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THE SIZE OF ADENYLATE CYCLASE AND GUANYLATE CYCLASE FROM THE RAT RENAL MEDULLA

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The size distribution of adenylate cyclase from the rat renal medulla solubilized with the nonionic detergents Triton X-100 and Lubrol PX was determined by gel filtration and by centrifugation in sucrose density gradients made up in H_2O or D_2O. The physical parameters of the predominant form in Triton X-100 are $s_{20,w}$, 5.9 S; Stokes radius, 62 A; partial specific volume ($\bar{v}$), 0.74 ml/g; mass, 159,000 daltons; f/f_o, 1.6; axial ratio (prolate ellipsoid), 11. For the minor form the values are: $s_{20,w}$, 3.0; Stokes radius, 28 A; mass, 38,000 daltons; f/f_o, 1.2. The corresponding values determined in Lubrol PX are similar.

The value for $\bar{v}$ for the enzyme indicates that it binds less than 0.2 mg detergent/mg protein. Since interactions with detergents probably substitute for interactions with lipids and hydrophobic amino acid side chains, these findings suggest that no more than 5% of the surface of adenylate cyclase is involved in hydrophobic interactions with other membrane components. Thus, most of the mass of the enzyme is not deeply embedded in the lipid bilayer of the plasma membrane.

Similar studies have been performed on the soluble guanylate cyclase of the rat renal medulla. In the absence of detergent, the molecular properties of this enzyme are: $s_{20,w}$, 6.3 S; Stokes radius, 54 A, $\bar{v}$, 0.75 ml/g; mass, 154,000 daltons f/f_o, 1.4; axial ratio, 7. The addition of 0.1% Lubrol PX to this soluble enzyme increases its activity two- to fourfold and changes the physical properties to: $s_{20,w}$, 5.5 S; Stokes radius, 62 A; $\bar{v}$, 0.74 ml/g; mass, 148,000 daltons; f/f_o, 1.6; axial ratio, 11. These results show that Lubrol PX activates the enzyme by causing a conformational change with unfolding on the polypeptide chain.

Guanylate cyclase from the particulate cell fraction can be solubilized with Lubrol PX but has properties quite different from those of the enzyme in the soluble cell fraction. It is a heterogeneous aggregate with $s_{20,w}$, 10S; Stokes radius, 65 A; mass about 300,000 daltons. The conditions which solubilize guanylate cyclase also solubilize adenylate cyclase and the two activities can be separated on the same sucrose gradient.

Mammalian cells contain two principal enzymes which cyclize purine nucleotides – adenylate cyclase and guanylate cyclase. The former is associated with the plasma membrane of mammalian cells. It occurs in all cells and is activated by a number of hormones. Activation of the enzyme can be easily demonstrated in broken cell preparations. Guanylate cyclase, on the other hand, is found both in association with particulate cell fractions and in the supernatant of cell homogenates. The distribution between these

forms varies from tissue to tissue, ranging from entirely particulate in the small intestine (1) to virtually entirely soluble in lung, spleen, and liver (2). In contrast to adenylate cyclase, it has been very difficult to demonstrate stimulation of guanylate cyclase by hormones in broken cell systems (3).

This paper describes studies on the physical properties of adenylate and guanylate cyclase from the rat renal medulla. With both enzymes, the object of the studies was to get some information about the nature of their interaction with cell membranes.

