Journal of Supramolecular Structure 4:205 (165)–220 (180) (1976)

SOME KINETIC AND CHROMATOGRAPHIC PROPERTIES OF DETERGENT-DISPERSED ADENYLATE CYCLASE

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Studies on the reaction kinetics and chromatographic properties of detergent-dispersed adenylate cyclase are described. Detergent-dispersed enzyme was prepared from whole rat cerebellum and from partially purified plasma membranes from rat liver.

Data were simulated to fit kinetic models for which an inhibitor is added in constant proportion to the variable substrate. Models were chosen to distinguish whether the adenylate cyclase reaction may be controlled by an inhibitory action of free ATP^{-4} (or $HATP^{-3}$) or by a stimulatory action of free divalent cations. The various kinetic models were then tested with the dispersed brain adenylate cyclase with both Mg^{++} and Mn^{++} and in two different buffer systems. The experimental data indicate that this enzyme has a distinct cation binding site, but exhibits no significant inhibition by $HATP^{-3}$ or ATP^{-4}.

The detergent-dispersed adenylate cyclase both from liver plasma membranes and from brain have been chromatographed on anion exchange material and have been subjected to gel filtration. The presence of detergent was required for elution of cyclase activity from DEAE-Sephadex but was not required when DEAE-agarose was used. Dispersed brain cyclase was also chromatographed on agarose-$NH(CH_2)_3NH(CH_2)_3$-NH_2 which exhibits both ionic and hydrophobic properties. Fifty percent of the applied activity was recovered with a fivefold increase in specific activity. The data suggest that the relative effectiveness of a given chromatographic procedure for detergent-dispersed adenylate cyclase may reflect the influence of both hydrophobic and ionic factors.

INTRODUCTION

Adenylate cyclase (ATP: pyrophosphate lyase, cyclizing; E.C.4.6.1.1.) is a nearly ubiquitous enzyme that catalyzes the formation of adenosine 3′, 5′-monophosphate (cAMP) from ATP (1–3). In mammalian tissues it is located principally in the plasma membrane, but is also found in subcellular fractions enriched in nuclei, smooth and rough microsomes, and in Golgi F1 fractions, but not in either mitochondria or cytosol (1–10). Typically, adenylate cyclase activity is increased by target organ specific hormones, fluoride ion, guanyl nucleotides, or various anions (1–3, 11, 12). We feel that the elucidation of the mechanisms by which these agents stimulate adenylate cyclase may be aided by investigations of the enzyme's kinetic behavior and by purification of the enzyme's various components.

Adenylate cyclase activity has been successfully dispersed by several nonionic detergents (1, 3, 13–22), but few detailed studies on the properties and purification of the dispersed enzyme have appeared (14, 17, 20). Although detergent dispersion has resulted in reductions in ATPase activity in some adenylate cyclase preparations (1, 15, 16), it has also resulted in enzyme preparations that were essentially insensitive to stimulatory agents (1, 12, 13, 15, 16). However, the low ATPase activity and the established stability of such preparations (15, 16) may make them useful for characterization of the chromatographic and kinetic properties of the adenylate cyclase "catalytic moiety." In this paper we describe some studies on the reaction kinetics of detergent-dispersed adenylate cyclase from rat cerebellum. Also, the detergent-dispersed cyclases from cerebellum and from rat liver plasma membranes were used for studies of some of the enzyme's chromatographic properties.

METHODS

Enzyme Preparation

Detergent-dispersed preparations of particulate adenylate cyclase were prepared as described elsewhere, from rat cerebellum (15, 23) and rat liver plasma membranes (16).

Adenylate Cyclase Assay

Brain adenylate cyclase activity was determined in a reaction mixture containing 5–500 μM ATP, 10–600 μM total $MnSO_4$ or 0.5–9 mM total $MgSO_4$, 50 mM glycylglycine, or 50 mM triethanolamine, pH 7.5, 1 mM 1-methyl, 3-isobutylxanthine, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, 8-[^{3}H] ATP (3–6 $\times$ 10^5 cpm) and 220 μg/ml dispersed brain protein in a final volume of 0.2 ml. Incubations were at 37°C and were linear for at least 2 min at each ATP concentration tested. The 8-[^{3}H] cAMP formed was purified by adsorption and ion exchange chromatography by minor modifications (12) of the QAE method of Schultz et al. (24).

Adenylate cyclase activity of intact or detergent-dispersed plasma membranes of rat liver was determined essentially as for brain above (see also reference 16) except that unlabeled substrate was used, the reaction volume was 0.5 ml, and incubations were for 5 min at 37°C. Unlabeled cAMP formed in the reaction was purified by Dowex-50 chromatography and assayed by the protein binding assay described previously (12, 15, 16).

Kinetic Theory

Adenylate cyclase kinetics were evaluated to determine whether the rate of substrate ($MgATP^{-2}$ or $MnATP^{-2}$) conversion to cAMP may be controlled by an inhibitory effect of free ATP (ATP^{-4} or $HATP^{-3}$) or by a stimulatory effect of uncomplexed divalent cation (Mg^{++} or Mn^{++}). The development of the various models, rate equations, and theory is described by Garbers and Johnson elsewhere (25). The models (see Table I) are based on those of Cleland et al. (26) where the effects of inhibitors on the shape of reciprocal plots were determined as a function of the variable substrate, when the inhibitor was always added in constant proportion to the variable substrate. From known stability constants for metal-ATP complexes and from appropriate conservation and rate

equations, it was possible to show that the Cleland approach could also be used for the adenylate cyclase reaction (25). Kinetic constants were determined by linear regression analysis of the slopes and intercepts of secondary plots as suggested by Cleland (27).

