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Soluble adenylate cyclase activity in *Neurospora crassa*

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A soluble form of adenylate cyclase was extracted from mycelia of *Neurospora crassa* wild-type strains. This enzyme activity was purified by chromatography on hexyl-amino-Sepharose, agarose and Blue Sepharose and preparative polyacrylamide-gel electrophoresis. On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, peak fractions from the later purification steps showed a main polypeptide band with an apparent molecular weight of about 66000. The following hydrodynamic and molecular parameters were established for the *Neurospora* soluble adenylate cyclase activity: sedimentation coefficient, 6.25 S; Stokes radius, 7.3 nm; partial specific volume, 0.74 ml/g; molecular weight, 202000; frictional ratio, 1.65. The isoelectric point of this enzyme activity was 4.65. The enzyme was not activated by GTP, [$\beta\gamma$-imido]GTP, fluoride or cholera toxin.

A membrane-bound adenylate cyclase activity [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] in the FGSC 1118 slime strain of *Neurospora crassa* has been described by workers in this laboratory (Flawiá & Torres, 1972*a*,*b*). In this mutant strain the characteristic polysaccharide wall of mycelial strains is absent. This adenylate cyclase activity has two interesting properties: first, it is Mn^{2+}-dependent, the activity with Mg^{2+} being negligible; secondly, it is insensitive to fluoride (Flawiá & Torres, 1972*a*). Such properties are also characteristic of other adenylate cyclases found in lower eukaryotic organisms, such as *Mucor rouxii* (Paveto *et al.*, 1975), *Saccharomyces cerevisiae* (Varimo & Londesborough, 1976), *Trypanosoma cruzi* (da Silveira *et al.*, 1977) and *Blastocladiella emmersoni* (Lopez Gomez *et al.*, 1978), as well as in cytosolic fractions of rat testicular germinal cells (Braun & Dods, 1975; Neer, 1978; Kornblihtt *et al.*, 1981).

The adenylate cyclase activity in *Neurospora* mycelial strains has not been studied in detail. Only two facts have been well established, namely the above-mentioned dependence on Mn^{2+} and the deficiency observed in strains carrying the *cr-1* (crisp) mutation (Flawiá *et al.*, 1977).

A better understanding of the function of adenylate cyclase activity associated with membranes from eukaryotic organisms requires some progress in the purification of this enzyme activity. Such progress, however, is hindered because of three main problems: the enzyme constitutes a small proportion of membrane protein; as an integral part of membranes, it requires detergents for solubilization; the enzyme activity is extremely unstable under reasonably mild conditions. In the case of soluble (Mn^{2+}-dependent) adenylate cyclase activity from testicular germinal cells, some success in the purification has been obtained in this laboratory by procedures that do not involve the use of detergents (Kornblihtt *et al.*, 1981).

The present paper reports that a soluble form of adenylate cyclase can be extracted from the mycelia of wild-type *Neurospora crassa* strains. The purification and characterization of this non-sedimentable or 'soluble' adenylate cyclase activity are described.

Experimental

Materials

Phenylmethanesulphonyl fluoride, Pipes (1,4-piperazinediethanesulphonic acid) and 1,6-diaminohexane were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Bio-Gel A-5m was from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and Trasylol was from Bayer (Leverkusen, West Germany). Sepharose 4B and Blue Sepharose CL-6B were purchased from Pharmacia (Uppsala, Sweden), and *o*-nitrophenyl β-D-glactoside was from Koch–Light Laboratories (Colnbrook, Bucks., U.K.). Hexyl-amino-Sepharose was prepared by the procedure of Cuatrecasas (1970).

The sources of other materials were as previously given elsewhere (Kornblihtt *et al.*, 1981).

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Strains and growth conditions

Unless otherwise indicated, the original St.Lawrence 74 wild-type strain of *Neurospora crassa* from this laboratory's stock was used. Other wild-type strains, FGSC 987 (St.Lawrence), FGSC 2218 (Rockefeller–Lindegren), FGSC 1757 (Abbot) and FGSC 691 (Emerson), as well as the adenylate cyclase-deficient mutant FGSC 488 (*cr-1*) (Flawiá *et al.*, 1977), were generously given by Dr. W. N. Ogata (Fungal Genetic Stock Center, Arcata, CA, U.S.A.).

Cultures were grown in Vogel's (1956) minimal medium containing 2% (w/v) sucrose and 2.5 μg of (+)-biotin/ml. Growth was at 30°C for 40h with shaking (120rev./min) in 2000ml Erlenmeyer flasks containing 500ml of medium.