In the renal medulla, as in other mammalian tissues, adenylate cyclase can be dislodged from the plasma membrane only by the use of detergents. The requirement for detergent to solubilize the enzyme suggests that some part of the adenylate cyclase surface must be in contact with lipids or with hydrophobic regions of membrane proteins. Detergents probably solubilize proteins from cell membranes by substituting for the surrounding hydrophobic membrane constituents (4). Therefore, the quantity of detergent bound by a solubilized membrane protein may be a function of how much of the protein's surface is hydrophobic. To test this hypothesis, one could study the detergent binding of a series of proteins known on other, independent, grounds to be deeply embedded in the lipid bilayer of their membranes and compare this to the detergent binding of freely soluble globular proteins with hydrophilic exteriors. Helenius and Simons (5) and Clarke and Farber (6) have carried out such studies and have found that proteins normally associated with lipids or proteins known to span the lipid bilayer bind detergent with a range of 0.2 mg/mg of Na^+/K^+ ATPase – 1.1 mg/mg of rhodopsin. A series of nine water soluble proteins bound a tenth or twentieth as much detergent, a virtually undetectable amount.

In these studies, detergent binding was measured directly with tritiated Triton X-100. This method cannot be used when the protein of interest is only a small fraction of the total protein and has not yet been significantly purified from any mammalian cell. Adenylate cyclase probably makes up only 0.005% of the plasma membrane protein or about 1 part in 500,000 of the total cell protein. Its abundance in the membrane is about the same as that calculated for the insulin receptor (7).

Fortunately, the nonionic detergents commonly used to solubilize active membrane enzymes, Triton X-100 and Lubrol PX, do have a property which allows one to measure the degree of binding by another means. This property is their partial specific volume which is markedly different from that of a typical protein. A complex of detergent and protein would have a partial specific volume ($\bar{v}$) which is intermediate between that of the protein and that of the detergent and which would be proportional to the fraction of each. In complex mixtures of proteins, it is possible to measure the partial specific volume of one component by comparing its rate to sedimentation in sucrose gradients made up in H_2O and D_2O. To obtain the value for $\bar{v}$ from such a pair of centrifugations one solves the following equation for $\bar{v}$:

$$s_D \left(\frac{\eta_D}{\eta_{20,w}}\right)\left(\frac{1-\bar{v}\rho_{20,w}}{1-\bar{v}\rho_D}\right) = s_{20,w} = s_H \left(\frac{\eta_H}{\eta_{20,w}}\right)\left(\frac{1-\bar{v}\rho_{20,w}}{1-\bar{v}\rho_H}\right),$$

(see footnote 1). On the right side of this expression is the formula for correcting the experimental sedimentation coefficient in H_2O to $s_{20,w}$, and on the left is the correction for the experiment in D_2O. As it stands, there are two unknowns, $s_{20,w}$ and $\bar{v}$. Since the same particle is being studied in both cases, the right and left sides may be set equal, eliminating $s_{20,w}$ and leaving $\bar{v}$ as the only unknown.[2] The values for s_H and s_D are the experimentally determined sedimentation rates in H_2O and D_2O. The density (ρ_H, ρ_D) and viscosity (η_H, η_D) of the sucrose gradients can be determined. This approach was originally described and theoretically justified by Edelstein and Schachman (8). The calculations used in the kind of experiment described here are based on the work of Clarke (9).

Such experiments were carried out with adenylate cyclase solubilized from the rat renal medulla with both Triton X-100 and Lubrol PX (10). The yield of activity was somewhat better with Lubrol PX than with Triton X-100, but since the studies with other membrane proteins were done in Triton X-100, a direct comparison is easier with this detergent. The results, however, are the same for both.

Figure 1 shows the sedimentation pattern of Triton X-100-solubilized adenylate cyclase in sucrose gradients in H_2O and D_2O containing 0.1% Triton X-100. The enzyme sediments in an identical fashion relative to the marker enzymes in the two gradients, indicating that the partial specific volume of adenylate cyclase is similar to theirs. The same is true when Lubrol PX-solubilized enzyme is analyzed in Lubrol PX containing gradients.

From sedimentation experiments one can obtain the value for $s_{20,w}$ and $\bar{v}$. To obtain the molecular weight one needs only the value for the Stokes radius of the enzyme, and this can be obtained by gel filtration on columns of Sepharose 4B together with marker enzymes of known Stokes radius. The results found with the Lubrol PX-solubilized enzyme are shown in Fig. 2. The pattern with Triton X-100 is the same except that there is no peak of activity in the void volume. The sucrose gradients shown in Fig. 1 represent the activity in peak B. Peak C is always very small.

