresponding hexose-6-phosphates which supports Lin's findings (110). This active transport system (79) could only be induced by extracellular hexose-6-phosphates (40).

L-Arabinose Transport

The L-arabinose transport system of *E. coli* has been well characterized by Englesberg and coworkers and Schleif. The enzymes for the L-arabinose metabolic pathway and the L-arabinose 'permease' are specified by distinct operons but yet are regulated by a common product of the C-gene (62). The inducible L-arabinose permease of *E. coli* B/r actively transports L-arabinose. The temperature dependent uptake of this sugar was found to be inhibited by D-xylose, D-fucose and D-galactose (74). Using the osmotic shock method of Heppel and coworkers (71, 73), Schleif (94) and Hogg and Englesberg (42) have purified an L-arabinose binding protein. Cirillo reported the existence of a temperature dependent 'mobile' sugar transport carrier system in the protozoa *Tetrahymena*. L-Arabinose uptake by facilitated diffusion was competitively in-

hibited by D-glucose (13).

Maltose Transport

Wiesmeyer and Cohn were the first to report on an inducible maltose transport system in bacteria by using an amylomaltose negative mutant. The temperature dependent uptake system was inhibited by sodium azide (109). Harris and Thompson found a maltose specific transport system in S. cerevisae. 2,4-Dinitrophenol, azide and fluoride were inhibitory to active transport of the sugar (38). Maltose, like most disaccharides, has been found not to be transported by a wide variety of mammalian tissue types studies thus far.

Lactose Transport

The lac y gene of the lac operon in E. coli codes for an inducible lactose transport protein (82). Fox and Kennedy isolated a membrane associated protein which in nearly all respects is similar to the lac permease (28). Further characterization of this β-galactoside permease has been carried out by Winkler and Wilson. Their results suggest that translocation and energy coupling to the transport process are two

separable entities. Energy coupling occurred at the level of exit rather than entry. Thus the effect of energy poisons on the lactose system is to increase the rate of efflux rather than to decrease the rate of influx, thereby accounting for the reduction in net accumulation of the substrate. They suggested that the same 'carrier' may be involved in both energy coupled transport and facilitated exchange (111). Lactose transport has also been reported for Streptococcus lactis (64).

Recent reports by Rosen using an E. coli mutant lacking the Mg^{++}-adenosine triphosphatase (89) and that of Kashket and Wilson using Streptococcus lactis (48) showed that the active transport of lactose or its analogues may possibly be coupled to a proton gradient as was first proposed by Mitchell (68).

Lester et al detected a lactose transport system in N. crassa. The active accumulation of lactose was inhibited by exogenously added D-galactose, but neither by ONPG nor methyl-β-D-galactoside. The entry of the sugar was temperature dependent and inhibited by sulfhydryl reagents such as ρ-chloromercuribenzoate and iodoacetate. Azide strongly retarded lactose uptake while arsenate and 2,4-dinitrophenol were only moderately inhibitory. Surprisingly,

11-deoxycorticosterone, a steroid hormone inhibited lactose uptake (60).

D-galactose Transport

Rotman et al detected four separate D-galactose permeation systems in E. coli K12 which they have designated as galactose permease, methyl galactoside permease, TMG I and TMG II permeases (90). However it is apparent in the literature that different terms have been assigned by different research groups to the same 'permease' in question. But it is generally accepted that there are four transport systems in E. coli capable of accumulating D-galactose. A D-galactose permease found in E. coli strain W4345 was reported by Rotman et al. It acts specifically on D-galactose (30, 90). D-galactose is also transported by the β-galactoside permease (47, 116), referred to as TMG I by Rotman et al (90). The so called melibiose permease characterized by Pardee and Prestige is capable of actively transporting D-galactose (75, 80). This 'permease' is designated as TMG II by Rotman et al (90). D-galactose uptake can also be accomplished using the methyl galactoside 'permease'. This 'permease' has a high affinity for this sugar (3). Rotman et al pointed out that the system studied by Horecker et al

(43, 44) and Buttin (8, 9) is identical to the methyl galactoside 'permease' (90).

A D-galactose binding protein was isolated by Anraku from the shock fluid of osmotically shocked E. coli (1). The uptake rate of D-galactose by 'shocked' cells was greatly reduced. Parnes and Boos contend that the D-galactose binding protein is a necessary component of the methyl galactoside transport system. Also this protein mediates only the entry, not the exit of D-galactose (76). A further report by Shen and Boos showed that the synthesis of the D-galactose binding protein and an increase in D-galactose transport in synchronized cells occurred only after cell division had been initiated. These two phenomena were not observed when the cells were 'elongating' but without dividing (95). The molecular relationship between this binding protein and the uptake process remains to be elucidated.

Sorbose Transport

Transport systems specific for sorbose have been reported by Cirillo in yeast (12) and in N. crassa by Crocken and Tatum (19). In the latter, L-sorbose is transported by facilitated diffusion. Its uptake

was competitively inhibited by D-glucose. This system bears some characteristics similar to those reported by Scarborough (93) and Neville *et al* (72). For example energy poisons inhibited transport of sugars. Further, two transport systems common to both D-glucose and L-sorbose are present, one, a glucose repressible active transport system and the other a facilitated uptake system.

Glycerol Transport

Sanno *et al* (91) and Cozzarelli *et al* (18) reported that glycerol uptake by *E. coli* K12 occurred by an inducible facilitated diffusion system. A similar inducible system was detected in *Ps. aeruginosa*. Osmotically 'shocked' cells failed to transport glycerol. A glycerol binding protein was found in the 'shock' fluid from induced cells. However, no binding activity was present when 'shock' fluid from either uninduced or transport negative mutants was assayed (102).

The multitude of data on transport processes show the diversity not only between procaryotes and eucaryotes, but also within each of these two groups. These differences must surely be of some evolutionary advantage to each individual organism.

MATERIALS AND METHODS

(I) Organism

The organism used in this study is a fresh water-mould Achlya sp. (1969), from Dr. J. S. Lovett, Purdue University.

(II) Media, Buffers and Chemicals

(a) G_2Y- 5 gm D-glucose, 0.5 gm yeast extract in one litre of distilled H_2O.

(b) PYG - 2 gm peptone, 2 gm yeast extract, 3 gm D-glucose in one litre of distilled water.

(c) (i) Buffer A - 5 mM tris-acetate, 5 mM D-glucose, 1 mM KCl and 1 mM NaCl, pH 7.0.

(ii) Buffer B - 10 mM tris-acetate, 10 mM D-glucose, 2 mM KCl and 2 mM NaCl, pH 7.0.

(iii) Buffer C - 1 mM tris-acetate, 2 mM KCl and 2 mM NaCl, pH 7.0.

(d) All amino acids used were the (L) optical isomers.

(e) All sugars used were D-sugars. All

chemicals used were obtained from Difco Laboratories, Calbiochem, Sigma Chemical Co. and J.T. Baker Chemical Co.

(f) Radioactive Isotopes were obtained from

(i) New England Nuclear

(^{3}H(G) 2-deoxy-D-glucose (1 mCi/23 µg)

(ii) Amersham/Searle

(^{14}C(U)) D-glucose (285 mCi/mmol.); (6-^{3}H) D-fructose (650 mCi/mmol.); (2-^{3}H) glycerol (500 mCi/mmol.); (2,3-^{3}H) L-alanine (34 Ci/mmol.); (ring-4-^{3}H) L-phenylalanine (12.5 Ci/mmol.); (methyl-^{3}H) L-methionine (100 mCi/mmol.); (^{3}H(n)) L-valine (31.6 Ci/mmol.); (2,5-^{3}H) L-histidine (58 Ci/mmol.); (^{3}H(G)) L-tryptophan (3.1 Ci/mmol.); (4,5-^{3}H) L-lysine monohydrochloride (250 mCi/mmol.); (2-^{14}C) uracil (62 mCi/mmol).

(III) Growth of Organism

Stock cultures were maintained on Cantino's PYG agar (11) slants and transfers made monthly. Germinated spores used in all experiments were obtained following growth and sporulation in liquid G_2Y medium. This was accomplished by transferring some mycelial strands from the agar slants into standard size plastic petri dishes containing 20 ml of G_2Y medium and incubated for 48 hr at 24C for complete growth and

sporulation.

To continue propagating cultures in this liquid medium, four hyphal mats were transferred aseptically into a flask containing 100 ml of distilled water, shaken vigorously and the mats removed using an inoculating needle. Two ml of this spore suspension was then used as inoculum for each 20 ml of G_2Y medium.

IV Preparation of Germinated and Ungerminated Spores

(a) For large scale production of spores, 10 ml of spore suspension prepared as described above was inoculated into individual Roux bottles containing 90 ml of G_2Y medium. Spores were harvested after a 40 hr incubation period at 24C by vigorously shaking each Roux bottle and then filtering the entire contents through eight layers of sterile cotton gauze.

b) One litre of the spore filtrate was diluted with 500 ml of deionized water and then incubated at 11.5 C for 18 hr as stationary cultures to permit the spores to germinate. The germinated spores, each with a hyphal protuberance about 100 µm long were harvested by filtering the germling suspension through nylon mesh (61 µm pore size). The trapped germlings

were carefully washed with the suspending buffer (one part buffer to two parts spore suspension v/v) and resuspended by everting the nylon mesh followed by gentle swirling in a beaker containing the appropriate buffer so as to dislodge the germinated spores. The cloth was washed with buffer from a squirt bottle and all the cell washes were pooled.

(V) Cell Density and Cell Volume Determinations

The cell density in the transport studies was adjusted with the suspending buffer to an optical density of 0.20 ± 0.02 at a wavelength of 700 nm using a Gilford recording spectrophotometer, model 2400. Osmotically shocked germlings were used at a cell density of 0.32 ± 0.02 O.D. (700 nm).

Viability estimates were about 75% for spores and about 95% for germlings (2).

(i) Cell number = 2.0×10^3 cells/ml

(ii) Diameter of germlings = 5 μm (d)

(iii) Length of germlings = 100 μm (ℓ)

(iv) Volume/cell = $\pi r^2 \ell$ where $r = {}^{d}/_{2}$

(VI) Transport Assays

(a) Incubation Time

To determine the initial reaction velocity of various substrates in the transport studies, a time dependent uptake experiment was first performed. The linear portion of the curve of substrate accumulated vs. time was assumed to represent the initial reaction rate. The incubation times for the transport assays were chosen to fall within the linear portion of the curve. The incubation times for amino acid and sugar uptake studies were 3 min and 2.5 min respectively.

(b) Assay Procedure

For amino acid and sugar transport assays, the germlings were suspended in buffer B and buffer B minus D-glucose respectively. The germlings, suspended in the appropriate buffer, were first preincubated for 10 min, then cycloheximide (2.8 μM) was added to minimize the effect of macromolecular synthesis on uptake studies. This was followed by a further 5 min incubation period before the experiments were initiated. Cycloheximide addition concerned amino acid transport, primarily.

The experiments were initiated as follows: One ml of the germling suspension was pipetted into one ml of the reaction solution containing labelled substrate, specified additions and water, mixed rapidly and then incubated for the specified time at room temperature. The reaction was terminated by rapidly filtering the entire incubation mix through 25 mm HAWP Millipore filters (0.45 μm pore size), then washed immediately with 15 ml of buffer A (minus D-glucose for sugar uptake studies). The filters were sucked dry, dissolved in 10 ml Bray's solution (6) and radioactivity monitored using either a Packard TriCarb liquid scintillation spectrometer or a Beckman LS-230 Liquid scintillation system. The entire filtration-washing procedure which was carried out with a Millipore filtration manifold attached to two vacuum pumps never exceeded 15 sec. The net accumulation of a substrate was obtained by substracting non-specific binding to 0.4% formaldehyde killed cells and background counts from the total radioactivity on the filters.

(c) Transport Assays for pH Studies

The cells were preincubated as described previously except that buffer C was used as the suspending medium. One ml of this cell suspension was added to one ml of the reaction mixture containing

10 mM tris-acetate at the various specified pH values and the substrate to be transported. The assay was carried out as described in (VI b).

(d) Transport Assays for Temperature Studies

The germling suspension and reaction mixtures were preincubated separately for 10 min in water baths at specified temperatures before the assays were carried out as described in (VI b).

(VII) Preparation of Osmotically Shocked Cells

Ungerminated spores were obtained as described in (IV a) except that the cells were collected by shaking the hyphal mats contained in 16 layers of cotton gauze in G_2Y medium. The spores were recovered from the spore suspension on Duralon filters (Millipore NSWP, 7 μm pore size) by vacuum filtration. The spores were then dislodged from the filters by everting the filters, spore side outwards, and then layering them inside large test tubes containing 20 ml of G_2Y medium. The contents of the test tubes were mixed vigorously, the spore suspension decanted and the process repeated thrice with fresh G_2Y medium. The spore suspension was diluted with G_2Y medium to a density of 10^5 spores per ml then incubated at 28C for 120 mins in a Bellco spinner flask with continu-

ous stirring. The flask-shaped germinated spores each about 30 µm long were recovered using the Duralon filter technique already described.

The germlings from one litre of suspension were collected on filters and resuspended in 50 ml of a sucrose medium consisting of 0.5 M sucrose, 0.05 M tris-acetate (pH 8.0) and 0.01 M EDTA. The germlings were incubated with constant shaking at 30C for 15 min following which they were collected and washed with 1 or 2 ml water, again using the Duralon filter technique.

The sucrose treated cells were shocked according to the technique of Nossal and Heppel (73). This was accomplished by pipetting 5 ml of ice-cold $MgCl_2$ (0.5 mM) into a test tube containing the filter-trapped germlings; this mixture was mixed vigorously for 30 sec and then incubated 10 min in an ice bath. The cell suspension was shaken periodically by hand during this incubation period. The osmotically shocked germlings were finally removed by centrifugation at 3000 x g for 5 min at OC. These cells were resuspended in the appropriate buffer at the O.D. specified under transport studies.

(VIII) Chromatographic Analysis

A suitable volume of the germling suspension was prepared and preincubated with stirring in buffer A (minus D-glucose and cycloheximide) as described previously. The experiment was initiated when the labelled substrate was added to the germling suspension. One millilitre samples of the uptake incubation mixture were removed at various intervals and filtered through 25 mm HAWP Millipore filters (0.45 μm pore size) using a Millipore filtration apparatus and then washed rapidly with 15 ml of Buffer A (minus D-glucose). The filter was removed and the trapped cells washed off the filter into a test tube with 4 ml of 70% ethanol at 95 C (72). The cell-ethanol mixture was heated for 10 min in a water bath kept at 95 C. The extracts were cooled, filtered through 25 mm HAWP Millipore filters (0.45 μm pore size) and washed with 2 ml of deionized water. The ethanol extract was lyophilized and resuspended in a drop of distilled water. The entire sample was applied to Whatman No. 1 paper and chromatographed in a descending system. Chromatography was terminated when the solvent front had migrated 40 cm from the origin. The sheets were then dried, each vertical sample strip (3 cm wide) cut into 1 cm lengths and

radioactivity determined as prescribed in (VI). Known markers were co-chromatographed with the samples. The solvent systems used were either 1-butanol-pyridine-water (10:3:3, v/v/v) or n-butanol-acetic acid-water (3:1:1, v/v/v).

RESULTS

(1) Determination of K_m's for D-Glucose and 2-Deoxy-D-Glucose

Rate-concentration plots show that D-glucose and 2-deoxy-D-glucose are actively transported by the fungus. Calculations show that the cells are able to accumulate the sugars intracellularly at least 15 fold over the exogenous concentration within 1 min. Thus these two sugars are transported against a concentration gradient. The apparent K_m's for D-glucose and 2-deoxy-D-glucose obtained from Lineweaver-Burk plots (63) of rate-concentration experiments as represented by Fig. 1 and 2 respectively are 4×10^{-5}M for D-glucose and 5×10^{-5}M for 2-deoxy-D-glucose.

Germlings obtained from cells grown in G_2Y medium failed to accumulate D-fructose and glycerol. When D-glucose was substituted with 5 mM D-fructose or 10% glycerol, the cells grew poorly although they could complete their life cycle. Also cells after two passages in either D-fructose or glycerol G_2Y were still unable to transport these metabolites.

Figure 1. Lineweaver-Burk plot of the inhibition of D-glucose transport (initial reaction rate) by 2-deoxy-D-glucose; (●), 0 mM; (o), 1.0 mM; (□), 2.5 mM; (△), 5 mM. The inset illustrates the K_i of 2-deoxy-D-glucose inhibition. The velocity v is expressed as the amount of substrate taken up by 1 ml cells/minute.

