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THE HLB DEPENDENCY FOR DETERGENT SOLUBILIZATION OF HORMONALLY SENSITIVE ADENYLATE CYCLASE

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The HLB dependency for the solubilization of membrane proteins and adenylate cyclase activity from a plasma membrane-enriched fraction from rat liver has been determined. The HLB (hydrophilic/lipophilic/balance) number of a detergent is an empirical measure of its relative hydrophobicity. Detergent HLB numbers vary systematically with the length of the ethylene oxide chain for a homologous series of detergents such as the Triton X series. These detergents have a constant hydrophobic moiety, octylphenyl, and a variable polar portion, polyethoxyethanol. Basal-NaF-epinephrine-, and glucagon-stimulated adenylate cyclase activities were solubilized in the HLB range of 16.8–17.4. Solubilization was most effective in 0.01 M Tris buffers at pH 7.5 containing 1–5 mM mercaptoethanol, 1 mM $MgCl_2$, and 0.1% Triton X-305. The detergent to membrane protein ratio used in these studies was 3:1.

Criteria for solubilization included lack of sedimentation at 100,000 X g, the absence of particulate material in the supernatant when examined by electron microscopy, and inclusion of hormonally sensitive adenylate cylcase activity in Sephadex G-200 gels. The apparent molecular weight of the solubilized enzyme was approximately 200,000 in the presence of Triton X-305. The solubilized enzyme was stimulated 5-fold by NaF, 7-fold by glucagon, and 20-fold by epinephrine compared to the particulate enzyme used in this study which was stimulated 10-fold, 3.4-fold, and 4-fold by NaF, epinephrine, and glucagon, respectively. The solubilized enzyme is stable for several weeks when stored at −60°C.

INTRODUCTION

Membrane-associated adenylate cyclase activities from a wide variety of animal tissues are stimulated by peptide hormones and/or catecholamines (1–4). This phenomenon has attracted a great deal of interest because of the crucial role that c-AMP plays in the control of cellular metabolism (5). A great deal of useful information about the adenylate cyclase system has been obtained using relatively crude preparations, such as partially purified plasma membranes and crude particulate preparations. However, solution of this problem will ultimately require solubilization, purification, and characterization of hormonally sensitive adenylate cyclase. Basal- and NaF-stimulated activities have been solubilized and are being characterized in several laboratories (6).

The most effective agents for solubilization of membrane enzymes have been non-ionic detergents. Unfortunately, there are literally thousands of detergents available commercially and there has not been, up until recently, a systematic method for screening detergents for solubilization studies. Umbreit and Strominger have found a striking correlation between detergent solubilization of several bacterial membrane enzymes and

detergent HLB numbers (7, 8). The HLB (hydrophilic/lipophilic/balance) number of a detergent is a measure of its relative hydrophobicity (9). For a homologous family of detergents such as the Triton X series, HLB numbers increase systematically with the length of the ethylene oxide chain. As the HLB number increases, polarity increases, the degree of hydration increases, the critical micelle concentration increases, and the detergent micelle size decreases. We have used the HLB classification as a guide in screening detergents for solubilization of adenylate cyclase from rat liver. In this report, the HLB dependency for solubilization of adenylate cyclase is described and some properties of the solubilized enzyme are discussed.

METHODS

Materials

Male or female Sprague-Dawley rats were purchased from Murphy Breeding Inc., Plainfield, Ind. Triton detergents were obtained from Sigma Chemical Co. and Rohm and Haas. Atlas G 1292, G 1790, Tween 40, and Tween 60 were supplied by Atlas Chemical Industries, and Neodol 25-9 was obtained from Shell Chemical Co. All detergents were technical grade and were used without further purification. Detergent HLB numbers are tabulated in Table I. Each of these detergents is a family of chain lengths giving an average HLB number. ATP, c-AMP, epinephrine, and glucagon were purchased from Sigma Chemical Co. [^{3}H] c-AMP and [^{3}H]-d, l-epinephrine were purchased from New England Nuclear. α[^{32}P] ATP was synthesized from [^{32}P] H_3PO_4 and isopropylidene adenosine (10).

