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Bovine Adrenocortical Adenylate Cyclase: Some Properties of the Solubilized, Fluoride-Activated Enzyme[1]

YOSHIO USHIJIMA, FRANCES M. FINN, AND KLAUS HOFMANN[2]

Protein Research Laboratory, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

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The activity of bovine adrenocortical plasma membrane adenylate cyclase can be maintained at 4°C in the presence of NaF. The half-life of the fluoride-stabilized enzyme is approximately 7 days. Maximal activation by fluoride requires approximately 20 min at 0°C and the level of activity attained is dependent on fluoride concentration. The enzyme from freshly harvested membranes can also be stimulated by $ACTH_{1-24}$ and Gpp(NH)p and the stimulatory effects of these two activators are additive. Prolonged exposure to either NaF or Gpp(NH)p precludes hormone activation. Optimal concentration for Gpp(NH)p activation is 10^{-4}–10^{-5} *M*. Treatment of the enzyme with a Tris–HCl buffer containing Lubrol-PX (1%), NaF, dithiothreitol, and $MgSO_4$ followed by sonication affords a preparation that does not sediment at 100 000 *g* in 1 hr. This material has a low specific activity; however, removal of the detergent on DEAE cellulose restores specific activity to its original level. A significant improvement in specific activity is observed following dialysis or ultrafiltration of the detergent-free 100 000-*g* supernatant. At this stage the enzyme can be lyophilized and stored at −70°C without loss of activity. The enzyme in the detergent-free 100 000-*g* supernatant behaves as a single peak that is included in Sepharose 6B. Comparison of the elution profile of the enzyme with profiles produced by a standard set of proteins suggests a molecular weight of 1×10^6. Hydrophobic chromatography of the detergent-free 100 000-*g* supernatant on *n*-hexyl Sepharose 4B results in a fivefold enhancement of specific activity.

INTRODUCTION

Adenylate cyclases are membrane-bound enzymes widely distributed in nature. Of particular significance is the observation that a number of polypeptide hormones activate the adenylate cyclase of specific target cells and that this activation appears to be an early step in the sequence of events through which these hormones regulate cellular metabolism. Hormone-sensitive adenylate cyclases are also activated by such diverse materials as fluoride ion, cholera toxin (*1*), and Gpp(NH)p[3] (*2*); however, the mechanisms through which these various agents activate the cyclase remain to be elucidated.

This paper is dedicated to the memory of S. Morris Kupchan, a dear friend and colleague in whose company I was privileged to spend many enjoyable hours.

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[2] Author to whom correspondence should be addressed.

[3] The abbreviations used are: ACTH = adrenocorticotropic hormone; $ACTH_{1-24}$ = a fully active synthetic peptide corresponding to the amino terminal 24 amino acids of ACTH; cAMP = 3′,5′-cyclic adenosine monophosphate; DTT = dithiothreitol; Gpp(NH)p = 5′-guanylylimidodiphosphate.

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Little progress has been achieved in the isolation of pure mammalian adenylate cyclases in the 14 years since their discovery. The subcellular location of the enzymes in the highly hydrophobic environment of the plasma membrane has hampered efforts to obtain a soluble preparation and, more importantly, gains in specific activity attained through purification are often offset by losses in enzyme activity due to the lability of the cyclase.

In the present study we describe a preparation of solubilized, detergent-free fluoride-activated bovine adrenocortical adenylate cyclase that can be stored in lyophilized form at −70°C without loss of activity. Having the capability to accumulate a reservoir of stable, solubilized enzyme to use as starting material for further purification steps is of value in isolation studies.

MATERIALS AND METHODS

Pyruvate phosphokinase (rabbit skeletal muscle, type II), *P*-enolpyruvate (trisodium salt, hydrate), DTT, thyroglobulin, hemoglobin, catalase, and sodium lauryl sulfate were purchased from Sigma. Cyclic AMP and [^{3}H] ATP were obtained from Schwarz–Mann, Lubrol-PX from Savco, DEAE cellulose (DE52) from Whatman, Gpp(NH)p from P-L Biochemicals, PM10 ultrafiltration membranes from Amicon Corporation, theophylline from Nutritional Biochemicals, and Sepharose 4B and 6B and blue dextran from Pharmacia Fine Chemicals. $ACTH_{1-24}$ was a gift from Dr. W. Rittel of Ciba–Geigy Corporation, Basel, Switzerland. Medium A consists of Tris–HCl, pH 7.3 (50 m*M*), containing $MgSO_4$ (5 m*M*), Na^+ (5 m*M*) and DTT (0.2 m*M*); Medium B is Tris–HCl, pH 7.3 (0.5 *M*), containing $MgSO_4$ (5 m*M*), NaF (5 m*M*), and DTT (0.2 m*M*).

