MATERIALS AND METHODS

MATERIALS

Agar and yeast extract were obtained from Difco., USA, DE-52 and GF/C filters (diameter 2.4 cms) were purchased from Whatman, UK. Cyclic AMP assay kit, containing (^{3}H) cAMP (26 Ci/mmole) and N(^{3}H)-acetyl-glucosamine (500 Ci/mmole) was procured from the Radiochemical Centre, Amersham, UK. (^{3}H) leucine (1.3 Ci/mmole) and ^{32}P (1 mCi/ml) was obtained from Bhabha Atomic Research Centre, Bombay, India. The following chemicals were purchased from Sigma Chemical Co., St. Louis, USA.: ATP, ADP, cAMP, BSA, casein, bovine heart cAMP dependent protein kinase, EDTA, Folin's reagent, DTT, glucosamine 6-phosphate, glucose 6-phosphate dehydrogenase, glycine, histone type II A, HEPES, 2-mercaptoethanol, N-acetyl-D-glucosamine, $NADP^{+}$, N, N' methylene-bis-acrylamide, NAD, N-ethylmaleimide, PPO, POPOP, potassium phosphate, dibasic, PIPES, protamine, p-dimethylamino-benzaldehyde, phosphoglucose isomerase, phosvitin, 3-phosphoglyceric phosphokinase, 3-phosphoglyceraldehyde dehydrogenase and Tris (hydroxy methyl) - amino methane. Coomassie brilliant blue R, Protein Reagent and TEMED were purchased from Biorad Inc., USA. Acrylamide was from Eastman Kodak Co., USA. Pharmacia fine Chemicals, Sweden, supplied Sepharose 6B and gel electrophoresis markers. Cyanogen bromide was supplied

by Fluka AG, Germany. All other chemicals were from commercial sources of highest purity available.

ORGANISMS

Saccharomyces cerevisiae 3059 and *Candida albicans* 3100 were obtained from the National Chemical Laboratory, Pune, India.

CULTURE CONDITIONS

Both *Saccharomyces cerevisiae* and *Candida albicans* were grown at 30°C for 48 hrs on slants containing

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

in distilled water. After growth the slants could be stored at 4°C upto two weeks.

In each instance, cells were transferred from a freshly made slant (not more than 72 hrs) to an inoculation medium containing 1.0% glucose, 0.5% peptone and 0.3% KH_2PO_4. Cells were grown for 17 hrs (*Candida albicans*) or 18 hrs (*Saccharomyces cerevisiae*) and 4.5 ml of cells were transferred from this to 100 ml growth medium containing 0.5% glucose, 0.5% peptone and 0.3% KH_2PO_4 to get an approximate A_{595} of 0.1.

Cells were then grown with shaking at 30°C for desired lengths of time.

The volume of the medium was usually 20% of the total volume of an Erhlenmeyer flask. All media were sterilized by autoclaving at 15 lbs/sq inch pressure for 15 min. Growth of the cells in the liquid culture was monitored turbidimetrically by measuring absorbance at 595 nm in a Bausch and Lomb colorimeter, Spectronic 20.

INDUCTION EXPERIMENTS

Cells were collected from growth medium in the mid-log phase, washed with 0.3% KH_2PO_4 and resuspended in 0.3% KH_2PO_4 containing 0.5% N-acetyl-glucosamine. The volume of induction medium was one-fifth of that of the growth medium and it contained 3 mM cAMP in the treated sample. After incubation at 30°C for various times cells were collected, washed and used to determine the rate of protein synthesis and N-acetyl-glucosamine uptake. For the assay of deaminase and kinase, cells were harvested, washed and homogenized in 50 mM potassium phosphate buffer (pH 7.6) containing 1 mM EDTA and 1 mM 2-mercaptoethanol (2 ml per gm wet weight of cells). Cells were disrupted by grinding with acid-washed sand in a mortar and pestle for 15 min. After centrifugation at 14,000 x g for 30 min, the supernatant, referred to as crude extract, was used for kinase and deaminase assay.

