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THE CHARACTERISTICS OF LUBROL-SOLUBILIZED ADENYLATE CYCLASE FROM RAT LIVER PLASMA MEMBRANES

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Summary

Adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) was solubilized from rat hepatic plasma membranes with Lubrol PX. The enzyme could be stabilized during solubilization by pretreating the membrane with glucagon and 5′-guanylimidodiphosphate (Gpp(MH)p) or Gpp(NH)p alone but not by glucagon alone or in the presence of GTP or other guanine nucleotides. The solubilized enzyme was purified 3—4-fold by molecular exclusion chromatography on Ultrogel AcA22; the presence both 0.01% Lubrol and 25% sucrose were required to maintain both the solubility as well as stability of the enzyme. The enzyme was purified from 70% of the other solubilized membrane proteins and 85% of the solubilized phospholipids, and was completely separated from 5′-nucleotidase. In addition, the enzyme was purified 2-fold with respect to non-specific nucleotide phosphohydrolase and pyrophosphohydrolase activities but not with respect to 'specific' GTPase activity.

No perceptible change in molecular size of partially purified adenylate cyclase was observed after it had been pre-activated in its membrane-bound form with hormone and Gpp(NH)p. The solubilized enzyme also retained its ability to be activated by the guanine nucleotide analogues Gpp(NH)p and $Gp(CH_2)pp$ with similar concentration and time-dependent characteristics displayed by the membrane-bound form of the enzyme.

Attempts to further purify the solubilized enzyme by a variety of size- and charge-based chromatography techniques proved unsuccessful. As an explanation for this phenomenon we suggest that, after solubilization with Lubrol,

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Abbreviations: App(NH)p, 5′-adenylimidodiphosphate; Gpp(NH)p, 5′-guanylylimidiphosphate; TES, *N*-tris (hydroxymethyl)methyl-2-aminoethane sulfonic acid; cyclic AMP, cyclic adenosine 3′,5-monophosphate; $Gpp(CH_2)p$, 5′-guanyl-β,γ-methylenediphosphonate; $Gp(CH_2)pp$, 5′-guanylyl-α,β-methylenediphosphonate.

adenylate cyclase is associated with other membrane proteins in the form of protein-lipid-detergent complexes. Because of this characteristic, we believe that calculations of the Stokes radii and molecular weights for Lubrol PX-solubilized forms of adenylate cyclase can yield unrealistic values for the intrinsic properties of the enzyme.

Introduction

Adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) systems in animal cells represent an important class of membrane-bound enzymes that are subject to regulatory control by numerous ligands, including hormones, prostaglandins, neurotransmitters, adenosine, and opiates. Many of these systems are regulated also by guanine nucleotides [1—8]. The goal of many investigators studying this enzyme is to purify its catalytic and regulatory components, and no reconstitute these components into the regulatory state found in the membrane. Numerous reports on such attempts have revealed certain problems in this venture. Even though most investigators have used non-denaturing, non-ionic detergents, such as Lubrol PX or Tritons, to solubilize the enzyme [9—19], solubilization still results in substantial loss of enzyme activity [11—19]. The apparent lability of the enzyme is usually cited as a major problem in its purification. It has been observed that pretreatment of membranes with the GTP analog, Gpp(NH)p, leads to a persistent, high activity state of the enzyme even after detergent solubilization of the enzyme [15,20, 21]. This procedure seems useful, therefore, for purifying the enzyme.

In this paper, we developed methods for stabilizing Lubrol PX-solubilized hepatic adenylate cyclase using Gpp(NH)p and high concentrations of sucrose so that applications of a wide variety of conventional techniques employed for purifying extrinsic membrane proteins or soluble proteins could be studied. We have observed that the lability of adenylate cyclase is not the sole limiting factor in the purification of the enzyme. We will describe other characteristics of the Lubrol PX-solubilized enzyme which raise questions about the applicability of neutral detergents in the solubilization and purification of this enzyme.

Materials and Methods

Materials. App(NH)p, Gpp(NH)p, and $Gp(CH_2)pp$ were obtained from International Chemical and Nuclear Corp., as was [α-^{32}P]ATP. $Gpp(CH_2)p$ was from Miles Labs. 3′-Deoxy GTP was kindly supplied by Drs. M. Cashel and E. Hamel. [γ-^{32}P]GTP was obtained from New England Nuclear Corp. Thyroglobulin, hemoglobin, β-galactosidase, and human γ-globulin were obtained from Sigma, while catalase was from Worthington Biochemical Corp. TES was a product of the Calbiochem Company. Ultrogel AcA22 was a product of the LKB Instrument Co.; Lubrol PX was a gift from ICN. DEAE-agarose, CM-agarose, and hydroxyapatite were obtained from Bio-Rad Labs., while alkyl-agarose of various lengths were obtained from Miles Lboratories.

Preparation of Hepatic Plasma membranes. Partially purified plasma membranes from rat liver were prepared by a modification of the procedure of

Neville [22] as previously described [23]. Membranes were stored in liquid nitrogen prior to use.

Glucagon and Gpp(NH)p-pretreatment. Liver plasma membranes were suspended to a concentration of approx. 1 mg/ml in 25 mM Tris · HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM App(NH)p, 10 μM cyclic AMP, 5 mM $MgCl_2$, 10 μM Gpp(NH)p, and $2 \cdot 10^{-7}$ M glucagon. This mixture was incubated either at 37°C for 10 min or 30°C for 20 min with frequent mixing. After pretreatment, the mixture was centrifuged in a Sorval RC2B at 49 500 $\times g$ for 30 min. The pellet was then resuspended in 10 mM TES, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, and 25% sucrose (Buffer A).

Preparation of soluble adenylate cyclase. All solubilization procedures were carried out at 0—4°C. Typically, rat liver plasma membranes were suspended to a concentration of 5—10 mg/ml in Buffer A. Lubrol PX (10%) was added subsequently to give a final concentration of 1%. This mixture was mixed for 30 s and centrifuged in a Sorvall RC2B at 49 500 $\times g$ for 30 min. After centrifugation the supernatant, containing the solubilized adenylate cyclase, was assayed directly or applied to an Ultrogel AcA22 column.

Ultrogel AcA22 gel filtration chromatography. The material solubilized from liver plasma membranes with Lubrol PX was chromatographed on Ultrogel AcA22 (fractionation range of $1 \cdot 10^6$—$6 \cdot 10^4$ daltons) in the presence of 10 mM TES, pH 7.5, 1 mM EDTA, 1 mM dithiothrietol, 25% sucrose, and 0.01% Lubrol PX (Buffer B). Chromatography was carried out at 0—4°C. Adenylate cyclase activity in the chromatographed fractions was stable to storage at —20°C for several weeks.

