TRANSPORT OF PURINE BASE(S) AND NUCLEOSIDE (S) IN CANDIDA ALBICANS CELLS

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T. VENUGOPALA RAO

SCHOOL OF LIFE SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
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C O N T E N T S

PREFACE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted so far, in part or full for any other degree or diploma of any university.

T. Venu Gopala Rao

T. VENUGOPALA RAO
Candidate

Rajendra Prasad

Dr. RAJENDRA PRASAD
Supervisor

P.C. Kesavan

Prof. P.C. KESAVAN
Dean

MARCH, 1982.

SCHOOL OF LIFE SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI 110067 INDIA.

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T.VENUGOPALA RAO

ABBREVIATIONS

A	:	Absorbance
ADP	:	Adenosine diphosphate
AMP	:	Adenosine monophosphate
ATP	:	Adenosine triphosphate
dADP	:	Deoxyadenosine diphosphate
dAMP	:	Deoxyadenosine monophosphate
dATP	:	Deoxyadenosine triphosphate
CTP	:	Cytidine triphosphate
dCTP	:	Deoxycytidine triphosphate
p-CMBS	:	p-Chloromercuribenzene Sulphonic acid
DNA	:	Deoxyribonucleic acid
GDP	:	Guanosine diphosphate
GMP	:	Guanosine monophosphate
GTP	:	Guanosine triphosphate
dGDP	:	Deoxyguanosine diphosphate
dGMP	:	Deoxyguanosine monophosphate
dGTP	:	Deoxyguanosine triphosphate
IDP	:	Inosine diphosphate
IMP	:	Inosine monophosphate
ITP	:	Inosine triphosphate
Ki	:	Kinase
NAD	:	Nicotinamide adenine dinucleotide
NADH	:	Reduced NAD^+

$NADP^+$	:	Nicotinamide adenine dinucleotide phosphate
NADPH	:	Reduced $NADP^+$
NEM	:	N-Ethylmaleimide
POPOP	:	Phenyl oxazolyl phenyl oxazolyl phenyl : 1;4 bis (2-(5 phenyl oxazolyl)-benzene
PPO	:	2,5 Diphenyloxazole
PRPP	:	5-Phosphoribosyl 1-pyrophosphate
RNA	:	Ribonucleic acid
R-1-P	:	Ribose - 1 - phosphate
TCA	:	Trichloroacetic acid
TTP	:	Thymidine triphosphate
UTP	:	Uridine triphosphate
XDP	:	Xanthosine diphosphate
XMP	:	Xanthosine monophosphate
XTP	:	Xanthosine triphosphate

INTRODUCTION

One of the important functions of the cell membrane is maintenance of selective permeability in both the temporal and physiological senses. The lipid bilayer impermeable to hydrophilic substances surrounding the cell retains all nutrients within the confines of the cell (Lieb and Stein, 1974). Metabolic waste products leave the cell, cells communicate with one another via chemical messengers and cells utilize a number of essential and useful nutrients present in the extracellular space. To make use of nutrients from extracellular space, cells have evolved mechanisms for the uptake of nutrient molecules which are unable to permeate through a lipid bilayer.

Eukaryotes possess two general types of mechanism for bringing small nutrient molecules into the cell: 1) an energy-dependent transport system able to establish a concentration gradient across the cell membrane (Christensen, 1975) and 2) a non concentrative transport system coupled with phosphorylation or phosphoribosylation of the substrate, by which the metabolic alteration prevents the nutrient from exiting the cell (Plagemann and Richery, 1974; Berlin and Oliver, 1975).

Among the many studies of nutrient uptake the study of nucleic acid precursors has acquired a new significance. This followed the discovery of specific membrane bound "carrier systems" (Berlin and Oliver, 1975) and the possible relationship of the uptake of nucleic acid precursors with cell growth or proliferation (Holley, 1972). In many instances, it has been observed that the rates of nutrient uptake have been related to the availability of exogeneous substrate, effect of inhibitors, and temperature. There are many observations that the rate of uptake of particular nutrient responds to the physiological demand. Thus, for example, the uptake of uridine in quiescent cells is increased soon after stimulation with serum or hormones (Cunningham and Pardee, 1969; Hare, 1972; Jimenez de Asua et al., 1974). The correlation between enhanced nutrient uptake and stimulation of cell growth or proliferation showed that modulation of nutrient uptake is mainly involved in the regulation of cell proliferation (Holley, 1972; Bhargava, 1977). Further it was observed that modulation of uptake is essentially a membrane phenomenon i.e. the messages are making impact on the cell membrane directly and causing altered permeability of the membrane toward specific nutrients. On the other hand, there is

considerable evidence that intracellular phosphorylation determines the rate of uptake of nucleosides and nucleobases under variety of conditions (Wohlhueter et al., 1976; Rozengurt et al., 1977; Marz et al., 1979).

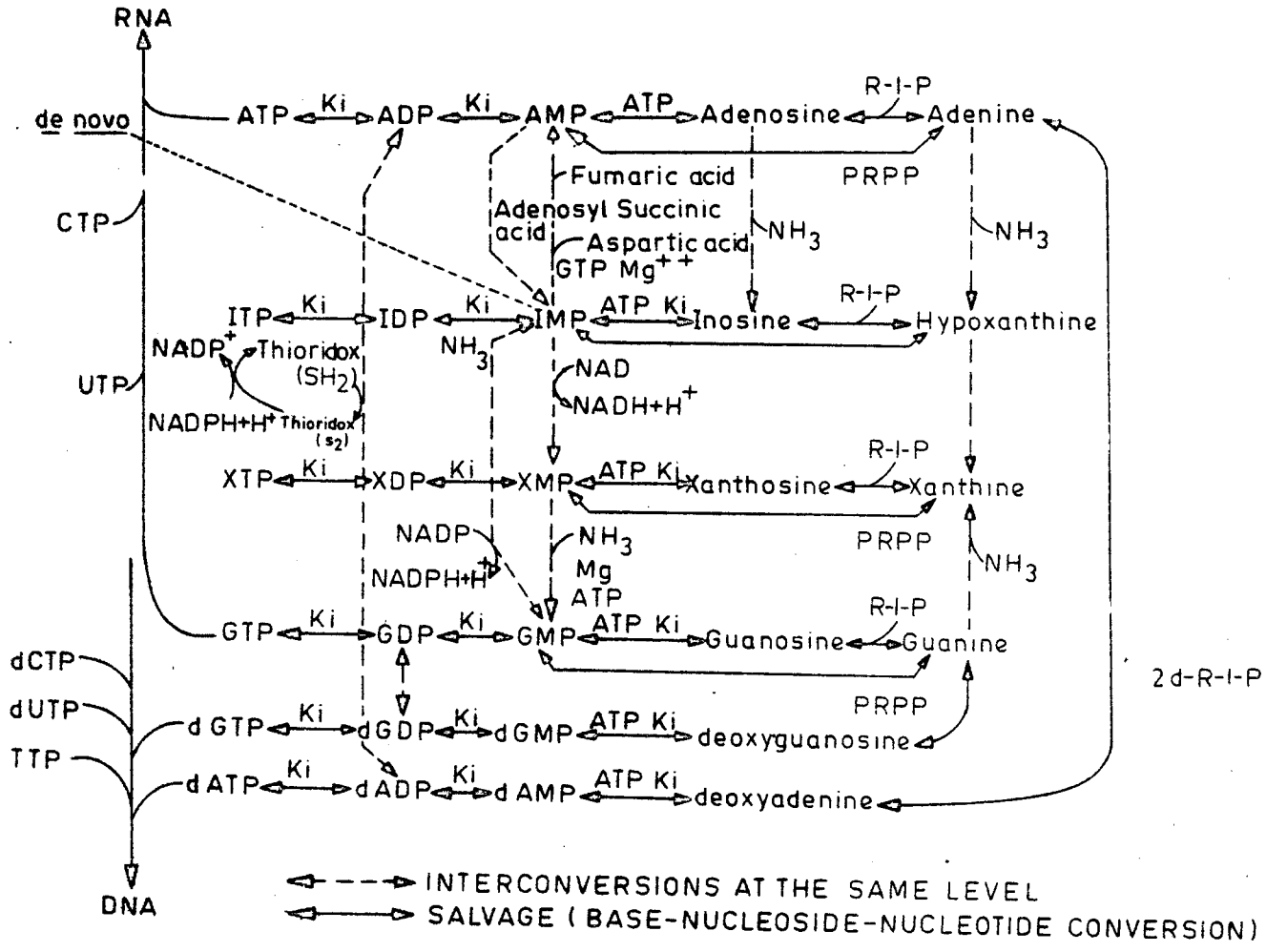
The control of purine utilization presents some unique problems since the availability of corresponding nucleotides depends on the interaction of two converging pathways : 1) the de novo synthesis of nucleotides (Buchanan and Hartman, 1959). and 2) The reutilization pathway through which free nucleobases are transferred to the phosphoribosyl moiety of 5-phosphoribosyl-1-pyrophosphate which builds up the level of nucleoside monophosphate (Kornberg et al., 1955). These nucleoside monophosphates are interconvertible among each other and this interconversion is itself under stringent enzymatic control (Berlin and Stadtman, 1966). Nucleoside monophosphates are also interconvertible with both the corresponding nucleoside diphosphates and triphosphates through the action of phosphotransferases as well as by different kinases. Furthermore, the ribonucleotides are convertible to deoxyribonucleotides through their [illegible] nucleoside diphosphates. Thus, there are a number of structurally related compounds (they

all share phosphoribosyl moieties) which are the "end products" of nucleobase pathways and which could conceivably act in retroinhibition. The conversion of free purines to their mononucleotide derivatives involves a transfer of the phosphoribosyl moiety of 5-phosphoribosyl-1-pyrophosphate to the purine base and is catalyzed by purine phosphoribosyltransferases in yeast (Mager and Magasanik, 1960), mammalian tissues (Murray, 1966) and bacteria (Berlin and Stadtman, 1966) (Fig. 1).

Uptake of Nucleosides

(a) In animal cells - The uptake of nucleosides has been studied in a variety of cultured animal cells e.g. Chick fibroblasts (Scholtissek, 1968; Steck et al., 1969), sea urchin egg cells (Piatigorsky and Whiteley, 1965), Ehrlich ascites tumor cells (Jacquez, 1962), erythrocytes (Templeton and Chilson, 1981), Novikoff rat hepatoma cells (Plagemann, 1971; Plagemann and Erbe, 1972; Plagemann et al., 1978; Wohlhueter et al., 1978; Marz et al., 1977, 1978), Chinese hamster ovary cells (Wohlhueter et al., 1978; Prasad et al., 1981) and Balb 3T3 cells (Hochstadt and Quinlan, 1976; Quinlan and Hochstadt, 1976; Prasad et al., 1981). In the case of animal cells

Fig. 1. METABOLIC PATHWAYS - PURINES



both mediated process (Plagemann and Richery, 1974) as well as group translocation (Quinlan and Hochstadt, 1976) have been shown to operate for the uptake of nucleosides.

