Freedman, T. B., Loehr, J. S., & Loehr, T. M. (1976) *J. Am. Chem. Soc. 98*, 2809–2815.
Goodgame, M., & Cotton, F. A. (1962) *J. Am. Chem. Soc. 84*, 1543–1548.
Gutteridge, S., & Robb, D. A. (1973) *Biochem. Soc. Trans. 1*, 519–520.
Gutteridge, S., & Robb, D. A. (1975) *Eur. J. Biochem. 54*, 107–116.
Himmelwright, R. S., Eickman, N. C., & Solomon, E. I. (1979) *J. Am. Chem. Soc. 101*, 1576–1586.
Himmelwright, R. S., Hepp, A. F., Eickman, N. C., Lerch, K., LuBien, C. D., Schoeniger, L. O., & Solomon, E. I. (1980) in *Comparative Study and Recent Knowledge on Quaternary Structure and Active Sites of Oxygen Carriers and Related Proteins*, Tours, France, Marcel Dekker, New York (in press).
Hoffman, B. M., & Petering, D. H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 637–643.
Horowitz, N. H., Fling, M., & Horn, G. (1970) *Methods Enzymol. 17A*, 615–620.
Hush, N. S., & Horbs, R. J. M. (1968) *Prog. Inorg. Chem. 10*, 259–486.
Jolley, R. L., Evans, L. H., Makino, N., & Mason, H. S. (1974) *J. Biol. Chem. 249*, 335–345.
Kannan, K. K., Liljas, A., Waara, I., Bergstên, P.-C., Lövgren, S., Strandberg, B., Bengtsson, U., Carlbom, U., Fridborg, K., Järup, L., & Petef, M. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 221–231.
Lane, R. W., Ibers, J. A., Frankel, R. B., Papaefthymiou, G. C., & Holm, R. H. (1977) *J. Am. Chem. Soc. 99*, 84–98.
Larrabee, J. A., & Spiro, T. G. (1979) *Biochem. Biophys. Res. Commun. 88*, 753–760.
Latt, S. A., & Vallee, B. L. (1971) *Biochemistry 10*, 4263–4270.
Lerch, K. (1976) *FEBS Lett. 69*, 157–160.
Lerch, K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3635–3639.
Lindskog, S. (1963) *J. Biol. Chem. 238*, 945–951.
Mason, H. S. (1965) *Annu. Rev. Biochem. 34*, 595–634.
May, S. W., & Kuo, J. (1978) *Biochemistry 17*, 3333–3338.
McMillin, D. R., Rosenberg, R. C., & Gray, H. B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4760–4762.
Nicolaus, R. A. (1968) *Melanins*, Hermann, Paris.
Palmer, G. (1967) *Methods Enzymol. 10*, 594–609.
Pfiffner, E., Dietler, C., & Lerch, K. (1980) in *Comparative Study and Recent Knowledge on Quaternary Structure and Active Sites of Oxygen Carriers and Related Proteins*, Tours, France, Marcel Dekker, New York (in press).
Ray, W. J., Goodin, D. S., & Ng, L. (1972) *Biochemistry 11*, 2800–2804.
Rich, P. R., Wiegand, N. K., Blum, H., Moore, A. L., & Bonner, W. D. (1978) *Biochim. Biophys. Acta 525*, 325–337.
Rosenberg, R. C., Root, C. A., & Gray, H. B. (1975) *J. Am. Chem. Soc. 97*, 21–26.
Rotilio, G., Calabrese, L., Mondovi, B., & Blumberg, W. E. (1974) *J. Biol. Chem. 249*, 3157–3160.
Schoot Uiterkamp, A. J. M., & Mason, H. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 993–996.
Simpson, R. T., & Vallee, B. L. (1968) *Biochemistry 7*, 4343–4350.
Suzuki, S., Mori, W., Kino, J., Nakao, Y., & Nakahara, A. (1980) *J. Biochem. (Tokyo) 88*, 1207–1209.
Sytkowski, A. J., & Vallee, B. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 344–348.
Vallee, B. L., & Wacker, W. E. C. (1970) *Proteins 5*, 94–128.
Vanneste, W. H., & Zuberbühler, A. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) pp 371–404, Academic Press, New York.
Van Wart, H. E., & Vallee, B. L. (1978) *Biochemistry 17*, 3385–3394.
Wang, M. R., & Hoffman, B. M. (1977) *J. Biol. Chem. 252*, 6268–6275.
Wilkins, R. G. (1971) *Adv. Chem. Ser. No. 100*, 111–134.

Manganese Ion Dependent Adenylate Cyclase Activity in Rat Testes: Purification and Properties†

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ABSTRACT: Testicular, soluble adenylate cyclase has been purified by anion-exchange chromatography, gel filtration, and isoelectric focusing. Upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis, peak fractions from the latter purification step showed only one polypeptide band with an apparent molecular weight of about 69 000. The following hydrodynamic and molecular parameters have been established for this enzyme: sedimentation constant, 4.3 S; Stokes radius, 3.95 nm; partial specific volume, 0.74 mL·g^{-1}; molecular weight, 74 000; frictional ratio, 1.4.