Bovine adrenocortical plasma membranes were prepared as described previously (*3*). Cyclic AMP was determined by a modification (*3*) of the method of Krishna et al. (*4*). Adenylate cyclase was determined essentially as described (*5*). The assay medium contained DTT (9.8 m*M*), [^{3}H] ATP (0.4 m*M*), Tris–HCl, pH 7.3 (30 m*M*), $MgSO_4$ (2.4 m*M*), theophylline (7 m*M*), pyruvate kinase (0.01 ml), and *P*-enolpyruvate (26 m*M*). Sodium fluoride, when used, was present at a concentration of 10 m*M*. Adenylate cyclase solutions containing 0.1 to 0.4 mg of protein in various buffers and detergents were added as indicated. Final volume of the assay solution was 0.82 ml. Specific activity denotes picomoles of cyclic AMP per milligram of protein per 15 min at 30°C. Protein was determined according to the method of Lowry et al. (*6*). Protein was precipitated from soluble samples or samples containing detergent by adding trichloroacetic acid to a final concentration of 8%. The precipitate was washed with 9 ml of 5% trichloroacetic acid. It was frequently necessary to centrifuge detergent-containing samples after the addition of the Folin–Ciocaltau reagent. For estimation of protein, 0.1 *N* sodium hydroxide containing sodium lauryl sulfate (0.75 mg/ml) was substituted for 0.1 *N* sodium hydroxide. Standards used to calibrate the Sepharose 6B column were assayed as follows: Blue dextran, thyroglobulin, and hemoglobin were detected by their absorbancies at 620, 280, and 418 nm, respectively; catalase was measured by the method of Beers (*7*).

Preparation of Soluble, Detergent-free Adenylate Cyclase

Unless otherwise noted, all operations were performed at 4°C. Plasma membranes (2 mg of protein/ml) stored frozen at −70°C in Tris–HCl, pH 7.3 (0.2 *M*), containing NaF (10 m*M*), $MgSO_4$ (10 m*M*), and DTT (2 m*M*) were thawed at 37°C. To the suspension an equal volume of a 2% aqueous solution of Lubrol was added. The diluted suspension was stirred for 15 min, and 30-ml aliquots were sonicated for 1 min at an ice-bath temperature at a setting of 30 on an Artek Sonic Dismembrator 300 using the small tip. The sonicate was centrifuged at 100 000 *g* for 1 hr (100 000-*g* supernatant). To the supernatant were added 3 vol of water and 1 vol of DEAE cellulose suspension (68.5 mg of DEAE cellulose/ml of 10 m*M* Tris–HCl buffer, pH 7.3). The suspension was stirred for 15 min and filtered *in vacuo*. The DEAE cellulose was resuspended in Medium A, a volume equivalent to four times that of the original supernatant, the suspension was stirred for an additional 5 min and filtered, and the filter cake was washed with 1 vol of Medium A. This process was repeated. The filter cake was resuspended in 2 vol of Medium A, and the suspension was centrifuged in a Sorvall RC2-B by decelerating the rotor once it had reached 10 800 *g*. The supernatant was removed by aspiration and discarded, and the DEAE cellulose was eluted with three portions of Medium B (each portion equivalent to one-third of the volume of the original supernatant). The suspensions were stirred at 4°C for 5 min and centrifuged as before. The combined supernatants were filtered to remove any remaining DEAE cellulose particles (DEAE eluates), and the filtrate was dialyzed overnight against 2 × 15 vol of Medium A (dialyzed enzyme). The volume of the dialyzed solution was reduced to 10% by filtration through a Diaflo ultrafiltration apparatus using a PM10 membrane (concentrate). The concentrate was centrifuged for 1 hr at 100 000 *g* (detergent-free 100 000-*g* supernatant). Specific activities and yields at various steps throughout a typical preparation are summarized in Table 1. Columns for hydrophobic chromatography were prepared according to the method of Shaltiel (*8*). The resins were stored at 4°C in 0.5% sodium azide and washed exhaustively with Medium A before use.

TABLE 1

YIELDS AND ACTIVITIES OF VARIOUS FRACTIONS FROM A TYPICAL PREPARATION OF DETERGENT-FREE SOLUBLE ADENYLATE CYCLASE[a]

	Protein		Adenylate cyclase activity		
Preparation	Total (mg)	Recovery (%)	Total	Recovery (%)	Specific activity
Plasma membranes	279	100	711 000	100	2548
DEAE eluates	53	19	197 000	28	3716
Dialyzed enzyme	49	18	287 000	40	5857
Concentrate	46	17	242 000	34	5260
Detergent-Free 100 000-*g* supernatant	30	11	145 000	20	4920

[a] For details see Materials and Methods.

RESULTS

Enzyme Stability

The instability of adenylate cyclase is the major impediment encountered during isolation studies. Thus, before attempting to solubilize the enzyme, conditions had to be established that would permit retention of activity over the time span necessary for solubilization and isolation experiments. The time course for enzyme inactivation was investigated by incubating membrane suspensions in sodium bicarbonate or in a sodium fluoride containing medium at 4°C (Fig. 1). Membranes stored in bicarbonate exhibited very low levels of activity after 22 hr of storage. In contrast, membranes stored in the presence of fluoride ions initially gained activity so that after 2.5 hr they had

