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# Solubilization and Other Studies on Adenylate Cyclase of Baker's Yeast

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1. Adenylate cyclase of *Saccharomyces cerevisiae* was sedimented from mechanically disintegrated preparations of yeast over an unusually wide range of centrifugal forces. 2. The enzyme was readily solubilized by Ficoll and by Lubrol PX. Lubrol caused a 2-fold activation. 3. Both particle-bound and Lubrol-solubilized enzyme had an apparent $K_m$ for ATP of 1.6mM in the presence of 0.4mM-cyclic AMP and 5mM-$MnCl_2$ at pH6.2 and 30°C. 4. The Lubrol-solubilized enzyme behaved on gel filtration as a monodisperse protein with an apparent mol.wt. of about 450000.

Adenylate cyclase activity in yeasts was first found in *Saccharomyces fragilis* by Sy & Richter (1972). Much higher activities of a $Mn^{2+}$-dependent enzyme with a slightly acid pH optimum were subsequently found in *Saccharomyces cerevisiae* (Londesborough & Nurminen, 1972), and it seems probable that fungal adenylate cyclases generally exhibit a $Mn^{2+}$ requirement and low pH optimum (Flawiá & Torres, 1972*a*; Paveto *et al.*, 1975). The enzyme of *Sacch. cerevisiae* is particulate, although its intracellular location is not known (Wheeler *et al.*, 1974). We now report the solubilization of the enzyme by the non-ionic detergent, Lubrol PX (a polyoxyethylene ether; Elworthy & Macfarlane, 1963), and a comparison of the solubilized enzyme and different particulate preparations. No convincing evidence was found for the existence of any adenylate cyclase activity not dependent on $Mn^{2+}$ ions in *Sacch. cerevisiae*.

## Materials and Methods

### *Buffers*

Yeast and subcellular fractions were suspended in Buffer T [25mM-Tris/HCl (pH7.2)/1mM-$MnCl_2$/0.3mM-EDTA/7mM-mercaptoethanol], with the additions shown in the text, except in the isolation of nuclei from spheroplasts, when Buffer N [20mM-potassium phosphate (pH6.7)/0.5mM-$MgCl_2$/0.1mM-EDTA] and Buffer P [10mM-potassium phosphate/0.5mM-$MgCl_2$/0.1mM-EDTA/20% (v/v) glycerol/5% (w/v) polyvinylpyrrolidone, final pH6.2] were used.

### *Disintegration and fractionation of yeast*

About 10g fresh wt. of commercial baker's yeast from our Rajamäki factories was suspended in 30ml of Buffer T containing any additions stated in the text. Duplicate 2ml samples were filtered through tared G4 glass sinters to collect yeast for dry-weight determinations. The rest of the suspension was mixed with 40ml of cold 0.2mm-diameter glass beads and homogenized for two 12min periods in the glass cup of a Mini mill (Gifford Wood Co., Hudson, NY, U.S.A.) surrounded by an ice/water mixture. The temperature of the suspension rose from 0°C to about 10°C, and over 90% of the cells were broken.

Glass beads were removed by filtration through a G1 glass sinter, and cell envelopes and any unbroken cells sedimented by centrifugation for 10min at 1000*g* (or 2000*g* where stated). This large sediment was washed with about 5 times its volume of buffer. Particulate material was sedimented from the combined supernatants by centrifugation for 45min at 34000*g* and/or 2h at 105000*g*.

### *Preparation and fractionation of spheroplasts*

Spheroplasts were prepared from $A_3$ yeast, which is a semi-aerobic stage in the commercial production immediately preceding the final commercial product, and with a succinate–cytochrome *c* reductase activity of 16$\mu$mol/min per g dry wt. of yeast. The yeast was preincubated with 0.1M-EDTA/0.35M-mercaptoethylamine, and then washed with and suspended in Buffer N containing 1.0M-sorbitol. Snail digestive enzyme (1ml of glusulase/5g fresh wt. of yeast) was added. Spheroplast formation was essentially complete after incubation for 1h at 30°C. Nuclei were partially purified from these spheroplasts by a modification of the method of Wintersberger *et al.* (1973). The spheroplasts were washed with Buffer N containing 1.0M-sorbitol and then suspended in Buffer N containing 18% (w/v) Ficoll (4ml/g fresh wt. of yeast). Remaining cells and unlysed spheroplasts were removed by centrifugation for 10min at 2000*g*, and

the lysate was then centrifuged for 45min at 34000*g*. We found that about 40% of the nuclei float in this centrifugation. They were recovered as a sediment by dilution of the floating material with Buffer N containing 18% Ficoll and re-centrifugation for 30min at 34000*g*. The two 34000*g* sediments (1st P and 2nd P, Table 1) were suspended in Buffer P containing 1.0M-sorbitol, and 7ml was layered on top of 10ml of Buffer P containing 2.6M-sorbitol, over 10ml of Buffer P containing 3.2M-sorbitol. After centrifugation for 1h at 150000*g* most of the nuclei had collected at the interface between the 2.6M- and 3.2M-sorbitol layers.

