Stimulation of Active Uptake of Nucleosides and Amino Acids by Cyclic Adenosine 3':5'-Monophosphate in the Yeast Schizosaccharomyces pombe*

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In conditions of glucose starvation, the maximum velocity of the mediated transport of nonmetabolized and metabolized amino acids, uridine, adenosine, and sucrose across the plasma membrane is stimulated by a factor of two by the addition of 1 mM adenosine 3':5'-monophosphate to Schizosaccharomyces pombe 972h- wild strain, to the glucose-super-repressed and derepressed mutants COB5 and COB6, and to Saccharomyces cerevisiae strain IL 216-IA. The mediated uptake of 2-n-deoxyglucose and the apparently nonmediated uptake of guanosine are not stimulated by the cyclic nucleotide. N6',O2'-Dibutyryl adenosine 3':5'-monophosphate is also efficient, whereas theophylline, guanosine 3':5'-monophosphate, 5'-AMP, ATP, and adenosine are ineffective.

The cellular ATP content of glycerol-grown S. pombe COB5 is about 10 nmol per mg of protein and is not decreased by further incubation in the starvation medium. The addition of 100 mM glucose markedly enhances transport without any increase of the cellular ATP content. The addition of antimycin A or Dio-9 decreases markedly both cellular ATP content and transport. The addition of 2.5 mM glucose to antimycin A-containing medium restores both transport and cellular ATP level, indicating that the ATP required for transport is not necessarily of mitochondrial origin. The uptake of 2-n-deoxyglucose is unaffected by the respiratory inhibitors.

Stimulation of uptake by cyclic adenosine 3':5'-monophosphate occurs only in glucose-deprived cells. The addition of 10 mM glucose elicits the disappearance of the stimulation and prevents the 30% decrease of the cellular adenosine 3':5' monophosphate content produced by glucose starvation. Adenosine 3':5'-monophosphate does not enhance the steady state ATP level but requires cellular ATP produced either by endogenous respiration or, in the absence of respiration blocked by antimycin A, by further addition of 2.5 mM glucose.

Stimulation of active uptake by adenosine 3':5'-monophosphate does not require protein synthesis because the addition of cycloheximide or anisomycin does not prevent the stimulation of L-leucine uptake.

In the absence of respiration, Dio-9, an ATPase inhibitor, suppresses instantaneously the cellular ejection of protons as well as the uptake of uridine and amino acids. It abolishes also the adenosine 3':5'-monophosphate-stimulated transport. In the presence of antimycin A, specific mitochondrial ATPase inhibitors such as venturicidin A do not inhibit metabolite uptakes and their stimulation by adenosine 3':5'-monophosphate. These results suggest that in these conditions, the target of Dio-9 is not the mitochondrial ATPase but a plasma membrane proton-translocating function generating an electrochemical gradient required for active transport. That adenosine 3':5'-monophosphate enhances the Dio-9-sensitive proton extrusion supports the view that the cyclic nucleotide might modulate the plasma membrane ATPase.

Cyclic adenosine 3':5'-monophosphate modifies the transport of ions, amino acids, and sugars across the plasma membrane of several animal tissues (1-6). It has been proposed that such stimulations of ion fluxes are related to stimulations of glycogenolysis and gluconeogenesis (2, 7). More recently, cyclic AMP was reported to interfere with the phosphorylation of a specific membrane protein which might be involved in the control of cellular permeability (8, 9). Modification of mem-

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brane permeability by cyclic AMP was also demonstrated in cell cultures (10–12). High intracellular cyclic AMP concentrations produced by serum deprivation in normal fibroblasts exert a negative control on growth and transport, possibly at the microtubule aggregation level (11). The regulatory processes, abolished in malignant transformed cells, can be partly restored by addition of exogenous cyclic AMP (10).

Control of cellular permeability by cyclic AMP has received much more attention in mammals than in yeast and in bacteria. Yeast, however, is a particularly suitable organism. It contains adenosine 3':5'-monophosphate, a specific phosphodiesterase, adenyl cyclase, and a cyclic AMP-dependent protein kinase (13–17). Elaborate genetic techniques are available and the results obtained with this simple eukaryote are more likely to be extended to mammalian cells than those obtained with bacteria. A convenient approach to study the role of cyclic AMP in bacteria as well as in fibroblasts has been the addition of the cyclic nucleotide to the culture medium (10, 18). Unfortunately, until now, few significant effects of exogenous cyclic AMP have been reported in yeast (19, 20). In a previous paper (21), we reported that exogenous cyclic AMP stimulates the incorporation of uridine into RNA in a glucose-supersensitive mutant COB5 (22) of the yeast Schizosaccharomyces pombe subjected to glucose deprivation. In the present paper, we show that this stimulation is most likely the result of a stimulated transport of uridine across the plasma membrane. Characterization of the active uptakes of nucleosides and of amino acids in several yeast strains under starvation conditions leads us to suggest a possible role of ATP and of a plasma membrane ATPase in the cyclic AMP-stimulated active transport of yeast.

