Detergent-dispersed Adenylate Cyclase from Rat Brain

EFFECTS OF FLUORIDE, CATIONS, AND CHELATORS

(Received for publication, March 5, 1973)

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SUMMARY

Some properties of particulate and detergent-dispersed preparations of adenylate cyclase from rat brain have been studied. The activity of the particulate enzyme was greater with Mn\(^{2+}\) than with Mg\(^{2+}\) and the activity in the presence of either cation was enhanced by F\(^-\) or nonionic detergents. In the presence of the non-ionic detergent Lubrol-PX, enzyme activity was further enhanced by dithiothreitol but inhibited by F\(^-\).

Adenylate cyclase activity was dispersed into a 27,000 × g for 20 min supernatant fraction by Lubrol-PX. Inclusion of dithiothreitol in the homogenizing medium facilitated the solubilization of enzyme activity. More total activity was extracted from cerebellum than from cerebrum. The activity of the dispersed enzyme was essentially unchanged by filtration (0.22-μm pore) or by centrifugation at 100,000 × g for 1 hour. Separation of the detergent from the enzyme by gel filtration resulted in a turbid fraction, the activity of which was increased by F\(^-\) or Lubrol-PX.

Detergent-dispersed adenylate cyclase was inhibited by Ca\(^{2+}\), the degree of inhibition depending on whether Mg\(^{2+}\) or Mn\(^{2+}\) was present. In the presence of 8 mM Mg\(^{2+}\), ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA) markedly decreased enzyme activity. The concentration causing 50% inhibition was dependent on protein concentration, but was usually about 30 μM. Inhibition by EGTA was completely prevented by the addition of an equimolar concentration of Ca\(^{2+}\), Mn\(^{2+}\), or Co\(^{2+}\), and was partially prevented by several other divalent cations. Gel chromatographic removal of EGTA from an inhibited enzyme completely restored activity. EDTA and several other metal-binding agents, at concentrations up to 1 mM, had little or no effect on adenylate cyclase activity. EGTA had no appreciable effect on either ATPase or cyclic 3':5'-nucleotide phosphodiesterase activities from brain or on adenylate cyclase activity prepared from partially purified plasma membranes from liver.

The data suggest that adenylate cyclase from brain requires some metal in addition to added Mg\(^{2+}\) for full expression of activity and that the metal is bound tightly to the enzyme.

Adenylate cyclase, which catalyzes the formation of adenosine 3':5'-monophosphate (cAMP)\(^1\) from ATP, has been characterized in particulate preparations derived from many sources (1-13). Numerous hormones, fluoride, detergents, and other agents have been shown to stimulate its activity (1, 7, 10). However, significant progress in the purification of the enzyme from mammalian tissues and evaluation of factors which modulate its activity have been hampered by the insolubility and instability of the enzyme. Although detergents have been shown to be capable of dispersing the enzyme from several mammalian sources, the solubilized enzyme has been studied in only a few instances (2, 13-18).

In this communication we report dispersion of a particulate adenylate cyclase from rat brain by a non-ionic detergent. The solubilized enzyme exhibited relatively high activity and stability. Its activity was diminished by fluoride, by several divalent cations, and by EGTA. The adenylate cyclase of rat brain appears to have a dual cation dependency.

EXPERIMENTAL PROCEDURE

Adenylate Cyclase Preparation and Activity Determination—Male Sprague-Dawley rats weighing 150 to 250 g were killed by decapitation. The whole cerebellum or cerebrum was immediately removed, placed in ice-cold homogenizing medium, and the tissue weight determined. The tissue was then homogenized on ice or at 4° in 0 volumes (relative to tissue weight) of homogenizing medium with three passes of a glass-Teflon motor-driven homogenizer, and the homogenate was centrifuged at 3,000 × g for 10 min. The resulting supernatant fraction was discarded and the pellet washed by resuspension and rehomogenization in 9 volumes of the homogenizing medium. This procedure of homogenization and centrifugation was done a total of three times. The third homogenate was designated the "washed particulate" preparation. Dispersed adenylate cyclase was prepared from these washed particles. The particles were collected by centrifugation at 3,000 × g for 10 min and the pellet was re-suspended and rehomogenized in 9 volumes of homogenizing medium identical with the initial one but containing Lubrol-PX, a nonionic detergent. This homogenate was centrifuged at 27,000 × g for 20 min, and the resulting supernatant fraction was removed and assayed for adenylate cyclase activity.

