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Cyclic GMP Synthesis and Function

Scott A. Waldman and Ferid Murad

Departments of Medicine and Pharmacology, Stanford University School of Medicine, and Veterans Administration Medical Center, Palo Alto, California 94304

I. Introduction

ALTHOUGH cyclic GMP was first described as a biological product more than 2 decades ago, the anticipated role of this cyclic nucleotide as another second messenger has been slow to develop. Many explanations could be offered for these delays, including its relatively low concentration in tissues. However, the presence of multiple isoenzyme forms of guanylate cyclase and their complex and unique mechanisms of regulation have no doubt been major contributors to the difficulties in this field. A detailed understanding of guanylate cyclase regulation has led to several proposed functions for cyclic GMP as summarized in this review.

In recent years these isoenzyme forms have been purified and characterized from several tissues. The discoveries that these enzyme forms could be activated with nitrovasodilators, nitric oxide, other free radicals, endothelium-derived relaxant factor (EDRF), *Escherichia coli* heat-stable enterotoxin (ST), and atrial natriuretic factor (ANF) have markedly stimulated the interests in cyclic GMP and have led to several proposed functions of the guanylate cyclase-cyclic GMP system.

This review will not consider some other major developments with cyclic nucleotide phosphodiesterases, cyclic GMP-dependent protein kinase, and identification of some of the latter enzyme's protein substrates. Clearly, cyclic GMP has emerged as one of the few fundamental second messengers. It has also become apparent that perturbation of one of these second messengers may alter the production, release, degradation, or action of the others. Complex interactions between cyclic GMP, cyclic AMP, calcium, and inositol phosphates have been described.

This review will summarize many of the data available regarding the synthesis of cyclic GMP by guanylate cyclase and several of its functions.

II. Cyclic GMP Synthesis

A. Guanylate Cyclase Isoenzymes

1. Tissue activities, subcellular distribution, and altered enzyme activities. Guanylate cyclase has been described in virtually all cell types and phyla examined. In most cells the enzyme exists as a polymorphic protein, with both cytosolic (soluble) and membrane-associated (particulate) forms existing in the same cell. The relative amounts of each of these forms within a cell vary with

the cell type, physiological state, and the protocol used for enzyme assay. Thus, in some tissues such as the normal adult liver (190, 187) or platelets (124, 23), guanylate cyclase is predominantly soluble. In other tissues, such as regenerating liver (194, 130), fetal liver (194), hepatomas and renal tumor (130, 50, 51, 65), intestinal mucosa (191, 276, 56), and retinal rod outer segments (200, 91, 92, 129, 68, 90), the enzyme is predominantly particulate. Furthermore, there are cell types, including C_6 rat glioma, B103 rat neuroblastoma (306), and sea urchin sperm (112, 108, 277, 132, 133), which appear to possess particulate guanylate cyclase exclusively. There appears to be no correlation between the subcellular localization of the enzyme and phylogeny. Soluble guanylate cyclase is the predominant form observed in the procaryote *E. coli* (237), while the particulate enzyme is the predominant form in Sendai virus (195) and the eucaryotic protozoans, such as *Tetrahymena pyriformis* (175, 202, 203) and *Paramecium tetraurelia* (297, 298, 196). It should be noted that, in the case of Sendai virus, it remains unclear whether particulate guanylate cyclase originates as a product of the viral or host genome (195).

The apparent relative distribution of guanylate cyclase between the soluble and particulate compartments is profoundly affected in most cells by the presence of detergents in the homogenizing medium or enzyme assay. Particulate guanylate cyclase activity can be increased as much as 3- to 12-fold with detergents, while much less activation of the soluble enzyme (30 to 100%) occurs. This has been observed with preparations from most tissues and cell cultures including kidney, liver, parotid, uterus, cerebellum, heart, sea urchin sperm, skeletal muscle, cerebral cortex, blood vessels, hepatomas, neuroblastomas, gliomas, and pheochromocytomas (190, 191, 306, 112, 108, 277, 132, 337, 334, 206, 336, 38, 269, 314, 315, 289, 254, 250, 342, 172, 145, 193, 100, 346, 98, 326, 214, 267). Increased activity in the presence of detergents correlates closely with release of enzyme from membranes into the soluble phase and has been attributed to the particulate guanylate cyclase being latent (190, 108, 277, 132, 337, 334, 206, 336, 38, 269, 314, 315, 289, 172, 145, 193, 100, 346, 98, 326, 214, 267). Whether increases in activity reflect solubilization of enzyme molecules normally sequestered in the membrane and unavailable to substrate or represent activation of enzyme is discussed in a later section. The alterations in physical and biochemical properties of both soluble and particulate guanylate cyclases exposed to detergents are also the subject of a later section.

Studies of the subcellular distribution of guanylate cyclase have indicated that there are at least more than two forms of this enzyme in most cell types. Particulate guanylate cyclase has been associated with plasmalemma, endoplasmic reticulum, Golgi (191, 192), and nuclear membranes (78) when these organelles were purified from homogenates of rat liver. Similarly, enzyme activity has been localized in the sarcoplasmic reticulum from heart (100), nuclear membranes from the uterus (305), mitochondria and synaptosomes from cerebellum (265), plasmalemma from parotid (77), skeletal muscle, heart, cerebral cortex (314, 315), murine plasmocytoma cells (24), rod outer segments from retina (200, 91, 92, 129, 68, 90), microvillus brush border from intestinal mucosa (276, 56, 57), the excitable ciliary membranes from *P. tetraurelia* (297, 298, 196), plasma membranes, endoplasmic reticulum, mitochondria and lysosomes from lymphoid cells (345), sea urchin sperm flagellar membranes (112, 108, 277, 132, 133), and Sendai virus nucleocapsids (195). Particulate fractions from murine plasmocytoma cells possess 90% of the total guanylate cyclase activity in this cell type (24). Inside-out plasma membrane vesicles isolated from these cells possess 9 times more enzyme activity when compared with right-side-out vesicles (24). This difference could be abolished by solubilizing guanylate cyclase from these membranes with detergents, suggesting that the catalytic portion of the particulate enzyme is localized on the inner surface of the plasmalemma in these cells (24). Thus, the particulate enzyme has been localized in most subcellular organelles in a variety of tissues and phyla. Whether there is also compartmentation of the soluble enzyme remains unknown.

In some cases, qualitative and quantitative differences in the regulation of guanylate cyclase activity can be correlated with differences in subcellular distribution. In liver, Ca^{2+} stimulates the soluble enzyme, inhibits the particulate enzyme associated with the cell surface, but has no effect on the particulate enzyme associated with microsomes (191, 192). Particulate guanylate cyclase obtained from homogenates of intestinal mucosa can be activated by the heat-stable enterotoxin of *E. coli* (ST) (157, 86, 140, 338). When these particulate preparations are subfractionated into microvillus brush border membranes and basolateral membranes, the enzyme is specifically enriched in the brush border and retains the capacity to respond to ST (157, 86, 140, 338). Atrial natriuretic factor also specifically activates the particulate but not the soluble isoenzyme from numerous tissues as discussed below.

Particulate guanylate cyclase also exhibits pleiomorphism within specific subcellular compartments. For example, this enzyme is enriched in particulate fractions of retinal rod outer segments and the microvillus brush border from intestinal mucosa (276, 56, 200, 91, 92, 129, 68, 90, 57). When particulate fractions from rod outer segments are subjected to detergent solubilization, a portion of the guanylate cyclase activity remains in a sedimentable form (91, 92). This guanylate cyclase appears to be localized to the axonemal appartus since the enzyme copurifies with these microtubular structures by differential and density gradient centrifugation (91, 92). The association between guanylate cyclase and the mi-

crotubule apparatus is such that enzyme cannot be readily solubilized with various detergents, salt, EDTA, or alkali (91, 92). Similarly, a portion of the enzyme associated with the microvillus brush border from intestinal mucosa is resistant to solubilization (335). In addition to the above agents, neither anionic detergents nor 1 M urea solubilized this form of guanylate cyclase from brush borders (335). In light of the abundance of cytoskeletal elements associated with the intestinal mucosa brush border, it appears that a third isoenzyme form exists in association with microtubular and/or filamentous structures (335). These data are in close agreement with previous reports of a detergent-resistant particulate guanylate cyclase in other tissues and suggest that this form may be present in many types of cells (91, 92, 335). It is interesting to note that, within the microvillus brush border of intestinal mucosa, the cytoskeletal form of particulate guanylate cyclase appears to be the isoenzyme that is activated by ST (335). Immunohistochemical localization of these isoenzymes at the electron microscopic level with antibodies specific for each of these isoenzyme forms would be of major interest.

2. Progress in the purification of multiple isoenzyme forms of guanylate cyclase. Much effort has been expended in developing protocols for purifying the different forms of guanylate cyclase. The soluble enzyme has been purified to apparent homogeneity from several sources, including liver (327, 33, 273), lung (112, 110, 226, 256, 26, 116, 117, 164), and brain (260, 359), and from rat and bovine species. These protocols utilize a variety of purification techniques including salt precipitation (327, 33, 226, 117, 164, 260, 359), isoelectric precipitation (33, 273, 110, 226, 260), gel filtration (327, 33, 273, 260, 359), ion-exchange chromatography (327, 33, 273, 117, 164, 260), hydrophobic chromatography (327, 33, 260), dye-ligand chromatography (273, 117, 164, 359), substrate affinity chromatography (110, 226, 260), and preparative polyacrylamide gel electrophoresis (327, 33, 110, 226, 117, 359). A recently described procedure employing monoclonal antibody affinity chromatography has proven to be rapid with a high yield of homogeneous enzyme (176). This procedure permits the purification of milligram quantities of soluble enzyme in a single chromatographic step (176). Results from these purification procedures are summarized in Table 1. The specific activity of soluble guanylate cyclase purified to apparent homogeneity varies between 100 and 1,000 nmol of cyclic GMP produced/min/mg protein. Recoveries of enzyme activity vary from 1 to 50% of the total starting activity, and from 5 μg to 2 mg of purified protein can be obtained with these procedures. In general, the enzyme requires about 1,000- to 10,000-fold purification from most tissues to achieve apparent homogeneity. The discrepancy between the magnitude of purification required to achieve homogeneity using rat lung soluble guanylate cyclase as the source probably reflects differences in the preparation and activity of the starting material. With the protocol yielding a 22,000-fold purification, the starting material was a low speed (30,000 $\times$ g $\times$ 30 min) supernatant fraction containing much particulate protein which depressed the specific activity of the starting material (110). Other protocols utilized a high speed (105,000 $\times$ g $\times$ 60 min) supernatant depleted of particulate proteins; these preparations exhibited a higher initial specific activity and, consequently, a lower overall purification (226).

The soluble enzyme appears to possess a heme prosthetic group that may mediate many of the activation phenomena characteristic of this enzyme (273, 116, 117, 164, 113). This porphyrin moiety is easily dissociated from the protein with mild treatments such as freeze-thawing (116, 117), acid conditions (pH 5.0) (116, 117), or ammonium sulfate precipitation (164). As a result, the soluble enzyme can be purified in two different forms, heme containing and heme deficient, with associated alterations in its biochemical characteristics (273, 116, 117, 164, 225). It is likely that protocols that incorporate isoelectric and salt precipitation steps (327, 33, 273, 110, 226, 117, 164, 260, 359) will result in preparations of guanylate cyclase that are heterogeneous or completely deficient in protein-associated heme (273, 116, 164, 225). The forms of soluble guanylate cyclase that contain or do not contain heme can be separated by ion-exchange or dye-matrix chromatography (118).

Sea urchin sperm membranes are a very rich source of particulate guanylate cyclase and have been used for purifying this enzyme to apparent homogeneity (108, 277). The original protocol, utilizing detergent extraction, GTP affinity chromatography, DEAE-Sephadex chromatography, and gel filtration, resulted in preparations which were 1200-fold purified and exhibited specific activities of 12 μmol of cyclic GMP produced/min/mg protein (108). These preparations yielded two protein-staining bands after sodium dodecyl sulfate (SDS) gel electrophoresis whose identity remained indeterminate (108). Most recently, this procedure has been modified to incorporate preparative polyacrylamide gel electrophoresis in place of gel filtration as a final step (277). This protocol yielded preparations with a final specific activity of 15 μmol/min/mg protein and a single band upon SDS gel electrophoresis (277).

Particulate guanylate cyclase from mammalian tissues has been attained in highly purified, albeit not homogeneous, preparations (337). Using rat lung membranes as a source, the enzyme has been purified 1000-fold using detergent solubilization and sequential affinity chromatography on concanavalin A-Sepharose, GTP-agarose, and Blue-Sepharose (337). These preparations exhibit specific activities of about 500 nmol/min/mg protein and about ten protein staining bands after SDS gel electrophoresis (337). More recently, this procedure has been modified yielding preparations with specific activities of

TABLE 1

Purification of guanylate cyclase from various sources

Tissue	Final specific activity*	% of total activity recovered	Total protein		Fold purification	Ref.
			Initial (g)	Final (mg)		
Soluble isoenzyme						
Rat liver	276	5	37.4	0.22	9,200	33
	880	1	7.7	0.005	17,000	327
Rat lung	693	2	251.0	0.250	22,000	110
	400	5	4.5	0.200	2,000	226
	432	53	15.9	3.500	2,400	176
Rat brain	72	8	13.8	1.000	1,200	359
Bovine brain	236	2	132.8	0.190	12,700	260
Bovine lung	610	3	241.6	1.000	15,000	117
	446	14	16.3	0.210	11,000	164
Particulate isoenzyme						
Rat lung	19,000	8	88.0	0.0052	15,000	206

* Nmol cGMP produced/min/mg protein.

1 to 5 μmol/min/mg protein (334). With further modification of this purification scheme for rat lung membranes, the enzyme has been purified about 15,000-fold with a specific activity of 19 μmol/mg/min (206). These preparations contain one major protein band (about 95% pure) after SDS gel electrophoresis (206).

It has been reported that particulate, but not soluble guanylate cyclase, can be activated by a variety of proteases (210, 209). Subsequently, it was demonstrated that the particulate enzyme could be solubilized by limited proteolysis with trypsin, with as much as 70% of the particulate enzyme released into the soluble phase by this technique (336, 209). This results in a fragment that is amenable to purification in the absence of detergents (336). Thus, the enzyme was purified 500-fold from rat liver particulate fractions after trypsin proteolysis using a combination of ammonium sulfate precipitation, GTP affinity chromatography, and preparative polyacrylamide gel electrophoresis (336). These preparations exhibited specific activities of 10 nmol/min/mg protein and 10 to 20 protein staining bands by SDS gel electrophoresis (336).

3. Physicochemical properties of soluble and particulate guanylate cyclase. a. PHYSICAL PROPERTIES. Soluble guanylate cyclase purified from a number of tissues exhibits similar molecular characteristics. Enzyme purified from rat lung and liver or bovine lung exhibited a Stokes radius of 44 to 46 Å by gel filtration and 35 Å by analysis on different percentages of polyacrylamide gels (337, 33, 117). The sedimentation constant ($S_{20,w}$) for preparations purified from rat or bovine lung was 7.0 S (337, 117). Thus, the molecular weight (M_r) of the soluble enzyme has been estimated to be about 150,000 (337, 33, 110, 256, 117). However, the molecular weight for the enzyme purified from bovine brain has been estimated by gel filtration to be about 270,000 (260). Isoelectric focusing of purified preparations from rat lung and brain has established a pI for this enzyme in the range of pH 6.0 (337, 256, 359).

When some soluble guanylate cyclase preparations purified from rat or bovine lung were subjected to SDS gel electrophoresis, a single M_r 72,000 protein staining band was obtained, suggesting that this enzyme is a dimer of two identical subunits (226, 256, 116, 117). However, other subunit patterns have been reported. Enzyme purified from rat lung has appeared as a single M_r 79,000 band (112), as a M_r 72,000 and 75,000 dimer (256), and as a M_r 74,000 and 79,000 dimer (110). The enzyme purified from rat brain exhibits M_r 61,000 and 66,000 protein staining bands when the enzyme is stored in the presence of glycerol and a reducing agent (359, 13). However, the mobility shifts to a single M_r 59,000 band upon removal of these stabilizing agents (359). These discrepancies in the subunit structure of the enzyme are probably due to proteolysis of some preparations during long and tedious purification procedures. When rat lung soluble enzyme is rapidly purified with monoclonal antibody affinity chromatography in the presence of protease inhibitors, the enzyme is a heterodimer with M_r 82,000 and 70,000 subunits (176). In the absence of protease inhibitors, the M_r 82,000 subunit, but not the M_r 70,000 subunit, diminishes and is recovered on SDS gels as a smaller M_r 68,000 or less fragment (176). Antibodies to the M_r 82,000 subunit fail to recognize the M_r 70,000 subunit but can recognize the proteolytic fragment (176). Antibodies to the M_r 70,000 subunit do not react with the M_r 82,000 subunit or proteolytic fragment. These antibodies can immunoprecipitate enzyme activity from crude or purified preparations, indicating that both subunits are contained in the holoenzyme (176). Preparations of the holoenzyme in which the M_r 82,000 subunit has been clipped with proteolysis, interestingly, possess activity and can be activated with some of the materials discussed below.