Mediated process - It has been established that the transport of nucleosides in several mammalian cell lines involves a saturable, non-concentrative mechanism consistent with carrier-mediated permeation. Such transport has been reported in Novikoff rat hepatoma cells and Chinese hamster ovary cells, for thymidine, uridine, deoxycytidine, cytosine arabinoside (Wohlhueter et al., 1976, 1979; Plagemann et al., 1978). All these studies were focused on the initial uptake of substrates rather on the longer time intervals, where the uptake process has been shown to depend on cellular metabolism. Kinetic data obtained by rapid sampling technique revealed that the cells incapable of metabolizing the transport substrate had low affinity (high K_m) as compared to the metabolizing cells. Kinetic analysis of thymidine transport in Novikoff cells provided evidence for the existence of a single carrier having broad specificity for purine nucleosides, pyrimidine nucleosides, deoxynucleosides and a number of nudleoside analogues. This broad specificity of carrier is based on inhibition of thymidine transport by other nucleosides and analogues (Wohlhueter et al., 1979). The

decrease in nucleosides uptake was reported in Novikoff rat hepatoma cells during the time intervals of 1 to 10 minutes. It was concluded that the decrease was mainly due to the metabolic unstability of nucleoside transporters (Plagemann et al., 1975). However, by using a newly developed "rapid sampling technique" Marz et al., (1978) have shown that the thymidine and uridine nucleosides transport increases linearly upto 10 seconds and start decreasing at later time points. It is concluded that intracellular phosphorylation determines the rate of uptake of thymidine and uridine for later time points i.e. after 10 seconds. Furthermore, in contrast to the earlier observation of Plagemann et al., (1975), it has been shown that the nucleoside transporters are metabolically stable and the decrease in the rate of nucleoside uptake beyond the time points longer than 10 seconds are mainly due to the decreased protein synthesis which reflected loss of nucleoside kinase activities (Marz et al., 1977 and 1978). These kinases rather than the membrane associated transport apparatus are the most likely sites for regulation of nucleoside uptake (Marz et al., 1978).

Scholtissek (1968) first suggested the role of

kinases in nucleosides uptake, and proposed a model "nucleoside - kinase complex" in which nucleosides uptake is mediated by the nucleoside-kinase complex in the membrane. But studies of thymidine uptake in normal and thymidine-kinase defficient hamster cells provided evidence that thymidine kinase regulates transport by interacting reversibly or irreversibly with membrane carrier (Schuster and Hare, 1970). However, its was observed that the kinase is rather limiting for intracellular utilization of nucleosides and not for its transport across the membrane (Hare, 1970).

Group translocation - Though it is not clear whether the cleavage of purine nucleosides is obligatory or even, the studies by using isolated vesicles from a variety of animal cell lines have provided evidence that the cleavage occurs to a significant extent (Hochstadt, 1974; Hochstadt and Quinlan, 1976; Quinlan and Hochstadt, 1974, 1976; Li and Hochstadt, 1976; Prasad et al., 1981). Studies of inosine uptake by membrane vesicles from mouse fibroblast cells indicated that the uptake of inosine involved a concomitant cleavage to ribose - 1 - phosphate and hypoxanthine. This mechanism involved a membrane localized purine nucleoside phosphorylase acting in a reaction during which hypoxanthine was released on the

exterior membrane surface while the ribose moiety was phosphorylated in the process of being transported across the membrane (Quinlan and Hochstadt 1976). This mechanism supported "group translocation". In membrane vesicles from polyoma-transformed hamster kidney cells, two distinct mechanisms for inosine uptake were found; carrier mediated diffusion as well as group translocation mechanism (Dowd et al., 1977).

Quinlan and Hochstadt (1976) proposed various models to demonstrate that the ribose moiety of inosine is accumulated as ribose - 1 - phosphate by plasma membrane vesicles isolated from SV-40 transformed Balb/C 3T3 cells. First model represents that the phosphorolysis takes place prior to the transport, such that ribose - 1 - phosphate itself serves as the actual transport substrate. Second model represents a mechanism in which phosphorolysis is carried out in a single step, referred to as a group translocation event. Here plasma membrane associated purine nucleoside phosphorylase which mediates the intravesiculation of ribose - 1-phosphate subsist the group translocation reaction. Third model explains the post-transport phosphorolysis. That is, inosine is transported into the vesicle intact and then is acted upon by intravesicular purine nucleoside

phosphorylase. The ribose - 1 - phosphate remains inside the vesicle, whereas the hypoxanthine is diffused out of the vesicle. The existence of this mechanism could mean that two separate molecules are there for transport event and metabolic event, which have different characteristics in terms of effector sensitivities and kinetic constants (Fig. 2).

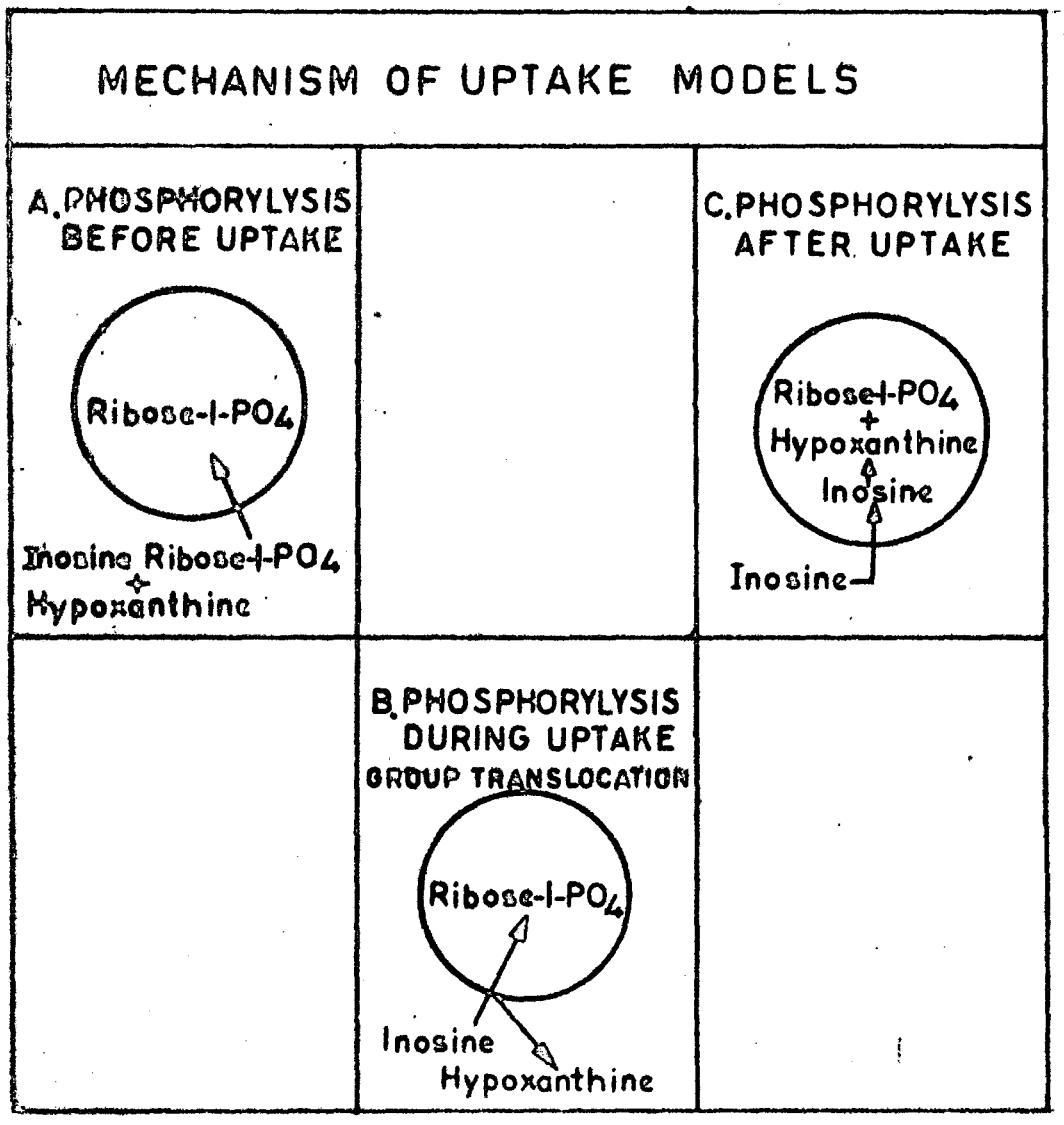
(b) In yeast and other fungi - A few studies have been carried out in yeast and other fungi detailing possible mechanisms of nucleic acid precursors transport. Various studies on transport mechanisms of nucleosides have demonstrated the lack of 'group translocation' mechanism in various yeasts and other fungi. There are reports that two distinct nucleoside transport systems are generated during the germination period of Neurospora crassa conidia: one was specific for purine nucleosides and other was common for both purine and pyrimidine nucleosides (John and Terry, 1970). In Streptomyces antibioticus the transport of adenosine was reported without cleavage which suggested mediated diffusion process. In Saccharomyces cerevisiae, however, the uptake of uridine nucleoside was shown to be an active process mediated via a specific permease (Losson et al., 1978). Studies on nucleosides transport in vacuoles

Fig. 2

A) FIRST MODEL

B) SECOND MODEL

C) THIRD MODEL



isolated from Saccharomyces cerevisiae showed that guanosine and adenosine transporting systems were saturable while the inosine transport was concentration dependent (Nagy, 1979).

Initially the accumulation of various purine compounds in yeast cells was observed by ultraviolet microscopy (Svihla et al., 1963). These observations demonstrated the presence of purine pools. The purine bases and nucleosides may all enter these purine pools prior to their utilization (Svihla et al., 1963). The existence of two distinct intracellular pools of purines i.e. a main pool, characterized by a rapid turnover, and a slowly metabolized storage pool was later confirmed by Cumming and Mitchison (1967). Depending on the yeast species, the differences in the pool contents were also observed (Cummins and Mitchison, 1967).

Uptake of Nucleobases

Exogeneous purine bases are utilized efficiently by cultured cells. In mammalian cells the salvage pathways comprise of 1) mediated permeation through the cell membrane followed by phosphoribosylation, which yields directly purine riboside monophosphates (Hochstadt and Dowd, 1973; Quinlan and Hochstadt, 1974;

Prasad et al., 1981) and 2) Carrier mediated, saturable, non-concentrative transport system where there is no change in transported substrate (Hawkins and Berlin, 1967; Plagemann and Erbe, 1972; Templeton and Chilson, 1981).

(a) In animal cells - Kinetic properties were observed for purine transport by rapid kinetic techniques in Novikoff rat hepatoma cells, Chinese hamster ovary cells and P-388 murine Leukemia cells (Zylka and Plagemann, 1975; Sixma et al., 1973). These studies were done in both cells, incapable of metabolizing the transport substrate, as well as in those for steady-state purine uptake in metabolically active cells. It was observed that the apparent K_m and V_{max} values of the transport were generally in the same order of magnitude, but somewhat higher than those of nucleosides. Furthermore, the affinity for carriers was very less (high K_m) in cells incapable of metabolizing the transport substrate than active metabolic cells. Competitive inhibition among the nucleobases implicated two different carriers: one specific for hypoxanthine and guanine, the other for adenine (Zylka and Plagemann, 1975; Sixma et al., 1973). In some cell lines differential inhibition by nitrobenzylthioinosine indicated that both purine and pyrimidine base carriers are kinetically distinct from the nucleoside transporters (Wohlhueter et al., 1978).