The values obtained from these studies are given in Table I. The molecular weight of the predominant form of adenylate cyclase is 157,000–159,000 daltons. It is a rather asymmetric molecule, as a number of membrane proteins have turned out to be (9). Most interesting is the fact that the partial specific volume, determined in detergent, is that of a typical water-soluble protein. This indicates that adenylate cyclase does not bind a detectable amount of detergent.

One can make an estimate of how much detergent binding would have been detected by these methods by making the following calculation. The standard error of the determination of $s_{20,w}$ is ± 0.2S. A shift of 1S between H_2O and D_2O gradients would have

[1] In this equation s_H and s_D are the experimentally determined sedimentation coefficients in H_2O and D_2O; η_H and η_D are the viscosities of the experimental media; $\eta_{20,w}$ is the viscosity of water at 20°C; ρ_H and ρ_D are the densities of the experimental media; $\rho_{20,w}$ is the density of water at 20°C; $\bar{v}$ is the partial specific volume of the particle.

[2] The equation given here is a simplification which neglects the possible effect of D_2O on detergent binding and on diffusion coefficient. These effects have been shown to be very small (9). For the explicit formulation see reference 9.

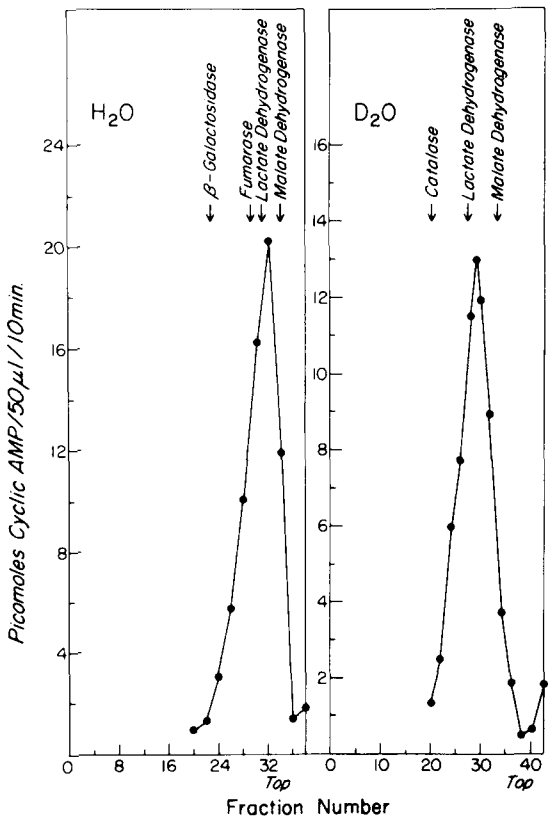

Fig. 1. Sedimentation pattern of Triton X-100-solubilized adenylate cyclase (100,000 × g supernatant); 5–20% sucrose gradient containing 0.1% Triton X-100 in H_2O or D_2O. 0.1 M Tris · HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol. The concentration of D_2O was 90%. The calibrating enzymes were added to the sample and centrifuged with it. Adenylate cyclase activity was measured by a modification of the method of Krishna et al. (17) and has been described (18). Assays were done in duplicate with NaF.

been readily detectable. Such a shift would give a value of $\bar{v}$ of 0.78 ml/g. Since the $\bar{v}$ of Triton X-100 is 0.94 ml/g (11) and that of an "average" protein is 0.74 ml/g, a value of $\bar{v}$ of 0.78 ml/g would represent the increase in partial specific volume due to the binding of 0.2 mg detergent/mg enzyme or, for example, 50 molecules of Triton X-100/molecule of enzyme. The area of a Triton X-100 molecule at an air-water interface is about $50A^2$.[3] If one assumes that adenylate cyclase is a sphere of 62 A radius, its surface area would be $48{,}000\,A^2$. Fifty molecules of Triton X-100 would then cover about 5% of the surface area. This is probably a high estimate, as a correction for the asymmetrical shape of the enzyme would increase its surface area.