MATERIAL AND METHODS

Yeast Strains

Most of the experiments were carried out with Schizosaccharomyces pombe COB5, a respiratory-proficient mutant selected on a cobalt sulfate-containing medium (22, 23). The chromosomal respiratory-deficient mutants from S. pombe 572a were also used: COB2, COB6, COB7, M126 (23, 24). Eighteen different strains of Saccharomyces cerevisiae with mitochondrial markers were gifts of Dr. Slonimsky: IL116-6C, IL116-5B, IL117-4C, IL117-7A, IL116-1A, IL116-1C, IL458-1A, IL458-1C, D11-1B, IL125-3A, IL125-2B, 55R5 3C/32, 516-10B, 5R5-5C/222, IL102-8D, IL126-3A, IL126-7A, 5S5-3C/1.

Culture Conditions

The YD medium contains 58 g of glucose and 20 g of yeast extract (Difco) per liter of water. The YD medium contains 36 g of glycerol, 1 g of glucose, and 20 g of yeast extract per liter of water. Both media were brought to pH 4.5 with HCl. Cultures were inoculated with 10^6 cells per ml from an overnight preculture and grown at 30°C with vigorous shaking. Cells were harvested in the exponential phase of growth by centrifugation at 4°C and washed with cold sterile water only when indicated.

Metabolite Uptake Measurements

Immediately after harvest, the cells were transferred into the starvation medium containing 20 mM Tris adjusted with citric acid to pH 4.5. Then, 5 × 10^7 cells per ml were preincubated for 15 min in the presence or absence of 1 mM cyclic AMP before introduction of the labeled compound. Incubations were carried out at 30°C with continuous shaking. At the indicated times, samples of 2.5 × 10^7 cells were diluted into 10 volumes of cold water and collected on Gelman filters (0.8 μm pore size) prewashed with 0.5% of the unlabeled compound. The cells were immediately washed with 20 ml of cold water and dried. The filters were introduced into glass vials containing 7 ml of a toluene mixture (21) and counted with a scintillation spectrometer. Incorporations of [5^-H]jirimycin into RNA and of L-[U-14C]leucine into proteins were measured as previously described (21).

Cellular cyclic AMP Measurements

Three grams of glycerol-grown cells (wet weight) were extracted with 6 ml of 10% cold trichloroacetic acid and ground for 2 min with 5 g of glass beads (0.45 μm diameter) in a Braun homogenizer at about 4°C. The supernatant was extracted five times with 2 volumes of diethyl ether to remove trichloroacetic acid and lipids. Ether was evaporated by boiling the extract for 2 min at 100°C, and the pH was brought to 6.5 with NaOH. One milliliter of the extract containing a trace of cyclic [G^-H]AMP to estimate the cyclic AMP recovery was purified by chromatography on a Dowex 50W-X4 column (5 cm high and 5 mm diameter) under 120 cm of H₂O pressure. Elution was carried out with water. The cyclic AMP cellular content was then determined by Gilman's method (20) with a Boehringer kit. A Dowex blank was carried out according to Otten et al. (29). The results were identical with those obtained by the method of Van den Berg et al. (27).

Cellular ATP Measurements

Five hundred milligrams of cells (wet weight) were collected on Millipore filters (0.8 μm pore size and 47 mm diameter) at 2°C and immediately dipped into 2 ml of 10% cold trichloroacetic acid. After 15 hours of trichloroacetic extraction, the cells were sonicated for 2 min and centrifuged (28). The supernatant was treated with diethyl ether as described above. The ATP content was evaluated by spectrophotometric measurements of NADPH produced by the addition of 15 mm glucose to the incubation medium containing 8 mM MgCl₂, 0.1 mM NADP, 1 unit of glucose-6-dehydrogenase and 7 units of hexokinase in 100 mm Tris-CHCl, pH 8.0. The cellular glucose-6-phosphate content was subtracted.