Assay of Adenylate Cyclase Activity

Adenylate cylcase activity was assayed at 30°C by the Krishna technique using either [^{3}H] ATP and [^{14}C] c-AMP or α-[^{32}P] ATP and [^{3}H] c-AMP (11). The assay mixture (50 μl) contained 5 mM $MgCl_2$, 1 mM ATP, 2 mM c-AMP, 20 mM creatine phosphate, 20 mM Tris-HCl, pH 7.6, 1 mg/ml creatine kinase. All samples were assayed in triplicate. When NaF, epinephrine, or glucagon were present they were at concentrations of 15 mM, 10 μM, and 1μM respectively.

Preparation of Rat Liver Plasma Membranes

Partially purified plasma membranes were prepared by the Neville method with one modification (12). The first homogenization was performed with a Dounce homogenizer having a 16-sec drop time. These membranes were stored at −60°C suspended in 1 mM $HaHCO_3$.

Solubilization of Membranes with Nonionic Detergents

Membrane samples were treated with 0.1% (wt/vol) detergent solutions in 0.01 M Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol. The ratio of detergent to membrane protein (wt/wt) was kept constant at 3.0 to 1.0. Detergent-membrane mixtures were incubated for 30 min at 4°C and gently agitated every 5 min. These solutions were then centrifuged at 100,000 × g for 1 hr at 4°C. The supernatants were carefully withdrawn, an aliquot was assayed for protein, and the remainder was concentrated by lyophilization and assayed for adenylate cyclase activity.

Epinephrine Binding Assay

Binding of [^{3}H]-d, l-epinephrine to membranes and solubilized preparations was measured using the Millipore filter assay (13). This particular technique measures epinephrine binding activity and is not intended to be an indicator of catecholamine receptor activities (14).

SDS Polyacrylamide Gel Electrophoresis

Membrane samples or detergent-solubilized extracts were frozen and lyophilized to dryness. The lyophilized samples were then resuspended in 100 μl of sample buffer containing 10 mM phosphate, pH 7.2, 1% SDS, 0.14 M β-mercaptoethanol, 10% (vol/vol) glycerol, and 0.002% bromphenol blue. After boiling for 5 min, the samples were run on 7.5% polyacrylamide gels. Lyophilization of membrane samples and detergent-solubilized extracts before SDS gel electrophoresis eliminated accumulation of protein at the origin of the gel which occurred when samples were not lyophilized. Gels were fixed in 5% sulfosalicylic acid-5% trichloroacetic acid for 1 hr at room temperature and stained for protein with 0.025% Coomassie blue in 25% isopropanol-10% acetic acid at room temperature overnight. Coomassie blue-stained gels were destained by soaking for 3 hr in 25% isopropanol-10% acetic acid followed by 10 hr in 5% methanol-5% acetic acid and then soaking for several days in 10% acetic acid. During all staining and destaining procedures gels were continuously rotated to insure uniform access of solvent.

Quantitation of Proteins on SDS Polyacrylamide Gels

Coomassie blue-stained gels were scanned at 540 nm using a Gilford linear transport system. The absorbance of each protein peak at 540 nm was found to be linearly proportional to the amount of that protein applied to the gel. Varying amounts of membrane protein or bovine serum albumin were run on SDS polyacrylamide gels and scanned for absorbance at 540 nm. For a single protein, such as bovine serum albumin, this relationship was linear up to 15 μg of bovine serum albumin added per gel. When SDS-solubilized rat liver plasma membrane was used, there was a linear relationship between total membrane protein added to the gel and the absorbance of individual protein bands up to 100 μg of total membrane protein. The integrated areas of protein bands could not be used for quantitation with mixtures of membrane proteins due to extensive overlap of individual bands. The maximum absorbance for individual bands could always be unambiguously determined. In order that this quantitation procedure be reliable for relative comparisons, it was necessary that samples be run simultaneously on parallel gels and destained under identical conditions.