[3]Rohm and Haas Technical Bulletin.

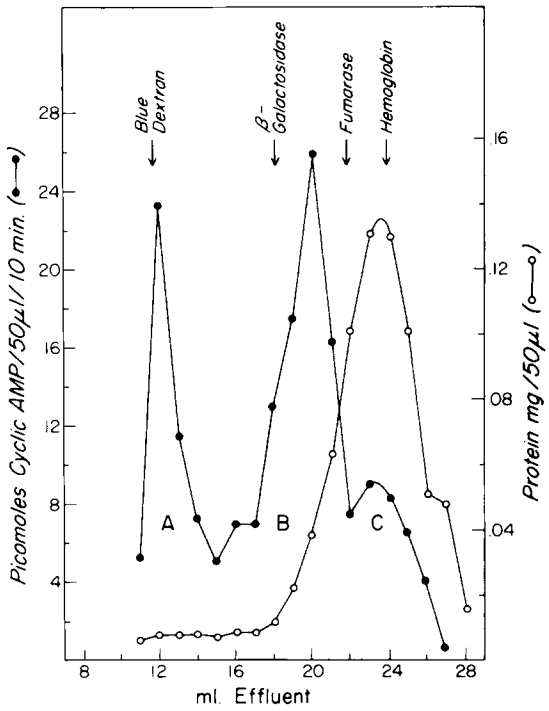

Fig. 2. Sepharose 4B gel filtration of adenylate cyclase solubilized with Lubrol PX. The method of solubilization has been described as has the method of assaying adenylate cyclase and calibrating enzyme activities (10). Filtration conditions: 1.0 X 29 cm column, sample volume, 2 ml; flow rate, 6 ml/hr; buffer 0.1% Lubrol PX; 0.1 M Tris.HCl, pH 7.6, 0.075 M sucrose, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol. Samples were assayed in duplicate for adenylate cyclase activity with NaF. Protein was measured by a modification of the method of Lowry et al. (19) as described by Bailey (20).

In fact, there was no difference between the sedimentation coefficient measured in H_2O and that in D_2O, and the $\bar{v}$ for adenylate cyclase of 0.74 ml/g is that of a "typical" soluble protein. It seems reasonable that the enzyme must bind some detergent or it would not require detergent for solubilization, but the amount must be very small indeed and the surface area covered must be a very small fraction of the total surface of the enzyme.

Such arguments lead one to the conclusion that most of the mass of the enzyme is not deeply embedded in the lipid bilayer of the plasma membrane. Taken together with the fact that adenylate cyclase used intracellular ATP as its substrate this suggests that the enzyme is attached only to the inner surface of the plasma membrane. Perhaps it represents another example of the arrangement described by Strittmatter et al. (12) for two other membrane-bound enzymes, cytochrome b5 and cytochrome b5 reductase. Both of these enzymes are held in place in the microsomal membrane by a small hydrophobic pedicle. Detergents are needed to liberate the holoenzyme, but controlled proteolysis will produce an enzymatically active fragment which is globular and water soluble and makes up some 70–80% of the mass of the enzyme.

TABLE I. Molecular Parameters of Solubilized Adenylate Cyclase

Physical parameter[1]	Triton X-100		Lubrol PX	
	B	C	B	C
Sedimentation coefficient, $s_{20,w}$ (S)	5.9 ± .2 (14)[2]	3.0	6.1 ± .2 (6)[2]	2.9
Stokes radius, a (A)	62 ± 3 (3)	28 ± 2 (3)	64 ± 2 (3)	34 ± 4 (3)
Partial specific volume, $\bar{v}$ (ml/g)	0.74 ± .01[3]		0.71[3]	
Molecular weight	159,000	38,000	157,000	40,000
Frictional ratio, f/f_o	1.6[4]	1.2	1.6[4]	1.4

[1]The values given are the mean ± 1 SE for the number of determinations shown in parenthesis.