UPTAKE OF N-ACETYL-GLUCOSAMINE

For the assay of N-acetyl-glucosamine permease (high affinity uptake system) according to the method of Singh et al. (127), cells were taken from the induction medium at various times, washed and resuspended in sterile water so as to give an A_{595} of 0.2 in a volume of 0.5 ml. Then 0.45 ml of this cell suspension was incubated at 30°C in a waterbath shaker. After preincubation for 50 sec at 30°C, 50 µl N(^{3}H) - acetyl-glucosamine (1 µCi/ml) was added to give a final concentration of 0.5 mM. After 5 min of incubation, 0.5 ml samples of the incubation mixture were diluted with sterile water, filtered on glass fibre filters and washed with 10-15 ml of cold sterile water. Filters were dried under infrared lamp, put into vials and counted, after addition of toluene based scintillation fluid, in a Packard Tri-carbliquid scintillation counter.

Composition of Scintillation fluid :

POPOP	-	200 mg
PPO	-	4 gm
Toluene	-	1 litre

N-ACETYL-GLUCOSAMINE ASSAY

This was done by the method of Reissig et al.(128).

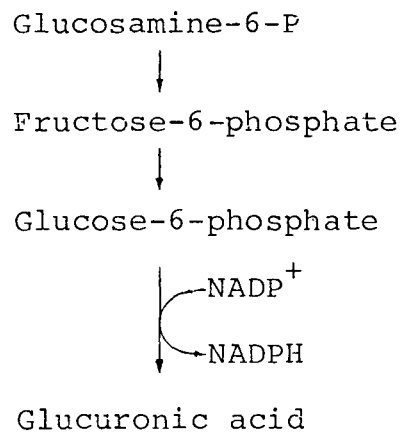
To 200 µl of sample, 200 µl of sodium tetraborate (0.8 M) was added and incubated in boiling water bath for 4 min. Then 2.5 ml of paradimethylaminobenzaldehyde (10% in glacial acetic acid containing 12.5% 10N HCl) diluted 1:9 with glacial acetic acid was added. After 10 min of incubation at 37°C, optical density was measured spectrophotometrically at 585 nm. The concentration was calculated from a standard plot.

GLUCOSAMINE-6-PHOSPHATE DEAMINASE ASSAY

Glucosamine-6-phosphate deaminase catalyses the conversion of glucosamine-6-phosphate to fructose-6-phosphate.

Glucosamine-6-P ⟶ Fructose-6-P + ammonia

Deaminase activity was assayed by coupling the enzyme to phosphoglucose isomerase and glucose-6-P dehydrogenase and following the formation of reduced pyridine nucleotide spectrophotometrically, according to the following reaction.



The assay was done according to a modified method of White and Pasternak (129). The following reagents were added to a quartz cuvette with a 1 cm light path in a final volume of 1 ml; 10 µl of 0.1 M glucosamine-6-P, 0.2 ml of 0.2 M potassium phosphate buffer (pH 7.6), 3 units of glucose-6-P dehydrogenase, 10 µl of 20 mM $NADP^+$, 8 units of phosphoglucose isomerase, 0.85 ml of 0.05 M phosphate buffer (pH 7.6) and enzyme fraction. The reaction was started by adding crude extract containing deaminase to the cuvette which was kept at 25°C in a Carl Zeiss PMQ II Spectrophotometer. The change in absorbance at 340 nm was followed upto five min. The blank contained all components except the enzyme and the value was subtracted from the experimental one to determine the activity.

MEASUREMENT OF PROTEIN SYNTHESIS IN INDUCED CELLS

At various times after incubation in the induction medium, 1 ml of cell suspension was taken out from the control (untreated) and treated (3 mM cAMP) induction media, and then labelled with (^{3}H)leucine (2 µCi/ml) for 5 min at 30°C in a water bath shaker. Radioactivity was then determined by precipitating with cold 5% TCA, boiling for 20 min in a water bath, plating on glass fibre filters, drying and counting in a toluene based scintillation fluid.

ASSAY OF GLUCOSE IN MEDIUM

According to the method of Nelson (130) reagents were prepared for the assay of glucose in the medium of cell culture.

Copper Reagent A : This contained 12.5 gm of sodium carbonate, 12.5 gm of potassium sodium tetraborate, 10.0 gm of sodium bicarbonate and 100.0 gm of sodium sulphate in 400 ml of water.