Sephadex G-200 Gel Chromatography

A particulate preparation of rat liver plasma membranes obtained from the second low speed spin (1,500 × g) of the Neville preparation (12) was solubilized with Triton X-305 as described above. After centrifugation for 1 hr at 100,000 × g, 4 ml of the supernatant was applied to a Sephadex G-200 column (2.5 × 95 cm). The column was eluted with 0.1% Triton X-305 in 0.01 M Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol at a flow rate of 1 ml/3 min. After the first 15 fractions, each fraction (4.5 ml) was frozen at −60°C. Fractions from the column were assayed for adenylate

cylcase activity in the presence of epinephrine using 20-μl aliquots as described above. The void volume of the column (Fig. 8) was determined using Blue Dextran applied to the column in 4 ml of the detergent containing buffer described above.

RESULTS AND DISCUSSION

HLB Dependency for Solubilization of Protein from Rat Liver Plasma Membranes

As a preliminary to solubilization of adenylate cyclase, the general HLB dependency for solubilization of rat liver membrane protein was examined. The detergents used and their HLB numbers are given in Table I. In the first experiment, membrane samples were treated with detergent solutions, as described in Methods, centrifuged at 100,000 × g for 1 hr, and the supernatants were assayed for protein by the Lowry method (15). The percentage of membrane protein solubilized as a function of HLB number is given in Fig. 1. Membrane protein was solubilized to one extent or another by all of these detergents. The plot in Fig. 1 exhibits a broad maximum in the HLB range 12–14 and another peak at 16–17.5. These data represent solubilization of total membrane protein and it was thought that the HLB dependency for solubilization of individual polypeptides might be more enlightening. Therefore, the HLB dependency for solubilization of 12 membrane polypeptides (identified by their mobility on SDS gels) was examined. This data is given in Fig. 2. The apparent molecular weights of these 12 components on SDS gels is given in Table II. Individual polypeptides were solubilized with pronounced HLB dependencies. Each curve shows two maxima; one in the HLB range 12–14 and a second at 16–17. Despite the similarities in these curves, it is clear that each polypeptide was solubilized with a unique HLB dependency. For example, component no.1 was maximally solubilized at an HLB no. of 13.1, whereas components nos. 2 and 5 were optimally solubilized at HLB numbers of 12.4 and 15.6. When attempting to solubilize and purify a specific membrane enzyme, it would be advantageous to use detergents in the 14–15.5 HLB range or at HLB numbers greater than 17 because less total membrane protein is

TABLE I. HLB Numbers of Detergents Used in Solubilization Studies

	Detergent	HLB Number[1]
1	Triton X-207	10.7
2	Atlas G 1790	11.0
3	Triton X-114	12.4
4	Neodol 25-9	13.1
5	Triton N-101	13.4
6	Triton X-100	13.5
7	Triton N-111	13.8
8	Triton CF-10	14.0
9	Triton X-102	14.6
10	Tween 60	14.9
11	Tween 40	15.6
12	Triton X-165	15.8
13	Triton X-67	16.8
14	Triton X-305	17.3
15	Triton X-405	17.9

[1] HLB numbers for commercial detergents are tabulated in McCutcheon's Detergents and Emulsifiers (20).

TABLE II. Molecular Weight Distribution of Polypeptides Solubilized[1] from Rat Liver Plasma Membranes

Components	Apparent molecular weight
1	263,000
2	240,000
3	214,000
4	186,000
5	166,000
6	126,000
7	79,400
8	63,100
9	52,500
10	44,700
11	40,000
12	31,000

[1] Polypeptides 1–12 were identified by virtue of their mobility on SDS gels.