As mentioned earlier that the uptake of nucleic acid precursors is dependent either on their utilization in nucleic acid synthesis or on their metabolic transformations. It seems that in some cases this is due to the fact that the uptake system is subject to feedback inhibition by its product (Berlin and Stadtman, 1966; Grenson, 1969). In other cases, the first metabolic enzyme might be directly involved in transport across the membrane. Similar conclusion was drawn by Hochstadt-Ozer and Stadtman (1971) for adenine uptake in Escherichia coli., which appeared to be mediated by the adenine phosphoribosyltransferase.

(b) In yeast cells - Purine transport systems have been demonstrated in the yeast Schizosaccharomyces pombe and Saccharomyces cerevisiae. Various studies in yeast and other fungi detailing possible mechanisms of purine bases have demonstrated the lack of "group translocation" mechanism. Two purine "permeases" were demonstrated in the exponential growth phase of Saccharomyces cerevisiae: 1) adenine permease 2) guanine permease; either recognizing hypoxanthine and some other analogues (Pickering and Woods, 1972). In Schizosaccharomyces pombe, an active transport system common for adenine, guanine and hypoxanthine was described (Pourquie, 1970).

On the other hand, it was shown that purine phosphoribosyltransferase activities have a positive effect on the rate of purine uptake in Schizosaccharomyces pombe (Housset and Nagy, 1977). However, it does not support "group translocation."

A specific cytosine uptake system distinct from both uracil and uridine uptake systems has been characterized on the basis of genetic, physiological and kinetic evidences in Saccharomyces cerevisiae (Grenson, 1969). The presence of a common uptake system shared by adenine, cytosine and hypoxanthine has been shown in Candida albicans and Saccharomyces cerevisiae which does not appear to be mediated by the phosphoribosyltransferase system (Polak and Grenson, 1973).

Significance

Mammals do not have a nutritional requirement for nucleobases and nucleosides because of the mammalian cells and tissues have the capacity to synthesize purine and pyrimidine nucleotides de novo. However, certain tissues lack the synthetic pathways for purines and are apparently dependent on external sources. The source may be either dietary or endogeneous production by other tissues (Pritchard et al., 1970; Murray et al., 1970;

Roux, 1973). Whether capable of de novo synthesis or not, most of the tissues and cells appear to be able to utilize purines, pyrimidines and nucleosides via salvage pathways. Even though, the cultured cell lines possess the de novo synthetic pathways, the energy cost to a cell to synthesize such nutrients de novo is enormous and would lead a markedly slowed growth rate. If exogeneous sources are available, they are utilized to apparent advantage, so that the cells can save comparable amount of energy which is necessary for de novo synthesis.

Studies on transport of nucleic acid precursors in yeast were mostly done by using Saccharomyces cerevisiae and Schizosaccharomyces pombe cells. In addition, not enough work on nucleic acid precursor's transport has been reported by using the rapid sampling technique in yeast. Studies in our laboratory have been directed towards the characterization of various transport systems of Candida albicans, a pathogenic yeast (Jayakumar et al., 1978, 1979, 1981). In the present investigation, we have characterized the mechanism of uptake of guanine and inosine. Studies have been undertaken to elucidate the mechanism of uptake by analyzing the intracellular products at short time and long time

intervals. It is demonstrated that both the substrates (guanine and inosine) are transported by a carrier mediated, saturable process and not by a group translocation mechanism.

MATERIALS AND METHODS

Agar, Peptone and yeast extract were obtained from Difco, Michigan, U.S.A. Purine bases, nucleosides, p-Chloromercuribenzene sulfonic acid (p-CMBS), N-ethylmaleimide (NEM), 2,5 - diphenyloxazole (PPO), 1, 4 bis 2-(5-phenyloxazolyl) benzene (POPOP) were obtained from Sigma Chemical Company, St. Louis, U.S.A. All other reagents were from commercial sources in the highest purity available. Uniformly ^{14}C labelled inosine (524 m Ci/m mol) and 8 - ^{14}C labelled guanine (51 m Ci/m mol) were obtained from Radiochemical Centre, Amersham, England. Maxflow filters (0.45 μm pore size) were obtained from Maxflow, Bombay, India.

Organism and Growth Conditions

Wild type *Candida albicans* 3100 strain was obtained from National Chemical Laboratory, Poona, India. The cultures were grown for 48 hrs on slants containing -

Yeast extract	- 1.0%
Peptone	- 2.0%
Sucrose	- 2.0%
Agar	- 2.0%

All slants were stored at 4°C for 3-4 weeks.

The yeast cells were transferred from a slant into a synthetic minimal medium containing glucose 0.5% (w/v); KH_2PO_4, 0.3% (w/v); $(NH_4)_2SO_4$, 0.3% (w/v); $MgSO_4$, 0.025% (w/v); $CaCl_2$, 0.025% (w/v) and biotin 0.001% (w/v). The cells were grown at 30°C. Cell growth was monitored turbidimetrically by reading the absorbance at 460 nm in a Bausch and Lomb Spectronic-20 Spectrophotometer. For uptake measurements cells growing in mid-exponential phase were harvested by centrifugation (1500 x g for 10 minutes), washed twice with sterile distilled water and suspended in the same before use.

Transport Assay

Yeast cells (180-250 µg protein/ml) in 16 µM Phosphate buffer (pH 6.5) were incubated in a reaction mixture of 0.25 ml. The reaction was initiated by the addition of 0.06 mM radioactive (8-^{14}C) guanine (1 µCi/umol) or 2mM radioactive (U-^{14}C) inosine (1 µCi/umol). At different time intervals, the reaction mixture was immediately diluted to 10-12 folds with chilled distilled water. The diluted suspension was filtered rapidly through a 0.45 µm maxflow filter disc (Maxflow, Bombay, India) and quickly washed three times with 12 ml of cold water. Total time took for washing

was approximately 10 sec. Constant washing procedures were maintained to prevent differences in uptake measurements. The filters were dried and radioactivity retained was counted in a Packard Scintillation Counter (Model 3381) using a Scintillation liquid containing 0.4% PPO (2,5 - diphenyloxazole) and 0.01% POPOP (1,4 - bis 2-(5-phenyloxazolyl benzene)).

Guanine being less soluble in water usually sticks to the filters. Therefore, the uptake measurements are difficult to study due to its trapping. To avoid this difficulty several washing procedures were attempted:

1. Prewashed water filters followed by cold water washings.

2. Prewashed water filters followed by 37°C water washings.

3. Prewashed guanine (500 μM to 660 μM) filters-followed by cold water (4-6°C) washings.

4. Prewashed guanine (500 μM to 660 μM) filters followed by room temperature water (22-25°C) washings.

5. Prewashed guanine (500 μM to 660 μM) filters followed by cold guanine (500 μM to 660 μM) water washings.

The trapping was mainly dependent on the radioactivity and concentration of guanine. Out of several methods, prewashed guanine filters followed by quick washings of room temperature water was found to be satisfactory which had minimum background. The same washing procedure was followed to prevent further variation.

Chromatographic Analysis of Intracellular Contents

After filtrations of reaction mixtures, the filters were transferred into 2 ml of boiling distilled water to elute the intracellular radioactivity. After boiling for 10 minutes the filters were removed, the procedure was repeated twice and the eluates were pooled to be lyophilized. Lyophilized samples were dissolved in 40 to 50 µl distilled water for chromatographic analysis.

Cellulose thin layer sheets (Eastman Chromagram with fluorescent indicator No.6065) were used to separate the intracellular accumulated products by using Butanol: Water : Propionic acid (12.5 : 8.7 :6.2 v/v) solvent system. The spots were visualized under UV lamp. The marked spots were cut into Scintillation vials which were counted in a Packard Scintillation

Counter, using toluene based scintillation fluid.

Competition Experiments

For competition experiments cells were pre-incubated with ten folds concentration of unlabelled bases and nucleosides for 10 minutes. The reaction was then initiated by adding the radioactive guanine or inosine. The reaction was terminated after 30 seconds for inosine and after 4 minutes for guanine.

Protein Estimation

Protein was estimated by the method of Bradford (Bradford, 1976). Yeast cells were boiled in 10% TCA for 30 minutes. After cooling, suspension was centrifuged and the supernatant discarded. After complete removal of TCA from the precipitate, it was dissolved in 0.1N NaOH. To suitable aliquotes (containing 50-100 µg protein) 5 ml of Coomassie Brilliant Blue - G solution (stock solution prepared by dissolving 100 mg of Coomassie Brilliant Blue in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid (v/v) is added in a final 200 ml volume; working solution prepared by adding 4 parts of distilled water to 1 part of stock and filtered), was added. Colour intensity was read after 10 minutes at 595 nm. Bovine serum albumin was used as standard protein.

RESULTS

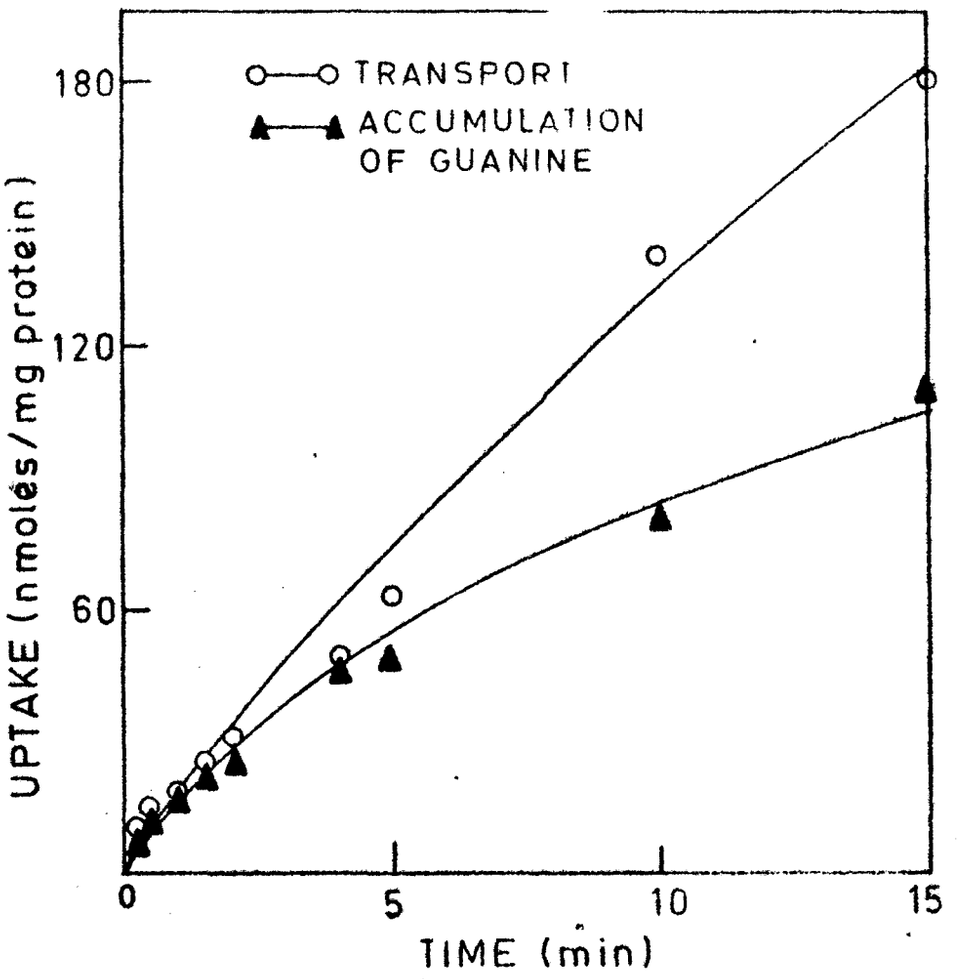
Uptake of Guanine in Candida albicans Cells

As can be seen in the Figure 1, an increase in the uptake of guanine was observed with time. Initially the rate of uptake was linear over a period of 2 minutes. To avoid any complications due to intracellular metabolism, the initial samples were taken out within seconds.