Enzyme assays. Adenylate cyclase was assayed according to the method of Salomon et al. [24]. The assay mixture, in 100 μl, contained 30 mM Tris · HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM creatine phosphate, 3.3 units of creatine phosphokinase, and the concentrations of ATP and cyclic AMP indicated in the legends to the figures and tables. The final specific activity of [α-^{32}P]ATP ranged from 20 to 200 cpm/pmol. Assays were performed for 10 min at 30°C.

GTPase activity was assayed as a modifcation of the method described by Abrams et al. [25]. Assays were performed in a medium very similar to that used for measuring adenylate cyclase: 25 mM Tris · HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM App(NH)p, 1 mM dithiothreitol, 10 μM cyclic AMP, and 50 μM [γ-^{32}P]GTP ($8 \cdot 10^3$ dpm/nmol). The assay volume was 100 μl and protein varied between 10 and 50 μg. Reactions were initiated by addition of the fraction, and after incubation at 30°C for 10 min, were stopped by addition of 10% trichoroacetic acid containing 60 mg/ml of activated charcoal (untreated powder form). This mixture was mixed several times over a period of 10 min and then centrifuged in an Eppendorf microfuge for 4 min. The pelleted charcoal contained the nucleotides present in the assay mixture, while the supernatant contained free ^{32}P. A 100-μl aliquot of the supernatant was counted in 8 ml of Aquasol. In some cases hydrolysis of GTP, GDP or Gpp(NH)p and the resulting products were determined by incubation of [^{14}C]GTP, [^{3}H]GDP or [^{3}H]Gpp(NH)p (10, 200, and 100 Ci/mol, respectively) in 100 μl of 20 mM Tris · HCl, pH 8.0, containing 0.1 mM App(NH)p, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 50 μM nucleotide. Reactions were carried out at 30°C for 10 min and stopped by the addition of 100 μl of 4% sodium dodecyl sulfate (SDS) and

20 mM EDTA followed by boiling for 3 min. Typically 2 μl of this mixture was then loaded on a polyethylene-imino-cellulose chromatography plate and co-chromatographed with a standard mixture of guanine nucleotides and guanosine. The plate was first developed in deionized water, dried, and developed again in 0.5 M LiCl/1.0 M formic acid. The spots were visualized under ultraviolet light, were cut-out, and extracted in a scintillation vial with 2 ml of 20 mM Tris · HCl, pH 7.5, containing 0.7 M $MgCl_2$. After shaking the vials for 10 min, 10 ml of Aquasol were added and the samples counted in a liquid scintillation spectrometer.

5-Nucleotidase was assayed according to the method of Avruch and Wallach [26] as modified by Luzio et al. [27]. Sodium β-glycerophosphate (2 mM) was included in the assay mixture to inhibit non-specific phosphatases.

Ultrogel AcA22 gel chromatographic markers were assayed as follows: hemoglobin by its adsorption at 540 nm, catalase as described in the Worthington Enzyme Manual [28], and thyroglobulin by protein determination [29].

Other methods. Protein was assayed by the procedure described by Lowry et al. [29] after reaction of dithiothreitol in the samples with sodium iodoacetate as described by Ross and Schatz [30]. Phospholipid analysis was conducted on samples extracted with chloroform/methanol according to the method of Bligh and Dyer [31]. Typically, when extracting liver plasma membrane preparations, 500 μl of a 5 mg protein/ml solution was carried through the extraction procedure. When Ultrogel AcA22 column fractions were analyzed, 200-μl samples (50—500 μg protein/ml) were extracted. A portion of the organic extract containing between 5 and 50 nmol of phospholipid was dried under N_2 and then ashed to produce inorganic phosphate with $Mg(NO_3)_2 \cdot 6H_2O$ [32]. After ashing, samples were resuspended in 0.5 ml of 0.5 M HCl, and heated in a boiling water bath for 15 min before assaying the samples for inorganic phosphate by the method of Itaya and Ui [33].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed as described by Neville and Glossman [34], using system J4179. The lower gel was made up to 6.4% acrylamide and 0.3% bisacrylamide while the stacking gel contained 3.2% acrylamide and 0.1% bisacrylamide. Molecular weight markers β-galactosidase, human γ-globulin and bovine serum albumin were run in parallel with samples of liver plasma membranes and Lubrol-solubilized fractions.

Preparation of affinity columns. Affinity columns containing either ATP or GTP linked to agarose by periodate oxidation of the ribose ring [35] were purchased from P-L Biochemicals. Affinity columns containing ATP linked to agarose through the N^6 of the purine ring were prepared as previously described [36]. GTP columns attached through the terminal phosphates to an agarose matrix were prepared according to a method supplied by Dr. Thomas Pfeuffer *.

* GTP coupled through the terminal γ-phosphate to ρ-phenylenediamine was subsequently attached to carboxypropylamino-Sepharose 4B by a method kindly provided prior to publication by Dr. Thomas Pfeuffer, Department of Physiological Chemistry, University of Wurzburg School of Medicine, 8700 Wurzburg, Federal Republic of Germany.

Results

Stabilization of solubilized adenylate cyclase

During detergent-solubilization of liver plasma membrane adenylate cyclase, 75% of the basal activity was lost upon exposure of the membrane to Lubrol PX (Table I). Pretreatment of membranes with glucagon and Gpp(NH)p stabilized the enzyme, as has been reported for other systems [15,21,37]. After pretreatment, total activity increased 40% upon exposure to detergent. In both cases, 90% of the remaining activity was found in the supernatant after centrifugation. The enzyme passed through a Millipore filter (GS, 0.22 μM), was included in an Ultrogel AcA22 molecular sieving column (see below), and remained in the supernatant after centrifugation at 109 000 $\times g$ for 90 min. The enzyme could be activated by Gpp(NH)p to varying extents depending upon pretreatment, or not. As reported in other systems [13,15–19,38], hormonal stimulation was lost after solubilization. This was true even if excess Lubrol PX, which interferes with glucagon binding to its receptor [39], was removed by chromatography on DEAE-agarose [10] or Ultrogel AcA22.