Regulation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], the enzyme that catalyzes the synthesis of adenosine cyclic 3′,5′-phosphate (cyclic AMP), has been the subject of studies documented in profuse literature. However, a better understanding of the function of this enzyme would require an appropriate knowledge of both its structural and molecular properties. Progress in the purification of adenylate cyclase from mammalian cell membranes has been hindered mostly by two facts: first, the enzyme constitutes a very small proportion of the total cell protein; second, enzymatic activity is extremely unstable under the reasonably mild conditions of some standard protein purification procedures.

Adenylate cyclase from brain (Stellwagen & Baker, 1976)

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and heart membranes (Homcy et al., 1978) has been purified by affinity chromatography. In the latter case, a previous resolution from some other hydrophobic proteins allowed a better purification on subsequent steps. On the other hand, "soluble" adenylate cyclase from *Brevibacterium liquefaciens* was purified to homogeneity following conventional procedures (Takai et al., 1974).

Results reported by Braun & Dods (1975) and confirmed by Neer (1978) indicate that in rat seminiferous tubules a large proportion of adenylate cyclase activity is not associated to membranes. Moreover, this enzyme is insensitive to gonadotropins, fluoride, and guanyl nucleotides, and it is strictly dependent on Mn^{2+}.

The present paper describes the purification and some molecular and hydrodynamic properties of the testicular, "soluble" adenylate cyclase.

Experimental Procedures

Materials

Rabbit muscle creatine kinase and lactic dehydrogenase were from Boehringer. ATP, cyclic AMP, creatine phosphate, horse heart cytochrome *c*, pig heart malate dehydrogenase, beef liver catalase, blue dextran, deuterium oxide, crystalline serum albumin, and Tris were purchased from Sigma. Sucrose and ammonium sulfate were from Schwarz/Mann. AG 50 W-X4 (200–400 mesh), Bio-Gel A 0.5 m (100–200 mesh), DEAE-cellulose (Cellex D), acrylamide, ammonium persulfate, *N,N'*-methylenebis(acrylamide), *N,N,N',N'*-tetramethylethylenediamine, sodium dodecyl sulfate, and glycine were purchased from Bio-Rad, 3-isobutyl-1-methylxanthine was from Aldrich, and Coomassie brilliant blue G and R250 were from Fluka. A low molecular weight calibration kit for polyacrylamide gel electrophoresis was obtained from Pharmacia, and poly(ethylenimino)cellulose sheets were from Baker.

[α-^{32}P]ATP was prepared as previously described (Torres et al., 1978), using ^{32}P-labeled inorganic phosphate purchased from the Radiochemical Center (Amersham).

Methods

Enzyme Purification. Unless otherwise indicated, all operations were performed at 2–4 °C.

(*Step 1*) *Crude Extract.* Testes were obtained from Wistar rats fed ad libitum with standard laboratory chow and weighing about 200 g. After being excised, the organs were placed in an ice-cooled beaker and immediately decapsulated and freed of blood vessels. The tissue was then homogenized in 3 volumes of an ice-cooled 50 mM Tris-HCl buffer solution, pH 7.5, containing 1 mM β-mercaptoethanol and 0.5 mM EDTA[1] (buffer A), using a SDT Tissuemizer (Tekmar Co.) provided with a 182 E shaft and generator. The homogenate was centrifuged at 7700*g* for 10 min, and the supernatant fluid thus obtained was centrifuged at 105000*g* for 60 min. The latter supernate was referred to as the "crude extract".

(*Step 2*) *Chromatography on DEAE-cellulose.* A DEAE-cellulose column (2.5 × 28 cm) equilibrated with buffer A was loaded with 200 mL of crude extract and washed with 900 mL of the same buffer solution. Elution was performed with a NaCl linear gradient from 0 to 0.7 M (1200-mL total volume) in buffer A. Fractions of 20 mL were collected at a rate of 2.2 mL/min. Adenylate cyclase activity eluted as a single peak at about 0.25–0.30 M NaCl. Fractions thus obtained were referred to as "DEAE-cellulose" and were stored at 0–2 °C.