Intracellular products of guanine - Intracellular products were identified during guanine transport as described in Materials and Methods by using Eastman chromatogram sheets (Fig. 1). The results showed that intracellular product which accumulated during the guanine transport was as guanine. The radioactivity recovered was between 90 and 95% upto first 2 min during the uptake, after that the concentration of guanine decreased with time. The decrease in concentration of guanine after 2 min indicated that it might be metabolized by hypoxanthine-guanine phosphoribosyltransferase linked system. It also appeared that intracellular metabolism starts only after few minutes.

Fig. 1 UPTAKE AND INTRACELLULAR ACCUMULATION OF GUANINE IN CANDIDA ALBICANS CELLS

Assay conditions were similar to those described in 'Materials and Methods.'



Accumulation of guanine at different stages of growth - In order to ascertain if there was any difference in the accumulation of guanine during various phases of Candida albicans growth, the uptake of guanine was followed at various stages of cell growth (Fig. 2 inset A, B and C). It was observed that the actively growing cells of mid-exponential phase accumulated more guanine as compared to the cells of other phases (Fig. 2 inset B). However, there was no noticeable difference in guanine accumulation between stationary and lag phase cells (Fig. 2 inset A and C). For all other studies mid-exponentially growing cells were used.

Effect of pH on the guanine uptake - The uptake of guanine was followed at various pH values using various buffers. It was found that initially at lower pH the uptake of guanine was increased with increasing pH but at high pH values (beyond pH 5) its accumulation decreased with increasing pH. The optimum pH was 5 for guanine uptake (Fig. 3).

Kinetics of guanine uptake - The apparent K_m value for guanine uptake was found to be 10 μM. The V_{max} value was 33 n moles/mg protein/min. The K_m value for guanine uptake in Candida albicans was much

Fig. 2 ACCUMULATION OF GUANINE AT DIFFERENT STAGES OF GROWTH OF CANDIDA ALBICANS CELLS

Cells were grown as described in 'Materials and methods" and harvested at indicated times. The assay conditions for uptake studies were similar to as described in 'Materials and Methods'. Insets A, B and C show the uptake of guanine at lag phase, mid-log phase, and stationary phase respectively.

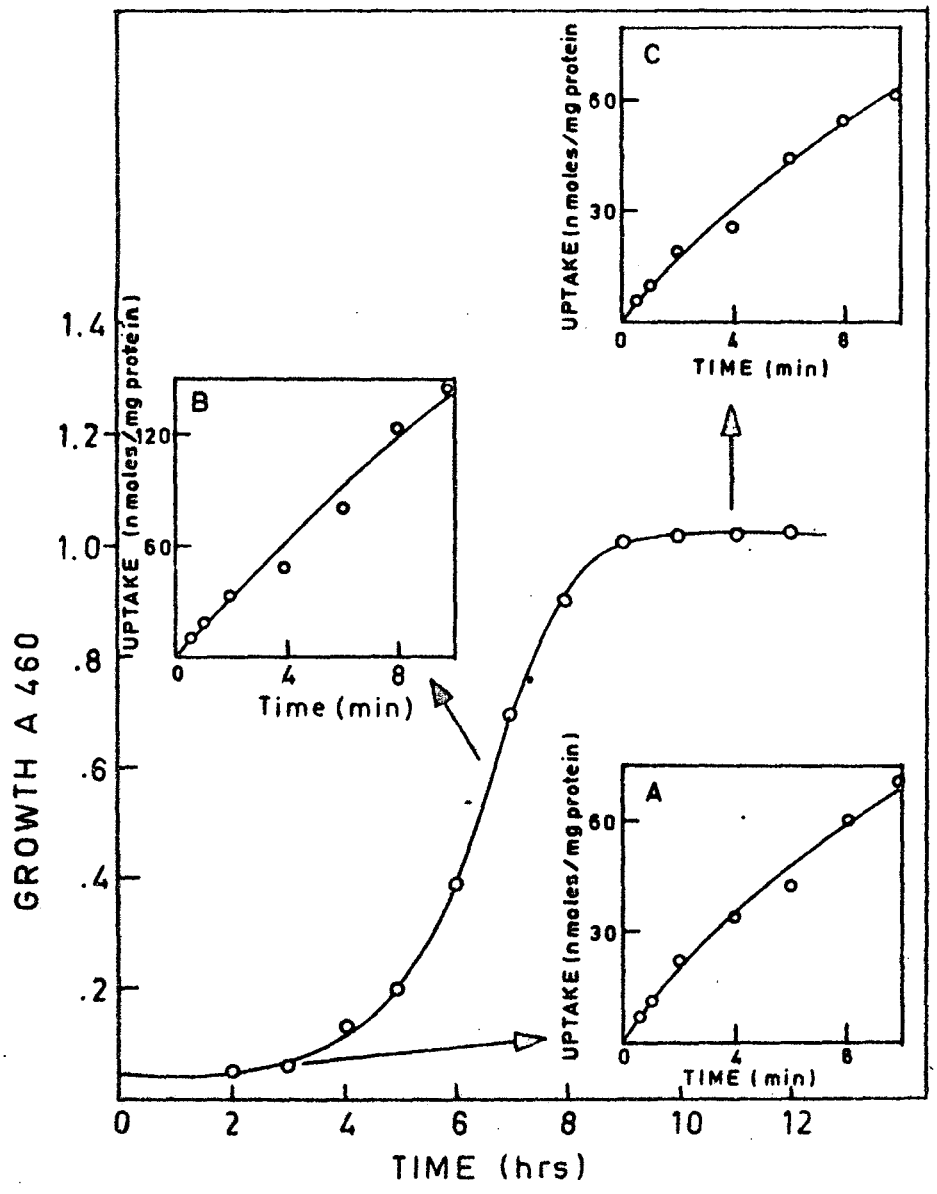
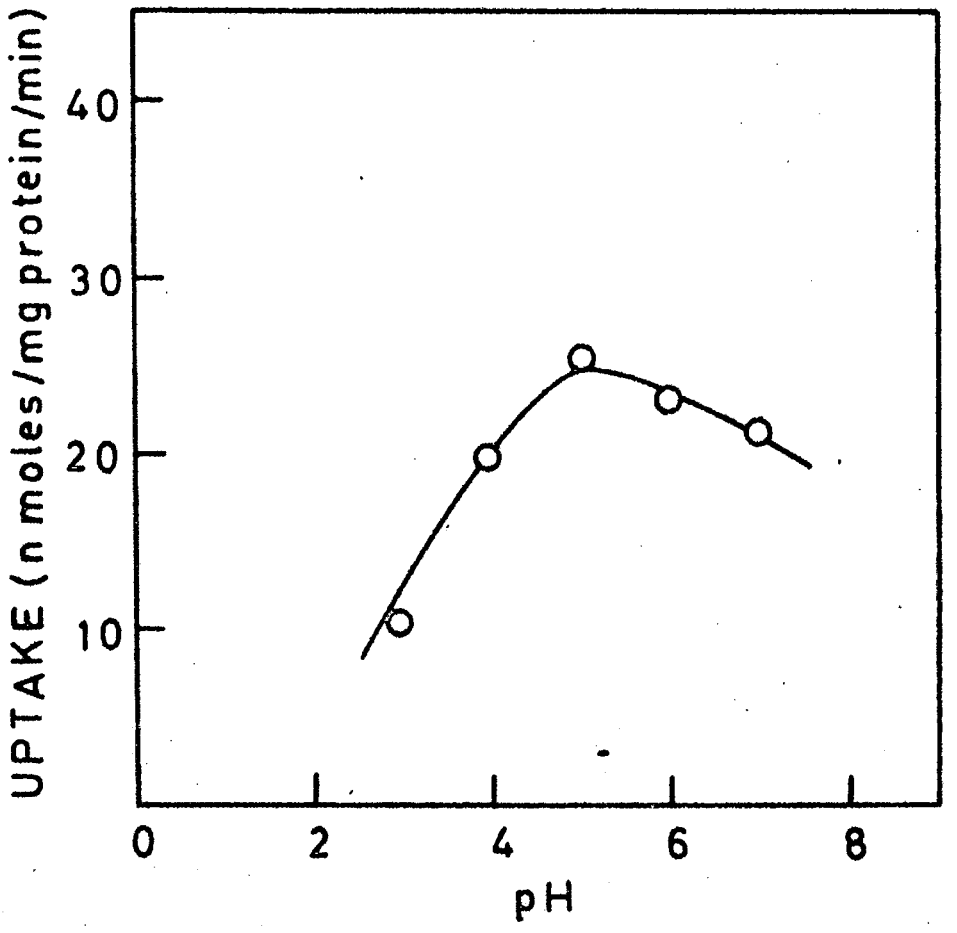


Fig. 3 EFFECT OF pH ON GUANINE UPTAKE BY CANDIDA ALBICANS CELLS.

Cells were suspended in citrate-phosphate buffer (24 mM) of indicated pH values. The uptake of guanine was then assayed as described in 'Materials and Methods.'



higher than the one reported for Schizosaccharomyces pombe (Housset and Nagy, 1977). The inset of Fig. 4 shows the plot of V/S for guanine uptake.

Competition experiments - In order to ascertain if the guanine uptake is mediated via a common or specific permease, the uptake was followed in presence of various other bases and nucleosides. It was observed that adenine and hypoxanthine affected the uptake of guanine maximally, where the inhibition in guanine uptake was between 90 and 92%. Thymine, a pyrimidine base and inosine, a purine nucleoside, had however, inhibited guanine accumulation between 56 and 69% only. On the other hand, uracil had no effect on its uptake, infact it showed 26% stimulation (Table 1).