We failed to observe any stabilizing effects of GMP and hormone (glucagon) on the liver adenylate cyclase system, of the sort reported for pigeon erythrocyte membranes [15]. Furthermore, pretreatment of membranes with glucagon, glucagon and GTP, or GTP alone, did not stabilize the enzyme during solubilization. After detergent solubilization, both untreated and pretreated (Gpp(NH)p and hormone) forms of adenylate cyclase lost 70–75% of their activity when diluted 5-fold and stored at 0–4°C overnight; enzyme stored in a

TABLE I

EFFECT OF LUBROL SOLUBILIZATION ON ADENYLATE CYCLASE ACTIVITIES

Pretreated membranes refer to liver plasma membranes which were subjected to the glucagon and Gpp(NH)p-pretreated conditions described in the Materials and Methods. Untreated membranes, suspended in parallel to a protein concentration of approx. 1 mg/ml in 25 mM Tris · HCl, pH 8.0, containing 1 mM dithiothreitol, were incubated at 0–4°C for 10 min and then centrifuged at 49 000 $\times g$ for 30 min. Both pretreated and untreated membranes were resuspended to a protein concentration of 7 mg/ml in buffer A. The membrane suspensions were adjusted to 1% with 10% Lubrol PX and centrifuged at 49 500 $\times g$ for 30 min to obtain solubilized adenylate cyclase. This procedure typically solubilized between 60 and 70% of the membrane protein. Prior to assay, all samples were diluted 1 : 5 in Buffer A. The concentration of ATP used in the adenylate cyclase was 1 mM, cyclic AMP was 0.4 mM, and Gpp(NH)p was 0.1 mM (where included). When assaying samples containing Lubrol PX, the final detergent concentration was 0.1%. When assaying pretreated membrane samples, the amount of Gpp(NH)p carried into the assay was estimated to be 0.2 μM based on the amount of Gpp(NH)p which normally binds to membranes under these conditions [55]. The data presented in this table are representative of more than five separate experiments. Results are expressed in pmol/10 min per 50 μl.

	Preparations	Adenylate cyclase activity	
		−Gpp(NH)p	+Gpp(NH)p
A.	Untreated membranes	20.8	72.1
B.	Untreated membranes + Lubrol PX	5.3	30.1
C.	Lubrol PX supernatant from untreated membranes	5.1	27.7
D.	Pretreated membranes	101.0	130.0
E.	Pretreated membranes + Lubrol PX	142.0	157.0
F.	Lubrol PX supernatant from pretreated membranes	126.0	139.0

concentrated form only lost 10—30% of its activity. A wide variety of compounds were tested for their ability to stabilize the enzyme to dilution. Those included substrate (App(NH)p), metal ions, product (cyclic AMP), activators (Gpp(NH)p), other proteins (serum albumin), salts (KCl), polyalcoholic compounds (glycerol, polyethylene glycol, and sucrose), and combinations of the above. Of these compounds, 25% sucrose stabilized best. In the presence of sucrose, adenylate cyclase typically lost only 30—40% of its activity when stored overnight at 0—4°C. Because of this observation, 25% sucrose was used throughout the purification procedures described in this study.

Ultrogel AcA22 chromatography

Fig. 1 illustrates the elution pattern of Lubrol-solubilized adenylate cyclase from pretreated liver membranes compared to that of several other solubilized components. With 0.01% detergent in the elution buffer, the solubilized enzyme was included in the column and could be separated from 70% of the solubilized membrane proteins. The specific activity of the enzyme increased 3—4-fold over that of the enzyme initially solubilized (Table II). During chromatography, 70% of the applied adenylate cyclase activity was recovered for both pretreated and untreated membrane sources of the enzyme. Adenylate cyclase activity eluted in the same position as thyroglobulin (R_s = 86 Å [40], molecular weight, 670 000 [41]) *. No detectable difference in elution position of adenylate cyclase was observed when the enzyme was obtained from membranes pretreated with Gpp(NH)p plus glucagon. However, a molecular weight change of less than 50 000 (difference of two column fractions) could not be easily detected on this column.

Fig. 2 compares the sodium dodecyl sulfate polyacrylamide gel electrophoresis patterns of the Ultrogel fractions containing adenylate cyclase activity with the patterns for liver plasma membranes and the fractions eluting in the void volume of the column. There is an evident purification of the adenylate cyclase fractions over the liver plasma membranes. An interesting feature of these gels is that the void material is enriched in low molecular weight components whereas the adenylate cyclase fraction is enriched in high molecular weight components. These findings gave the first evidence that the adenylate cyclase fraction contains a rather distinct class of proteins from those proteins that associated with the larger aggregates appearing in the void volume. As will be shown below, there are also distinct classes of enzymes in the two major fractions.

Phospholipid eluted both with the void volume fraction, with the fractions containing adenylate cyclase, and in fractions which eluted after the adenylate cyclase peak. The later fractions were enriched in detergent, as determined by analyzing the elution pattern of [^{14}C]acetyl Lubrol PX. The amount of phospholipid associated with the adenylate cyclase fraction was 290 nmol lipid phosphate/mg protein vs. 540 nmol lipid phosphate/mg protein in intact

* A R_s of 86 Å would be correlated with a calculated molecular weight of 400 000—450 000 if the enzyme were asymmetric with a frictional ratio of 1.7 (assuming no solvation) and a partial specific volume of 0.74 as suggested by Neer [12] for the solubilized adenylate cyclase from rat renal medulla.

