Solubilization, Stabilization, and Partial Purification of Brain Adenylate Cyclase from Rat†

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ABSTRACT: Membrane-bound adenylate cyclase from rat brain has been solubilized with Lubrol-PX. The soluble enzyme is nonsedimentable at 165,000*g* for 2 hr, is filterable through 0.22 μ Millipore filters and is responsive to F^-. The solubilized adenylate cyclase has been stabilized for at least 7 days by the addition of 5 mM NaF and 5 mM $MgSO_4$. The enzyme has an estimated molecular weight of 800,000 which was obtained on the basis of a calibrated Bio-Gel A-15 m (agarose) column. The enzyme has been purified 18-fold representing the removal of over 99% of the starting protein. Addition of fractions removed during purification did not increase enzyme activity. The partially purified adenylate cyclase has ATPase and adenosine 3′,5′-cyclic monophosphate phosphodiesterase activities associated with it. The material obtained after ion exchange chromatography and gel filtration has low mobility on electrophoresis in acrylamide gels. Electrophoresis of this material in 1% sodium dodecyl sulfate, which results in disaggregation of the protein and total loss of catalytic activity indicates that at least eight components are present in the active enzyme preparation. Whether these enzymes were present together in the native state as a regulatory unit at the membrane or whether they have associated during the course of purification remains to be determined, particularly since solubilized guinea pig brain Na–K-ATPase is reported to have a molecular weight of 775,000.

Adenylate cyclase catalyzes the conversion of adenosine triphosphate to cAMP,[1] and plays a pivotal role in mediating the effects of a wide variety of hormones (Sutherland *et al.*, 1965). The physiological importance of adenylate cyclase has long been recognized and its properties as a particulate, hormone-responsive membrane-associated enzyme have been extensively described and are the subject of investigation (Bitensky *et al.*, 1968; Chase and Aurbach, 1968; Pohl *et al.*, 1971).

Extensive purification of adenylate cyclase has been hampered by its resistance to solubilization and its lability during storage and processing. Levey's (1970) success in solubilizing myocardial adenylate cyclase with Lubrol-PX, a nonionic detergent, stimulated our efforts to solubilize, stabilize, and purify adenylate cyclase from the brain, a tissue rich in adenylate cyclase activity. A portion of these studies has been presented in abstract form (Swislocki, 1972).

Methods

Adenylate cyclase activity was measured by the method of Krishna *et al.* (1968). Incubations were carried out at 30° for 15 min in 0.04 M Tris-HCl (pH 7.4), containing 1 mM ATP, 1 mM $MgCl_2$, and 10 mM theophylline. Each reaction contained 2–3 μCi of [α-^{32}P]ATP obtained from International Chemical and Nuclear Corp. After incubation 0.5 mg of cAMP was added to each tube and reactions were terminated by boiling for 3 min. [α-^{32}P]cAMP was purified by Dowex 50 (H^+) chromatography and $ZnSO_4$–$Ba(OH)_2$ precipitation (Krishna *et al.*, 1968). Recovery of labeled cAMP was followed spectrophotometrically at 260 nm of added cAMP.

Tissues of the rat were removed after decapitation and immediately homogenized in 0.2 M Tris-HCl (pH 7.4), containing 0.25 M sucrose and 1 mM EDTA–$MgCl_2$ (Levey, 1970). Lubrol-PX was added to the buffer, as will be indicated, to solubilize the enzyme. Protein was determined either by absorbance at 280 nm or by the method of Lowry *et al.* (1951). The detergent formed a precipitate during color development in the latter procedure which did not interfere if removed by centrifugation before spectrophotometry. Phospholipid analysis was by the method of Skipski and Barclay (1969). Acrylamide gel electrophoresis was performed by the method of Davis (1964) and electrophoresis in sodium dodecyl sulfate by the technique of Lenard (1970).

Lubrol-PX was a gift of Imperial Chemical Industries. The ^{14}C-labeled detergent was generously provided by Dr. Gerald S. Levey. Ficoll and Blue Dextran were obtained from Pharmacia, Bio-Gel A-15m (agarose) from Bio-Rad Labs and DE-52 (diethylaminoethylcellulose) from Whatman. Beef heart cAMP phosphodiesterase, thyroglobulin, and pyruvate kinase were obtained from Sigma. Nucleotides were from P-L Laboratories. All other chemicals were also reagent grade and were obtained from various commercial sources.