Soluble guanylate cyclase purified from bovine lung contains both heme and copper (113). When enzyme purified more than 7,000-fold was subjected to spectro-

scopic analysis, three absorption maxima at 433 nm, 550 nm, and 565 nm were observed (113). These absorption maxima could be shifted by exposing the protein to carbon monoxide or nitric oxide (113). These findings are characteristic of the α, β, and Soret bands of hemoproteins; pyridine hemochrome determinations on these preparations confirmed this (113). Atomic absorption spectroscopy of the enzyme revealed the presence of iron, which is associated with the heme moiety, and copper (113). It is unlikely that these findings are due to contamination of the protein preparations, since heme and copper were present in stoichiometric concentrations of 1 mol of iron and copper per mol of enzyme after four purification steps (113). The role of these moieties in regulating the activity of soluble guanylate is discussed in a later section.

Estimates of the size of particulate guanylate cyclase have ranged from 200,000 to 900,000 daltons (337, 38). Most studies place the particulate enzyme in the molecular weight range of 300,000 to 400,000 (269, 126). Recently, this enzyme has been purified about 10,000- to 15,000-fold from rat lung (334, 206). These preparations exhibit a Stokes radius of about 55 Å and an $S_{20,w}$ of about 7 to 10 S, therefore having a molecular weight in the range of 200,000 to 300,000, in close agreement with previous estimates (337, 269, 126, 255, 257). It must be noted that several factors complicate these types of analyses. For example, these determinations are made in the presence of nonionic detergents above their critical micellar concentrations. Micelles of these detergents can be very large (M_r 50,000 to 100,000) (147) and thus influence the biophysical properties of the associated proteins. After SDS gel electrophoresis of purified rat lung particulate guanylate cyclase, a predominate M_r ~120,000 to 130,000 protein band was obtained (206). From data currently available, it cannot be concluded whether the particulate isoenzyme exists in the membrane as a monomer or dimer of this subunit.

Preparations of particulate guanylate cyclase purified to apparent homogeneity from sea urchin sperm also yield a single M_r 135,000 protein-staining band after SDS gel electrophoresis (277). The sedimentation coefficient was 6.8 S, and the Stokes radius was 51 Å from which an estimated molecular weight of 157,000 was calculated. These values are in close agreement with those obtained with preparations of particulate guanylate cyclase purified from rat lung (334, 206). It is interesting to note that, when preparations from sea urchin sperm were treated with endoglycosidase H, an enzyme which removes carbohydrate moieties from glycoproteins, the subunit molecular weight shifted from 135,000 to 72,000 on SDS gels (277). Thus, the size of the peptide is similar to that reported for soluble guanylate cyclase purified from mammalian tissues (226, 256, 116, 117, 176) and may suggest some homology between these proteins.

These data also demonstrate the glycoprotein nature of the particulate enzyme. Particulate guanylate cyclase from sea urchin sperm or mammalian tissues exhibits an affinity for immobilized lectins, such as concanavalin A and wheat germ agglutinin, and can be eluted from these resins in a specific manner with the appropriate sugars, such as α-methylmannoside and N-acetyl-D-glucosamine, respectively (112, 277, 337, 334, 206). Also, particulate preparations purified from sea urchin sperm exhibited a periodic acid-Schiff (PAS)-positive staining protein band on acrylamide gels, indicating the presence of carbohydrate on the enzyme (277). In contrast, soluble enzyme from mammalian tissues does not bind to immobilized lectins and yields a PAS-negative protein band on acrylamide gels (112, 277, 337). Carbohydrate analysis of the particulate guanylate cyclase purified from sea urchin sperm has suggested that N-acetylglucosamine, mannose, galactose, and 2-aminoerythritol constitute about 40% (w/w) of the enzyme (277).

Particulate guanylate cyclase can also be solubilized from membranes using limited proteolysis with trypsin (336, 209). This enzyme is a proteolytic cleavage product of the native holoenzyme and, consequently, should exhibit different physical properties compared with the native enzyme. Indeed, crude preparations of trypsin-solubilized particulate guanylate cyclase from rat liver plasma membranes exhibited a molecular weight of about 140,000 compared with 200,000 to 300,000 for the detergent-solubilized enzyme (108, 277, 337, 334, 206, 336, 38, 269, 210, 209, 13, 126, 255, 257). However, when this trypsin-solubilized fragment was purified 500-fold, a considerably smaller fragment was obtained (336). This fragment had a Stokes radius of 40 Å, a sedimentation coefficient of 4.6 S, and an estimated molecular weight of about 80,000 (336). These values are smaller than those obtained for native, detergent-solubilized particulate guanylate cyclase, and, in fact, compare quite closely to those obtained for the subunits of the soluble enzyme purified from lung or liver (226, 256, 116, 117, 176). Similar results were obtained with crude trypsin-solubilized preparations from rat liver plasma membranes when proteolysis was conducted in the presence of Mn-GTP (16). It appears that the presence of substrate, Mn-GTP, confers protection on the proteolyzed enzyme preventing degradation of the solubilized protein to active fragments with molecular weights smaller than 68,000 (16).

b. KINETICS AND ALTERATIONS BY ACTIVATORS AND INHIBITORS. Kinetic analyses of soluble guanylate cyclase purified from a number of tissues suggest complex mechanisms for catalytic regulation. Unactivated enzyme exhibits classical Michaelis-Menten kinetics when Mn^{2+} or Mg^{2+}-GTP is used as substrate. This is true for enzyme purified from rat liver (327), bovine liver (273), rat lung (110, 226, 225, 25, 27), bovine lung (116, 164, 171, 352), and rat brain (359). Substrate affinities (K_m) vary from 5 (116) to 50 μM (327) with Mn^{2+}-GTP and

from 20 (116) to 150 μM (25) with Mg^{2+}-GTP as substrate. Hill plots of 1.0 have been obtained with GTP as substrate and either cation cofactor, suggesting that a single class of substrate binding sites exists in purified, unactivated enzyme which are not interactive (27). Studies with competitive inhibitors of enzyme activity have demonstrated that guanylate cyclase purified from rat lung has at least two domains at the nucleotide binding site (27). One recognizes the purine ring of guanine and is inhibited by other purine derivatives, such as ATP or the 2′,3′-dialdehyde derivative of GTP (27). The other binds phosphate moieties and can be inhibited by the addition of an extra phosphate group beyond the γ-position in the native substrate (27). Studies with oxygen-18-labeled isomers of GTP and soluble guanylate cyclase purified from bovine lung suggest that catalysis proceeds by way of a single direct displacement reaction, with the pyrophosphate-leaving group in the nucleotide being displaced by the 3′-hydroxyl group on the ribose (301). These data are supported by similar studies utilizing α-$^{18}O_1$-GTP as the substrate, which identified the α-phosphoanhydride bond as the site of cleavage during the cyclizing reaction (344). Analysis of the reaction of soluble guanylate cyclase purified from rat liver by nuclear magnetic resonance (NMR) spectroscopy demonstrated that only pyrophosphate and cyclic GMP in amounts corresponding to the amount of GTP utilized were produced by this enzyme (328).

The linear Michaelis-Menten kinetics exhibited by soluble guanylate cyclase can be altered by a variety of conditions. For example, guanylate cyclase can utilize several GTP analogues as substrates (27). Thus, GTP-γ-S, GMP-P(NH)P, and GMP-P(CH_2)P support the formation of cyclic GMP (cGMP) with either Mn^{2+} or Mg^{2+} as the cation cofactor at 10 to 75% of the rate observed with GTP (27). Unlike GTP, these analogues exhibit curvilinear kinetics with Eadie-Hofstee transformations, suggesting at least two substrate binding sites, one with a K_m of 25 to 70 μM and the other corresponding to 3 μM (27). Hill coefficients for these analogues were less than one, in contrast to GTP, suggesting multiple substrate binding sites interacting with negative cooperativity (27).

Guanylate cyclase can be inhibited by reversible formation of mixed disulfide bonds with agents such as cystamine and cysteine (337, 336, 25). Treatment of soluble guanylate cyclase purified from rat lung with cystamine led to a decrease in V_{max} and the appearance of multiple substrate binding sites (25). Control enzyme exhibited a K_m of 25 μM and 150 μM for Mn^{2+}- and Mg^{2+}-GTP, respectively (25). In the presence of cystamine, the enzyme exhibited curvilinear Eadie-Hofstee plots with K_ms of 30 and 2 μM for Mn^{2+}-GTP and 140 and 2 μM for Mg^{2+} GTP (25). These data suggest that more than one GTP site is present in purified enzyme preparations but that mixed disulfide formation or the use of guanine nucleotide analogues is needed to demonstrate the apparent cooperativity (25, 27).

Guanylate cyclase can be activated by a variety of agents that lead to the formation of nitric oxide. We have coined the term "nitrovasodilators" for this group of agents, which includes azide, nitrite, hydroxylamine, nitroglycerin, nitroprusside, nitrosamines, nitrosoureas, etc. The effects of these agents are discussed in greater detail in a later section. Treatment of soluble guanylate cyclase purified from rat lung with nitroprusside results in a 15-fold increase in V_{max} and the apparent appearance of multiple substrate binding sites (225). Control enzyme exhibited K_ms of 13 and 140 μM with Mn^{2+}- and Mg^{2+}-GTP, respectively (225). In the presence of nitroprusside, curvilinear Eadie-Hofstee plots were obtained with K_ms of 13 and 3 μM with Mn^{2+}-GTP and 100 and 22 μM with Mg^{2+}-GTP (225). These data are in close agreement with those obtained with mixed disulfide formation (25) and with GTP analogues (27) and suggest that two or more interactive substrate binding sites exist in purified preparations of soluble guanylate cyclase which can be regulated by mixed disulfide formation and enzyme activation. As mentioned, the soluble enzyme is a heterodimer, and each monomer could possess different, potentially interacting, sites for substrate binding. Alternatively, there may be different populations of enzyme molecules in purified preparations which exhibit different kinetic properties. It is interesting to note that alterations in the kinetic properties of guanylate cyclase upon activation by agents, such as sodium nitroprusside, have not been a uniform observation. Enzyme purified from bovine lung exhibited an increase in V_{max} with no change in K_m or cooperativity when exposed to sodium nitroprusside (116). Similar preparations in another laboratory exhibited increases in V_{max} with agents such as protoporphyrin IX and nitric oxide, but no change in K_m or cooperativity, with Mn^{2+}-GTP as the substrate (164, 171, 352). Studies conducted with Mg^{2+}-GTP showed a modest decrease in K_m from about 100 to about 60 μM (164, 171, 352). Differences between these studies and those cited previously could be due to differences in the tissue source of the enzyme, guanylate cyclase assay, or enzyme purification techniques. These differences are not due to the absence of a heme cofactor required for maximum activation in preparations of enzyme from rat lung (225, 25, 27), since multiple substrate binding sites can be observed in the presence of exogenously added heme (225).

In contrast to soluble guanylate cyclase, crude preparations of the particulate enzyme exhibit curvilinear kinetic plots, suggesting positive cooperativity with respect to GTP, and require higher concentrations of substrate for half-maximal activity. Apparent S 0.5 values reported for several tissues vary from 70 to 200 μM with Hill coefficients of 1.4 to 1.7. Tissues examined include rat lung (337, 334, 38), rat heart (190, 191), rat kidney (43), rat small intestine (57), rat cerebral cortex (314,

315), calf uterus (305), rabbit skeletal muscle (314, 315), and sea urchin sperm (108). These data suggest that particulate guanylate cyclase possesses multiple substrate-binding sites that interact with positive cooperativity when the enzyme resides in its native membrane environment.

The characteristic positive cooperativity exhibited by particulate, membrane-bound guanylate cyclase can be altered by several different treatments. Extraction of particulate preparations with detergent yields solubilized guanylate cyclase exhibiting classic Michaelis-Menten kinetics with Hill coefficients of about 1.0. This has been observed with rat lung (337, 206), rat liver (unpublished observation), mouse mammary gland (289), and sea urchin sperm (112, 108, 277). Similarly, when guanylate cyclase is solubilized from particulate preparations obtained from rat liver by limited proteolysis with trypsin, the enzyme displays typical linear kinetics (336, 16). Thus, positive cooperativity appears to be a characteristic of guanylate cyclase in its native membrane environment. This may reflect the ability of membrane lipids to facilitate intramolecular interactions requisite for positive cooperativity. However, this may also reflect the influences of other membrane components with secondary effects on enzyme activity. For example, particulate guanylate cyclase solubilized by limited proteolysis with trypsin exhibited striking heat lability even at 32°C (16). The enzyme could be rendered heat stable by the addition of Mn-GTP (16). Temperature lability at low substrate concentrations could result in the appearance of positive cooperativity, even though this may not be an intrinsic characteristic of the enzyme. In fact, trypsin-solubilized enzyme exhibited linear kinetics over very short incubation periods (16).

Highly purified particulate guanylate cyclase prepared from rat lung utilized GTP analogues as substrates in a fashion similar to the soluble enzyme purified from the same tissue (337, 27). The nucleotide analogues GTP-γ-S and GMP-P(NH)P supported particulate guanylate cyclase activity at 20 and 2% of the maximum rate achieved with GTP, respectively, when Mn^{2+} was used as the cation cofactor (337, 27). The purified particulate enzyme demonstrated linear kinetic plots and a K_m of about 100 to 130 μM with Mn^{2+}-GTP. However, curvilinear plots with the two most linear portions yielding K_m values of 100 to 600 μM and 15 to 30 μM were obtained with the GTP analogues (337). When these data were analyzed by the method of Hill, a coefficient of 1.0 was obtained with GTP, while coefficients less than 1.0 were obtained with the GTP analogues (337). These data agree closely with those obtained with the soluble enzyme purified from the same tissue (27). They suggest that both particulate and soluble guanylate cyclase possess multiple nucleotide binding sites which are identical and noninteractive when GTP is used as the substrate, but which interact with negative cooperativity when GTP analogues are used as substrates (337, 27).

4. Studies with antibodies to guanylate cyclase. Several laboratories have generated polyclonal antibodies against guanylate cyclase purified from a variety of sources. Rabbits immunized with soluble guanylate cyclase purified from bovine brain yielded serum which gave a single precipitin line on Ouchterlony gels when tested against crude and purified bovine brain soluble guanylate cyclase (260). These polyclonal antibodies inhibited guanylate cyclase activity in crude supernatant fractions prepared from a variety of rat tissues including brain, liver, lung, heart, and kidney (260). These antibodies also inhibited soluble guanylate cyclase from mouse brain, liver, lung, and heart and mouse neuroblastoma cells NIE (178, 260). Preparations of particulate guanylate cyclase from various rat and mouse tissues did not cross-react with these antibodies as judged by the absence of a precipitin band on Ouchterlony gels, the lack of inhibition of cyclase activity, and the inability to immunoprecipitate solubilized particulate guanylate cyclase using goat anti-rabbit second antibody (260). Polyclonal antibodies were also generated against highly purified sea urchin sperm particulate enzyme (109). High concentrations of these antibodies inhibited preparations of sea urchin spermatozoa particulate guanylate cyclase and particulate enzyme from rat heart, lung, spleen, kidney, and liver while no cross-reactivity was observed with crude preparations of soluble guanylate cyclase from these rat tissues (109). In contrast, it has been reported that polyclonal antibodies raised against highly purified rat brain soluble guanylate cyclase cross-reacted with the particulate enzyme from rat brain (359).

Monoclonal antibodies generated against soluble guanylate cyclase purified from rat lung or brain have also been developed. A number of monoclonal antibodies developed against purified soluble isoenzyme from rat lung do not inhibit guanylate cyclase activity and cross-react with enzyme from rat brain cortex, liver, heart, kidney, and testes (226, 256, 26). These antibodies also cross-react with soluble isoenzyme prepared from beef and pig lung, show partial cross-reactivity with mouse lung enzyme, and show no cross-reactivity with soluble guanylate cyclase from rabbit or human tissue (226, 256, 26). These antibodies did not cross-react with particulate guanylate cyclase from any tissue tested (337, 336, 226, 256, 26). Nakane and Deguchi reported the production of monoclonal antibodies directed against rat brain soluble guanylate cyclase which cross-react with preparations of soluble guanylate cyclase from several rat tissues but not with brain enzyme from mouse, rabbit, chick, or monkey (261). They also reported that these monoclonal antibodies did not cross-react with particulate guanylate cyclase from various rat tissues, in close agreement with results obtained with monoclonal antibodies generated against rat lung soluble enzyme (226, 256, 26). These

data indicate that the different isoenzyme forms of guanylate cyclase are antigenically dissimilar (337, 336, 226, 256, 26, 261).

Monoclonal antibodies directed against rat lung soluble guanylate cyclase have been used for immunocytochemical localization of guanylate cyclase in rat brain sections (3, 2, 262). Staining for guanylate cyclase was observed within neuronal perikarya and proximal dendrites of rat caudate putamen complex, neocortex, and cerebellum (3, 2, 262). Nuclear staining was uniformly absent in all tissue sections. Immunocytochemical patterns observed with monoclonal antibodies to guanylate cyclase were identical to those observed with antibodies against cyclic GMP and cyclic GMP-dependent protein kinase (3, 233). The immunocytochemical staining patterns for cyclic AMP were different from those obtained for cyclic GMP or guanylate cyclase (3). These data are consistent with biochemical studies that support a cellular selectivity of cyclic GMP (233, 127, 37, 52, 4), cyclic GMP phosphodiesterase (112, 116), and cyclic GMP-dependent protein kinase (233). In contrast, Zwiller et al. (362) have reported a more ubiquitous localization of guanylate cyclase by polyclonal antibody immunohistochemistry in cerebellum. Such disparities may be ascribed to methodological differences and the use of antibodies with differing specificities. It is not clear whether the soluble, particulate, or both isoenzyme forms of guanylate cyclase were being stained in these latter studies, since the antibody used showed cross-reactivity with the two forms of the enzyme (362). The immunocytochemical data obtained with monoclonal antibodies suggest that a pool of soluble guanylate cyclase exists that is closely associated with intraneuronal pools of cyclic GMP, cyclic GMP-phosphodiesterase, and cyclic GMP-dependent protein kinase.