(*Step 3*) *Ammonium Sulfate Fractionation and Bio-Gel Chromatography.* DEAE-cellulose fractions were pooled (about 80 mL) and precipitated by the slow addition of 34.3 mL of a neutralized, saturated ammonium sulfate solution (30% saturation). Precipitation was performed in an ice-cooled beaker, with magnetic stirring, and the ammonium sulfate solution was at room temperature. The pellet obtained by centrifugation at 10000*g* for 10 min was discarded, and the supernatant solution (1 volume) was precipitated with 0.75 volume of the ammonium sulfate solution (60% saturation). After centrifugation, the pellet was dissolved in about 5 mL of buffer A and dialyzed for 3 h against the same buffer solution. The dialyzed fraction (7.0 mL, 14 mg of protein/mL) was loaded on a Bio-Gel A 0.5-m (100–200 mesh) column (2.5 × 87 cm) equilibrated with 0.15 M NaCl in buffer A (buffer B). Fractions of about 7.0 mL were collected at a rate of 1.7 mL/min. The active fractions with the highest specific activity (21 mL) were combined and precipitated by the addition of 1.5 volumes of a saturated ammonium sulfate solution (60% saturation) as indicated above. After centrifugation, the precipitate was dissolved in a minimum volume of buffer A (about 0.7 mL) and dialyzed against the same buffer solution for 6 h. The fraction thus obtained was referred to as "Bio-Gel".

(*Step 4*) *Isoelectric Focusing.* Focusing of the enzyme protein was performed in an Ampholine (LKB) pH range between 5.0 and 5.6. Since Ampholine solution for such a narrow range is not commercially available, it was obtained by a previous isoelectric focusing carried out as follows: column, 110 mL (LKB) Ampholine (pH range 4–6); final concentration in column, 4%; polarity, anode at the bottom; anode solution, sucrose (15 g), H_2O (12 mL), and 1 M phosphoric acid (4 mL); cathode solution, 1 M NaOH (2.5 mL) and H_2O (7.5 mL); dense gradient solution, sucrose (27 g), H_2O (28.9 mL), and 40% Ampholine (pH range 4–6) (8.1 mL); light electrode solution, sucrose (2.7 g), H_2O (50.3 mL), and 40% Ampholine (pH range 4–6) (2.7 mL); initial current, 12 mA; initial voltage, 800 V; final current, 6 mA; final voltage, 1600 V; time, 16 h; cooling temperature, 4 °C. After the pH gradient was formed, the column content was pumped out, and those fractions from 5.0 to 5.6 pH units were pooled (about 23 mL, with a sucrose concentration of 25%).

Isoelectric focusing of the Bio-Gel enzyme preparation was performed in a 110-mL column as follows: Ampholine (pH range 5.0–5.6); final concentration in column, 1%; polarity, anode at the bottom (anode and cathode solutions as indicated above); dense gradient solution, 60% sucrose solution (w/v) (37.5 mL) and Ampholine (pH range 5.0–5.6) (16.5 mL); light gradient solution, 2.8% sucrose solution (w/v) (48.5 mL) and Ampholine (pH range 5.0–5.6) (5.5 mL); initial current, voltage, and power, 2 mA, 1600 V, and 3.2 W, respectively; cooling temperature, 4 °C. After 6 h, the current was interrupted, and 3 mL of the gradient solution was pumped out from the middle of the column and mixed with 1 mL of the Bio-Gel fraction. After the density was adjusted by the addition of solid sucrose, the sample was injected into the middle of the gradient column. Isoelectric focusing of the enzyme protein was then performed under the same conditions for 16 h. Final current, voltage, and power were 1.7 mA, 1800 V, and 3.2 W, respectively. Fractionation was carried out at a rate of 2 mL/min; 2-mL fractions were collected.

[1] Abbreviatons used: DEAE-cellulose, diethylaminoethylcellulose; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate.

Estimation of Molecular and Hydrodynamic Parameters. (*1*) *Sucrose Density Gradient Centrifugation.* Sedimentation studies were performed in 5–20% sucrose linear gradients (w/v) made in H_2O or D_2O containing 50 mM Tris-HCl, pH 7.5, 1 mM β-mercaptoethanol, 0.5 mM EDTA, and 0.15 M NaCl. Gradients were overlaid with 0.3 mL of a DEAE-cellulose preparation containing 3 mg of protein/mL, or a mixture of calibrating proteins.

Centrifugations were carried out at 2 °C at 45 000 rpm for 16 h, using a Beckman SW 56 rotor. Fractions (about 0.2 mL) were collected by pumping from the bottom at a rate of 1.0 mL/min.

(*2*) *Gel Filtration.* A Bio-Gel A 0.5-m (100–200 mesh) column (0.9 × 75 cm; Glenco, precission bore) was equilibrated and eluted with buffer B. Conditions were as follows: sample, DEAE-cellulose preparation, 1.0 mL (containing 3 mg of protein/mL); flow rate, 1.1 mL/min; fraction volume, 1.0 mL; temperature, 2–5 °C.

(*3*) *Calibrating Proteins.* Calibrating proteins were added to the samples (gradient centrifugation or gel filtration) at the following concentrations: catalase (beef 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.

(*4*) *Calculation of Molecular and Hydrodynamic Parameters.* Calculation of sedimentation coefficients ($s_{20,w}$) was performed according to Martin & Ames (1961). These data enabled the estimation of the partial specific volume ($\bar{v}$) as described by Meunier et al. (1972) and Clarke (1975). Determination of Stokes radius (*a*) was carried out by comparing the elution volume of the adenylate cyclase from Bio-Gel columns with that of calibrating proteins as described by Laurent & Killander (1964). The molecular weight (M_r) and the frictional ratio (f/f_o) were calculated according to the equations described by Tanford (1961). Hydrodynamic parameters of calibrating proteins have been summarized by Haga et al. (1977).