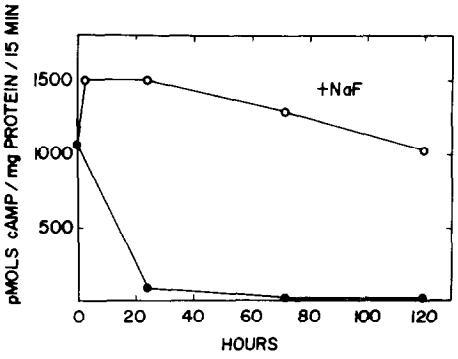

FIG. 1. Rate of deactivation of bovine adrenocortical plasma membrane adenylate cyclase. Membrane suspensions (4 mg of protein/ml) in 1 m*M* bicarbonate, pH 7.5, were diluted with 4 vol of 1 m*M* sodium bicarbonate (●), or 4 vol of a solution containing Tris–HCl, pH 7.3 (0.1 *M*), $MgSO_4$ (5m*M*), NaF (5 m*M*), and DTT (1 m*M*), (○) and were kept at 4°C. Aliquots (0.4 ml each) were withdrawn at various times and assayed for cyclase activity in the prsence of NaF (see Materials and Methods).

approximately 140% the activity of the starting material. Thereafter the rate of deactivation was relatively slow. After 5 days the activity had fallen to 68% of the maximal value.

Activation by Fluoride

The rate of stimulation of the enzyme by fluoride ion was next investigated. The results of preincubation at 0°C of freshly harvested plasma membranes with 1 or 5 m*M* sodium fluoride (Fig. 2) indicate that fluoride ion activation is a slow process at 0°C. The 15-min incubation at 30°C employed routinely for adenylate cyclase assay is not sufficient to stimulate the enzyme fully. A preincubation period of at least 20 min at 0°C in the presence of 5 m*M* fluoride is required to obtain maximal stimulation. The concentration of fluoride ion determined the degree of stimulation attained.

Detailed studies on the optimum concentrations of fluoride and magnesium for activation of adenylate cyclases from various tissues have been reported (9–11); however, it should be noted that MgF_2 readily precipitates from solutions containing these two ions since the solubility of MgF_2 is approximately 1.2 m*M* at 18°C. The influence of the presence of membrane proteins on the effective concentration of

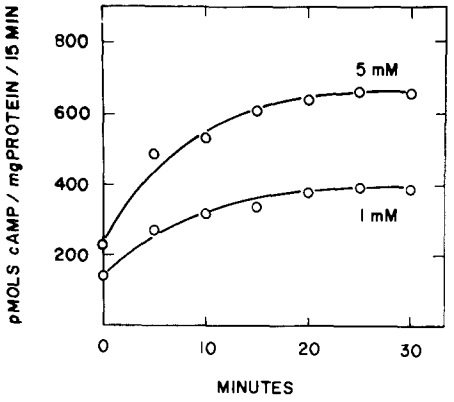

FIG. 2. Rate of activation of membrane adenylate cyclase by NaF. Plasma membranes (4 mg of protein/ml) were preincubated at 0°C in a medium containing NaF (1 or 5 m*M*), Tris–HCl, pH 7.3 (50 m*M*), $MgSO_4$ (5 m*M*), and DTT (0.2 m*M*). Aliquots 0.1 ml) were removed at the times indicated and assayed for adenylate cyclase activity in the usual manner (Materials and Methods) in the absence of added fluoride.

magnesium and fluoride ions in solution has not been measured. In view of this uncertainty the concentrations of magnesium sulfate and sodium fluoride are based on the amount of the salts added and are not meant to imply final concentrations.

The reversibility of NaF activation was investigated by dialyzing membranes, preincubated with NaF, in the presence or absence of fluoride (Fig. 3). Plasma membranes dialyzed for 20 hr against Tris–HCl buffer containing DTT retained

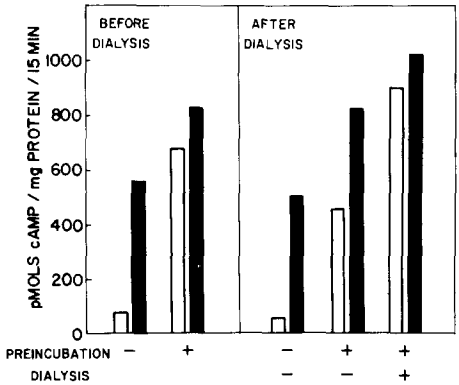

FIG. 3. Effect of dialysis on sodium fluoride activation of adenylate cyclase. Left panel: Plasma membranes suspended in 1 m*M* bicarbonate, pH 7.5 (4 mg of protein/ml), were divided into two portions. To one of these an equal volume of Tris–HCl, pH 7.3 (0.2 *M*), containing $MgSO_4$ (10 m*M*) and DTT (2 m*M*), was added (preincubation minus); to the other portion, an equal volume of the same solution but containing NaF (10 m*M*) was added (preincubation plus). Both suspensions were kept at an ice-bath temperature for 1 hr when aliquots were assayed for adenylate cyclase activity in the presence (black bars) or absence (white bars) of NaF (10 m*M*). Right panel: The remaining portions of the samples were dialyzed at 4°C against 2 × 100 vol of Tris–HCl, pH 7.3 (0.1 *M*), containing $MgSO_4$ (5 m*M*) and DTT (1 m*M*) in the presence (dialysis plus) or absence (dialysis minus) of NaF (5 m*M*). After 20 hr aliquots were assayed for adenylate cyclase.

approximately 80% of their NaF-sensitive activity. Plasma membranes preincubated with fluoride and dialyzed in the absence of fluoride can be reactivated to their original level by the addition of NaF to the assay medium. It should be noted that fluoride activation is partially reversible, since the enzyme preincubated with fluoride, dialyzed without fluoride, and assayed without the addition of fluoride was not as active after dialysis as before. The continued presence of fluoride both in the preincubation and dialysis solutions results in complete retention of activity.