*Extraction of the 105000g sediment with Lubrol*

A portion of the sediment was suspended in the appropriate buffer to a final volume of 7ml and placed in the cup of a Mickle disintegrator (The Laboratory Engineering Co., Gomshall, Surrey, U.K.) with 6.5ml of cold 1mm-diameter glass beads. The mixture was shaken at 5°C and maximum speed for 1–30min as indicated in the text, and then poured through a G1 glass sinter to remove the beads. The beads were washed with 1.5ml of buffer. The combined filtrates were then centrifuged for 1 or 2h at 105000*g*.

*Assays*

Enzyme activities were measured at 30°C. Adenylate cyclase was determined by a modification of the method previously described (Londesborough & Nurminen, 1972). Reaction mixtures contained 100mM-Pipes [piperazine-*NN'*-bis-(2-ethanesulphonic acid)]/KOH, pH6.2, 5mM-$MnCl_2$, 1.6mM-$MgCl_2$, 10mM-phosphoenolpyruvate (potassium salt), pyruvate kinase (0.25mg/ml), 2mM-[8-$^{14}$C]ATP (1200c.p.m./nmol) and 0.4mM-cyclic AMP. Reactions were started by addition of sufficient enzyme to cause the linear production of cyclic [$^{14}$C]AMP with time for about 20min. A milliunit (munit) of adenylate cyclase was defined as the amount of enzyme which produces 1nmol of cyclic AMP/min under these conditions. At suitable intervals (30s–40min) 0.1ml samples of the reaction mixture were added to 0.1ml of 75% (v/v) ethanol containing 1mM-cyclic AMP and 1mM-adenine in glass-stoppered centrifuge tubes, and heated in boiling water for 1min. The tubes were cooled in ice, centrifuged at 1000*g*, and 60$\mu$l portions of the supernatants applied to thin-layer plates (20cm×20cm) prepared from silica gel $GF_{254}$ suspended in 5% (w/v) $Na_2B_4O_7$,10$H_2O$, as described by Upton (1970). Five samples were run on each plate. The plates were developed first with 50% (v/v) ethanol until the solvent reached the top of the plate, and then dried and developed in the opposite direction with water-saturated butan-1-ol. Nucleoside 5′-tri-, di- and mono-phosphates, AMP (2′- and 3′-) and nucleosides migrate much more slowly than cyclic AMP in 50% ethanol (Upton, 1970; Londesborough, 1976). Adenine and hypoxanthine migrate immediately behind cyclic AMP, but are removed by the water-saturated butan-1-ol, in which solvent cyclic AMP is immobile. The cyclic AMP spots were located under u.v. light, scraped off the plates and suspended in 1ml of water. After centrifugation, 0.8ml of each supernatant was added to 15ml of scintillation mixture, prepared as described by Bray (1960) but without the secondary scintillant. Tubes were stored in the dark for several hours and radioactivity was then determined in a Packard Tri-Carb 2420 liquid-scintillation counter, by using a wide discriminator setting.

The t.l.c. procedure of Upton (1970) used here requires a minimum of equipment and can be completed in 5h. At least 90% of the radioactivity found in the cyclic AMP spots was due to cyclic [$^{14}$C]AMP. In one experiment, used reaction mixtures in which less than 1% of the [$^{14}$C]ATP had been converted into cyclic AMP were chromatographed and the cyclic AMP spots extracted with water as described above. Combined extracts were treated with 12$\mu$g of a highly purified soluble cyclic AMP phosphodiesterase from yeast (Londesborough, 1974) of specific activity 20$\mu$mol/min per mg of protein. After re-chromatography, 86% of the radioactivity in the original extracts (1800c.p.m.) was recovered, and 90% of this recovered radioactivity co-chromatographed with 5′-AMP. The cyclic AMP and adenine spots each contained 3% of the recovered radioactivity.

Protein was determined by the biuret reaction (Gornall *et al.*, 1949) on samples dialysed against 20mM-potassium phosphate, pH7.0, and incubated with 5% (w/v) sodium deoxycholate. It was assumed that 1mg of protein in 3ml of assay mixture caused an $E_{540}$ of 0.1$cm^{-1}$.