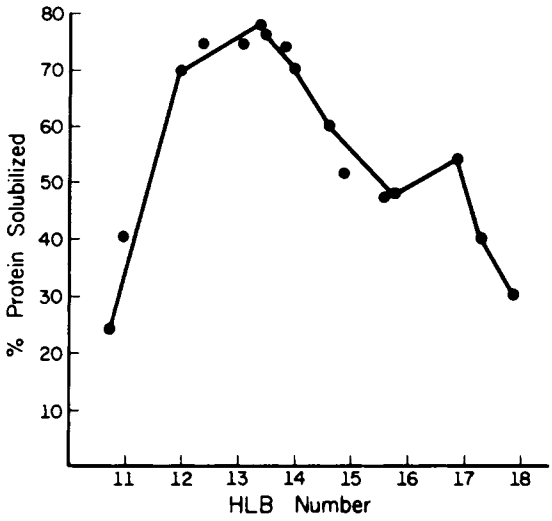

Fig. 1. HLB dependencies for solubilization of total membrane protein from rat liver plasma membranes. A sample of membranes containing 1 mg of protein was treated with 3 ml of detergent solution containing 0.1% (wt/vol) detergent in 0.01 M Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol for 30 min at 4°C. The sample was then centrifuged at 100,000 × g for 1 hr and the supernatants were assayed for protein by the Lowry method (15). Protein solubilized is expressed as the percentage of protein solubilized relative to the total amount of membrane protein submitted to solubilization. The detergents used and their HLB numbers are given in Table I.

solubilized by these detergents. Thus, if a membrane enzyme can be successfully solubilized in the HLB ranges described above, it may be possible to achieve significant purification of the enzyme during the solubilization step. In addition, these data suggested that detergent HLB numbers might serve as a useful general guide when screening detergents for solubilization of a specific membrane activity. Therefore, a series of Triton X detergents representing a broad HLB range (10.7–17.9) were tested for their ability to solubilize adenylate cyclase in a hormonally sensitive form.

The HLB Dependency for Solubilization of Adenylate Cyclase

The Triton detergents listed in Table I were examined for their effectiveness in solubilizing adenylate cyclase from partially purified rat liver plasma membranes. It is extremely important to emphasize that success in solubilizing adenylate cyclase depends strongly on the properties of the particulate preparation used for solubilization. The plasma membrane-enriched preparation used in these studies had basal-, NaF-, epinephrine- and glucagon-stimulated activities of 0.45, 4.58, 1.52, and 1.97 nmoles/mg/10 min, respectively. We have found considerable variation in adenylate cyclase activities from one preparation to another, particularly with epinephrine-stimulated activities. Wallach has estimated that the yield of plasma membranes obtained using the general Neville preparation is approximately 10% (17). This low yield, coupled with the heterogeneity of cell types obtained with crude rat liver homogenates probably accounts for the variability in enzyme activities observed with different membrane samples.

The HLB dependencies for solubilization of basal-, NaF-, epinephrine-, and glucagon-stimulated adenylate cyclase activities are given in Figs. 3–6. Basal activity was solubilized most effectively in the lower HLB range (Triton X-207 and X-114), and in the range from