Activation by Gpp(NH)p

Adrenal plasma membrane cyclase is stimulated by low concentrations of Gpp(NH)p (Table 2). The activation by the guanyl nucleotide is not as high as that achieved either

TABLE 2

ACTIVATION OF MEMBRANE ADENYLATE CYCLASE BY Gpp(NH)p[a]

Activators	Specific activity
None	67
$ACTH_{1-24}$	373
NaF	438
Gpp(NH)p	212
$ACTH_{1-24}$ + Gpp(NH)p	575
NaF + Gpp(NH)p	440

[a] Plasma membranes were assayed for adenylate cyclase activity as described (Materials and Methods). In addition to the usual components the assay mixture contained $ACTH_{1-24}$ (3.98 μM), NaF (10 mM), Gpp(NH)p (10^{-5} M), or various combinations of activators as indicated.

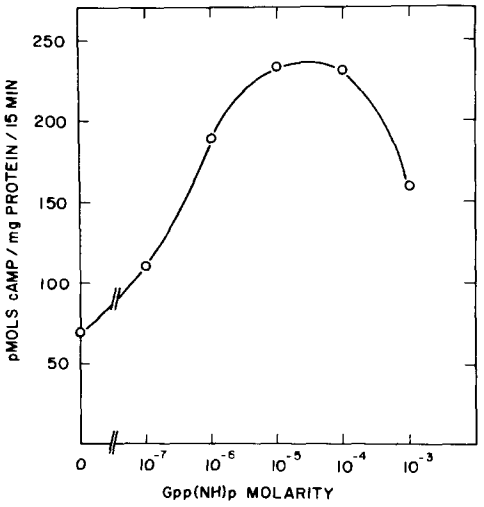

FIG. 4. Concentration dependency of Gpp(NH)p stimulation of membrane adenylate cyclase, Plasma membranes were assayed for adenylate cyclase activity in the presence of varying amounts of Gpp(NH)p.

with $ACTH_{1-24}$ or NaF. The combination of hormone and nucleotide results in an increase in activity to a level equivalent to the sum of activities achieved when either activator is used alone. Addition of Gpp(NH)p does not increase the activity of NaF stimulated enzyme. The highest degree of activation by Gpp(NH)p is observed when the nucleotide concentration in the assay medium is between 10^{-5} and 10^{-4} *M* (Fig. 4).

TABLE 3

EFFECT OF Gpp(NH)p ON PREPARATION OF SOLUBLE ADENYLATE CYCLASE[a]

Sample	Activator	Protein (%)	Adenylate cyclase activity	
			Total	Specific activity
I		100		
	None		2240	123
	$ACTH_{1-24}$		13 900	763
	Gpp(NH)p		12 100	661
	$ACTH_{1-24}$ + Gpp(NH)p		24 600	1350
II		100		
	None		792	43
	$ACTH_{1-24}$		2940	161
	Gpp(NH)p		6240	342
	$ACTH_{1-24}$ + Gpp(NH)p		10 100	553
III		18.7		
	$ACTH_{1-24}$		4700	1380
	Gpp(NH)p		4680	1370
	$ACTH_{1-24}$ + Gpp(NH)p		4510	1320
IV		14.7		
	None		231	86
	$ACTH_{1-24}$		238	89
	Gpp(NH)p		436	162
	$ACTH_{1-24}$ + Gpp(NH)p		673	251

[a] Plasma membranes (4 mg of protein/ml) suspended in 1 m*M* sodium bicarbonate, pH 7.5, were divided into four equal aliquots. One of these (I) was assayed immediately for adenylate cyclase activity. Aliquot (II) was stored in bicarbonate at 4°C. The third aliquot (III) was solubilized, the detergent was removed, and the volume of the soluble sample was reduced to 10% by filtration through a Diaflo ultrafiltration membrane (PM10). Each step in this procedure was performed in the usual manner except that Gpp(NH)p (10^{-5} *M*) was substituted for NaF. The fourth aliquot (IV) was treated the same as sample III but in this case both NaF and Gpp(NH)p were omitted. Adenylate cyclase assays were performed on samples II, III, and IV 18 h after the assays of sample I. Adenylate cyclase assays were performed in the presence of the activators shown below. $ACTH_{1-24}$, when present, was 3.98 μM and Gpp(NH)p was 10^{-5} *M*.

Higher levels of the nucleotide are inhibitory. Like sodium fluoride (see below), Gpp(NH)p has little influence on the amount of protein solubilized by Lubrol, but it preserves enzyme activity in the soluble material. In most cases (Table 3 samples I, II and IV), the effects of $ACTH_{1-24}$ and Gpp(NH)p were additive. However, when Gpp(NH)p was present throughout the solubilization and concentration steps (sample III), no further increase in activity was observed on addition of $ACTH_{1-24}$.