Enzyme purification

All operations were performed at 2–5°C.

Step 1. Crude extract. Mycelia from wild-type strains were filtered on a Buchner funnel, rinsed with water, weighed and frozen with liquid N_2. The material was ground in a mortar in the presence of liquid N_2 and further homogenized with 2.0ml/wet wt. of mycelium of cold buffer A [50mM-Tris/HCl buffer, pH7.5, containing 0.5mM-EDTA, 1mM-dithiothreitol, 1mM-spermidine and 5% (v/v) glycerol] containing 0.5M-NaCl in an SDT Tissumizer (Teckmar Co., Cincinnati, OH, U.S.A.) provided with a 182E shaft and generator. This is a mechanical high-frequency dispersion apparatus whose generator recirculates the suspension. A rapid homogenization was obtained by the combined effects of shear, impact, collision and cavitation. Mycelial debris were discarded by centrifugation at 12000*g* for 10min, and the supernatant fluid was further centrifuged at 105000*g* for 120min. The supernatant fluid thus obtained was dialysed overnight against 40vol. of buffer A. This dialysed preparation is referred to as 'crude extract'.

Step 2. Chromatography on hexylamino-Sepharose. The column (3cm × 20cm), equilibrated with buffer A, was loaded with 1000ml of the crude extract (adenylate cyclase activity 14pmol/min per mg of protein; 3mg of protein/ml). After a washing with about 500ml of this buffer solution, the column was eluted with 1000ml of a 0–0.7M-NaCl linear gradient in the same buffer solution. Fractions (25ml) were collected at a rate of 2ml/min. Adenylate cyclase activity was eluted as a single nearly symmetrical peak, at about 0.37M-NaCl. Fractions corresponding to this peak, referred to as 'hexylamino-Sepharose fractions', were pooled, concentrated by ultrafiltration in an Amicon cell (50ml) provided with a PM-30 membrane and stored at 0–2°C.

Step 3. Gel filtration. Pooled and concentrated hexylamino-Sepharose fractions (7ml; 25mg of protein/ml) were loaded on a Bio-Gel A-5m (100–200 mesh) column (2.5cm × 87cm) equilibrated with buffer A. Fractions (about 7ml) were collected at a rate of 2.5ml/min. The fractions with the highest specific activity, referred to as 'Bio-Gel fractions', were combined and stored at 0–2°C.

Steps 4 and 5a. Blue Sepharose chromatography steps. The column (0.9cm × 15cm), equilibrated with buffer A, was loaded with 12–15ml of the Bio-Gel fractions (1.5mg of protein/ml). After a washing with about 30ml of the equilibrium buffer, the column was eluted with a 0–0.8M-NaCl linear gradient (120ml) in buffer A. Fractions (5ml) were collected at a rate of 1.5ml/min. Adenylate cyclase activity was eluted as a broad peak between 0.2M- and 0.6M-NaCl. Fractions having the highest specific activity were immediately pooled, dialysed against buffer A for 3h and loaded on the same Blue Sepharose column equilibrated with 20mM-Tris/HCl buffer, pH7.5. The column was eluted with a 0–0.8M-NaCl linear gradient (100ml) made in the latter buffer solution.

Step 5b. Preparative polyacrylamide-gel electrophoresis. Electrophoresis was performed for 10h at 4°C on a Prep-Disc (Canalco) equipment, provided with a PD-2/70 upper column, by following the details given in the Instruction Manual and in accordance with the following conditions: stacking gel (1.0cm high), 3.5% (w/v) acrylamide in 50mM-Tris/HCl buffer, pH7.2; separating gel (4cm high), 5% (w/v) acrylamide in 0.37M-Tris/HCl buffer, pH8.4; electrode buffer, 30mM-Tris/glycine buffer, pH8.2; elution buffer, 0.37M-Tris/HCl buffer, pH8.4, containing 10% (v/v) glycerol; elution rate, 15ml/h (2.5ml per fraction). Electrophoresis was performed at constant current (10mA). The fractions from the first chromatography on Blue Sepharose that had the highest specific activity were concentrated by ultrafiltration on an Amicon PM-30 membrane; the sample (1ml) loaded on to the column contained 0.5mg of protein/ml.