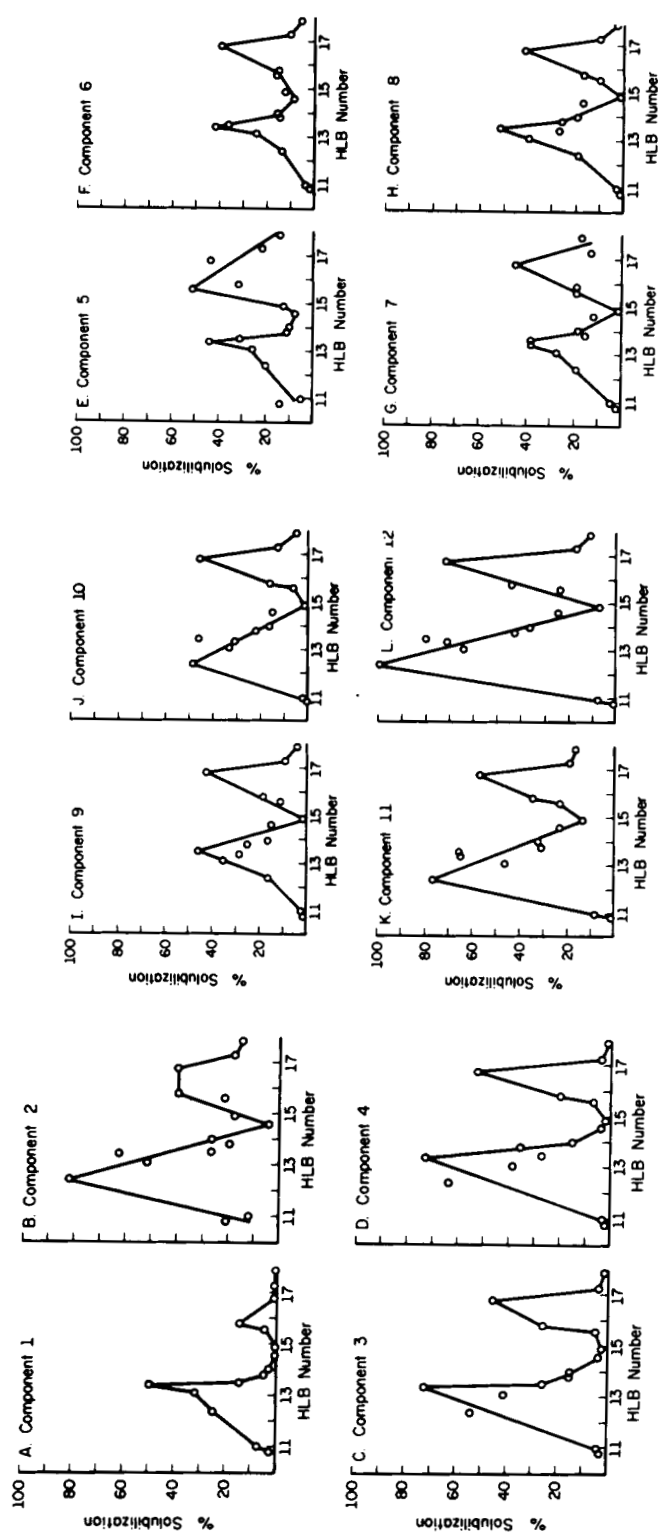

Fig. 2. HLB dependency for solubilization of individual polypeptides from rat liver plasma membranes. Components 1–12 (A–L) are 12 polypeptides identified by their mobility on SDS polyacrylamide gels. Membrane samples containing 100 μg of protein were treated with 0.1% solutions of the 15 detergents listed in Table I. The 100,000 × g supernatant from each detergent extract and one whole membrane sample containing 100 μg of protein were run on 7.5% polyacrylamide gels in the presence of SDS, stained with Coomassie blue, and scanned at 540 nm. All gells were destained under identical conditions and the amount of each component solubilized was compared to the amount of that peptide in the control which consisted of 100 μg of membrane protein solubilized with 1% SDS and directly run on a SDS polyacrylamide gel.

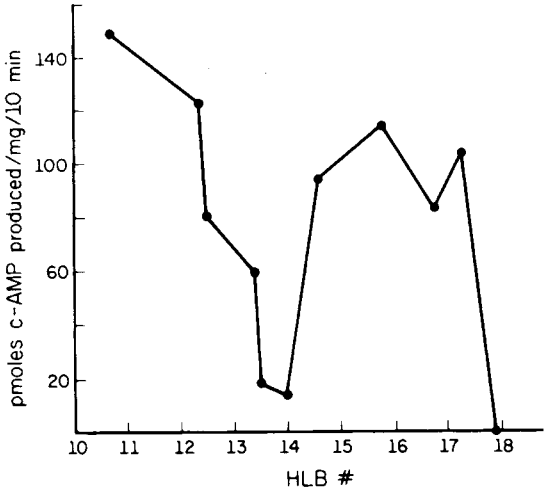

Fig. 3. The HLB depen 'ency for detergent solubilization of basal adenylate cyclase activity. Partially purified rat liver plasma membranes were solubilized with the various detergents listed in Table I by the procedure described in Methods. The 100,000 × g supernatants were concentrated (5- to 10-fold) by lyophilization, frozen overnight at −60°C, and assayed for adenylate cyclase activity as described in Methods. The amount of solubilized protein used in each assay varied from 10 to 40 μg of protein.