DNA was determined as described by Burton (1956).

*Gel filtration*

Sepharose 6B or Sephadex G-200 was equilibrated with Buffer T containing 0.3M-KCl with or without 0.1% Lubrol, and columns (2.6cm×37cm) were built and used at 5°C. Fractions (2–4ml) were collected with a drop counter.

*Hydroxyapatite adsorption*

Hydroxyapatite was prepared as described by Levin (1962), and stored at 113mg/ml in 20mM-potassium phosphate, pH7.2, containing 1mM-$MgCl_2$. After addition of 1ml of hydroxyapatite to 18ml of a Sepharose 6B eluate containing 2.4munits of adenylate cyclase and 5.3mg of protein in Buffer T containing 0.3M-KCl and 0.1% Lubrol, the hydroxyapatite

was sedimented at 12000*g*, and 1.9munits of adenylate cyclase were recovered by washing the sedimented hydroxyapatite three times with 0.5ml portions of 0.4M-potassium phosphate, pH7.5, containing 0.1% Lubrol. The combined extracts were dialysed against Buffer T containing 0.3M-KCl and 0.1% Lubrol before assay.

*Materials*

NEN Chemicals G.m.b.H. (Dreieichenhain, West Germany) supplied the [8-$^{14}$C]ATP. Other nucleotides, nucleosides and purines, and phosphoenolpyruvate were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sepharose 6B and Dextran Blue 2000 were from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Horse heart cytochrome *c*, ox serum albumin and ox liver catalase were purchased from Sigma, and rabbit muscle pyruvate kinase and *Escherichia coli* β-galactosidase were from Boehringer und Soehne G.m.b.H. (Mannheim, West Germany). The β-galactosidase contained an impurity which behaved like serum albumin on gel filtration and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Glucose concentrations in growth media were measured with glucose oxidase, by using the Blood Sugar Test Programme supplied by Boehringer. Glusulase was from Endo Laboratories Inc. (Garden City, NY, U.S.A.).

The Lubrol used was Lubrol PX from Sigma. Silica gel $GF_{254}$ (E. Merck, Darmstadt, West Germany), polyvinylpyrrolidone (Fluka A.G., Buchs, Switzerland) and Ficoll (Pharmacia) were obtained from the suppliers indicated.

## Results

*Fractionation of spheroplast lysates in* 18% *Ficoll*

Adenylate cyclase activity is present in the sediments collected at 1000, 34000 and 105000*g* and in the 105000*g* supernatant of yeast mechanically disintegrated in the Mini mill (Table 2). Similar results have been reported for yeast disintegrated with a Mickle disintegrator (Londesborough & Nurminen, 1972), and with that disintegration procedure as much as 40% of the total activity is sometimes found in the well-washed 1000*g* sediment (K. Varimo, unpublished work). This unusually wide sedimentation pattern would be expected if adenylate cyclase were located in the plasma or nuclear membranes, because both these membranes fragment during mechanical disintegration of yeast. Many of the remaining nuclei and pieces of plasma membrane still attached to the cell walls sediment at 1000*g* in the cell-envelope fraction (Nurminen *et al.*, 1970), but smaller fragments of both membranes contaminate the mitochondrial and microsomal fractions.

The distribution of adenylate cyclase during the isolation of nuclei from spheroplasts by the method of Wintersberger *et al.* (1973) is shown in Table 1. Evidently Ficoll causes extensive solubilization of adenylate cyclase. The soluble supernatants (1st S and 2nd S) together contained 54munits/g dry wt. of yeast, compared with 55munits observed directly in the lysate, and a total recovered activity of 74munits. Recovery of 135% of the lysate activity and the higher specific activity in fraction 2nd S than in 1st S suggest that solubilization by Ficoll causes activation, and is a relatively slow process. Fraction 1st S was diluted

Table 1. *Distribution of adenylate cyclase during the isolation of nuclei*

Spheroplasts of $A_3$-stage yeast were allowed to lyse in 18% Ficoll, pH6.7, and fractionated by differential centrifugation as described in the Materials and Methods section.