Results

Several tissues of the rat were examined for Lubrol-solubilized adenylate cyclase activity. As seen in Table I, brain yielded the largest amount of solubilized enzyme. The concentration of adenylate cyclase in brain was 13 times that in heart and 27 times that in liver. The enzyme in all of these tissues could be readily solubilized at 0.02, 0.1, and 0.2 M Lubrol. On the basis of the data in Table II, 0.1 and 0.2 M Lubrol gave a higher yield of solubilized brain enzyme as judged by specific activity of the supernate of the 105,000*g* and 165,000*g* fractions. Thereafter 0.1 M Lubrol was used to solubilize the enzyme. Stirring the enzyme for up to 6 hr in several Lubrol concentrations did not increase the degree of solubilization as judged by activity remaining in the 165,000*g* supernatant.

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[1] Abbreviation used is: cAMP, adenosine 3′,5′-cyclic monophosphate. Lubrol-PX is an ethylene oxide condensate of dodecanol.

TABLE I: Distribution and Solubilization of Adenylate Cyclase in Brain, Liver, and Heart of the Rat.[a]

	Adenylate Cyclase Activity		
	Homogenate	105,000*g* Supernatant	% Solubilized
Brain	11.26[b]	6.49	58
Liver	0.42	0.27	64
Heart	0.85	0.61	72

[a] Tissue of the rat, 1 g/10 ml of buffer, was homogenized in 0.2 M Tris-HCl (pH 7.4), 1 mM EDTA, 0.25 M sucrose, 0.02 M Lubrol-PX, and 1 mM dithiothreitol, and centrifuged at 10,000*g* for 15 min. The supernatant therefrom was recentrifuged at 105,000*g* for 2 hr and assayed for enzyme activity. [b] All values in nmol of cAMP/mg of protein per 15 min.

The fate of Lubrol was examined by following the efflux of ^{14}C-labeled Lubrol during dialysis. Half of the labeled Lubrol was removed by overnight dialysis against two changes of 500 volumes each of buffer. The remainder of the detergent was removed by ion-exchange chromatography during subsequent purification of the enzyme.

The Lubrol-solubilized brain enzyme was compared to the particulate enzyme for sensitivity to F^- and Ca^{2+}. Solubilization does not alter the sensitivity of brain cyclase to F^- stimulation (Table III). Both the particulate enzyme isolated without detergent as a 10,000*g* pellet (McCune *et al.*, 1971) and the solubilized enzyme in the 105,000*g* supernatant can be stimulated with 2 mM NaF. The qualitative response to Ca^{2+}, however, is modified. Ca^{2+} stimulated the enzyme in the particulate state but inhibited it after solubilization.

To evaluate the degree of solubilization the 105,000*g* supernatant was filtered through 0.22 μ Millipore filters. All of the enzyme activity was found in the filtrate, further demonstrating that the adenylate cyclase was removed from its resident site in the membrane.

The solubilized enzyme was subjected to gel filtration on Bio-Gel A-15m on a 1.4 × 90 cm column in 0.1 M Tris-HCl

TABLE II: Effect of Lubrol-PX Concentration on Brain Adenylate Cyclase Solubilization.[a]

	Lubrol-PX		
	0.02 M	0.1 M	0.2 M
Homogenate	10.8[b]	8.4	8.6
10,000*g* supernatant	10.5	8.6	8.6
10,000*g* pellet	15.2	3.8	10.6
105,000*g* supernatant	2.7	10.6	9.4
105,000*g* pellet	26.5	5.0	2.3
165,000*g* supernatant	1.8	11.7	10.6
165,000*g* pellet	19.2	9.0	11.0

[a] Brain was homogenized in the buffer described in Table I containing, in addition, Lubrol-PX, as indicated; dithiothreitol was omitted. [b] nmol of cAMP/mg of protein per 15 min.

TABLE III: Effects of Fluoride and Calcium on Particulate and Soluble Adenylate Cyclase from Rat Brain.

	Adenylate Cyclase Act. (nmol of cAMP/mg of Protein per 15 min)	
Membranes[a]	0.418 ± 0.051[b]	
Plus 2 mM NaF	0.610 ± 0.035	<0.05[c]
Plus 0.025 mM $CaCl_2$	1.226 ± 0.155	<0.01
105,000*g* supernatant	2.168 ± 0.03	
Plus 2 mM NaF	2.809 ± 0.075	<0.001
Plus 0.025 mM $CaCl_2$	1.534 ± 0.0014	<0.001

[a] Membranes from brain were prepared by the methods of McCune *et al.* (1971) and employed as obtained in a 10,000*g* pellet which was resuspended in 50 mM Tris-HCl (pH 7.5). Samples of 76 μg of protein were used for assay. The 105,000*g* supernatant of solubilized enzyme was obtained as described exept that NaF and $MgSO_4$ were omitted from the homogenizing buffer. Adenylate cyclase in the supernatant was assayed on 90 μg of protein sample. [b] Mean ± SE based on triplicate determinations. [c] *p* value.