(*5*) *$NaDodSO_4$–Polyacrylamide Gel Electrophoresis.* Samples to be electrophoresed were first dialyzed overnight, at 4 °C, against 10 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl and then for 6 h against the same buffer without NaCl. Samples were then lyophilized and heated for 5 min at 100 °C with a denaturating mixture containing 2% sodium dodecyl sulfate (w/v), 5% β-mercaptoethanol (v/v), 20% glycerol (v/v), 0.06 M Tris-HCl buffer, pH 6.8, and 0.002% bromophenol blue (w/v). Gel slabs were prepared essentially as described by Laemmli (1970). Unless otherwise indicated, conditions were as follows: sample volume, 0.05 mL; protein, 10–100 μg; stacking gel, pH 6.8, 4% (w/v) acrylamide, height 1.5 cm, width 1.5 mm, well width 8 mm; separating gel, pH 8.8, 10% (w/v) acrylamide, height 8 cm, width 1.5 mm; electrophoresis conditions, current 20 mA, time 6–7 h, temperature 15 °C, Bio-Rad Model 220 cell, power source LKB Model 2103. Calibration standards were phosphorylase *b* (rabbit muscle), albumin (bovine serum), ovalbumin (egg white), carbonic anhydrase (bovine erythrocytes), and trypsin inhibitor (soybean). Gels were fixed and stained with an aqueous solution containing 25% methanol (v/v), 8% acetic acid (v/v), and 0.2% Coomassie brilliant blue G or R250 (w/v) and destained with an aqueous solution containing 25% methanol and 8% acetic acid.

Enzyme Assays. (*1*) *Adenylate Cyclase.* The standard incubation mixture contained 50 mM Tris-HCl buffer, pH 7.5, 0.2 mM 3-isobutyl-1-methylxanthine, 1 mM cyclic AMP (cAMP), 2.5 mM $MnCl_2$, 0.5 mM [α-^{32}P]ATP (specific activity 100–300 cpm/pmol), 2 mM phosphocreatine, 0.2 mg of creatine kinase, and enzyme fraction (0.5–100 μg of protein). The volume was 0.1 mL. Incubations were performed at 37 °C for 2.5–10 min, and reactions were stopped by the addition of 0.1 mL of a solution containing 12.5 mM [^{3}H]-cAMP (specific activity 3800 cpm/μmol) plus 40 mM ATP and heating in a boiling water bath for 3 min (Rodbell, 1967). Each sample was adjusted to 1 mL with water, and cyclic AMP was purified according to the sequential column procedure (Dowex-50 and alumina) described by Salomon et al. (1974).

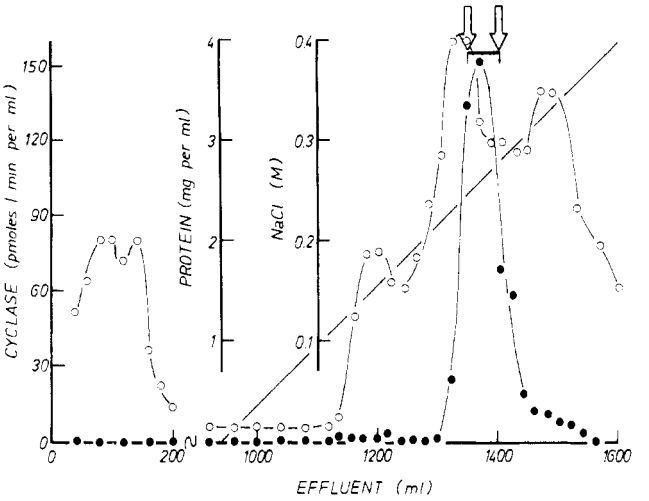

FIGURE 1: DEAE-cellulose column chromatography of a testicular crude extract. (●) Adenylate cyclase activity; (○) protein. Conditions were as indicated under Experimental Procedures.

(*2*) *Catalase.* The activity was assayed at room temperature following the disappearance of hydrogen peroxide by the conversion of iodide to iodine, according to the method of Terenzi et al. (1971).

(*3*) *Lactic and Malic Dehydrogenases.* The activities were measured by NADH consumption following the disappearance of its absorbance at 340 nm as it is described in the Worthington Enzyme Manual (Worthington Biochemical Corp., 1972).

Other Analytical Procedures. Protein was assayed following the procedure of Lowry et al. (1951) with crystalline serum albumin as standard. Cytochrome *c* was measured by its absorbance in the Soret band (410 nm). Periodic acid–Schiff (PAS) staining of polyacrylamide gels was performed according to Fairbanks et al. (1971).