| | DNA yield (%) | Protein/DNA (mg/mg) | Adenylate cyclase (munits/g dry wt. of yeast) | Adenylate cyclase (munits/mg of protein) |
|---|---|---|---|---|
| Spheroplast lysate | (100) (1.3mg/g of yeast) | 160 | 54.6 | 0.30 |
| First Ficoll centrifugation | | | | |
| 2000*g* Precipitate | 14 | 130 | 6.5 | 0.26 |
| 34000*g* Precipitate (1st P) | 42 | 58 | 2.9 | 0.093 |
| Soluble supernatant (1st S) | 4 | 1700 | 36.8 | 0.40 |
| 34000*g* Float (1st F) | 34 | 154 | — | — |
| Re-centrifugation in Ficoll of '1st F' | | | | |
| 34000*g* Precipitate (2nd P) | 34 | 34 | 4.3 | 0.26 |
| Soluble supernatant (2nd S) | 0.4 | 3600 | 17.3 | 0.85 |
| 34000*g* Float (2nd F) | 3.6 | 370 | 5.9 | 0.30 |
| '1st P' and '2nd P' centrifuged through sorbito | | | | |
| 2.6M/3.2M-sorbitol interface | 17 | 28 | 1.0 | 0.15 |

Table 2. *Effect of Lubrol on the yield and centrifugal distribution of adenylate cyclase in yeast homogenates*

Commercial baker's yeast was disintegrated in Buffer T containing the indicated additions. Total activities (munits/g dry wt. of yeast) and specific activities (munits/mg of protein) in fractions obtained by differential centrifugation are shown. N.D., Not determined.

| Addition | Whole homogenate | 1000*g* Sediment | 34000*g* Sediment | 105000*g* Sediment | 105000*g* Supernatant | 105000*g* Lipid layer |
|---|---|---|---|---|---|---|
| None | | | | | | |
| Total activity | 49 | 5.1 | 19 | 12 | 8.9 | 0.2 |
| Specific activity | N.D. | N.D. | 0.20 | 0.23 | 0.12 | 0.03 |
| 0.1% Lubrol/0.3 M-KCl | | | | | | |
| Total activity | 115 | N.D. | 11 | 18 | 71 | 10 |
| Specific activity | N.D. | N.D. | 0.14 | 0.58 | 0.66 | 0.56 |

with an equal volume of water and centrifuged for 2h at 105000*g*. About 20% of the protein but only 11% of the adenylate cyclase sedimented, the remainder being recovered in the optically clear supernatant. Thus at least 65% of the total activity was solubilized when spheroplasts were allowed to lyse in 18% Ficoll, without use of mechanical disintegration procedures. The total particulate activity was 19 munits/g dry wt. of yeast, including the sedimentable activity in fractions 1st S and 2nd S, but excluding that in the 2000*g* precipitate. Fraction 2nd P and the material purified through the sorbitol gradient contained 22 and 5.3%, respectively, of this particulate activity, and 34 and 17% of the total DNA. Therefore, of the remaining particulate adenylate cyclase, not more than 65–30% can be located in the nuclei, and possibly less, since the nuclear preparations were only 50–60% pure, assuming a protein/DNA ratio of 17 for yeast nuclei (Wintersberger *et al.*, 1973).

*Solubilization by Lubrol*

Table 2 shows the effect of adding Lubrol to the buffer in which commercial baker's yeast was homogenized. The yield increased 2-fold in the presence of 0.1% Lubrol and 0.3 M-KCl, and 60% of the total activity remained in the optically clear 105000*g* supernatant. A lipid layer was separately removed from above this supernatant, and contained a further 9% of the activity. Lubrol alone caused similar effects (not shown), but KCl was usually included because this reagent itself slightly increased the proportion of non-sedimentable activity.

Adenylate cyclase activity in the 105000*g* supernatant obtained as described above appeared to be soluble, because it was eluted from Sephadex G-200 (equilibrated with Buffer T containing 0.1% Lubrol and 0.3 M-KCl) immediately after the void volume. However, it tended to aggregate into a sedimentable form. For example, when the 105000*g* supernatant obtained in the presence of 0.1% Lubrol/0.3 M-KCl shown in Table 2 was stored for 12 days at −20°C and then thawed and centrifuged for 2h at 105000*g*, only 23% of the original activity remained in the supernatant. However, 69% of the original activity was recovered in the sediment, with specific activity (1.23 munits/mg of protein) twice that of the original supernatant. Apparently the activation by Lubrol was not simply a result of solubilization.