Stabilization of Adenylate Cyclase by Fluoride Ion During Solubilization

The experiments discussed thus far show that the presence of fluoride ion activates and at the same time stabilizes the adenylate cyclase. In order to gain more insight into the stabilizing effect of fluoride ion the experiments shown in Table 4 were conducted. Two plasma membrane samples (II and III) were solubilized in the same manner with the exception that in one case NaF was omitted. Sodium fluoride has no effect on the amount of protein that is solubilized by Lubrol; however, its presence is apparently

TABLE 4

STABILIZATION OF ADENYLATE CYCLASE BY SODIUM FLUORIDE ION DURING SOLUBILIZATION[a]

Sample	Activator	Protein (%)	Adenylate cyclase activity	
			Total	Specific activity
I		100		
	None		565	76
	$ACTH_{1-24}$		1240	167
	NaF		6130	828
II		16.7		
	NaF		2800	2250
III		15.6		
	None		264	228
	$ACTH_{1-24}$		277	241
	NaF		594	515

[a] Plasma membranes (4 mg of protein/ml) suspended in 1 m*M* sodium bicarbonate, pH 7.5, were divided into three equal aliquots. One of these (I) was assayed immediately for adenylate cyclase activity. A second aliquot (II) was solubilized, the detergent was removed, and the volume of the soluble samples was reduced to 10% by ultrafiltration through PM10 membranes. The third aliquot (III) was treated exactly as sample II, except that sodium fluoride was omitted throughout the preparative procedures. Adenylate cyclase assays were performed on samples II and III 18 hr after the assays of sample I. Assays were performed in the presence of the activators shown below. $ACTH_{1-24}$ when present, was 3.98 μM and sodium fluoride was 10^{-2} *M*.

essential for optimal recovery of enzyme activity. Less than 10% of the total fluoride-sensitive activity remained when fluoride was absent throughout the procedure as compared with 45% recovery in its presence. The sample solubilized without sodium fluoride lost all sensitivity to $ACTH_{1-24}$. Diminished responsiveness to fluoride stimulation was also evident in this sample.

Effect of Lubrol on Enzyme Activity

During the development of isolation procedures enzyme solutions containing various amounts of Lubrol had to be assayed. It was therefore necessary to determine the effect of Lubrol on adenylate cyclase activity. The results presented in Fig. 5 indicate that Lubrol inhibits the enzyme. Enzyme activity was further decreased when samples were incubated with detergent for 1 hr; however, when the enzyme was stored for 1 to 3 days

at 4°C in the presence of 0.01% Lubrol, activity did not decrease below the values observed after 1 hr of exposure. Removal of the Lubrol by DEAE cellulose according to the procedure of Levy (*12*) resulted in an increase in specific activity. In view of this

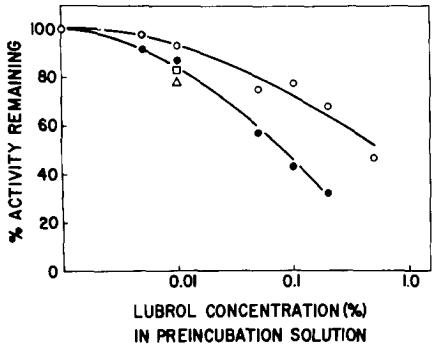

FIG. 5. Effect of Lubrol on adenylate cyclase activity. Aliquots (0.2 ml) of the detergent-free 100 000-*g* supernatant were mixed with equal volumes of aqueous Lubrol solutions to achieve the concentrations shown. The solutions were assayed in the presence of NaF immediately (○) or after standing at 4°C for 1 (●), 24 (□), or 72 hr (△).

finding, the effect of Lubrol concentration on adenylate cyclase solubilization was investigated using the yield and specific activity of the detergent-free DEAE eluate as criteria for evaluating the relative merits of the procedures (Table 5). Although the overall yield of cyclase activity is not markedly improved when higher Lubrol concentrations are employed, the specific activity of the Lubrol-free enzyme obtained

TABLE 5

EFFECT OF LUBROL CONCENTRATION ON SOLUBILIZATION OF PLASMA MEMBRANES[a]

Preparation	0.15% Lubrol-PX			1.0% Lubrol-PX		
	Specific activity	Recovery (%)		Specific activity	Recovery (%)	
		Protein	Cyclase		Protein	Cyclase
Plasma membranes	2370	100	100	2370	100	100
Sonicate	1310		55	285		12
100 000*g* pellet	1940	39	32	256	35	4
100 000*g* supernatant	1060	63	28	345	65	10
DEAE eluates	1600	18	12	2305	17	16

[a] For experimental details see Materials and Methods.

under these conditions is considerably higher than with 0.15% Lubrol. Still higher detergent concentrations, e.g. 2% (data now shown) did not increase the yield and appeared to irreversibly inactivate the enzyme. A second exposure of the 100 000-*g* pellet to 1% Lubrol also failed to increase the yield of solubilized cyclase.