Cellular Amino Pool Measurements

First, 4 × 10^8 cells were incubated at 0°C in 2 ml of trichloroacetic acid for 1 hour. After centrifugation, the supernatant was treated with diethyl ether as described above and was evaporated. The samples were dissolved in 0.2 m sodium citrate buffer, pH 2.2, and aliquots were applied to a Hocaco amino acid analyzer (Hocaco Co., London). Analysis was carried out with a single column system using stepwise elution.³

Chemicals

Radioactive Products—[2,8^-3H]adenosine, cyclic [G^-H]adenosine 3':5'-monophosphate, ammonium salt (33 Ci per mmol), L-[2,3^-H]jaspartic acid (24 Ci per mmol), 2-D-[G^-H]deoxycytidine (7.2 Ci per mmol), [2-H]glycine (8.6 Ci per mmol), [8-H]guanosine (6.15 Ci per mmol), L-[U-14C]leucine (305 mCi per mmol), [4,5^-H]lysine (55 Ci per mmol), [7,8^-14C]glucose (300 mCi per mmol), L-[G^-H]tryptophan (2.1 Ci per mmol), [5^-H] Juridine (28 Ci per mmol), and L-[G^-H] valine (1.3 Ci per mmol) are from the Radiochemical Centre, Amersham. ²

Antibiotics and Inhibitors—Antimycin A is from Boehringer; anisomycin is from Pfizer Laboratories; cycloheximide is from Sigma; carbonyl-β-cyclodextrin-o-chlorophenol is from Calbiochem; N,N'-dicyclohexylcarbodiimide is from British Drug House Laboratories; Dio-8 was purchased from Koninklijke Nederlandse Stichting Stierrup, Delft, The Netherlands; venturicidin A was a gift from Dr. J. Mattoon, Johns Hopkins University of Baltimore. When the inhibitor is diluted in ethanol, an equal volume of ethanol is added to the control.

Enzymes—Glucose-6-P-dehydrogenase (n-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.48), 140 units per mg and hexokinase (ATP:hexose-6-phosphate transferase, EC 2.7.1.1) 140 units per mg, were from Boehringer. Adenosine 3':5'-monophosphoronic radiosotope dilution test for determination of adenosome 3':5'-monophosphate concentration is from Boehringer, as well as adenosome 3':5'-monophosphoronic acid and guanosine 3':5'-monophosphoronic acid.

RESULTS

Stimulation of Uridine and L-Leucine Uptakes by Cyclic AMP—Glycerol-grown cells of Schizosaccharomyces pombe COB5 were collected in the exponential phase of growth and transferred into a buffer without glucose and nitrogen. In this

³The performance of amino acid analyses by Dr. R. Crighton is gratefully acknowledged.
condition of starvation, the uptake of added uridine was linear for more than 5 min and markedly stimulated within 30 s by the addition of 1 mM adenosine 3'-5'-monophosphate (Fig. 1A). A slow incorporation of uridine into RNA began 3 min after the uptake. This incorporation which accounts for only 10% of the total uptake was also enhanced by cyclic AMP. Similar stimulations were obtained for L-leucine uptake and incorporations were obtained for L-leucine uptake and incorporation (Fig. 1B). These results suggest that cyclic AMP acts primarily on the cell membrane transport. However, a slight but distinct effect on RNA synthesis itself cannot be excluded. The stimulation of transport might be due to a stimulated metabolism of uridine or L-leucine, eliciting a decreased internal pool and reduced feed-back control of the uptake (29). The intracellular amino acid pool however is not significantly modified during the 20-min incubation in the presence of cyclic AMP (Table I). Moreover, we shall see below that the uptake of α-aminoisobutyric acid, a nonmetabolized amino acid, is also enhanced by cyclic AMP (Table II). We can therefore conclude that cyclic AMP stimulates the uptake process itself, and that the increased incorporations of uridine into RNA, and of L-leucine into proteins are mainly the result of stimulated transport across the cell membrane.

Cyclic AMP Specificity—N\textsuperscript{6},O\textsuperscript{4'-Dibutyryl cyclic AMP is commonly used instead of adenosine 3'-5'-monophosphate, as it is more efficient in fibroblasts and mammals. The main reason for this higher efficiency is that N\textsuperscript{6},O\textsuperscript{4'-dibutyryl cyclic AMP inhibits cyclic AMP phosphodiesterase and prevents the intracellular cyclic AMP degradation, just as theophylline does (30). In S. pombe however, we do not find any difference of efficiency between the two cyclic nucleotides (Fig. 2). Theophylline (1 mM to 20 mM) even in the presence of exogenous cyclic AMP, does not affect the transport (data not shown). Whether exogenous theophylline prevents cyclic AMP degradation in S. pombe is unknown, but we must note that the purified cyclic AMP phosphodiesterase of Saccharomyces cerevisiae is inhibited only partially by concentrations of theophylline as high as 10 mM (16). ATP, 5'-AMP, adenosine, and guanosine 3'-5'-monophosphate are not stimulatory in S. pombe (Fig. 2).