In order to obtain preparations of solubilized adenylate cyclase which did not readily aggregate, the sediment collected between 2000*g* and 105000*g* from yeast disintegrated in Buffer T was extracted with Lubrol by shaking in a Mickle disintegrator as described in the Materials and Methods section. The 105000*g* sediment was always stored at −20°C for at least 15h before extraction with Lubrol. Trials with a shaking time of 30 min and a protein concentration of 4.6 mg/ml showed that addition of 0.1 or 0.5% Lubrol to the buffer increased the recovery of activity from 34 to 61 and 100% respectively, and increased the non-sedimentable proportion of the recovered activity from 13 to 42 and 55%. With a shaking time of only 1 min, and a protein concentration of 11.6 mg/ml, the recovery of activity in the absence of Lubrol was improved. Under these conditions the recovered activity in the presence of Lubrol was twice that in the absence of Lubrol, and 25% higher than the starting activity (Table 3). At 0.3% Lubrol (i.e. a Lubrol/protein wt. ratio of 0.26) 58% of the recovered activity did not sediment at 105000*g*. Neither the total amount, nor the sedimentability, of the recovered activity changed significantly between 0.3 and 5.0% Lubrol, although more protein was solubilized by the higher concentration. These results suggested that a part of the adenylate cyclase was relatively resistant to solubilization by Lubrol. The sediments obtained in the experiment of Table 3 were stored at −20°C, thawed, and adjusted to about 8 mg of protein/ml and the same Lubrol concentration as used in the first extraction. The suspensions were treated in the Mickle disintegrator as before. Between 82 and 99% of the initial activities were recovered, and at 0.3, 1.0 and 5.0% Lubrol, 5.7, 6.1 and 10.6 munits/g dry wt. of yeast were still sedimented at 105000*g*. In a similar experiment the 105000*g* sediment was extrac-

Table 3. *Solubilization of adenylate cyclase by increasing concentrations of Lubrol*

The sediment collected between 2000 and 105000*g* from commercial baker's yeast disintegrated with the Mini mill in Buffer T was suspended in Buffer T and stored for 6 days at −20°C. Portions of thawed material were centrifuged for 1 h at 105000*g* (18% of the activity remained in the supernatants). The sediments ('Starting sediments') were suspended in Buffer T containing 0.3 M-KCl and the indicated amounts of Lubrol to a final protein concentration of 11.6 mg/ml. The suspensions were shaken for 1 min in a Mickle disintegrator, as described in the Materials and Methods section, and then centrifuged for 2 h at 105000*g*. The sediments were suspended in Buffer T, and the total activity (munits/g dry wt. of yeast) and protein contents (mg/g dry wt. of yeast) determined. Specific activities are munits/mg of protein.

| | Starting sediments | Lubrol extractions | | | |
|---|---|---|---|---|---|
| | | No Lubrol | 0.3% Lubrol | 1.0% Lubrol | 5.0% Lubrol |
| Sediment | | | | | |
| Total activity | 27 | 14.4 | 13.9 | 14.6 | 13.6 |
| Protein | 96 | 80 | 70 | 74 | 68 |
| Specific activity | 0.28 | 0.18 | 0.20 | 0.20 | 0.20 |
| Supernatant | | | | | |
| Total activity | — | 2.9 | 20 | 20.5 | 19 |
| Protein | — | 8.0 | 19 | 22 | 31 |
| Specific activity | — | 0.36 | 1.06 | 0.93 | 0.61 |
| Recovery (%) | (100) | 65 | 126 | 131 | 121 |

ted three times with 1,0% Lubrol, without freezing between extractions. At each step a smaller proportion of the remaining activity was solubilized, and there was an apparently insoluble residue of about 7 munits/g dry wt. of yeast.

The gel-filtration behaviour on Sepharose 6B of adenylate cyclase solubilized with 1.0% Lubrol is shown in Fig. 1. Two small peaks of activity, probably associated with particulate material, emerged soon after the Dextran Blue 2000 exclusion volume, but 90% of the applied activity was recovered in a symmetrical peak 50 ml later. Comparison with the behaviour of the marker proteins indicates an apparent mol.wt. of about 450000 for detergent-dispersed adenylate cyclase. Most of the protein solubilized by Lubrol was considerably smaller than this. Similar results were obtained when material extensively dialysed against Buffer T containing 0.3 M-KCl and 0.1% Lubrol was applied to Sepharose 6B equilibrated with Buffer T containing 0.3 M-KCl but no Lubrol. However, Lubrol appeared in the eluate fractions containing adenylate cyclase and solubilized protein, although both the fractions immediately before adenylate cyclase, and those after the main peak of protein, did not contain Lubrol.