Other Methods

The concentrations of $MnSO_4$ and $MgSO_4$ were verified to be within 1% by atomic absorption spectrometry. Protein was determined essentially as described by Lowry et al. (28) and appropriate corrections were made for the possible influence of detergents and other reagents on the assay. All materials were obtained from commerical sources with the exception of the protein kinase used for the assay of cAMP, which was prepared by established procedures (29, 30).

RESULTS

Kinetic Models for Adenylate Cyclase

The earlier studies of Drummond and co-workers (31–33) and of Birnbaumer et al. (34) suggested that in addition to the substrate, $MgATP^{-2}$, adenylate cyclase required free cation (Mg^{++}) for activity. Birnbaumer et al. (34) and later Perkins (3) suggested that hormones and fluoride ion might activate adenylate cyclase by decreasing the K_{diss} for Mg^{++}, but Drummond and co-workers indicated that activation occurs completely through changes in V_{max} (31–33). However, it was suggested in a more recent re-evaluation of some of these data (32, 34) by de Haën (35) that the previously observed kinetic behavior of adenylate cyclase could be explained entirely by a potent inhibition by free ATP^{-4} (or $HATP^{-3}$). Furthermore, it was concluded that hormone or fluoride ion stimulation of adenylate cyclase was due to changes in the sensitivity of the enzyme to inhibition by free ATP rather than to changes in the enzyme's sensitivity to activation by free cation (35). No evidence for a cation binding site was found (35). However, data (32, 34) used by de Haën to construct his model were not in a form that would permit a reliable kinetic distinction to be made between a requiste inhibitory site for free ATP or a requisite stimulatory site for free cation (see Fig. 1).

It is apparent from Fig. 1 that theoretical curves derived from either of two models may fit the data of Drummond et al. (32). The solid line represents the de Haën model (ATP is an inhibitor) and the dashed line was derived from the following rate equation for the case where Mg^{++} is an activator:

$$v = \frac{V_{max}(Mg^{++})(MgATP^{-2})}{(K_{iMg^{++}})(K_{MgATP}) + K_{MgATP}(Mg^{++}) + K_{Mg}(MgATP^{-2}) + (Mg^{++})(MgATP^{-2})}$$

for which $K_{iMg^{++}}$ = dissociation constant for Mg^{++}, K_{Mg} and K_{MgATP} are the respective Michaelis constants for Mg^{++} and $MgATP^{-2}$, and V_{max} is the maximal velocity.

In these instances (3, 32, 34), either the total magnesium concentration was fixed and total ATP was varied, or ATP was fixed and magnesium varied. However, in consideration of the known stability constants of $MgATP^{-2}$ and $MgHATP^{-1}$, this method results in

TABLE I. Kinetic Models for Adenylate Cyclase

Substrate	Activator	Inhibitor
1. $Me-ATP^{-2}$	None	None
2. $Me-ATP^{-2}$	Free Me^{++}	None
3. $Me-ATP^{-2}$	None	Free ATP^{-4} or $HATP^{-3}$
4. $Me-ATP^{-2}$	Free Me^{++}	Free ATP^{-4} or $HATP^{-3}$

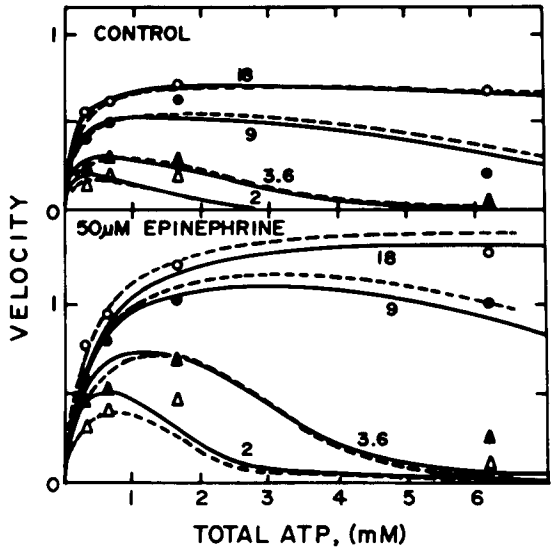

Fig. 1. A comparison of theoretical curves representing different kinetic models for cardiac adenylate cyclase. The total ATP concentration was varied as indicated on the abscissa at each of several fixed Mg^{++} concentrations (numbers next to each curve) both without (control, upper panel) and with 5×10^{-5} M epinephrine (lower panel). The data points are from Drummond et al. (32) as redrawn by de Haën (35). The solid curves are from de Haën (35) and represent the theoretical curves derived from a model where there is a requisite inhibition by free ATP and there is no Mg^{++} site. The dashed lines represent the theoretical curves obtained with values derived from the rate equation shown in the text for which there is no inhibition by free ATP and free Mg^{++} is a requisite activator.

simultaneous changes in the concentration of free Mg^{++}, and in the ratios of $MgATP^{-2}$ to ATP^{-4} and of $MgHATP^{-1}$ to $HATP^{-3}$. The unacceptability of this approach to kinetic analysis in general has been discussed by Cleland et al. (26) and with regard to adenylate cyclase studies by Garbers and Johnson (25).

The earlier kinetic studies of adenylate cyclase were also complicated by the fact that ATP-regenerating systems were required to prevent extensive hydrolysis of ATP by ATPase activity present in these particulate preparations (1–3, 31). ATP-regenerating systems, employing either creatine phosphate and creatine kinase or phosphoenolypyruvate and pyruvate kinase, may also interact with ATP and free cation, thereby making more difficult the interpretation of kinetic data obtained in their presence. However, an ATP-regenerating system was not required for the present kinetic analysis of adenylate

cyclase. This was possible for two reasons: first the detergent-dispersed adenylate cyclase of rat cerebellum was contaminated with less ATPase activity than were the brain particles prior to dispersion; and secondly, the apparent K_m for ATP of the cyclase was much lower than that of the ATPase (15, 23).