Stability of Detergent-free Enzyme to Lyophilization

The concentrate was dialyzed prior to lyophilization to reduce the salt concentration (Table 6). The lyophilized powders can be stored at least 1 month at −70°C without loss of activity; however, some activity losses were observed on repeated reconstitution and relyophilization.

TABLE 6

EFFECT OF LYOPHILIZATION ON ADENYLATE CYCLASE ACTIVITY[a]

Preparation	Protein		Adenylate cyclase activity		
	Total (mg)	Recovery (%)	Total	Recovery (%)	Specific activity
Plasma membranes	61.4	100	162 000	100	2640
Concentrate	8.0	13.1	33 400	20.6	4160
Dialyzed sample	7.8	12.7	30 500	18.8	3900
Lyophilized sample					3440
					3890[b]
					2700[c]

[a] Detergent-free concentrate (20 ml) was dialyzed overnight against 2 liters of 5 m*M* Tris–HCl, pH 7.3, containing NaF (0.5 m*M*), $MgSO_4$ (0.5 m*M*), and DTT (0.5 m*M*), and aliquots of the dialyzed solution (8 ml) each were lyophilized. For adenylate cyclase assay, the lyophilized samples were dissolved in 8 ml of 1 m*M* DTT.

[b] The lyophilized sample was assayed after storage for 1 month at −70°C.

[c] The lyophilized sample was dissolved, relyophilized and stored at −70°C until assayed.

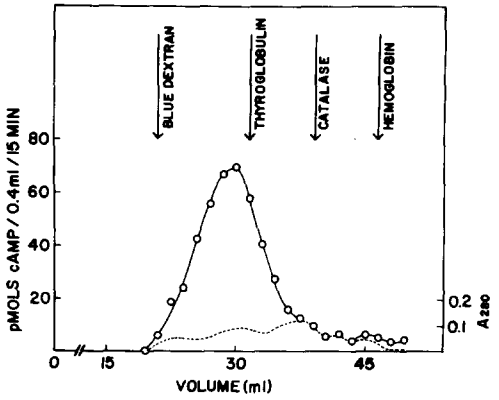

FIG. 6. Gel filtration of detergent-free adenylate cyclase on Sepharose 6B. The volume of a solution of solubilized, detergent-free adenylate cyclase was reduced by ultrafiltration to a final protein concentration of 1.4 mg/ml. An aliquot of this sample (1 ml) was applied to a Sepharose 6B column (1 × 60 cm) which had been equilibrated with Medium A, and the column was eluted with the same solution. Fractions (1.5 ml each) were collected and assayed for 280-nm absorbing material (– – –) and adenylate cyclase activity (O——O). Standards used to calibrate the column were chromatographed under the same conditions prior to application of the adenylate cyclase sample. The standards were assayed as indicated in Materials and Methods.

Gel Filtration

When the enzyme concentrate was chromatographed on Sepharose 6B, adenylate cyclase activity appeared as a single peak with an elution volume slightly smaller than that required for thyroglobulin (Fig. 6). Only 50% of the activity was recovered. The material applied to the column had not been subjected to centrifugation at 100 000 *g* before application and presumably contained aggregates. Centrifugation of the concentrate to prepare the 100 000-*g* detergent-free supernatant in a typical experiment produced a pellet containing 41% of the total cyclase activity. The specific activities of the enzyme in the supernatant and pellet were approximately equal.

Hydrophobic Chromatography

When the detergent-free 100 000-*g* supernatant was filtered through a series of alkyl Sepharose columns, the results shown on Fig. 7 were obtained. It is apparent that the *n*-hexyl and *n*-octyl Sepharoses retain the bulk of the enzyme activity; however, the *n*-hexyl column had the advantage that approximately 40% of nonenzyme protein was not adsorbed. These experiments suggested that chromatography of the enzyme on *n*-hexyl Sepharose columns might result in significant purification. A preparative scale

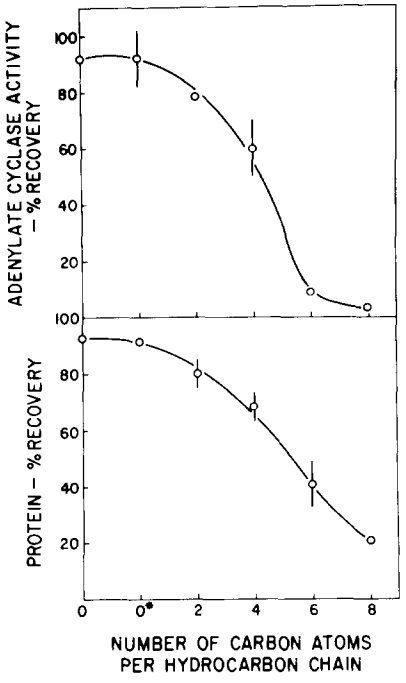

FIG. 7. Exploratory hydrophobic chromatography of detergent-free 100 000-*g* supernatant. Columns (0.4 × 3.0 cm) of Sepharose 4B (O); cyanogen bromide-activated Sepharose 4B (O*); and alkyl Sepharoses containing hydrocarbon chains varying from two to eight carbon atoms were equilibrated with Medium A. Aliquots of the detergent-free 100 000-*g* supernatant (1 ml each, containing 0.6 mg of protein; specific activity 3450) were applied to each column, and the columns were developed with Medium A. Fractions (3 ml each) were collected and assayed for protein and adenylate cyclase activity. Vertical lines indicate standard deviation.