[2]Since there was no difference between $s_{20,w}$ determined from gradients in H_2O and D_2O, the values were combined.

[3]The calculation of $\bar{v}$ was made on three pairs of gradients in Triton X-100 and one pair in Lubrol PX.

[4]The frictional ratio was calculated assuming solvation to be 0.2 g solvent/g protein (22).

If adenylate cyclase is indeed attached by a rather small hydrophobic segment to the inner surface of the membrane, and if hormones do bind to specific protein receptors which are exposed to the outer surface of the cell membrane, then one might expect that these proteins will have a large hydrophobic surface in order to span the lipid bilayer and interact with adenylate cyclase. In fact, the partial specific volume of the insulin receptor from fat and liver cells measured in Triton X-100 by Cuatrecasas (13) and the gonadotropin receptor from testis and ovary studied by Dufau and his co-workers (14) is high. This high value for $\bar{v}$ would be explained by the binding of 100–200 molecules of detergent / molecule of receptor.

Before leaving adenylate cyclase, I would like to add a word about the 40,000 molecular weight fragment which is the last small peak to elute from Sepharose 4B (see Fig. 2). Its relation to the predominant peak is unclear at the moment. It may be a proteolytic fragment, although I could not generate this fragment by treating the major peak with trypsin or pronase. It could represent a subunit of the enzyme. Which possibility is correct, if either, is difficult to know until the enzyme is purified and chemically characterized.

I would like to turn now to the other purine cyclase in renal cells, guanylate cyclase. This enzyme occurs with an activity at least as great as or greater than that of adenylate cyclase, although the concentration in the renal medulla of its product, cyclic GMP, is about 20–30 times lower than that of cyclic AMP (3). In the renal medulla about 70% of the activity is freely soluble, being found in the 100,000 × g supernatant when a homogenate made in dilute Tris buffer is centrifuged. The remaining activity is found in the pellet and distributes itself similarly to adenylate cyclase when the pellet is analyzed by discontinuous density gradient centrifugation. Both the soluble and the membrane-bound enzyme are stimulated by detergent and to about an equal extent.

Much less is known about guanylate cyclase than about adenylate cyclase. We set out to define some of the physical properties of guanylate cyclase for two main reasons. First, there is the intriguing question of the relationship between adenylate cyclase and guanylate cyclase. Perhaps these enzymes are interconvertible, as was suggested by Illiano and Cuatrecasas (15), and represent the same protein which changes its substrate specificity depending upon whether or not it is associated with a membrane. This hypothesis cannot be rigorously tested until both activities have been purified and chemically characterized. However, one can make a start at the characterization of guanylate cyclase by determining the size and shape of the enzyme both in its soluble form and when it is solubilized with detergent from the particulate form. The second reason for being interested in the physical properties of guanylate cyclase is to elucidate the nature of the interaction of the enzyme with detergent. This could give information about the interaction of guanylate cyclase with hydrophobic cell components.

There are a number of ways in which detergent might affect the measured activity of an apparently soluble enzyme. First, the enzyme might not be truly soluble but be contained in small, nonsedimentable membrane vesicles. By breaking these up, the detergent would make the substrate more available to the enzyme. Second, the detergent might provide a hydrophobic, membrane-like environment either by forming micelles into which the enzyme might insert or by binding in large quantity to the surface of the enzyme. Finally, the detergent might activate by binding to a few specific sites causing a conformational change in the enzyme. These possibilities can be distinguished from one another by determining the physical properties of the enzyme in the presence and the absence of detergent.