Determination of molecular and hydrodynamic parameters

Enzyme sample. A 27ml portion of the crude extract was passed through a DEAE-cellulose column (28cm × 2.5cm) equilibrated with buffer A. After a washing with about 200ml of this buffer, the column was eluted with a 0–0.5M-NaCl linear gradient (480ml) in the same buffer. Adenylate cyclase activity was eluted as a single peak at about 0.27M-NaCl. Fractions having maximal adenylate cyclase activity, referred to as 'DEAE-cellulose fractions', were pooled (40ml) and precipitated by the addition of 17.2ml of a neutralized saturated $(NH_4)_2SO_4$ solution. After centrifugation, the supernatant fluid (1vol.) was precipitated with 0.75vol. of the saturated $(NH_4)_2SO_4$ solution. The pellet ob-

tained after centrifugation was dissolved in about 1 ml of buffer B (buffer A containing 0.15 M-NaCl) and dialysed for 3 h against the same buffer solution. Samples of this enzyme preparation were used for centrifugation insucrose density gradients or for gel filtration as described below.

Sucrose-density-gradient centrifugation. Sedimentation studies were performed in 5–20% (w/v) sucrose linear gradients in buffer B made up in ^1H_2O or ^2H_2O, by the procedure described elsewhere (Kornblihtt *et al.*, 1981).

Gel filtration. A Bio-Gel A-5 m (100–200 mesh) column (0.9 cm × 75 cm; Glenco, precision bore) was equilibrated and eluted with buffer B. Conditions were as follows: sample volume, 1.0 ml; flow rate, 2 ml/min; temperature, 2–5°C. The elution volume of each particular compound was expressed as the ratio of the elution volume of this substance to that of bacterial suspension (K_{el}).

Calibrating proteins. Calibrating proteins were added to the samples (gradient centrifugation or gel filtration) at the following concentrations: β-galactosidase (*Escherichia coli*), 50 μg/ml; catalase (bovine liver), 100 μg/ml; lactate dehydrogenase (rabbit muscle), 30 μg/ml; malate dehydrogenase (pig heart), 10 μg/ml; cytochrome *c* (horse heart), 2 mg/ml. The void volume in Bio-Gel columns was determined by using a suspension of *Rhizobium meliloti* containing 6×10^8 bacteria/ml.

Calculation of molecular and hydrodynamic parameters. Calculations were performed as previously described (Kornblihtt *et al.*, 1981).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Electrophoresis was performed in gel slabs containing 10% (w/v) acrylamide by the procedure described elsewhere (Kornblihtt *et al.*, 1981). Gels were fixed and stained with an aqueous solution containing 25% (v/v) methanol, 8% (v/v) acetic acid and 0.2% Coomassie Brilliant Blue R250 and destained with an aqueous solution containing 25% (v/v) methanol and 8% (v/v) acetic acid. In some cases gels were restained with $AgNO_3$ (Oakley *et al.*, 1980).

Enzyme assays

Adenylate cyclase. Standard assays of adenylate cyclase activity were performed as previously described (Kornblihtt *et al.*, 1981).

Other enzyme assays. Catalase, lactate dehydrogenase and malate dehydrogenase activities were assayed by the procedures described previously (Kornblihtt *et al.*, 1981).

β-Galactosidase activity was measured at room temperature with *o*-nitrophenyl β-D-galactopyranoside as substrate, as described in the *Worthington Enzyme Manual* (Worthington Biochemical Corp., 1972).

Other analytical procedures

Isoelectric focusing of the hexylamino-Sepharose or Bio-Gel preparations was performed in an Ampholine (LKB, Stockholm, Sweden) pH range between 3.5 and 6.5 as previously described (Kornblihtt *et al.*, 1981). Before electrophoresis, samples were dialysed against buffer A for 3 h.

Table 1. *Distribution of adenylate cyclase activity in fractions from Neurospora mycelial extracts*
The freeze-dried mycelium (about 50 mg) was homogenized in 2 ml of buffer A with or without NaCl (0.5 M), phenylmethanesulphonyl fluoride (5 mM) or Trasylol (1000 units/ml). Homogenates were centrifuged at 10000 *g* for 10 min, and the supernatant fluids thus obtained were centrifuged at 105000 *g* for 60 min. Fractions thus obtained were resuspended in the same buffer as that used during homogenization. Other conditions were as indicated in the Experimental section.