Monoclonal antibodies directed against soluble guanylate cyclase purified from rat lung have also been utilized to develop a direct immunoradiometric assay for this protein (227). Two antibodies that recognize different antigenic determinants on the enzyme, as determined by competitive binding studies, and which are of different IgG subclasses (IgG1 and IgG2a) were used for this immunoassay (227). Specific monoclonal antibody (IgG2a) was preabsorbed to *S. aureus* membranes, purified soluble guanylate cyclase standards or samples of interest were incubated with this complex, and the amount of enzyme absorbed was quantified with the addition of the second specific monoclonal (^{125}I-IgG1) antibody (227). Nonspecifically absorbed ^{125}I-IgG1 antibody was removed by washing at pH 6.0, a step that preferentially removes IgG1 compared to IgG2a from Protein A (227). This direct double determinant tandem immunoradiometric assay correlated closely with the quantity of enzyme activity in a variety of samples (227). Direct quantification of enzyme protein with this assay yielded an estimate of the theoretical maximum specific activity for soluble guanylate cyclase, which was about 350 nmol of cyclic GMP formed/min/mg protein, in close agreement with reported values (33, 273, 110, 226, 117, 164, 260, 359). This assay has been successfully used to quantify soluble guanylate cyclase in a variety of rat tissues and cell lines and is capable of measuring as little as 70 fmol of the enzyme protein (227). This assay is unaffected by agents which influence guanylate cyclase activity, such as sodium nitroprusside and cystamine, indicating that quantification of the enzyme may be performed regardless of the activation state of the enzyme (227).

Monoclonal antibodies generated against soluble guanylate cyclase purified from lung also have been used for immunopurification of the enzyme (176). Antibodies (IgG1) against the enzyme were immobilized on cyanogen bromide-activated Sepharose 4B resin. Immunoaffinity resin was loaded with crude rat lung supernatants, washed with high salt buffers, and eluted with 3 M urea. Soluble guanylate cyclase purified by immunoaffinity chromatography yielded an enzyme with a molecular weight of 150,000 with M_r 82,000 and 70,000 subunits on SDS gel electrophoresis (176). The specific activity of these homogeneous preparations was 430 and 50 nmol cyclic GMP produced/mg/min with Mn^{2+}-GTP or Mg^{2+}-GTP as substrate (176). This rapid purification procedure permits the generation of milligram quantities of homogeneous enzyme that can be activated by nitroprusside or arachidonic acid (176). Thus, detailed physiochemical modifications of soluble guanylate cyclase with activation should now be possible.

5. Regulation of soluble and particulate guanylate cyclase. a. ACTIVATION BY NITROVASODILATORS, RELATED COMPOUNDS, AND FREE RADICALS. In early studies examining the kinetic properties of soluble and particulate guanylate cyclase, GTPase inhibitors were added to maintain substrate concentrations constant (187, 245, 258). Sodium azide, a GTPase inhibitor, activated crude soluble guanylate cyclase obtained from homogenates of rat liver in a dose-dependent manner (187, 245, 258). In these early experiments, activation was observed with guanylate cyclases obtained from many, but not all, tissues. Activation was observed with soluble guanylate cyclase from bovine brain and guinea pig tracheal smooth muscle (187, 254, 180, 189), guinea pig taenia coli (180), rat liver (187, 51, 254, 245, 258, 130, 189, 178), cerebral cortex (187), adipocytes (224), and renal cortex and medulla (187, 50, 254, 43, 189, 62). Crude particulate preparations were also activated by azide in rat liver (187, 254, 189, 178), kidney (187, 189), cerebral cortex (187, 254, 180, 189, 188), and cerebellum (187, 254, 180, 189). Most notably, certain tissues did not respond to azide with an activation of guanylate cyclase, including rat heart, spleen, pancreas, adrenal, skeletal muscle, and lung (187, 254, 180, 189, 178).

Activation of guanylate cyclase has also been observed

with other agents including $NaNO_2$, hydroxylamine, phenylhydrazine (187), nitroglycerin (178), nitrosoguanidine and nitrosamines (63), nitrosurea (333), 4-nitroquinolone 1-oxide (66), isosorbide dinitrate, pentaerythritol tetranitrate, and amylnitrate (138, 169). In general, nitrocompounds and their derivatives do not demonstrate the tissue specificity for activation of guanylate cyclase that is characteristic of azide and hydroxylamine (187, 254, 250, 245, 258, 189, 178, 63, 333, 66, 138, 169, 55). All of these compounds are potential sources of nitric oxide under the appropriate conditions (178, 148, 319, 183, 354). Thus, nitric oxide has been proposed as the proximal activator of guanylate cyclase by these agents, and its formation may represent the common pathway by which most of these agents influence enzyme activity (254, 250, 255, 258, 178, 5). Since all of these compounds can lead to the formation of nitric oxide and can relax vascular (and nonvascular) smooth muscle, we have coined the term "nitrovasodilators" for this diverse group of inorganic and organic agents. Nitric oxide activates soluble guanylate cyclase from virtually all tissues tested. Maximum activation by nitric oxide is similar to that achieved with other agents activating guanylate cyclase (257, 258, 5). Furthermore, activation with nitric oxide is not additive to that by other nitrocompounds or their derivatives (257, 258, 5). These data support the hypothesis that activation of guanylate cyclase by nitrovasodilators is mediated by nitric oxide formation which may occur enzymatically or nonenzymatically from these precursor compounds.

Generation of nitric oxide from azide or hydroxylamine suggests an explanation for the tissue specificity and other characteristics of the activation of guanylate cyclase by azide. Although sodium azide activates crude preparations of rat liver soluble guanylate cyclase, this effect is lost upon partial purification of the enzyme (250, 257, 245, 258, 246). Activation of the partially purified liver enzyme by azide could be restored by addition of a factor isolated from crude liver supernatant fractions (250, 257, 245, 258, 178, 246). This factor was heat labile, nondialyzable, and chymotrypsin sensitive, and it could be separated from soluble guanylate cyclase by chromatography on DEAE-cellulose (245, 258, 246). The factor had a molecular weight of about 200,000 and copurified with catalase (246). Addition of azide and either the activator factor or catalase to azide-insensitive preparations resulted in activation of guanylate cyclase (245, 258, 178, 246). Horseradish peroxidase or some cytochromes could substitute for the factor or catalase in mediating the activation of guanylate cyclase by azide (258, 246). Catalase, and presumably other metalloproteins containing heme or flavin moieties and involved in oxidation-reduction reactions, can catalyze the oxygen-dependent conversion of azide and hydroxylamine to nitric oxide (319, 183, 246, 244). Indeed, azide activation of crude liver guanylate cyclase was oxygen dependent (246) and exhibited a lag phase that could be eliminated by preincubating azide together with enzyme (187, 245, 246). In contrast, agents that can spontaneously generate nitric oxide, such as nitroprusside, or nitric oxide itself do not demonstrate a dependence upon O_2, a time lag, or tissue specificity in effects on guanylate cyclase (254, 250, 257, 245, 258, 246).

The tissue specificity exhibited by azide for activation of guanylate cyclase could also be explained by the presence of specific inhibitors. Crude soluble guanylate cyclase from heart and lung is not activated by azide and its addition to incubations of liver guanylate cyclase inhibited the activation of this enzyme by azide (187, 245, 244). Inhibitory activity was nondialyzable, heat labile, and resistant to trypsin treatment (258, 244). Chromatography of heart or lung supernatants by gel filtration separated the inhibitory materials from guanylate cyclase, which could now be activated by azide (258, 244). The inhibitory materials cochromatographed with hemoglobin and myoglobin (258, 244). Addition of these agents, which are ferro (Fe^{2+})-metalloproteins, but not their ferric (Fe^{3+}) forms, such as methemoglobin, to preparations of azide-sensitive enzyme preparations resulted in inhibition of azide, $NaNO_2$, nitroprusside, and nitric oxide activation (258, 244). Presumably these heme-containing compounds in their Fe^{2+} form inhibit the production of or scavenge free nitric oxide. Thus, the tissue specificity of azide and hydroxylamine activation of guanylate cyclase appear to be functions of the presence or absence of metalloproteins containing heme or flavin prosthetic groups that can alter the generation or accumulation of nitric oxide.

As suggested by the above data, the activation of guanylate cyclase by nitro compounds and their derivatives probably involves at least one, and probably several, oxidation-reduction events. It has been pointed out that nitric oxide does not require protein activators or oxygen for its effects on guanylate cyclase (254, 250, 257, 245, 258, 178, 246). Indeed, activation with this agent was similar in air or nitrogen atmospheres and was decreased in an atmosphere of 95% O_2/5% CO_2 (5). Similarly, oxidizing agents such as hydrogen peroxide, methylene blue, or $Ke_3Fe(CN)_6$ inhibited, whereas reducing agents like ascorbate, cysteine, glutathione, and dithiothreitol promoted, activation of guanylate cyclase by these agents (187, 225, 257, 178, 5, 32). Similarly, guanylate cyclase purified to homogeneity from rat liver or lung could be activated as much as 30- to 40-fold by direct exposure of incubations to nitric oxide (33, 32). The dose response to nitric oxide activation was shifted to the left with purified, as compared to crude, enzyme preparations (32). Also, activation was markedly affected by the concentration of purified protein in the incubations with less activation observed at higher protein concentrations (33). Furthermore, activation of purified enzyme by nitric oxide was affected by several oxidation-reduction agents.

Methemoglobin, dithiothreitol, bovine serum albumin, and sucrose potentiated the activation, whereas hemoglobin inhibited the activation of purified guanylate cyclase (32). These data suggest that oxidation-reduction agents may affect activation at several points. For example, oxidizing agents and O_2 could be inhibiting activation by converting nitric oxide to less effective higher oxides of nitrogen, such as nitrogen dioxide; reducing agents might be potentiating activation by preventing this oxidation (178, 5, 32). Also, these agents could be altering the oxidation-reduction state of the enzyme directly which may have a profound effect on its ability to be subsequently modulated by nitric oxide. Finally, these compounds could be altering a key prosthetic group, such as heme, which might mediate the activation of guanylate cyclase by nitric oxide (254, 250, 116, 117, 164, 225, 118, 257, 245, 258, 178, 5, 246, 244, 32, 45, 162, 325, 48).

Activation of guanylate cyclase with nitro compounds and their derivatives results in striking alterations in the properties of the enzyme. Unactivated native enzyme demonstrates a specific preference for Mn^{2+} compared to Mg^{2+}; activity may be 4- to 10-fold higher in the presence of Mn^{2+}-GTP compared to Mg^{2+}-GTP (33, 110, 226, 256, 189). Upon activation of guanylate cyclase with azide, nitric oxide, and nitroso compounds, the enzyme can utilize either Mn^{2+} or Mg^{2+} equally well (189). Also, the enzyme loses its requirement for excess free Mn^{2+} but not free Mg^{2+} for expression of maximum activity (189). Activation increases the V_{max} with both Mn^{2+}-GTP and Mg^{2+}-GTP as substrate, with a greater effect observed when Mg^{2+} is the cation cofactor (33, 273, 110, 226, 256, 225, 352, 189). The K_m for Mn^{2+}-GTP remains virtually unchanged, whereas that for Mg^{2+}-GTP decreases from about 150 to about 40 μM (273, 164, 225, 352, 189). Indeed, before activation guanylate cyclase demonstrates a single, noninteractive binding site when Mg^{2+}-GTP is used as the substrate (225, 27). However, upon activation, the enzyme demonstrates at least two binding sites for Mg^{2+}-GTP with K_ms of about 100 and 20 μM that appear to interact in a negatively cooperative fashion (225, 25, 27). These kinetic characteristics are virtually identical to those observed in the presence of other guanine nucleotide analogues (27) and inhibitory concentrations of disulfides (25). Native guanylate cyclase does not catalyze the formation of cyclic AMP from ATP. Yet activated enzyme can form cyclic AMP from Mg^{2+}-ATP at a rate that is 1 to 15% that of the formation of cyclic GMP from Mg^{2+}-GTP by activated enzyme (247). Enzyme activity catalyzing the formation of cyclic GMP and cyclic AMP in the presence of azide coeluted after chromatography of crude rat liver supernatant fractions by gel filtration, and parallel losses of the formation of both cyclic nucleotides were obtained by heat denaturation (247). GTP inhibits the formation of cyclic AMP, whereas ATP inhibits the formation of cyclic GMP (247). Thus, it appears that both cyclic nucleotides can be formed by a single soluble enzyme, guanylate cyclase, when it is activated by nitrovasodilators. The significance of this alternate mechanism for cyclic AMP synthesis remains unknown.

The ability of nitro compounds and their derivatives to activate particulate in addition to soluble guanylate cyclase has not been conclusively determined. Early studies demonstrated activation of particulate guanylate cyclase by azide in rat liver (187, 254, 189, 178), kidney (187, 189), cerebral cortex (187, 254, 189, 178, 188), and cerebellum (187, 254, 189, 178). Similarly, nitric oxide, sodium nitroprusside, hydroxylamine, and nitroglycerin activated particulate guanylate cyclase in rat liver, cerebral cortex, and cerebellum (250, 178). Particulate guanylate cyclase in membranes isolated from homogenates of rat lung was stimulated 300- to 400-fold by nitric oxide (30). This activation was apparently dependent upon the oxidation-reduction state of the membranes, requiring a balance between the oxidation and reduction of key sulfhydryl groups (30). However, a problem with these studies is the use of unwashed membranes for determinations of enzyme activation. Adventitious and entrapped cytosolic proteins are common contaminants of membrane preparations; as much as 50% of the guanylate cyclase activity in high speed particulate fractions prepared from homogenates of liver or lung can be removed with successive hypo- and hypertonic washes and recognized by monoclonal antibodies directed against soluble guanylate cyclase (337, 334, 206, 336). Thus, the activation of particulate guanylate cyclase observed with various nitro compounds and their derivatives could be due to contamination of those membrane preparations with soluble enzyme. Particulate guanylate cyclase in rat liver membranes can be washed free of soluble contaminants, solubilized by limited proteolysis with trypsin, and purified about 500-fold (336, 210, 209). Both washed membranes and partially purified trypsin-solubilized particulate enzyme could be activated 3- to 4-fold with nitric oxide (336). Most recently, cell lines of neural origin have been identified that appear to contain only particulate guanylate cyclase (306). Virtually all of the guanylate cyclase in homogenates of these cells sedimented at 225,000 $\times$ g, bound to concanavalin A-Sepharose after detergent solubilization, and was activated with hemin (306, 337, 334, 342). Furthermore, soluble enzyme could not be detected with a sensitive immunoradiometric assay using two monoclonal antibodies directed against the soluble enzyme (306, 227). In homogenates and high speed particulate fractions obtained from these cultured cells, sodium nitroprusside activated guanylate cyclase about 3- to 4-fold, in close agreement with previous results in other preparations (306, 336, 254, 250, 178). To date, this is the strongest evidence that particulate guanylate cyclase can be regulated by nitro compounds and their derivatives. We have found that detergents inhibit the activation of soluble or particulate guanylate

cyclase by a variety of agents including nitrovasodilators, *E. coli* enterotoxin, and atrial natriuretic factor as discussed below. Since detergents are required for solubilization and purification of the particulate isoenzyme and removal of any contaminating soluble enzyme, this important question about nitrovasodilator activation of the particulate isoenzyme cannot be readily resolved.

The direct interaction of nitric oxide with crude (178, 5), partially purified (5), and purified guanylate cyclase (33, 32) and its postulated role as the mediator of activation of this enzyme by nitrovasodilators suggest that guanylate cyclase might be regulated by free radicals in vivo (254, 250, 255, 257, 258). Indeed, partially purified soluble guanylate cyclase from rat liver was activated by incubating in the presence of superoxide dismutase, which converts superoxide anion to hydrogen peroxide (254, 250, 257, 258, 248). Activation was potentiated by incorporating nitrate reductase, a flavoprotein catalyzing the formation of superoxide anion from molecular oxygen, into incubations with superoxide dismutase and guanylate cyclase (248). Inhibitors of superoxide dismutase, such as glutathione or KCN, also inhibited activation of guanylate cyclase (248). Free radical scavengers, such as butylated hydroxyanisole, catecholamines, or hydroquinone, also inhibited this activation (248). Activation could not be achieved by direct addition of H_2O_2, and catalase inhibited the effects of superoxide dismutase on guanylate cyclase (248). These data suggest that both the superoxide anion and H_2O_2 are required for activation of guanylate cyclase (248). These compounds can react by the Haber-Weiss or Fenton reactions to form hydroxyl free radicals, a postulated activator of guanylate cyclase in vivo (254, 250, 257, 258, 248). While this hypothesis is consistent with the effects of hydroxyl free radical scavengers and catalase on activation of guanylate cyclase by superoxide dismutase and the potentiating effects of nitrate reductase, the generation and/or effects of other reactive species of oxygen cannot be excluded. Hydrogen peroxide has been demonstrated to activate soluble guanylate cyclase from lung, probably by a similar mechanism (347). The formation of superoxide anion, hydrogen peroxide, and hydroxyl radical is influenced by many processes and agents in vivo (318, 99). Phagocytosis by polymorphonuclear leukocytes results in a burst of oxidative activity resulting in superoxide ion and hydroxyl radical formation and, possibly, changes in cyclic GMP levels (318, 163). Also, arachidonate metabolism and prostaglandin formation can increase levels of hydroxyl radical, and these processes are associated with elevated levels of cyclic GMP as well as increases in guanylate cyclase activity (124, 80, 76, 343). Thus, it is possible that physiological, hormonal, and autocoid regulation of guanylate cyclase activity and cyclic GMP formation may occur via mechanisms involving oxidative processes generating reactive free radicals that interact directly with guanylate cyclase.