Kinetics of Dispersed Adenylate Cyclase from Rat Cerebellum

To determine which of these two models may be correct, we have derived expected kinetic patterns of reciprocal plots for the four general enzyme models shown in Table I (see also references 25, 26). Those mechanisms involving ATP inhibition were evaluated for linear and hyperbolic inhibition that was competitive, noncompetitive, or uncompetitive. For the mechanisms involving activation by Mg^{++}, both equilibrium-ordered and random mechanisms were considered (25). The various models were then fit to data obtained with the dispersed brain enzyme.

Reciprocal plots of adenylate cyclase activity are shown in Figs. 2–4 as functions of substrate ($MgATP^{-2}$ or $MnATP^{-2}$) and free (excess) cation (Mg^{++} or Mn^{++}) concentrations. For Fig. 2 free Mg^{++} and MgATP were used at the indicated concentrations, in a reaction mixture containing glycylglycine buffer, bovine serum albumin, and dithiothreitol. These are agents known to form complexes with cations and have been used in earlier studies with this enzyme (15). The data obtained (Fig. 2), whether plotted as a function of free Mg^{++} (upper panel) or of MgATP (lower panel), resulted in a pattern of linear plots that intersects to the left of the ordinate.

In an analogous experiment with Mg^{++} and MgATP (Fig. 3) in which only triethanolamine buffer was used, a similar pattern of lines resulted. Triethanolamine does not readily form cation complexes. Thus, bovine serum albumin, dithiothreitol, and glycylglycine did not greatly affect the general kinetic behavior of the enzyme (Figs. 2 and 3).

When MnATP and free Mn^{++} were used with triethanolamine buffer (Fig. 4), the pattern of lines obtained from the double-reciprocal plots is similar to those shown previously for magnesium (Figs. 2 and 3). Again, the lines intersect to the left of the ordinate and show no tendency to be concave upward (e.g., linear competitive inhibition as in model 4, Table I) nor concave downward (e.g., hyperbolic competitive inhibition as in model 3, Table 1; see also references 25, 26). In other experiments (not shown), kinetics were also studied with Mn^{++} and MnATP in a reaction mixture containing dithiothreitol, bovine serum albumin, and glycylglycine. The resulting plots were qualitatively similar to those shown in Fig. 4.

The slopes and intercepts of the lines obtained in the experiments shown in Figs. 2 and 3 were replotted as functions of the respective fixed concentrations of free Mg^{++} or MgATP (Fig. 5). The resulting secondary plots are also linear, regardless of which buffer was used and whether plotted as a function of free Mg^{++} or MgATP (Fig. 5). The secondary plots from experiments with Mn^{++} and MnATP were likewise linear (not shown). From the slopes and intercepts of such secondary plots, the true Michaelis constants and dissociation constants of the reactants have been calculated (Table II). The data show agreement in the values obtained with either buffer system and indicate that for the dispersed brain adenylate cyclase Mn^{++} and MnATP are far more effective reactants than are Mg^{++} and MgATP.

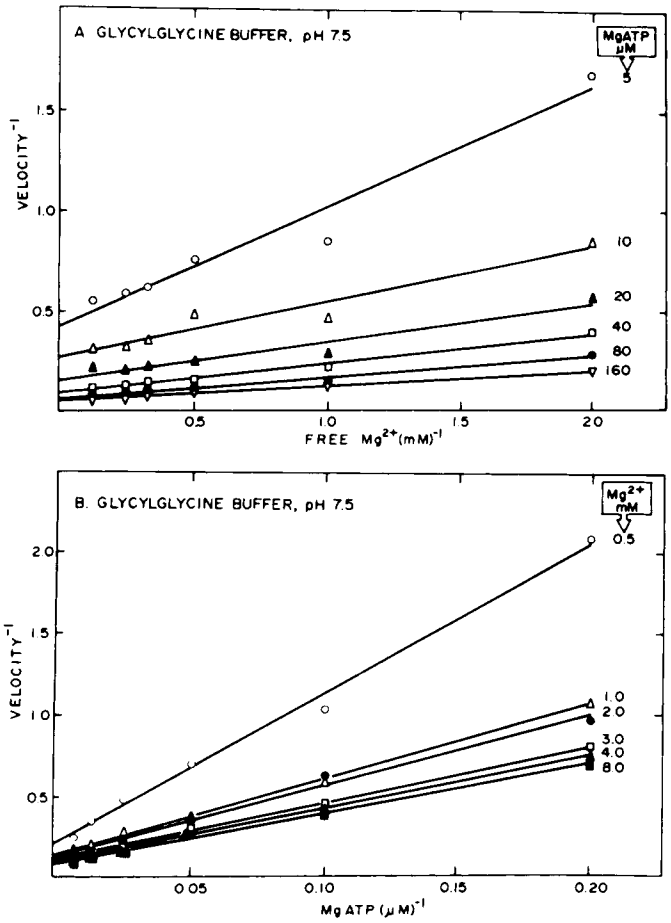

Fig. 2. Reciprocal plots showing the effects of Mg^{++} and MgATP on adenylate cyclase activity with a glycylglycine buffer system. Detergent-dispersed cerebellar adenylate cyclase was prepared and assayed as described in Methods. The reaction mixture contained 50 mM glycylglycine, pH 7.5, 1 mM dithiothreitol, and 1 mg/ml bovine serum albumin. Dimensions for $(\text{velocity})^{-1}$ are $(\text{nmole cAMP})^{-1}$ $(2\ \text{min})^{1}(\text{mg protein})^{1}$. Values represent the average from triplicate adenylate cyclase incubations. Upper panel: reciprocal plot of activity vs free Mg^{++} at each MgATP concentration indicated (numbers next to lines). Lower panel: reciprocal plot of activity vs MgATP at the indicated free Mg^{++} concentrations.