*Catalytic properties of particle-bound and Lubrol-solubilized enzyme*

In the presence of 0.4 mM-cyclic AMP, the apparent Michaelis constant for ATP was 1.6 mM both for enzyme bound to the sediment collected between 2000*g* and 105000*g* and for enzyme solubilized from this sediment with Lubrol (Fig. 2). Inhibition was observed above 2 mM-ATP with all enzyme samples tested (shown only for the solubilized enzyme). At 8 mM-ATP this inhibition was decreased, but not abolished, by increasing the concentration of $MnCl_2$ from 5 to 10 mM. Results obtained with the adenylate cyclase activity in the cell-envelope fraction collected at 2000*g* are also shown in Fig. 2. The apparent Michaelis constant (2 mM) was slightly higher with this material. Because it contains less cyclic AMP phosphodiesterase activity than the 105000*g* sediment (Londesborough, 1975), linear progress curves could be obtained in the presence of only 0.1 mM-cyclic AMP. Neither the initial rates nor the Michaelis constant were changed.

Table 4 shows the effect of replacing the 5 mM-$MnCl_2$ in the assay system by an extra 5 mM-$MgCl_2$ at pH 6.2 and 7.0. The four different kinds of enzyme preparation tested behaved similarly, within the error of measurement of the very small rates in the absence of $Mn^{2+}$ ions.

## Discussion

We have not been able to find evidence for any adenylate cyclase with a low specificity for $Mn^{2+}$ in *Sacch. cerevisiae*. Enzyme in the cell envelope had a slightly less stringent requirement than did the rest of the particle-bound enzyme (Table 4), but this may represent greater difficulty in the removal of endogenous $Mn^{2+}$ from preparations containing cell walls. These two particulate fractions and Lubrol-solubilized enzyme and the Lubrol-insoluble residue all responded similarly to change of pH from 6.2 to 7.0. After Lubrol treatment, both the solubilized enzyme and the insoluble residue retained a high $Mn^{2+}$ specificity. We reported (Londesborough &

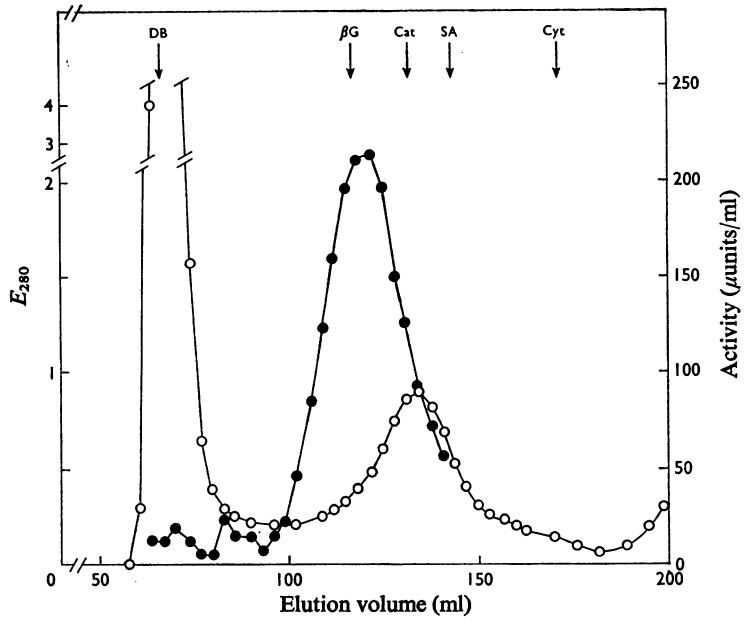


Fig. 1. *Gel filtration of Lubrol-solubilized adenylate cyclase on Sepharose 6B*

The 105000*g* sediment was extracted in the Mickle disintegrator with Buffer T containing 0.3M-KCl and 1% Lubrol, and centrifuged for 1h at 105000*g*. A 4ml sample of the resulting supernatant was applied to Sepharose 6B equilibrated with Buffer T containing 0.3M-KCl and 0.1% Lubrol. Arrows show the exclusion volumes of Dextran Blue 2000 (DB, mol.wt. 2000000), β-galactosidase (βG, mol.wt. 520000), catalase (Cat, mol.wt. 240000), serum albumin (SA, mol.wt. 68000) and cytochrome *c* (Cyt, mol.wt. 12400) in the absence of Lubrol. The exclusion volumes of β-galactosidase and serum albumin were unaltered in the presence of Lubrol. ○, $E_{280}$; ●, activity.

Nurminen, 1972) that 10mM-NaF slightly activated adenylate cyclase at pH7.0, whereas Wheeler *et al.* (1974) found a small inhibition at pH6.2. We now find that, at both pH6.2 and 7.0, 10mM-NaF has no effect on the initial rates of reaction with either $Mn^{2+}$ or $Mg^{2+}$ as added cation (K. Varimo & J. Londesborough, unpublished work). However, NaF much improved the linearity of progress curves at pH7.0, presumably by inhibiting adenosine triphosphatase, which is more active at the higher pH.