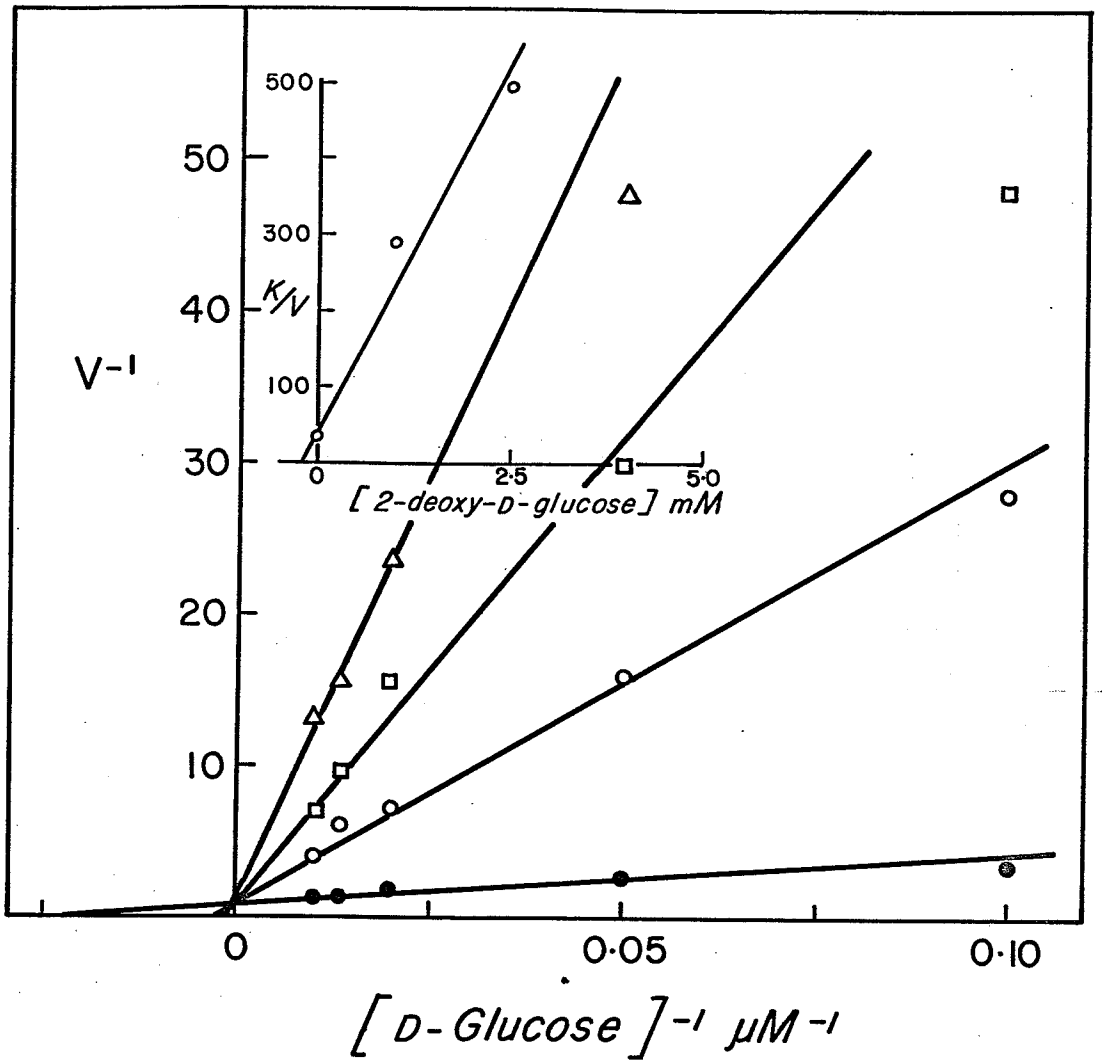
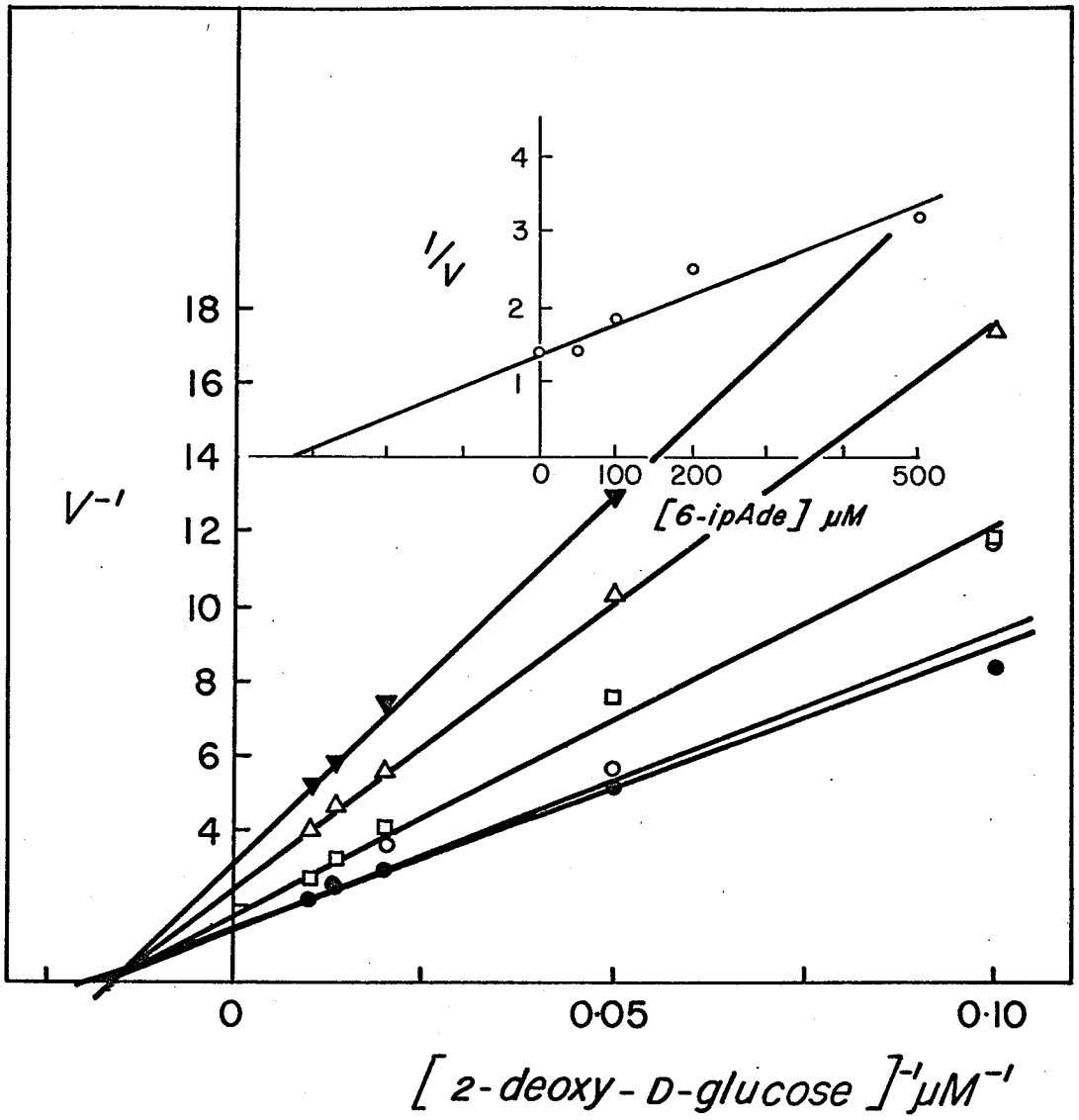

V-1
50
40
30
20
10
0
0·05
0·10
[D-Glucose]-1 µM-1
500
300
K/V
100
0
2·5
5·0
[2-deoxy-D-glucose] mM

Figure 2. Lineweaver-Burk plot of the inhibition of 2-deoxy-D-glucose transport (initial reaction rate) by 6ipAde; (●), 0 μM; (○), 50 μM; (□), 100 μM; (△), 200 μM; (▼), 500 μM. The inset illustrates the K_i of 6ipAde inhibition.


V⁻¹
18
16
14
12
10
8
6
4
0
0·05
0·10
[2-deoxy-D-glucose]⁻¹ μM⁻¹
1/V
4
3
2
1
0
100
200
500
[6-ipAde] μM

D-glucose was not transported by osmotically shocked cells. The fact that numerous proteins associated with bacterial transport systems are easily dislodged by 'shock' treatment (40) led to a search for a D-glucose binding protein in the osmotic shock fluid. However equilibrium dialysis experiments failed to detect such an entity.

(2) Competition Studies

Competition studies were carried out to determine the kind of stereospecificity that is exhibited by the D-glucose transport carrier. D-glucose transport was competitively inhibited by D-mannose, D-galactose, 2-deoxy-D-glucose and D-xylose as shown by Lineweaver-Burk plots (63) in Fig. 3, 4, 1 and 5 with K_i's of 5.8 x 10^{-4}M, 1.55 x 10^{-3}M, 1.7 x 10^{-3}M and 3.7 x 10^{-3}M respectively. Very slight inhibition of glucose transport was observed when tested with phosphoenolpyruvate, glucose-6-phosphate and α-methyl-D-glucoside. D-fructose, D-fucose, sucrose, α-methyl-D-mannopyranoside and N-acetyl-D-glucosamine displayed no competition with D-glucose uptake. The D-glucose transport system seems quite specific, transporting only D-glucose, 2-deoxy-D-glucose, D-galactose, D-mannose and D-xylose. As a comparison, D-glucose, 6-deoxy-D-glucose, 2-deoxy-D-

Figure 3. Lineweaver-Burk plot of the inhibition D-glucose transport (initial reaction rate) by D-mannose; (●), 0 mM; (□), 1.0 mM; (△), 2.5 mM; (▼), 5.0 mM. The inset illustrates the K_i of D-mannose inhibition.

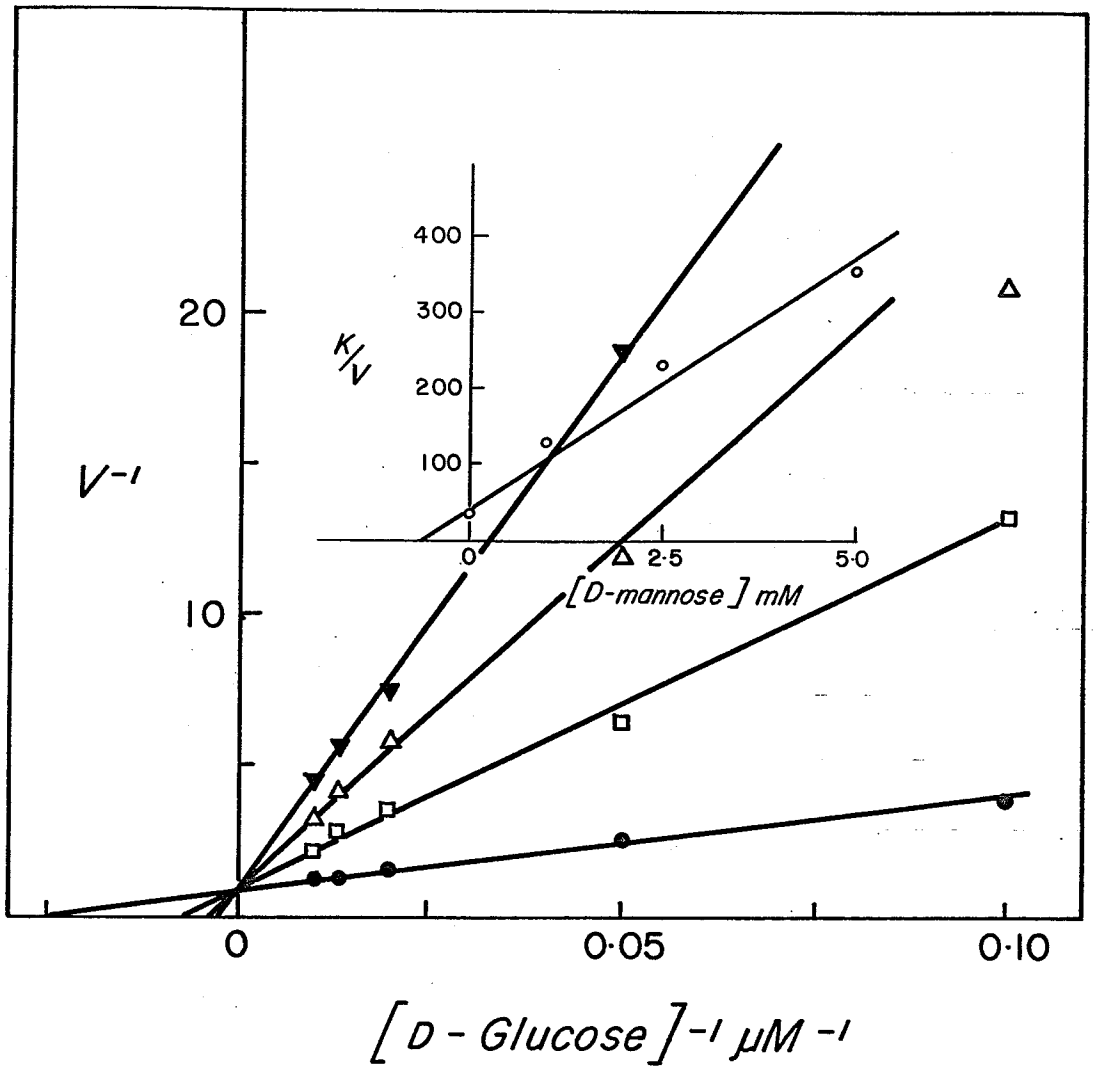

V⁻¹
20
10
0
0·05
0·10
[D - Glucose]⁻¹ μM⁻¹
K/V
400
300
200
100
0
2·5
5·0
[D-mannose] mM

Figure 4. Lineweaver-Burk plot of the inhibition of D-glucose transport (initial reaction rate) by D-galactose; (●), 0 mM, (○), 1.0 mM; (□), 2.5 mM; (△), 5.0 mM. The inset illustrates the K_i of D-galactose inhibition.

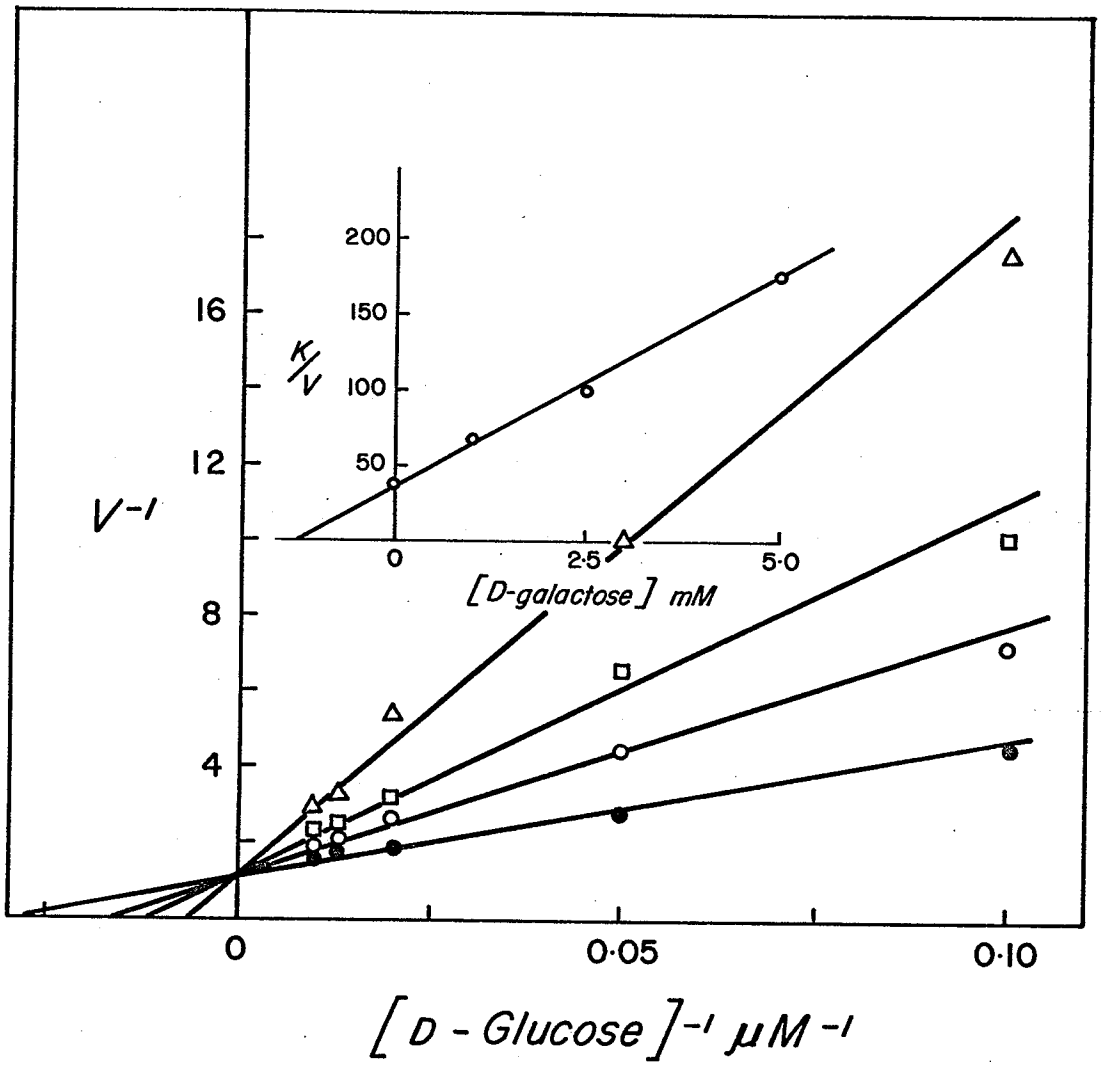
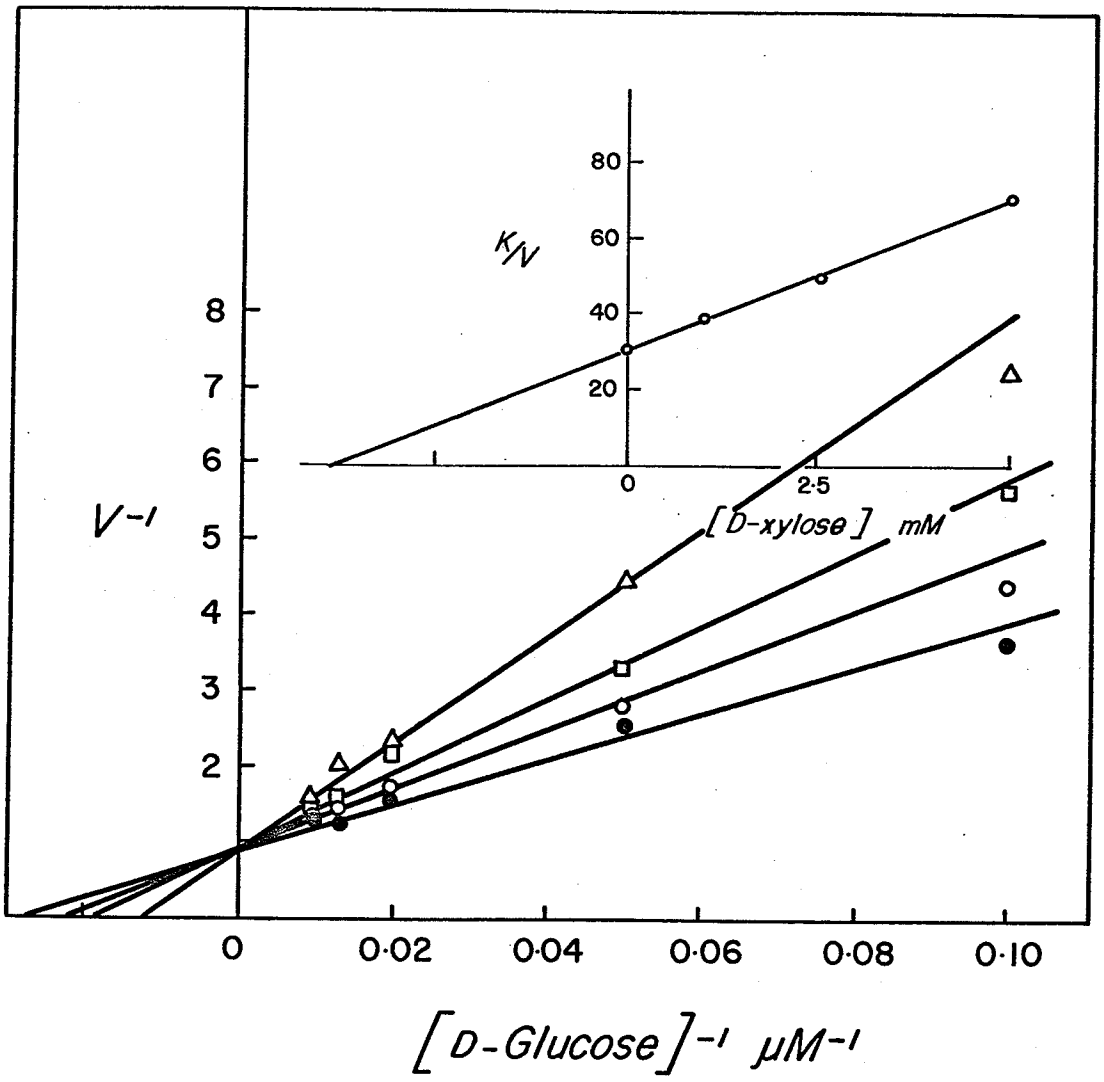