Chromatography of Detergent-Dispersed Adenylate Cyclase

The earliest attempts to chromatograph dispersed adenylate cyclase were reported by Sutherland et al. (1) who studied DEAE-cellulose chromatography of cyclase dispersed by Triton X-100. However, the elution of the enzyme from the DEAE cellulose required the presence of detergent (1). This requirement for detergent was apparently not observed by Levey (14) in studies of cardiac cyclase dispersed by Lubrol-PX (also a nonionic detergent), nor by Neer in similar studies with dispersed renal adenylate cyclase (17). In fact, DEAE-cellulose chromatography of dispersed cardiac cyclase has been reported to separate completely the enzyme from the nonionic detergent (14). An additional problem

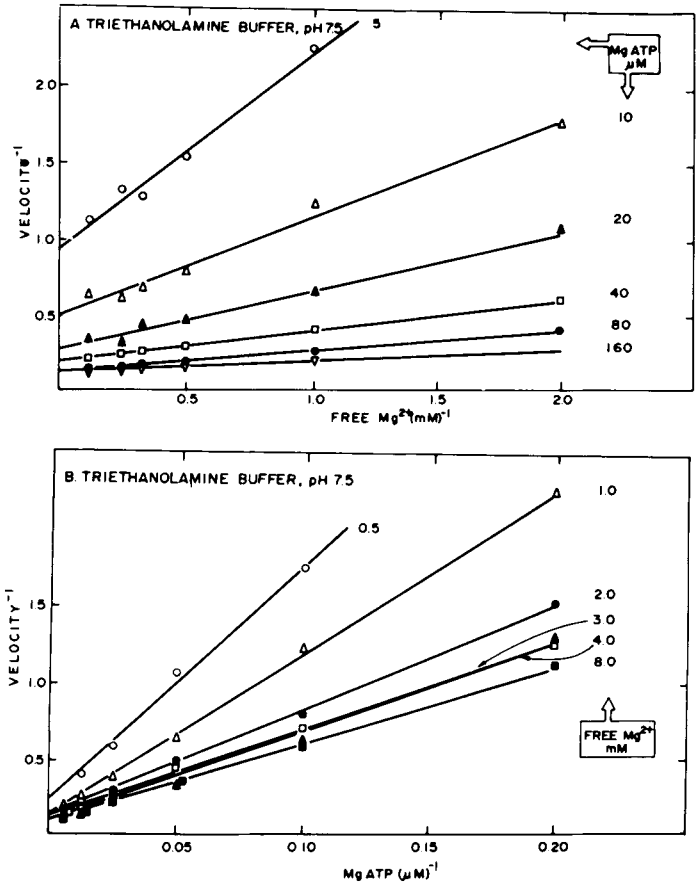

Fig. 3. Reciprocal plots showing the effects of Mg^{++} and MgATP on adenylate cyclase activity with a triethanolamine buffer system. Detergent-dispersed enzyme was prepared and assayed as described in Methods, except that 50 mM triethanolamine, pH 7.5, was used in the reaction mixture instead of glycylglycine. There was no bovine serum albumin or dithiothreitol present. Dimensions for $(\text{velocity})^{-1}$ are $(\text{nmole cAMP})^{-1}(2\text{ min})^{1}(\text{mg protein})^{1}$. Values are averages from triplicate adenylate cyclase incubations.

typically encountered with DEAE chromatography of adenylate cyclase was the loss of significant amounts of the applied activity (1, 14). We have conducted some experiments that bear on both of these problems.

Detergent-dispersed adenylate cyclase from liver was chromatographed in the absence and presence of 0.1% Lubrol-PX on DEAE-Sephadex (Fig. 6). Consistent with the earlier observations of Sutherland et al. (1), we also found that detergent was required for the elution of dispersed adenylate cyclase from DEAE-Sephadex. Figure 6 shows that without detergent, essentially no hepatic adenylate cyclase activity could be eluted with NaCl concentrations up to 1 M. In the presence of 0.1% Lubrol-PX, enzyme activity was readily eluted with 300 mM NaCl in fractions that were slightly turbid.

In similar experiments with dispersed brain adenylate cyclase (Fig. 7), it is apparent that the salt elution in the absence of detergent did not inactivate the enzyme. Activity was readily eluted by the subsequent addition of Lubrol-PX. In other experiments (not