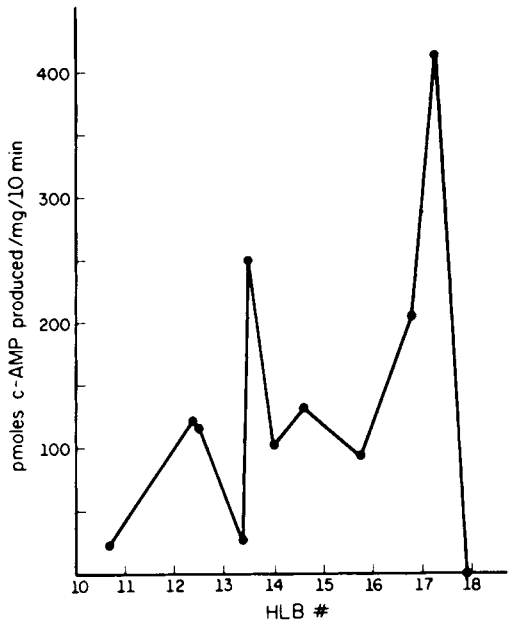

Fig. 4. The HLB dependency for the detergent solubilization of NaF-stimulated adenylate cyclase activity. Experimental details are given in the legend to Fig. 3.

14.6 to 17.3. Solubilization of NaF-, epinephrine-, and glucagon-stimulated activities was most effective in the HLB range from 16.9 to 17.3. The effectiveness of Triton X-305 as a solubilizing agent for hormone-stimulated adenylate cyclase was striking and was, in our hands, the most effective detergent for solubilization of adenylate cyclase. Triton X-305

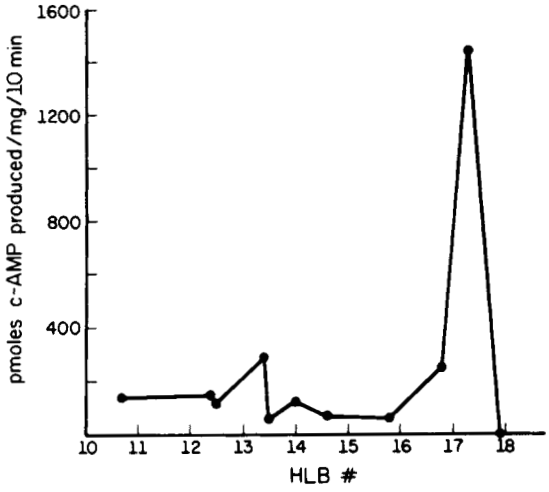

Fig. 5. The HLB dependency for solubilization of epinephrine-sensitive adenylate cyclase activity. Experimental details are given in the legend to Fig. 3.

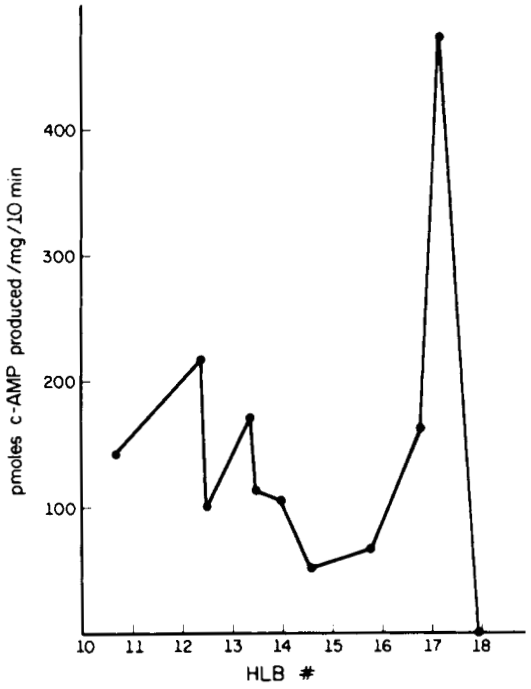

Fig. 6. The HLB dependency for solubilization of glucagon-stimulated adenylate cyclase activity. Experimental details are given in the legend to Fig. 3.