V⁻¹
16
12
8
4
0
0·05
0·10
[D - Glucose]⁻¹ μM⁻¹
K/V
200
150
100
50
0
2·5
5·0
[D-galactose] mM

Figure 5. Lineweaver-Burk plot of the inhibition of D-glucose transport (initial reaction rate) by D-xylose; (●), 0 mM; (o), 1.0 mM; (□), 2.5 mM; (Δ), 5.0 mM. The inset illustrates the K_i of D-xylose inhibition.


V⁻¹
8
7
6
5
4
3
2
0
0·02
0·04
0·06
0·08
0·10
[D-Glucose]⁻¹ μM⁻¹
K/V
80
60
40
20
0
2·5
[D-xylose] mM

glucose, D-xylose and D-mannose inhibited 3-0-methyl-D-glucose transport in N. crassa (72).

(3) Temperature and pH Effects on D-Glucose Transport

Sugar transport was temperature dependent as illustrated in Fig. 6 for D-glucose. The optimum temperature for uptake was 30 C. The rate of transport dropped very rapidly on either side of this optimum and transport was totally abolished at temperatures either below 4 C or above 50 C.

Fig. 7 shows the pH dependency of sugar transport. In the range tested, uptake was enhanced with increasing pH up to pH 6.5, the optimum for D-glucose uptake. Further increases in pH resulted in a fairly rapid decline in uptake rates. For example at pH 9.0 the rate of D-glucose transport was only 40% of that at the optimum pH.

(4) Effect of Energy Poisons on Transport

Azide, cyanide, 2,4-dinitrophenol and CCCP which are inhibitors of electron transport and uncouplers of oxidative phosphorylation inhibited transport of D-glucose. CCCP was the most potent of the four poisons

Figure 6. Influence of temperature on the transport of D-glucose; (⊙), 50 μM; (◬), 100 μM D-glucose.

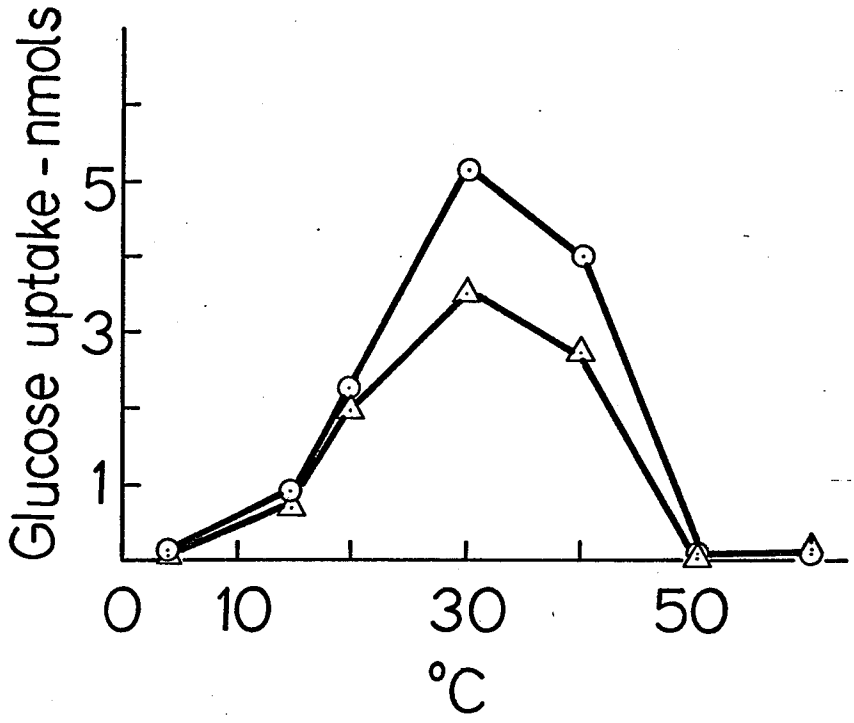

Glucose uptake - nmols
5
3
1
0
10
30
50
°C

Figure 7. Influence of pH on the transport of D-glucose; (⊙), 50 μM; (●), 100 μM D-glucose.

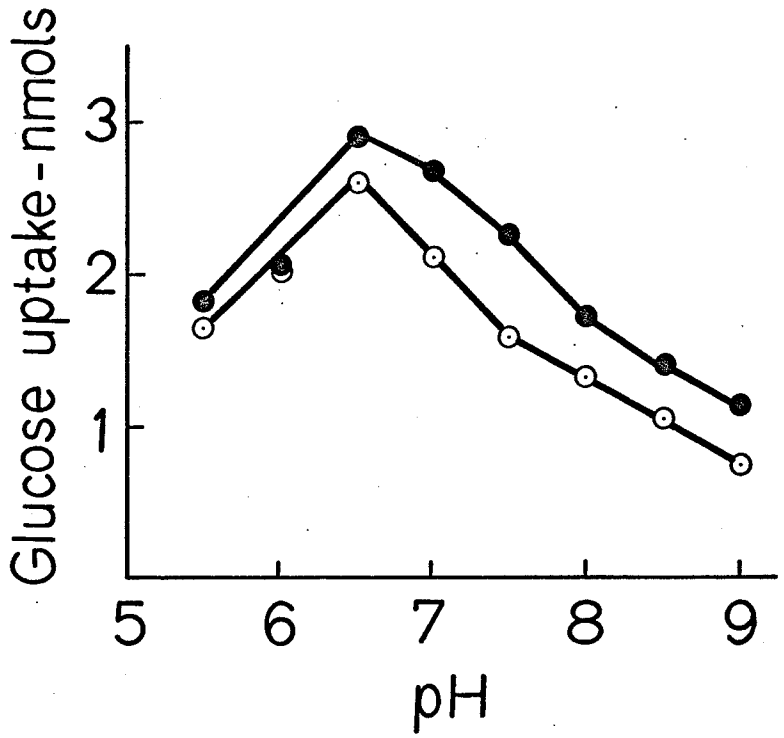

Glucose uptake-nmols
3
2
1
5
6
7
8
9
pH

Table II. Effect of energy poisons on D-glucose transport in Achlya sp.

Inhibitor	Conc. (mM)	% Inhibition of Uptake
Na azide	1.0	69
KCN	1.0	64
2,4 DNP	1.0	87
CCCP	0.1	94
NaF	1.0	0
Na arsenite	1.0	55

tested. It is interesting to note that CCCP has been shown to interfere with both electron transfer and proton conductance (37). Other energy inhibitors such as NaF had no effect on D-glucose transport at the concentration tested. Sodium arsenite however did inhibit the uptake of the sugar. The results are summarized in table II. Preincubation of cells for 5 min in 2.8 μM cycloheximide had no effect on sugar and amino acid uptake. Cycloheximide at this concentration was found to inhibit protein synthesis completely and RNA synthesis, partially (10).

(5) Sulfhydryl Group Reagents and Active Transport

Thiol group-reacting agents tested inhibited D-glucose transport to varying degrees. This is summarized in table III. I_2, even when used at very low concentrations was the most potent of the three inhibitors tested. I_2 and Hg^{++} inhibition of D-glucose transport was maximal at the concentrations cited in the table.

Table III. Effect of sulfhydryl group reagents on D-glucose transport in Achlya sp.

Inhibitor	Conc. of Inhibitor	% Inhibition of D-glucose Uptake
NEM	5×10^{-4}M	66
Hg^{++}	1×10^{-4}M	95
I_2	2×10^{-6}M	95

Reversal of the effect of the sulfhydryl reagents with DTT proved to be complex. Not only did I_2 and NEM react with cell components in an apparently irreversible manner, but DTT at a concentration of only 10 μM activated D-glucose transport by 15%. This seems to be similar to the effect which thiols have on D-glucose transport in mammalian tissues (54, 21).

Competition studies using I_2 as the inhibitor were carried out to determine whether -SH groups of the sugar carrier are involved in the binding and transport of D-glucose. As shown in Fig. 8, I_2 inhibition of D-glucose transport was non-competitive. Also a plot of 1/v vs $[I_2]$ was nonlinear; but a plot of 1/v against $[I_2]^2$ was linear (inset of Fig. 8).

I_2 and Hg^{++} were found previously to inhibit D-glucose when used at micromolar concentrations. However 6ipAde and xanthine, which are themselves inhibitory to D-glucose transport were able to overcome the I_2 and Hg^{++} inhibition. These observations are illustrated by Fig. 9, 10 and 11.

Figure 8. Lineweaver-Burk plot of the inhibition of D-glucose transport (initial reaction rate) by I_2; (●), 0 μM; (o), 0.75 μM; (□), 1.0 μM; (Δ), 2.0 μM. The plot of 1/v vs $(I_2)^2$ is shown in the inset.

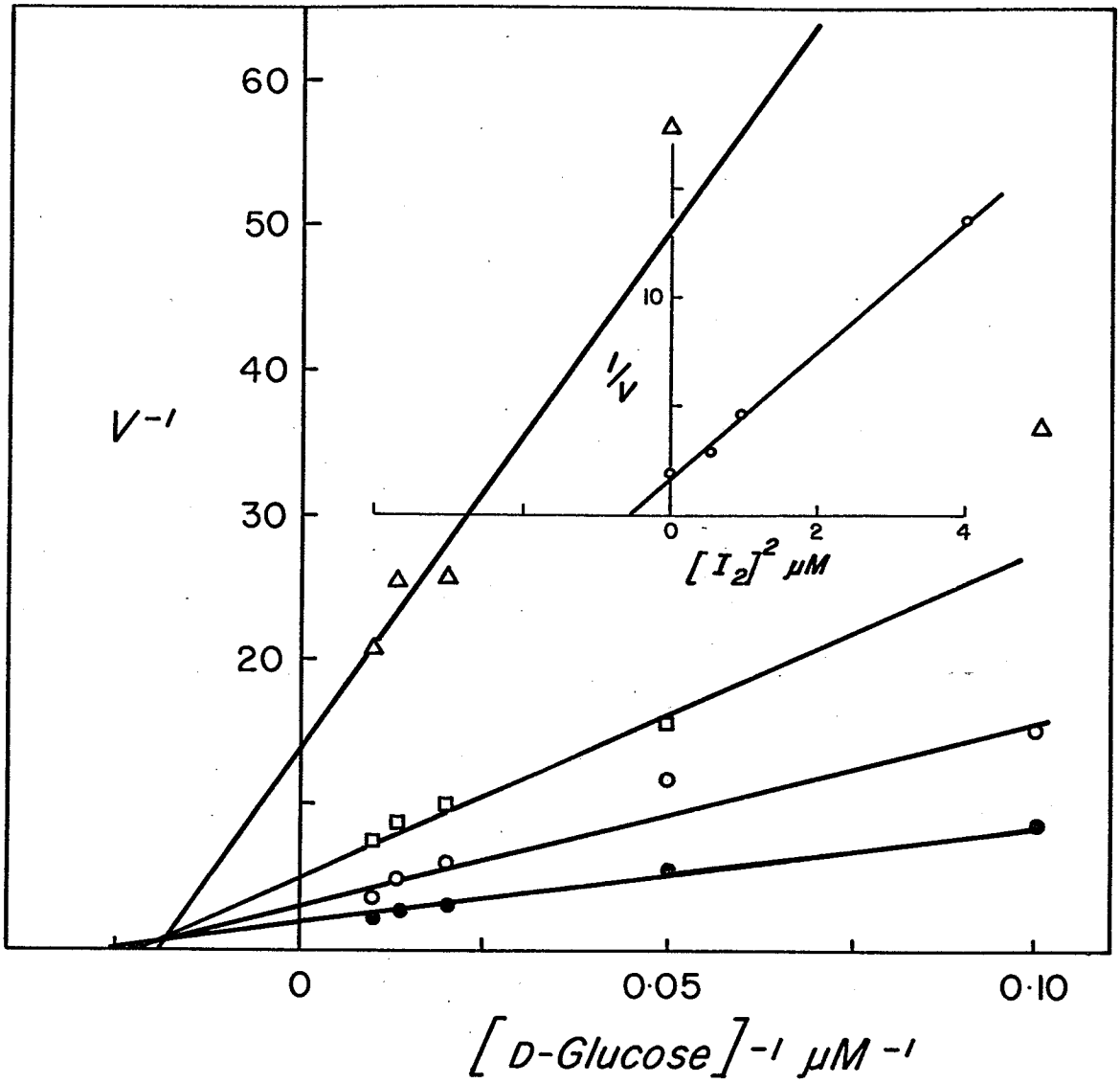

60
50
40
30
20
V⁻¹
0
0·05
0·10
[D-Glucose]⁻¹ μM⁻¹
10
1/V
0
2
4
[I₂]² μM

Figure 9. Inhibition of D-glucose transport (initial reaction rate) by I_2 (0 to 250 μM) and its reversal with xanthine; (o), 0 mM; (■), 0.5 mM; (Δ) 1.0 mM. The final concentration of D-glucose used was 50 μM.

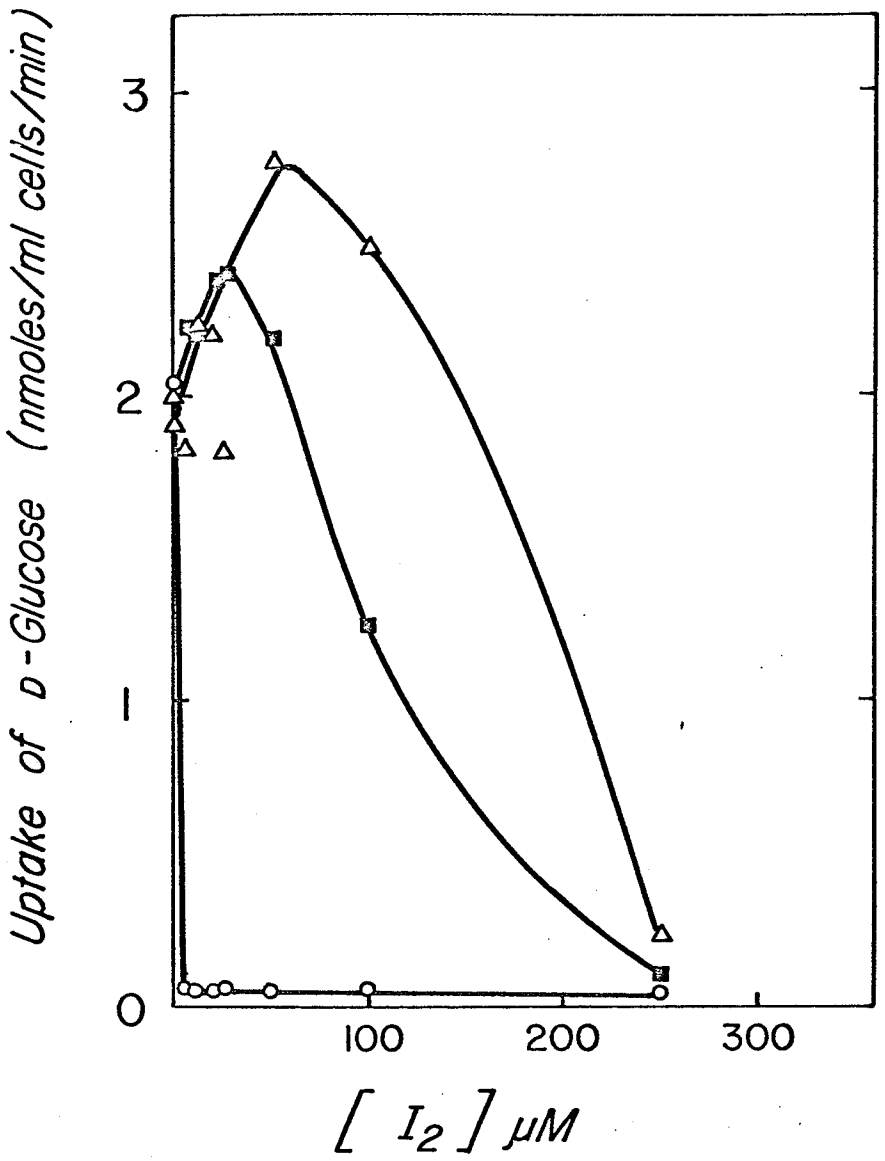

Uptake of D-Glucose (nmoles/ml cells/min)
3
2
1
0
100
200
300
[I2] μM

Figure 10. Inhibition of D-glucose transport (initial reaction rate) by I_2 (0 to 5 μM) and its reversal with 6ipAde; (●), 0 μM; (o) 100 μM. The final concentration of D-glucose used was 50 μM.