Figure 3 shows the distribution of activity of guanylate cyclase in two sucrose gradients, one with and one without 0.1% Lubrol PX. There is a difference of about 1 Svedberg unit in the sedimentation coefficient of the enzyme under these two conditions. Without detergent the enzyme has a value of 6.3S while the same sample sedimented in a sucrose gradient containing detergent has a value of 5.5S.

This is not due to dissociation into subunits because, as is shown in Figs. 4 a and b, in the presence of detergent the Stokes radius of the enzyme increases from 54 A to 62 A. These findings eliminate the first possibility listed above because soluble enzyme does not exist as a large aggregate such as a membrane vesicle and the detergent does not make it smaller.

Such a combination of findings could be brought about by two kinds of events. First, the enzyme might bind a large amount of detergent or insert into the detergent micelle. This would increase the mass of the particle and its Stokes radius. It would also increase the partial specific volume, hence slowing the rate of sedimentation. Second, the detergent could cause a conformational change such that the enzyme would become more asymmetric. It would then have a larger Stokes radius and a smaller sedimentation coefficient, but there would be no change in its mass or in its partial specific volume. In this case, the enzyme might bind some detergent but in an amount too small to cause a measurable change in the last two parameters.

To decide between these alternatives, the partial specific volume of the soluble guanylate cyclase was measured in the same way as that of adenylate cyclase by comparing the rate of sedimentation in gradients made up in H_2O and D_2O both with and

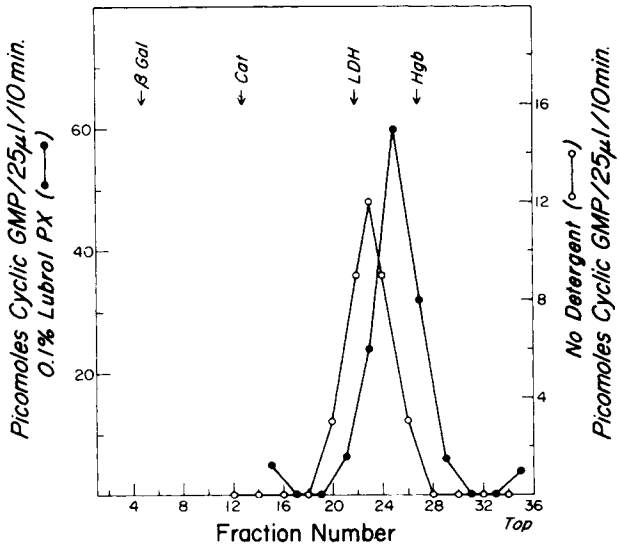

Fig. 3. Sedimentation of supernatant guanylate cyclase in sucrose gradients with and without 0.1% Lubrol PX. The patterns of activity from two separate sucrose gradients are superimposed. The 5–20% sucrose gradients were made up in 0.1 M Tris. HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 90% D_2O. Samples were assayed in duplicate for guanylate cyclase activity by an isotope dilution method with lobster tail cyclic GMP binding protein. This method has been described (21). The calibrating enzymes were included in the sample applied.

without Lubrol PX. Both with and without detergent the partial specific volume of guanylate cyclase is the same as that of a typical soluble protein. It cannot therefore bind detergent in large amounts. The effect of detergent must be to cause unfolding of the polypeptide chain.

The measurement of the partial specific volume allows the explicit calculation of the molecular weight of the enzyme in the presence and absence of detergent. The mass of the enzyme is the same in both cases. The values obtained are given in Table II.

One can now compare the physical properties of supernatant guanylate cyclase in detergent with those of membrane-bound adenylate cyclase solubilized with detergent. The two enzymes are remarkably similar in size and shape.

Adenylate cyclase is known to be a membrane enzyme. It is possible that guanylate cyclase, which resembles it so much in physical properties, is also membrane associated in vivo but differs from adenylate cyclase in the ease with which it is dislodged from the membrane. There is an argument, admittedly speculative, which one can make in favor of such a proposal. This argument is based on the fact that guanylate cyclase is activated by detergents which may be thought of as lipid analogs. In fact, Limbird and Lefkowitz (16) reported stimulation of cardiac guanylate cyclase with phospholipids. Proteins which interact with lipids are, with the exception of the plasma lipoproteins, generally membrane bound.