Uptake of Inosine in Candida albicans Cells

To avoid any complication due to intracellular metabolism the uptake of inosine was also done at earlier time points. As can be seen from the figure (Fig. 5) that the uptake of inosine was linear for first two minutes. However, at later points, it showed slight saturation. The total accumulation of inosine was 60 n moles/mg protein/15 minutes.

Fig. 4 LINEWEAVER-BURK PLOTS OF GUANINE UPTAKE IN CANDIDA ALBICANS CELLS.

All the assay conditions were similar to as described in 'Materials and Methods' with the exception that different concentrations of guanine was used. The reaction was terminated after 30 seconds during which time it was linear. K_m and V_{max} values were calculated from Lineweaver-Burk plots (1/v vs 1/s). Inset shows plot of v/s.

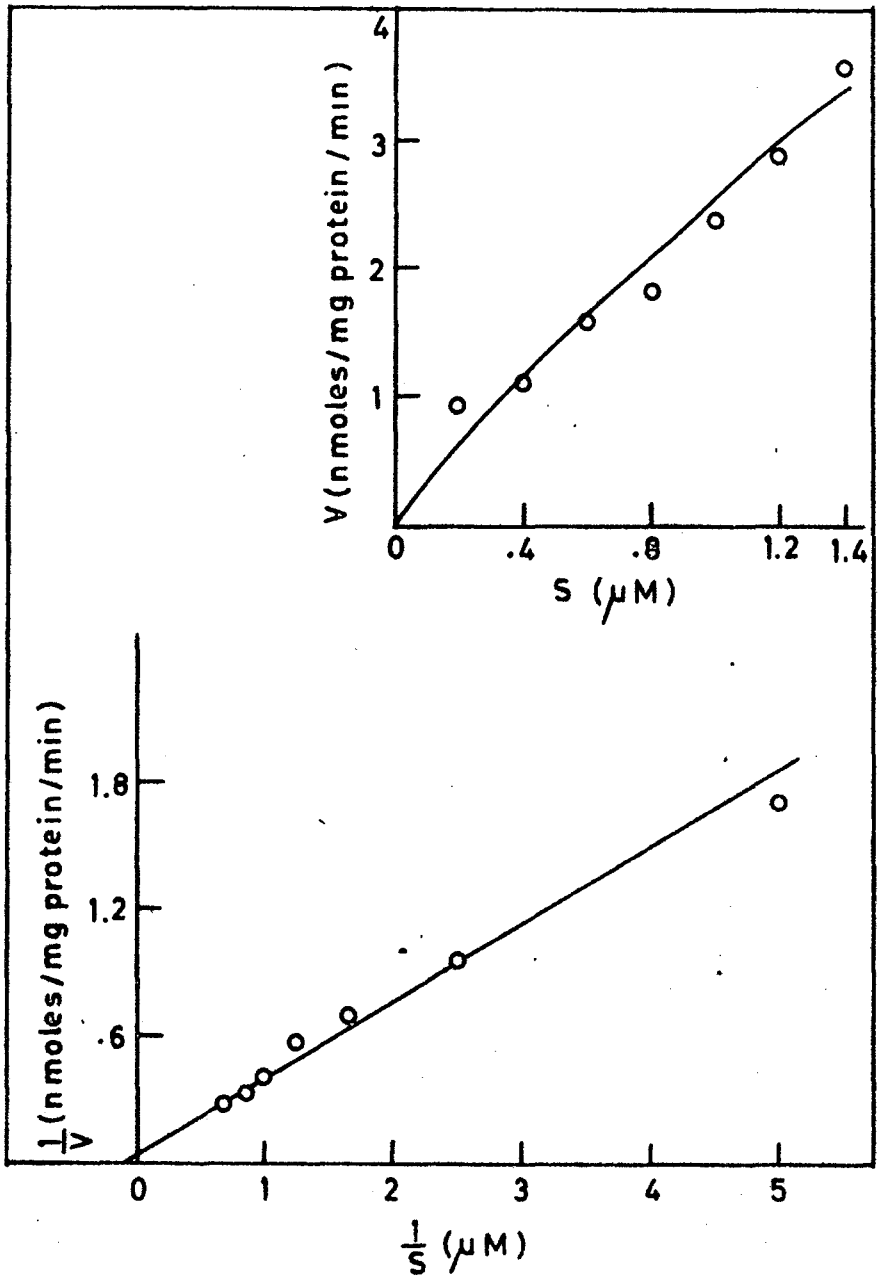


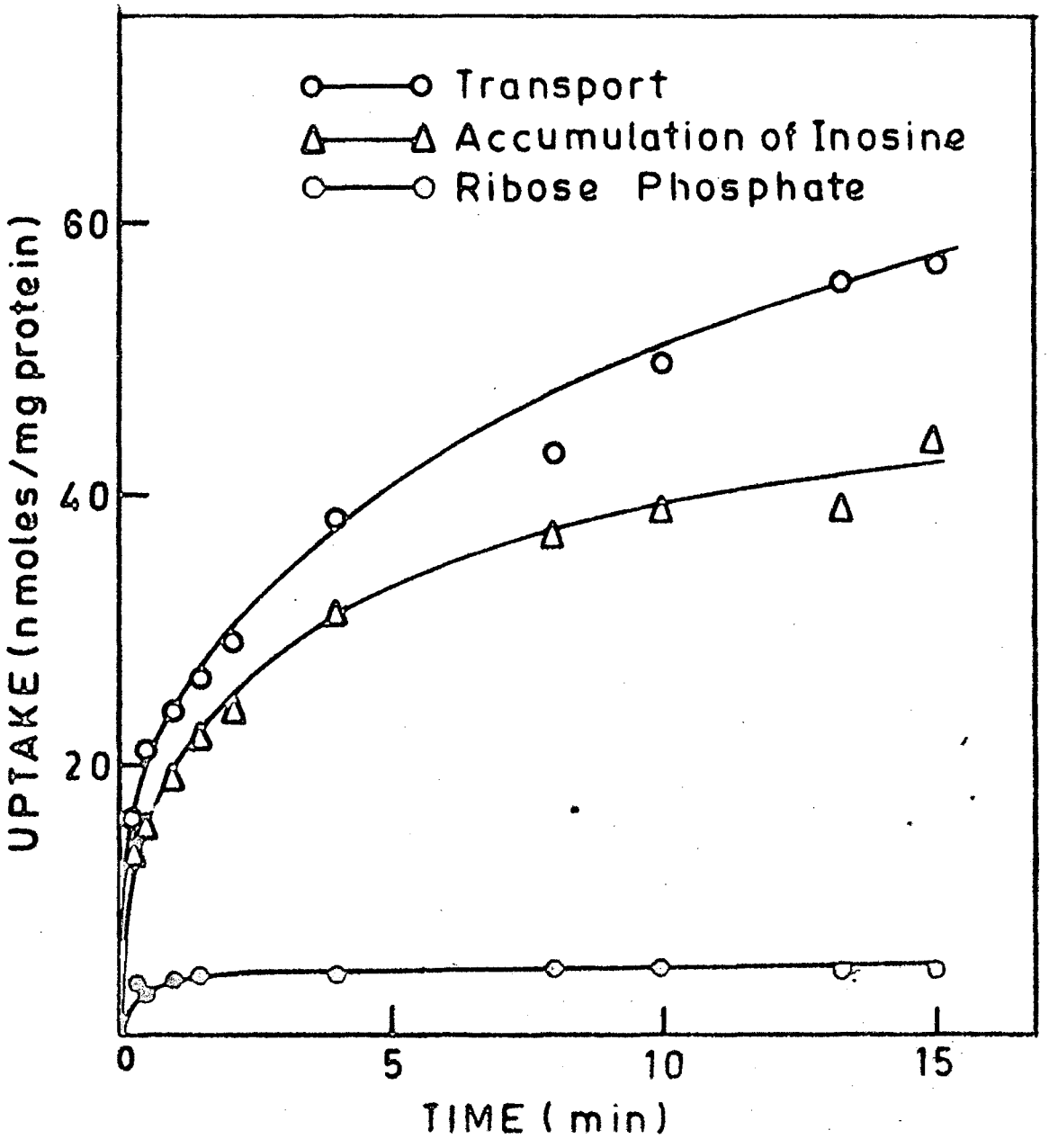
TABLE - 1

THE EFFECT OF ADENINE, HYPOXANTHINE, INOSINE, THYMINE AND URACIL ON GUANINE TRANSPORT

Inhibitor	% inhibition	% stimulation
Adenine	92.4	-
Hypoxanthine	89.6	-
Thymine	56.7	-
Inosine	69	-
Uracil	-	26

Fig. 5 UPTAKE AND INTRACELLULAR ACCUMULATION OF INOSINE IN CANDIDA ALBICANS CELLS.

Assay conditions were similar to those described in 'Materials and Methods.'



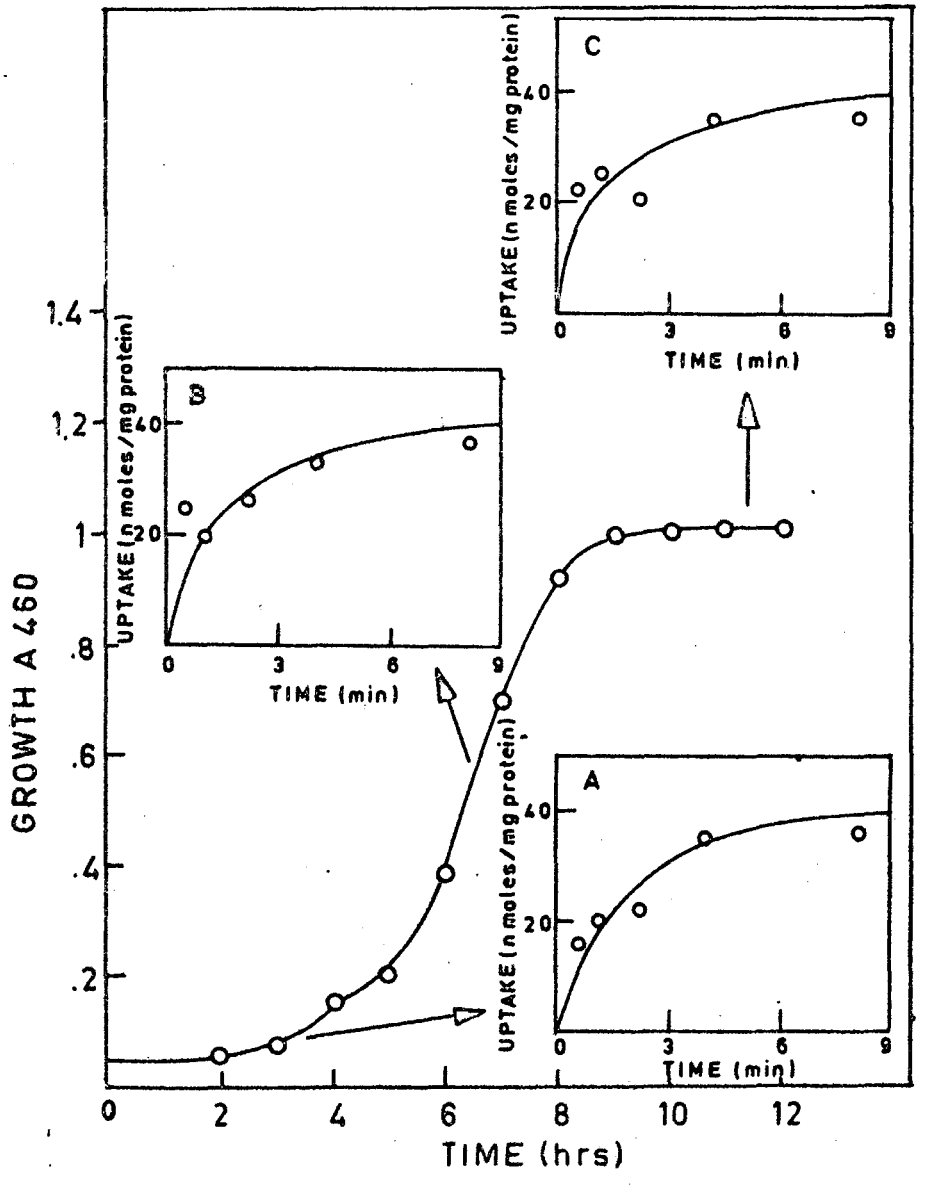
Intracellular products of inosine uptake - On analysing the intracellular products during accumulation of inosine, it was observed that for few minutes most of the radioactivity could be recovered in the form of free inosine (90-95%). However, at later points the percentage of radioactivity recovered was reduced between 60 and 70%. Ribose - 1 - phosphate which is formed due to the action of nucleoside phosphorylase, did not have any noticeable increase in radioactivity during the course of uptake (Fig. 5).