Phenylhydrazine has been reported to be a weak activator of guanylate cyclase (187). However, in contrast to the nitric oxide or hydroxyl free radical pathways of activation described previously, this compound appears to generate a phenyl free radical that activates the enzyme (170). Activation of guanylate cyclase by phenylhydrazine is potentiated by flavin nucleotides that enhance the formation of phenyl radicals (170, 307), while inclusion of antioxidants and free radical scavengers or anaerobic conditions prevented the activation of guanylate cyclase by phenylhydrazine (170). Hydrazine alone could not activate the enzyme, supporting a role for the phenyl moiety in this event (170). As with nitric oxide, activation of guanylate cyclase by phenylhydrazine is dependent upon the presence of heme in enzyme preparations (170). This requirement may be due to the ability of heme to catalyze the formation of the phenyl radical from phenylhydrazine (170, 307). All of these data indicate that guanylate cyclase is uniquely regulated and that it can be activated in a reversible manner with various free radicals and oxidizing agents. The physicochemical mechanisms involved in the activation by this diverse group of agents will undoubtedly share many common features or may be identical. However, the precise mechanism(s) involved in this oxidation and activation will require additional studies. The activation of guanylate cyclase with azide, nitric oxide, and related agents is spontaneously reversible (187, 5, 32). The activation and inactivation of purified enzyme in the absence of other macromolecules or enzymes indicate that significant covalent modifications of the protein, such as phosphorylation, adenylation, etc., are not involved in this activation or inactivation process.

b. ROLE OF PORPHYRINS IN THE REGULATION OF GUANYLATE CYCLASE. It was recognized early on that porphyrins in their free form or as hemoproteins could have profound effects on the regulation of guanylate cyclase activity. Indeed, activation of this enzyme by sodium azide and hydroxylamine demonstrated a requirement for hemoproteins, such as catalase, cytochrome *c*, cytochrome reductase, or peroxidase, and could be inhibited by other hemoproteins including hemoglobin and myoglobin (187, 254, 250, 245, 258, 246, 244). Similarly, agents such as hemoglobin also inhibited the activation of guanylate cyclase by nitric oxide (187, 254, 250, 258, 244). The first suggestion of a specific role for porphyrins in the regulation of guanylate cyclase was the role of catalase in the generation of nitric oxide from azide (254, 250, 245, 258, 246). It had been demonstrated much earlier that, in the presence of hydrogen peroxide, catalase could catalyze the formation of the nitric oxide radical from sodium azide (319, 183). Hydroxylamine could also activate guanylate cyclase (187, 178) and substitute for azide in the presence of catalase and a hydrogen peroxide-generating system (49). Of greater interest was the requirement for the formation of a complex

between nitric oxide and catalase (49). Indeed, the capacity for azide or hydroxylamine to activate guanylate cyclase was closely correlated with their ability to form NO-catalase (49). These data suggest that the hemoprotein is important not only for the oxidative conversion of the precursor compounds to nitric oxide but also as a free radical acceptor (49). The heme-nitric oxide complex appears to be the actual moiety regulating the activity of guanylate cyclase.

Similar results were obtained with a variety of other agents including N-methyl-N′-nitrosoguanidine, nitroprusside, and nitrite (45). In the case of these agents, conversion to nitric oxide occurs nonenzymatically (259, 308, 17, 198, 67). Partial purification of hepatic soluble guanylate cyclase results in loss of responsiveness to these agents, as well as to nitric oxide (45, 49). Responses were partially restored by the addition of free hematin, hemoglobin, methemoglobin, or catalase to those preparations (45). Activation was potentiated by the incorporation of a reducing agent into these incubations which facilitates the generation of nitrosyl-heme from hemoproteins and agents such as nitroprusside by maintaining the heme iron in the ferrous (Fe^{2+}) form (178, 45, 259, 308, 17, 198). Preformed nitrosyl-hemoglobin, generated by reacting nitric oxide with methemoglobin or hemoglobin, maximally activated these preparations of partially purified guanylate cyclase (45, 49). Activity could not be increased by subsequent addition of agents, such as nitric oxide or sodium nitroprusside (45, 49). Preformed nitrosyl-hemoglobin was 10-fold more potent in activating guanylate cyclase than the nitro compounds or their derivatives (45). Other preformed nitrosyl-hemoproteins, including nitrosyl-catalase and cytochrome P450 also were effective in activating the enzyme (45, 49, 67). Activation of partially purified guanylate cyclase by preformed nitrosyl-hemoproteins was not potentiated by reducing agents, supporting the suggestion that reducing agents affect the conversion of the parent nitro compounds to nitrosyl-heme complexes (45, 49). These data suggest that activation of guanylate cyclase by nitro compounds and their derivatives occurs through reductant-dependent formation of nitrosyl-porphyrin complexes.

The ability to explore further these questions was expedited by the development of different protocols for obtaining homogeneous preparations of soluble guanylate cyclase from various tissues. The first preparations of enzyme purified from rat liver and lung were apparently devoid of heme by spectrophotometric criteria (33, 273, 110, 226, 32). These preparations demonstrated a marked insensitivity to nitric oxide, with activations of only 2- to 4-fold in contrast to crude preparations which could be activated 30- to 50-fold (33, 5, 32). Activation was potentiated in these preparations by including hemoglobin and dithiothreitol into incubations (32). Using a different purification protocol, soluble guanylate cyclase was purified from bovine lung in a form containing heme (116, 117, 113, 118). In these studies, enzyme containing heme could be separated from enzyme without heme by ion-exchange chromatography (116, 117, 113, 118). Guanylate cyclase possessing heme retained its ability to be activated by nitroprusside while enzyme without heme could not be similarly activated (116, 117, 113, 118). Subsequently, other protocols for purification have been employed that confirm the presence of heme as a prosthetic group in soluble guanylate cyclase purified from lung (171). It appears that earlier observations of a lack of heme in the responsiveness to nitric oxide of purified soluble guanylate cyclase were due to variability in the retention of heme during different purification protocols (33, 116, 117, 164, 113, 118, 32). Procedures which have been demonstrated to be effective in removing the heme moiety from the enzyme include ammonium sulfate precipitation, ion-exchange chromatography, isoelectric precipitation, isoelectric focusing, native polyacrylamide gel electrophoresis, and freeze-thawing (33, 116, 117, 164, 113, 118, 32, 45, 48). Furthermore, the sequence of steps used during purification appears to be important since ion-exchange chromatography prior to ammonium sulfate precipitation did not result in loss of heme from the enzyme (117). Whether heme is a normal prosthetic group of soluble guanylate cyclase in vivo or whether their association is an artifact of purification remains unknown. It is virtually impossible to obtain tissues for enzyme purification free of contaminating blood and hemoglobin.

As indicated above, soluble guanylate cyclase can be purified from several tissues with or without heme. The heme-deficient form can be reconstituted with heme by reacting purified enzyme with hematin in the presence of an excess of reducing agent such as dithiothreitol (164, 168). The interaction between enzyme and porphyrin is of high affinity since heme remained associated with guanylate cyclase during extensive dialysis or gel filtration (164). Heme-deficient guanylate cyclase demonstrated modest or little activation by agents such as nitric oxide, S-nitrosopenicillamine, or nitroprusside (33, 273, 110, 226, 256, 164, 32, 325, 48). However, these agents markedly activated guanylate cyclase purified or reconstituted with heme (273, 164, 225, 48). Also, addition of heme to heme-deficient purified guanylate cyclase restored the ability of the enzyme to be activated by these agents (273, 226, 164, 32, 325, 48). Preformed nitrosyl-heme maximally activated heme-deficient guanylate cyclase in the absence of additional free heme (273, 164, 352, 325, 48, 347, 318, 99). Agents that bind to the heme moiety, such as sodium cyanide, block the ability of free heme to restore nitroprusside activation of heme-deficient preparations of enzyme (225, 352). Cyanide was also found to block activation of the enzyme by azide, nitroprusside, or nitric oxide in earlier studies with crude or partially purified preparations (187, 178, 5). These

data support the hypothesis that activation of soluble guanylate cyclase by nitro compounds and their derivatives involves the interaction of the nitric oxide free radical with a heme moiety associated with the enzyme. This nitrosyl-porphyrin complex directly mediates the activation of guanylate cyclase. Indeed, this may be a general mechanism for regulation of soluble guanylate cyclase, since activation by phenylhydrazine, which occurs through a phenyl free radical as discussed above, is also dependent upon the presence of heme in preparations of soluble guanylate cyclase purified from bovine lung (170). Although the mechanism by which the nitrosyl-heme complex activates guanylate cyclase remains unknown, a model has been presented (352). Protoporphyrin IX, the demetalated precursor of ferroprotoporphyrin IX (heme), markedly activates guanylate cyclase purified from lung or liver (273, 164, 225, 352). Maximum activation with this agent was similar to that obtained with nitro compounds in the presence of heme, and the affinity of the enzyme for this porphyrin was about nanomolar (171, 352). Increases in V_{max} and decreases in K_m for Mg^{2+}-GTP observed with enzyme activated with nitrosyl-heme were also observed with protoporphyrin IX (171, 352). Protoporphyrin IX can activate heme-containing, heme-deficient, and heme-reconstituted enzyme (273, 164, 352), and this activation was unaffected by iron-complexing agents such as cyanide (273, 352). Insertion of iron into protoporphyrin IX converts this activator into an inhibitor of basal and nitro-activated guanylate cyclase (254, 342, 273, 164, 225, 257, 171, 352, 258, 5, 244, 32, 45, 162, 48). Heme competitively inhibited the activation of guanylate cyclase by protoporphyrin IX, suggesting that these porphyrins compete for a common binding site on the enzyme (352). Protoporphyrin forms a stable complex with guanylate cyclase, since this complex does not dissociate during gel filtration or dialysis (352). These data suggest that activation of guanylate cyclase by nitro compounds or protoporphyrin occurs through common pathways. Indeed, electron paramagnetic resonance studies have demonstrated that the binding of nitric oxide to ferroprotoporphyrin IX (heme) results in weakening of coordinate bonds holding the iron in the plane of the protoporphyrin molecule (199, 174, 252). This complex with the metal out of the plane of the protoporphyrin molecule resembles protoporphyrin IX. Thus, nitric oxide may convert the heme prosthetic group of guanylate cyclase into a protoporphyrin-like molecule, resulting in enzyme activation (352). Another simplistic view is that the heme group inhibits or prevents activation of the enzyme until nitric oxide complexes with the iron, resulting in a molecule that resembles protoporphyrin IX. However, it is unlikely that this is the entire mechanism. An activation-enhancing factor was partially purified from hepatic soluble fractions by isoelectric precipitation, ammonium sulfate precipitation, and ion-exchange chromatography (273). This heat-stable factor potentiated the activation of soluble guanylate cyclase by protoporphyrin IX about 2-fold (273). Also, rat thoracic aorta can be desensitized by pretreatment with nitroglycerin, resulting in a decrease in the amount of relaxation that can be elicited from those vessels by agents such as sodium nitroprusside (10, 185, 9, 286, 341). Such pretreatment of tissues with nitrovasodilators also results in molecular desensitization at the level of the soluble guanylate cyclase (9, 341). Soluble guanylate cyclase prepared from desensitized blood vessels demonstrates a diminished response to agents such as sodium nitroprusside, nitroglycerin, and nitric oxide compared with enzyme obtained from control vessels (9, 341). This desensitization is stable to partial purification of the enzyme with monoclonal antibodies directed against soluble guanylate cyclase. In contrast, guanylate cyclase from vessels pretreated with nitroglycerin demonstrated a 2- to 5-fold enhancement of activation by protoporphyrin IX compared to enzyme from control vessels (341). This potentiation of protoporphyrin IX activation was lost upon partial purification of the enzyme with monoclonal antibody (341). These data support the suggestion that activation of guanylate cyclase by protoporphyrin IX may involve other unknown factors. Furthermore, data obtained with vascular guanylate cyclase suggest that protoporphyrin IX activates the enzyme in this tissue by a mechanism dissimilar to that of nitric oxide, nitroglycerin, or sodium nitroprusside (341). Obviously, additional studies with enzyme purified from different tissues after several conditions of activation and inhibition are required in order to elucidate the mechanisms of activation by nitro compounds and porphyrins.

The ability of particulate guanylate cyclase to be regulated by porphyrins has also been described. Particulate guanylate cyclase in intact plasma membranes or after trypsin solubilization from homogenates of rat liver was activated 6- to 10-fold by protoporphyrin IX (208). Meso- and deuteroprotoporphyrin IX, hematoporphyrin, and protoporphyrin III also activated, while uroporphyrin III and hemin had no effect on particulate guanylate cyclase (208). Activation was optimal when Mg^{2+}-GTP was used as the substrate (208). In contrast, washed particulate preparations from C6 rat glioma and B103 neuroblastoma cells and from rat lung were activated 3- to 15-fold by hemin (342). Activation was observed only with Mg^{2+}-GTP as substrate and was unaffected by protease inhibitors indicating that this activation was not due to limited proteolysis (336, 342, 210, 209). Protoporphyrin IX alone had no effect on these preparations of particulate guanylate cyclase and did not compete for activation by hemin (342). Hemin activation was not affected by metal-complexing agents such as cyanide, azide, or fluoride but was almost completely inhibited by reducing agents such as sodium borohydride (342). These data suggest that hemin activation of particulate guanylate

cyclase requires the iron moiety to be in the ferric (Fe^{3+}) form, although it may be complexed with other ligands (e.g., cyanide) (342). Since the activation by hemin was unaltered during purification of the particulate enzyme from rat lung, it seems unlikely that other factors are required, and a direct interaction of the oxidized porphyrin with the enzyme probably occurs (342). The discrepancies between these observations (342) and those made with rat liver plasma membranes (208) could be a result of several factors. It has been demonstrated that a significant amount of guanylate cyclase in crude membrane preparations may represent contamination with soluble enzyme, and this could explain the observed activations. The more recent studies were performed with particulate preparations obtained from cultured cell lines devoid of soluble guanylate cyclase as well as with preparations of particulate guanylate cyclase purified 1000-fold from rat lung, also devoid of soluble contaminants (306, 337, 342). The finding that particulate guanylate cyclase activity can also be regulated by heme derivatives suggests that the soluble and particulate isoenzymes may be regulated in a coordinated fashion by porphyrin compounds. Activation of particulate guanylate cyclase occurs at concentrations of hemin resulting in profound inhibition of basal, protoporphyrin-activated, and nitric oxide-activated soluble enzyme (254, 342, 273, 164, 225, 257, 171, 352, 258, 32, 45, 162, 48). In contrast, lower concentrations of this porphyrin are required for activation of the soluble enzyme by free radicals including nitric oxide and phenyl radicals, while having little effect on the particulate enzyme (342, 33, 273, 110, 226, 256, 164, 225, 352, 32, 45, 162, 170).

c. ROLE OF THIOLS IN THE REGULATION OF GUANYLATE CYCLASE. As indicated above, important mechanisms underlying the regulation of guanylate cyclase involve oxidation-reduction reactions. Examples of such regulation include enzyme activation by Mn^{2+} (29), nitrovasodilators and nitric oxide (254, 250, 116, 117, 164, 225, 118, 257, 352, 258, 178, 244, 32, 45, 162, 325, 48), fatty acids and their oxidized metabolites (149, 131, 64), dehydroascorbate (142), oxygen and its free radical products (23, 248, 347), and the phenyl free radical (170). It has been suggested that these agents activate guanylate cyclase through a common mechanism involving free radical formation and oxidation of the enzyme. Since sulfhydryl groups can undergo oxidation-reduction reactions, and thiol-disulfide conversions have been demonstrated to regulate protein structure and function, it is reasonable to suggest that the targets for oxidation-reduction regulation on guanylate cyclase are sulfhydryl groups.

It was recognized early that agents that modify free sulfhydryl groups could alter basal and activated guanylate cyclase activity. Alkylating agents which covalently modify free thiol groups, such as maleimide, N-ethylmaleimide, and ethacrynic acid, inhibit basal soluble guanylate cyclase activity and activity stimulated by preincubation, sodium nitroprusside, nitrosyl-heme complexes, streptozotocin, nitrosourea, nitric oxide, nitrosoguanidine, nitrite, azide, and dehydroascorbate (178, 45, 168, 142, 22, 79, 324, 64, 44). Similarly, mixed disulfide formation with agents such as cystine, cystamine, and 5,5'-dithiobis(2-nitrobenzoic acid) also results in inhibition of basal and activated enzyme activity (187, 337, 336, 273, 25, 178, 168, 22, 79, 324). Indeed, soluble guanylate cyclase purified from rat lung was inhibited in a concentration- and time-dependent fashion by preincubating with cystamine (25). Studies with [^{35}S]cystamine resulted in incorporation of radioactivity into the enzyme with a concentration and time dependence similar to that of inhibition (25). Both radiolabeling and inhibition of activity were reversed by dithiothreitol, which converts mixed disulfides to free thiols (25). These data demonstrate that free thiols on soluble guanylate cyclase which are available for complexing with alkylating agents or mixed disulfide formation are critical for expression of basal and stimulated activity. These data also suggest that thiol-disulfide oxidation and reduction could be an important mechanism for regulating guanylate cyclase activity.