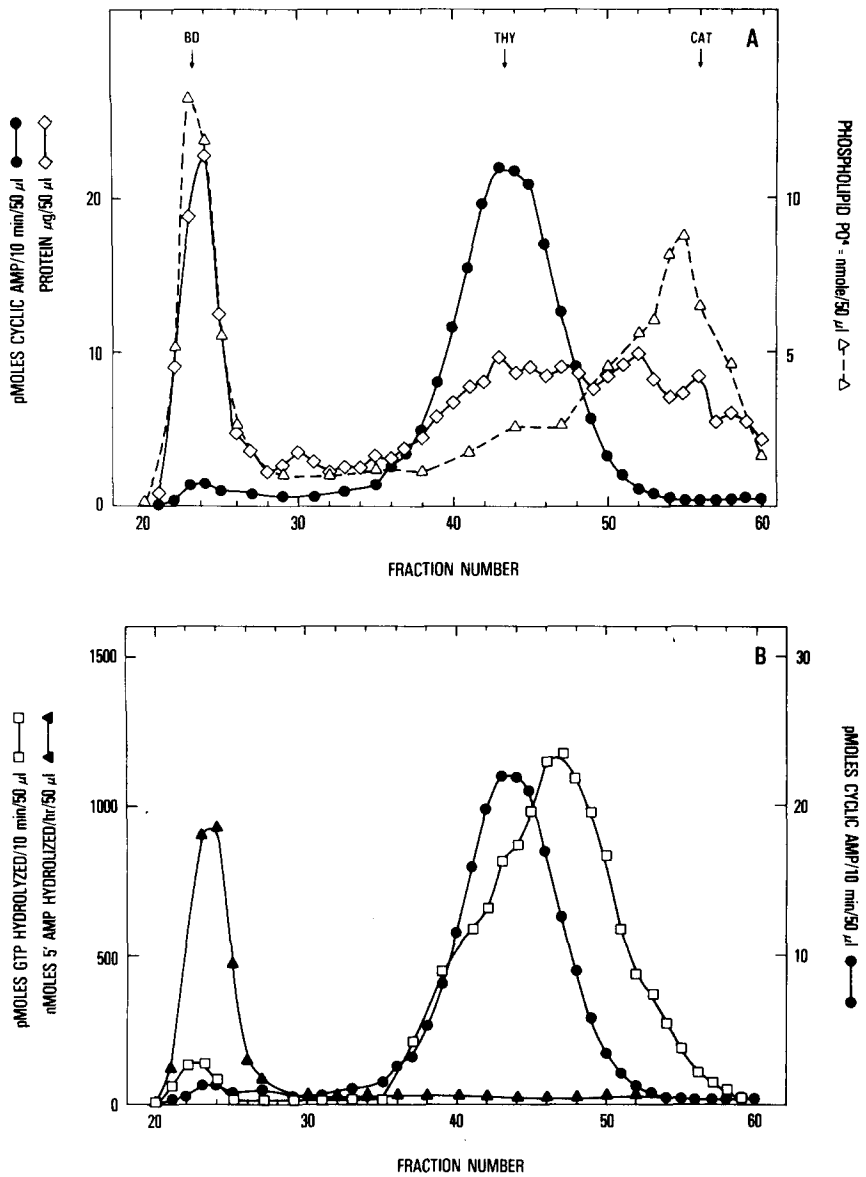

Fig. 1. Ultrogel AcA22 elution profile. In panel A membranes were pretreated with glucagon and Gpp(NH)p and solubilized with Lubrol PX, as described in Materials and Methods. The Lubrol extract (0.8 ml) was applied to an Untrogel AcA22 column (2 × 23 cm) eluted with Buffer B. 1-ml fractions were collected and assayed for adenylate cyclase (0.1 mM ATP, 40 μM cyclic AMP, and 10 μM Gpp(NH)p in assay mixture), phospholipid, and protein, as described in Materials and Methods. The elution positions of blue dextran (BD), thyroglobulin (THY), and catalase (CAT) are demonstrated on this figure. Hemoglobin eluted in Fraction 75 (not shown). These marker proteins were assayed as described in Materials and Methods. Panel B illustrates the elution positions from the Ultrogel AcA22 column of 5′-nucleotidase, acenylate cyclase, and "specific" GTPase determined as described in Materials and Methods.

plasma membranes. However, the types and distribution of phospholipids present in the adenylate cyclase fraction did not differ from the membrane phospholipid [22]. Thus, there was no apparent enrichment in a particular class of

TABLE II

PARTIAL PURIFICATION OF LUBROL PX-SOLUBILIZED ACENYLATE CYCLASE ON ULTROGEL AcA22

Pretreated membranes refer to liver plasma membranes treated exactly as described in Materials and Methods. Untreated membranes were suspended, in parallel, to 2—5 mg/ml in 10 mM TES, 1 mM EDTA, and 1 mM dithiothreitol prior to centrifugation at 49 000 × *g* for 30 min. This washing procedure was carried out at 0—4°C. Both treated and untreated membranes were then resuspended to 5—10 mg/ml in Buffer A. The Lubrol extract (8—10 ml) was then applied to an Ultrogel AcA22 column (3 × 38 cm) which was then eluted with Buffer B. 3-ml fractions of the column eluate were collected. This table compares the specific activities of adenylate cyclase obtained by assaying the samples in an assay mixture containing 0.1 mM ATP, 40 μM cyclic AMP and, where included, 10^{-5} Gpp(NH)p. Results are expressed in pmol/10 min per mg.

Preparations	Adenylate cyclase activity	
	—Gpp(NH)p	+Gpp(NH)p
Untreated membranes	189	595
Lubrol PX supernatant from untreated membranes	67	352
Peak Ultrogel fractions from untreated membranes	259	1164
Pretreated membranes	328	557
Lubrol PX supernatant from pretreated membranes	498	500
Peak Ultrogel fractions from pretreated membranes	1470	1860 *

* These values, as the others in this table, represent the average of duplicate determinations from a single experiment. Fold purification over the Lubrol PX-solubilized enzyme was always between 3- and 4-fold throughout experiments but final specific activities varied depending upon the specific activities of the enzyme in the original membranes and the assay conditions. In five preparations the activity ranged from 1.5 to 2.5 nmol/10 min per mg protein when assayed with 0.1 mM ATP and 10 mM Gpp(NH)p and 8—12 nmol/10 min per mg protein when assayed with 1 mM ATP and 10 mM Gpp(NH)p.

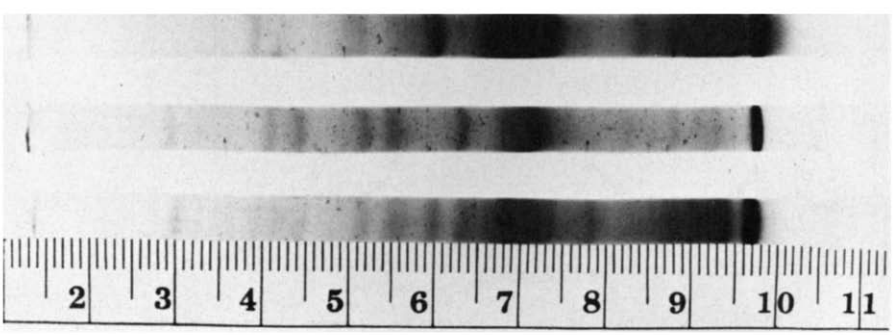

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of liver plasma membranes, partially purified adenylate cyclase from Ultrogel AcA22, and the void volume components from Ultrogel AcA22 chromatography. The peak fractions eluting in the void volume and adenylate cyclase-enriched fractions were dialyzed 15 h against 5 mM TES, pH 7.6, and concentrated by lyophilization. These samples and liver plasma membranes were then solubilized in 1% sodium dodecyl sulfate-buffer for electrophoresis according to the method of Neville and Glossman [34]. The gel on the left contains 70 μg of liver plasma membrane protein, the middle gel 70 μg of adenylate cyclase-enriched fractions, and the right gel contains 70 μg of the void volume protein. The gels were stained with Coomassie Blue [57]. Protein standards, β-galactosidase, human γ-globulin, and bovine serum albumin were run in parallel to calibrate the gels. Marker points of 5, 6.5, and 8 would represent the migration position of proteins of 100 000; 50 000; and 25 000 daltons, respectively, on these gels. The heavy band at the bottom of the gel (9.7 marker point) represents the tracking dye front.

phospholipids associated with the adenylate cyclase-containing fraction.