Accumulation of inosine at different steges of growth - Fig. 6 (inset A, B and C) shows the uptake of inosine in lag, midlog and stationary phase of Candida albicans cells growth. As compared to guanine uptake, the uptake of inosine was not affected by the growth stages of Candida albicans. It was observed that uptake of inosine was more or less similar in all phases. For further studies, the mid-exponentially growing cells were used.

Effect of pH on inosine uptake - When the uptake of inosine was followed at various $_{p}H$ values using various buffers, it was found that the uptake of inosine was increased with increasing $_{p}H$ values but at higher $_{p}H$ values (beyond $_{p}H$ 6) the uptake was

Fig. 6 ACCUMULATION OF INOSINE AT DIFFERENT STAGES OF GROWTH OF CANDIDA ALBICANS CELLS.

Cells were grown as described in 'Materials and Methods' and harvested at indicated time. The assay conditions for uptake studies were as described in 'Materials and Methods.' Insets A, B and C show the uptake of inosine at lag phase, mid-log phase and stationary phase respectively.



rapidly decreased (Fig. 7). The optimum pH for inosine uptake was 6.

Kinetics of inosine uptake - The apparent K_m for inosine uptake was found to be 420 µM. The V_{max} value was 27 n moles/mg protein/min. The inset of Fig.8 shows the plot of V/S for inosine uptake.

Competition experiments - In order to asses the multiplicity of inosine transport, the uptake of inosine was done in presence of ten folds concentration of adenine, hypoxanthine, guanine and thymine. The uptake of inosine was inhibited by all these four bases. 1/V vs 1/S plot in presence of these bases exhibited that the inhibition was associated with changes in apparent K_m values. The K_m for inosine was 420 µM and in the presence of adenine, hypoxanthine, guanine and thymine was increased to 1300 µM, 800 µM, 1300 µM and 650 µM respectively (Fig. 9 and 10). Thus inosine uptake was competitively inhibited by all these bases.

Effect of sulfhydryl reagents on inosine uptake - The -SH group is one of the most reactive and ubiquitous ligands in the biological systems. It is involved in many membrane functions. The various sulfhydryl reagents

Fig. 7 EFFECT OF pH ON INOSINE UPTAKE BY CANDIDA ALBICANS CELLS

Cells were suspended in citrate-phosphate buffer (24 mM) of indicated pH values. The uptake of inosine was then assayed as described in 'Materials and Methods.'

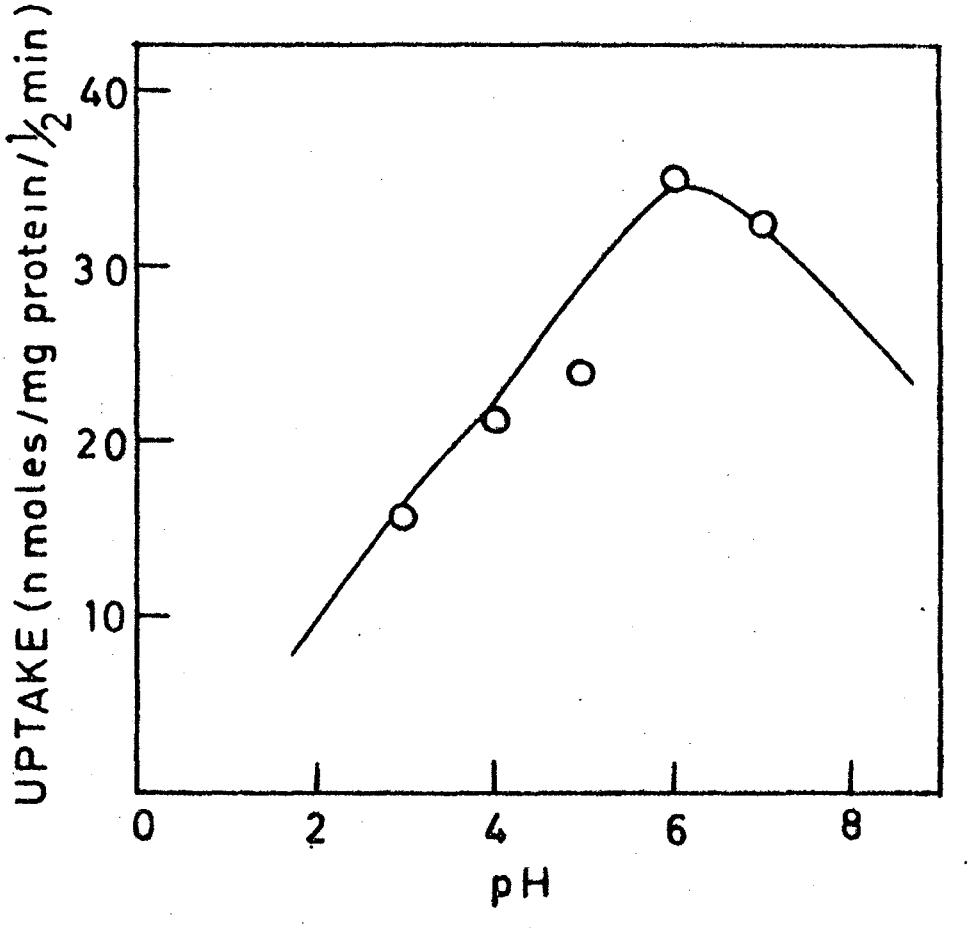


Fig.8 LINEWEAVER-BURK PLOTS OF INOSINE UPTAKE IN CANDIDA ALBICANS CELLS

All conditions were similar to those described in 'Material and Methods' except different concentrations of inosine was used. The reaction was terminated after 30 seconds. K_m and V_{max} were calculated from Lineweaver-Burk plots (1/v vs 1/s). Inset shows the v/s plot.

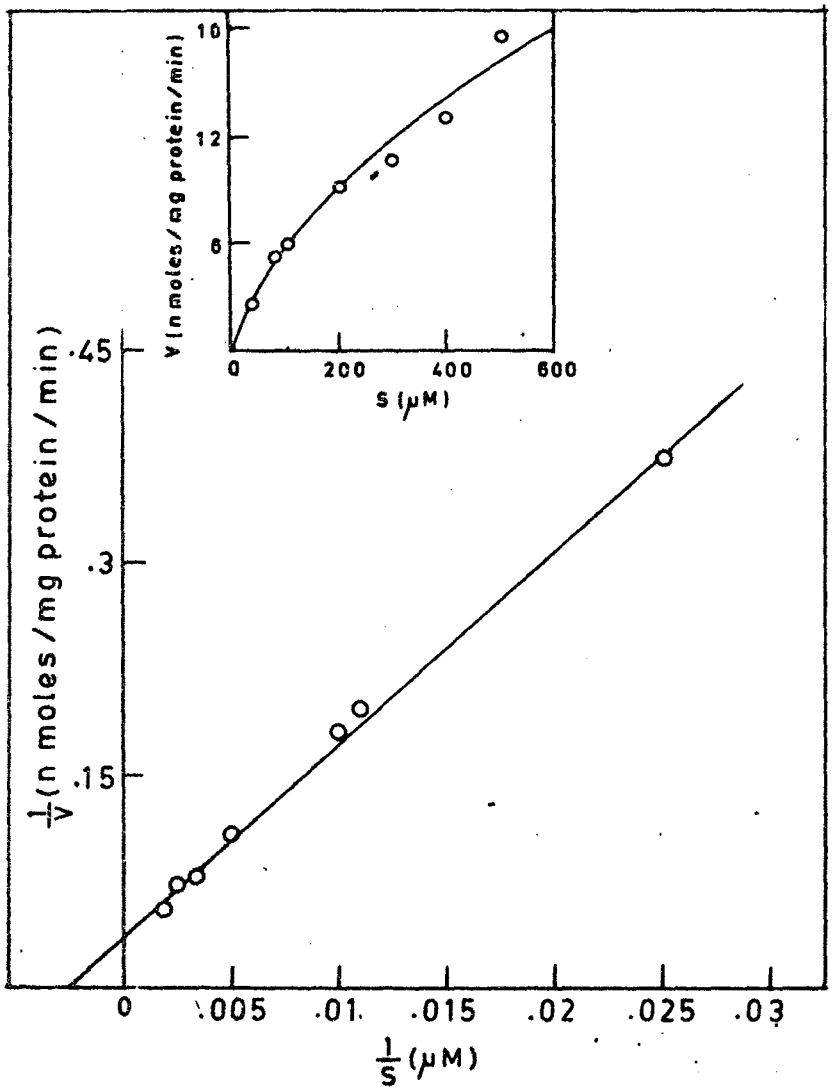


Fig. 9 LINEWEAVER-BURK PLOTS OF INOSINE UPTAKE IN CANDIDA ALBICANS CELLS IN THE PRESENCE OF ADENINE AND HYPOXANTHINE.

Competition experiments were done in a reaction mixture containing cells (20-25 µg protein), 10 mM buffer (pH 6), 20 mM adenine or hypoxanthine in a final volume of 0.1 ml. The reaction mixture incubated for 10 minutes and reaction was initiated by adding 2 mM U-^{14}C-inosine (1 µCi/1 µmole).