Copper Reagent B : This was prepared by dissolving 15 gm of copper sulphate in 100 ml of water containing 1-2 drops of concentrated sulphuric acid.

For the estimation of glucose, to 0.4 ml of the sample 0.4 ml of copper reagent mixture (25 parts of A mixed with 1 part of B by volume) was added and it was heated for 20 min in a water bath. The tubes were then cooled and 0.4 ml of arsenomolybdate solution was added. The volume was made upto 3 ml with water and optical density was taken at 510 nm.

ASSAY OF CYCLIC AMP IN CELLS

Cyclic AMP was assayed in the cells under different conditions by competitive binding assay, using radioactive cyclic AMP (^{3}H) by the method of Gilman (131). The measurement is based on the competition between unlabelled cAMP

and a final concentration of (^{3}H)cAMP, for binding to a protein which has high specificity for cAMP. The amount of labelled protein - cAMP complex formed is inversely related to the amount of unlabelled cAMP present in the assay. The concentration is determined by comparison with a linear standard curve.

In a total volume of 150 µl, 50 µl of sample (cell extract) 50 µl of (^{3}H)cAMP (180 pmoles containing approximately 5 µCi) and 100 µl of binding protein or sample was added and all the tubes were vortexed for about 5 seconds. The tubes were then put into ice at 2-4^{o}C and left for 2 hrs. At the end of the incubation, 100 µl of charcoal reagent in water was added to each tube. The charcoal suspension had to be kept stirring continuously. The tubes were vortexed and centrifuged and 200 µl of supernatant was taken. To it, 200 µl of 3N HCl was added and then counted in Bray's scintillation fluid.

Composition of Bray's fluid in 5 litres :

Napthalene	-	300 g
PPO	-	20 g
POPOP	-	1.0 g
Methanol (absolute)	-	500 ml
Ethylene glycol	-	100 ml

Volume made up with 1,4 - Dioxane.

CYCLIC (^{3}H)AMP UPTAKE STUDIES

Cyclic (^{3}H)AMP accumulation was measured by the following method. Cells ($\approx$ 8 x 10^6 cells/ml) harvested at midlog phase were washed and resuspended in water. Cyclic (^{3}H)AMP (2.5 μCi/ml) was then added to give a final concentration of 0.1 mM. At various times after the addition of cAMP, 0.5 ml aliquots were taken, immediately filtered on GF/C filters and washed twice with ice cold water. The filters were dried and the radioactivity was determined by scintillation counting. A sample taken immediately after adding cAMP allowed the measurement of any radioactivity trapped on the cell surface after washing. This blank value was subtracted from the experimental sample.

PROTEIN ASSAY

Protein was assayed in the induction experiments by the method of Lowry et al. (132).

In the protein kinase experiments, protein was assayed by the method of Bradford (133) using Biorad protein reagent with BSA as standard.

SYNTHESIS OF γ (^{32}P) LABELLED ATP

Gamma labelled ATP was prepared essentially according to the method of Glynn and Chappel (134). To 0.5 ml of ^{32}P solution containing hydrochloric acid, the following reagents

were added in the given order : 1.5 μmoles of solid Tris for each μmole of hydrochloric acid present (≈ 91 mg); 0.5 ml of a cofactor solution containing 2 mM EDTA (sodium salt), 2.5 mM ATP (sodium salt), 2.5 mM ADP (sodium salt), 2.5 mM 3 phosphoglyceric acid and 0.1 mM NAD; 0.25 ml of 0.5 M Tris base; 0.25 ml of 0.5 M Tris-HCl (pH 8.1); 1 μl of 2-mercaptoethanol; 20 units of 3 phosphoglyceric phosphokinase; 6 units of 3 phosphoglyceraldehyde dehydrogenase; 0.05 ml of 50 mM magnesium chloride. After mixing properly the reaction mixture was incubated at 37°C for about 1-2 hrs. Then it was loaded on a Dowex (1 x 10)Cl^- form, column (0.5 x 1 cm) charged with 10 ml of 1 N HCl and washed with water. The column was then washed with a solution of 20 mM ammonium chloride in 0.2 N HCl (about 30 ml) followed by about 20-30 ml of water. The $\gamma(^{32}P)$ATP was then eluted from the column by 0.25 N HCl on ice. The pH of the eluent was maintained at near pH 7.0 by the addition of 0.5 ml of 1 M Tris for every 2.0 ml of eluent. The labelled ATP was eluted in about 15 ml of 0.25 N HCl, the pH adjusted to 7.0 and stored frozen in aliquots.