Thin-layer and paper chromatographies of substrate and products from adenylate cyclase incubation mixtures were performed according to Randerath & Randerath (1967) and Paladini & Leloir (1952), respectively.

Results

Comments on Adenylate Cyclase Purification by DEAE-cellulose and Gel Permeation Chromatography. Figure 1 shows the elution pattern of testicular adenylate cyclase from a DEAE-cellulose column. The enzyme eluted as a single peak at about 0.25–0.30 M NaCl. The enzyme preparation was further fractionated with ammonium sulfate and filtered through a Bio-Gel A 0.5-m column. A rather symmetrical activity peak was obtained from this column with a K_{el} (elution volume/void volume) of about 1.8 (Figure 2).

The major problem during these purification steps was the loss of enzyme activity. Recovery was not improved either by addition of glycerol (up to 30%), increase in β-mercaptoethanol concentration, or its omission or by changing the pH of the elution buffer.

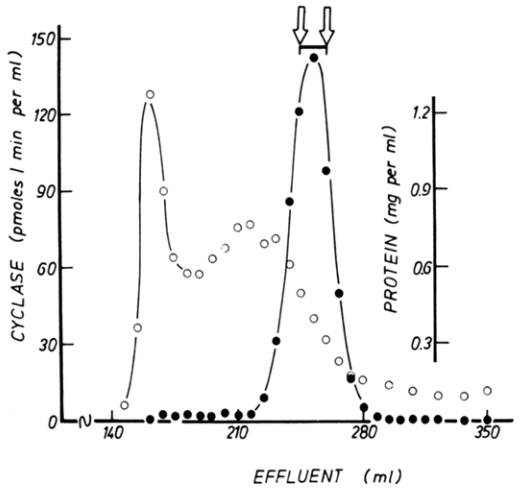

FIGURE 2: Preparative Bio-Gel column chromatography of a testicular DEAE-cellulose preparation fractionated with ammonium sulfate. (●) Adenylate cyclase activity; (○) protein. Conditions were as indicated under Experimental Procedures.

Table I: Purification of Testicular Adenylate Cyclase[a]

fraction	protein (mg)	cyclase activity: total (pmol/min)	cyclase activity: specific [pmol min^{-1} (mg of protein)$^{-1}$]
crude extract (105000*g* supernate)	1900	15800	7
DEAE-cellulose (peak fractions)	315	10000	30
Bio-Gel (peak fractions)	14	3286	235
isoelectric focusing (peak fractions)	0.02[b]	240	12000

[a] Peak fractions were marked by arrows in Figures 1, 2, and 4. [b] Protein concentration was estimated by densitometric scannings (530 nm) of polyacrylamide gels stained with Coomassie brilliant blue R250 with bovine serum albumin as standard.

Because of such a problem, the first two purification steps had to be performed in less than 30 h. Recovery of activity after gel filtration was about 30% of that in the crude extract (Table I). The enzyme of the Bio-Gel preparation showed maximal stability at 2–4 °C.

Figure 3 (lanes 2 and 3) shows peptide patterns obtained after $NaDodSO_4$–polyacrylamide gel electrophoresis of peak fractions from DEAE-cellulose and Bio-Gel column chromatographies.

Isoelectric Focusing of Adenylate Cyclase. Preliminary experiments on isoelectric focusing of testicular adenylate cyclase were performed by using an Ampholine pH range from 4 to 6. This procedure resolved the enzyme activity, focusing at pH 5.35, from a large peak of more acidic proteins. Recovery of enzyme activity was rather poor. It accounted for about 4% of the activity loaded in the focusing column. $NaDodSO_4$–polyacrylamide gel electrophoresis of the active fraction from this isoelectric focusing revealed the presence of protein bands with mobilities corresponding to molecular weights of about 100 000, 80 000, 69 000, 52 000, and 43 000 (results not shown).

A better purification was obtained when the enzyme preparation was electrofocused with an Ampholine pH range between 5.0 and 5.6. Enzyme activity was distributed between pH 4.8 and 5.55, with a main peak at pH 5.5 (Figure 4). This might indicate that selection of an appropriate and narrow Ampholine pH range is important for resolution of adenylate