Fig. 1B illustrates the comparison of the elution pattern of adenylate cyclase activity with the patterns obtained for 5′-nucleotidase and 'specific' GTPase activities. 5′-Nucleotidase activity was detected only in the void fraction. 'Specific' GTPase activity (assayed in the presence of 0.1 mM App(NH)p, 50 μM [α-^{32}P]GTP, and other adenylate cyclase reagents) was included in the Ultrogel column and eluted as a rather broad band which peaked just behind the adenylate cyclase peak fraction. When GTPase activity was similarily measured in the absence of App(NH)p, 50% of the activity was then observed in both the void and included fractions. We interpret these findings as suggesting that the adenylate cyclase fraction is enriched with specific GTPase (either nucleotide phospho- or pyrophosphohydrolases) relative to non-specific nucleotidases that appear to be enriched in the void volume fraction from the Ultrogel column. Subsequent analysis of the breakdown products obtained when [^{14}C]GTP, [^{3}H]GDP, or [^{3}H]Gpp(NH)p were used as the substrate rather than [α-^{32}P]-GTP, demonstrated an enrichment of GTPase activity in the adenylate cyclase fractions while those fractions eluting after adenylate cyclase were enriched in GDPase activity. Since [^{3}H]Gpp(NH)p was not a substrate for any of these fractions, there appeared to be very little guanine nucleotide pyrophosphohydrolase activity in the included column fractions in contrast to the fractions in the void.

It should be emphasized that retardation of the included fraction containing adenylate cyclase and GTPase activities required the presence of Lubrol during chromatography; in its absence, 50—60% of these activities and the other proteins with which they co-eluted were shifted to the void volume. The elution position was not changed by increasing the detergent concentration above 0.01% *.

The use of other purification procedures

Subsequent to Ultrogel chromatography a variety of other purification procedures were studied in order to further purify adenylate cyclase. The enzyme proved unstable after $(NH_4)_2SO_4$ precipitation (enzyme activity precipitated between 40 and 50% saturation), precipitation at pH 5, or during isoelectric focusing [42] in the presence of 0.01% Lubrol (the pH gradient ranged from 3 to 10).

Fig. 3 illustrates the elution patterns of partially purified adenylate cyclase fraction rechromatographed on columns of DEAE-agarose, hydroxyapatite or columns containing hydrophobic amines [43,44]. The invariant correspondence of adenylate cyclase activity and protein patterns is striking and demonstrates the close association of the proteins obtained from the Ultrogel column. This association included 'specific' GTPase activity (data not shown). None of the enzyme activities or proteins adsorbed to CM-agarose columns. Use of higher detergent concentrations in the elution buffer failed to cause separation of proteins on any of the columns tested.

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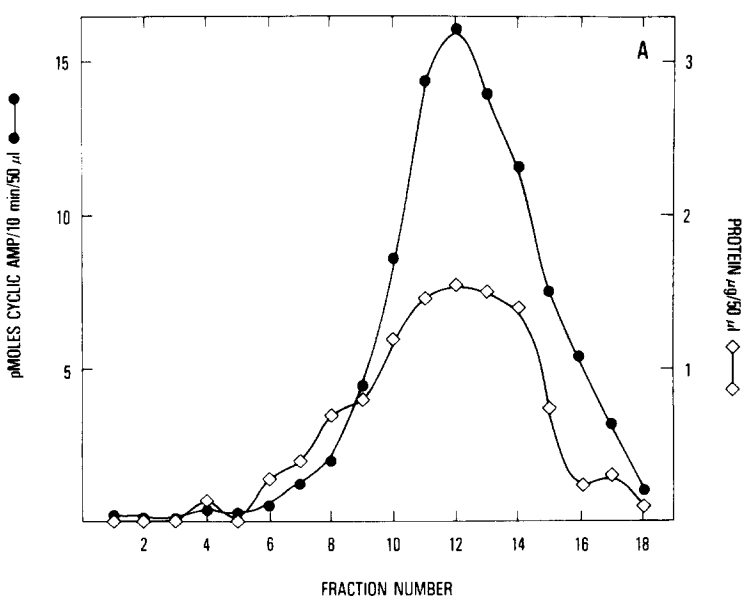
* Gel exclusion chromatography was normally carried out in the presence of 25% sucrose to stabilize enzymatic activity. Preliminary experiments using Sepharose 6B failed to demonstrate any change in the migration properties of the enzyme in the absence of this high sucrose concentration.

Affinity chromatography was also explored as a potential technique for purification. Neither adenylate cyclase nor any of the other proteins would specically adsorb to ATP affinity columns attached through the ribose ring or the N^6 position of the purine ring, or to GTP-affinity columns attached through either the ribose or terminal phosphate groups. Any small adsorption that did occur was non-specific and likely related to the ionic and hydrophobic interactions with the hydrophobic and charged groups used for attaching the nucleotides to the agarose matrix.

Activation of partially purified adenylate cyclase by guanine nucleotides

The above studies indicated that the enzyme could not by purified more than 3—4-fold and appears to be a complex aggregate of proteins phospholipids, and detergent (see Discussion). It was of interest to determine whether this still complex form of the enzyme would retain the characteristics of guanine nucleotide activation seen with the enzyme in its membrane-bound form. Fig. 4 shows the effects of Gpp(NH)p, $Gp(CH_2)pp$, $Gpp(CH_2)p$, 3′-deoxyGTP, and GTP on the activity of the partially purified enzyme obtained from non-treated membranes. The dose vs. response relationships for these nucleotides (insufficient 3′-deoxyGTP was available for a complete concentration curve) was essentially the same as that reported previously with intact liver membranes [45,46].