Sy & Richter (1972) found the adenylate cyclase content of *Sacch. fragilis* to change by more than 20-fold depending on the growth conditions, and, in particular, they were unable to detect any activity in cells grown either under anaerobic conditions or on 10% glucose. In contrast, *Sacch. cerevisiae* grown in $O_2$-free $N_2$ contained 40% as much adenylate cyclase as did the de-repressed cells grown under vigorous aeration used in most of the present work (K. Varimo, unpublished work). Sy & Richter (1972), however, measured adenylate cyclase activity at 30°C and pH7.0 in the presence of 0.5mM carrier cyclic AMP, but without added $Mn^{2+}$ ions. In view of the very low activities that they found (the maximum they reported was 10pmol/min per mg of protein in a spheroplast lysate, which is 30-fold smaller than shown in Table 1), it is probable that the adenylate cyclase of *Sacch. fragilis* also has a specific $Mn^{2+}$ requirement, in common with the enzymes of baker's yeast and at least some other fungi (Flawiá & Torres, 1972*a*; Paveto *et al.*, 1975). It would be interesting to know whether the severe repression of adenylate cyclase in *Sacch. fragilis* represents a species difference from *Sacch. cerevisiae*, or whether the large variations in activity found by Sy & Richter (1972) would not be observed if adenylate cyclase were measured in the presence of $Mn^{2+}$ ions. The low activities observed in the absence of $Mn^{2+}$ would easily be disturbed by cyclic AMP phosphodiesterase activity, which is more than 1000pmol/min per mg of protein at 0.5mM-cyclic AMP in baker's yeast (Londesborough, 1975).

The distribution of enzyme among fractions obtained by differential centrifugation of preparations of mechanically disintegrated yeast is unusually wide (Table 2). Wheeler *et al.* (1974) have shown that yeast adenylate cyclase does not co-sediment with heavy fragments of the plasma membranes through sucrose density gradients. Our attempts to demonstrate a nuclear location for the enzyme were inconclusive, because of the extensive solubilization caused by

Ficoll. A substantial proportion of the remaining particulate activity was found in fractions in which nuclei accounted for 50–60% of the protein. Examination of purer nuclei is not likely to be fruitful until a method has been developed to isolate them without solubilizing adenylate cyclase. This may prove difficult, since, in contrast with the mammalian enzymes, yeast adenylate cyclase is rather weakly bound by subcellular particles, and a variety of agents cause at least partial solubilization. For example, 20% of the activity of the 105000*g* sediment was solubilized by standing for 1h at 0°C in Buffer T containing 1M-sucrose, and 40% was solubilized by standing for 45min at 30°C in Buffer T containing 0.3M-KCl (K. Varimo & J. Londesborough, unpublished work). Adenylate cyclase of *Tetrahymena pyriformis* is also solubilized by sucrose solutions (Kassis & Kindler, 1975).

Stable solubilized preparations were obtained by extracting the 105000*g* sediment with Lubrol. Some 20% of the original activity could not be solubilized either by increasing the concentration of Lubrol from 0.3 to 5.0% (Table 3) or by repeated extractions with 1.0% Lubrol. However, the catalytic activity of the insoluble residue was not distinguishable, by a number of criteria, from the solubilizable activity. Possibly some of the adenylate cyclase is not accessible to Lubrol because of the macroscopic structure of the disrupted and sedimented membranes. Lubrol activated adenylate cyclase by about twofold. This is probably due to unmasking of previously inaccessible enzyme, since the activation persisted after reaggregation, and the Michaelis constants of solubilized and particle-bound enzymes were the same (Fig. 2). Solubilization without change in the Michaelis constant is consistent with a model in which adenylate cyclase is rather loosely particle-bound through parts of the protein not close to the catalytic site.

Lubrol-dispersed adenylate cyclase was eluted from Sepharose 6B immediately after the β-galactosidase exclusion volume, with an apparent mol.wt. of about 450000 (Fig. 1). This probably refers to a molecule containing bound Lubrol.