\(^1\)The abbreviations used are: cAMP, adenosine 3':5'-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; CIITA, cyclohexanediaminetetraacetic acid.
The homogenizing medium contained 0.25 M sucrose, 0.1 M glycylglycine buffer, pH 7.5, 2 mM MgCl₂, 1 mM EGTA, and 3 mM dithiothreitol. Lubrol-PX was used at a final concentration of 1% (w/v). Variations in the homogenizing medium and in the homogenization and centrifugation procedures were used on occasion as indicated.

The adenylate cyclase activity was determined by incubation of the appropriate protein fraction in a reaction mixture containing 4 mM ATP, 8 mM divalent cation (either Mg²⁺ or Mn²⁺), 50 mM glycylglycine buffer, pH 7.5, 10 mM theophylline, and, unless otherwise indicated, 1 μg per ml of bovine serum albumin and 1 mM dithiothreitol. Prior to assay, the enzyme fraction was diluted between 5- and 20-fold with the appropriate homogenizing medium. The diluted enzyme fraction constituted 10% of the assay incubation volume which was 0.5 ml. The reaction was initiated by the addition of enzyme. The incubation was for 5 min at 37°. An ATP-regenerating system was found to be unnecessary under these conditions of high substrate and low protein concentrations, since cyclase activity was the same at either 2 or 4 mM ATP and in some of the preparations tested, particulate or dispersed, was more than 10% of the substrate hydrolyzed. ATPase was measured from γ-3²P ATP essentially according to the method of Sugino and Miyoshi (19).

The reaction was terminated with 0.2 ml of 0.3 N HCl containing 5,000 to 10,000 cpm of [G-3H]cAMP (24 Ci per mmole) for measuring loss of sample during its fractionation. The sample was then placed in a boiling water bath for 5 min, chromatographed, lyophilized, and assayed. The cAMP formed was determined by the luciferase luminescence assay (20), or the protein kinase binding assay (21). When the luciferase luminescence assay was used, the samples were processed as previously described (22). For the protein kinase binding assay the samples were chromatographed on a single cation exchange column prior to assay. (The column was 0.8 x 10 cm, Bio-Rad AG50-X8, 100 to 200 mesh, H⁺ form, previously equilibrated with 0.1 N HCl, with which the sample was also eluted.) The correlation coefficient for 102 samples assayed by both procedures was 0.97. The samples that were compared were prepared under a wide variety of conditions and had concentrations covering a 50-fold range.

Protein was determined essentially according to Lowry et al. (23) with appropriate correction being made for the interference or contribution of the homogenizing medium.

Materials—The reagents for the assay of cAMP by luciferase luminescence were purchased from E. I. DuPont de Nemours and Co. The protein kinase used in the binding assay for cAMP was prepared from beef muscle essentially according to published procedures (21, 24). Bovine serum albumin was added to the dialyzed protein kinase prior to storage to give a final concentration of 1 mg per ml and was again added (20 to 40 μg per tube) to the binding assay incubation mixture. These steps enhanced the capacity of the protein to bind cAMP. Lubrol-PX was a gift of ICI America, Stamford, Conn. Lubrol-WX was a gift of Dr. E. J. Landon. [G-3²H]cAMP (24 Ci per mmole) was obtained from New England Nuclear Corp. EGTA was from Eastman Organic Chemicals. CDTX was from Matheson. Crystalline-lyophilized bovine serum albumin was obtained from Sigma and dithiothreitol from Calbiochem. Sephadex G-200 and G-25 were from Pharmacia. Chelex-100 (100 to 200 mesh, Na⁺ form, from Bio-Rad) was washed by extensive rinsing with alternating solutions of 1 N HCl and 1 N NaOH as described by Brostrom et al. (25). The resin was used to remove contaminating metals from the reagents used in several experiments. The reagents, at concentrations between 3- and 300-fold greater than those finally used, were individually passed over a Chelex 100 column (1.5 x 15 cm). ATP (0.1 μM) was successively passed over two such columns.