**Table I**

| Amino acids       | Control | + Cyclic AMP | + Anti-mycin A |
|-------------------|---------|--------------|---------------|
| Aspartic acid     | 26.0    | 27.0         | 27.5          |
| Threonine         | 5.5     | 4.0          | 4.5           |
| Serine            | 26.3    | 22.0         | 18.0          |
| Glutamine         | 110.4   | 96.0         | 98.5          |
| Glycine           | 4.1     | 3.0          | 4.5           |
| Alanine           | 27.1    | 20.3         | 25.0          |
| Valine            | 15.0    | 13.0         | 10.0          |
| Methionine        | 1.8     | 1.5          | 1.9           |
| Isoleucine        | 2.7     | 2.3          | 1.9           |
| Leucine           | 7.6     | 6.0          | 6.8           |
| Tyrosine          | 0.9     | 0.9          | 0.1           |
| Phenylalanine     | 5.7     | 4.5          | 4.8           |
| Lysine            | 60.0    | 43.3         | 45.0          |
| Arginine          | 59.0    | 52.0         | 51.5          |
| Total amino acids | 353     | 295          | 304           |

**Table II**

| Amino acids | K\textsubscript{m} (nmol/min/mg protein) | V\textsubscript{max} (nmol/min/mg protein) |
|-------------|----------------------------------------|------------------------------------------|
| Adenosine   | 2.0                                   | 900                                      |
| Guanosine   | >5.0                                  | Not determined                           |
| Uridine     | 0.014                                 | 90                                       |
| Aspartic acid | 5.0                             | 230                                      |
| Threonine   | 1.0                                   | 290                                      |
| Methionine  | 0.55                                  | 480                                      |
| Isoleucine  | 2.0                                   | 590                                      |
| Leucine     | 0.20                                  | 800                                      |
| Tyrosine    | 0.25                                  | 690                                      |
| Phenylalanine | 3.0                                | 150                                      |
| Lysine      | 3.3                                   | 15,300                                   |
| Arginine    | 0.30                                  | 830                                      |
| Succrose    | 3.3                                   | 1,660                                    |
The effects of exogenous cyclic AMP are thus more specific in *S. pombe* than in fibroblasts where other adenine derivatives interfere with membrane transport (10). 

**Strain Specificity and Growth Conditions**—The stimulation of the uptake of uridine by cyclic AMP occurs only in certain strains and only under certain culture conditions. The uptake of uridine is stimulated by cyclic AMP in glycerol-grown cells of the wild strain *S. pombe* 972h− submitted to glucose starvation. In similar conditions the cyclic nucleotide fails to increase the uptake in 19 different glucose-grown strains of *S. cerevisiae*. Only the strain LI126-1A (a ura3−, α−, C−) was clearly sensitive to the cyclic nucleotide. In the wild strain, *S. pombe* 972h− grown on 320 mM glucose and harvested either in exponential phase or in stationary phase of growth, a glucose shift down does not induce stimulation of uptake by cyclic AMP. Similarly, no stimulation is observed with three glucose-grown respiratory-deficient mutants, COB2, COB7, and M126. Curiously, in glucose-grown as well as in glycerol-grown cells, the uptake of uridine is stimulated by cyclic AMP both in the glucose-derepressed mutant of *S. pombe* COB6 and in the glucose-superrepressed mutant COB5 (22).