Adenylate cyclase from *Sacch. cerevisiae* closely resembles the enzyme from *Neurospora crassa* (Flawiá & Torres, 1972*a*,*b*). Both enzymes have a

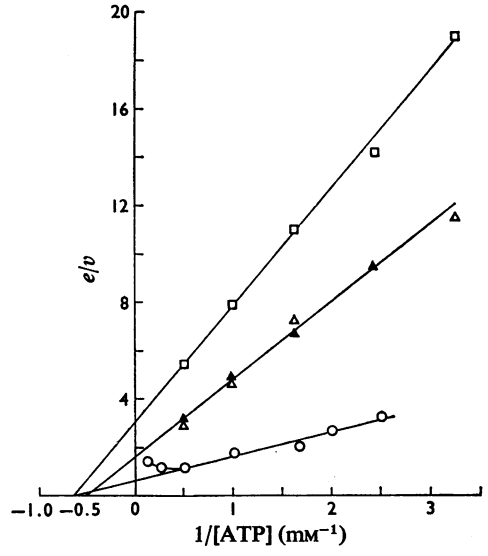


Fig. 2. *Michaelis constants of particle-bound and solubilized adenylate cyclase*

Initial rates (*v*/*e*) determined in standard assay mixtures containing 0.31–8mM-ATP are expressed as nmol/min per mg of protein for the 2000*g*–105000*g* sediment (□) and for enzyme solubilized from this sediment with Lubrol, chromatographed on Sepharose 6B, and concentrated with hydroxyapatite as described in the Materials and Methods section (○). For enzyme in the 2000*g* sediment, initial rates measured in the presence of (△) 0.4mM- or (▲) 0.1mM-cyclic AMP are expressed as nmol/min per 60mg dry wt. of yeast.

Table 4. *Effect of $Mn^{2+}$ and $Mg^{2+}$ on the activity of some adenylate cyclase preparations*

Activities were measured at the pH and bivalent-metal-ion concentrations shown, with other components as in the standard assay. Enzyme preparations were dialysed for 4h against two changes of 25mM-Tris/HCl, pH7.2, containing 7mM-mercaptoethanol, 0.3mM-EDTA and 1mM-$MgCl_2$, and were the 2000*g* sediment, the sediment between 2000 and 105000*g*, and the first soluble extract and final insoluble residue after three extractions of this sediment with 1% Lubrol. The activities at pH6.2, with 5mM-$MnCl_2$, have been set at 100. Abbreviations: N.D., not determined; Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N*′-yl)ethanesulphonic acid.

| | 2000*g* Sediment | 105000*g* Sediment | 1% Lubrol supernatant | 3×1% Lubrol insoluble residue |
|---|---|---|---|---|
| pH6.2, Pipes buffer | | | | |
| 1.6mM-$MgCl_2$, 5mM-$MnCl_2$ | 100 | 100 | 100 | 100 |
| 6.6mM-$MgCl_2$, no $MnCl_2$ | 7 | 2.6 | N.D. | N.D. |
| pH7.0, Hepes buffer | | | | |
| 1.6mM-$MgCl_2$, 5mM-$MnCl_2$ | 36 | 29 | 39 | 32 |
| 6.6mM-$MgCl_2$, no $MnCl_2$ | 2.9 | 2.4 | 1.0 | 1.2 |

specific $Mn^{2+}$ requirement and a pH optimum of about 6.0. They are unaffected by NaF, and are particle bound but easily solubilized by Lubrol. A 7.1 S molecule (about 150000 daltons) is the dominant species observed when Lubrol-dispersed enzyme from *N. crassa* is centrifuged through sucrose density gradients, but variable amounts of 14.5 and 17.5 S material were also observed (Flawiá & Torres, 1972*c*). In a detailed kinetic study of the effects of $Mn^{2+}$ and ATP, Flawiá & Torres (1972*b*) found only small differences between the membrane-bound and Lubrol-dispersed enzymes from *N. crassa*. They concluded that MnATP was the true substrate. In the presence of a 0.5mM excess of $MnCl_2$, the particulate and solubilized enzymes had Michaelis constants for MnATP of 1.1 and 0.6mM respectively. The *Sacch. cerevisiae* enzyme has $K_m$ 1.6mM for ATP in the presence of a constant concentration of 5mM-$MnCl_2$ (Fig. 2).

Three other fungal adenylate cyclases have been reported. The enzyme from *Mucor rouxii* is $Mn^{2+}$-specific and unaffected by NaF (Paveto *et al.*, 1975). *Coprinus macrorhizus* (Uno & Ishikawa, 1973) and *Aspergillus niger* (Wold & Suzuki, 1974) both contain apparently soluble adenylate cyclase activity in the presence of $MgCl_2$, but the effect of $Mn^{2+}$ on these enzymes has not been tested. It appears at present that the fungal adenylate cyclases, including those of yeasts, more closely resemble the bacterial enzymes, some of which are $Mn^{2+}$-dependent and only weakly membrane-bound (Ide, 1971), than the mammalian enzymes, which are generally $Mg^{2+}$-dependent and difficult to solubilize.

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