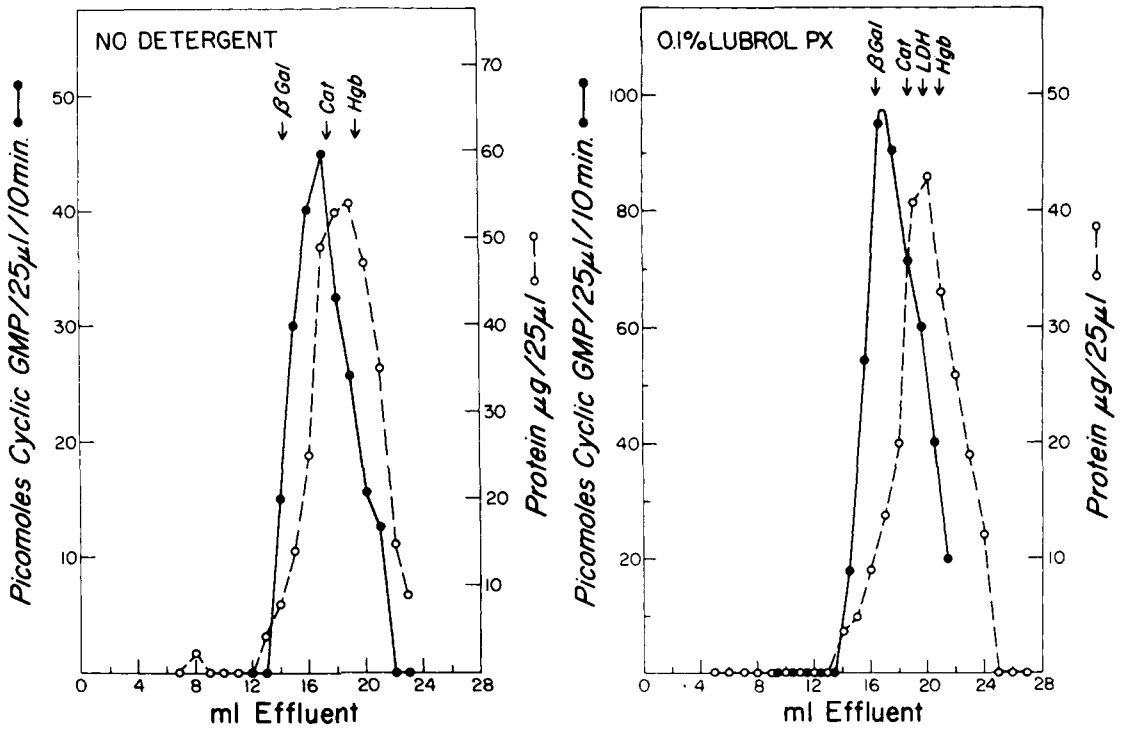

Fig. 4. a and b. Sepharose 4B gel filtration of supernatant guanylate cyclase with and without 0.1% Lubrol PX. Filtration conditions: 1.0 X 29 cm column, sample volume, 1 ml; flow rate, 7 ml/hr; buffer, 0.075 M sucrose, 0.1 M Tris. HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol with or without detergent; 4°C. Samples were assayed in duplicate for guanylate cyclase activity by the method described in reference (21). The protein was measured by the method of Lowry et al. (19) as modified by Bailey (20). The calibrating enzymes were included in the sample.