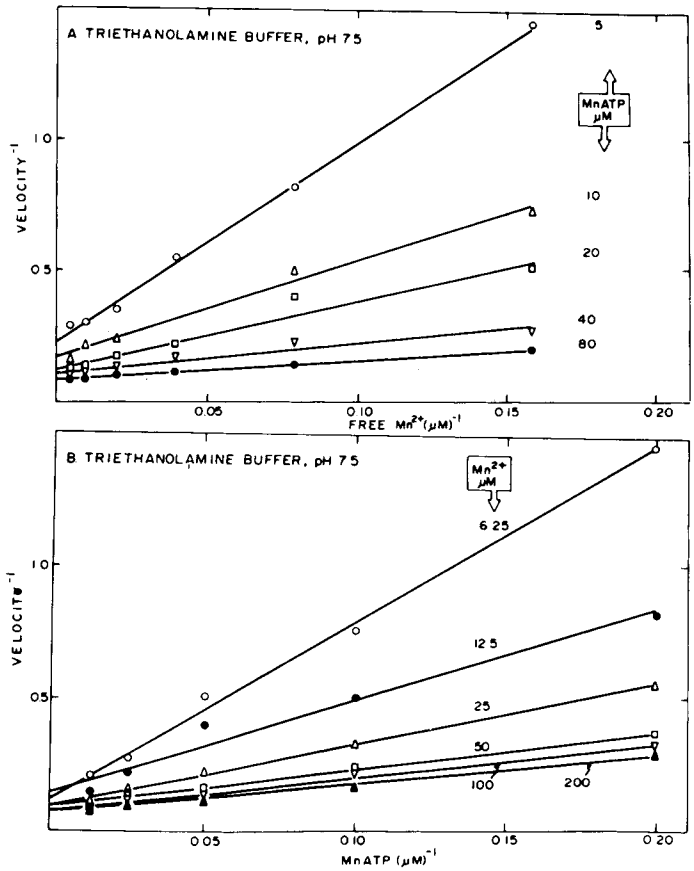

Fig. 4. Reciprocal plots of the effects of Mn^{++} and MnATP on adenylate cyclase activity with a triethanolamine buffer system. Detergent-dispersed brain cyclase was prepared and assayed as described in Methods and for Fig. 3. Dimensions for $(velocity)^{-1}$ are $(nmole\ cAMP)^{-1}(2\ min)^{1}$ $(mg\ protein)^{1}$ and those for both free $(Mn)^{-1}$ and $(MnATP)^{-1}$ are $(\mu M)^{-1}$. Values are averages from triplicate adenylate cyclase incubations.

shown) up to 2 M NaCl was used (without detergent) and no measurable activity was eluted from the DEAE-Sephadex. The elution sequence shown here (Fig. 7) has one advantage in that significant quantities of protein were removed from the preparation that otherwise would have cochromatographed with cyclase activity.

Typically with brain adenylate cyclase, a significant percentage of the applied activity did not bind to the DEAE-Sephadex and appeared in the wash-through fractions. This failure to bind was not seen with dispersed liver cyclase applied to DEAE-Sephadex (Fig. 6), nor with dispersed brain cyclase applied to DEAE-agarose (Fig. 8).

Though detergent was required for elution of adenylate cyclases from the DEAE-Sephadex system, DEAE-agarose (DEAE-BioGel A, 100–200 mesh, from Bio-Rad) may be used for the chromatography of dispersed cerebellar adenylate cyclase in the absence of detergent (Fig. 8). In contrast with DEAE-Sephadex columns above, little or no enzyme activity was eluted in the wash-through fractions from DEAE-agarose. Furthermore, in the fractions containing enzyme activity, no turbidity was observed. The recovery of

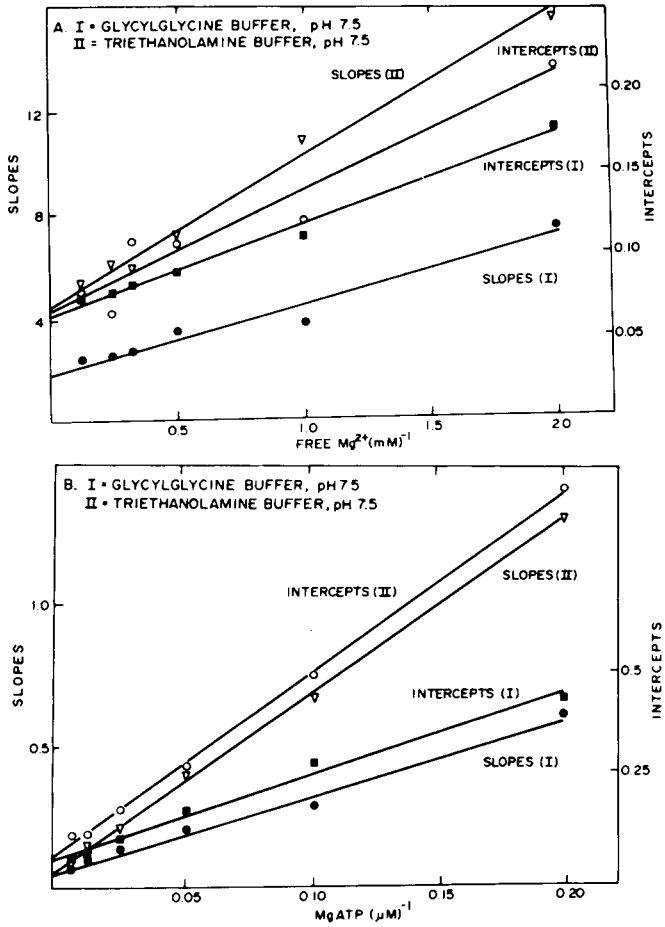

Fig. 5. Secondary plots of slopes and intercepts from the primary reciprocal plots of Figs. 2 and 3. Replots of data obtained with the glycylglycine (Fig. 2) or triethanolamine (Fig. 3) buffer systems are as indicated. Upper panel: slopes and intercepts from the lower panels of Figs. 2 and 3 replotted as a function of $(\text{free Mg})^{-1}$. Lower panel: slopes and intercepts from the upper panels of Figs. 2 and 3 replotted as a function of $(\text{MgATP})^{-1}$.

applied activity from DEAE-agarose was typically between 60% and 80% with an increase in specific activity of up to fivefold.

In other experiments (not shown), we have further subjected brain adenylate cyclase, previously chromatographed on DEAE-agarose, to filtration on Sepharose 4B in the absence of detergent. The enzyme activity emerged in a single peak with an elution volume relative to the void volume of 1.23. For comparison, large absorbance (280 nm) peaks were observed at 1.00, 1.54, and 3.46. These preliminary observations would suggest a relatively large molecular weight for this adenylate cyclase. But in view of the fact that the enzyme is still very impure and that hydrophobic and other factors may significantly influence its chromatographic behavior, conclusions from such evidence alone regarding molecular size are probably premature.