RESULTS

Effects of Fluoride and Detergents on Particulate Adenylate Cyclase—The activity of the washed particulate adenylate cyclase was greater with Mn²⁺ than with Mg²⁺ (Table I). In the presence of either cation, fluoride and several nonionic detergents each increased enzyme activity. These observations are generally consistent with those of other investigators (4, 6, 7, 12, 13). Lubrol-PX and Lubrol-WX were consistently more stimulatory at a concentration of 0.1% than were either Triton X-100 or Brij-35. In addition, the presence of each of these detergents the stimulatory effect of fluoride was inconsistently observed. For succeeding studies Lubrol-PX was used almost exclusively.

The effect of Lubrol-PX on the activity of particulate adenylate cyclase from cerebellum and cerebrum was determined. The control activities of the washed particulate enzyme from cerebellum and cerebrum were statistically comparable (Fig. 1). In both tissues the optimal concentration of Lubrol-PX was about 0.1% (w/v).

The inclusion of 10 mM dithiothreitol in the reaction mixture further stimulated cyclase activity in the presence of detergent but not in its absence (Fig. 1). Comparable effects of dithiothreitol were also observed with the cerebral enzyme (not shown).

The effect of fluoride on particulate adenylate cyclase activity in the presence of detergent was studied further as shown in

### Table I

Effects of several agents on washed particulate adenylate cyclase from rat cerebellum

Washed particulate adenylate cyclase was obtained from five pooled cerebellums as described under “Experimental Procedure.”

The homogenizing medium also contained 1 mg per ml of bovine serum albumin. Final detergent concentration was 0.1% (w/v) or v/v, as appropriate) and protein was 65 μg per ml. The incubation was for 10 min at 37°. Values represent mean ± S.E. from four incubations.

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>Fluoride</th>
<th>Control</th>
<th>Lubrol-PX</th>
<th>Lubrol-WX</th>
<th>Triton X-100</th>
<th>Brij-35</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0</td>
<td>4.77 ± 0.30</td>
<td>22.5 ± 1.5</td>
<td>21.5 ± 1.8</td>
<td>17.0 ± 0.8</td>
<td>13.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6.31 ± 0.32</td>
<td>18.1 ± 1.1</td>
<td>21.4 ± 0.5</td>
<td>15.9 ± 0.8</td>
<td>16.7 ± 0.6</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>0</td>
<td>8.85 ± 0.59</td>
<td>29.2 ± 1.6</td>
<td>33.0 ± 1.4</td>
<td>24.4 ± 1.3</td>
<td>22.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12.5 ± 1.1</td>
<td>30.4 ± 1.3</td>
<td>38.9 ± 2.2</td>
<td>25.2 ± 1.0</td>
<td>28.0 ± 1.7</td>
</tr>
</tbody>
</table>
Fig. 1. Effect of Lubrol-PX on particulate adenylate cyclase from rat cerebrum and cerebellum. The washed particulate adenylate cyclase was prepared as described under "Experimental Procedure." The homogenizing medium contained only sucrose, glycyglycine buffer, MgCl₂, and EDTA. The preparations were made either from four pooled cerebellums or from one cerebrum. Both were assayed at 70 µg of protein per ml in the absence of bovine serum albumin. Detergent and dithiothreitol (DTT) were present only during the incubation. The data represent the average values ± S.E. obtained from three incubations.

Fig. 2. In the absence of detergent, fluoride produced a concentration-dependent stimulation and then inhibition of cyclase activity. However, in the presence of 0.1% Lubrol-PX, which itself increased enzyme activity about 5-fold, fluoride only produced inhibition. Inhibition of cyclase from other sources by high concentrations of fluoride has been reported although its mechanism is not certain (4, 11).