**Substrate Specificity**—In fibroblasts, the uptake of leucine, uridine, and 2-deoxyglucose is inhibited by adenosine 3'5'-monophosphate (10-12). In jejunal mucosa, only the transport of basic amino acids is enhanced by cyclic AMP (5), whereas in kidney cortex, the uptake of all of the amino acids is stimulated (4). It was therefore of interest to determine the effect of cyclic AMP on the uptake of various metabolites in *S. pombe* COB5. Under starvation conditions, the uptakes of glycerol, five distinct L-amino acids, α-aminobutyric acid, adenosine, uridine, sucrose, and 2-deoxyglucose show saturation kinetics (Fig. 3), each with a distinct *Kₐ* (Table II). These transports are likely mediated by specific "carriers" as in other yeasts (31-33). The uptake of guanosine is very low, and its apparent *Kₐ* is too high to be determined. The rates of uptake of neutral, acidic and basic L-amino acids, α-aminobutyric acid, adenosine, uridine, and sucrose are stimulated twice to three times by cyclic AMP, whereas the uptake of 2-deoxyglucose and guanosine is not modified. In all cases, cyclic AMP stimulates the maximum velocity of entry and does not modify the apparent *Kₐ* (Table II). Variable results have been reported in mammals. In kidney cortex, cyclic AMP stimulates the *Vₘₐₓ* of α-methylglucoside uptake, whereas it decreases the apparent *Kₐ* of amino acid entry in jejunal mucosa (5, 6). Our results show that in *S. pombe* the maximum velocities of the uptakes of metabolites as different as uridine, amino acids, and sucrose, most likely mediated by distinct carriers, are all stimulated by cyclic AMP. Interaction of cyclic AMP at the level of a specific carrier can thus be excluded. Because the apparent affinities of the metabolite uptakes are not modified, a direct effect of the cyclic nucleotide on the binding of a general "carrier system" is rather unlikely.

**Stimulation of Uptake by Cyclic AMP Requires Both Glucose Deprivation and Intracellular ATP**—All of the preceding experiments were performed with glucose-starved yeast cells. In these conditions, the uptake is low and markedly enhanced by cyclic AMP. Increasing the glucose concentration from 0 to 10 mM in the incubation medium enhances the uptake of uridine up to a plateau (Fig. 4A). The extent of stimulation by cyclic AMP is most pronounced in the absence of glucose, and gradually decreases and finally disappears at higher glucose concentrations (Fig. 4A). Starvation conditions are therefore required for obtaining the stimulation of uptake by cyclic AMP.

During the incubation of resting cells in the starvation medium, ATP continues to be produced by an active endogenous respiration and its intracellular content remains as high as in growing cells (Table III). The addition of respiratory inhibitors such as antimycin A and of oxidative phosphorylation inhibitors such as N,N'-dicyclohexylcarbodiimide or Dio-9 (34, 35) leads to a drastic fall in the cellular ATP level with a concomitant decrease of uridine uptake and disappearance of the stimulation by cyclic AMP (Table III and Table IV, Figs. 2 and 3).
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Charomyces pombe COB5. After a 15 min preincubation period in increasing glucose concentrations and 5 PCi of 10

starvation medium, cells were further incubated for 5 min with

In the experiment shown in (B), 30 s before the introduction of glucose

Uptake measurements were described under "Material and Methods.

Incubation was further carried out for 5 min as described in (A).

Resting cells in starvation medium

Growing cells

as described under "Material and Methods.

were added and the incubation was further carried out for 10 min. The cells

absence of cyclic AMP. The inhibitors or glucose or both were then

were collected on Millipore filters and the ATP content was measured

as described under "Material and Methods."
Experiment 1), does not modify significantly the intracellular pool of any amino acid in S. pombe within a 10-min glucose starvation period (Table I). The main role of ATP therefore is not to reduce the negative feed-back control. It is more likely that ATP might be involved in the formation of a “rich energy” compound (36) or of a potential gradient participating to the active transport across the plasma membrane. The chemiosmotic Mitchell’s hypothesis proposes that in microorganisms, the driving force for active transport is created by an electrochemical gradient (positive potential outside) generated across the plasma membrane by electrogenic extrusion of protons into the external medium (37, 38). It was suggested by Conway (39) that in yeast an oxidation-reduction pump is implicated in proton extrusion. More recently, it has been assumed that a plasma membrane ATPase is involved in the active extrusion of protons into the external medium (40).

Although several lines of evidence support this view in bacteria and fungi (41-44), little supporting evidence is available in yeast. The following experiments were carried out in order to investigate the effects of cyclic AMP on proton extrusion in S. pombe.

**Stimulation of Proton Extrusion by Cyclic AMP**—COB5 cells were preincubated in the starvation medium in the presence or absence of 1 mM cyclic AMP for 15 min prior to the addition of 10 μM antimycin A to block the respiration and of 5 mM glucose to allow the cells to glycolyze (Fig. 5). After a lag of 1 min, protons were extruded at the rate of about 2 μeq per min and per g of protein. A slight but reproducible increase of proton extrusion (about 0.5 μeq per min and per g of protein) was produced by cyclic AMP. In the same conditions, the stimulation of L-leucine uptake by cyclic AMP (50 μM in the external medium) attained 0.1 μmol per min and per g of protein. The stimulation of proton extrusion seems thus significant especially because the extruded protons were measured after dilution in the large external volume and as the acidification might be considerably amplified in the space localized between the cell membrane and the wall. The proton-conducting agent carbonylcyanide-m chlorophenylhydrazone inhibited both transport (Table IV, Experiment 2) and proton extrusion. It did not however inhibit specifically the cyclic AMP-stimulated transport, suggesting that cyclic AMP does not produce the electrochemical gradient.