The suggestion that "hydrophobic" properties of some enzymes may be exploited

TABLE II. Kinetic Constants for Detergent-Dispersed Cerebellar Adenylate Cyclase

Kinetic parameter	KINETIC CONSTANTS: BUFFER SYSTEM Glycylglycine 50 mM, pH 7.5 (μM)	Triethanolamine 50 mM, pH 7.5 (μM)
K_{MnATP}	7.1	8.6
$K_{Mn^{2+}}$	2.3	4.2
$K_{i(MnATP)}$	202	100
$K_{i(Mn^{2+})}$	64	49
K_{MgATP}	29	62
$K_{Mg^{2+}}$	830	860
$K_{i(MgATP)}$	48	91
$K_{i(Mg^{2+})}$	1,350	1,390

for enzyme purification was made by Shaltiel and Er-el (36) in studies on the "hydrophobic chromatography" of glycogen synthase. Figure 9 represents an experiment in which dispersed brain adenylate cyclase was chromatographed on agarose to which a side chain was attached (Affinose 102 from Affinity Systems). The side chain, $-NH(CH_2)_3-NH-(CH_2)_3-NH_2$, exhibits both ionic and hydrophobic properties. When the dispersed cyclase was applied, most of the protein (absorbance at 280 nm) washed through the column and emerged in turbid fractions. However, enzyme activity was retarded and later eluted with $MnSO_4$ in fractions that appeared clear. Overall recovery of applied activity was about 50% and specific activity was increased about fivefold. Thus, it may be possible to achieve some degree of purification of adenylate cyclase, and perhaps of other membrane constituents, by utilizing chromatographic materials with varying degrees of hydrophobic and ionic properties.

DISCUSSION

The kinetic studies of the dispersed cerebellar adenylate cyclase indicate that the probable true substrate for the reaction is the cation-ATP^{-2} complex and that the kinetic properties of the enzyme are qualitatively similar for both Mg^{++} and Mn^{++} in either of two buffer systems tested (Figs. 2–5). In addition, the linear intersecting reciprocal plots, whether as a function of free cation or of cation-ATP, suggest a bi-reactant sequential mechanism in which free cation is a requisite activator. That the secondary replots (Fig. 5) were also linear implies that this mechanism is correct and that there is no detectable inhibition by uncomplexed ATP^{-4} or $HATP^{-3}$. In conclusion, model 2 from Table I is the most probable kinetic model for this adenylate cyclase under the conditions tested.

It should be emphasized, though, that one cannot necessarily extrapolate the above conclusions to all adenylate cyclases under all assay conditions. It is conceivable that the dispersion by detergent significantly altered the kinetic properties of this enzyme. It is also possible that with 0.1% detergent present during the assay, the presumed micellar detergent-protein-enzyme may behave kinetically quite differently from the enzyme residing in an intact membrane structure. It is of interest to note, though, that in other studies not reported here, it has been shown that this model (model 2 from Table I) may

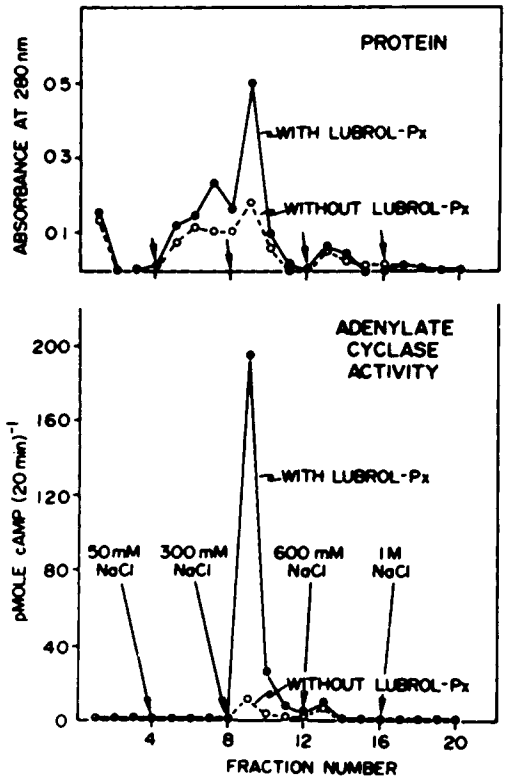

Fig. 6. Effect of detergent on the chromatography of dispersed liver adenylate cyclase on DEAE-Sephadex. The DEAE-Sephadex (A-25, from Pharmacia) was first equilibrated with medium containing 50 mM glycylglycine, pH 7.5, 3 mM dithiothreitol, 1 mM NaF, and either no detergent or 0.1% Lubrol-PX, a nonionic detergent. Liver plasma membranes were prepared and dispersed essentially as previously described (16). The dispersed membranes (2.8 ml; 2–3 mg protein) were applied to each of two DEAE-Sephadex columns (0.9 × 10 cm); one with and one without detergent. The columns were then washed with 17 ml of the respective elution media, followed by elution media containing the indicated NaCl concentrations. The salt concentrations were selected on the basis of previous experiments in which the enzyme was eluted from DEAE-Sephadex by a continuous gradient of NaCl. In this experiment the gradient was discontinuous with each step consisting of four 5-ml fractions. Upper panel: protein elution as monitored by absorbance at 280 nm. Lower panel: adenylate cyclase activity. The solid line represents chromatography with 0.1% Lubrol-PX and the dashed line represents elution without detergent. Values represent the average from duplicate enzyme incubations.

also obtain for particulate cardiac adenylate cyclase when a phosphoenolpyruvate-pyruvate kinase regeneration system was used (25). One might then speculate that if this model for the adenylate cyclase reaction were correct, stimulation of reaction velocity by hormones and other agents may be due in part to an alteration in the enzyme's sensitivity to free cation that is mediated by the action of other membrane constituent(s). Our observations would be consistent with this mechanism, suggested earlier by Birnbaumer et al. (34) and Perkins (3), but would be in contrast with the more recent interpretations and studies of de Haën (35) and Rodbell and his co-workers (37, 38).