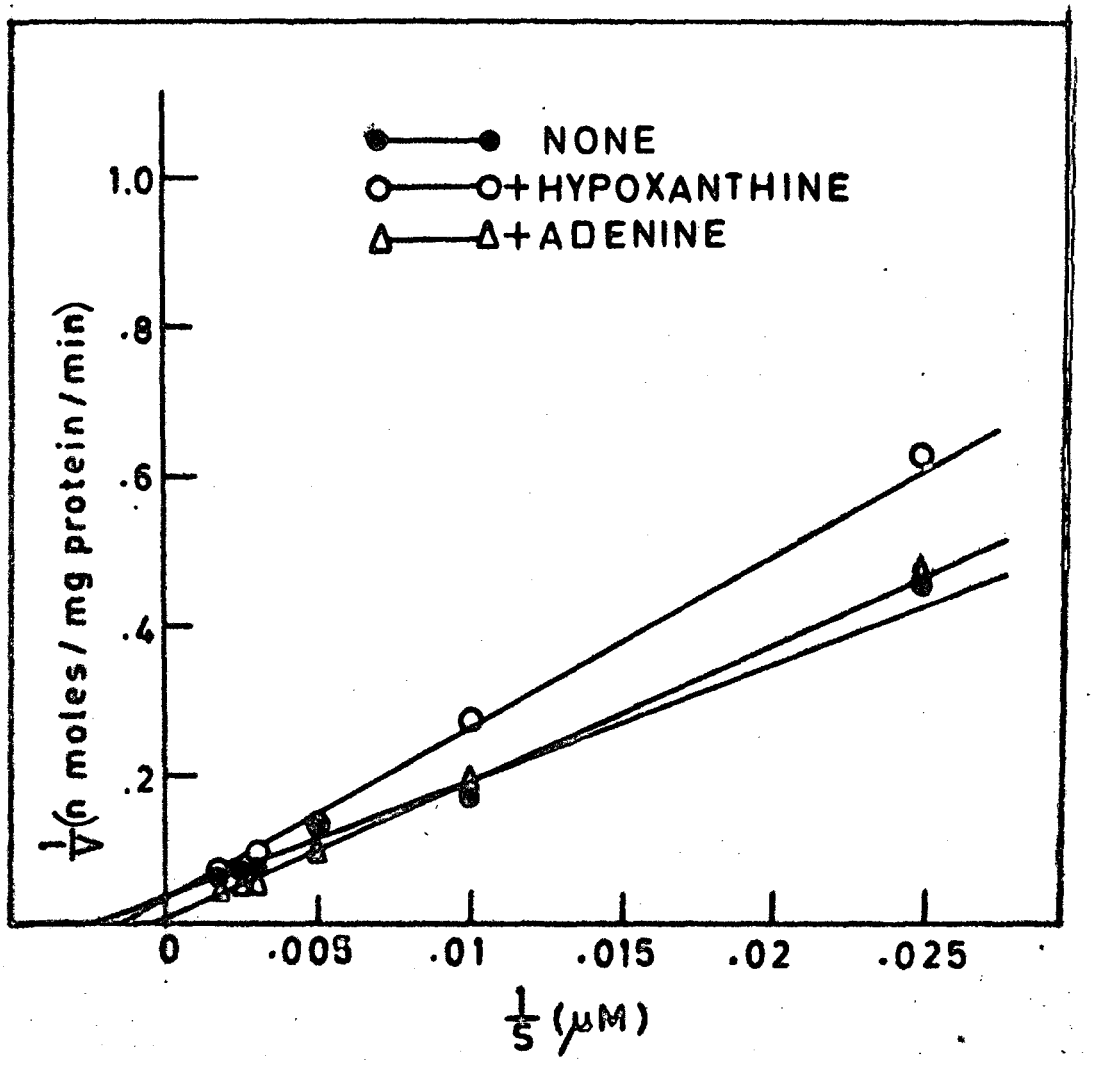
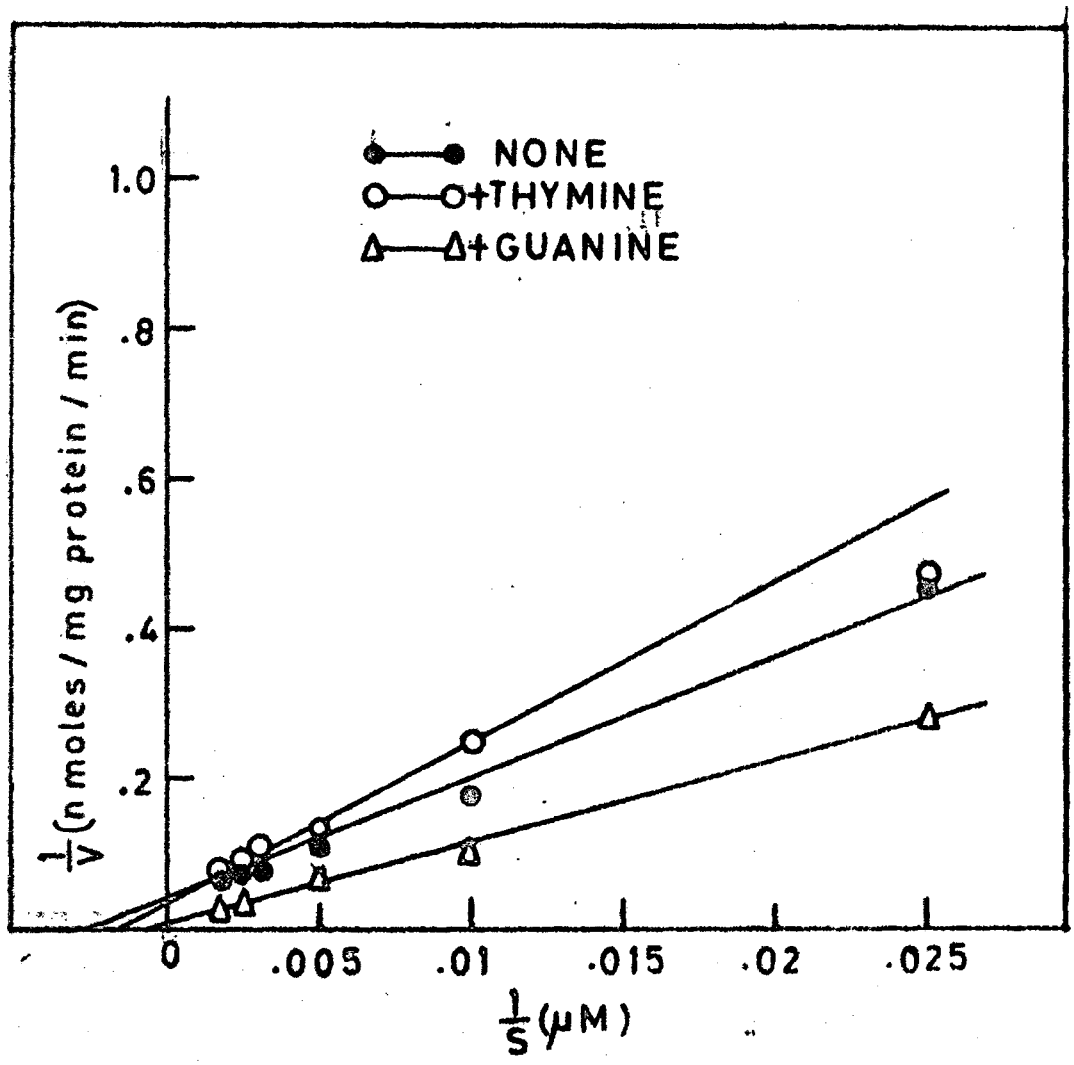


Fig. 10 LINEWEAVER-BURK PLOTS OF INOSINE UPTAKE IN CANDIDA ALBICANS CELLS IN THE PRESENCE OF GUANINE AND THYMINE.

Competition experiments were done in a reaction mixture containing cells (20-25 µg protein), 10 mM buffer (pH 6), 20 mM guanine or thymine in a final volume of 0.1 ml. The reaction mixture incubated for 10 minutes and reaction was initiated by adding 2 mM U-^{14}C inosine (1 µCi/1 µmole).



e.g. N-ethylmaleimide (NEM), p-chloromercuribenzene sulfonic acid (p-CMBS) are effectively used in the transport studies to demonstrate their involvement in membrane transport (Chan and Cossins, 1976). In order to ascertain the involvement of -SH groups in inosine uptake, the effect of both NEM and p-CMBS on inosine transport was investigated. It was found that both NEM and p-CMBS inhibited the uptake of inosine (Fig. 11). The effective concentrations for NEM and p-CMBS were found to be 60 μM and 100 μM respectively (Fig. 12 A and B).

Fig. 11 EFFECT OF NEM AND p-CMBS ON INOSINE UPTAKE IN CANDIDA ALBICANS CELLS

Cells were preincubated for 10 minutes with 60 µM NEM and 100 µM p-CMBS. Radioactive inosine (2mM) (1 µCi/1 µmole) was added to study the uptake as described in 'Materials and Methods.'