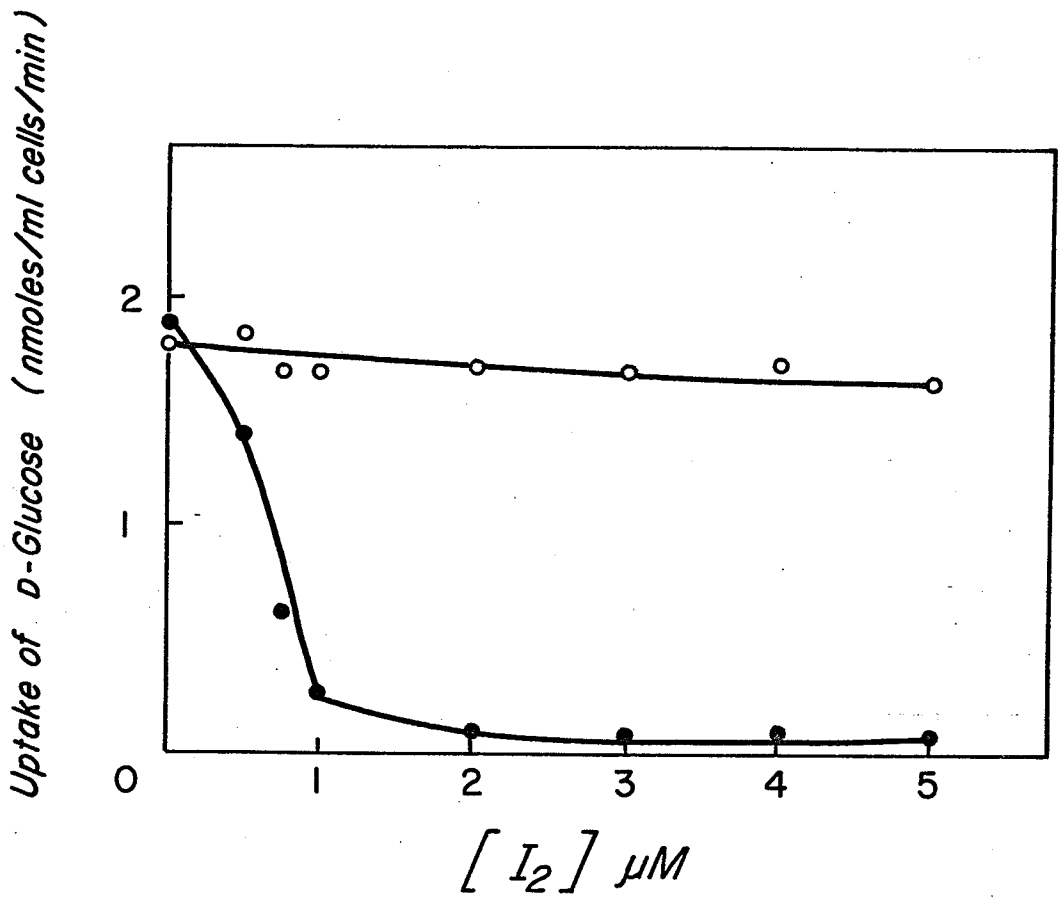
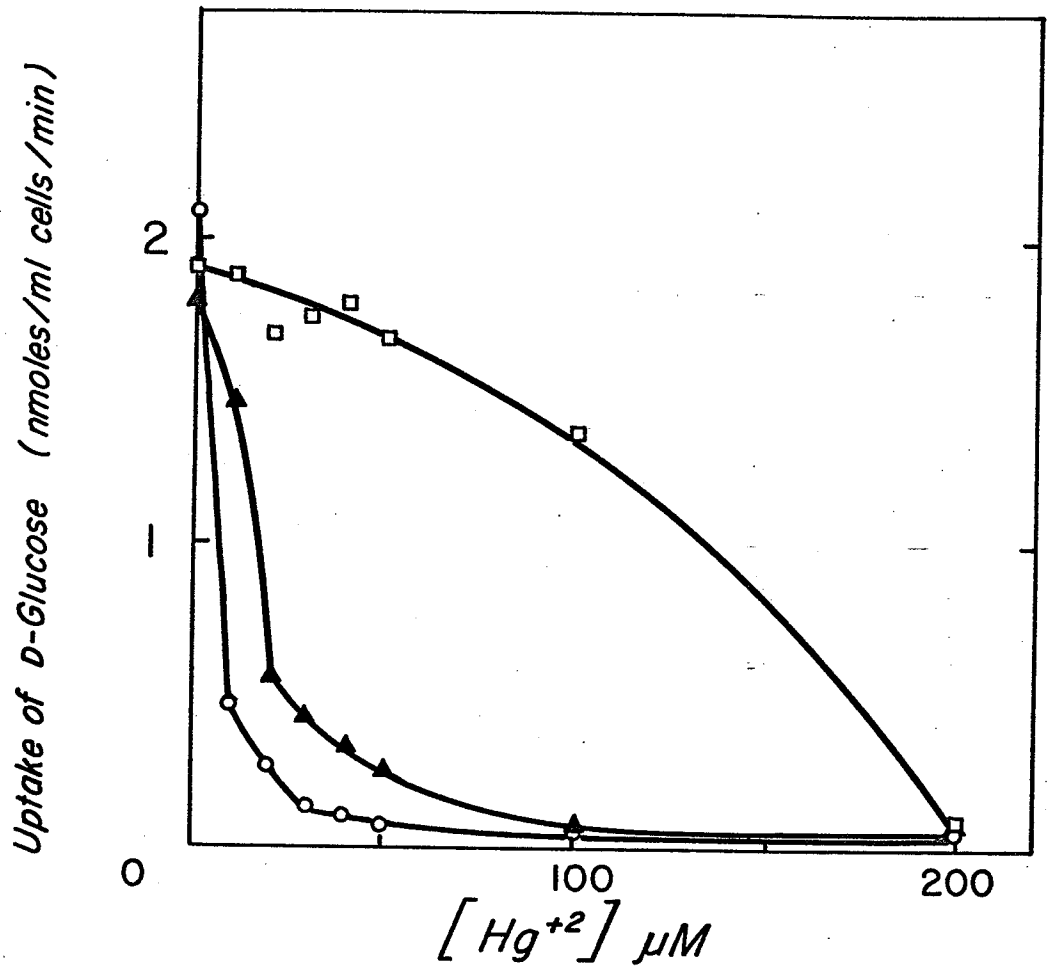

Uptake of D-Glucose (nmoles/ml cells/min)
2
1
0
1
2
3
4
5
[I2] μM

Figure 11. Inhibition of D-glucose transport (initial reaction rate) by Hg^{++} (0 to 200 μM) and its reversal with xanthine and 6ipAde; (○) Hg^{++} only; (▲), 50 μM 6ipAde; (□), 100 μM xanthine. The final concentration of D-glucose used was 50 μM.


Uptake of D-Glucose (nmoles/ml cells/min)
2
1
0
100
200
[Hg+2] μM

(6) Fate of Transported Sugars

Fig. 12 shows the chromatographic profiles of the cellular extracts made after the cells had been allowed to accumulate D-glucose for very short periods of time. As can be seen, the main products are glucose-6-phosphate and that constituting the slow migrating peak which may possibly be glucose-1, 6-diphosphate. Free D-glucose occurred in small amounts compared to the other intermediates. About 30% of the transported sugar was not extractable with ethanol.

Fig. 13 illustrates the profile when 2-deoxy-D-glucose was used instead of D-glucose. It shows that this sugar, like D-glucose, is also metabolized. Unlike that obtained for D-glucose, the main constituents of the extracts appear to be the free sugar with two slower migrating minor peaks. There seems to be another small peak chromatographing at around 20-22 cm from the origin. No further peaks were observed beyond 25 cm of the chromatogram (not included in the graph).

Figure 12. Chromatographic profiles of the intermediates of transported D-glucose after incubation periods of (▲), 1 min; (■), 2 min; (o), 4 min. The final concentration of D-glucose used was 100 μM.

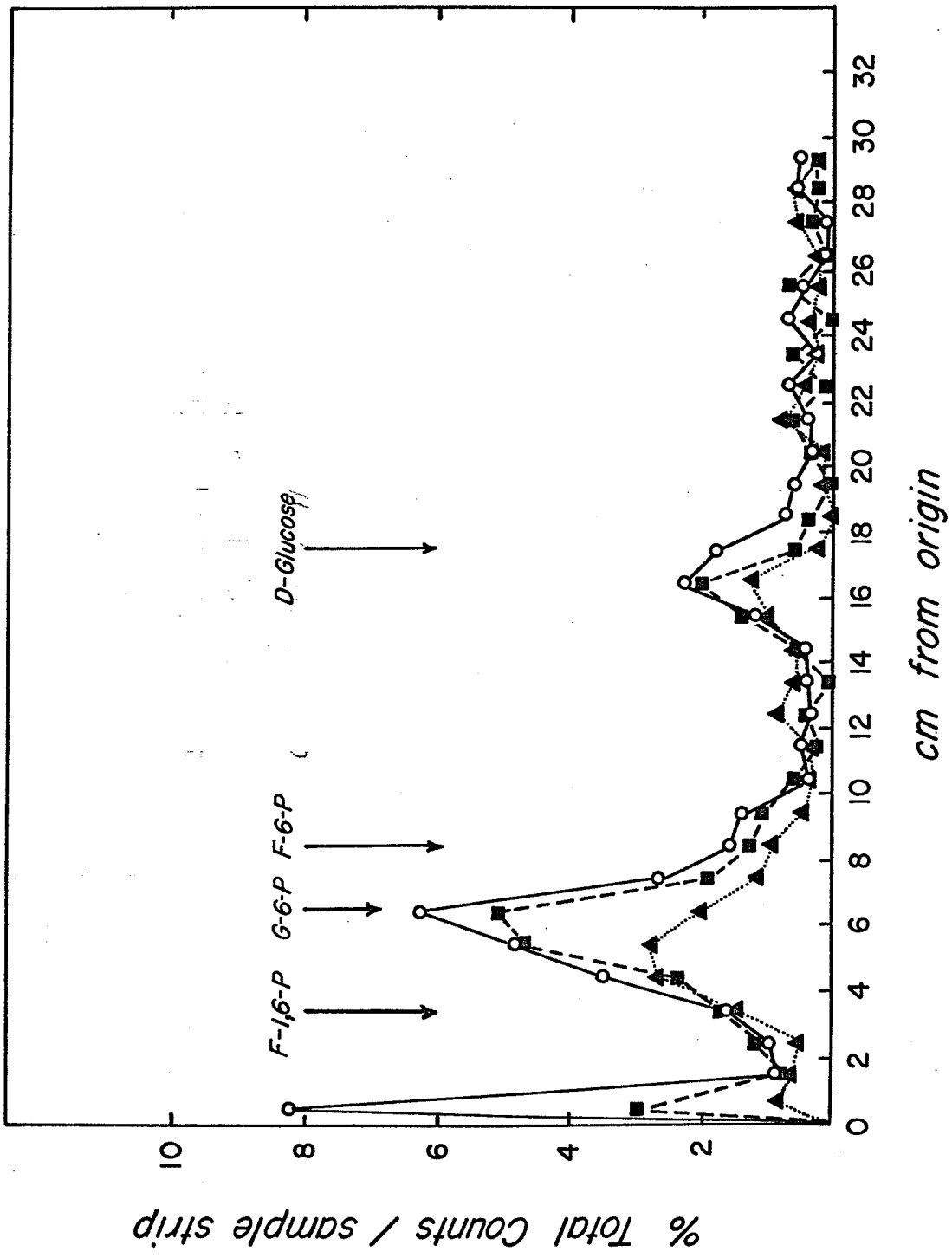
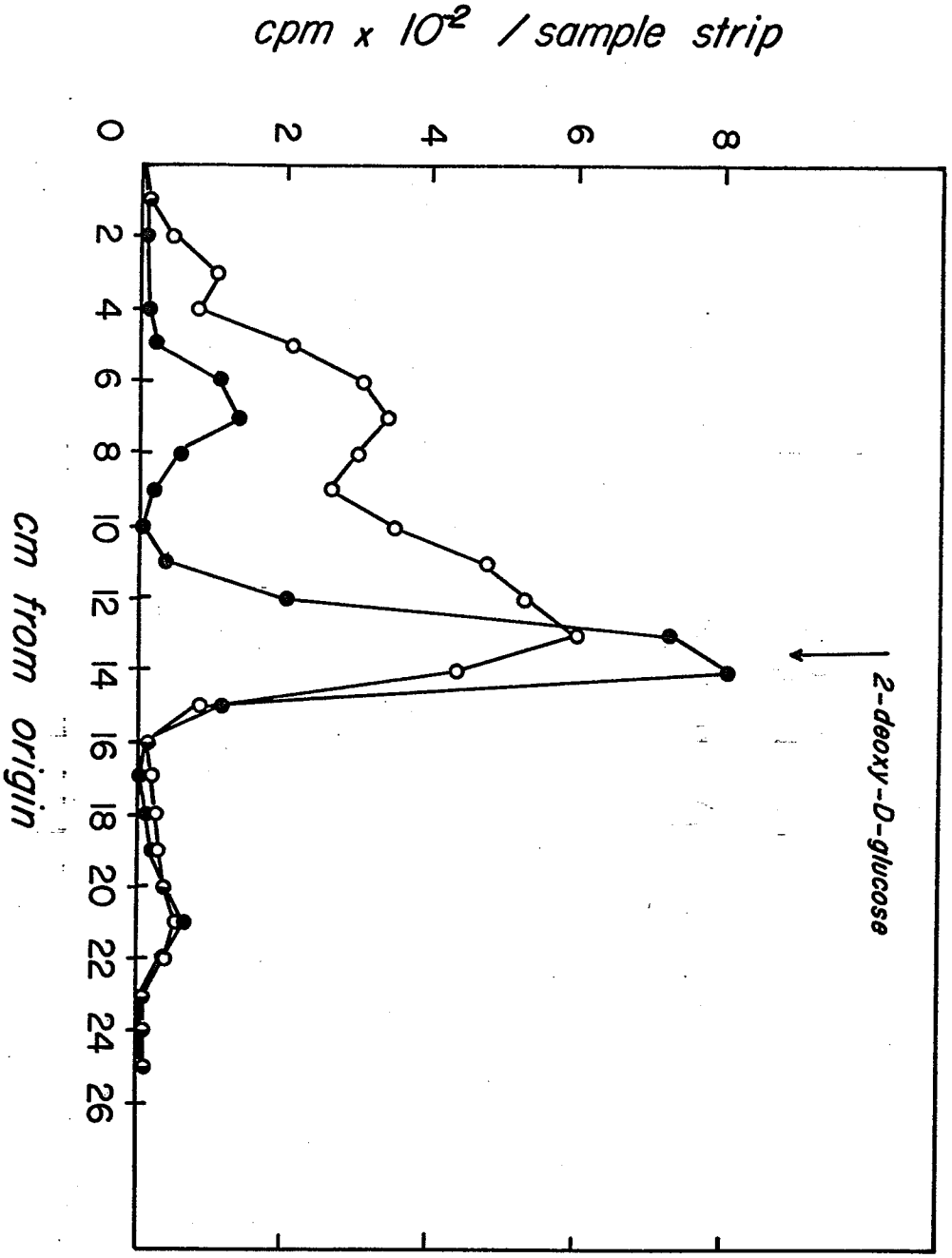

% Total Counts / sample strip
cm from origin
F-1,6-P
G-6-P
F-6-P
D-Glucose

Figure 13. Chromatographic profiles of the intermediates of transported 2-deoxy-D-glucose after incubation periods of (●), 1 min; (o), 2 min. The final concentration of 2-deoxy-D-glucose used was 100 μM.


cpm x 10² / sample strip
0
2
4
6
8
cm from origin
2
4
6
8
10
12
14
16
18
20
22
24
26
2-deoxy-D-glucose

(7) Citrate Inhibition of Amino Acid and Sugar Transport

Time course uptake experiments show that the transport of L-lysine, L-methionine, L-alanine and D-glucose was terminated very rapidly following the addition of 1×10^{-2}M sodium citrate (see Fig. 14 and 15). Sodium chloride and sodium acetate at concentrations of 2×10^{-2}M had no effect on D-glucose transport. An efflux of L-alanine was observed, as was the case for thymidine (10), following the addition of citrate. Other divalent metal cation chelators like EDTA (0.1 mM) and EGTA (0.5 mM) terminated transport of amino acids (10). Lineweaver-Burk plots (63) show that citrate inhibition of the transport of D-glucose, L-alanine, L-histidine, L-lysine, L-phenylalanine, L-tryptophan and L-valine (Fig. 16, 17, 18, 19, 20, 21, 22 respectively) was non-competitive. Citrate, which was used at concentrations varying between 1×10^{-3}M to 1×10^{-2}M seemingly affected only the rate of uptake but not the apparent Km's. Citrate, EDTA and EGTA which are all strong chelators of divalent cations are not normally taken up by the cells (10).