Some initial studies of the chromatographic properties of dispersed adenylate cyclase have been described briefly. Anion exchange chromatography was often accompanied with loss of enzyme activity. This loss may be due to enzyme inactivation. On the other

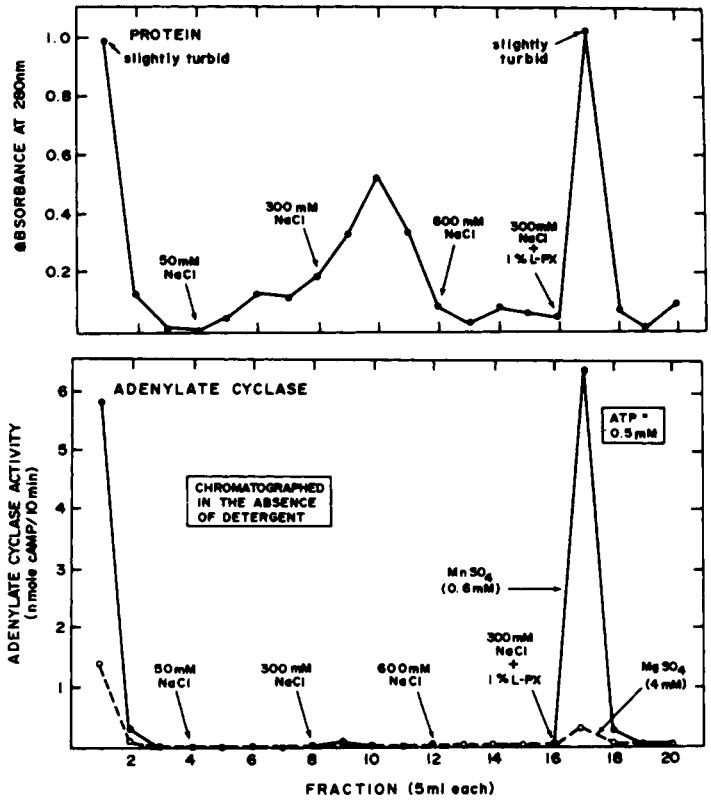

Fig. 7. Effect of detergent on the elution of dispersed cerebellar adenylate cyclase from DEAE-Sephadex. Detergent-dispersed adenylate cyclase was prepared as described (23) and was applied (500 μl, 11 mg protein) to a DEAE-Sephadex (A-25) column (0.9 X 10 cm) that had been previously equilibrated with medium containing 50 mM glycylglycine, pH 7.5, and 3 mM dithiothreitol. Following application of the enzyme, the column was eluted first with 19.5 ml of the same elution medium (above) and then with a discontinuous NaCl gradient as indicated. Each successive gradient step consisted of four 5-ml fractions. Upper panel: protein elution as monitored by absorbance at 280 nm. Lower panel: adenylate cyclase activity, assayed with 0.5 mM ATP and either 4 mM $MgSO_4$ (dashed line) or 0.6 mM $MnSO_4$ (solid line). Values are the averages from duplicate enzyme incubations.

hand, in view of the recent observations of Brostrom (39), one might speculate that during chromatography a hypothetical activator(s) may have been separated from the catalytic moiety. However, there is little evidence to support this latter suggestion.

It is difficult to interpret the differences observed in the requirement (or lack thereof) for detergent in the elution of adenylate cyclase from DEAE columns (Figs. 6–8). It would appear that the nature of the support material (to which the DEAE is attached) and its exchange capacity may play significant roles in the chromatography of the dispersed cerebellar adenylate cyclase. DEAE-Sephadex (A25) beads are relatively tight structures (low porosity) with high ion exchange capacity (ca. 3.5 meq/g dry) and with low molecular weight exclusion limits. But perhaps also of importance, these beads contain ether linkages and therefore relatively hydrophobic regions. DEAE-agarose (BioGel A) beads are more porous, with a nominal exclusion limit of 1.5×10^7 daltons, have a