is a relatively polar nonionic detergent and most detergents examined for solubilization of adenylate cyclase have had HLB numbers in the 12–15 HLB range (6, 18). It is interesting that although all of the adenylate cyclase activities were solubilized in the HLB range from 16.8 to 17.4, basal- and hormone-stimulated activities show different HLB profiles. This might suggest that these various adenylate cyclase activities are attributable to different molecules or that these activities are differentially inhibited to varying extents by the detergents examined. However, the existence of different forms of adenylate cyclase cannot be confirmed unambiguously until purified preparations are obtained. The HLB dependency for solubilization of an epinephrine binding activity was also examined (Fig. 7). This activity was also solubilized with highest specific activity in the 16–17.5 HLB range. It should be emphasized, however, that this binding activity was not the catecholamine receptor coupled to adenylate cyclase but was most probably nonreceptor proteins, such as catecholamine 0-methyl transferase or the proteins responsible for uptake of catecholamines (14). However, it is interesting that this binding activity was solubilized with a pronounced HLB dependency.

Properties of Solubilized Adenylate Cyclase

Failure to sediment at centrifugal forces $\geqslant$ 100,000 X g for extended periods of time is a necessary but not sufficient criterion for solubilization of a membrane enzyme. The 100,000 X g supernatant was examined for particulate material with the electron microscope. At a magnification of 10,000 this sample was virtually free of discernible particles (19). In addition, the solubilized enzyme was submitted to Sephadex G-200 gel chromatography. Hormonally sensitive adenylate cyclase activity was included in the gel with a molecular weight of approximately 200,000 (Fig. 8). These data, taken collectively, indicate that hormonally sensitive adenylate cyclase was indeed solubilized and not merely associated with dispersed membrane fragments.

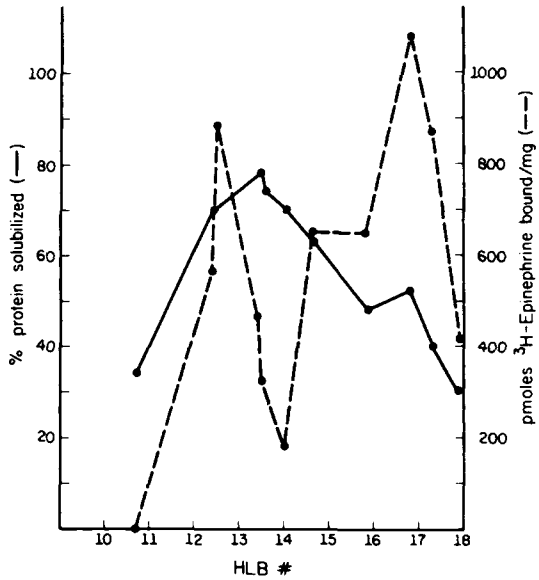

Fig. 7. The HLB dependency for solubilization of epinephrine binding activity from rat liver membranes. Experimental details for solubilization conditions are given in the legend to Fig. 3 and the epinephrine binding assay is described in Methods.