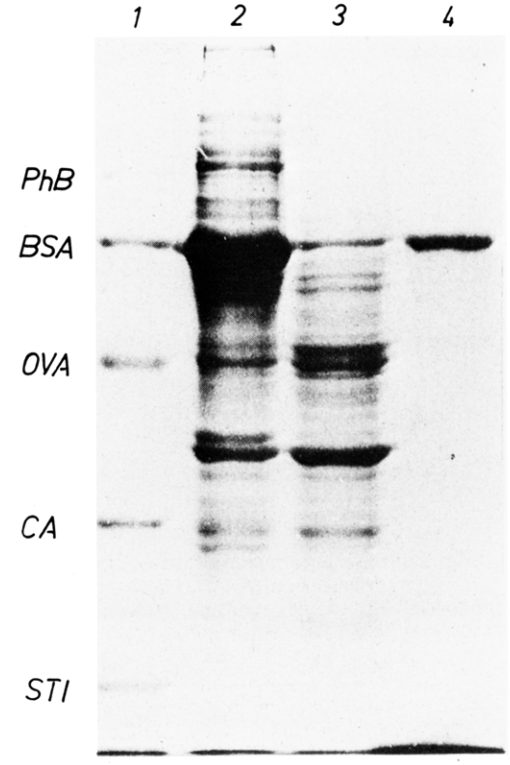

FIGURE 3: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of fractions from various purification steps of testicular adenylate cyclase. (Lanes 1–4) Calibrating proteins, DEAE-cellulose (100 μg of protein), Bio-Gel (50 μg of protein) and peak fractions from isoelectric focusing (10 μg of protein) in an Ampholine range between pH 5.0 and 5.6, respectively. PhB, phosphorylase *b*; BSA, bovine serum albumin; OVA, ovalbumin; CA, carbonic anhydrase; STI, soybean trypsin inhibitor.

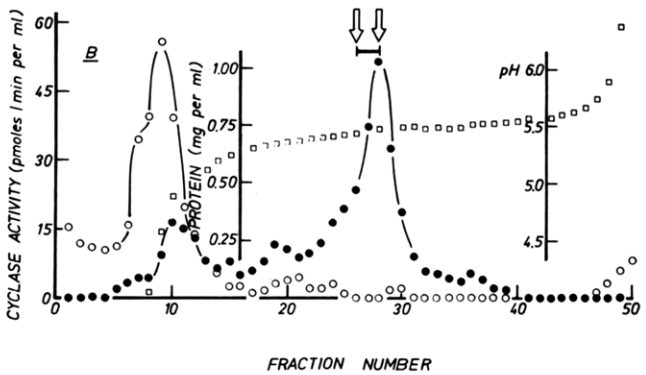

FIGURE 4: Isoelectric focusing of a testicular Bio-Gel preparation fractionated with ammonium sulfate and dialyzed. Ampholine range was between pH 5.0 and 5.6. (□) pH; (●) adenylate cyclase activity; (○) protein. Conditions were as indicated under Experimental Procedures.

cyclase from other acidic proteins. Figure 3 (lane 4) shows that the enzyme activity peak resolved in a pH range between 5.0 and 5.6 presented only one polypeptide band with an apparent molecular weight of about 69 000, upon electrophoresis on a $NaDodSO_4$–10% polyacrylamide gel at pH 8.8. A similar molecular weight was estimated for such a peptide band in 6.5% acrylamide gels performed at the same pH. This might suggest that under those conditions the protein had free mobility. Densitometric scans of the gels showed no other significant staining with Coomassie brilliant blue G or R250. Moreover, no evidence indicative of the presence of carbohydrates associated with the single polypeptide band was obtained by staining the gels with the periodate–Schiff reagent (results not shown).

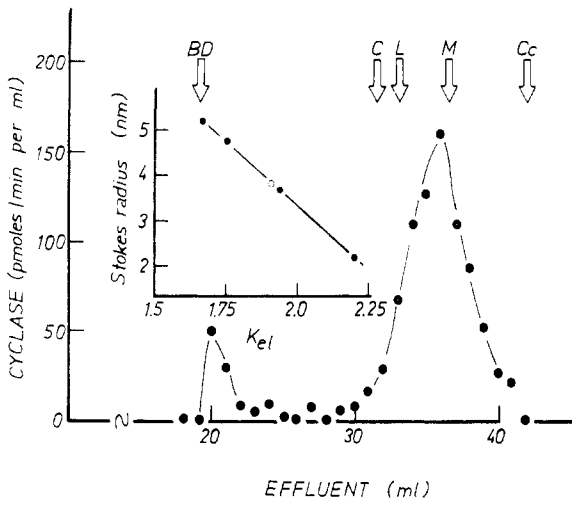

FIGURE 5: Analytical Bio-Gel column chromatography of a DEAE-cellulose preparation. The inset shows the relationship between K_{el} and Stokes radii for calibrating proteins (●) and cyclase (○). Conditions were as indicated under Experimental Procedures and in the text. BD, blue dextran; C, Catalase; L, lactic dehydrogenase; M, malic dehydrogenase; Cc, cytochrome *c*.

Table II: Hydrodynamic and Molecular Parameters of Testicular Adenylate Cyclase

parameter	value
$s_{20,w}$ (S)	4.3 ± 0.10
a (nm)	3.95 ± 0.05
$\bar{v}$ (mL·g^{-1})	0.74
M_r	74000
f/f_o	1.4

On the other hand, total recovery of enzyme activity in this electrofocusing step was 20% of the activity loaded in the column. Specific activity in the peak fractions was about 12 nmol min^{-1} (mg of protein)$^{-1}$, which represents a 1700-fold purification over the crude extract.