ISOLATION OF PROTEIN KINASE FROM YEAST

Yeast cells from 3-4 litres of culture medium were harvested and broken by grinding in a mortar and pestle in 20 mM Tris -HCl buffer (pH 7.5) containing 1 mM DTT. After centrifugation at 3000 rpm in a Remi centrifuge, the super-

natant was collected and centrifuged again at 14,000 x g in a K-24 centrifuge for 30 min. The clear supernatant was referred to as the crude extract. The crude extract was loaded on a DE-52 column (2.5 x 10 cm), previously equilibrated with Tris-HCl-DTT buffer (pH 7.5) and washed with same buffer till all the unabsorbed proteins were removed. Then proteins were eluted with a linear gradient of KCl, 0-0.5 M in the same buffer. About sixty fractions, each containing 4 ml, were collected and assayed for protein kinase activity.

ASSAY OF PROTEIN KINASE ACTIVITY

The standard reaction mixture contained in 50 µl : 25 mM HEPES-KOH (pH 7.2), 4 mM magnesium acetate, 0.4 mM γ-(^{32}P)ATP ($\approx$ 300 cpm/pmole), 30 µg Bovine serum albumin, 50 µg Histone type II A and 10 µM cAMP wherever indicated.

The reaction was started by adding 30 µl of DE-52 column fraction (120-150 µg) and reaction mixture was incubated at 30^{o}C for 8 min with shaking. To stop the reaction, 1 ml of 10% TCA was added in cold. The samples were then boiled in a boiling water bath, after keeping in cold for 30 min precipitates were collected on GF/C filters, washed with 50 ml of cold 5% TCA containing 5 mM sodium phosphate. Filters were dried and counted in a toluene based scintillation fluid in a Beckman Scintillation Counter.

ASSAY FOR DETERMINATION OF ENDOGENOUS SUBSTRATE FOR PROTEIN KINASE

For determination of endogenous substrates, according to the method of Uno et al. (135) the assay volume was 100 µl which contained 50 mM PIPES (pH 7.0) and 5 mM magnesium chloride and 1 µm cAMP where indicated. The mixture was incubated at 30°C for 4 min with shaking and reaction was stopped by adding a solution containing 9% SDS, 0.03 M Tris-HCl (pH 7.0), 6% 2-mercaptoethanol, 3 mM EDTA and 15% glycerol. This was then boiled for 5 min and used for SDS polyacrylamide gel electrophoresis, given below.

SDS - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was done according to the method of Laemmli with minor modifications (136). Running gel was made of 10% acrylamide, and 0.27% N-N'methylene bis-acrylamide, 0.1% SDS and 0.375 M Tris-HCl buffer (pH 8.8). The various components were mixed, deaerated and polymerization was initiated by adding 0.025% TEMED and 0.025% ammonium persulphate. The solution was quickly poured into 7 cm gel tubes and water layered on top. After polymerization was over, stacking gel solution containing 3% acrylamide, 0.1% SDS and 0.125 M Tris-HCl (pH 6.8) was poured and allowed to polymerize. The running buffer was composed of Tris (0.3%) glycine (1.42%) buffer (pH 8.3) and 0.1% SDS. After loading the samples, stacking was carried out at 1 mA per gel in constant current mode

and electrophoresis was done using a current of 3 mA per gel at room temperature, till the marker dye reached about 1 cm from the bottom of the gel tube.

After electrophoresis was completed gels were put into 10% TCA to fix the protein, sliced, dried and counted in a toluene based scintillation fluid.