Figure 14. Time course studies of L-lysine and D-glucose transport and the inhibition of uptake by citrate; (●), 0 mM citrate; (O), 10 mM citrate. Citrate was added at the times indicated by the arrows. 50 µl of ^{3}H-L-lysine and ^{14}C-D-glucose were added to cell suspensions of 20 ml (final volume) to initiate the reactions. The experimental procedures are as described in 'Methods' under chromatographic analysis with the exception that the total radioactivity within whole cells was monitored directly after washing.

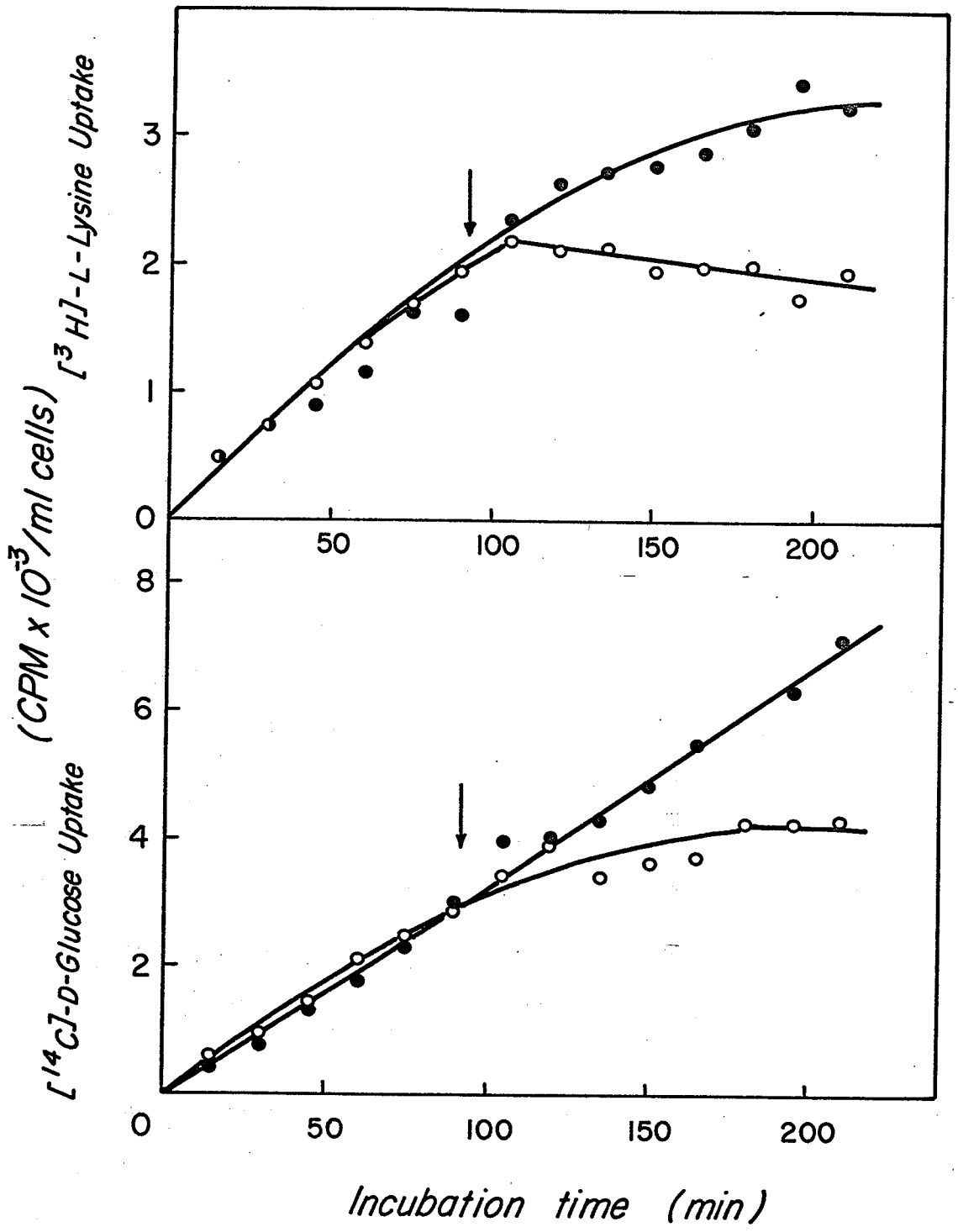

[3H]-L-Lysine Uptake
[14C]-D-Glucose Uptake
(CPM x 10-3/ml cells)
0
1
2
3
4
6
8
50
100
150
200
Incubation time (min)

Figure 15. Time course studies of L-methionine and L-alanine transport and the inhibition of uptake by citrate; (●), 0 μM citrate; (O), 10 mM citrate. Citrate was added at the times indicated by the arrows. 50 μl of ^{3}H-L-methionine and ^{3}H-L-alanine were added to cell suspensions of 20 ml (final volume) to initiate the reactions. The experimental procedures are as described in 'Methods' under chromatographic analysis with the exception that the total radioactivity within whole cells was monitored directly after washing.

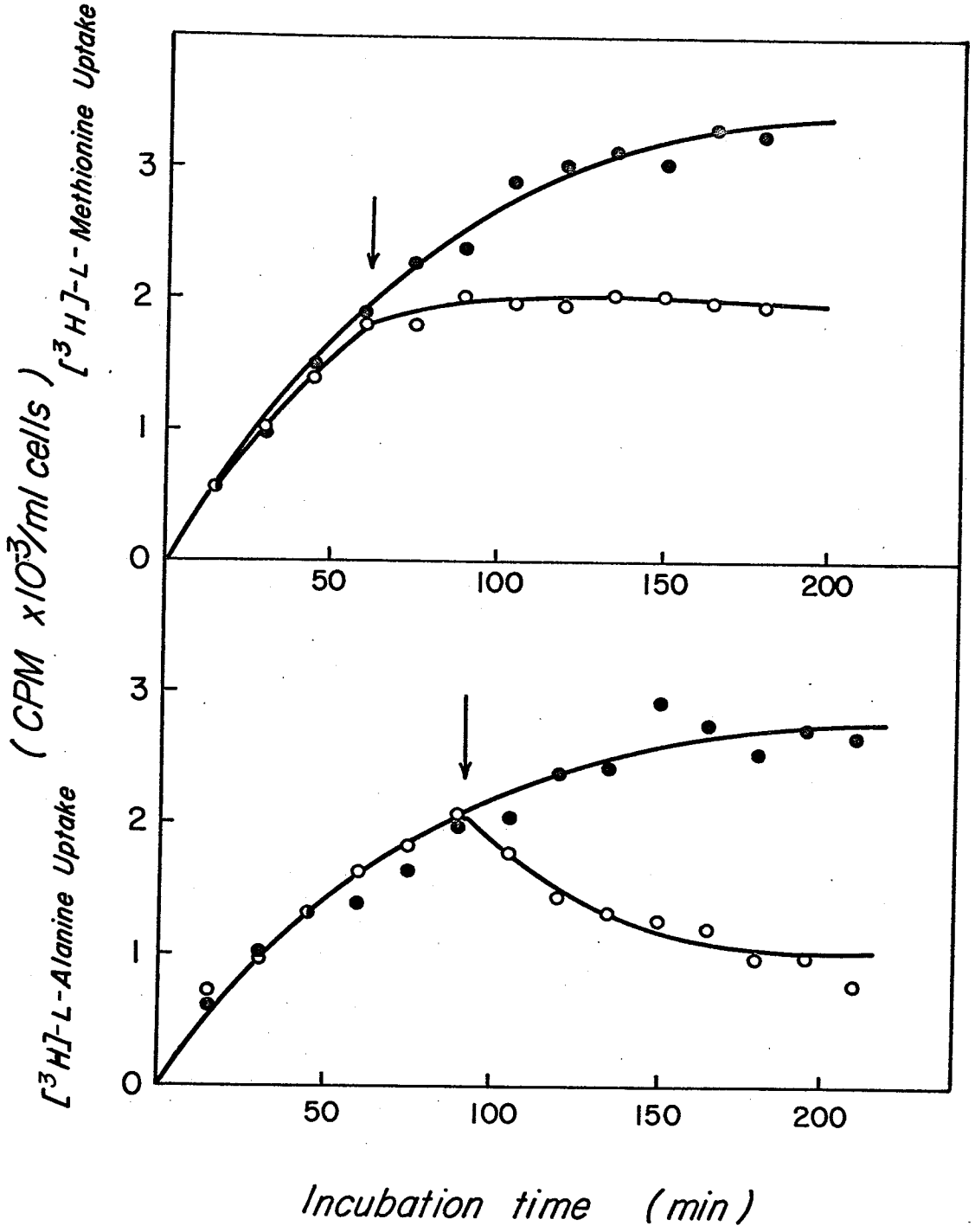

[³H]-L-Alanine Uptake
[³H]-L-Methionine Uptake
(CPM x10⁻³/ml cells)
0
1
2
3
50
100
150
200
Incubation time (min)

Figure 16. Lineweaver-Burk plot of the inhibition of D-glucose transport (initial reaction rate) by citrate; (●), 0 mM; (○), 1 mM; (□), 2 mM; (△), 5 mM; (▼), 10 mM. The inset illustrates the K_i of citrate inhibition.

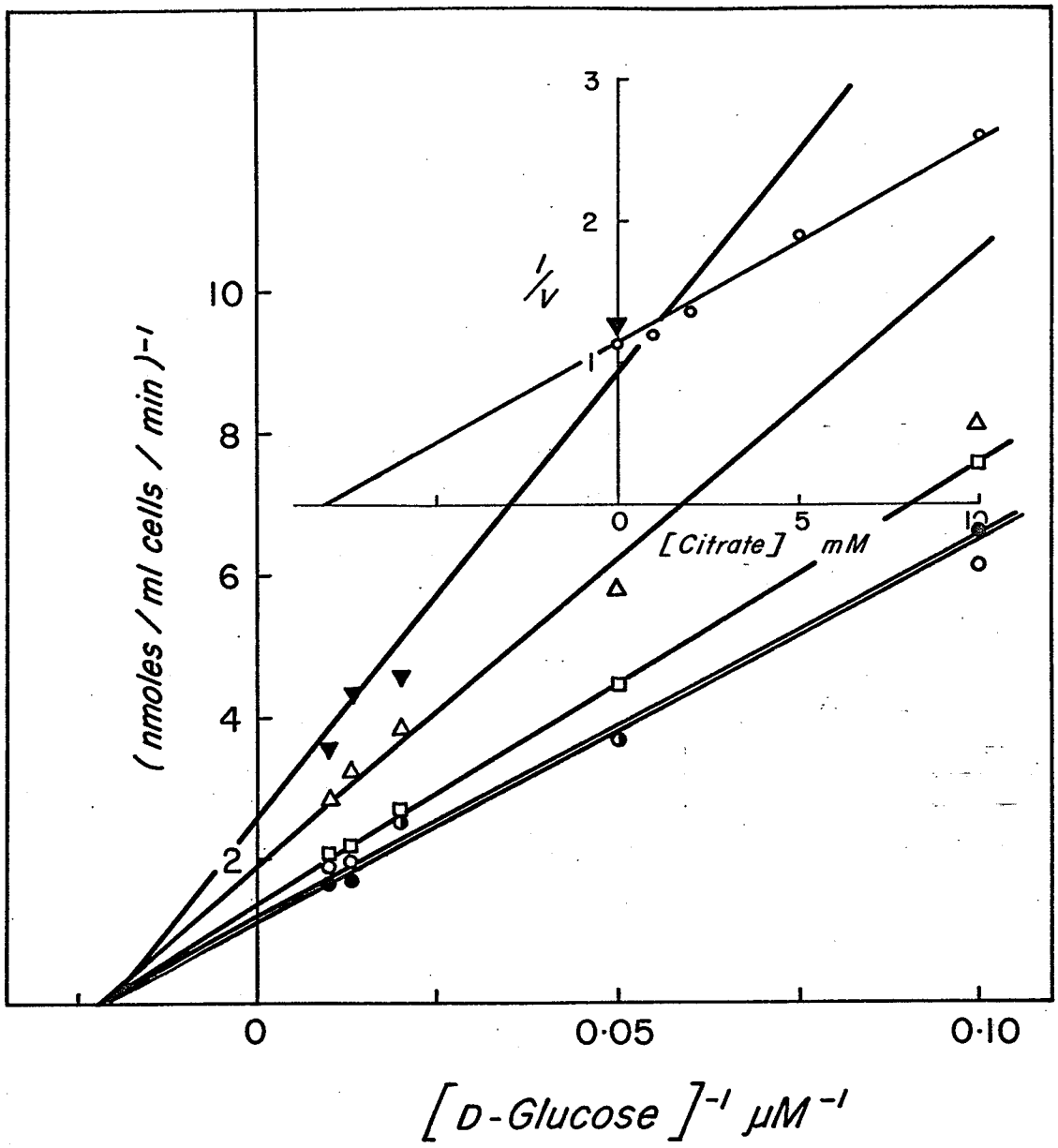

3
2
1/v
1
0
5
10
[Citrate] mM
10
8
6
4
2
(nmoles / ml cells / min)-1
0
0·05
0·10
[D-Glucose]-1 μM-1

Figure 17. Lineweaver-Burk plot of the inhibition of L-alanine transport (initial reaction rate) by citrate; (●), 0 mM; (○), 1 mM; (□), 2 mM; (△), 5 mM; (▼), 10 mM. The inset illustrates the K_i of citrate inhibition.

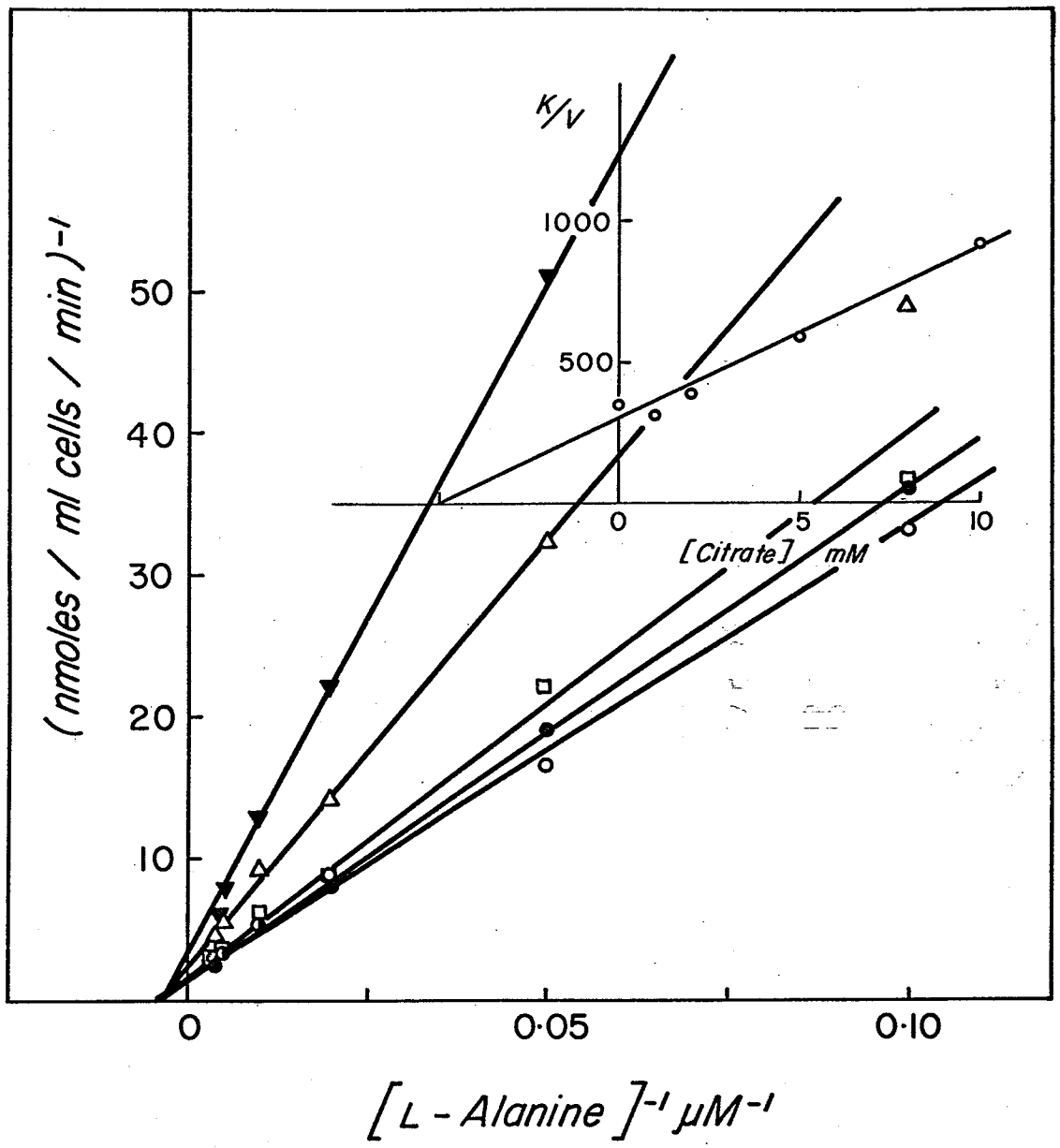

K/V
1000
500
0
5
10
[Citrate] mM
(nmoles / ml cells / min)-1
50
40
30
20
10
0
0·05
0·10
[L - Alanine]-1 μM-1

Figure 18. Lineweaver-Burk plot of the inhibition of L-histidine transport (initial reaction rate) by citrate; (●), 0 mM; (○), 1 mM; (□), 2 mM; (▼), 5 mM; (△), 10 mM. The inset illustrates the K_i of citrate inhibition.