In other experiments (data not shown), the identical time dependence and reversibility characteristics observed for Gpp(NH)p and $Gp(CH_2)pp$ activation of the partially purified enzyme as reported for the membrane-bound form of the enzyme [45,46]. Thus, in terms of potency, kinetics of activation, and reversibility, the behaviour of the enzyme has not been modified by the procedures used for purification.



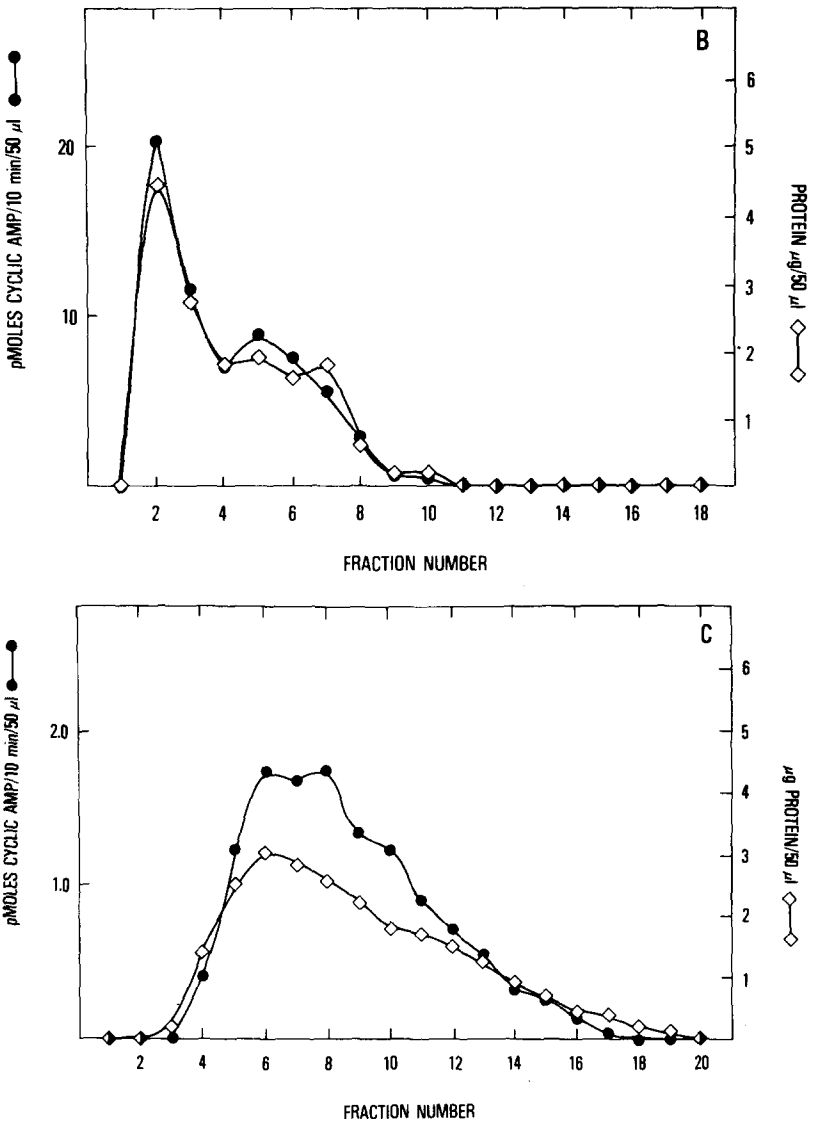


Fig. 3. Chromatography of Ultrogel-purified adenylate cyclase on various ion-exchange resins. (A) 2 ml of pretreated adenylate cyclase after ultragel chromatography (specific activity of 9.4 nmol/10 min per mg, 700 µg protein) was applied to 1 ml bed volume of DEAE-agarose equilibrated with Buffer B. The column was washed with 2 ml of elution buffer and then developed using a linear gradient (30 ml) of elution buffer containing 0—150 mM NaCl. Adenylate cyclase was measured in an assay mixture containing 1 mM ATP, 0.4 mM cyclic AMP and 0.1 mM Gpp(NH)p. Protein was assayed as described in Materials and Methods. The recovery of enzymatic activity was 80%. (B) 2 ml of pretreated enzyme (specific activity of 6.7 nmol/10 min per mg, 400 µg or protein) was applied to 1.0 ml bed volume of ω-aminoakyl agarose (8-carbon arm length). The column was washed with the elution buffer described in A and developed using a linear gradient (30 ml) of elution buffer containing 0—1.0 M NaCl. Adenylate cyclase and protein were assayed as described above. Recovery of enzymatic activity was 90%. (C) 2 ml of pretreated enzyme (specific activity of 1.1 nmol/10 min per mg, 600 µg protein) was applied to 7.0 ml of hydroxyapatite equilibrated with the elution buffer described above and developed using a linear gradient (20 ml) of elution buffer containing 0—200 mM potassium phosphate. Adenylate cyclase was measured in an assay mixture containing 0.1 mM ATP, 40 µM cyclic AMP, and 10 µM Gpp(NH)p. Only 30% recovery of enzymatic activity was obtained from this column, presumably due to inhibition of activity by phosphate present in the column eluates.

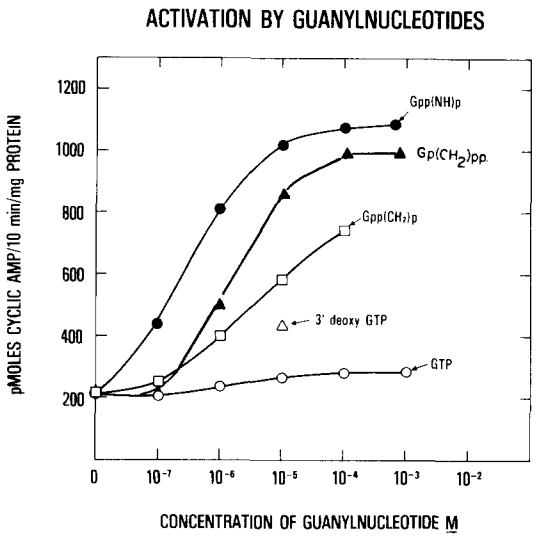


Fig. 4. Activation of partially purified adenylate cyclase by guanine nucleotides. Adenylate cyclase was determined in an assay mixture containing 0.1 mM ATP, 40 μM cyclic AMP, and the indicated amount of guanine nucleotide, as described in Materials and Methods.