Additions to buffer A	Fraction	Total activity (pmol/min)	Specific activity (pmol/min per mg of protein)
None	Homogenate	180	—
	10000 *g* sediment	52	18
	105000 *g* sediment	80	50
	105000 *g* supernatant	62	16
NaCl (0.5 M)	Homogenate	160	—
	10000 *g* sediment	38	16
	105000 *g* sediment	33	23
	105000 *g* supernatant	90	21
NaCl (0.5 M) + phenylmethanesulphonyl fluoride (5 mM)	Homogenate	151	—
	10000 *g* sediment	35	15
	105000 *g* sediment	30	23
	105000 *g* supernatant	90	22
NaCl (0.5 M) + Trasylol (1000 units/ml)	Homogenate	148	—
	10000 *g* sediment	36	13
	105000 *g* sediment	28	23
	105000 *g* supernatant	88	21

Details of other analytical procedures were also as given elsewhere (Kornblihtt *et al.*, 1981).

Results

Existence of a 'non-sedimentable' form of adenylate cyclase in mycelial extracts

Mycelia from St.Lawrence 74 wild-type strain were homogenized in a 50mM-Tris buffer (buffer A) and the extract was subjected to two successive centrifugations. As shown in Table 1, one-third of the total adenylate cyclase activity was found in the 105000*g* supernatant fluid. The proportion of this enzyme activity in the 105000*g* supernatant was increased by up to 60% when 0.5M-NaCl was included in the extraction buffer.

The possibility that a 'non-sedimentable' form of adenylate cyclase might arise after proteolysis of a membrane-bound enzyme could be discounted by the fact that the presence of two different proteinase inhibitors in the extracting buffer did not alter enzyme distribution (Table 1).

Similar evidence was obtained with mycelia from the other *Neurospora* wild-type strains FGSC 927, FGSC 2218, FGSC 1757 and FGSC 691. Percentages of adenylate cyclase activities found in the corresponding 105000*g* supernatants, after homogenization with buffer A, varied from 20 to 40. When these supernatant fractions were further centrifuged at 140000*g* for 3h, adenylate cyclase activities remained in the supernatants. In addition, adenylate cyclase activity in all the fractions obtained from mycelia of the FGSC 488 strain was negligible. In this strain, carrying the *cr-1* (crisp) morphological mutation, mycelial concentrations of cyclic AMP were 10-fold or more lower than in wild-type strains (Terenzi *et al.*, 1976). Such evidence indicates that both 'membrane-bound' and 'non-sedimentable' or 'soluble' adenylate cyclase activities are affected to the same extent by a mutation at the *cr-1* locus (results not shown).

Comments on the purification of the Neurospora soluble adenylate cyclase

Neurospora mycelia were extracted with a buffer solution containing 0.5M-NaCl, and the 'soluble' adenylate cyclase that remained in the 105000*g* supernatant was subjected to purification procedures often employed with other soluble proteins.

Loss of enzyme activity was the major problem encountered during the purification of *Neurospora* adenylate cyclase. The best recovery of enzyme activity was obtained when spermidine, glycerol and dithiothreitol were present in the buffer solutions used. Because of this problem all purification steps had to be performed in less than 3 days. Maximal enzyme stability was obtained by storage at 2–4°C.

During preliminary attempts to purify this enzyme

Table 2. *Purification of the Neurospora soluble adenylate cyclase*

For full details see the Experimental section.

Fraction	Protein (mg)	Total activity (pmol/min)	Specific activity (pmol/min per mg of protein)
Crude extract	3000	43000	14
Hexylamino-Sepharose fraction	220	30000	136
Bio-Gel fraction	20	12000	600
1st Blue Sepharose fraction	0.5	2700	5400
2nd Blue Sepharose fraction	<0.1	1200	12000

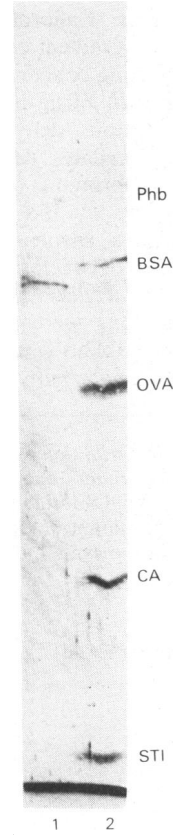

Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the peak fraction from the second Blue Sepharose column chromatography*

1, Enzyme sample (<1 μg of protein); 2, calibrating proteins (Phb, phosphorylase *b*; BSA, bovine serum albumin; OVA, ovalbumin; CA, carbonic anhydrase; STI, soya-bean trypsin inhibitor). The gel was stained with $AgNO_3$. Conditions were as indicated in the Experimental section.

activity DEAE-cellulose was used instead of hexyl-amino-Sepharose. However, the latter chromatographic support was eventually preferred because enzyme recovery and purification were better than those obtained with DEAE-cellulose.