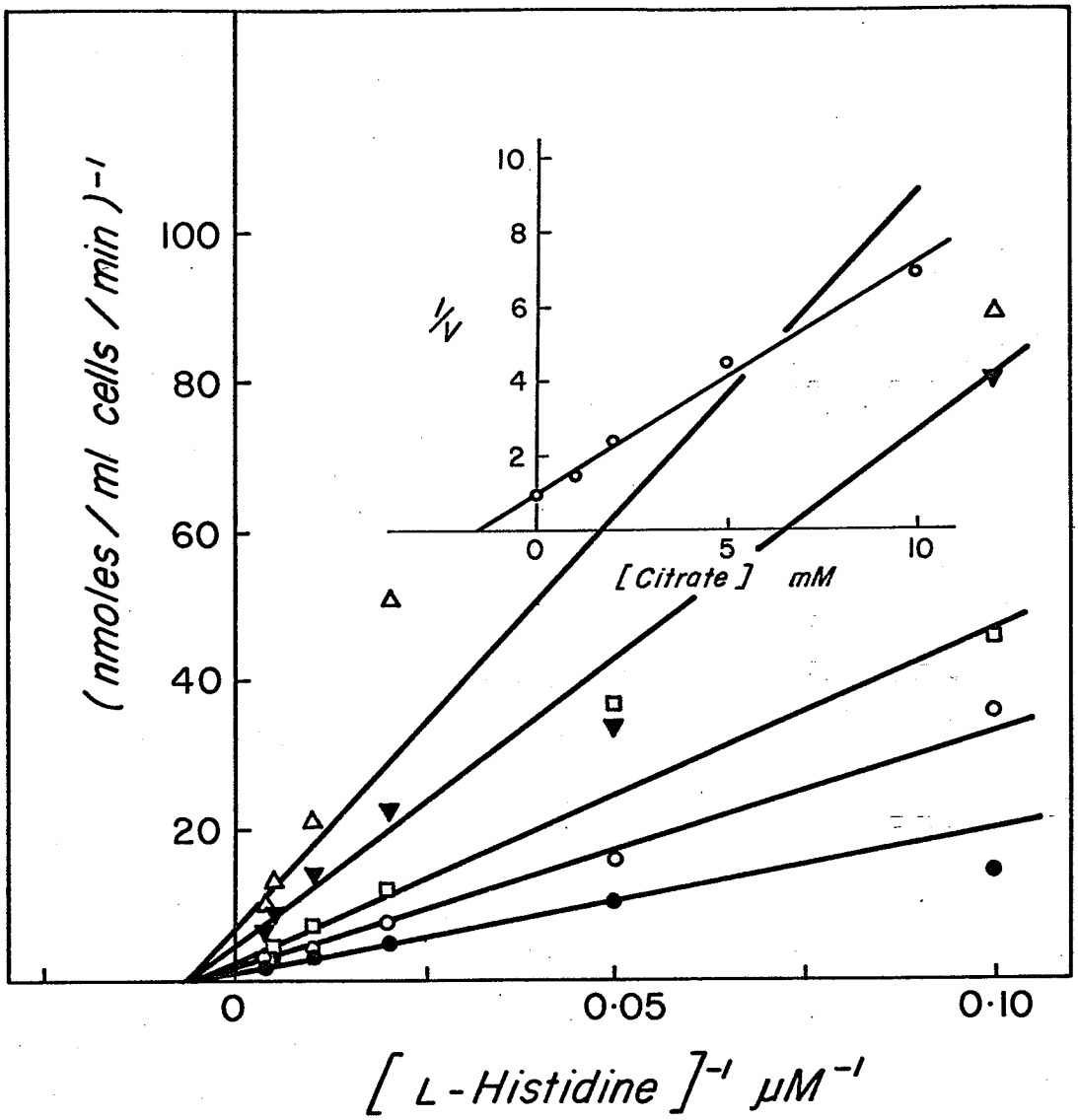

(nmoles / ml cells / min)⁻¹
100
80
60
40
20
0
0·05
0·10
[L-Histidine]⁻¹ μM⁻¹
1/V
10
8
6
4
2
0
5
10
[Citrate] mM

Figure 19. Lineweaver-Burk plot of the inhibition of L-lysine transport (initial reaction rate) by citrate; (●), 0 mM; (○), 2 mM; (□), 5 mM; (△) 10 mM. The inset illustrates the K_i of citrate inhibition.

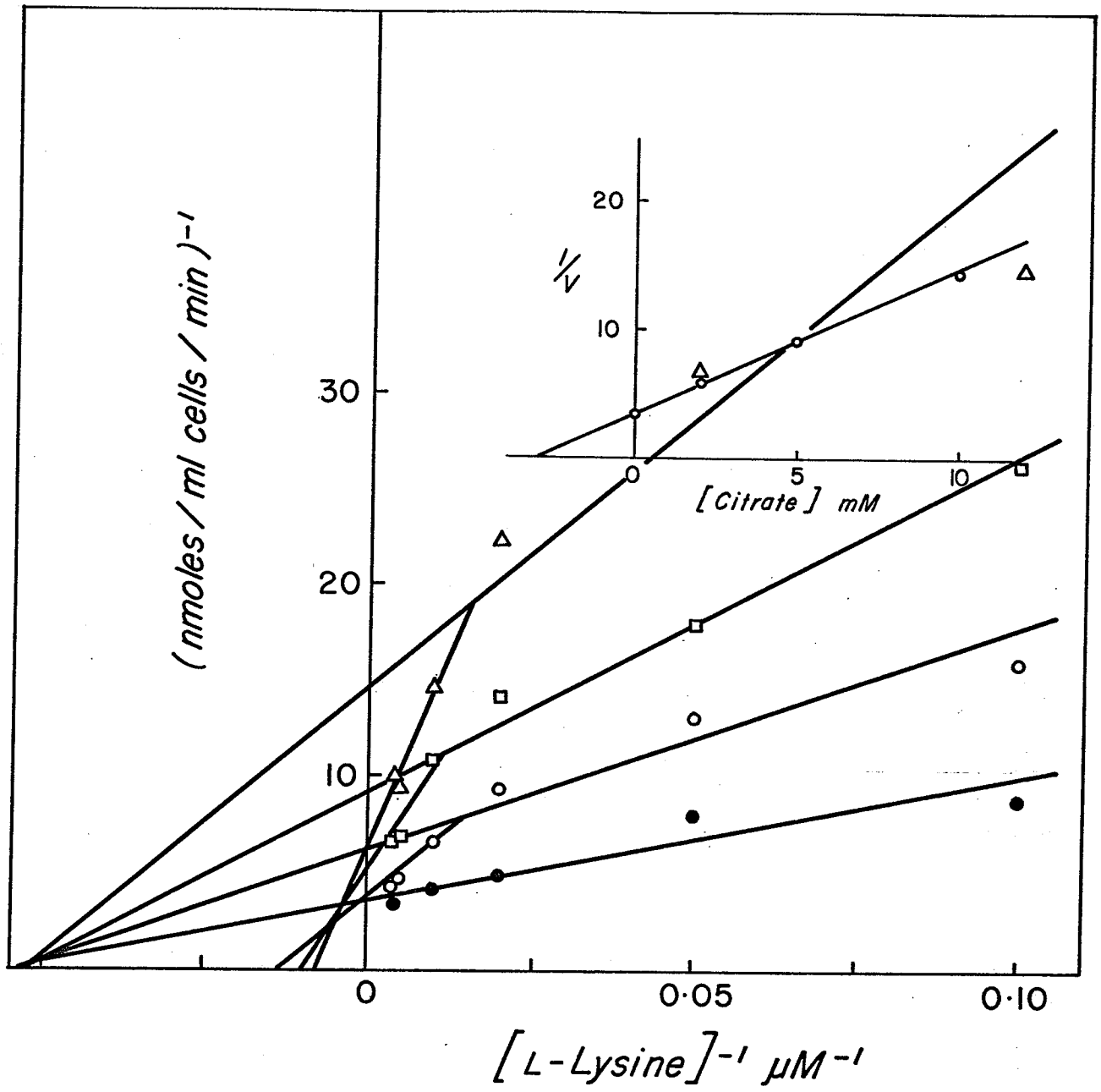

(nmoles / ml cells / min)⁻¹
30
20
10
0
0·05
0·10
[L-Lysine]⁻¹ μM⁻¹
1/V
20
10
0
5
10
[Citrate] mM

Figure 20. Lineweaver-Burk plot of the inhibition of L-phenylalanine transport (initial reaction rate) by citrate; (●), 0 mM; (○), 1 mM; (□), 2 mM; (△), 5 mM; (▼), 10 mM.

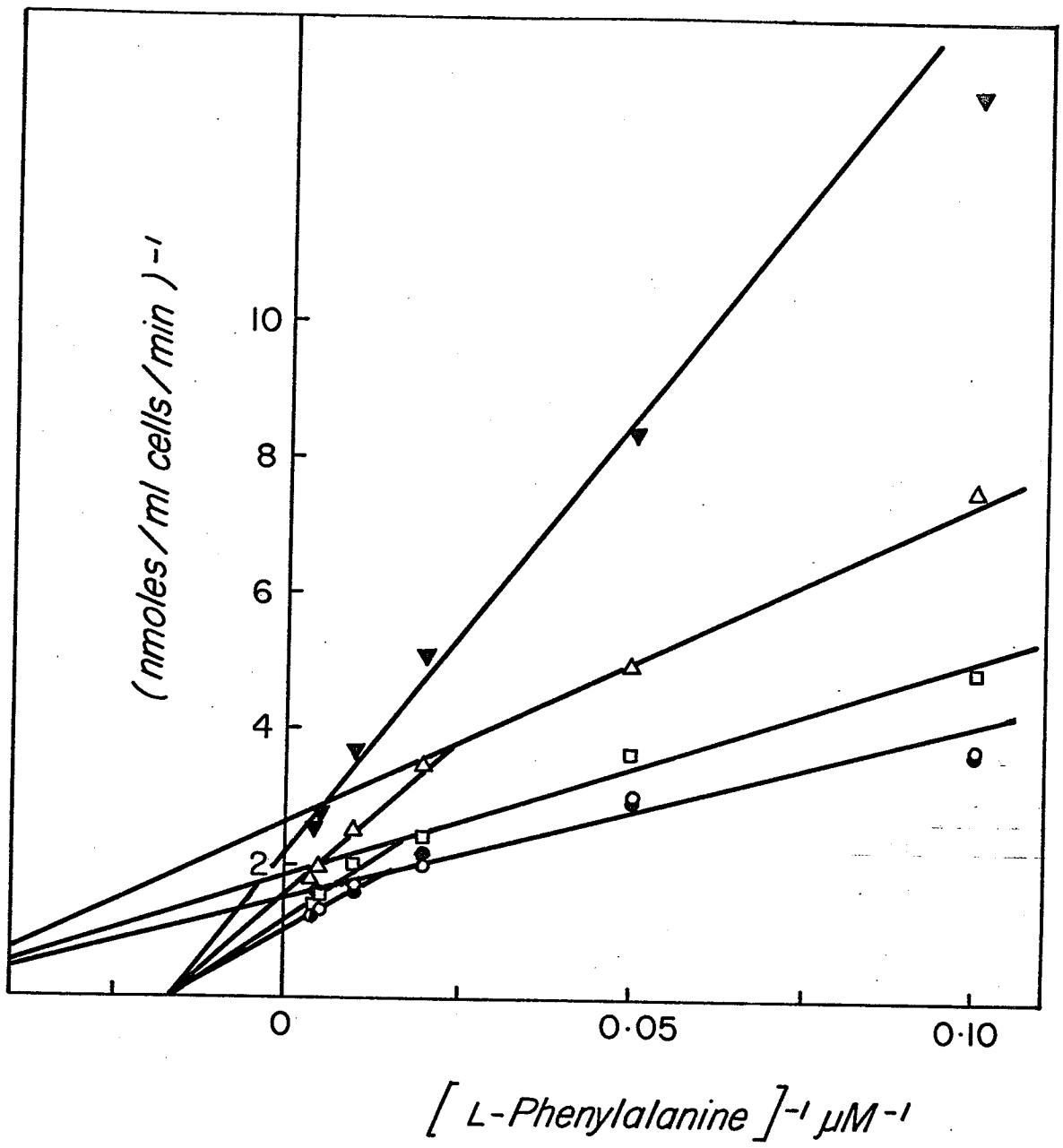

(nmoles / ml cells / min)-1
10
8
6
4
2
0
0·05
0·10
[L-Phenylalanine]-1 μM-1

Figure 21. Lineweaver-Burk plot of the inhibition of L-tryptophan transport (initial reaction rate) by citrate; (●), 0 mM; (○), 1 mM; (□), 2 mM; (Δ), 5 mM; (▼), 10 mM. The inset illustrates the K_i of citrate inhibition.

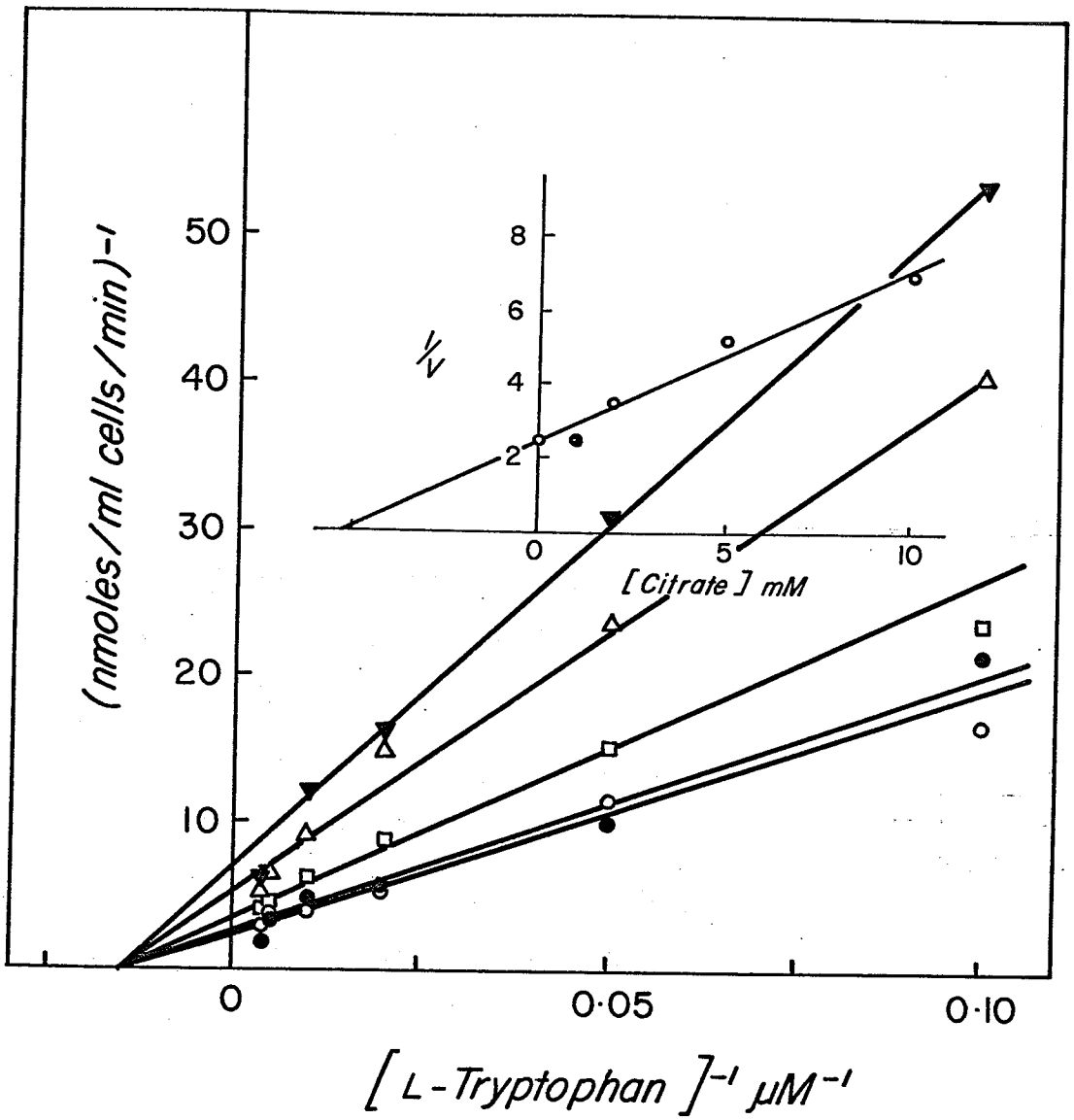
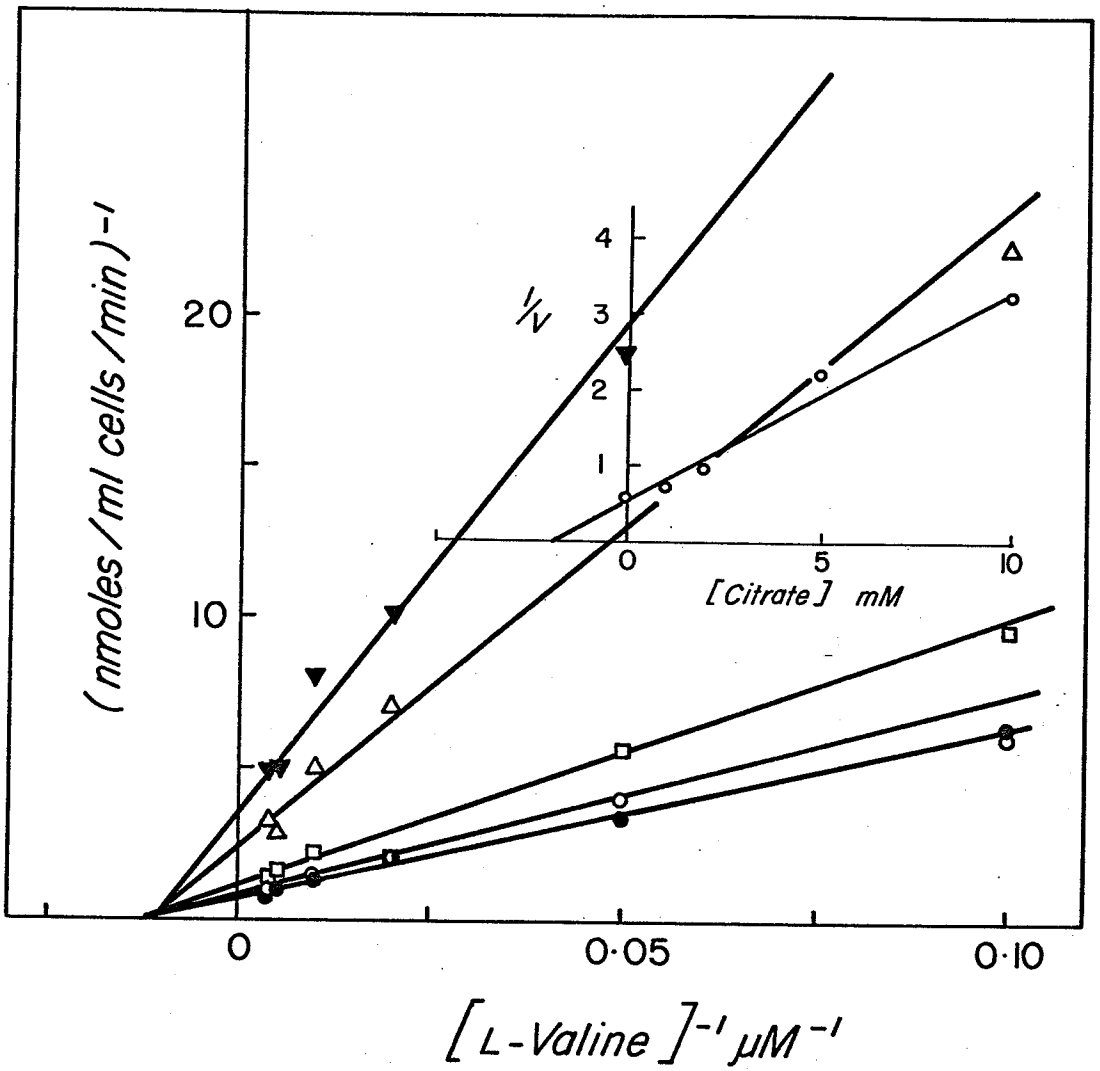