Agents that oxidize or reduce sulfhydryl groups markedly alter basal and stimulated guanylate cyclase activity. Dithiothreitol and other reducing agents inhibited basal guanylate cyclase in crude supernatant fractions from a variety of tissue. In contrast, up to 5 mM dithiothreitol had no effect on basal enzyme activity in crude soluble fractions from rat liver (168, 79). Some reports have demonstrated that enzyme purified to homogeneity from rat liver or lung exhibits an increase in basal activity in the presence of dithiothreitol (225, 32). Others have reported that similar preparations exhibit little alteration in basal activity in the presence of this reducing agent (25, 162, 48). The apparent contrast in the effects of reducing agents on basal activity probably reflects the oxidation state of the enzyme at the time of assay. It has been demonstrated that oxidation of guanylate cyclase by preincubation in an oxygen atmosphere can result in activation followed by inhibition of the enzyme that can be prevented with dithiothreitol (23, 347, 142, 22). Since enzyme can become oxidized during tissue homogenization, subcellular fractionation, or protein purification depending upon the buffers and conditions used, the effects of dithiothreitol would be expected to be variable depending upon the oxidation state of the enzyme. Dithiothreitol may then activate, have no effect, or inhibit basal activity depending on whether the enzyme is oxidized, in a "ground" state, or reduced, respectively.

Reducing agents also have profound effects on the activation of soluble guanylate cyclase. Dithiothreitol and other reducing agents potentiate the activation of crude and purified soluble enzyme by the nitrovasodilators, related compounds, and nitric oxide (187, 273, 164,

225, 178, 138, 32, 45, 162, 48, 168, 22, 64, 44, 161, 166, 165, 240). In contrast, activation by preincubation in an oxygen atmosphere is prevented or reversed by these reducing agents (23, 142, 22). Similarly, activation with nitric oxide can be reversed or prevented by dithiothreitol (295). Activation by fatty acids or their oxidized metabolites is also inhibited by dithiothreitol or reduced glutathione (149, 131). These data suggest a role for the reversible oxidation-reduction of sulfhydryl groups in regulating the activation state of the enzyme. Low or modest concentrations of reducing agents could maintain thiol groups of the enzyme in the reduced state and decrease basal activity. This may result in an apparent marked activation of the enzyme with an oxidizing agent that converts these thiols to disulfides. Alternatively, high concentrations of reducing agents may interact with the activating oxidant and/or prevent oxidation of the thiol groups. The possible participation of thiol groups in contaminating proteins during incubation cannot be readily assessed.

Other agents also exhibit profound effects on basal and activated guanylate cyclase activity. Diamide, oxidized glutathione, iodosobenzoic acid, oxidized coenzyme A, pantethene, and periodate all inhibit basal activity and nitric oxide activation of soluble guanylate cyclase (273, 25, 178, 168, 324). In contrast, dehydroascorbate activates soluble guanylate cyclase from spleen cells (142). Diamide or oxidized glutathione caused a rapid and irreversible loss of nitric oxide-stimulated enzyme activity with either crude or purified soluble guanylate cyclase from rat lung (31). These data suggest that, while oxidation of sulfhydryl groups is associated with activation of soluble guanylate cyclase, the enzyme can be "overoxidized" resulting in irreversible loss of basal and stimulated activity. The lability of the enzyme during purification and prolonged storage may also be related to excess oxidation and irreversible loss of enzyme activity, since such losses can be minimized with various reducing agents.

Studies with thiol reactive agents have suggested that more than one free thiol group on the enzyme is involved in regulating its activity. As discussed above, preincubation of soluble guanylate cyclase in an oxygen atmosphere results in activation followed by inactivation of the enzyme (23, 347, 142, 22). Activation and inactivation are dependent upon the presence of oxygen and can be blocked or reversed by dithiothreitol or reduced glutathione, suggesting that these effects are due to the reversible oxidation-reduction of thiol groups on the enzyme. However, activation can be dissociated from inactivation by alterations in temperature, pH, or alkylating agents, indicating that these effects are regulated by different sulfhydryl groups (142, 22). Also, enzyme activated by preincubation and subsequently inactivated by dithiothreitol possesses higher activity than enzyme prepared and stored in the presence of this reducing agent. These data suggest that guanylate cyclase obtained after dithiothreitol reversal of activation by preincubation is different from dithiothreitol-stabilized enzyme (22). It has been suggested that alterations of guanylate cyclase activity by preincubation may involve two sulfhydryl sites: one mediating activation and the other inactivation of the enzyme (22).

That more than one oxidizable site could be mediating the activation of the soluble enzyme was suggested by studies with guinea pig spleen cells. In these studies, guanylate cyclase could be activated by preincubation in an oxygen atmosphere or with dehydroascorbate (142). Both activations were reversible by dithiothreitol, again suggesting that these effects were mediated by sulfhydryl oxidation-reduction (142). However, activation by preincubation in an oxygen atmosphere was additive to that of dehydroascorbate, suggesting that the sulfhydryl groups involved in these activations may be different (142). In addition, low concentrations of the thiol alkylating agent N-ethylmaleimide had a minimal effect on basal enzyme activity and prevented dehydroascorbate activation, while higher concentrations also inhibited basal activity (142). Similar observations have been made in other tissues. Alkylating, oxidizing, and disulfide reagents at low concentrations can inhibit activation of soluble guanylate cyclase by nitro compounds without altering basal activity, whereas higher concentrations inhibit both basal and activated enzyme activity (178, 45, 168, 149, 131, 142, 79, 44). Taken together, these data suggest that multiple sulfhydryl groups participate in the regulation of guanylate cyclase activity.

Further support for the suggestion that multiple sulfhydryl groups regulate guanylate cyclase was obtained with crude and purified soluble guanylate cyclase from rat lung. In these studies, activation of guanylate cyclase by nitric oxide was reversible over time (225, 5, 31). The rate of inactivation was dependent upon temperature and the presence of thiol reducing agents, such as dithiothreitol or reduced glutathione (31). Increases in temperature or reductant concentration facilitated the reversal of nitric oxide activation. Enzyme activated by nitric oxide and subsequently inactivated with dithiothreitol could be reactivated by reexposure to nitric oxide (225, 31). In contrast, activation reversed by incubating at increased temperatures could not be recovered and, in fact, resulted in an irreversible loss of guanylate cyclase activity. This irreversible loss of enzyme activity was prevented by incubating with reductant and is similar to the inactivation in an oxygen atmosphere described above (344, 142, 22). Incubation of guanylate cyclase with diamide or oxidized glutathione resulted in the irreversible inactivation of nitric oxide-stimulated as well as basal enzyme activity (273, 25, 178, 168, 324). These data suggest that several sulfhydryl groups on guanylate cyclase influence enzyme activity. Oxidation of one group is associated with activation by nitric oxide, and its

reduction by dithiothreitol results in activation reversal (31). Another sulfhydryl group regulates basal enzyme activity, and oxidation by oxygen, diamide, or oxidized glutathione results in irreversible inactivation of nitric oxide-stimulated or basal enzyme activity (31). This inactivation can be blocked but not reversed by incorporating a thiol reductant into incubations (225, 31).

At least one of these reactive sulfhydryl sites on the enzyme may be located at the catalytic site (168, 44). While basal guanylate cyclase activity and responsiveness to nitric oxide, 5-nitrosocysteine, and nitrosyl-hemoglobin could be blocked by incubating enzyme with thiol oxidants, alkylating agents, or disulfides, preincubating enzyme with excess substrate (Mg-GTP) or the activating agent protected the enzyme against inactivation (168). Basal and stimulated guanylate cyclase activity was also sensitive to agents that react with vicinal thiol groups such as arsenite (44, 31, 173, 94, 311) and $CdCl_2$ (44, 31, 310). These agents inhibited basal guanylate cyclase activity and responsiveness to azide, nitroprusside, nitrosoguanidine, and nitrite, while dimercaprol potentiated inhibition of activity by arsenite (44). Dimercaprol alone was an inhibitor of guanylate cyclase, competing with Mg-GTP presumably for the active site of the enzyme (168). These data suggest that vicinal thiol groups located near the active site influence basal and agonist-stimulated guanylate cyclase activity. However, additional studies to map the thiol groups on the protein and determine their role in basal and stimulated activity are required.

Thiol reactive agents could also alter the stimulation of guanylate cyclase by various activators through mechanisms independent of reactive groups on the enzyme. For example, reducing agents such as dithiothreitol, cysteine, or reduced glutathione facilitate the generation of reactive intermediates from nitrovasodilators (138, 162, 79, 161, 166, 165). For example, nitrite formation from nitroglycerin or amylnitrate is increased in the presence of cysteine (138, 148, 166, 165). Nitrite may then be converted to HONO and subsequently nitric oxide, under appropriate conditions (138, 148, 295, 84). Similarly, thiols promote the liberation of nitric oxide from solutions of nitroguanidine (165). However, it may not be the generated nitric oxide that activates guanylate cyclase, but, rather, the reaction products of reduced thiols and the reactive nitric oxide intermediate (138, 162, 79, 161, 165). Formation of these complexes can be demonstrated in reactions between thiols such as cysteine, dithiothreitol, penicillamine, and reduced glutathione and nitric oxide, nitrite, nitroprusside, nitrosoguanidine, nitroglycerin, isosorbide dinitrate, and pentaerythritol tetranitrate (148, 162, 79, 161, 166, 165). Studies with nitroprusside and nitrosoguanidine demonstrated that the S-nitrosothiol derivatives were about 100 times more potent than the nitro compounds alone in activating guanylate cyclase (166). Agents that inhibit activation by nitro compounds and nitric oxide such as methylene blue (178), ethacrynic acid, methemoglobin, and thiol oxidants also inhibited activation by S-nitrosothiols (138, 131). These data support the hypothesis that S-nitrosothiols are involved in guanylate cyclase activation (138, 162, 79, 161, 166, 165). However, other data have been presented that contradict this hypothesis. In these studies, preformed S-nitrosothiols did not activate soluble guanylate cyclase purified from rat liver or lung unless heme was added to incubations (273, 164, 48). Preformed S-nitrosothiols decomposed rapidly (138, 48), and fully decomposed mixtures retained the ability to activate guanylate cyclase when heme was present (48). These enzyme preparations also respond to nitric oxide alone (48). In contrast, purified enzyme deficient in heme could not be activated by nitric oxide or S-nitrosothiols (48). At neutral pH, S-nitrosothiol formation between nitroprusside, which spontaneously releases nitric oxide (138, 165), and cysteine could not be detected (48). Furthermore, preformed nitrosyl-heme activated purified, heme-deficient enzyme in the absence of any reducing agent (48). These data suggest that, while S-nitrosothiols may activate heme-containing enzyme, they are not obligate intermediates; rather, it is nitrosyl-heme that acts in this capacity.

Another mechanism by which thiol reductants can alter guanylate cyclase activation involves the formation of nitrosyl-heme complexes (45, 48). Formation of nitrosyl-heme from reactions of hematin or hemoproteins with nitrocompounds such as nitroprusside, nitric oxide, nitrosoguanidine, or nitrite is greatly facilitated by reducing agents or anaerobic conditions (45, 259, 308, 17, 198). Reducing agents increase the rate of this reaction by maintaining the heme iron in the Fe^{2+} state (48, 259, 308, 17, 198). The nitric oxide-heme complex is more stable than the free nitric oxide radical which could increase its effect as a guanylate cyclase activator (45, 48).

The role of sulfhydryls in regulating particulate guanylate cyclase activity is less well understood. Crude particulate guanylate cyclase could be inhibited by thiol reactive agents including disulfides, alkylating agents, and oxidation-reduction agents (336, 44, 144). Crude hepatic particulate preparations were inhibited by arsenite, arsenite plus dimercaprol, iodoacetamide, N-ethylmaleimide, and *p*-hydroxymercuribenzoate (44). Yet, $CdCl_2$ had no inhibitory effect on these preparations (44). Similar results were obtained with particulate guanylate cyclase solubilized from hepatic plasma membranes with trypsin (144). However, particulate guanylate cyclase solubilized from rat liver membranes by limited proteolysis with trypsin and partially purified was inhibited in a concentration- and time-dependent fashion by mixed disulfide formation with cystamine (336). This inhibition was reversible by dithiothreitol similar to that described for the purified soluble enzyme (336,

25). Also, particulate guanylate cyclase solubilized with detergent from rat lung membranes and purified more than 1000-fold was inhibited in a concentration- and time-dependent manner by cystamine (337). Interestingly, cystine, a potent inhibitor of the soluble enzyme purified from rat lung tissue (25), had little effect on particulate guanylate cyclase purified from that tissue (337). Further studies concerning the role of sulfhydryls in regulating particulate guanylate cyclase await the availability of larger quantities of homogeneous protein.

d. REGULATION OF GUANYLATE CYCLASE BY FATTY ACIDS AND DETERGENTS. It has been suggested that unsaturated fatty acids and their metabolites constitute the physiological regulators of guanylate cyclase activity and cellular levels of cyclic GMP with a variety of stimuli (254, 126, 257, 258, 248, 131, 142). Activation of crude preparations of soluble guanylate cyclase by fatty acids and their metabolites has been demonstrated in a variety of tissues including rat heart (229), human platelets (124, 149), guinea pig spleen cells (131), lung (139), and myometrium (219), rat kidney medulla (47), and rat liver (309). Similar observations have been made with soluble guanylate cyclase purified from rat liver (33), rat and bovine lung (228, 115), and human platelets (8) as well as crude preparations from other tissues (6, 288). Generally, activation of soluble guanylate cyclase has been observed with unsaturated fatty acids and their oxidized metabolites. While arachidonic acid activated purified preparations of soluble guanylate cyclase from rat and bovine lung (228, 115) and rat liver (33) 2- to 3-fold, other unsaturated fatty acids including linoleate and oleate could activate the purified enzyme. Crude preparations of soluble guanylate cyclase from human platelets were activated by arachidonic acid as well as dihomolinolenic acid and polyunsaturated 22-carbon fatty acids (124). Structure-function studies indicated that maximal enzyme activation was obtained with 20-carbon fatty acids possessing a 1,4,7-octatriene group with its first double bond in position 6 (124). It is interesting to note that this structural determinant is also required for the substrate specificity of cyclooxygenase. However, activation occurred with soluble fractions, whereas cyclooxygenase is localized in microsomes (124). Furthermore, aspirin, a cyclooxygenase inhibitor, had no effect on activation (124).

While the above studies suggest that polyunsaturated fatty acids activate soluble guanylate cyclase directly, other studies suggest that metabolism of the unsaturated fatty acid may be important. Soluble guanylate cyclase from spleen cells was activated by prostaglandin endoperoxides and fatty acid hydroperoxides 3- to 5-fold (131). Activation was prevented or reversed by thiol reductants, suggesting a role for the oxidation of enzyme-related thiol functions in this process (131). Since these oxidized fatty acids are potential generators of the hydroxyl free radical and perhaps other reactive oxygen species (80) which may activate soluble guanylate cyclase (248), it has been suggested that these generated radicals mediate this activation of soluble guanylate cyclase (254, 250, 255, 257).

A role for the metabolism of unsaturated fatty acids in the activation of soluble guanylate cyclase has also been suggested by other studies. In human platelets, greater than 90% of the guanylate cyclase present is soluble (124). Enzyme purified from human platelets was activated directly by fatty acid peroxides (8). In platelet homogenates, activation was observed with the unsaturated fatty acids oleic, linoleic, linolenic, eicosenoic, eicosodienoic, and arachidonic (149). In contrast, saturated fatty acids such as stearic had no effect (149). Activation by unsaturated fatty acids was potentiated by exogenous lipoxygenase and was proportional to the concentration of the resultant fatty acid peroxides rather than the parent unsaturated fatty acids (149). Similarly, soluble guanylate cyclase was activated by arachidonic acid in homogenates, but not in supernatant fractions of guinea pig lung (139). As stated previously, cyclooxygenase is present in microsomes but not in cytosolic preparations. Inhibitors of cyclooxygenase such as indomethacin, aspirin, and meclofenamate inhibited this activation in the homogenate, supporting the suggestion that this process was dependent upon metabolism of arachidonate by cyclooxygenase (139). However, guanylate cyclase in supernatant fractions could also be activated by arachidonate in the presence of exogenous lipoxygenase, and this could be prevented by lipoxygenase, but not cyclooxygenase inhibitors (139). Thus, metabolites of arachidonic acid produced by either lipoxygenase or cyclooxygenase may mediate activation of soluble guanylate cyclase in this tissue (139, 219). These observations are supported by those in rat kidney in which activation of guanylate cyclase and elevation of cyclic GMP levels in cells stimulated by exogenous arachidonic acid are apparently mediated by the products of the cyclooxygenase pathway in the inner medulla and by the lipoxygenase pathway in the cortex (47).

The observations, that soluble guanylate cyclase can be activated directly by unsaturated fatty acids in some tissues but requires oxidative metabolism of those fatty acids in other tissues, appear contradictory. However, as noted earlier, soluble guanylate cyclase has a heme prosthetic group (116, 117, 113, 118). It has been suggested that heme-containing proteins can oxidize unsaturated fatty acids in the presence of oxygen, expressing lipoxygenase-like activities (317, 205, 349). The reaction involves the formation of a heme-hydroperoxide complex and the generation of free radicals (317, 205). Therefore, soluble guanylate cyclase possessing a heme prosthetic group may be capable of lipid peroxidation, converting unsaturated free fatty acids to hydroperoxides and free radicals that are known to activate the enzyme. The role of the heme prosthetic group of guanylate cyclase in

mediating unsaturated fatty acid oxidation and enzyme activation remains to be clarified.