Guanylate cyclase in the particulate cell fraction can be solubilized with 1% Lubrol PX. The values for the sedimentation coefficient, Stokes radius, and approximate molecular weight for this form of the enzyme are given in Table II. These values are all approximate because the peak of guanylate cylase activity which is solubilized from the particulate cell components is very broad, indicating that the enzyme is heterogeneous. The broadness of the peak made it meaningless to calculate partial specific volume by comparing sedimentation rates. The heterogeneity shown by guanylate cylcase is not simply the result of some nonspecific aggregation in the process of solubilization. The evidence for this is that the procedure used to solubilize guanylate cyclase solubilizes adenylate cyclase as well. Adenylate cyclase in the same sucrose gradient sediments at the rate expected from the studies described above (see Fig. 5).

These findings show that there are circumstances under which adenylate cyclase and guanylate cyclase activities can be separated. Furthermore, the two activities can coexist both in a soluble state, as in the sample analyzed in Fig. 5, and in a membrane-bound state. If they are the same protein, incorporation into a membrane is not the factor which controls substrate specificity.

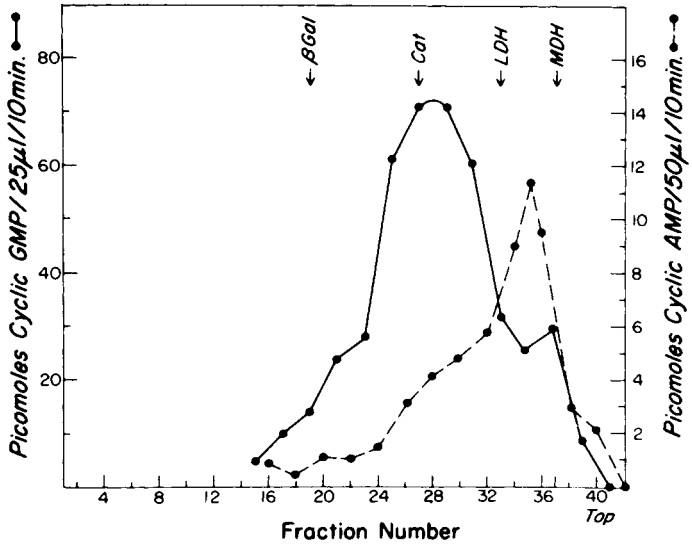

Fig. 5. Sedimentation pattern of guanylate cyclase and adenylate cyclase solubilized with 1% Lubrol PX from the pellet obtained when a homogenate of rat renal medulla (made up in 0.1 M Tris. HCl, pH 7.6, 0.075 M sucrose, 10 mM $MgCl_2$, 1mM EDTA, and 1 mM dithiothreitol) was centrifuged at 100,000 X g for 45 min. The composition of the gradients was the same as those described in Fig. 3 except that H_2O was used. Adenylate cyclase was assayed with NaF. The calibration enzymes were included in the sample applied.

TABLE II. Molecular Parameters of Guanylate Cyclase

Physical parameter[1]	Soluble, no detergent	Soluble, 0.1% Lubrol PX	Solubilized from particulate form
Sedimentation coefficient, $s_{20,w}$ (S)	6.3 ± 0.1 (6)[2]	5.5 ± 0.1 (6)[2]	10 ± 2 (3)
Stokes radius, a (A)	54 ± 2 (3)	62 ± 2 (5)	65
Partial specific volume, $\bar{v}$ (ml/g)[3]	0.75 ± 0.01 (2)	0.74 ± 0.01 (2)	
Molecular weight	154,000	148,000	300,000
Frictional ratio f/f_o[4]	1.4	1.6	
Axial ratio (prolate ellipsoid)[5]	7	11	

[1]The values given are the mean ± 1 SE for the number of determinations shown in parenthesis except for duplicates where the range is given.

[2]Since there was no difference between $s_{20,w}$ determined in H_2O and D_2O, the values were combined.

[3]The calculation of $\bar{v}$ was made on two pairs of gradients with detergent and two pairs without.

[4]The calculation of f/f_o was made assuming a solvation factor of 0.2 g solvent/g protein.

[5]Calculated from the graph given by Tanford (22).

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