(nmoles/ml cells/min)⁻¹
50
40
30
20
10
0
0·05
0·10
[L-Tryptophan]⁻¹ μM⁻¹
1/v
8
6
4
2
0
5
10
[Citrate] mM

Figure 22. Lineweaver-Burk plot of the inhibition of L-valine transport (initial reaction rate) by citrate; (●), 0 mM; (○), 1 mM; (□), 2 mM; (△), 5 mM; (▼), 10 mM. The inset illustrates the K_i of citrate inhibition.


(nmoles/ml cells/min)⁻¹
20
10
0
0·05
0·10
[L-Valine]⁻¹ μM⁻¹
1/v
4
3
2
1
0
5
10
[Citrate] mM

(8) Inhibition of Sugar Transport by Cytokinins

Cytokinins, a class of plant growth hormones, were found to inhibit D-glucose and 2-deoxy-D-glucose transport. Of the various natural and synthetic cytokinins tested, 6-hexylaminopurine, 6ipAde and 6-benzyladenine were the most potent. Table IV lists the potency of those purine compounds that were tested. Fig. 23 and 24 show the effective inhibitory ranges of 6-hexylaminopurine and 6ipAde on D-glucose transport. Both inhibition patterns are very similar. Small increases in concentration of these plant hormones above 1×10^{-5}M resulted in very pronounced reduction of D-glucose uptake. cAMP, a purine derivative, had no effect on sugar transport. Fig. 25 and 2 show the inhibition patterns of D-glucose and 2-deoxy-D-glucose transport. 6ipAde as inhibitor had a K_i value of 1.82×10^{-4}M and 3.25×10^{-4}M for D-glucose and 2-deoxy-D-glucose. In both cases the inhibition was non-competitive. The cytokinin inhibited uptake of D-glucose was effective throughout the pH range tested (5.5 to 9.0). However it was slightly more effective between pH 6 to 7.5 as shown in Fig. 26.

Table IV. Effect of various purine compounds on D-glucose transport.

Agent (conc. of 250 μM)	% Inhibition of D-glucose transport
Histaminopurine	0
Zeatin	0
Dimethylaminopurine	0
Methylaminopurine	0
Butoxypurine	40
cAMP	0
Propylmercaptopurine	40
Benzyladenine	42
6ipAde	52
Hexylaminopurine	81
Xanthine	44
6ipAdo	0

Figure 23. Inhibition profile of D-glucose transport (initial reaction rate) in the presence of varying concentrations of hexylaminopurine. The final concentration of D-glucose used was 50 μM.

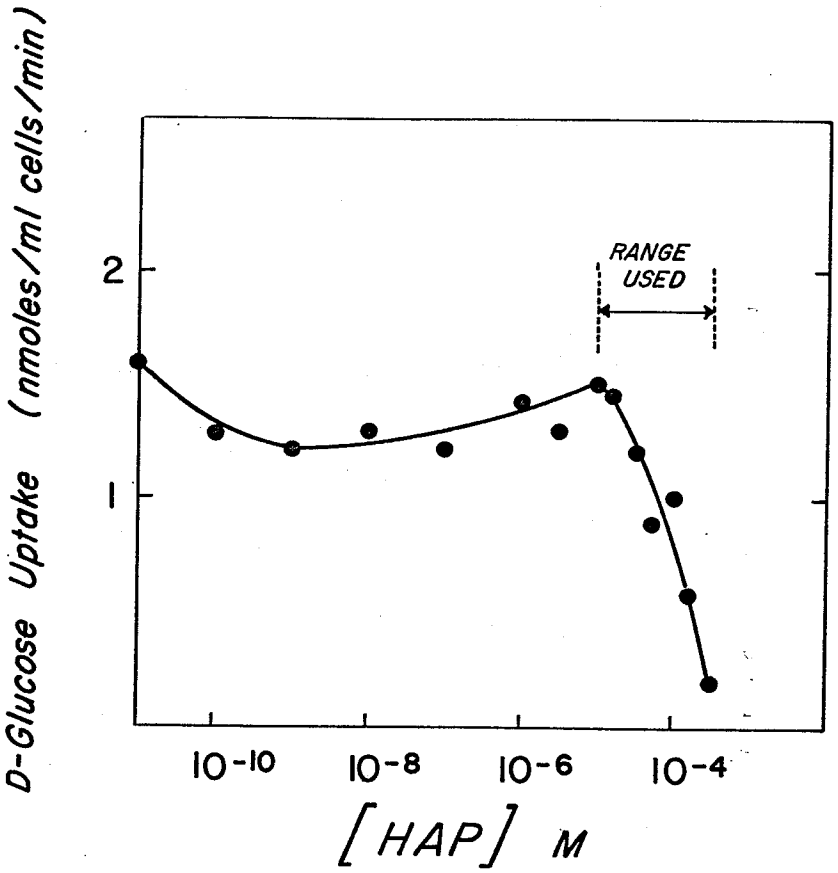

D-Glucose Uptake (nmoles/ml cells/min)
2
1
RANGE USED
10-10
10-8
10-6
10-4
[HAP] M

Figure 24. Inhibition profile of D-glucose transport (initial reaction rate) in the presence of varying concentrations of 6ipAde. The final concentration of D-glucose used was 50 μM.

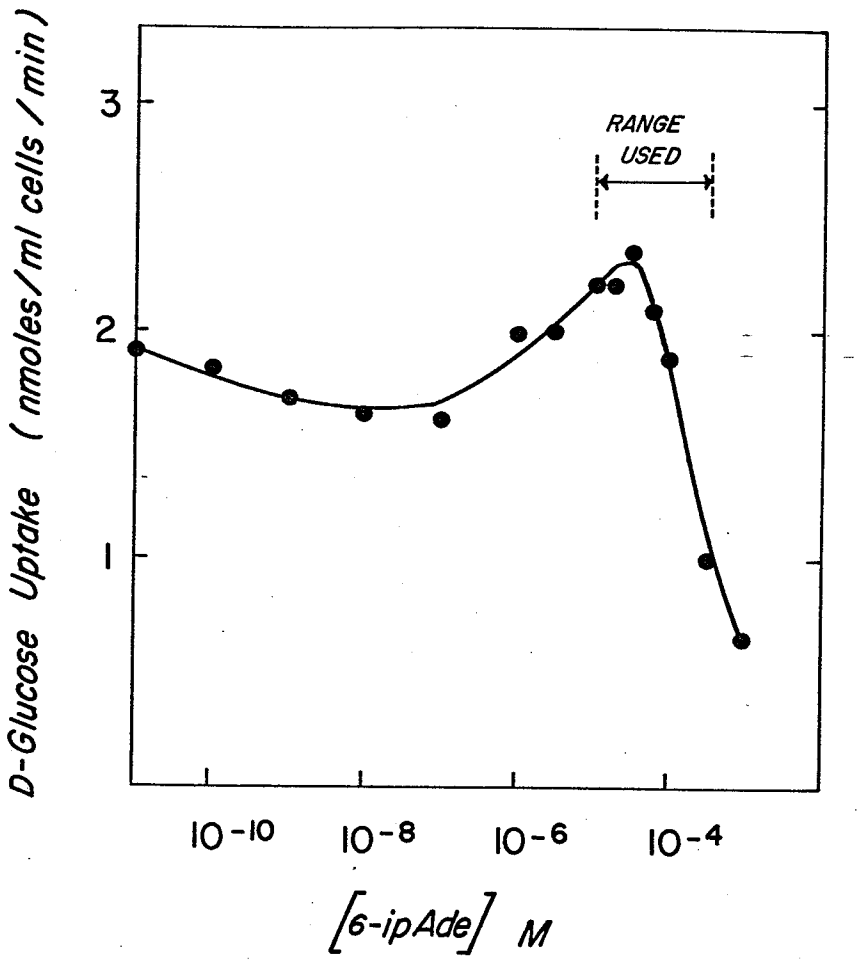

D-Glucose Uptake (nmoles/ml cells / min)
3
2
1
RANGE
USED
10-10
10-8
10-6
10-4
[6-ipAde] M

Figure 25. Lineweaver-Burk plot of the inhibition of D-glucose transport (initial reaction rate) by 6ipAde; (●), 0 μM; (○), 50 μM; (□), 100 μM; (△), 200 μM; (▼), 500 μM. The inset illustrates the K_i of 6ipAde inhibition.

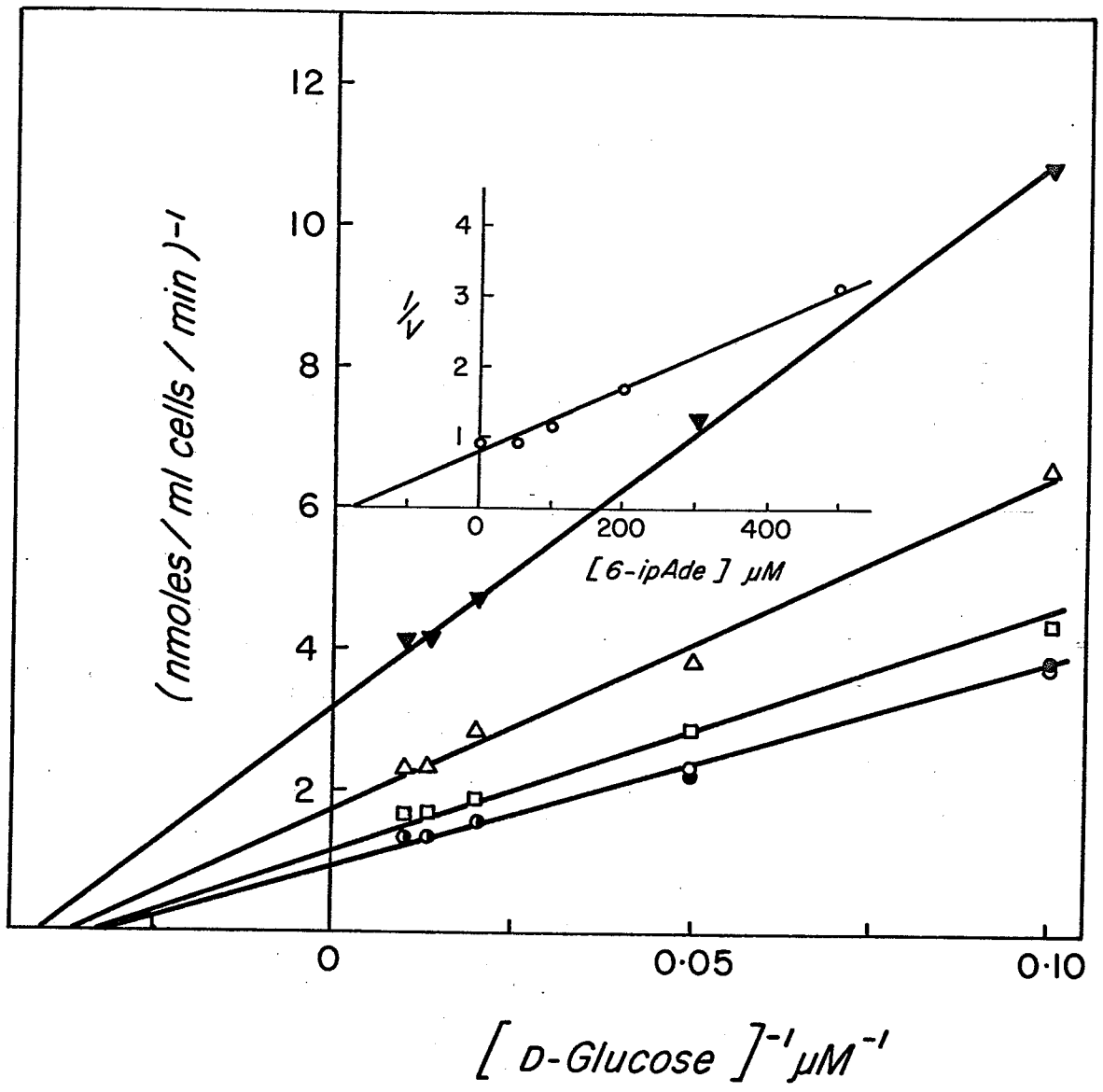
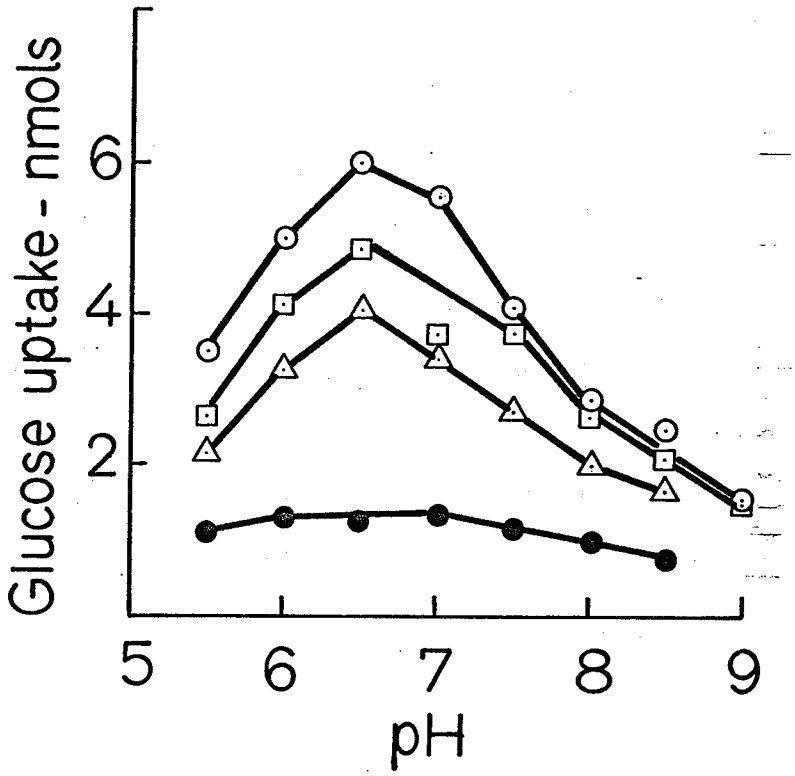

12
10
8
6
4
2
0
0·05
0·10
(nmoles / ml cells / min)-1
[D-Glucose]-1 µM-1
1/v
4
3
2
1
0
200
400
[6-ipAde] µM

Figure 26. Inhibition profiles of D-glucose transport (initial reaction rate) by 6ipAde at various pH; the concentrations of 6ipAde used are, (⊙), 0 μM; (⊡), 50 μM; (△), 100 μM; (●), 500 μM. The final concentration of D-glucose used was 100 μM.


Glucose uptake - nmols
2
4
6
5
6
7
8
9
pH

DISCUSSION

(1) Determination of Km's for D-Glucose and 2-Deoxy-D-Glucose

The kinetic studies as illustrated by Fig. 1 and 2, indicate that the D-glucose transport system not only has a high affinity for D-glucose and 2-deoxy-D-glucose but is also able to accumulate these metabolites against a concentration gradient. This system is of great advantage to the organism since D-glucose is an important metabolite and one would expect only low levels of this sugar in the natural fresh-water environment of this fungus.