TABLE IV: Stabilization of Solubilized Adenylate Cyclase with NaF and $MgSO_4$.[a]

165,000*g* Supernatant	Additions	Adenylate Cyclase Act. (nmol of cAMP/mg of Protein per 15 min)
Fresh	None	1.440 ± 0.077[b]
Fresh	5 mM NaF and 5 mM $MgSO_4$	5.633 ± 0.254
Stored 4°, 24 hr	None	0.288 ± 0.005
Stored 4°, 24 hr	5 mM NaF and 5 mM $MgSO_4$	1.684 ± 0.019
Stored 4°, 24 hr in 5 mM NaF and 5 mM $MgSO_4$	None	5.225 ± 0.148
Dialyzed 24 hr *vs.* 0.1 M Tris-HCl (pH 7.5)	5 mM NaF and 5 mM $MgSO_4$	6.647 ± 0.074
Brought to 5 mM NaF and 5 mM $MgSO_4$; dialyzed *vs.* 0.1 M Tris-HCl (pH 7.5) containing 5 mM NaF and 5 mM $MgSO_4$	None	6.730 ± 0.039
Brought to 5 mM NaF and 5 mM $MgSO_4$ and dialyzed *vs.* 0.1 M Tris-HCl (pH 7.5)	None	1.084 ± 0.033
As above	5 mM NaF and 5 mM $MgSO_4$	3.560 ± 0.079

[a] The solubilized adenylate cyclase was prepared in the homogenizing buffer in 0.1 M Lubrol-PX. Samples containing 60–70 μg of protein were assayed as indicated. Additions of NaF and $MgSO_4$, when made, were just prior to assay. [b] Mean ± SE of triplicate determinations.

TABLE V: Stability of Solubilized Adenylate Cyclase during Storage and Purification.[a]

	Day 1	Day 2	Day 5
Homogenate	2.43[b]		
17,000*g* supernatant	4.09		
165,000*g* supernatant	5.33		
Dialyzed 165,000*g* supernatant		5.00	
Dialyzed 165,000*g* supernatant recentrifuged at 165,000*g*	6.14	6.09	5.05
DE-52 pool	7.51	6.34	
DE-52 concentrate		5.49	

[a] Adenylate cyclase was prepared in the homogenizing buffer with 0.1 M Lubrol-PX to which were added 5 mM NaF and 5 mM $MgSO_4$. The enzyme was kept at 4° during a 7-day period from homogenization of tissue to the assay of the DE-52 concentrate. [b] nmol of cAMP/mg of protein per 15 min.

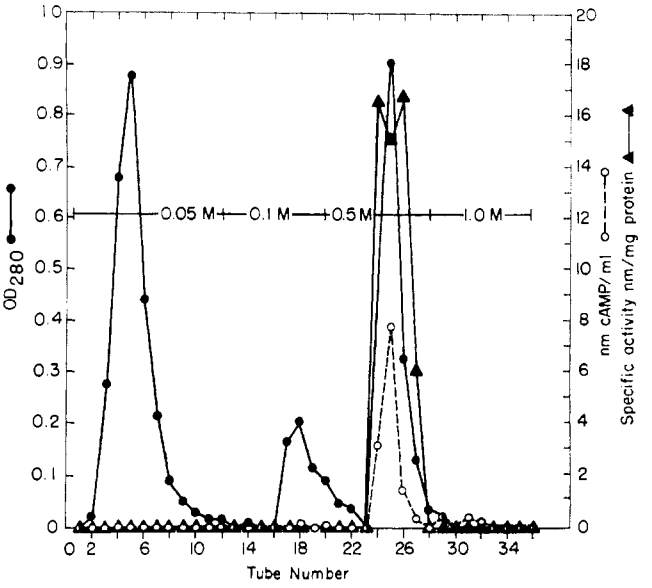

FIGURE 1: Ion-exchange chromatography of solubilized adenylate cyclase. The 165,000*g* supernatant was dialyzed against 50 mM Tris-HCl (pH 7.5), containing 5 mM NaF and 5 mM $MgSO_4$ and 30 ml of the enzyme containing 1.3 g of protein was applied to a 1.3 × 30 cm column of DE-52 equilibrated with the buffer. The enzyme was eluted with 0.05, 0.1, 0.5, and 1.0 M Tris-HCl (pH 7.5) in 5-ml fractions: (●) OD_{280}; (○) nmol of cAMP/ml; (▲) nmol of cAMP/mg of protein.