Concentration of enzyme samples before gel-permeation chromatography was performed either by precipitation with $(NH_4)_2SO_4$ or by ultrafiltration. The latter procedure was preferred because enzyme recovery was much higher.

Conditions for adsorption of enzyme on Blue Sepharose varied from one preparation to another. Therefore in every case it was necessary to test for the optimum conditions. Although adsorption of enzyme on the support increased at lower pH values (e.g. 50mM-Pipes buffer, pH6.5), purification and recovery decreased when chromatography was performed at such pH values.

The overall recovery of enzyme activity after the second Blue Sepharose chromatography was about 2–3%; alternatively the enzyme yield from the preparative disc electrophoresis was about 0.1–0.2% of the activity in crude extracts. After these purification steps, the amount of protein in the fractions showing adenylate cyclase activity was undetectable by the method of Lowry *et al.* (1951). A better evaluation of the protein concentration in the fractions was made in sodium dodecyl sulphate/polyacrylamide gels stained with $AgNO_3$ (Oakley *et al.*, 1980). If it is assumed that enzyme protein (but not activity) was quantitatively recovered after these purifiction steps, the extent of purification of crude extracts would be about 10^4-fold. Table 2 summarizes some of these results.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, fractions having adenylate cyclase activity from the first or the second chromatography on Blue Sepharose showed several bands after staining with Coomassie Brilliant Blue R250 and $AgNO_3$. The main band had a mobility slightly lower than that of bovine serum albumin (Fig. 1). This principal band was also observed in fractions having adenylate cyclase activity from the preparative gel electrophoresis (results not shown).

Hydrodynamic and molecular characteristics of Neurospora adenylate cyclase

Gel filtration. Fig. 2 shows the elution pattern of *Neurospora* adenylate cyclase from a Bio-Gel A-5m column. $K_{el.}$ for adenylate cyclase was 1.8, corresponding to a Stokes radius of 7.3nm (inset).

Sucrose-density-gradient centrifugation. Results of the centrifugation of *Neurospora* adenylate cyclase through sucrose density gradients made in ^1H_2O or ^2H_2O are shown in Fig. 3. The sedimentation coefficients in both gradients had the same value, 6.25S (inset), indicating that adenylate cyclase and the calibrating proteins have essentially the same partial specific volume, 0.74ml/g. The latter value is characteristic of globular 'soluble' proteins.

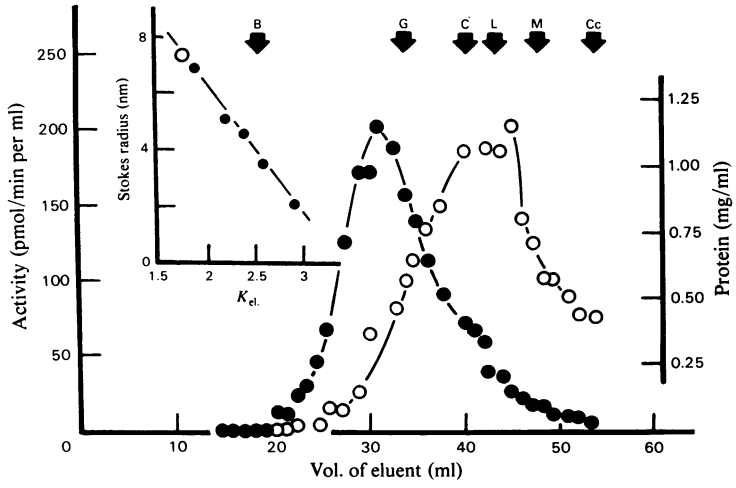

Fig. 2. *Analytical Bio-Gel column chromatography of a Neurospora DEAE-cellulose preparation fractionated with* $(NH_4)_2SO_4$

The inset shows the relationship between $K_{el.}$ and Stokes radii for calibrating proteins and cyclase. ●, Adenylate cyclase activity; O, protein. Conditions were as indicated in the Experimental section. Key: B, bacteria; G, β-galactosidase; C, catalase; L, lactate dehydrogenase; M, malate dehydrogenase; Cc, cytochrome *c*.