Particulate guanylate cyclase can also be activated by fatty acids. Membranes obtained from BALB 3T3 fibroblasts contain guanylate cyclase that is activated by both saturated and unsaturated fatty acids (343, 6). Of the saturated fatty acids examined, myristic acid had the highest activity, with stimulation diminishing as the hydrocarbon chain length changed (343). The greatest stimulation was observed with unsaturated fatty acids including oleic, linoleic, and arachidonic (343, 6). It was concluded from these studies that enzyme activation may reflect a general ability of fatty acids to interact with proteins and lipids in membranes (343, 6). Similar results were obtained with guanylate cyclase in membranes of isolated rat fat cells, cerebral cortex (159), liver (6, 159), synaptic plasma membranes, heart, small intestine, adrenal medulla, lung (7), and colonic mucosa (46). In contrast to the soluble enzyme, the activation of particulate guanylate cyclase in these studies appeared to be independent of fatty acid metabolism to oxidized analogues. Thus, activation by unsaturated fatty acids was not dependent upon an O_2 atmosphere and was unaffected by inhibitors of cyclooxygenase (6, 46). Also, agents that facilitate fatty acid oxidation, including lipoxygenase, resulted in inhibition of enzyme activation by fatty acids (6). Conversely, agents that prevent fatty acid oxidation shift the dose-response curve of fatty acid activation to the left (6).

The above data suggest that fatty acids activate particulate guanylate cyclase by direct protein-lipid interactions. However, several studies have also demonstrated an effect of oxidized fatty acids on particulate guanylate cyclase. Guanylate cyclase in particulate fractions from rat or guinea pig myometrium was activated by unsaturated fatty acids and their hydroperoxy derivatives (219, 218). Activation by fatty acids was not dependent upon their conversion to hydroperoxy derivatives, nor were free radical scavengers effective in blocking activation. These data also suggest that unsaturated fatty acids and their hydroperoxy derivatives activate the particulate enzyme by direct interaction with the protein (219, 218). Similarly, fatty acid hydroperoxides activated particulate guanylate cyclase obtained from medulla and cortex of rat kidney (47) and liver (309).

The molecular mechanisms underlying activation of guanylate cyclase by fatty acids remain unknown. While it has been suggested that a specific fatty acid binding domain exists on both soluble and particulate guanylate cyclase that modified enzyme activity (124, 219, 6, 218), binding studies with purified enzyme preparations have not been performed. Also, it has been suggested that the soluble enzyme possesses a hydrophobic site with a fatty acid bound to it, since agents that readily scavenge fatty acids, such as bovine serum albumin and lactoglobulin, inhibit crude and partially purified soluble guanylate cyclase in a concentration-dependent fashion (348). Furthermore, both soluble and particulate isoenzymes bind to hydrophobic affinity resins, demonstrating the presence of at least one exposed hydrophobic site on these proteins (206, 33, 348, 111). Alternatively, fatty acids may bind nonspecifically to hydrophobic sites on these proteins, and acting similarly to detergent amphiphiles, they may increase enzyme activity. A third possibility is that fatty acids, upon conversion to their oxidized metabolites, result in the generation of oxygen free radicals, that are known activators of guanylate cyclase as described above. Indeed, oxidation of fatty acids might be catalyzed by the heme moiety of guanylate cyclase (114). However, recent studies demonstrated that activation of purified cyclase possessing heme was achieved directly by arachidonic acid and was not dependent upon further oxidation of this fatty acid (114). These data argue against a requirement for heme-dependent or -independent oxidation in activation of guanylate cyclase by fatty acids (114). The importance of sulfhydryl groups in fatty acid regulation of guanylate cyclase is also being explored. Agents such as 2-mercaptoethanol, dithiothreitol, glutathione, and N-ethylmaleimide inhibit activation of guanylate cyclase by unsaturated fatty acid hydroperoxides and prostaglandin endoperoxides (149, 131). Similarly, N-ethylmaleimide blocks activation of soluble guanylate cyclase from liver by fatty acid hydroperoxides (309). These data suggest that oxidation of critical free thiol groups on the enzyme is important in modulation of guanylate cyclase activity by fatty acids (149, 131, 309). Whether this is true for both forms of the enzyme and all fatty acids that modulate guanylate cyclase is currently being studied.

As indicated above, fatty acids may activate guanylate cyclase by merely acting as detergents. Indeed, detergents themselves have profound effects on these isoenzymes. Nonionic detergents, such as Lubrol-PX and Triton-X-100, activate soluble and particulate guanylate cyclase in all tissues examined (190, 191, 306, 112, 108, 277, 132, 337, 334, 336, 38, 269, 314, 315, 289, 254, 250, 342, 172, 145, 193, 100, 346, 98, 326, 214, 267, 341, 146). In general, the soluble enzyme is activated 50 to 100% whereas the particulate enzyme can be activated as much as 10-fold (190, 191, 306, 112, 108, 277, 132, 337, 334, 336, 38, 269, 314, 315, 289, 254, 250, 342, 172, 145, 193, 100, 346, 98, 326, 214, 267, 146). Activation of the soluble enzyme from rat renal medulla with Lubrol-PX has been associated with decreases in the sedimentation coefficient, Stokes radius, and molecular weight of the protein (269). Activation of the particulate enzyme by detergents has been correlated with solubilization of this protein from membranes (190, 108, 277, 132, 337, 334, 336, 38, 269, 314, 315, 289, 145, 193, 100, 346, 98, 326, 214, 267). Indeed, in most tissues, 70 to 90% of the particulate guanylate cyclase can be solubilized by nonionic detergents (190, 108, 277, 132, 337, 334, 336, 38, 269, 314, 315,

289, 145, 193, 100, 346, 98, 326, 214, 267). The particulate fractions obtained from intestinal mucosa and retinal rod outer segments are notable exceptions, since only small amounts of the particulate enzyme in these tissues can be solubilized by treatment with anionic detergents (200, 91, 92, 335). Activation of soluble and particulate guanylate cyclase by detergents also is associated with an increase in the ability of these enzymes to utilize Mg^{2+}- compared to Mn^{2+}-GTP as substrate, as discussed in an earlier section. We have used the decrease in the ratio of enzyme activity assayed with Mg^{2+}-GTP versus Mn^{2+}-GTP as an assessment of enzyme activation with various agents and/or purification (254, 140, 33, 255, 257, 189).

When detergents were tested for their ability to activate the particulate enzyme, the relative potency observed was: Lubrol-PX > Triton X-100 > Triton X-67 > Tween 20 (214). In these studies activation of particulate guanylate cyclase was correlated with the HLB number which represents the relative ratio of hydrophilic to hydrophobic region in a surfactant (214, 147). Optimal activity for the Triton detergents for stimulation of particulate guanylate cyclase was observed in a fairly narrow range of HLB values between 12.5 to 15 (214). These values are similar to those obtained for activation and solubilization of other membrane-bound enzymes (214). Thus, it was suggested that activation of the particulate enzyme reflects solubilization of that protein (214). It should be noted that, although the nonionic detergents can solubilize and activate particulate guanylate cyclase at lower concentrations, these surfactants can inhibit activity and solubilization at higher concentrations (267). Triton X-100 solubilized 80% of the particulate guanylate cyclase activity from rabbit heart at concentrations of 0.2 to 0.7 μmol/mg protein (267). However, only 20% was solubilized at concentrations greater than 0.7 μmol/mg (267).

Other surfactants also affect particulate guanylate cyclase activity. Deoxycholate, an anionic detergent, can activate the enzyme at low concentrations, but inhibits at higher concentrations (214). Inhibition can be reversed by separating detergent and protein by gel filtration chromatography (214). Similarly, the bile salts deoxycholate, cholate, chenodeoxycholate, and their taurine or glycine conjugates activated or inhibited particulate guanylate cyclase depending on their concentration (160). Particulate guanylate cyclase from rat lung and 3T3 mouse fibroblasts was also activated by lysolecithin. Stimulation was correlated with solubilization of enzyme activity, suggesting that the effects of lysolecithin were probably due to its surfactant properties (214, 304). However, others have reported activation of particulate guanylate cyclase in neuroblastoma cells by lysolecithin in the absence of solubilization of this enzyme (360).

In contrast to the particulate enzyme, the same surfactants exhibited different potencies for activation of soluble guanylate cyclase. The soluble enzyme from rat lung was activated by detergents in the following order: Tween 20 > Lubrol-PX > Triton X-67 > Triton X-100 (214). It is interesting to note that the soluble enzyme activated by preincubation in the presence of oxygen was inhibited by these detergents in the reverse order of potency (214). It was suggested that the soluble enzyme has two sites that interact with nonionic detergents and mediate the activation of this protein (214). It was proposed that one site is available in the enzyme in its basal state and mediates activation, while the second site becomes available only after activation and mediates inhibition (214). Soluble guanylate cyclase was inhibited by ionic detergents such as deoxycholate (214). However, unlike particulate guanylate cyclase, these effects were not reversed by subsequent removal of the inhibitory detergent (214).

The observations that lipids and detergents can alter particulate and soluble guanylate cyclase activity suggest that endogenous surfactants and amphiphiles might be modulators of these isoenzymes in vivo. Lysophospholipids, fatty acids, and their oxidized metabolites are products of the pathways initiated by the action of phospholipase A_2 on membrane phospholipids. This calcium-dependent enzyme catalyzes the release of fatty acids from phospholipids, resulting in the production of free fatty acids and lysophospholipids (216, 270, 97). The free fatty acid, which is most often unsaturated and frequently arachidonic acid, can then be further metabolized by cyclooxygenase or lipoxygenase to prostaglandin endoperoxides or fatty acid hydroperoxides (216, 270, 97). These compounds and the immediate products of phospholipase A_2 activity activate particulate and soluble guanylate cyclase as reviewed above.

Phospholipase A_2 activated guanylate cyclase when introduced into incubations of whole cells or enzyme assays. Guanylate cyclase in homogenates of rat glial cells was activated 3- to 4-fold when these cells were preincubated in the presence of phospholipase A_2 (361). In parallel studies, activation by phospholipase A_2 was observed with particulate but not soluble guanylate cyclase prepared from rat brain (361). Activation of guanylate cyclase by phospholipase A_2 also was observed in particulate preparations of guinea pig tracheal smooth muscle (100), mouse mammary glands (289), skeletal muscle (314, 315), cerebral cortex (249), and neuroblastoma cells (360). Activation was not due to solubilization of the particulate enzyme, but rather seemed to be a result of alterations in the lipid composition of cellular membranes (289, 361). These data suggest that phospholipase A_2 may specifically regulate the activity of the particulate isoenzyme.

The ability of phospholipase A_2 to regulate soluble guanylate cyclase has been demonstrated directly in a series of reconstitution studies (228). Snake venom phospholipase A_2 was incorporated into the interior of re-

sealed human erythrocyte ghosts. Addition of soluble guanylate cyclase purified from rat lung on the outside of the resealed ghosts resulted in activation of the enzyme (228). Activation of guanylate cyclase was dependent upon the concentration of phospholipase A_2 inside the ghosts and was blocked in a dose-dependent fashion by phospholipase A_2 inhibitors, such as quinacrine or *p*-bromphenacyl bromide (228). Phospholipase A_2 had no direct effect on soluble guanylate cyclase (228). Free fatty acids released from red cell ghosts by phospholipase A_2 are retained in the membrane (ref. 228; unpublished observations). Indeed, only membranes from ghosts containing phospholipase A_2 activated soluble guanylate cyclase, while supernatant fractions from these preparations had no effect (ref. 228; unpublished observations). Scavengers of fatty acids such as bovine serum albumin blocked activation in this reconstituted system (ref. 228; unpublished observations). Similarly, enzymes that metabolize polyunsaturated fatty acids, such as lipoxygenase, also blocked activation. Soluble guanylate cyclase was activated directly by arachidonic acid or by red cell membranes preincubated with arachidonate mimicking the effects of phospholipase A_2. These data demonstrate in a reconstituted system with purified components that the action of phospholipase A_2 on cellular membranes can result in modulation of soluble guanylate cyclase. Furthermore, these data demonstrate that this regulation is not dependent upon further oxidative metabolism of the released fatty acid, since the product of phospholipase A_2 action, arachidonic acid, mimicked the effects of the lipid metabolic enzyme on guanylate cyclase, while lipoxygenase inhibited these effects. Finally, these data suggest that soluble guanylate cyclase can be regulated by membrane-localized events and that activation does not require the release from the membrane of soluble factors.

Similar results have been obtained in studies with rat or rabbit platelet membranes (115). Incubation of these membranes with calcium resulted in the release of soluble factors that activated soluble guanylate cyclase purified from bovine lung (115). These factors were identified as arachidonic and linoleic acids (115). Presumably, these fatty acids were released from membranes by the action of phospholipase A_2 due to the calcium dependence of fatty acid release and its inhibition by trifluoperazine and mepacrine, although this was not demonstrated directly (115). Further oxidative metabolism of the released fatty acids for activation of guanylate cyclase was not required, since indomethacin and oxyphenbutazone did not alter the effects of the fatty acids (115). These studies demonstrate that unsaturated fatty acids can be released from membranes in concentrations that are sufficient to directly activate soluble guanylate cyclase (115). That fatty acids which activate the soluble enzyme were released as soluble factors in these studies while they were retained within the red cell ghosts in the previously described studies (228) may be due to differences in the lipid composition of these membranes.

The above studies suggest that both soluble and particulate guanylate cyclase may be regulated by phospholipase A_2 action on membrane phospholipids. It is significant that many hormones, autocoids, and toxins elevate cyclic GMP in their target tissues in a calcium-dependent fashion. As discussed above, in some tissues these effects can be mimicked by incubation with activators of phospholipase A_2 and prevented by inhibitors of that enzyme. These data and those discussed above concerning the ability of various products of lipid metabolism to regulate guanylate cyclase activity suggest the following hypothesis. Extracellular signal molecules interacting at their appropriate cell surface receptors stimulate phospholipase A_2 probably by raising intracellular concentrations of calcium. Phospholipase A_2 releases unsaturated fatty acids and lysophospholipids that can directly activate either soluble or particulate guanylate cyclase. Alternatively, unsaturated fatty acids can be metabolized by lipoxygenase or cyclooxygenase, and the resulting fatty acid hydroperoxides or prostaglandin endoperoxides may then activate guanylate cyclase. Most hormones that elevate cellular levels of cyclic GMP fail to activate guanylate cyclase in cell-free preparations. This might be due to the inefficiency of coupling of the ligand-receptor interaction to phospholipase A_2 activation. This coupling inefficiency might result from the inability of the hormone to raise the calcium concentration in cell-free preparations sufficiently to stimulate phospholipase A_2. Questions for the future include the relative importance of the particulate and soluble isoenzymes in hormone-mediated lipid regulation of guanylate cyclase, the relationship of receptor-ligand interactions to lipid metabolism and guanylate cyclase activity, and the identification of the lipid activators of guanylate cyclase. While the effects of various hormones and/or toxins on cyclic GMP accumulation in intact cells can be decreased or prevented with inhibitors of phospholipase A_2, lipoxygenase, and/or cyclooxygenase, these experiments must be interpreted cautiously due to the lack of specificity of most or all the inhibitors available. The effects of some hormones and toxins on cyclic GMP accumulation are discussed below.

The activity of particulate guanylate cyclase can also be related to the physiochemical state of the lipids within membranes. Studies of the temperature dependence of guanylate cyclase in rat intestinal microvillous membranes demonstrated a break in the slope of Arrhenius plots that correlated with the lipid thermotropic transition in these membranes (28). Gramicidin-S, melittin, and alomethicin also activated particulate guanylate cyclase obtained from rat lung and heart (215, 213). These effects were thought to result from their preferential interaction with and reorganization of membrane phospholipids (215, 213), rather than activation of a phos-

pholipase A_2, since inhibitors of phospholipase had no effect on the activity of these peptides (213). These observations may explain the ability of bee venom to activate particulate guanylate cyclase in rat liver, lung, heart, kidney, ileum, and cerebellum, since mellitin makes up 50% of the dry weight of been venom (332). Mellitin has been demonstrated to interact electrostatically with the polar head groups of phospholipids and to intercalate directly into the hydrophobic core of membranes (215). Similarly, alomethicin and gramicidin-S have been demonstrated to interact with membrane phospholipids (215). These agents have been associated with alterations in phospholipid packing densities, lipid phase transitions, and disruption in membrane structure (215). Also, agents that sequester cholesterol in membranes such as filipin, digitonin, cereolysin, and streptolysin-O activated rat lung particulate guanylate cyclase (211, 212). These agents bind to cholesterol making it unavailable for interaction in the membrane lipid phase (211, 212). Since cholesterol is a membrane fluidity "buffer," these agents may alter membrane fluidity and particulate guanylate cyclase activity (211, 212). The physiological importance of alterations in the physicochemical properties of cellular membranes in the regulation of guanylate cyclase remains unknown.

e. EFFECTS OF *E. COLI* HEAT-STABLE ENTEROTOXIN, ATRIAL NATRIURETIC FACTOR, ENDOTHELIAL-DERIVED RELAXANT FACTOR, AND OTHER MATERIALS. The soluble or particulate isoenzyme forms of guanylate cyclase can also be activated by hormones, toxins, and other factors. The effects of *E. coli* heat-stable enterotoxin (ST), atrial natriuretic factor (ANF), and endothelial-derived relaxant factor (EDRF) on guanylate cyclase activity and cyclic GMP levels in various tissues will be presented below in order to correlate these effects with some functions and avoid a redundant presentation of these effects.