PAGE AND SDS-PAGE OF CASEIN KINASE

For the detection of purity of casein kinase the sample was electrophoresed on a 7.5% neutral polyacrylamide slab gel. Stacking gel was made of 3% acrylamide. The components of the SDS containing gel were the same as that given above and in this case the sample was run on a slab gel. Stacking was done for both gels at 60 volts and electrophoresis at 120 volts, in the constant voltage mode. The sample buffer for SDS-PAGE had the following composition :

Tris-HCl	-	0.625 M (pH 6.8)
SDS	-	2%
Glycerol	-	10%
2-Mercaptoethanol	-	5%
Bromophenol blue	-	0.001%

Samples were boiled before running on the gel for 2 mins in a boiling water bath.

In both cases the gels were fixed in 10% TCA for 5-6 hrs after the run and stained in a staining solution (0.25% Coomassie brilliant blue in 45% methanol and 9% acetic acid) for 18-20 hrs. The gels were then destained in a destaining solution (20% methanol and 7.5% acetic acid). Pharmacia gel electrophoresis markers were used for determination of molecular weight.

PREPARATION OF CASEIN-SEPHAROSE FOR AFFINITY CHROMATOGRAPHY

Affinity matrix was made according to the method of Porath (137). Sepharose 6B was used as the matrix. About 15 gm of sepharose 6B was suspended in 20 ml of 4 M phosphate buffer (pH 12.0). At first the Sepharose 6B was activated with cyanogen bromide (CNBr) and then the substrate (casein) was bound to it.

The gel was then washed with same buffer on a sintered glass funnel. Next, the gel was suspended in 30 ml of 4 M phosphate buffer (pH 12.0) and 20 ml of CNBr solution (100 mg/ml) was added to it over a period of 2 min, slowly with gentle shaking at 0-5^{o}C. It was then incubated with gentle shaking at 5^{o}C for 10 min and then washed with cold water until it reaches pH 7.0.

The gel was then transferred to a coupling buffer of 0.1 M sodium bicarbonate (pH 8.9) containing 0.5 M sodium chloride and the ligand at a concentration of 5 mg/ml of

gel and rotated gently on a water bath shaker at 30°C for 24 hrs. The gel was then washed with an excess of the same coupling buffer till the filtrate from the funnel had no protein.

The protein in the gel was detected by Bradford method. The gel was stored in 0.02% sodium azide at 4°C.

ASSAY FOR ACTIVATION OF SEPHAROSE 6B WITH CNBr

To 10-20 mg of dry polysaccharide or the activated gel 1-2 ml of the qualitative reagent (described below) was added. After slight shaking even less than 5 nmoles of CNBr or cyante ester can be detected by the formation of red-purple colour, which develops within 30 sec and becomes maximum within 30 min.

COMPOSITION OF THE QUALITATIVE REAGENT

12 ml pyridine was slowly mixed with 25 ml concentrated hydrochloric acid. To this 0.5 gm of barbituric acid was added and the volume was made upto 20 ml with distilled water. It was stirred for 10 min to get a clear solution and stored in a dark bottle.

CASEIN KINASE ASSAY

Casein kinase was assayed essentially by the same procedure as the general protein kinase assay. In this case, however, no BSA was used in the assay to make sure

that only the substrate i.e. casein is being phosphorylated. Incubation was carried out either in presence or absence of cAMP upto 10 min and then the reaction was stopped. The specific activity of γ -(^{32}P)ATP was $\simeq$ 68 cpm/pmole.

For characterisation of the enzyme in various assays, different substrates, different ions or different concentrations of substrates were used. The inhibitors were used in concentrations mentioned in the TableX.

PURIFICATION OF CASEIN KINASE BY AFFINITY CHROMATOGRAPHY

Cells were harvested from 3-4 litres of rapidly growing culture at midlong phase, and washed with sterile water. The cells were broken using sterile glass beads (0.45 - 0.5 mm diameter) by vortexing for 6-10 min in cold in a buffer containing 20 mM Tris-HCl (pH 7.5) and 1 mM 2-mercaptoethanol. After centrifugation at 15,000 rpm, for 45 min in Sorval RC5 (SS34 roter), the supernatant was loaded on a casein-sepharose 6B column (1 x 8.5 cm) previously equilibrated and washed with the same buffer. After washing the column thoroughly to remove all the unbound proteins, the bound protein was eluted with the same buffer containing 0.5 M KCl. The peak fraction was used for assaying the activity, and the column was made ready for reuse by eluting any remaining protein with the buffer containing 2 M KCl.