Cells grown in a medium with D-glucose as the sole carbon and energy source failed to transport D-fructose and glycerol. As described in the results, D-fructose did not compete with D-glucose uptake. These two observations suggest that D-fructose transport may be via an inducible transport system. Thus the presence of D-glucose either inhibits or prevents the induction of the synthesis of the transport moieties for D-fructose uptake possibly by the phenomenon known as catabolite repression. This was

not borne out by further experiments as described in the results. It is possible that D-fructose and glycerol are transported by a very slow facilitated diffusion process. In any case, these two metabolites serve as very poor carbon sources for the growth of Achlya.

D-Glucose failed to be transported by osmotically shocked cells. One could ascribe this to a loss of specific sugar carrier(s) from the cell membrane. However no binding components for D-glucose were found in the shock fluid. The cells were not killed by such a mild treatment as the capacity for transport was recovered after the 'shocked' cells had been incubated in growing medium for 8 to 12 hr. Presumably, components vital for active transport processes removed by osmotic shock treatment are regenerated during this period. An alternative explanation for the failure of 'shocked' cells to take up D-glucose is that some membrane component essential for a functional D-glucose transport system is being removed from the cell membrane. A Ca^{++} binding glycopeptide is indeed easily dislodged by osmotic shock treatment. The possible relationship between this Ca^{++} sequestrator and D-glucose transport will be considered in the pertinent sections of this "Discussion".

(2) Competition Studies

D-Glucose, D-mannose and D-galactose are diastereomers while 2-deoxy-D-glucose is an analogue of D-glucose. The competition studies as illustrated by Fig. 3, 16, 1 and 5, showed that the three hexoses and the pentose, D-xylose, competitively inhibited D-glucose uptake, suggesting that these sugars all share a common transport system with D-glucose. Considering the molecular structures of all the sugars tested, one can infer that carbons one and two of five and six carbon sugars are important in conferring specificity for recognition by the D-glucose transport system. Substitution of the hydroxyl group on carbon 1 with a methyl group completely abolished the ability of sugars like α-methyl-D-glucoside and α-methyl-D-mannopyranoside to compete with D-glucose. Similar results were obtained from studies in yeast (14) and Aspergillus nidulans (14). A ketone function or a substitution of the hydroxyl group of carbon 2 will result in a loss of activity. This is clearly illustrated when D-glucose transport was not inhibited in the presence of either D-fructose or N-acetyl-D-glucosamine. This is in agreement with the results obtained by Romano (84). In marked contrast, transport of a hexose like L-sorbose bearing a ketone

function at carbon 2 was competitively inhibited by D-glucose in yeast (12) and N. crassa (19). Finally, the hydroxyl group on carbon 6 is probably essential for activity. Glucose-6-phosphate and D-fucose both failed to inhibit D-glucose transport. This aspect will be amplified below. Considering the stereospecificity exhibited by the D-glucose transport system, and the effects of -SH group reactants, pH, temperature effects, it is conceivable that the D-glucose transport process is protein mediated.

(3) Temperature and pH Effects on D-glucose Transport

When attempting to define a protein catalysed reaction, two useful parameters to consider are the effects of temperature and pH on the reaction rate. It is generally agreed that transport of sugars and amino acids through biological membranes are protein mediated processes (17, 86). Enzymes are sensitive to temperature changes. On the one hand, extreme temperatures will result in the denaturation of the enzyme leading to a loss in activity while on the other increasing temperatures generally results in an increase in activity. Consideration of both these factors are essential in examining enzyme catalysed reactions. Results obtained appear to indicate that

D-glucose transport is a protein mediated process. The rate of D-glucose transport increased with increasing temperatures, reaching an optimum at 30 C as is shown in Fig. 6. The shape of the left arm of the curve of Fig. 6 can be ascribed to an increase in activity of the transport system in question owing to increasing temperatures. Enzyme kinetics predict that increases in temperature beyond 30 C would result in further enhancement of the rate of transport of D-glucose. Such was not the case as illustrated by Fig. 6. A rapid denaturation of the protein(s) involved in D-glucose transport is the most likely explanation for this observation. Other possibilities include the denaturation of other temperature sensitive membrane located proteins, leading to distortion of the cell membrane. This membrane distortion may have an unfavourable steric effect on the D-glucose carrier system and thus impair its binding and transport functions. Alternatively, the structural integrity of the cell membrane may be lost resulting in the leakage of some of the accumulated sugar.

Similarly, enzymes are extremely sensitive to pH. The effects of pH changes, among others, include alterations in protein configuration, substrate

binding to the enzyme and on reactivity on catalysis. The bell shaped curve of Fig. 7 is characteristic of a plot of the initial velocity of an enzymic reaction as a function of pH. These results show that the D-glucose transport system of this fungus is sensitive to pH changes.

These two phenomena support the idea that specific proteins may possibly be involved in the transport of sugars in Achlya sp. In contrast, the pH and temperature optima (pH 4 to 7, temperature, 37 to 50 C) for transport of 3-0-methyl-D-glucose by N. crassa were found to encompass fairly wide ranges (72).

(4) Effect of Energy Poisons on Transport

The ability of various organisms to accumulate metabolites against a concentration gradient indicates that 'energy' input is required for active transport. The mechanism of energy coupling to transport processes has become a field of intensive research.

The phosphoenolpyruvate phosphotransferase system first characterized by Roseman and coworkers (45) represents a novel system by which sugars are

transported by 'vectorial transphosphorylation', with phosphoenolpyruvate being the initial phosphate donor. Other transport models have also been proposed. Among others, Kaback and Barnes (46) contend that β-galactoside transport in bacteria is coupled to respiration and that ATP derived from oxidative phosphorylation is not an intermediate in the coupling of respiration to transport. The origin of another model stems from the basic ideas of the chemiosmotic hypothesis (68); namely that the accumulation of substrates may occur as cotransport with protons and the process being driven by a membrane potential. Of interest is the recent report of a proton dependent cotransport system for D-glucose in N. crassa (98). However there are shortcomings in these and other models which have been put forward.

The effects of energy poisons on D-glucose transport by Achlya are listed in table II. Both azide and cyanide inhibit electron transfer while 2,4-dinitrophenol and CCCP not only uncouple oxidative phosphorylation but also conduct H^+ very specifically across artificial and biological membranes (37). The results in table II show that the uncouplers were more effective than electron transfer inhibitors

in inhibiting sugar transport. Sodium arsenite also inhibited the uptake of D-glucose. This respiratory inhibitor has a strong affinity for -SH groups of various enzymes. The only definitive conclusion which can be drawn from the results is that any perturbation of the energy generating system in this fungus will inhibit sugar transport. This satisfies one of the definitions of active transport i.e. the requirement for input of metabolic 'energy'. Sodium fluoride inhibits many Mg^{++} requiring enzymes, some of which are enolase and phosphoglucomutase. Both enzymes are important in the context of glycolysis. The fact that sodium fluoride (1 mM) failed to inhibit D-glucose transport suggests that D-glucose metabolism and transport in our kinetic studies may be two independent processes.

Cells for transport studies were always preincubated for 5 min with 2.8 μM cycloheximide before starting the experiments. This was carried out to minimize the effects, if any, of intracellular macromolecular metabolism on transport. Cycloheximide, at the concentration used, was found to inhibit RNA and protein synthesis (10). However, the transport of sugars and amino acids were not affected in the presence of cycloheximide thus implying that the transport processes of these metabolites does not

require continued protein and RNA synthesis and that the transporters (if they are proteins) may be reasonably stable in the membrane.

(5) Sulfhydryl Group Reacting Agents And Active Transport

The use of sulfhydryl group reacting agents was to gain some insight into the involvement of -SH groups in D-glucose transport. Using an *in vivo* system makes detailed analysis of results difficult. This is further complicated by the use of sulfhydryl group reacting agents which are not absolutely specific for -SH groups.

The three reagents tested, NEM, Hg^{++} and I_2, all react with -SH groups. NEM undergoes a rapid and essentially irreversible reaction with -SH groups at low concentrations. However this reagent does react slowly with other groups like peptide $-NH_2$ and imidazole residues when used at high concentrations (99). Hg^{++} and I_2 are also sulfhydryl group reagents, though not showing the kind of specificity that NEM has for such groups. Table III shows that NEM is the least potent in inhibiting D-glucose uptake even when used at a much higher concentration than either Hg^{++} or I_2. One

of the many possible explanations is that Hg^{++} and I_2 may be penetrating more rapidly through the cell membrane than the larger NEM moiety and thus can react not only with -SH groups accessible from the outside of the cell membrane but also with those accessible only from within. Another likely explanation is that Hg^{++} and I_2, owing to their lack of specificity may be reacting with other cell membrane sites not attacked by NEM.

The non-competitive nature of I_2 inhibition of D-glucose transport as shown in Fig. 8 is consistent with covalent modification of the D-glucose transport system. Also the linear curve obtained from a plot of $1/v$ vs $[I_2]^2$ suggests that I_2 is acting at more than one site, all of which may possibly be located on the cell membrane. Either one or more of these sites may be on a membrane-associated entity, the cell wall Ca^{++} binding glycopeptide and which is related to active D-glucose transport. This aspect will be further discussed in sections (8) and (9) of this "Discussion".

Haguenauer-Tsapis and Kepes (34) claim that NEM specifically inactivates the E. coli membrane bound phosphoenolpyruvate phosphotransferase enzyme II component resulting in the loss of sugar transport ability. In the human erythrocyte sugar transport system, the

essential residues of the sugar carrier have been identified as a sulfhydryl and an amino group with an imidazole residue possibly being involved (5).

The mechanism(s) by which cytokinins reverse the I_2 and Hg^{++} effect on D-glucose transport seems complex since both types of agents inhibit D-glucose transport by themselves (see table III and fig. 25 and 2). Studies by LéJohn et al show that the possibility of Hg^{++} and Ca^{++} complexing with 6ipAde can be excluded (58). I_2 at pH 7.0 does react with 6ipAde, but not with xanthine. Also 6ipAde and xanthine are able to negate the Hg^{++} effect on D-glucose transport (see fig. 11) suggesting that the reversal of I_2 inhibition by 6ipAde, despite some complex formation, is not an artifact.

(6) Fate of Transported Sugars

Chromatographic and kinetic studies were carried out to gain some insight into the D-glucose transport process. Even in the absence of cycloheximide, D-glucose and 2-deoxy-D-glucose especially are not metabolized very rapidly. However the main difference between D-glucose and its analogue is

that 2-deoxy-D-glucose is accumulated mainly as the free sugar while glucose is not. This is shown in Fig. 12 and 13. It is possible that an intracellular glucokinase has a greater affinity for the accumulated D-glucose than its analogue.

So far the novel phosphoenolpyruvate phosphotransferase system has been positively detected only in some bacteria (88). It is difficult to argue for the presence of such a system in this fungus. An enhancement of D-glucose transport was not observed in the presence of PEP as would have been expected since PEP is the primary phosphate donor in the phosphoenolpyruvate phosphotransferase system. Sodium fluoride, a strong inhibitor of enolase failed to inhibit D-glucose transport as described earlier. Also sodium azide which inhibited the transport of D-glucose in Achlya was not inhibitory to the phosphoenolpyruvate phosphotransferase system of bacteria (49).

An alternative process reported by van Steveninck (106, 107) is the necessity for phosphorylation of a sugar during translocation. If phosphorylation of the sugar is required for it to be transported, then analogues of those sugars which share the D-glucose transport system but which cannot

be phosphorylated will not compete with D-glucose uptake. α-D-Fucose (6-deoxy-D-galactose), which lacks a free hydroxyl group on carbon 6 not only failed to compete with D-glucose transport (as reported in section 2 of the results) but the sugar itself was not transported by the cells. Thus a transport process similar to that found in yeast (106, 107) may be operative in this primitive fungus. In order to support the presence of either a phosphoenolpyruvate phosphotransferase system or this phosphorylation phenomenon, one has to account for the observation that 2-deoxy-D-glucose is accumulated mainly as the free sugar. A possible explanation is that the sugar is dephosphorylated after being transported. This is a rather wasteful process. It could well be that D-glucose transport in Achlya is mediated by a proton dependent cotransport system as described recently by Slayman and Slayman for N. crassa (98). The presence of intracellular phosphorylated sugars can then be ascribed to the activity of the intracellular glucokinase rather than to its metabolism during uptake.

(7) Citrate Inhibition of Amino Acid and D-Glucose Transport

Ca^{++} has been found to be essential for both

growth and metabolism throughout the developmental life cycle of Achlya (10, 32). Mg^{++}, Mn^{++}, Fe^{++} and a few other divalent cations could not replace Ca^{++} in overcoming citrate inhibition of growth (10). Also citrate inhibition of amino acid transport could be reversed by the addition of exogenous Ca^{++} (10). Citrate, a tricarboxylic acid, is a good chelating agent of divalent cations. It is most likely that citrate deprives the organism of Ca^{++} through chelation. The impermeability of the cell membrane to citrate (10) suggests that this chelator may be removing Ca^{++} from sites located on the outer surface of the cell membrane. Similar rationale will support the effects of EDTA and EGTA on metabolite transport. Furthermore, citrate non-competitively inhibited transport of D-glucose and all the amino acids tested. This is illustrated by Fig. 16, 17, 18, 19, 20 and 22. Only the rates of uptake of these metabolites were affected while their affinities for their respective transport carriers remained unchanged. Also, short term rate-concentration studies clearly indicated that citrate inhibited only active transport processes (59). Thus it is conceivable that Ca^{++} is a vital component of the metabolic energy system which drives the various active transport systems. Ca^{++} could also be an essential

component for maintaining the integrity of the cell membrane and thus for its proper functioning. Ca^{++}, as suggested by Cameron and LéJohn play other important intracellular roles in Achlya besides that in transport (10).

(8) Inhibition of Sugar Transport by Cytokinins

It is well documented that not all known cytokinins are effective in every plant system tested (29). Also, in many instances the type of response evoked is concentration dependent (29). The inhibition of D-glucose transport by cytokinins appears to be specific (as illustrated by table IV). For example, 6ipAdo was ineffective as compared to 6ipAde, the only structural difference being that the former has a ribose moiety. Surprisingly, zeatin [N^6-(trans-4-hydroxy-3-methylbut-2-enyl) adenine] was not inhibitory at the concentration used. Fig. 26 shows that the pH dependent-D-glucose uptake system was inhibited by 6ipAde throughout the pH range tested. This implies that this cytokinin is affecting some physiological process of the fungus. Fig. 25 and 2 show that 6ipAde noncompetitively inhibited the transport of D-glucose and 2-deoxy-D-glucose. Also the cytokinin

effect on active transport systems is very rapid (LéJohn and Stevenson, in press). These results indicate that cytokinins are acting on other membrane sites distinct from those involved with sugar transport.

Cytokinins might act by removing Ca^{++} from the Ca^{++} binding glycopeptide (58). This change could in turn affect the structural integrity of the cell membrane and thereby impair the various transport systems within it. It is not known whether this organism produces such plant growth hormones. If it does, then they may be produced when the extracellular medium can no longer support vegetative growth (e.g. low sugar and amino acid concentrations) and sporulation is induced. The Ca^{++} taken up (58) could be used for cell wall and membrane assembly.

(9) Involvement of Ca^{++} In Citrate And Cytokinin Inhibition Of Sugar Transport.

Ca^{++} is an indispensible element in the diet of this fungus (10). Characterization of the Ca^{++} transport process in this organism (58) revealed a two component system consisting of a low molecular weight Ca^{++} binding glycopeptide located in the cell wall-membrane complex and a Ca^{++} transport carrier resident in the cell membrane.

Citrate, as discussed, is a strong chelator of divalent cations. Also this tricarboxylic acid inhibited noncompetitively the active transport of sugars, amino acids (see Fig. 16, 17, 18, 19, 20, 21 and 22) (96) and nucleosides (100). Cytokinins inhibited transport of such metabolites and precursors in a similar fashion (100, 96) (see Fig. 2 and 25). Furthermore, both these inhibitors appear to affect only membrane functions. This is supported by the fact that citrate does not penetrate the cell membrane (10). In the case of cytokinins, the inhibition of metabolite uptake is rapid (LéJohn and Stevenson, in press) but yet after as long as a 30 min incubation (using labelled 6ipAde) little or no cytokinin was transported into the cell. Most of the cytokinin taken up was bound to the cell wall-membrane complex (LéJohn and Stevenson, in press). These facts suggest that the citrate and cytokinin effects may be mediated through their regulation of the amount of Ca^{++} made available to the cells. Ca^{++} is probably an element that is used in the active transport systems in this fungus. Deprivation of Ca^{++} could then lead to either a cessation of energy generation or an uncoupling of the transport process from the metabolic energy system

which drives it. Cytokinins, besides being intimately involved with the Ca^{++} binding glycopeptide (58), may also be affecting other membrane sites distinct from those involved in transport (Stevenson and LéJohn, in press).

CONCLUSION

I have shown that D-glucose and 2-deoxy-D-glucose, but not D-fructose and glycerol are transported by D-glucose grown cells of Achlya. The germlings are able to accumulate sugars against a concentration gradient, which implies the presence of an active transport system. This is further supported by the fact that metabolic poisons inhibited the transport process.

D-glucose transport is temperature and pH dependent. Competition studies demonstrated that the D-glucose transport system is stereospecific. Also sulfhydryl reagents like Hg^{++}, I_2 and NEM inhibited transport. These observations strongly suggest that D-glucose may be transported by a protein-mediated system.

D-glucose was rapidly phosphorylated when transported. Based on the data obtained, it would be quite speculative to suggest the possible mechanism(s) by which Achlya transports D-glucose and other related sugars.

Cytokinins play very important regulatory roles in the growth of this organism by exerting their effects, at least, through two membrane located entities; a Ca^{++} sequestering glycopeptide and a Ca^{++} uptake system.

It is realized that <u>in vitro</u> studies using the isolated D-glucose transport system are essential to further support the findings reported in this thesis. However the difficult task involved in isolating such systems is well appreciated.

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