The products of the cyclase reaction catalyzed by peak fractions from the latter isoelectric focusing column were characterized by paper and thin-layer chromatographies. Cyclic AMP was the only radioactive substance produced whether the ATP generating system, unlabeled ATP, or cyclic AMP plus 3-isobutyl-1-methylxanthine was omitted or not from the reaction mixture. In addition, maximal cyclic AMP production by this enzyme preparation did not require the ATP generating system, a phosphodiesterase inhibitor, or unlabeled cyclic AMP in the assay mixtures.

Hydrodynamic and Molecular Parameters of Adenylate Cyclase. (*1*) *Gel Filtration.* Figure 5 shows the elution pattern of testicular adenylate cyclase from an analytical Bio-Gel A 0.5-m column. The position of markers is also shown. K_{el} for adenylate cyclase was 1.8, corresponding to a Stokes radius of 4.0 nm (inset). Results shown in Figure 5 were obtained with a sample containing a DEAE-cellulose preparation mixed with calibrating proteins. However, the elution position was essentially the same when standards and the enzyme preparations (DEAE-cellulose, Bio-Gel, or isoelectric focusing) were separately filtered.

(*2*) *Sucrose Density Centrifugation.* Figure 6 shows the results of centrifugation of testicular adenylate cyclase through sucrose gradients made in H_2O or D_2O. The sedimentation constants in both gradients had the same value, 4.3 S (inset), indicating that cyclase and calibrating proteins have essentially the same partial specific volume, 0.74 mL·g^{-1}. The latter value is characteristic of globular "soluble" proteins.

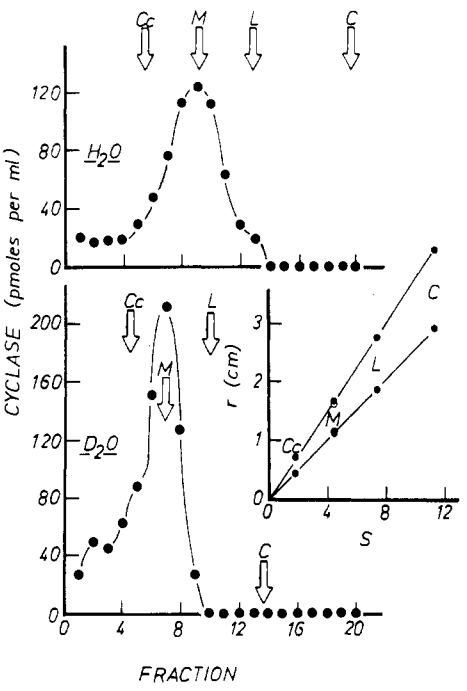

FIGURE 6: Sucrose density gradient centrifugation of a DEAE-cellulose preparation. Gradients were made in H_2O or D_2O. The inset shows the relationship between mobility in the gradient and sedimentation constants for calibrating proteins (●) and cyclase (○). Conditions were as indicated under Experimental Procedures and in the text. Abbreviations were as in Figure 5.

(*3*) *Molecular Parameters.* Table II shows the hydrodynamic parameters of testicular adenylate cyclase. Results correspond to, at least, five different experiments and enzyme preparations. From these data, a molecular weight of 74000 and a frictional ratio of 1.41 were calculated.

Discussion

This paper reports a rapid procedure for the obtainment of a testicular, "soluble" cyclase preparation of a reasonable purity. An important perspective of this work is the obtainment of antibodies against this protein which, in turn, would enable some progress in the purification of other adenylate cyclases. Scarce yield of enzyme activity is, however, the main deficiency of the reported procedure.

On the other hand, about 10000-fold purification of crude extracts would be achieved, if it is considered that enzyme protein (but not activity) is quantitatively recovered after the last purification step. The turnover number for the enzyme should be 5–15 min^{-1} by starting from crude extracts with a specific activity between 7 and 20 pmol min^{-1} (mg of protein)$^{-1}$ and a molecular weight of 70000.

Hydrodynamic and molecular parameters of testicular adenylate cyclase reported in this paper differ from those found by Neer (1978). Using a crude enzyme preparation (105000*g* supernate), this author found values for the Stokes radius and sedimentation constant which are smaller than those reported in the present paper. Data on the Stokes radius presented here are more coincident with those reported by Braun (1974).

Evidence presented in this paper shows a satisfactory correlation between the molecular weight of the native enzyme, calculated from hydrodynamic parameters, and the mobility of denaturated pure enzyme in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This might indicate that testicular soluble adenylate cyclase is in fact a monomeric protein. The structure of this enzyme differs from that of the "soluble" cyclase purified by Takai et al. (1974) from *Brevibacterium liquefaciens*. The latter seems to be a dimer with

a molecular weight of about 92 000, which consists of two subunits of identical molecular weight.