(pH 7.5) containing 5 mM NaF and 5 mM $MgSO_4$, and 2-ml fractions were collected. The adenylate cyclase activity appeared in tube 14, after Blue Dextran was eluted in tube 8. Particulate matter also preceded adenylate cyclase. The remainder of the solubilized protein appeared in more retarded fractions.

Stability and Sensitivity. As seen in Table IV the enzyme activity of the 165,000*g* supernate was increased fourfold in F^- and Mg^{2+}. Overnight storage of the enzyme without F^- and Mg^{2+} led to an 80% loss of activity which could be recovered in part by addition of F^- and Mg^{2+}. Activity was maintained whether the enzyme was stored in F^- and Mg^{2+} or dialyzed against F^- and Mg^{2+} overnight. Removal of F^- and Mg^{2+} by dialysis led to loss of activity which could be restored in part by addition of the ions.

The enzyme retains 80% of its activity over a 5-day period when stored in 5 mM F^- and Mg^{2+} (Table V). Reassays of several fractions demonstrate that the dialyzed, recentrifuged 165,000*g* supernatant and the 0.2 M peak from DE-52 (see below) ion-exchange chromatography retained most of their activity over this time period when stored at 4°. In the presence of 5 mM F^- and Mg^{2+} the solubilized enzyme was protected from losses of activity by freeze-thawing.

Ion-Exchange Chromatography and Gel Filtration. Adenylate cyclase was subjected to ion-exchange chromatography on DEAE-cellulose. Gradient elution with 0.1–0.5 M Tris (pH 7.4) at five times the column volume resulted in the spreading of the enzyme activity over the entire elution profile. Stepwise elution, however, provided a method of separation of several active components. As seen in Figure 1, the activity appears in the 0.5 M Tris-HCl fraction. Two prior peaks, eluted with 0.05 and 0.1 M Tris buffer did not contain any enzyme activity, nor was any activity present in the region eluted with 1.0 M Tris. Elution with a stepwide schedule at concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 M Tris-HCl buffer yielded activity in all but the 0.4 M fraction. Adenylate cyclase was present in the 0.2 and 0.3 M peaks with the highest specific activity located in the 0.2 M peak.

Further purification was achieved by gel filtration. The 0.2 M peak from the DEAE chromatography was concentrated with Ficoll. Up to a 20-fold reduction in volume could be achieved without significant loss of enzyme activity. Further reduction in volume resulted in reaggregation and formation of insoluble components concomitant with losses in adenylate cyclase activity.

The material concentrated with Ficoll was applied to a Bio-Gel A-15m column which was calibrated with Blue Dextran, thyroglobulin, and pyruvate kinase, which have, respectively, molecular weights of 2,000,000, 669,000, and 237,000. A plot of log molecular weight *vs.* elution volume yielded a linear slope from which the molecular weight of the solubilized adenylate cyclase could be estimated. Adenylate cyclase activity appeared in tubes 14–15; the enzyme has an apparent molecular weight of 800,000.

The following enzyme purification scheme was adopted and is based on the studies which were carried out to determine optimal conditions for yield and stability of enzyme. Rat brain (10 g/100 ml) was homogenized in 0.2 M Tris-HCl (pH 7.4), containing 0.25 M sucrose, 1 mM EDTA-$MgCl_2$, 5 mM NaF, 5 mM $MgSO_4$, and Lubrol-PX (0.1 M). The homogenate was centrifuged at 27,000*g* for 10 min. Most of the activity was in the supernatant and the pellet was discarded. The supernate was recentrifuged at 165,000*g* for 2 hr and the nonsedimentable fraction was dialyzed overnight against two changes of 500 volumes each of 50 mM Tris-HCl (pH 7.4), containing 5 mM NaF and $MgSO_4$. The F^- and Mg^{2+} were added, unless specified otherwise, to all buffers to stabilize the enzyme (see Table IV). The dialyzed nonsedimentable fraction was recentrifuged at 165,000*g* for 2 hr to remove opalescent, inactive material which had formed during dialysis.