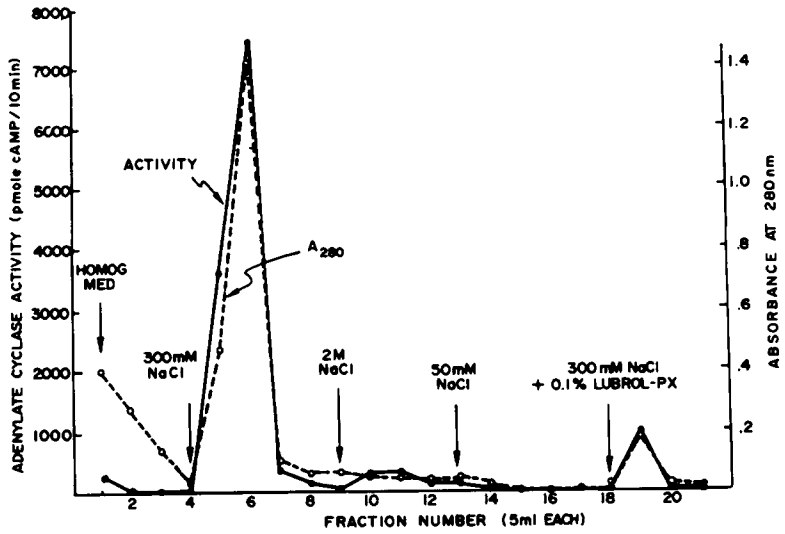

Fig. 8. Chromatography of detergent-dispersed adenylate cyclases from cerebellum on DEAE-agarose in the absence of detergent. Detergent-dispersed adenylate cyclase was prepared as described (23) and applied (500 μl, 11 mg protein) to a DEAE-agarose (DEAE-BioGel A, 100–200 mesh, from Bio-Rad) column (0.9 X 10 cm) that had been previously equilibrated with 50 mM glycylglycine, pH 7.5, and 3 mM dithiothreitol. Following application of the enzyme, the column was eluted with 19.5 ml of the same equilibration medium and then with a discontinuous NaCl gradient as indicated. Each successive step consisted of four 5-ml fractions. Activity is represented by the solid line and absorbance at 280 nm by the dashed line. Activity was measured with 0.5 mM ATP and 0.6 mM $MnSO_4$. Values are the averages from triplicate enzyme incubation.

relatively low ion exchange capacity (ca. 0.01 meq/ml wet), and do not contain ether linkages. Any or all of these factors may account for the observed differences (Figs. 8 and 9) in the chromatography of adenylate cyclase from brain. It is conceivable, for example, that the detergent-protein micelles that contain adenylate cyclase actually enter the agarose beads and are thereby more effectively disrupted. More effective disruption might be characterized by a lesser tendency for the eluted proteins to reaggregate following chromatography. The lesser exchange capacity of the DEAE-agarose (compared with DEAE-Sephadex) may actually preclude the binding of other dispersed membrane material that would later cochromatograph with adenylate cyclase activity. The cochromatography of such material may account for the turbidity commonly observed in fractions containing cyclase activity following DEAE-Sephadex chromatography. With the Affinose column (Fig. 9), turbid material was readily separated from the enzyme activity. Clearly, a fuller understanding of the chromatographic properties of adenylate cyclase, and of other dispersed membrane components as well, must await more detailed studies. Even so, it seems reasonable to consider that for detergent-dispersed adenylate cyclase, the relative effectiveness of a given chromatographic procedure may reflect the influence of both hydrophobic and ionic factors. The use of "hydrophobic" chromatography may be particularly applicable to the purification of membrane-bound enzymes.

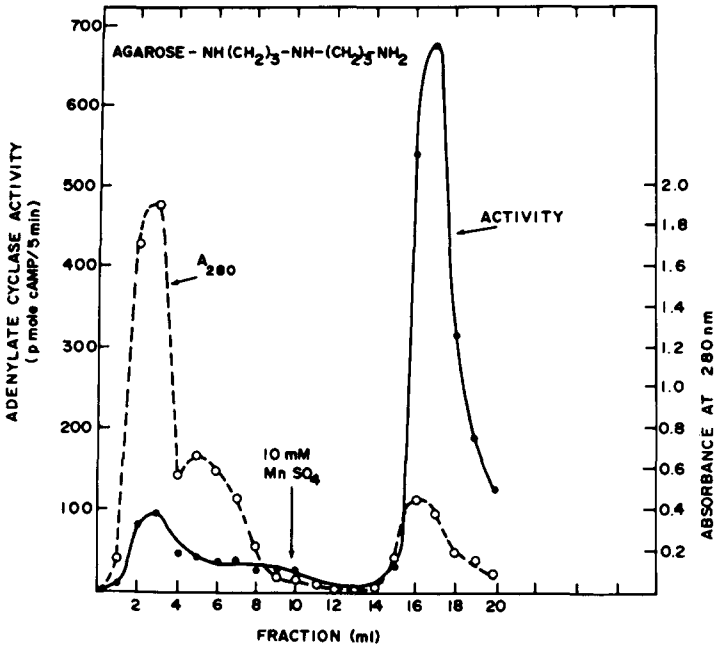

Fig. 9. "Hydrophobic" chromatography of detergent-dispersed adenylate cyclase from rat brain. Detergent-dispersed adenylate cyclase was prepared as previously described (15). The enzyme (2 ml, 5.8 mg protein) was applied to a column of agarose-NH-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2 (Affinose 102 from Affinity Systems) that had been previously equilibrated with medium containing: 100 mM glycylglycine, pH 7.5; 250 mM sucrose; 3 mM dithiothreitol; 1 mM EDTA; and 1% Lubrol-PX. Following application of the enzyme, the first 2 ml were discarded and then the column was washed with 10 ml of the above medium. Enzyme activity was eluted with 10 mM $MnSO_4$. 1-ml fractions were collected. The dashed line represents absorbance at 280 nm and the solid line represents activity. Activity was determined as described in Methods except that the reaction mixture contained 10 mM theophylline instead of 1 mM 1-methyl, 3-isobutylxanthine, 4 mM ATP, and 8 mM $MgSO_4$. The final concentration of $MnSO_4$ in the assay of all fractions was 1 mM. cAMP formed was measured by the protein binding assay as described in Methods. Values represent the averages from duplicate enzyme incubations.

ACKNOWLEDGMENTS

The authors wish to thank Drs. C.R. Park and J.G. Hardman for their generous encouragement and support throughout the studies. We are very grateful to Ms. Janette Welden and Ms. Janice Suddath for their excellent technical assistance.

This work was supported by grants HE 08332, AM 07462, and GM 16811 from the United States Public Health Service and USPHS grant HD 05797 from the Vanderbilt Population Center. Simon J. Pilkis is a Career Investigator of the Howard Hughes Medical Institute.

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