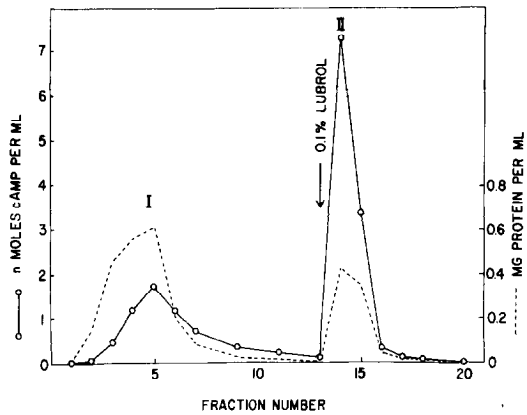

FIG. 8. Hydrophobic chromatography of soluble adenylate cyclase on *n*-hexyl Sepharose 4B. The detergent-free 100 000-*g* supernatant (12 ml containing 9.53 mg of protein, specific activity 5340) was applied to an *n*-hexyl Sepharose 4B column (1.0 × 6.5 cm) previously equilibrated with Medium A, and fractions (3 ml each) were collected at a flow rate of 6 ml/hr. After 12 fractions had been collected, the eluant was changed to Medium A containing Lubrol (0.1%). Protein and adenylate cyclase activity were determined in the eluates.

experiment with this column (Fig. 8) showed that the attachment of the enzyme to the resin is very firm as it was not possible to desorb cyclase activity with Tris–HCl buffers of higher ionic strength, e.g., Medium B or Medium B containing 40% ethylene glycol or glycine buffer, pH 9.3 (0.1 *M*), or Medium A containing sodium thiocyanate (not shown). Developing the column with Medium A containing 0.1% Lubrol eluted the enzyme. The specific activity of the detergent-free 100 000-*g* material applied to the column was 5340. The most active fractions in peaks I and II exhibited specific activities of 2870 and 17 200, respectively. The specific activity of the enzyme corresponding to peak II is a minimum figure since it has not been corrected for inhibition by Lubrol. Recovery of enzyme was usually in excess of 100%; however, this figure is subject to error since it is based on the assumption that the Lubrol concentration throughout peak II was 0.1%, and recoveries have been corrected for this concentration of detergent.

DISCUSSION

A current model concerning the molecular architecture of hormone-sensitive adenylate cyclases envisions a recognition site (receptor) on the cell surface and a catalytic site (cyclase) located on the inside of the plasma membrane. These hypothetical components, it is speculated, communicate *via* a "transducer."

We and others (*3*, *13*) have observed that activation of bovine adrenocortical membrane cyclase by fluoride ion precludes activation by ACTH, thus once fluoride is included in the medium it is no longer possible to demonstrate hormone sensitivity. We have shown also (*14*) that peptides that competitively inhibit the hormone-stimulated membrane cyclase have no effect on the fluoride-stimulated species and have concluded that activation by these agents proceeds via different mechanisms. The same

conclusions have been reached by other investigators (*15*). In regard to the stability of the enzyme from the bovine adrenal cortex membranes, we have consistently observed that hormone sensitivity is the most labile component of the system and have suggested that loss of hormone sensitivity may be the result of disruption of the communication between receptor and cyclase. This suggestion was based on the observation that both hormone binding and fluoride-activated cyclase activity are much more stable to freezing than is hormone sensitivity. Roy et al. (*16*) have recently shown by monitoring both adenylate cyclase activity and [Lys^8] vasopressin binding to solubilized porcine kidney medullary plasma membranes that the soluble enzyme failed to respond to hormone stimulation, although 30% of the hormone receptor and 100% of the cyclase were solubilized.

The actual mechanisms involved in the activation of the cyclase by fluoride, cholera toxin, or Gpp(NH)p are unknown, but it is generally assumed that the activated species represents the catalytic portion of the cyclase complex. Based on this assumption and because of the stability of the fluoride-activated cyclase, we have undertaken a study of the properties of the fluoride-activated enzyme. The availability of the catalytic subunit in a highly purified state may contribute to a better understanding of the complete adenylate cyclase system.

Conflicting results have been reported regarding the role of fluoride ion in the dispersion of adenylate cyclases. Pastan et al. (*17*) have described a preparation of adenylate cyclase obtained by passing an adrenal tumor particulate through a French pressure cell in a medium containing phosphatidyl ethanolamine and sodium fluoride. In the absence of fluoride little enzyme activity appeared in the 105 000-*g* supernatant prepared from this extract. The authors concluded that little or no solubilization occurred in the absence of fluoride. From these experiments it was not clear whether fluoride exerted a direct effect on solubilization or whether active enzyme failed to appear in the supernatant because of enzyme instability in the absence of the anion. Conversely Swislocki and Tierney (*18*) reported that the presence of 25 m*M* NaF produced a marked decrease in the amount of renal kidney membrane protein solubilized by Lubrol-WX or passage through a French pressure cell. Therefore measurements of the amount of protein and adenylate cyclase solubilized from adrenal membranes in the presence or absence of fluoride were conducted to test the effect of fluoride on solubilization in this system (Table 4). The amount of protein solubilized was not significantly different when fluoride was present. Only when fluoride was present throughout the experiment was specific activity significantly improved (sample II).