The yield of supernatant from 10 g of brain, approximately 90–95 ml containing 3.5–4 g of protein, was applied to a 2.5 × 40 cm column for ion-exchange chromatography on DEAE (Whatman DE-52). After washing out unadsorbed material with 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM F^- and Mg^{2+} the column was developed with 0.1, 0.2, 0.3, 0.4, and 0.5 M buffer. The 0.2 M peak and the 0.3 M peak, which

contained enzyme activity were concentrated with Ficoll. Further purification of the 0.2 M peak was achieved by gel filtration on Bio-Gel A-15m columns (1.4 × 90 cm) of 2.0-ml portions of the DE-52 concentrates. The columns were eluted with 0.1 M Tris-HCl buffer and 2-ml fractions collected. Adenylate cyclase appeared in tubes 13–14 and was utilized for further characterization.

The enzyme obtained from Bio-Gel A-15m was examined for the specificity of the adenylate cyclase reaction by incubating the enzyme as in the assay procedure and purifying the reactants on Dowex 50 (H^+). The $BaSO_4$ precipitation step was omitted. The cAMP peak from the Dowex 50 (H^+) column was subjected to descending paper chromatography in ethanol–ammonium acetate (7:3) (Swislocki, 1970). The cAMP spot was eluted and half the material incubated with purified beef heart cAMP phosphodiesterase (Butcher and Sutherland, 1962) for 10 min at 30° in 0.05 M Tris-HCl (pH 7.4) containing 1 mM $MgSO_4$. The reactants were chromatographed and the radioactivity in the paper strips detected at 1-cm intervals by liquid scintillation counting. Results indicated that the putative cAMP produced by partially purified adenylate cyclase was converted to 5′AMP by added phosphodiesterase.

The enzyme present in the peak tube of the Bio-Gel column was examined for sensitivity to F^- and Ca^{2+}; 5 mM F^- increased activity of the enzyme by 40% while 0.025 mM $CaCl_2$ was without effect.

Acrylamide gel electrophoresis indicates that the enzyme is associated with a fraction that has low mobility in 7.5% gels (Figure 2). The enzyme was, however, free of the electrophoretic species of protein that were retarded by the Bio-Gel. Electrophoresis of the same fractions in 1% sodium dodecyl sulfate, which resulted in disaggregation of the protein and total loss of catalytic activity indicates that at least eight components are present in the active enzyme preparation. It may very well be that the aggregation occured during lyophilization which was done to concentrate the protein for the purpose of electrophoresis.

To determine whether some of the proteins associated with the adenylate cyclase had catalytic activity to adenosine phosphates the adenylate cyclase fraction was qualitatively examined by incubating the enzyme fractions with ^{14}C-labeled ATP, ADP, 5′AMP, and cAMP. Analysis of chromatographed reactants indicated that enzymatic activity other than adenylate cyclase was present. cAMP phosphodiesterase is present as added cAMP was converted to 5′AMP. Added ATP was converted to ADP. On this basis at least ATPase and cAMP phosphodiesterase are associated with the adenylate cyclase fraction.

Phospholipids in the adenylate cyclase fraction obtained from gel filtration on Bio-Gel were characterized by thin-layer chromatography (Skipski and Barclay, 1969) of chloroform–ethanol (3:1) extracts of the enzyme. Phosphatidylcholine was identified as the most predominant phospholipid present.