Dispersion of Adenylate Cyclase—Detergents have been used to disperse adenylate cyclase from membranes and particles from several tissues (2, 13-18). The use of Lubrol-PX to solubilize adenylate cyclase was first reported by Levey (17) using cardiac tissue. The effects of several concentrations of Lubrol-PX on the dispersion of protein and adenylate cyclase from washed particulate cerebellar preparations are depicted in Fig. 3. The optimal concentration of Lubrol-PX for the dispersion of the enzyme was 1%. Further increasing the detergent concentration increased the dispersion of protein, decreased cyclase specific activity, and apparently decreased total activity. Decreased total activity at detergent concentrations greater than 1% (i.e. 0.1% in the assay) is consistent with the inhibitory effect seen in Fig. 1.

In experiments not shown 4% of the activity was lost when the dispersed cyclase was passed through a 0.22-µm pore diameter filter (Millipore) and 13% when the filtered enzyme was centrifuged at 100,000 × g for 1 hour.

The presence of dithiothreitol in the homogenizing medium affected the dispersion by detergent as shown in Fig. 4. At a concentration of 0.3 mM dithiothreitol a measurable increase in specific activity of the dispersed enzyme was observed; with an optimal increase at 3 mM dithiothreitol. This stimulatory effect of dithiothreitol was presumably due to a stabilizing effect on the enzyme during its assay (cf. Fig. 1 and below). In addition to this effect on enzyme activity dithiothreitol also increased the amount of detergent-solubilized protein (Fig. 4). Similar effects of reducing thiols on the extraction of protein from membranes have been observed by other investigators (26, 27) and have been interpreted as being due to the thiol’s facilitating the disruption of disulfide linkages within membrane proteins and thereby aiding their disruption by detergents or other agents.

The dispersion of adenylate cyclase from cerebrum and cerebellum was compared and is represented in Table II. In both cases about half the protein and most (>85%) of the total enzyme activity of the washed particles was dispersed by detergent into the 27,000 × g for 20 min supernatant fraction. The total adenylate cyclase activity in both washed particulate and in solubilized preparations of cerebellum was consistently greater than that found in cerebrum.

Stability of Detergent-dispersed Adenylate Cyclase—Adenylate cyclase, prepared and assayed in the absence of either dithiothreitol or bovine serum albumin, exhibited decreasing activity with time. The inclusion of both agents in the reaction mixture permitted the reaction to be linear for up to 20 min. The effects of bovine serum albumin and dithiothreitol were more than addi-
Some of the properties of adenylate cyclase from rat cerebellum by Lubrol-PX. The data represent the mean ± S.E. of adenylate cyclase activity determined on three to six separately homogenized and dispersed tissue preparations, as described under "Experimental Procedure," involving one cerebellum each at each concentration of dithiothreitol in the homogenizing medium tested. Each preparation was assayed for cyclase activity by duplicate incubations. The dithiothreitol concentration represents that present in the homogenizing medium. Bovine serum albumin was not present in the reaction mixture. Protein concentrations ranged from 10 to 30 μg per ml. Total activity (○-----○); specific activity (●-----●); and protein (□-----□).

Dispersed adenylate cyclase was stable to quick freezing and thawing repeated four times; after 16 times the enzyme lost about 25% of its initial activity. Neither bovine serum albumin nor dithiothreitol affected appreciably the enzyme's stability to freezing and thawing. The dispersed adenylate cyclase from brain was stable to storage for up to 3 months at -70°.

Preincubation of adenylate cyclase at 0° for 4 hours caused the loss of 30% of the initial activity. This was prevented by inclusion of bovine serum albumin and dithiothreitol in the homogenizing medium. Preincubation of the dispersed enzyme at 37° in the presence of 1% Lubrol-PX caused a rapid loss of activity (half-life approximately 6 min) followed by a slower rate of inactivation (half-life approximately 4 hours). The rate of over-all inactivation was retarded by the presence of dithiothreitol and bovine serum albumin by a factor of about 2. Rapid inactivation of dispersed adenylate cyclase at 37° was not observed in the presence of 0.1% Lubrol-PX if both bovine serum albumin and dithiothreitol were present.