A functional relationship between stimulations of active uptake by cyclic AMP and proton extrusion is suggested by their parallel appearance under different conditions. For instance, cyclic AMP fails to enhance both uptake and proton extrusion in glycerol-grown cells of S. pombe COB5 transferred into a medium containing 50 mM glucose, or in glucose-grown cells of the wild strain transferred into the starvation medium (Fig. 5).

**Inhibition of Uptake by Dio-9**—Dio-9, an antibiotic of unknown structure, is a potent inhibitor of the ATPase of chloroplasts, yeast mitochondria, and Streptococcus faecalis, and does not significantly affect the production of glycolytic ATP (32, 45, 46). In rat liver mitochondria, Dio-9 inhibits oxidative phosphorylation (46). COB5 cells were incubated in the starvation medium for 10 min in the presence or absence of 1 mM cyclic AMP. Antimycin A was then added to block the respiration and glycolysis was induced by 2.5 mM glucose. In these conditions, stimulation of the uptake of uridine by cyclic AMP was observed. Further addition of 25 μg of Dio-9 per ml of cell suspension blocked instantaneously the uptake of uridine both in the control and in the cyclic AMP-treated cells (Fig. 6 and Table IV, Experiment 2). The inhibition of uridine uptake by low concentrations of Dio-9 is more pronounced in cyclic AMP-treated cells; the apparent K_0 for Dio-9 is 5 μg per ml in the presence of cyclic AMP and 5 μg per ml in the control. Dio-9 also inhibits the extrusion of protons into the external medium (data not shown). In these experiments, the inhibition
of uptake by Dio-9 is not the result of inhibition of mitochondrial ATP production, because prior to the addition of Dio-9, the cellular respiration was totally abolished by antimycin A. The intracellular ATP level is unaffected by Dio-9 in nonrespiring cells inhibited by antimycin A (Table III). In the same conditions, only high (possibly nonspecific) concentrations of N,N'-dicyclohexylcarbodiimide (1 mM), another ATPase inhibitor (46) are inhibitory (Table IV, Experiment 2). Venturicidin A, an inhibitor of the mitochondrial membrane-associated ATPase (47) is totally inactive in antimycin A-treated cells, although it has some inhibitory effects on the uptake of uridine in respiring cells (Table IV, Experiment 3). In addition, we found that the cyclic AMP-insensitive uptake of 2-deoxy-glucose is not inhibited by Dio-9 in the presence of antimycin A (Table IV, Experiment 4). These findings suggest that cyclic AMP stimulates indirectly or directly a Dio-9-sensitive function of the plasma membrane involved in active transport. In S. faecalis, Dio-9 inhibits active K+ uptake most likely driven by a plasma membrane ATPase (43). Such an ATPase is likely present in yeast (48) and is also Dio-9-sensitive.

Effect of Protein Synthesis Inhibitors on Cyclic AMP-stimulated Transport—Stimulation of uptake is best expressed after a 15-min preincubation of the cells in the presence of cyclic AMP. Protein synthesis occurring during such preincubation might be required for the expression of the cyclic AMP effect. We have therefore investigated the effect of protein synthesis inhibitors on the uptakes of leucine and uridine. Anisomycin and cycloheximide which block protein synthesis at the elongation step (49) decrease partially and equally the uptake of L-leucine in cyclic AMP-treated cells and in the control (Table V). The stimulation by cyclic AMP does not require newly synthesized proteins during starvation. It must be noted that in these conditions, the leucine and total amino acid pool are decreased by less than 20%, making rather unlikely a negative feed-back control of the amino acid uptake.

On the other hand, anisomycin and cycloheximide only slightly inhibit the uptake of uridine in the control (Table V). Curiously, the extent of the stimulation is markedly decreased by cycloheximide but slightly enhanced by anisomycin. This result, possibly related to the stringent control of RNA synthesis and to the stimulation of uridine incorporation by cycloheximide that we have described in starved S. pombe COB5, deserves further investigations (91).