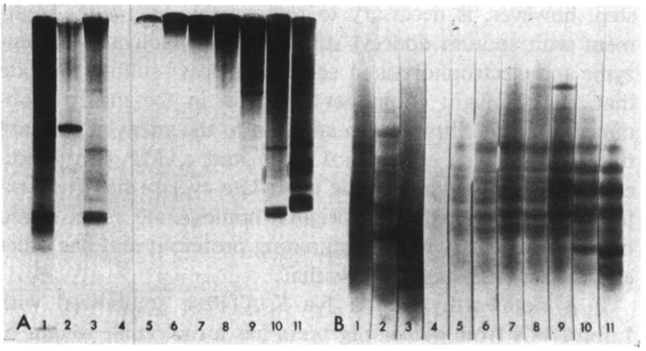

FIGURE 2: Acrylamide gel electrophoresis of solubilized adenylate cyclase, without sodium dodecyl sulfate (A) and with sodium dodecyl sulfate (B). Gels 1–11 in each of the two figures are as follows; 1, 165,000*g* supernatant; 2 and 3, respectively, are the peaks eluted with 0.2 and 0.3 M Tris buffer from DE-52 chromatography; 4, the void volume from Bio-Gel gel filtration; 5, fraction 9 of the Bio-Gel; 6, 7, and 8 correspond to Bio-Gel fractions 13, 14, and 15 and which contain adenylate cyclase activity. Gels 9, 10, and 11 are the more retarded portions of the gel filtration eluates which correspond to Bio-Gel fractions 17, 19, and 21. Electrophoresis without sodium dodecyl sulfate was performed on samples that were dialyzed against 1 mM Tris-HCl (pH 7.4), lyophilized, and then dissolved with water to contain 100–150 μg of protein/sample. Electrophoresis with sodium dodecyl sulfate was performed on samples dialyzed against 1 mM potassium phosphate (pH 7.4), containing 0.1 mM EDTA and 1 mM mercaptoethanol (Lenard, 1970). After lyophilization the samples were dissolved in 10 mM potassium phosphate buffer containing 10% sodium dodecyl sulfate and heated in a boiling-water bath for 3 min. Samples of 100–150 μg of protein were applied to each gel.

Discussion

The present study shows that rat brain adenylate cyclase can be solubilized by 0.1 M Lubrol-PX, a nonionic detergent. The solubilized material is nonsedimentable at 165,000*g* for 2 hr, is filterable through 0.22 μ Millipore filters, and is sensitive to F^-. These findings confirm some of the observations of Levey (1970) who obtained a soluble, nonsedimentable, filterable, F^--sensitive, glucagon-unresponsive adenylate cyclase from cat myocardium after solubilization with Lubrol-PX.

Our molecular weight estimate, based on a calibrated gel filtration column (Bio-Gel A-15m) indicated that the rat brain enzyme has a molecular weight of approximately 800,000. These findings are in contrast to the estimate of 100,000–200,000 reported by Levey (1970) for the cat heart enzyme which appeared in the void volume after Blue Dextran on Sephadex G-200 gel filtration.

Initial efforts at purification were hampered by the lability of the solubilized brain enzyme to storage and processing at 4°. We found that 5 mM NaF and $MgSO_4$ stabilized the solubilized brain adenylate cyclase. Under these conditions the solubilized enzyme retains 80% of its activity for at least 7 days, sufficient for a number of purification and characterization procedures. Lin (1971) reported that bovine brain cortex treated with 2% digitonin had a half-life of 6 weeks when both 10 mM NaF and 5 mM dithiothreitol were included in the extract; most of the adenylate cyclase activity was retained after dialysis to remove the NaF. In our hands when NaF and $MgSO_4$ were omitted or removed by dialysis the Lubrol solubilized brain adenylate cyclase lost activity (Table IV).

Quantitative data on the degree of purification as measured by increase in specific activity of the enzyme do not by themselves indicate as high a degree of purification as might be anticipated considering the removal of over 99% of protein. The material obtained after gel filtration exhibits an 18-fold increase in specific activity over that found for the enzyme in brain homogenates. Levey (1971) has obtained a twofold increase in cat heart adenylate cyclase specific activity associated with a 50–75% loss of total activity after ion-exchange chromatography. Similar losses of activity occur after ion-exchange chromatography of the brain adenylate cyclase. This

step, however, is necessary to remove the detergent. Treatment with sodium dodecyl sulfate which inactivates the enzyme and electrophoresis in sodium dodecyl sulfate indicate that at least eight components remain in the material obtained after gel filtration. In addition to the adenylate cyclase this fraction also contains ATPase and cAMP phosphodiesterase activity. Thus, while adenylate cyclase has been extensively purified over the original homogenate as measured by relative paucity of contaminating protein it still has other enzyme activities associated with it.

It is noteworthy that a Na–K-ATPase solubilized with Lubrol-WX from guinea pig brain has a molecular weight of 775,000 (Medzihradsky *et al.*, 1967). Whether adenylate cyclase and ATPase exist in association in the native state or whether they have aggregated in the course of purification is of interest and remains to be determined.

It is possible that the low specific activity of the enzyme as seen throughout the purification procedure is due to removal or denaturation of essential components. It has been reported that phospholipids are required for adenylate cyclase activity (Levey, 1971). Indeed, the addition of phosphatidylserine to solubilized cat heart adenylate cyclase restores glucagon responsiveness (Levey, 1971). We can increase, in part, the specific activity of the rat brain enzyme by addition of phosphatidylserine (N. I. Swislocki, unpublished observations). It is not clear at this juncture how other phospholipids and other membrane components stabilize the enzyme or render it responsive to hormones. In our hands addition of various fractions that have been removed from adenylate cyclase during purification has not restored or increased brain adenylate cyclase activity.

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