A persistent activation of erythrocyte adenylate cyclase by the GTP analog, Gpp(NH)p, has recently been reported (*19–21*). The Gpp(NH)p-treated enzyme was less subject to inactivation by detergents or exposure to elevated temperatures than the native cyclase. Consequently we have conducted experiments to compare the activation of adrenal cyclase by $ACTH_{1-24}$, NaF, and Gpp(NH)p.

Cyclase stimulation by $ACTH_{1-24}$ and Gpp(NH)p apparently proceeds by different and noninteracting mechanisms in bovine adrenocortical plasma membranes since the effects of the two activators are additive (Table 2). Londos et al. (*2*) were able to stimulate adenylate cyclase with Gpp(NH)p in particulates prepared from a mouse adrenal tumor line despite the lack of a functional ACTH receptor in these cells.

Fluoride stimulation usually precludes stimulation by another activator and is considered to produce full activation of the cyclase; however, as is apparent from the results presented in Table 2, a combination of $ACTH_{1-24}$ and Gpp(NH)p elicits a specific activity higher than is produced by NaF. When NaF and Gpp(NH)p are added together the activity is not greater than with fluoride alone.

Like fluoride, Gpp(NH)p stabilizes the enzyme (Table 3). When the guanyl nucleotide is present during solubilization, however, the added stimulus usually provided by $ACTH_{1-24}$ is absent. Regardless of the activator or combination of activators the level of stimulation is the same. The presence of Gpp(NH)p during solubilization does not prevent loss of cyclase sensitivity to hormone stimulation and thus does not appear to offer advantages from the point of view of enzyme purification over the fluoride activated species.

Preliminary attempts to obtain a soluble cyclase preparation with Triton X-100 or Triton X-305 resulted in very poor recoveries of protein in the 100 000-*g* supernatant; somewhat better results were obtained with sodium cholate (not shown). Johnson and Sutherland (*22*) reported that Lubrol-PX at concentrations up to 0.3% significantly enhanced adenylate cyclase activity in a rat cerebellum particulate. The enzyme from bovine adrenal cortex behaves quite differently toward this detergent. The addition of Lubrol-PX to the detergent-free 100 000-*g* supernatant (Fig. 5) inhibited enzyme activity. This effect was more pronounced when the samples were incubated at 4°C for 1 hr. Thus the inactivation of the cyclase appears to be a relatively slow process. Furthermore, inhibition is reversible as is evident from the marked enhancement of activity that accompanies Lubrol removal (Table 5).

Levy (*12*) has shown, with radioactive Lubrol-PX, that the detergent can be removed quantitatively by adsorbing the solubilized preparation to DEAE cellulose. When DEAE cellulose was mixed with the Lubrol-containing 100 000-*g* supernatant the adrenal enzyme was retained and could be eluted with Medium B.

In the procedure described in the experimental section approximately 40% of the enzyme activity was recovered from the DEAE following dialysis (Table 1). Using higher concentrations of Tris did not improve the yield nor did further elution with Medium B.

A tentative molecular weight of 1×10^6 has been derived for the detergent-free enzyme by comparison of its elution profile on a Sepharose 6B column with the profiles produced by a standard set of proteins. The molecular weight of solubilized ACTH sensitive adenylate cyclase froma mouse adrenal tumor has been estimated to be 3 to 7 $\times 10^6$ (*17*).

From the preparative point of view it is important to obtain a detergent-free solubilized cyclase that can be stored without loss of activity. Such a preparation was obtained when the concentrate was dialyzed against a solution containing Tris–HCl, NaF, $MgSO_4$, and DTT and lyophilized. In this state the enzyme was stable for long periods of time when stored at −70°C (Table 6). The lyophilized material provides a convenient starting material for further purification attempts.

Since the cyclase, in its natural environment, is embedded in a hydrophobic lipid matrix, we felt that hydrophobic chromatography would be ideally suited for purification. A screening study carried out with alkyl Sepharose 4B columns (Fig. 7) suggested that the *n*-hexyl derivative was the most desirable resin since it retained most

of the enzyme while a sizeable proportion of the protein failed to adsorb. In the techniques described by Shaltiel (*8*) materials are usually desorbed with salt gradients. Our attempts to displace the cyclase from the *n*-hexyl column under a variety of conditions were unsuccessful. We reasoned that surface active agents would be more likely to destabilize the hydrophobic interactions between the enzyme and the alkyl Sepharose and hence buffers containing 0.1% Lubrol-PX were used. As is apparent from inspection of Fig. 8, the Lubrol-containing buffer readily desorbed a cyclase fraction exhibiting a threefold higher specific activity than the applied material. Based on the results shown on Fig. 5, it can be calculated that the detergent-free enzyme in the Lubrol eluate can be expected to exhibit a specific activity of approximately 26 000.

The purification scheme described in this communication results in an approximately eightfold enhancement of specific activity as compared with the starting material, i.e., purified adrenocortical plasma membranes.

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