**Table V**

**Effect of protein synthesis inhibitors on cyclic AMP-stimulated transport**

| Additions      | Uptake |       |       |
|----------------|--------|-------|-------|
|                | L-leucine | Uridine |       |
|                | Control  | + cyclic AMP | Control  | + cyclic AMP |
|                | nmol/min/g protein |       | nmol/min/g protein |
| None           | 124     | 236   | 29    | 61    |
| Cycloheximide  | 93      | 160   | 24    | 39    |
| None           | 90      | 215   | 30    | 60    |
| Anisomycin     | 48      | 145   | 24    | 63    |

**Decrease of Cyclic AMP Concentration during Starvation—**

In order to determine whether the stimulation of active transport by exogenous cyclic AMP reflects a prior physiological decrease in the intracellular cyclic AMP level during starvation, we measured the variations of the internal cyclic AMP concentration under different conditions. The concentration of adenosine 3':5'-monophosphate in glycerol-grown S. pombe COB5 harvested in the exponential phase of growth, calculated from four different experiments, is about 2.5 nmol per g of dry weight. In cells further incubated for 30 min or 1 hour in the starvation medium, the internal cyclic AMP concentration is decreased only by about 30% (Table VI). On the other hand, if the cells are incubated for the same period in the presence of 5 mM glucose, no decrease of the cellular cyclic AMP concentration can be detected. Can this slight cyclic AMP concentration decrease account for the drastic increase of metabolite uptake produced by the addition of cyclic AMP to the starvation medium? Our results are in agreement with the small variations of the cyclic AMP level found in fibroblasts or in jejunal mucosa under different physiological conditions (5, 11). These data indicate either that the cyclic AMP target is located at the outside of the plasma membrane or that several cyclic AMP pools are present in the cell.

**DISCUSSION**

Our data show that exogenous adenosine 3':5'-monophosphate prevents the negative control exerted by glucose starvation on the uptake of a number of metabolites across the plasma membrane in resting cells of S. pombe COB5. In order to investigate the role of cyclic AMP in the active transport of glucose-starved yeast cells, it was necessary to study first active transport itself, a very poorly documented function in yeast resting cells.

**Role of ATP in Active Transport of Resting Cells—**

Our data show that in glucose-starved cells of S. pombe COB5, previously grown on glycerol, metabolites are actively transported. These resting cells contain about 10 nmol of ATP per mg of protein produced by high endogenous respiration. The addition of respiratory and oxidative phosphorylation inhibitors dramatically decreases both cellular ATP content and uptake. Further addition of 2.5 mM glucose to the incubation medium restores both cellular ATP content and active uptake. We found however that higher glucose concentrations enhance several times the rate of active transport but do not lead to any further increase of the steady state ATP level. These data do not support the idea of a physiological negative control of the amino acid uptake by glucose starvation, as was previously proposed (91).

**Table VI**

**Decrease of cellular cyclic AMP concentration during starvation in Schizosaccharomyces pombe COB5**

Glycerol-grown cells harvested in exponential phase of growth were incubated for 30 min in the absence of glucose and nitrogen or with 5 mM glucose (Experiment 1). In Experiment 2, cells were incubated for 1 hour in a medium containing 0.1% yeast extract (pH 4.5) in the absence or presence of 5 mM glucose. Other experimental conditions were described under "Material and Methods.

| Conditions | Cellular cyclic AMP concentration |
|------------|----------------------------------|
|            | Experiment 1 | Experiment 2 |
| No incubation | 2.7          | 2.4          |
| Incubation  | 2.2          | 1.8          |
| + 5 mM glucose | 3.0          | 2.4          |
not mean necessarily that ATP is not involved in active transport in yeast, as proposed by Kotyk et al. (50). They probably reflect a lowering utilization of intracellular ATP in resting cells than in growing cells and strict control of its steady state level by balanced production and utilization rates.

Furthermore, our data show that in the absence of respiration, inhibited by antimycin A, the active transport is instantaneously inhibited by Dio-9, a potent inhibitor of membrane ATPase in several organisms (35, 42, 45). According to Mitchell’s chemiosmotic hypothesis (37, 38), ATPase might couple hydrolysis of ATP to proton translocation across the plasma membrane, the proton gradient being the driving force for active uptake. In this respect, it must be noted that in absence of respiration, Dio-9 inhibits the extrusion of protons in *S. pombe* COB5. However, it has not yet been established whether proton extrusion is electronegative in yeast. According to Conway and Brady (51) the appearance of protons in the plasma membrane ATPase level. That mitochondrial ATPase is not involved in the inhibition of cellular transport by Dio-9 in the presence of antimycin A is further supported by the absence of the effect of inhibitors of the mitochondrial ATPase, such as venturicidin A in the same conditions.