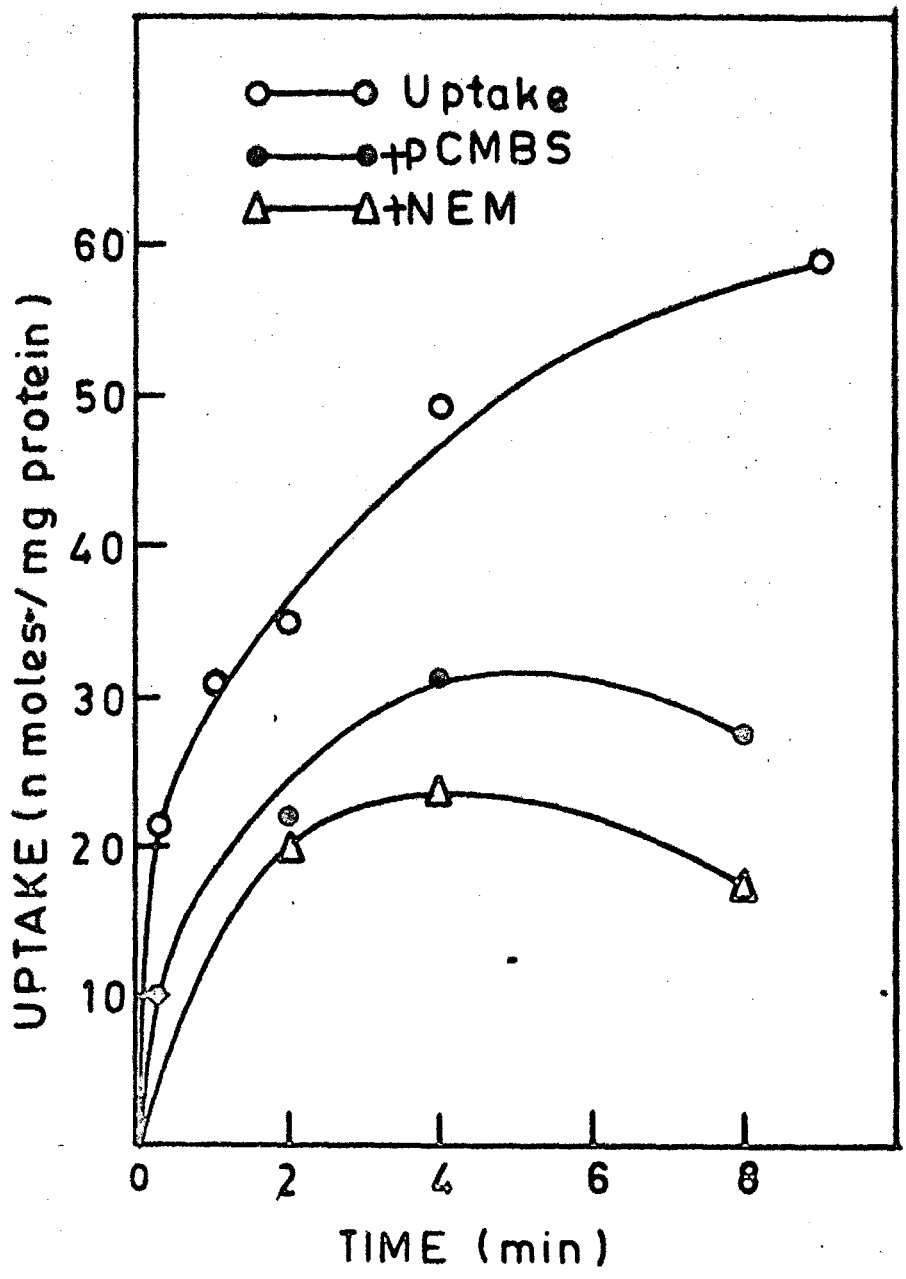
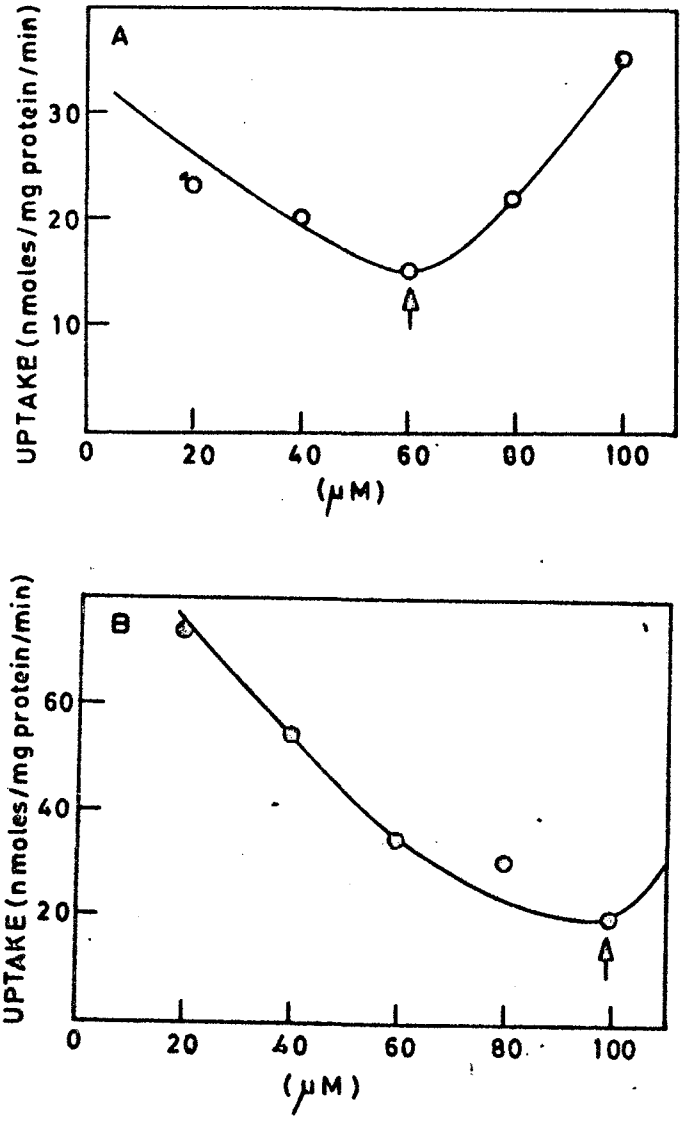


Fig. 12 A & B EFFECT OF VARIOUS CONCENTRATIONS OF NEM AND p-CMBS ON INOSINE UPTAKE

Cells were preincubated for 10 minutes with different concentrations of NEM & p-CMBS before initiating the uptake. Assay conditions were as described in 'Materials and Methods'.
(o) NEM
(●) p-CMBS



DISCUSSION

The uptake mechanisms for nucleic acid precursors have been characterized to a greater extent in both eukaryotes and prokaryotes. The conclusions made by Hochstadt and her group supports groups translocation of purine bases mediated by membrane bound phosphoribosyltransferases. In addition, for various nucleosides the cleavage of nucleosides by specific nucleoside phosphorylases followed by translocation of ribose moiety and base was described as a possible mechanism in bacteria and some of the mammalian cell lines (Hochstadt and Stadtman, 1971; Hochstadt and Quinlan, 1976). However, by using rapid sampling method Plagemann et al., (1978) suggested that in mammalian cells no group translocation process exist, but rather saturable, carrier mediated transport processes were prevalent (Wohlhueter et al., 1976, 1978, 1979, 1980; Plagemann et al., 1978). This controversy led many investigators to find out and support different types of mechanisms in different cell lines. The studies on intact cells, isolated vesicles and organelles, concluded three different types of mechanisms in the case of animal cells. 1) non-mediated diffusion 2) facilitative diffusion 3) group translocation.

So far the uptake studies that have been done in yeast cells did not support group translocation. There are several supporting observations for common transport systems for some purine bases and nucleosides in yeast cells (Grenson, 1969; Polak and Grenson, 1973; Housset and Nagy, 1977; Foret and Reichert, 1978). The transport of the purine bases, pyrimidine bases and nucleosides described as saturable, facilitative diffusion. However, the literature shows paucity with reference to nucleosides and their bases transport in Candida species. Since the aim of our laboratory is to characterize various groups of transport systems in Candida and other yeasts, to see their involvement in growth control and cell division of this organism, we have attempted to study a few characteristics of guanine, a purine base and inosine, a purine nucleoside uptake in Candida albicans cells.

Our results of guanine and inosine uptake confirms the mechanism of mediated transport taking place via a facilitative diffusion process. The mechanism is characterized by analysing the intracellular product at rapid intervals of time. Fig. 1 showed the intracellular accumulated product during guanine uptake which was mostly unmetabolized during its transport.

The recovery of the transported solute was between 95 and 100% for initial first one to two minutes. However, at later points, the radioactivity recovered in unmetabolized solute was decreased. If one studies the uptake of such bases during later times, one would conclude erroneously that a group translocation may exist in Candida cells. However, since we terminated the reactions within few seconds, the facilitated diffusion could be demonstrated and confirmed.

Our results of inosine transport also do not support the cleavage of nucleoside by its phosphorylase into ribose moiety and its base. The results of intracellular accumulation (Fig. 5) showed that inosine was accumulated as inosine during its transport. As in the case of guanine uptake, the level of accumulation of inosine was linear for first two minutes and radioactivity recovered was between 90 and 95% within that time. This observation suggested the following:

1) Transported inosine remains as free inosine upto 2 minutes.

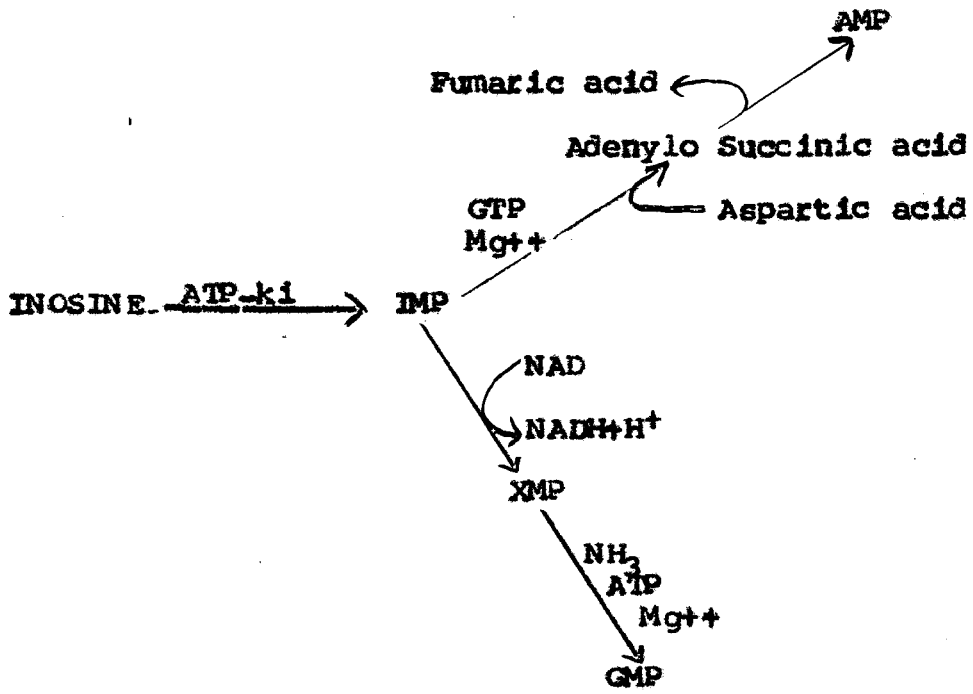
2) It is clear that nucleoside phosphorylase is not acting on inosine during its transport i.e. cleavage is not occuring in Candida albicans during the inosine transport as suggested for several mammalian

cell lines and bacteria (Quinlan et al., 1976; Prasad et al., 1981; Rader and Hochstadt, 1976).

The ribose-1-phosphate level during the transport of inosine did not increase with time. This may suggest that transported inosine did not undergo cleavage in the cell by phosphorylase i.e. it is not converting into

$$\text{inosine} \xrightarrow{\text{phosphorylase}} \text{hypoxanthine} + \text{ribose-1-phosphate}$$

However, the decrease in the level of accumulation at later time points could be due to its conversion into its monophosphates e.g. AMP and GMP as given below



The results of competition experiments showed maximum inhibition of guanine uptake by adenine and hypoxanthine suggesting a common transport system for all these purine bases. Similar common transport system has been reported for adenine, guanine, hypoxanthine and cytosine in Saccharomyces cerevisiae (Reichert et al., 1975; Chevallier et al., 1975), for adenine, guanine and hypoxanthine in Schizosaccharomyces pombe (Pourquie, 1970), for adenine, hypoxanthine and other analogues in Saccharomyces cerevisiae (Pickering and Woods, 1972). In addition inhibition by thymine and inosine also suggested that they might share the same permease. Results of inosine uptake suggested that adenine, hypoxanthine, guanine and pyrimidine base thymine compete with it. It suggested a common transport system for adenine, hypoxanthine, gauanine, thymine and inosine.

It is clear from the results that Candida cells have very interesting transport systems of purine bases and nucleosides. This study is a first step towards the characterization of various other transport systems. We would like to make use of plasma membrane vesicles isolated from yeast cells for future studies since the complications due to intracellular matabolism

could then be completely excluded. The role of these transport processes in cell growth and division is another interesting area of future research.

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* Original not seen.

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