Effects of Fluoride and Detergent on Gel-filtered Adenylate Cyclase—Under appropriate conditions some of the properties of particulate adenylate cyclase that were lost by detergent dispersion could be regained by gel filtration of the enzyme. Washed particulate adenylate cyclase was dispersed, filtered, and applied to a Sephadex G-200 column. In the absence of detergent the enzyme was eluted in a turbid fraction.2 Protein, turbidity (optical density at 550 nm), and adenylate cyclase activity each exhibited a peak equivalent to the column void volume. The washed particulate enzyme had an activity of 1.00 ± 0.10 n mole (5 min) and was stimulated about 2-fold by 10 mM fluoride. The dispersed enzyme had a specific activity 8 times that of the particulate enzyme but was inhibited 90% by 10 mM F-. However, the gel-filtered, "reaggregated" adenylate cyclase exhibited a specific activity (1.17 ± 0.01) comparable with that of the washed particulate preparation and was again stimulated by 10 mM fluoride about 2-fold. The response of the gel-filtered enzyme to F- was dependent on the amount of detergent used to solubilize the enzyme. At low ratios (e.g. 0.2) of detergent to protein (milligrams of detergent used to disperse milligrams of washed particulate protein), fluoride stimulated the gel-filtered enzyme, whereas at higher ratios (e.g. 1.2 to 1.6) fluoride had little or no effect on enzyme activity.

The activity of the gel-filtered adenylate cyclase was also stimulated by detergent as shown in Fig. 5. At low detergent concentrations (0.01%) the stimulatory effects of fluoride were observed, whereas at higher concentrations (0.03%) cyclase activity in the presence of both fluoride and detergent was less than that observed with detergent alone. The optimally stimulatory concentration of Lubrol-PX was 0.1% (cf., Fig. 1). Moreover, simultaneous with the increase in enzyme activity, the addition of detergent (to 0.1%) produced a rapid 60 to 70% decrease in turbidity (optical density at 550 nm) of the gel-filtered enzyme.

Effects of Cations on Detergent-dispersed Adenylate Cyclase—Inasmuch as the usually characteristic stimulation of particulate adenylate cyclase by fluoride was not observed following dispersion of the enzyme, it seemed possible that the detergent may

### TABLE II

Comparative dispersion of adenylate cyclase from cerebellum and cerebrum.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein</th>
<th>Adenylate cyclase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/g tissue</td>
<td>Control Lubrol-PX (0.1%)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>Cerebrum</td>
<td></td>
</tr>
<tr>
<td>Washed particulate</td>
<td>49.8 ± 1.3</td>
<td>1.50 ± 0.12</td>
</tr>
<tr>
<td>Lubrol-PX supernatant</td>
<td>23.3 ± 1.7</td>
<td>24.2 ± 1.7</td>
</tr>
<tr>
<td>Washed particulate</td>
<td>50.0 ± 1.0</td>
<td>1.05 ± 0.05</td>
</tr>
<tr>
<td>Lubrol-PX supernatant</td>
<td>29.6 ± 0.4</td>
<td>10.7 ± 0.4</td>
</tr>
</tbody>
</table>

* Total activity was calculated from the specific activity of washed particles assayed in the presence of 0.1% Lubrol-PX and from the control activity of the Lubrol-PX supernatant preparation.
FIG. 5. The effects of F− and Lubrol-PX on gel-filtered, “re-aggregated” adenylate cyclase. Whole cerebrums from three rats were pooled and the washed particulate enzyme was prepared as described under “Experimental Procedure” except that all centrifugations were at 12,000 x g for 10 min. The washed particulate pellet was dispersed with an equal volume of homogenizing medium containing 1% Lubrol-PX. The solubilized enzyme was filtered (0.22-μm pore, Millipore) and 1 ml of the filtrate applied to a Sephadex G-200 column (0.9 x 28 cm), equilibrated, and run with homogenizing medium which did not contain detergent. Protein concentration was 35 μg per ml. Values represent the mean ± S.E. from three incubations.