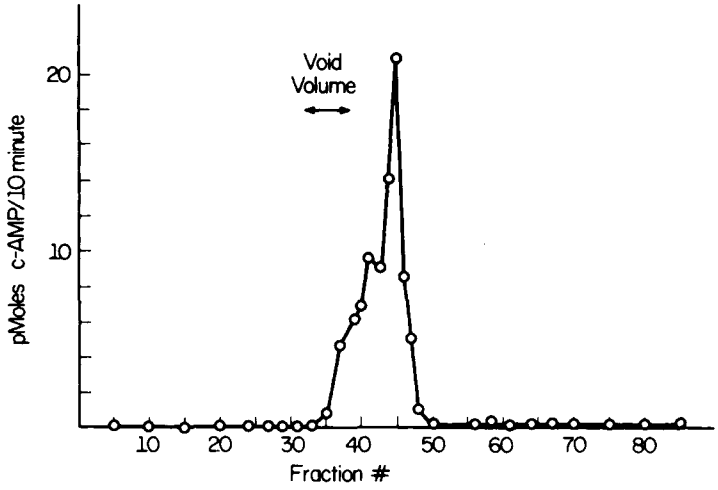

Fig. 8. Sephadex G-200 gel chromatography of solubilized adenylate cyclase activity. A sample of particulate rat liver membranes obtained from the second low speed pellet of the Neville preparation (12) was solubilized with Triton X-305 as described in Methods. 4 ml of the 100,000 × g supernatant was applied to a Sephadex G-200 column (2.5 × 95 cm) and eluted with 0.1% Triton X-305 in 0.01 M Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol. 20 μl aliquots were assayed for adenylate cyclase activity in the presence of epinephrine as described in Methods.

The stability of the solubilized enzyme has not been systematically examined. However, this activity was stable when stored at −60°C for several weeks and activity was retained after concentration by lyophilization or ultrafiltration on UM-10 filters. As yet, no attempt has been made to remove detergent from this preparation. Repeated freezing and thawing of the solubilized enzyme preparation resulted in total loss of basal- and hormone-stimulated activities. In preliminary studies, solubilized adenylate cyclase activity was subjected to several purification procedures which included gel chromatography on Sephadex G-200, an acidic pH step (pH 4.8) in which activity was retained in the supernatant, and precipitation by 20% ammonium sulfate. The purifications obtained by these three methods were 50-fold, 2.5-fold and 3-fold, respectively (16). These preliminary results are encouraging since they demonstrate that the solubilized enzyme can be manipulated in various ways without losing hormonal sensitivity.

Solubilization of Adenylate Cyclase Using Different Forms of Rat Liver

The solubilization studies described thus far were done using partially purified rat liver plasma membranes. It is expected that solubilization of adenylate cyclase will be most effective using plasma membrane preparations rather than cruder particulate preparations. The data in Table III support this conclusion. Solubilization of adenylate cyclase was attempted using three different forms of rat liver (minced liver, low speed pellets from the Neville preparation, and partially purified plasma membranes). In all cases, the solubilizing buffer contained 0.1% Triton X-305, 1–5 mM mercaptoethanol, and 1 mM $MgCl_2$. The advantage in using partially purified plasma membranes is evident from the data in Table III. The specific activities of the enzyme solubilized from plasma membranes were 10- to 20-fold greater than that solubilized from the Neville low speed pellets. Furthermore, the degree of hormone stimulation was much greater when the enzyme was solubilized from plasma membranes. The observation that the degree of

TABLE III. Solubilization of Adenylate Cyclase Using Different Forms of Rat Liver

	[1] Minced liver	Stimulation over basal	Low speed pellet	stimulation over basal	Plasma membrane	Stimulation over basal
Basal	0.0016	–	0.031	–	0.11	–
NaF	0.0024	1.5X	0.058	1.9X	0.59	5.4X
Epinephrine	0.0026	1.6X	0.102	3.3X	2.21	20X
Glucagon	0.0021	1.3X	0.063	2.0X	0.77	7X

[1] Adenylate cyclase activities expressed in nmoles/mg/10 min. Three forms of rat liver (minced liver, low speed pellet from Neville preparation, partially purified plasma membranes) were treated with 0.1% Triton X 305 in 0.01 M Tris, pH 7.5, containing 1 mM mercaptoethanol and 1 mM $MgCl_2$ as described in Methods. In each case the 100,000 × g supernatant was assayed for adenylate cyclase activity.

hormone stimulation obtained with enzyme solubilized from different forms of rat liver suggests that perhaps the more heterogeneous preparations contain inhibiting contaminants which suppress hormone stimulation in solubilized extracts. The heterogeneity of these solubilized preparations makes it difficult to interpret these differences in hormone stimulation, but they do illustrate that the properties of the particulate preparation are a crucial determinant for successful solubilization of hormonally sensitive adenylate cyclase.

The availability of solubilized, hormonally sensitive adenylate cyclase should be of great value in defining the mechanism for hormonal stimulation of adenylate cyclase. The next major technical barrier will be purification of the solubilized enzyme in sufficient quantity for future studies.

ACKNOWLEDGMENTS

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