**Role of ATP in Cyclic AMP-stimulated Transport**—The stimulation of uptake produced by the addition of glucose to starved cells occurs without any increase of the cellular ATP level. Similarly, in the same starving conditions, the stimulation of uptake produced by adenosine 3':5'-monophosphate is not accompanied by any increase of the cellular ATP content. However, in cells depleted of ATP by the addition of antimycin A, 2.5 mM glucose is able to restore both high cellular ATP level and active transport. In the absence of glucose, cyclic AMP is unable to induce and to stimulate active uptake in nonrespiring cells. This result makes it rather unlikely that cyclic AMP raises the production of ATP, although it increases the V_{max} of the uptake of a number of metabolites and enhances proton extrusion when sufficient cellular ATP is available.

**Primary Target of Cyclic AMP**—We have shown previously that cyclic AMP stimulates the incorporation of uridine into RNA during glucose derepression of the glucose-super-repressed mutant COB5 (21). In the present paper, we show that the stimulation of the cellular uptake of uridine is more pronounced and precedes the stimulation of uridine incorporation into RNA. These results suggest that the stimulation of incorporation of the labeled precursors into macromolecules is the consequence of the stimulated uptake. That the stimulation by cyclic AMP of active uptake is not secondary to stimulations of RNA or protein synthesis is further indicated by the stimulation of uptake of α-aminoisobutyric acid, a nonmetabolized amino acid, and of sucrose which is not an immediate precursor of RNA or protein.

At least two general hypotheses can be put forward to explain the role of cyclic AMP in the active transport across yeast plasma membrane.

1. Cyclic AMP might stimulate the glycolytic flux. It might activate, for instance, phosphofructokinase like in mammals (1). The cyclic nucleotide however is inactive on yeast phosphofructokinase (53). Cyclic AMP might also stimulate the degradation of lipids or of storage sugars such as trehalose and glycogen. In several mammalian tissues, stimulatory effects of cyclic AMP on ionics movements seem to be related to glucose production (2, 7). It also has been reported recently that in bakers’ yeast, cyclic AMP stimulates the degradation of trehalose in cell-free extracts (54). Stimulated degradation of carbohydrates should yield glycolytic ATP in the absence of respiration. However, we cannot detect any increase by cyclic AMP of the cellular ATP level in the presence of antimycin A and active uptake is not stimulated by cyclic AMP in ATP-depleted cells of *S. pombe* COB5.

2. Cyclic AMP might act directly at the plasma membrane level. It has been recently proposed that in several animal tissues cyclic AMP acts on the phosphorylation of a specific protein involved in active transport (8, 9). No experimental data support or contradict this attractive view in yeast. Among the membrane proteins, specific carriers are excluded as direct targets of cyclic AMP, because similar stimulations of the uptake are observed with chemically unrelated metabolites such as sugars, amino acids, and nucleosides.

On the other hand the plasma membrane ATPase is a possible membrane target for cyclic AMP. The cyclic nucleotide stimulates proton extrusion in *S. pombe* COB5, and in the absence of respiration, Dio-9, an ATPase inhibitor, strongly inhibits the uptake of metabolites as well as the extrusion of protons, and their stimulations by cyclic AMP. In kidney cortex, the stimulation of the uptake of amino acids by cyclic AMP is inhibited by ouabain, a mammalian plasma membrane ATPase inhibitor (4). It has been suggested that in perfused rat liver, the Na^+ + K^+ -dependent ATPase might be involved in the cyclic AMP-stimulated efflux of Na^+ occurring against an electrochemical gradient and generating membrane hyperpolarization (7). Recently however it has been reported that cyclic AMP inhibits the (Na^+ + K^+ ) (sodium and potassium ion-activated) ATPase in plasma membrane preparations (55). The possibility of modifications by cyclic AMP of the plasma membrane ATPase activity deserves further consideration in *S. pombe*.

Much further work is obviously required to elucidate in molecular terms the primary site of action of cyclic AMP. We feel however that the modification of cellular uptakes of metabolites by the addition of cyclic AMP to intact yeast cells provides a valuable new experimental tool to study not only the mode of action of cyclic AMP but also the control of cellular active transport. The use of yeast permits indeed a variety of elaborated genetical and physiological approaches which are not conceivable at the present time with higher eukaryotes.

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Stimulation of active uptake of nucleosides and amino acids by cyclic adenosine 3':5'-monophosphate in the yeast Schizosaccharomyces pombe.

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