FIG. 6. The effect of Mn²⁺ and Mg²⁺ on dispersed cerebellar adenylate cyclase activity. Four cerebellums were pooled and the detergent-dispersed adenylate cyclase was prepared as described under “Experimental Procedure,” in the presence of 1 mg per ml of bovine serum albumin. The reaction mixture contained either Mn²⁺ or Mg²⁺ at the concentrations indicated. Protein concentration was 43 μg per ml. Values represent the mean ± S.E. from three incubations.

FIG. 7. The effect of Ca²⁺ on dispersed cerebellar adenylate cyclase activity. Ten cerebellums were pooled and homogenized in 9 volumes of medium containing 3 mM dithiothreitol and 0.1 M glycylglycine buffer, pH 7.5. The whole homogenate was centrifuged at 1000 x g for 10 min. The supernatant fraction was collected and sedimented at 16,000 x g for 10 min. This rehomogenization and sedimentation was repeated once. The fourth pellet was then homogenized in 9 volumes of medium containing 1% Lubrol-PX, the suspension centrifuged at 27,000 x g for 20 min, and the supernatant fraction collected. The reaction mixture contained no bovine serum albumin. The homogenizing media and the reagents used in the incubation mixture, with the exception of Mg²⁺ and Mn²⁺, had each been previously chromatographed on Chelex-100. Protein concentration was 16 μg per ml. Values represent the mean ± S.E. from three incubations.

These data are essentially consistent with observations reported by other investigators (1-5, 7-10, 12, 13) using either particulate or dispersed enzyme.

The effects of Ca²⁺ on detergent-dispersed adenylate cyclase activity are shown in Fig. 7. As has been seen by other workers (3-5, 12), in the presence of Mg²⁺ (8 mM), Ca²⁺ diminished enzyme activity by 50% at concentrations between 100 and 200 μM. However, in the presence of Mn²⁺ (8 mM), Ca²⁺ was without effect at concentrations up to 1 mM (Fig. 7), but produced a 40% decrease in activity at 10 mM (not shown). In contrast to the observations of Bradham et al. (28, 29), no concentration of Ca²⁺ tested (between 100 nM and 10 mM) had a stimulatory effect.

In another experiment (not shown) the dispersed adenylate cyclase prepared as described for Fig. 7, was incubated with 10 mM EGTA for 30 min at 0°C, then was applied to a Sephadex G-25 column (0.9 x 25 cm) equilibrated and run with medium (3 mM dithiothreitol, 1% Lubrol-PX, and 50 mM glycylglycine buffer, pH 7.5) that had also been treated with Chelex-100. This enzyme preparation was inhibited 22% by 10 μM CaCl₂ and 50% by 100 μM CaCl₂.

Effects of Chelators on Detergent-dispersed Adenylate Cyclase—At concentrations in excess of 10 μM, EGTA markedly inhibited detergent-dispersed adenylate cyclase (Fig. 8). At the protein concentration used in these experiments, the EGTA concentration exhibiting half-maximal inhibition was about 30 μM; 100 μM EGTA inhibited 90%. The inhibition of adenylate cyclase by these concentrations of EGTA was not observed when 8 mM Mn²⁺
was used instead of Mg++. Significant diminution of enzyme activity in the presence of 8 mM Mn++ was observed only at concentrations of EGTA greater than 1 mM, due presumably to the decrease in free Mn++ (cf. Fig. 6).

The effects of several other metal-binding agents on cyclase activity were determined. Cysteine, 8-OH-quinoline, 1,10-phenanthroline, ethylenediamine, inorganic pyrophosphate, CDTA, and EDTA inhibited adenylate cyclase by no more than 20% at concentrations up to 1 mM. In one experiment a solubilized cyclase preparation was incubated with Chelex-100 in an intermittently stirred slurry at 0°C for 2 hours with no demonstrable loss of enzyme activity.