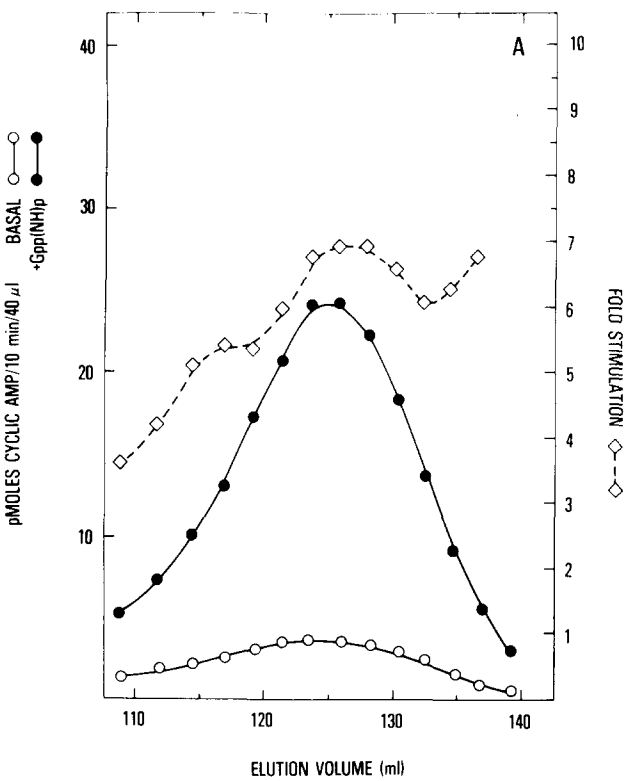


Fig. 5. Comparison of the fold stimulation by Gpp(NH)p of column fractions from an Ultrogel AcA22 column. Column chromatography after Lubrol solubilization of untreated membranes was carried out as described for Table II. Adenylate cyclase was measured in an assay mixture containing 0.1 mM ATP, 40 μM cyclic AMP, in the presence and absence of 10 μM Gpp(NH)p.

Finally, a phenomenon observed repeatedly with the untreated enzyme was a systematic variation in the degree of stimulation attained with Gpp(NH)p in the column fractions; i.e. those fractions eluting first appear to have less susceptibility to activation by Gpp(NH)p than those eluting later. A representative experiment is shown in Fig. 5.

Discussion

The studies presented in this paper were undertaken to employ conventional protein purification techniques for the isolation of Lubrol-solubilized adenylate cyclase. Our conclusions are that such an approach can yield only a partial enzyme purification *. The question is why? We believe it is naive to assume that this is because all the proteins present in the adenylate cyclase-enriched fractions have similar size and charge properties. In fact, the protein-banding pattern of this fraction on sodium dodecyl sulfate polyacrylamide gels indicated that it contained polypeptide components having a wide variety of molecular weights; this certainly argues against a similarity of protein sizes. A more logical explanation is that these proteins are associated together in the form of detergent-phospholipid-protein complexes. The extremely close, invariant association of protein and adenylate cyclase profiles under a wide variety of charge and hydrophobic chromatographic conditions (for example, see Fig. 3) would certainly support this hypothesis. In actuality this possibility has some interesting implications for the organization of functionally associated proteins within the membrane and for the mechanism by which Lubrol PX solubilizes such components from membranes. Studies presented elsewhere [47] have demonstrated that after partial purification, solubilized adenylate cyclase still has guanine nucleotide regulatory and hormone receptor components associated with it. This suggests that components of adenylate cyclase may exist in the membrane as specialized tightly associated units which are solubilized by Lubrol PX as discrete structures rather than as individual proteins. This possibility has precedence from other studies showing that neutral detergents solubilize lipid-protein-detergent complexes from mitochondria [48], chloroplasts [49,50], and the sarcoplasmic reticulum [51,52].

The physical properties of Lubrol-solubilized adenylate cyclase (Stokes radii, sedimentation coefficients, hydrophobicity, and molecular weight) have been reported prior to complete purification of this enzyme [12]. Our observations suggest that these measured physical parameters may reflect a lipid-protein-detergent complex rather than the intrinsic physical properties of adenylate cyclase. This problem becomes even more apparent when one considers that

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* Subsequent to completion of this manuscript, a report appeared stating that adenylate cyclase solubilized from rat liver plasma membranes by Lubrol PX had been purified to "homogeneity" as attested by a single protein band on SDS-polyacrylamide gels [56]. After "purification" the enzyme displayed no increase in specific activity over the crude solubilized material, the yield was less than 1% and the enzyme would no longer respond to Gpp(NH)p or fluoride. We find it strange that the specific activity obtained during this "purification" was not significantly greater than that we obtained in our "partial purification" and suggest that, in this case again, adenylate cyclase is but a minor component of an aggregate of which the single protein band observed on SDS polyacrylamide gels is the major constituent.

the enzyme is probably but a fraction of the total protein in the complex. That is, judging from the highest specific activity obtained for the partially purified liver enzyme (1.2 nmol/min per mg) and assuming that its turnover number is that of the highly purified bacterial adenylate cyclase [54], the enzyme comprises less than 0.1% of the protein present in the adenylate cyclase-containing fraction from the Ultrogel column. The idea of determining the physical properties of Lubrol-solubilized adenylate cyclase becomes even more difficult when one considers that properties, such as Stokes radii on Ultrogel columns depend upon the concentration of detergent used in the column elution buffer. For example, in the absence of detergent we have observed that Lubrol-solubilized adenylate cyclase aggregates and elutes in the void volume of the column. Thus, knowledge of the aggregation characteristics of the enzyme further suggests that caution must be used in assigning values to the intrinsic physical properties of the enzyme.

A final interesting aspect of this study concerns the findings that the partially purified enzyme retains the characteristics of Gpp(NH)p and Gp(CH_2)pp activation which have been observed in the intact liver membrane. In addition, the fraction is also depleted of non-specific nucleotide phosphohydrolases and pyrophosphohydrolases and, as shown elsewhere [47], also of Gpp(NH)p-binding sites that are not involved in adenylate cyclase activation. Its enriched content of specific GTPases may help to resolve the question of the possible role of GTPase in adenylate cyclase regulation [55,58]. Thus, though far from purified, the adenylate cyclase-enriched fraction can be used as a model for testing certain limited questions such as the relation of the glucagon receptor to this fraction and the role of guanine nucleotides in the activation process. These are questions that are currently being studied in our laboratory.