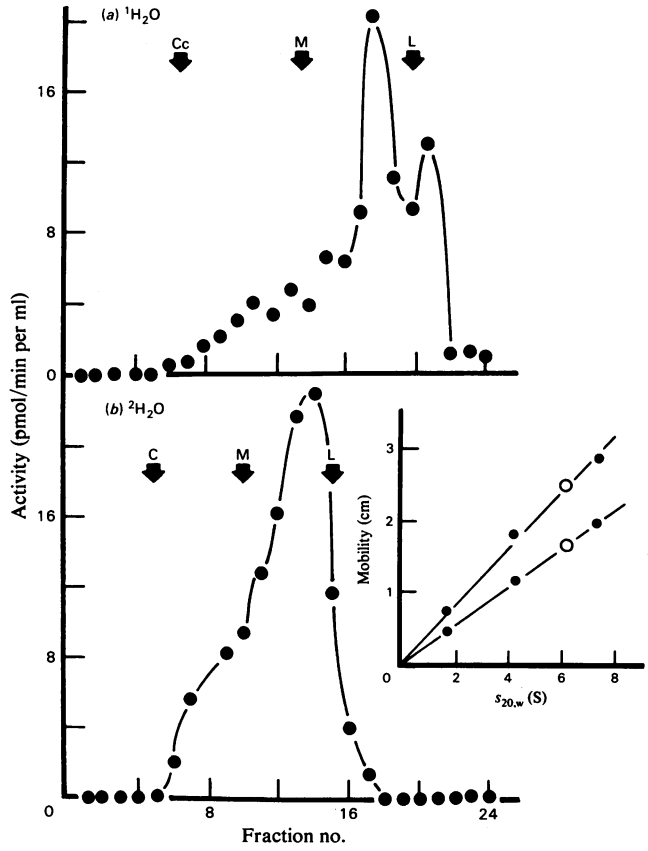

Fig. 3. *Sucrose-density-gradient centrifugation of a Neurospora DEAE-cellulose preparation fractionated with* $(NH_4)_2SO_4$

Gradients were made in ^1H_2O (*a*) or ^2H_2O (*b*). The inset shows the relationship between mobilities in the gradients and sedimentation coefficients for calibrating proteins and adenylate cyclase. Conditions were as indicated in the Experimental section. The key is as for Fig. 2.

Table 3. *Hydrodynamic and molecular parameters of Neurospora soluble adenylate cyclase*

For full details see the text.

Parameter	Value
Sedimentation coefficient (S)	6.2 ± 0.1
Stokes radius (nm)	7.3 ± 0.05
Partial specific volume (ml/g)	0.74
Molecular weight	202000
Axial ratio	1.65
Isoelectric point	4.6

Molecular parameters. Table 3 shows hydrodynamic and molecular parameters of *Neurospora* adenylate cyclase. Results are the average for at least six different experiments and enzyme preparations. From the experimental values a molecular weight of 202000 and a frictional ratio of 1.65 were calculated.

Isoelectric focusing. Enzyme activity was distributed between pH4.5 and 5, with a main peak at pH4.6.

Kinetic properties

The activity of the *Neurospora* soluble adenylate cyclase in assay mixtures containing Mg^{2+} instead of Mn^{2+} was negligible. In addition, fluoride, GTP and [$\beta\gamma$-imido]GTP did not activate this enzyme. On the other hand, preincubation of a crude extract or other purified preparations of this adenylate cyclase with cholera toxin (pretreated or not with dithiothreitol) in the presence or absence of NAD^+ did not influence the activity (results not shown).

The behaviour of the soluble adenylate cyclase activity as a function of Mn^{2+}–ATP or Mn^{2+} concentration was similar to that described for the membrane-associated enzyme (Flawiá & Torres, 1972*b*).

Discussion

The results indicate that *Neurospora* adenylate cyclase can be obtained as a soluble entity from an extract prepared with buffer solutions of high ionic strength. In addition, a simple procedure for purification of this soluble adenylate cyclase is reported. After the last purification steps enzyme fractions showed a main band with an apparent molecular weight of about 66000. This value is similar to that reported for the soluble form of adenylate cyclase from rat testis (Kornblihtt *et al.*, 1981).

Molecular and hydrodynamic properties of *Neurospora* soluble adenylate cyclase are strikingly similar to those reported for the catalytic subunit of this enzyme activity from lymphoma S-49 cells (Ross *et al.*, 1978). It is noteworthy that *Neurospora* cyclase and the catalytic protein from an S-49 variant are dependent on Mn^{2+} and insensitive to fluoride and guanine nucleotides.

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