Many other hormones, steroids, autocoids, and miscellaneous agents have also been reported to alter guanylate cyclase activity in cell-free preparations. It is not the intent of this review to summarize all of the work with guanylate cyclase. Furthermore, some of these single reports have not been confirmed or cannot be repeated. The complexity of the regulation of guanylate cyclase activity, as summarized above, would suggest that some of these reports may be dealing with interesting artifacts that could lead to important additional information about the regulation of these isoenzymes if these studies were confirmed and pursued in some systematic detail.

III. Some Functions of Cyclic GMP in Model Systems

A. Intestinal Secretion

Sodium and chloride transport across intestinal epithelium is regulated by toxins produced by a variety of bacteria often resulting in a secretory diarrhea. Indeed, above the age of 2, enterotoxigenic *E. coli* may be responsible for the majority of infectious diarrhea in the world (36, 85). *E. coli* causes diarrhea by elaborating two different toxins: one heat labile and the other heat stable (141, 292, 291, 290). The heat-labile toxin (LT) of *E. coli* is a multimeric protein structurally and antigenically similar to cholera toxin, and both stimulate intestinal secretion by activating adenylate cyclase and elevating intracellular levels of cyclic AMP (39, 152, 121, 122, 123). In contrast, the heat-stable toxin (ST) of *E. coli* is a low-molecular-weight peptide which stimulates secretion by activating guanylate cyclase and elevating intracellular levels of cyclic GMP (157, 86, 140, 338, 335, 278). Levels of cyclic GMP were elevated 30-fold when ST was inoculated into the small intestines of rats, rabbits, pigs, or suckling mice, standard bioassay systems used to detect this toxin (157, 86, 140, 338). Cyclic nucleotide accumulation was dose dependent and had a time course that compared favorably with that of secretion induced by ST (157, 86, 140, 338). Ligated rabbit ileal loops and suckling mice inoculated with the cyclic GMP analogue, 8-bromo-cyclic GMP, exhibited a secretory response that was quantitatively and qualitatively similar to exposure to ST (157). Furthermore, 8-bromo-cyclic GMP elicited an electrical response in intact rabbit intestinal mucosa similar to that induced by ST (86). Thus, a role for cyclic GMP in the induction of secretion by ST is suggested by the accumulation of this cyclic nucleotide in intestinal tissue exposed to toxin and the ability of analogues of this cyclic nucleotide to mimic the toxin by stimulating secretion and electrical activity in intestinal mucosa.

As pointed out above, cellular levels of cyclic GMP are regulated by the rate of synthesis by guanylate cyclase and the rate of hydrolysis by cyclic nucleotide phosphodiesterase. ST activates guanylate cyclase 7- to 10-fold in a dose-dependent fashion in homogenates of intestinal mucosa cells isolated from rat or rabbit ileum, and there is no evidence for an effect of ST on phosphodiesterase (140, 338, 335). Whereas guanylate cyclase is equally distributed between a soluble and particulate isoenzyme form in most cells, about 95% of the enzyme is particulate in intestinal mucosa (190, 187, 191, 276, 56). ST specifically activates particulate, compared to soluble, guanylate cyclase in isolated membranes prepared from intestinal mucosa cells (86, 140, 338, 335, 278). In addition, there appears to be a unique form of particulate guanylate cyclase in intestinal mucosa that is tightly bound to membrane constituents (335). This enzyme is resistant to solubilization by conventional techniques including exposure to high salt concentrations, ionic and nonionic detergents, and urea and may be covalently associated with membrane cytoskeletal components (335). Up to 50% of the particulate enzyme in intestinal mucosa appears to be this tightly bound form (335). A similar form of particulate guanylate cyclase resistant to solubilization in the mammalian retinal rod outer segment axoneme apparatus has been reported (91, 92). When mem-

branes prepared from homogenates of intestinal mucosa cells were sequentially extracted with high salt and detergents, guanylate cyclase resistant to solubilization retained the ability to be maximally activated by ST (335). These data permit the speculation that ST is coupled to a unique form of particulate guanylate cyclase resistant to solubilization and possibly covalently associated with membrane cytoskeletal components (335).

The actions of ST on particulate guanylate cyclase and intestinal secretion are mediated by specific protein receptors (335, 119, 321, 96, 74, 75, 207). Radiolabeled ST specifically bound in a time- and dose-dependent fashion to intestinal mucosa cells or isolated brush border membranes (119, 96). Binding was saturable and could be competitively inhibited by unlabeled ST. Dissociation constants for binding of ST to its receptor ranged from 10^{-11} to 10^{-8} M (119, 96). Kinetics of receptor-ST interaction compared closely with kinetics of ST activation of guanylate cyclase, with a 50% effective dose (ED_{50}) for enzyme activation of 10^{-11} to 10^{-8} M ST (338, 335, 119, 96). Tissue specificity of the actions of ST correlates with the distribution of specific receptors. To date, receptors have been identified only in small intestinal mucosa and rat basophilic leukemia cells, and ST has been demonstrated to stimulate fluid and electrolyte secretion and histamine release in these cell types, respectively (140, 278, 321, 75). In close agreement with studies of particulate guanylate cyclase, a portion of ST receptors in intestinal cells is solubilized with various detergents, while much of the receptor resists extraction and remains associated with the insoluble residue (335, 207). Both the soluble and insoluble receptor preparations retain their ability to bind ST with kinetic characteristics similar to native receptors in intact membranes (335, 207). However, only the receptors resisting extraction and associated with the insoluble residue remain coupled to guanylate cyclase, mediating maximal activation of this enzyme (335). In contrast, solubilized enzyme and receptor are not coupled, with no evidence of enzyme activation by toxin in detergent-extracted preparations (335). Guanylate cyclase and ST receptor which are solubilized by detergents from intestinal membranes are independent proteins easily separated by several chromatographic techniques including GTP-agarose affinity chromatography, gel filtration, sucrose density gradient centrifugation, or isoelectric focusing (335, 207). Thus, it appears that both particulate guanylate cyclase and the ST receptor are independent proteins closely associated, possibly covalently, with insoluble membrane components (335). It may be this tight association that confers a particular spatial organization required for receptor-enzyme coupling (335).

Although activation of particulate guanylate cyclase and elevation of cyclic GMP levels in intestinal cells play a central role in mediating toxin-induced secretion, little is known concerning the specific events coupling ST-receptor interaction to enzyme activation. Early studies suggested that enzyme activation might be closely coupled to arachidonic acid metabolism. Chlorpromazine, a compound that inhibits phospholipase A_2, inhibited ST-induced secretion in suckling mice (134). Similarly, this compound inhibited toxin activation of guanylate cyclase in intestinal membranes (140). Indomethacin, an inhibitor of prostaglandin synthetase (293, 217, 93), inhibited the secretory response and activation of guanylate cyclase stimulated by ST (140, 134). Inhibition with indomethacin was not observed after administering 8-bromo-cyclic GMP, suggesting that these compounds alter toxin-mediated secretion by inhibiting activation of guanylate cyclase (134).

These data suggest that ST-receptor interaction activates a phospholipase A_2, inducing the release of arachidonate, which is metabolized by prostaglandin synthetase to an activator of guanylate cyclase (134, 320, 294, 238). However, subsequent studies suggest that this is not the case. The rate of release of arachidonic acid from intestinal cells was unchanged by incubation with ST (74). Also, there were no qualitative or quantitative differences in phospholipids, neutral lipids, or free fatty acids extracted from cells pretreated with ST compared to control cells (74). ST did not activate intestinal cell membrane phospholipase A_2 when that enzyme was directly assayed (335). Also, ST did not alter the content of prostaglandins E_2 or F_2 or thromboxane B_2 in intestinal cells or their membranes, compared to controls (74). Furthermore, inhibitors of phospholipase A_2 and cyclooxygenase inhibited both basal and ST-stimulated guanylate cyclase, explaining the original observations of the effects of these inhibitors on ST-induced secretion.

The mechanism by which the ST receptor is coupled to activation of particulate guanylate cyclase is unknown. One current hypothesis suggests that the receptor and cyclase are organized in a specific spatial arrangement imposed by the brush border cytoskeletal system, which is required for functional coupling (335). Indeed, these membranes are especially rich in cytoskeletal components including actin, myosin, and intermediate filaments, which remain as insoluble residue after exhaustive detergent extraction (73, 34, 151). It is especially interesting to note that the cyclic GMP-dependent protein kinase in intestinal mucosa cells also shows similar localization and solubility characteristics with the ST receptor and particulate guanylate cyclase (335, 58, 59, 330). Thus, this kinase is localized to brush border membranes where it is tightly associated with cytoskeletal components (58, 59, 330). Furthermore, this kinase phosphorylates a M_r 25,000 protein in brush borders that may mediate ion fluxes in intestinal mucosa (58, 59). These data suggest that ST-induced secretion may be mediated by functional complexes composed of the receptor, guanylate cyclase, cyclic GMP-dependent protein kinase, and the kinase substrate. These may be tightly associated

with the brush border cytoskeletal apparatus which confers the appropriate organization and spatial arrangement required for functional coupling of the components.

B. Retinal Phototransduction

An interesting area of investigation that provides an understanding of the complex cascades underlying information transfer and of the role of cyclic GMP as an intracellular messenger is visual phototransduction. In this system, light in the form of photons is converted to electrical impulses by the specialized cells of the retina, the rods and cones. It has recently become evident that this process involves the complex interactions of an array of membrane and cytosolic proteins, ions, and messengers to regulate the steps in vision.

The best studied model for visual transduction is the vertebrate rod. In this cell, the enzymatic machinery regulating routine intracellular processes, such as metabolism, is confined to the rod inner segment, while the components involved in visual transduction are concentrated in the rod outer segment (ROS) (156, 82, 300). The ROS is a modified cilia composed of a stack of about 2000 membrane discs surrounded by plasma membrane and attached to the inner segment by a thin stalk (156, 82, 300). The discs contain the proteins responsible for receiving and converting photons to intracellular signals, while the plasma membrane contains the proteins that convert these signals into electrical impulses.

The first step in phototransduction involves light activation of rhodopsin. Rhodopsin is a M_r 40,000 transmembrane protein that comprises 90% of the integral protein of disc membranes (70). It contains a chromophore, 11-*cis*-retinal, embedded in the protein core near the center of the disc membrane (322). Light converts this chromophore to the all-*trans*-isomer, from which follows a series of interconversions for the rhodopsin molecule, ultimately resulting in its hydrolysis to opsin and all-*trans*-retinal (155). One of the intermediates in this process, metarhodopsin II, appears to be the form important for information transfer in phototransduction (81, 18, 19).

Photoisomerization and conversion of rhodopsin to metarhodopsin II cause conformational alterations in structures that result in the ability of this protein to activate the next component in the visual cascade, transducin. This component is a peripheral disc protein composed of three subunits (204, 312). It is an oligomeric GTP-binding protein that is structurally and functionally related to the guanine nucleotide binding proteins regulating hormone receptor-adenylate cyclase systems (312, 101, 20, 313, 1, 21, 239, 331, 268, 177, 120). In the inactivated state, the α-subunit of transducin contains GDP and is associated with the β- and γ-subunits (312, 101). Upon interaction with metarhodopsin II through an unknown mechanism, the α-subunit exchanges GDP for GTP, resulting in a decrease in the affinity of the α-subunit for the β- and γ-subunits of transducin and for rhodopsin (312, 101). The activated α-subunit-GTP complex is released, permitting metarhodopsin II to activate more transducin molecules (312, 101). The released α-subunit activates the next protein in the phototransduction cascade, cyclic nucleotide phosphodiesterase (312, 101). This portion of the cycle is terminated by the GTPase activity inherent in the α-subunit, resulting in conversion of GTP to GDP and reassociation of the three transducin subunits to form inactivated transducin (312, 101).

Cyclic nucleotide phosphodiesterase is a membrane protein associated with discs and also consists of three subunits in the inactivated state (12). It is specific for cyclic GMP hydrolysis compared to cyclic AMP (241). In the dark, this enzyme appears to be inhibited by its γ-subunit, a M_r 11,000 protein (241, 158). Transducin apparently activates the phosphodiesterase by overcoming the inhibition imposed by the γ-subunit (102). It remains unknown whether transducin displaces the γ-subunit from phosphodiesterase or complexes this inhibitory subunit to render it inactive.

As in other cell systems, the concentration of cyclic GMP in ROS reflects the relative activities of the enzymes guanylate cyclase and cyclic nucleotide phosphodiesterase. As demonstrated in intestinal mucosa cells, guanylate cyclase probably exists as three distinct isoenzymes in ROS: a cytosolic, soluble form; a particulate and easily extractable form that is predominantly associated with membrane; and a particulate form resistant to solubilization that is associated with the axoneme complex of ROS (91, 92). Although these enzymes are responsible for the production of the intracellular messenger that is central to visual transduction, little is known concerning their regulation or relative contribution to the cyclic nucleotide pool in ROS. Indeed, it remains to be determined if light has any effect on guanylate cyclase activity in ROS. In contrast, ROS cyclic GMP phosphodiesterase is profoundly activated by light, as indicated above. This enzyme demonstrates first-order rate constants in the same range as carbonic anhydrase and acetylcholinesterase, enzymes with the highest turnover numbers reported (158, 102).

Activation of ROS phosphodiesterase results in a large increase in turnover of cyclic GMP. Studies utilizing ^{18}O incorporation into guanylnucleotides following hydrolysis of cyclic GMP demonstrated a 5-fold increase in turnover of cyclic GMP as a result of light activation of phosphodiesterase (125). Interestingly, studies of changes in absolute concentration of cyclic GMP in ROS in the presence of light demonstrated only a 10 to 20% decrease in cyclic nucleotide concentration (128, 186, 353, 42). These studies measured alterations in total cyclic GMP content in ROS and would not detect transient changes in cyclic nucleotide in the microenvironment of the plasma membrane (128, 186, 353, 42). Also, the actual concentration of free cyclic GMP available for

information transfer may be considerably lower than the total cyclic GMP content of the ROS. Indeed, there are high affinity cyclic GMP binding sites on several protein components of the ROS, particularly on the phosphodiesterase, which are noncatalytic (357, 356). Thus, the actual concentration of free cyclic GMP may be much less than the total ROS concentration of this cyclic nucleotide, and small changes of 10 to 20% observed in total cyclic GMP levels in ROS may represent much greater changes in the free cyclic nucleotide pool available as an intracellular messenger.

It was not until recently that a role in phototransduction for transient decreases in cyclic GMP concentrations in ROS was elucidated. Previous studies demonstrated that cyclic GMP depolarized ROS plasma membranes when injected intracellularly, whereas light induced a transient decrease in ROS cyclic GMP and hyperpolarization of the plasma membrane (143, 323, 242). These changes in membrane potential are due predominantly to fluxes in the flow of sodium through specific plasma membrane channels (14, 15). Thus, these data suggest that light closes sodium channels while cyclic GMP opens them. Further evidence supporting this hypothesis was obtained in studies of ion fluxes across vesicles derived from ROS (41, 35). In these studies, the flux of sodium was increased by concentrations of cyclic GMP normally found in ROS (41, 35). These alterations in ion flux were independent of the addition of ATP or GTP, suggesting a direct effect of the cyclic nucleotide on ion channels rather than an indirect effect through a protein kinase and protein phosphorylation (41, 35). Most recently, definitive evidence for a role of cyclic GMP in directly modulating sodium channels in ROS was obtained using patch-clamp techniques to study ion fluxes through individual channels (83, 263). In these studies, cyclic GMP directly opened sodium channels in the absence of added ATP or GTP (83, 263). This effect was completely reversible with closing of sodium channels when membranes were washed free of cyclic GMP (83, 263). Thus, cyclic GMP appears to be the intracellular regulator of sodium channel function in ROS.

The above studies suggest the following mechanism for phototransduction in ROS. In the dark, phosphodiesterase is not activated, cyclic GMP concentrations are elevated, and sodium channels are open, producing the depolarization commonly observed in nonilluminated ROS. Light causes photoisomerization of rhodopsin, stimulating GDP-GTP exchange, activation of transducin, and subsequent activation of phosphodiesterase. Activated phosphodiesterase, in turn, decreases the free cyclic GMP concentration, resulting in the hyperpolarization commonly observed in the illuminated ROS. These changes in ion fluxes and polarization are ultimately used to regulate signalling between the rod and the next several elements in the visual pathway. Of course, this outline is somewhat simplistic, since other ions and proteins have been found which are probably important in the fine tuning of this system. However, visual transduction in the ROS is an excellent example of a system in which cyclic GMP plays a critical and integral role in the flow of information through the cell.

Since light causes a rapid and marked increase in the hydrolysis of cyclic GMP with only a modest decrease in cyclic GMP levels, it must be assumed that cyclic GMP synthesis is also markedly increased. The mechanism(s) whereby light increases guanylate cyclase activity and cyclic GMP synthesis (as well as hydrolysis) is unknown. It is tempting to speculate that a photochemical free radical product or one of the several proteins discussed above may be responsible for activating one or more isoenzyme forms of guanylate cyclase in the ROS.