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References

1 Rodbell, M., Krans, H.M.J., Pohl, S.L. and Birnbraumer, L. (1971) J. Biol. Chem. 246, 1872—1876
2 Rodbell, M., Birnbaumer, L., Pohl, S.L. and Krans, H.M.J. (1971) J. Biol. Chem. 246, 1877—1882
3 Goldfine, I.D., Roth, J. and Birnbaumer, L. (1972) J. Biol. Chem. 247, 1211—1218
4 Krishna, G., Harwood, J.P., Barber, A.J. and Jamieson, G.A. (1972) J. Biol. Chem. 247, 2253—2254
5 Wolff, J. and Cook, G.H. (1973) J. Biol. Chem. 248, 350—355
6 Bockaert, J., Roy, C. and Jard, S. (1972) J. Biol. Chem. 247, 7073—7081
7 LeRay, F., Chambaut, A. and Hanoune, J. (1972) Biochem. Biophys. Res. Commun. 48, 1385—1390
8 Harwood, J.P., Low, H. and Rodbell, M. (1973) J. Biol. Chem. 248, 6239—6245
9 Stellwagen, E. and Baker, B. (1976) Nature 261, 719—720
10 Levy, G.S. (1970) Biochem. Biophys. Res. Commun. 38, 86—92
11 Swislocki, N.I. and Tierney, J. (1973) Biochemistry 12, 1862—1866
12 Neer, E.J. (1974) J. Biol. Chem. 249, 6527—6531

13 Pilkis, S.J. and Johnson, R.A. (1974) Biochim. Biophys. Acta 341, 388—395
14 Ryan, J. and Storm, D.R. (1974) Biochem. Biophys. Res. Commun. 60, 304—311
15 Pfeuffer, T. and Helmreich, E.J.M. (1975) J. Biol. Chem. 250, 867—876
16 Swislocki, N.I. and Tierney, J. (1975) Arch. Biochem. Biophys. 168, 455—462
17 Queener, S.F., Fleming, J.W. and Bell, N.H. (1975) J. Biol. Chem. 250, 7586—7593
18 Roy, C., Rajerison, R., Bockaert, J. and Jard, S. (1975) J. Biol. Chem. 250, 7885—7894
19 Johnson, R.A., Garbers, D.L. and Pilkis, S.J. (1976) J. Supramol. Struct. 4, 205 (165)—220 (180)
20 Londos, C., Salomon, Y., Lin, M.C., Harwood, J.P., Schramm, M., Wolff, J. and Rodbell, M. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3087—3090
21 Schramm, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 2232—2237
22 Neville, D.R. (1968) Biochim. Biophys. Acta 154, 540—552
23 Pohl, S.L., Birnbaumer, L. and Rodbell, M. (1971) J. Biol. Chem. 246, 1849—1856
24 Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541—548
25 Abrams, A., Baron, C. and Schnebli, H.P. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.), Vol. XXXIII, pp. 428—439
26 Avruch, J. and Wallach, D.F.H. (1971) Biochim. Biophys. Acta 233, 334—347
27 Luzio, J.P., Newby, A.C. and Hales, C.N. (1976) Biochem. J. 154, 11—21
28 Worthington Enzyme Manual (1972) p. 41, Worthington Biochemical Corp., Freehold, N.J.
29 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
30 Ross, E. and Schatz, G. (1973) Anal. Biochem. 54, 304—306
31 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
32 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769—775
33 Itaya, K. and Ui, M. (1966) Clin. Chim. Acta 14, 361—366
34 Neville, Jr., D.M. and Glossman, H. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.), Vol. XXXIII, pp. 92—102, Academic Press, New York
35 Lamed, R., Levin, Y. and Wilcheck, M. (1973) Biochim. Biophys. Acta 304, 231—235
36 Lindberg, M. and Mosbach, K. (1975) Eur. J. Biochem. 53, 481—486
37 Cuatrecasas, P., Jacobs, S. and Bennett, V. (1975) Proc. Natl. Acad. Sci. U.S. 72, 1739—1743
38 Neer, E.J. (1973) J. Biol. Chem. 248, 3742—3744
39 Giorgio, N.A., Johnson, C.B. and Blecher, M. (1974) J. Biol. Chem. 249, 428—437
40 Handbook of Biochemistry (Sober, H.A., ed.) 2nd edn., pp. C62—C66, The Chemical Rubber Co., Cleveland, Ohio
41 Pavelich, M.J. and Hammes, G.G. (1973) Biochemistry 12, 1408—1412
42 Vesterberg, O. (1971) in Methods in Enzymology (Jakoby, W.B., ed.), Vol. XXII, pp. 389—412, Academic Press, New York
43 Er-el, Z., Zaidenzaig, Y. and Shaltiel, S. (1972) Biochem. Biophys. Res. Commun. 49, 383—390
44 Shaltiel, S. and Er-el, Z. (1973) Proc. Natl. Acad. Sci. U.S. 70, 778—781
45 Salomon, Y., Lin, M.C., Londos, C., Rendell, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 4239—4245
46 Londos, C., Lin, M.C., Welton, A.F., Lad, P.M. and Rodbell, M. (1977) J. Biol. Chem., submitted for publication
47 Welton, A.F., Lad, P.M., Newby, A.C., Yamamura, H., Nicosia, S. and Rodbell, M. (1977) J. Biol. Chem., in the press
48 Kagawa, Y. (1972) Biochim. Biophys. Acta 265, 297—338
49 Anderson, J.M. and Boardman, N.K. (1966) Biochim. Biophys. Acta 112, 403—421
50 Shibuya, I., Horda, H. and Mauro, B. (1968) J. Biochem. Tokyo 64, 571—576
51 Nelson, N. and Neuman, J. (1972) J. Biol. Chem. 247, 1817—1824
52 MacLennan, D.H. (1970) J. Biol. Chem. 245, 4508—4518
53 Meissner, G. and Fleisher, S. (1974) J. Biol. Chem. 249, 302—309
54 Takai, K., Kurashina, Y., Suzuki-Hort, C., Okamoto, H. and Hayaishi, O. (1974) J. Biol. Chem. 249, 1965—1972
55 Salomon, Y. and Rodbell, M. (1975) J. Biol. Chem. 250, 7245—7250
56 Swislocki, N.I., Magnuson, T. and Tierney, J. (1977) Arch. Biochem. Biophys. 179, 157—165
57 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
58 Cassel, D. and Selinger, Z. (1976) Biochim. Biophys. Acta 452, 538—551