In addition, data presented here confirm that the molecular weight of the testicular enzyme is smaller than those reported for the membrane-bound cyclases solubilized with detergents. The latter ranged from about 114×10^3 to 270×10^3 (Neer, 1974, 1978; Haga et al., 1977; Asbury et al., 1978; Stellwagen & Baker, 1976).

The testicular enzyme is the only nonsedimentable adenylate cyclase found in mammalian tissues, but little is known about its regulation and analogies with membrane-bound cyclases. The role of this enzyme in testicular germ cells is an important point to be elucidated.

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References

Asbury, R. F., Hope-Cook, G., & Wolff, J. (1978) *J. Biol. Chem. 253*, 5286–5292.

Braun, T. (1974) in *Hormone Binding and Target Cell Activation in the Testis* (Dufau, M. L., & Means, A. R., Ed.) p 237, Plenum Press, New York.

Braun, T., & Dods, R. F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1097–1101.

Clarke, S. (1975) *J. Biol. Chem. 236*, 1372–1379.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Haga, T., Haga, K., & Gilman, A. G. (1977) *J. Biol. Chem. 252*, 5776–5782.

Homcy, C., Wrenn, S., & Haber, E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 59–63.

Laemmli, V. K. (1970) *Nature (London) 227*, 680–685.

Laurent, T. C., & Killander, J. (1964) *J. Chromatogr. 14*, 317–330.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372–1379.

Meunier, J. C., Olsen, R. W., & Changeux, J. P. (1972) *FEBS Lett. 24*, 63–68.

Neer, E. J. (1974) *J. Biol. Chem. 249*, 6527–6531.

Neer, E. J. (1978) *J. Biol. Chem. 253*, 5808–5812.

Paladini, A. C., & Leloir, L. F. (1952) *Biochem. J. 51*, 426–430.

Randerath, K., & Randerath, E. (1967) *Methods Enzymol. 12*, 323–348.

Rodbell, M. (1967) *J. Biol. Chem. 242*, 5744–5750.

Salomon, Y., Londos, C., & Rodbell, M. (1974) *Anal. Biochem. 58*, 541–548.

Stellwagen, E., & Baker, B. (1976) *Nature (London) 261*, 719–720.

Takai, K., Kurashina, Y., Susuki-Hori, C., Okamoto, H., & Hayaishi, O. (1974) *J. Biol. Chem. 249*, 1965–1972.

Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp 364–396, Wiley, New York.

Terenzi, H. F., Roselino, E., & Passeron, S. (1971) *Eur. J. Biochem. 18*, 342–350.

Torres, H. N., Flawia, M. M., Hernaez, L., & Cuatrecasas, P. (1978) *J. Membr. Biol. 43*, 1–18.

Worthington Biochemical Corporation (1972) *Worthington Enzyme Manual*, pp 7–12, Freehold, NJ.

Preparation and Activation of a Spin-Labeled Pepsinogen†

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ABSTRACT: A porcine pepsinogen derivative has been prepared, using 3-[[(2,5-dioxo-1-pyrrolidinyl)oxy]carbonyl]-2,5-dihydro-2,2,5,5-tetramethyl-1*H*-pyrrolyl-1-oxy with 3 spin-labels/protein molecule. Two are located in the peptide that is released first (1–16) and the other is in the secondarily removed sequence (17–44). Activation of the labeled zymogen is a faithful model of native pepsinogen activation because the two processes are closely related in rate, pH dependence of rate, and sites of peptide bond cleavage. The ESR signal associated with the bound label changes during activation due to release of the activation peptide. The rate of release (0.5 min^{-1} at pH 2, 22 °C) is an order of magnitude slower than is the rate of activation, i.e., cleavage of the peptide bond that holds the peptide to the enzyme. Activation in the presence of pepstatin, however, results in peptide release (2 min^{-1} at pH 2, 22 °C) nearly as fast as activation occurs. At pH values between 2.5 and 3, there is a lag in the change of the ESR signal following acidification. This indicates accumulation of an intermediate with pH 8.5 labile activity that still has its activation peptide attached. The strong temperature dependence of the rate of activation (26 kcal/mol) is reflected neither in reported characteristics of pepsin catalysis nor in the measured rate of release of the activation peptide from the enzyme (13 kcal/mol).

Activation of porcine pepsinogen occurs when the zymogen is brought to pH values below 4. The first well-described activation event is cleavage of the Leu_{16}–Ile_{17} peptide bond, generating pseudopepsin (abbreviated as ϕ-pepsin), which is enzymatically active (Dykes & Kay, 1976; Christensen et al., 1977). This step is unimolecular. This and subsequent self-processing of ϕ-pepsin by bimolecular processes cleaves a total of 44 amino acid residues from pepsinogen (Al-Janabi et al., 1972). Both the rate of activation of pepsinogen and the rate of proteolysis by pepsin are pH dependent, apparently controlled by a group with a pK of 2.1 that must be protonated for activity (Bull & Currie, 1949). Although pepsinogen is quite acidic, having a pI of 3.7 (Herriott, 1938), pepsin with

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