C. Smooth Muscle Relaxation

It was about 10 yr ago that a relationship between smooth muscle relaxation and cyclic GMP was established. This relationship was initially suggested by the fortuitous observation of the effects of nitrovasodilator compounds on cyclic GMP levels in tissues and on guanylate cyclase in cell-free systems. As indicated earlier in this chapter, activation of guanylate cyclase was observed when azide was added to crude enzyme preparations to inhibit GTPase activity (187). In addition, other agents, including nitroprusside, nitroglycerin, hydroxylamine, sodium nitrite, nitric oxide, etc., were demonstrated to activate guanylate cyclase and increase cyclic GMP levels in a wide variety of tissues (187, 255, 257, 258, 180, 178, 188, 5). These agents also elevated cyclic GMP in a variety of vascular and nonvascular smooth muscle preparations, including bovine tracheal smooth muscle, guinea pig tracheal smooth muscle, guinea pig taenia coli, ductus deferens, aorta, coronary, mesenteric, femoral, and umbilical arteries (180, 138, 169, 10, 185, 9, 282, 135, 136, 154, 232, 181, 182, 179, 299, 284, 230, 201, 137, 279, 11, 87, 69, 189, 283). Elevations of intracellular cyclic GMP with those agents were associated in a dose- and time-dependent fashion with relaxation of many of these smooth muscle preparations. The elevations in intracellular concentrations of cyclic GMP stimulated by these agents preceded the associated relaxation, consistent with a cause-and-effect relationship between these events.

As described earlier, these agents, now termed "nitrovasodilators," are thought to mediate smooth muscle relaxation by activating guanylate cyclase and increasing intracellular concentrations of cyclic GMP. While the precise mechanism of enzyme activation remains unknown, as discussed above, it has been suggested that these agents are all capable of generating nitric oxide spontaneously or enzymatically and that this free radical is the proximal activator of guanylate cyclase (255, 257, 178, 5, 258). Indeed, nitric oxide activates crude or homogeneous preparations of soluble guanylate cyclase with half-maximal activation observed at nanomolor

concentrations of this radical (5, 32). Activation of the enzyme may involve interaction of the nitric oxide radical with critical free thiols and/or a heme prosthetic group on the enzyme, as discussed earlier.

The relative roles of the isoenzyme forms of guanylate cyclase in cyclic GMP elevation and relaxation in smooth muscle stimulated by the nitrovasodilators remain unclear. These agents have profound effects on soluble guanylate cyclase, with activations up to 200-fold as reviewed earlier. However, there have been some reports of activation of particulate guanylate cyclase with these agents. One problem with these studies concerns the soluble guanylate cyclase that is inevitably trapped as a contaminant in crude preparations of membranes yielding artifactual activations of particulate preparations with nitrovasodilators. Another problem concerns the profound activation of partially purified preparations of particulate guanylate cyclase by the detergents required for membrane solubilization. This activation renders the enzyme unresponsive to further activation by other agents. Thus, the question of the responsiveness of particulate guanylate cyclase to nitrovasodilators remains unresolved.

A role for guanylate cyclase and cyclic GMP in mediating smooth muscle relaxation stimulated by nitrovasodilators is supported by studies using inhibitors of various processes in the cascade. Agents that inhibit activation of guanylate cyclase by nitrovasodilators, such as butylated hydroxyanisole, hydroquinone, methylene blue, hemoglobin, methemoglobin, myoglobin, cyanide, and ferricyanide, also inhibit smooth muscle relaxation and/or accumulation of cyclic GMP (180, 178, 138, 135, 136, 154, 258, 201, 137). Similarly, agents that directly inhibit guanylate cyclase, such as those interacting with sulfhydryl groups like cystamine, also inhibit nitrovasodilator-induced relaxation and accumulation of cyclic GMP in rat aorta (25, 178, 279). Furthermore, inhibitors of cyclic GMP phosphodiesterase directly relax bovine tracheal smooth muscle, aorta, and coronary artery and result in accumulation of cyclic GMP in these tissues (154, 182, 236, 153, 235). In addition, these agents potentiate nitrovasodilator-induced smooth muscle relaxation and accumulation of cyclic GMP. Consistent with these observations, cyclic GMP analogues have been demonstrated to directly relax various types of smooth muscle, and this effect can be potentiated by phosphodiesterase inhibitors (182, 284, 230).

Studies of vascular smooth muscle desensitized with nitroglycerin also support a role for guanylate cyclase and cyclic GMP in relaxation (10, 185, 9, 286, 341, 251). In these studies rat aortas pretreated in vivo or in vitro with nitroglycerin are desensitized to relaxation by further treatment with the same agent. This treatment not only results in tissue refractory to nitroglycerin-induced relaxation, but also decreases cyclic GMP accumulation stimulated by reexposure to this agent. In addition, relaxation and cyclic GMP accumulation induced by sodium nitroprusside were inhibited by nitroglycerin-induced desensitization, supporting the suggestion of a common mechanism of action for these agents. Interesting, soluble guanylate in crude or partially purified preparations from rat aorta desensitized with nitroglycerin was refractory to reactivation by nitroglycerin or sodium nitroprusside (9, 341). These data support the suggestion that soluble guanylate cyclase and cyclic GMP represent the proximal mediators of smooth muscle relaxation stimulated by the nitrovasodilators.

Relaxation of blood vessels by the nitrovasodilators occurs in the presence or absence of an intact endothelium; hence, these agents have been termed endothelium independent. In contrast, agents such as acetylcholine, histamine, bradykinin, the calcium ionophore A23187, ATP, and thrombin require an intact endothelium in order to relax blood vessels (284, 106, 103, 107, 105, 285, 253, 281, 104). In fact, in the absence of the endothelium, some of these agents become vasoconstrictors, depending on the blood vessel and species being tested. As with the nitrovasodilators, the endothelium-dependent vasodilators elevate intracellular concentrations of cyclic GMP in a dose- and time-dependent manner, which precedes relaxation (282, 284, 153, 285, 253, 281). It is known that these agents interact with specific receptors on the endothelium and cause the synthesis and/or release of an endothelial-derived relaxant factor (EDRF) (106, 103, 107, 105, 285, 253, 281, 104, 40). However, the structure of this factor is unknown because of its short half-life and low concentration (105, 104, 40). While studies using inhibitors of phospholipase and lipoxygenase have suggested this compound may be an oxidized lipid product, these studies should be interpreted with caution because of the nonspecific nature of these inhibitors (285).

The above data suggest that endothelium-independent and -dependent vasodilators stimulate vascular relaxation through a common pathway involving activation of guanylate cyclase and accumulation of cyclic GMP. Data supporting these suggestions have come from a variety of studies. As discussed above, blood vessels can be made tolerant to the effects of nitrovasodilators by pretreatment with nitroglycerin (9, 286, 341, 251). Such treatment inhibits relaxation, accumulation of cyclic GMP, and activation of guanylate cyclase by agents such as nitroglycerin and sodium nitroprusside (9, 286, 341, 251). Similarly, desensitization of rat aorta with nitroglycerin also inhibits relaxation and accumulation of cyclic GMP stimulated by acetylcholine (286, 251). This "cross-tolerance" between endothelium-dependent and -independent vasodilators and the direct effects, which are observed with immunopurified soluble guanylate cyclase, support the suggestion that both these classes of agents share, as a final common pathway, activation of soluble guanylate cyclase and accumulation of cyclic GMP. Recent studies have provided additional support for this

hypothesis. Soluble guanylate cyclase was introduced into the lumen of vessel segments (95), or segments were added to guanylate cyclase incubations (167). The addition of endothelium-dependent vasodilators increased guanylate cyclase activity and cyclic GMP synthesis only when the endothelium was intact, while nitroprusside increased enzyme activity with or without endothelium present. These studies suggest that EDRF directly activates the soluble isoenzyme form of guanylate cyclase. The similarities of the effects of EDRF and nitrovasodilators with regard to guanylate cyclase activation and the cross-tolerance that develops as discussed above suggest that the mechanisms of activation of guanylate cyclase by these agents are identical (253). Indeed, the lability of EDRF indicates that this factor may be a reactive oxidized material that can give rise to a free radical that oxidizes and activates guanylate cyclase analogous to nitric oxide (253). For these reasons and other data reviewed previously (285, 253), we have proposed that EDRF is the equivalent of the "endogenous nitrate" or "endogenous nitrovasodilators" (253). The half-life of EDRF of about 6 to 30 s, depending upon conditions, would indicate that EDRF is itself not a free radical but can probably lead to the formation of a free radical and guanylate cyclase activation much like the nitrovasodilators (253).

Further support for this hypothesis comes from studies of cyclic nucleotide kinases and protein phosphorylation. Sodium nitroprusside and acetylcholine activate cyclic GMP-dependent protein kinase in a dose- and time-dependent manner in rat aorta (95). Activation of the kinase correlates closely with accumulation of cyclic GMP and vascular relaxation. In addition, alterations in the phosphorylation state of cellular proteins can be studied by incubating vascular tissue in ^{32}P to prelabel ATP pools, followed by addition of various agents to be studied. Using this approach, sodium nitroprusside, 8-bromo-cyclic GMP, and a variety of endothelium-dependent vasodilators including acetylcholine, the calcium ionophore A23187, ATP, thrombin, and trypsin induced identical patterns of phosphorylation of proteins in rat aorta (282, 285, 253, 281, 280, 71, 72). Incorporation of ^{32}P was dependent upon the concentration of relaxing agent used. Removal of the endothelium altered the phosphorylation pattern observed with the endothelium-dependent agents, while the pattern obtained with the nitrovasodilators or 8-bromo-cyclic GMP was unaltered (282, 285, 253, 281, 280, 71, 72). The pattern of phosphorylation observed with the endothelium-dependent and -independent agents and 8-bromo-cyclic GMP was different when compared with that induced with isoproterenol or dibutyryl AMP, although some similarities in phosphorylation patterns did occur. The endothelium-dependent and -independent agents decreased the amount of phosphorylation of myosin light chain, alterations known to correlate with vascular relaxation (71, 72). These data suggest a model for vascular relaxation induced by nitrovasodilators and endothelium-dependent agents which includes activation of soluble guanylate cyclase, accumulation of cyclic GMP, activation of cyclic GMP-dependent protein kinase, and dephosphorylation of myosin light chain (71, 72). Whether myosin light chain dephosphorylation occurs by alterations in the activities of myosin light chain kinase and/or phosphatase is currently a focus of much research. Additionally, identifying the other proteins whose phosphorylation state is altered as a result of exposure to these agents is being pursued. Recent studies with the effects of nitrovasodilators and cyclic GMP on vascular smooth muscle calcium-ATPase activity (275) and calcium concentrations (197) suggest that cytosolic calcium concentrations influence the contraction-relaxation process with these agents. This could be responsible for altered myosin light chain kinase activity and myosin light chain dephosphorylation observed in the above studies (71, 72).

Recently, a third class of vasodilators acting through cyclic GMP has been described, the atrial natriuretic factors (ANF). These low-molecular-weight, heat-stable peptides are produced as a preprohormone and stored as a prohormone in granules in atrial myocytes that are released upon stimulation of cardiac volume receptors (264, 355, 234, 54, 53). They have a pleiomorphic effect, causing natriuresis, diuresis, vasodilation, and inhibition of aldosterone and vasopressin secretion to regulate blood pressure, intravascular fluid, and electrolyte balance (234, 54, 53, 61, 271). Vasodilation induced by these peptides is associated with elevation in cyclic GMP in both a dose- and time-dependent manner (286, 351, 287, 272, 350, 231). These increases in cyclic GMP are associated with activation of cyclic GMP-dependent protein kinase (89). Relaxation, accumulation of cyclic GMP, and activation of protein kinase occur in the presence or absence of the endothelium (351, 287, 272, 350, 231, 89). Interestingly, the atrial natriuretic factors specifically activate only the particulate form of guanylate cyclase without altering soluble guanylate cyclase from a variety of tissues, including blood vessels (351, 340, 339). These data suggest that, like the nitrovasodilators and endothelium-dependent vasodilators, the atrial natriuretic factors activate guanylate cyclase, resulting in the accumulation of cyclic GMP which mediates relaxation through activation of cyclic GMP-dependent protein kinase and alterations in the phosphorylation of cellular proteins. These studies were the first to demonstrate a role for the particulate guanylate cyclase in regulating vascular relaxation. It is of interest to note that, in the experiments with desensitized rat aorta, tissues tolerant to nitrovasodilator and endothelium-dependent vasodilator-induced relaxation and accumulation of cyclic GMP demonstrated no alterations in these parameters when exposed to the atrial natriuretic factors (286). In addition, tolerance resulting in desensitization of soluble

guanylate cyclase to activation by nitrovasodilators had no effect on activation of particulate guanylate cyclase from the same tissues (341). These data are consistent with a role for separate and independently regulated pools of guanylate cyclase which both contribute to accumulation of cyclic GMP and vascular smooth muscle relaxation.

The mechanism by which the atrial natriuretic factors activate particulate guanylate cyclase remains unknown. There are specific receptors for these peptides in many tissue preparations which demonstrate affinities for the ligand in the nanomolar range (60, 266, 150, 296, 223, 243, 358, 303, 221, 329, 220, 274, 302, 316, 222). Although Scatchard analyses suggest a single class of receptors, other studies demonstrate receptor heterogeneity. Thus, if membranes are incubated with iodinated atrial natriuretic factor and a cross-linking agent, and if proteins are subsequently separated by SDS-polyacrylamide gel electrophoresis, radiolabeled proteins with molecular weights of 66,000, 130,000, and, occasionally, 180,000 are observed (358, 303, 221, 329, 220, 274, 302, 316, 222). Also, structure-activity studies comparing binding affinity and potency to elevate intracellular concentrations of cyclic GMP suggest at least two populations of receptors (206, 223, 243, 358, 303, 221, 329, 220, 222). One population is coupled to particulate guanylate cyclase, while the other may be coupled to a different second messenger system (206, 221, 220, 274, 302, 316, 222). However, these different receptor types have similar binding affinities for the various peptides. The properties of these receptors have recently been reviewed (222). Most recently, highly purified preparations of the M_r 130,000 and 180,000 receptors have been obtained from rat lung and bovine and rat adrenal (206, 274, 316). These preparations possess both receptor binding and particulate guanylate cyclase activities, which copurify in an almost identical manner during purification (206, 274, 316). These data suggest that the receptor binding and guanylate cyclase activities may reside on the same transmembrane glycoprotein (274, 316). Alternatively, these activities may reside on different proteins of similar molecular weight with high affinity for each other to account for their copurification. Nevertheless, it seems apparent that there is heterogeneity in the population of atrial natriuretic factor receptors, with the high-molecular-weight receptor most likely being coupled to particulate guanylate cyclase and mediating vascular relaxation. Fig. 1 illustrates the various classes of cyclic GMP-coupled vasodilators and their postulated mechanisms of action (253).

IV. Conclusions

While many unanswered questions remain with the guanylate cyclase-cyclic GMP system, considerable progress has been made during the past decade. The multiple isoenzyme forms of guanylate cyclase have undoubtedly added to some of the difficulties encountered. These isoenzymes can be regulated in a variety of ways as summarized in this review. The activation of the enzyme with free radicals has, to date, remained a unique mechanism of regulation that presumably will be utilized by other enzyme systems.

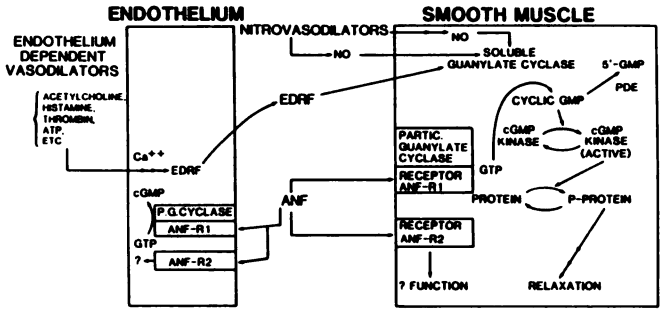

FIG. 1. Proposed mechanisms of action of nitrovasodilators, endothelium-dependent vasodilators, and atrial natriuretic factors on cyclic GMP synthesis and vascular smooth muscle relaxation. Endothelium-dependent vasodilators act at specific receptors on endothelial cells which lead to the release of a relaxant factor (*EDRF*). Nitric oxide (*NO*) derived from the nitrovasodilators and EDRF activate soluble guanylate cyclase, resulting in cyclic GMP accumulation in smooth muscle cells. Atrial natriuretic factors bind to both endothelial and smooth muscle cells. They induce relaxation independent of the endothelium by activating particulate guanylate cyclase which is coupled with a specific receptor designated *ANF-R1*. The function of the receptor not coupled to guanylate cyclase (*ANF-R2*) and the role of ANF-mediated cyclic GMP generation in the endothelial cell are unknown. In smooth muscle cells, cyclic GMP can activate cyclic GMP-dependent protein kinase (*G-Kinase*) or be degraded by phosphodiesterase (*PDE*). Altered phosphorylation of cell proteins by the catalytic unit of *G-Kinase* may lead to vascular smooth muscle relaxation.

A systematic examination of these isoenzyme forms has led to an understanding of cyclic GMP functions in several systems. Most notably the effects of cyclic GMP in smooth muscle relaxation, intestinal secretion, and retinal phototransduction have been exciting and rewarding. The role of the guanylate cyclase-cyclic GMP system in these processes should permit us to develop new and innovative therapeutic approaches to some disorders, such as enterotoxigenic diarrhea, hypertension, vasospasm, etc.

Nitrovasodilators, EDRF, atrial natriuretic factor, and *E. coli* heat-stable enterotoxin are probably but a few of the many agents that can regulate guanylate cyclase activity. The effects of these agents have stimulated considerable interest in cyclic GMP.

In spite of the significant progress in recent years, many important old questions remain and even more are raised. Optimistically, with the new information and sophisticated technologies available, it should be possible to make considerable progress in these areas in the next decade.

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