The effects of several metals on EGTA-inhibited detergent-dispersed adenylate cyclase are shown in Table III. EGTA (100 μM) inhibition was completely prevented by the presence of equimolar concentrations of Ca++, Co++, and Mn++, and was partially prevented by Ni++, Sr++, Zn++, Fe++, and Cu++, even though these latter metals were to a greater or lesser degree inhibitory by themselves. In experiments not shown (in which the order of addition of divalent cation and EGTA was reversed from that shown in Table III) 100 μM Mn++, Co++, or Ca++ were added to a cyclase inhibited 90% by 100 μM EGTA and were found to restore enzyme activity completely.

The data thus far presented (Fig. 8 and Table III) suggest that EGTA inhibits adenylate cyclase either by forming a complex with (and thereby making ineffective) some metal in the reaction mixture which is required for activity or by directly interacting with a metal bound to the enzyme. It is evident from Fig. 9 that the degree of inhibition of adenylate cyclase by EGTA was dependent on the protein concentration in the reaction mixture. The inhibition of the enzyme observed at a constant concentration of EGTA (30 μM) was progressively decreased as the protein concentration was increased. In the presence of 100 μM EGTA no measurable increase in specific activity was observed until a very high protein concentration was reached (312 μg per ml). These observations would suggest that EGTA interacts directly with an enzyme-bound metal.

To test this hypothesis, attempts were made to remove the presumed metal from the enzyme preparation. In a typical experiment, dispersed adenylate cyclase was incubated with 10 mM EGTA for 30 min at 0°C and then applied to a Sephadex G-25 column (see above). The cyclase activity was initially 41.9 ± 0.8 nmole of cAMP formed (5 min)-1 (mg of protein)-1, and following treatment with EGTA and chromatography was 40.7 ± 0.9, and was again inhibited 50% by 30 μM EGTA. Similar results were observed when the enzyme was prepared and assayed with reagents previously chromatographed on Chelex-100.

In other experiments, EGTA (100 μM) was without effect on the recovery of [3H]cAMP (10 μM) added to the adenylate cyclase incubation mixture (ATP omitted) either in the presence of Mg++ or Mn++. Neither EGTA (100 μM) nor Ca++ (100 μM) had an appreciable effect on ATPase activity in these preparations when the activity was determined in the presence of either Mg++ or Mn++. The inhibition of cAMP formation by EGTA thus
Adenylate cyclase has been dispersed by detergent from particulate preparations from rat brain and exhibited relatively high specific activity and stability. The effect of fluoride on particulate adenylate cyclase was altered by the presence of detergent. Apparently fluoride exerts two effects on the enzyme, one stimulatory and one inhibitory (cf. Fig. 2). The distinction between these two actions was evidently enhanced by the presence of detergent; the stimulatory action being precluded and the inhibitory effect remaining. The mechanism by which F⁻ exerts its stimulatory effect is not known. Fluoride is a well known inhibitor of numerous enzymes (30). It is conceivable that its stimulation is the result of an inhibitory action at some other interrelated site (or sites). Others have suggested that the inhibitory effect of fluoride may be due to its ability to form a complex with Mg²⁺ (4).

The data further suggest that some of the effects of detergent on adenylate cyclase are to a degree reversible (Figs. 1, 2, and 5). Both the washed particulate enzyme and the enzyme which was dispersed by detergent and then chromatographed on Sephadex G-200 in the absence of detergent were turbid and were stimulated by F⁻ or detergent. Both preparations were also inhibited by F⁻ in the presence of detergent. The mechanisms underlying the reappearance of the stimulatory actions of both of these agents are not entirely clear.

EGTA is a chelator which has a relatively low affinity for Mg²⁺ compared with that for Ca²⁺ (31), and accordingly has often been considered to be a "calcium-specific" chelator (association constants for Mg-EGTA = 10⁻¹⁹; Ca-EGTA = 10⁻²⁶).⁴ While this is essentially only true when its binding to calcium and magnesium is compared in the absence of other metals, EGTA has nevertheless been a useful tool in establishing the Ca²⁺ dependence of some enzymes (25, 32). EGTA has been observed to enhance basal activity of particulate adenylate cyclase prepared from fat (33), cardiac tissue (5), and partially purified membranes from liver; yet it partially inhibited the enzyme from brain (28, 29).⁵ The mechanism of the inhibitory effect of EGTA is not understood but it appears to be quite specific for this chelator, even though the affinities for metals of some of these metal-binding agents are comparable to those of EGTA (31, 34). Perhaps the most significant distinction between EDTA, for example, and EGTA, with respect to their metal-ligand affinities, is that the association constant for Mg-EDTA (10⁶.⁸⁹ (Ref. 31)) is about three orders of magnitude greater than that for Mg-EGTA. Disregarding the binding of Mg²⁺ by ATP, which would only decrease Mg²⁺ to 4 mM, the concentration of uncomplexed EGTA with an initial concentration of 100 µM in the presence of 8 mM Mg²⁺ would be about 8 × 10⁻⁸ M. Under similar conditions the concentration of free EDTA would be about 3 × 10⁻¹¹ M. But with 8 mM Mn²⁺ and 100 µM EDTA (Kₐ₉₉ (Mn EDTA) = 10¹³.² (Ref. 31)) the concentration of free EDTA would be less than 10⁻¹⁵ M and therefore may account for the lack of effect of EGTA in the presence of this cation.

The partial inhibition by EGTA of particulate adenylate cyclase from brain observed by Bradham et al. (28, 29) and by Perkins and Moore⁶ was reversed by the addition of Ca²⁺ and led to the suggestion that there may be a direct role for Ca²⁺ in modulating cyclase activity (28). While this is an intriguing idea in view of the apparent involvement of Ca²⁺ in cellular processes (35), the data presented in this communication would suggest that Mn²⁺, Co²⁺, or other metals may also be candidates for a role in the control of adenylate cyclase. Because of the high metal-EGTA stability constants, any of the metals shown in Table III would significantly reduce the concentration of free EGTA and therefore presumably account for their ability to prevent or to reverse the inhibition by EGTA. Moreover, the lack of stimulatory effect of Ca²⁺, under normal assay conditions and also in preparations which had been treated with EGTA and gel filtration to remove contaminating and loosely bound metals, would suggest that added Ca²⁺ exerts only an inhibitory influence on the brain adenylate cyclase. It is possible that very tightly bound Ca²⁺ may be involved in modulating the enzyme's activity. Particulate (28) and dispersed⁷ preparations from brain contain relatively large amounts of bound Ca²⁺.

The complete reversal of the inhibitory effect of EGTA on adenylate cyclase by gel filtration of the EGTA-inhibited enzyme would indicate that the factor (or factors) with which the chelator interacts is not readily removed from the preparation. By itself, the fact that increasing protein concentration reversed the inhibitory effect of EGTA does not completely rule out the possibility that the chelator inhibits by interacting with a contaminating metal in the reaction mixture. It is possible that Chelex-100 treatment of reagents does not satisfactorily remove the contaminating metal and at higher protein concentrations the metal is bound to the protein with an affinity greater than that for EGTA. However, it would be expected that some of the other metal-binding agents tested which have low affinity for Mg²⁺ and high affinity for other metals would also have exerted some degree of inhibition at lower concentrations.

The evidence presented here suggests that a metal in addition to added Mg²⁺ is essential for full expression of brain adenylate cyclase activity. It remains possible that EGTA inhibits adenylate cyclase in some manner independent of its metal-binding properties. In either event, EGTA may prove to be a useful tool, in conjunction with detergent dispersion, in evaluating the factors that modulate adenylate cyclase activity as well as in the eventual purification of the enzyme.

Acknowledgments—We are grateful to Ms. Janette Welden and Mr. Joseph Campbell for their excellent technical assistance.

REFERENCES

7. A. Johnson and E. W. Sutherland, unpublished observations.