A kinetic characterization of the regulation of purified soluble guanylate cyclase from bovine lung by protoporphyrin IX and hemeatin is reported. Purified guanylate cyclase was isolated with heme and had specific activities (μmol of cGMP/min/mg of protein) of 0.1–0.2 and 0.3–0.6 in the presence of excess MgGTP and MnGTP, respectively, in the absence of added activators. Protoporphyrin IX, nitric oxide (NO), and NO-heme increased the Vₘₐₓ up to 40-fold and decreased the Kₘ for GTP (from 100 to 45–55 μM) in the presence of excess Mg²⁺. However, in the presence of excess Mn²⁺ the Vₘₐₓ was increased only slightly and the Kₘ for GTP was unchanged. Protoporphyrin IX resembled NO and NO-heme also in lowering the Kₘ and Kᵥ for uncomplexed metal. This close similarity in the interactions of these activators with guanylate cyclase suggests that a common form of activated enzyme is generated.

Hemin, in excess of 1.5 μM, inhibited guanylate cyclase activity. Smaller concentrations of hematin competitively inhibited protoporphyrin IX (Kᵢ = 0.35 μM), suggesting that both porphyrins compete for a common binding site on guanylate cyclase. The apparent Kᵢ for protoporphyrin IX (8–38 nm) varied directly as a function of the guanylate cyclase concentration under the assay conditions employed. The equilibrium dissociation constant of the guanylate cyclase-protoporphyrin IX complex was estimated by Scatchard analysis to be 1.4 nM. The stoichiometry of binding was estimated to be 0.92 mol/mol of holoenzyme. Cyanide and certain oxidants inhibited guanylate cyclase activation by NO, NO-heme, and nitroso compounds without affecting activation by protoporphyrin IX or unactivated enzyme.

These observations suggest that protoporphyrin IX, NO-heme, and perhaps other activators regulate guanylate cyclase by similar mechanisms. Moreover, protoporphyrin IX and heme may be important biological regulators of guanylate cyclase activity.

Guanylate cyclase (EC 4.6.1.2) from the soluble fraction of mammalian cells can be activated by NO¹ and several clinically employed nitrogen oxide-containing vasodilators (1–4). The latter compounds are capable of releasing or forming NO and markedly elevate cyclic GMP levels in smooth muscle, platelets, and other tissues (6–13). The rapidly developing view by some, that cyclic GMP may be associated with the vascular smooth muscle relaxant and platelet antiaggregatory effects of this important class of pharmacologic agent (7–15) has stimulated research on their mechanism of activation of guanylate cyclase. Recent studies from several laboratories suggested that guanylate cyclase activation by NO and related agents is dependent in part on the presence of heme and thiols (4, 5, 16–19). NO reacts with heme to form NO-heme, which has been shown to bind to (19) and activate (16–19) guanylate cyclase. Thiols appear to enhance or be required for guanylate cyclase activation by nitroglycerin, amyl nitrite, NaNOₓ, and sodium nitroprusside (4, 12). The latter observations may be explained by a reaction between these nitrogen oxide compounds and thiols to form S-nitrosothiols, which are potent activators of guanylate cyclase, elevate tissue levels of cyclic GMP, relax vascular smooth muscle, and inhibit platelet aggregation (4, 5, 12, 13, 18, 19).

One of the problems associated with elucidating the mechanism of guanylate cyclase activation by NO and nitro compounds is that such agents are not known to occur naturally in mammalian tissues. This has raised questions as to the physiological regulation of guanylate cyclase. Our recent observation that protoporphyrin IX, which is the naturally occurring immediate precursor to heme, is a potent activator of purified guanylate cyclase (20) may help to resolve this problem. Further, micromolar concentrations of heme inhibit unactivated and activated guanylate cyclase (20).

Our original observation that protoporphyrin IX activates guanylate cyclase was made with heme-deficient purified enzyme.³ The objective of that experiment was to ascertain whether the iron of heme is required for the binding of heme and NO to guanylate cyclase. The binding of heme to guanylate cyclase was recently reported (16, 19). A mixture of protoporphyrin IX and guanylate cyclase was passed through a gel filtration column in order to separate enzyme from unbound protoporphyrin IX. The column void volume was scanned in the visible wavelength range of 320 to 700 nm and subsequently tested for guanylate cyclase activity. A positive identification of bound protoporphyrin IX was made and the enzyme was recovered from the column in the maximally activated state. Additional experiments with heme-containing guanylate cyclase revealed that protoporphyrin IX markedly activated the enzyme at nanomolar concentrations and had a Kₘ of 15–30 nM (20). The objective of the present study was to employ a kinetic approach to characterize the mechanism of guanylate cyclase activation by protoporphyrin IX. In addition, experiments were conducted to compare and contrast the characteristics of enzyme activation by protoporphyrin IX, NO, NO-heme, and nitroso compounds.

¹ This work was supported in part by Grant AM 17692 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

² L. J. Ignarro and M. S. Wolin, unpublished observations.

³ Recipient of National Research Service Award HL 06225.
EXPERIMENTAL PROCEDURES

Materials—Reagents for the guanylate cyclase assay have been described (14, 18). Chromatographically pure protoporphyrin IX, hematin, and bovine serum albumin (fatty acid-free) were purchased from Sigma. The sources, preparation and handling of NO, NO-heme, heme, nitroprusside, and S-nitroso-N-acetylpenicillamine have been described previously (12, 14, 18). The approximate molar concentration of 1 μl of NO gas in 1 ml of aqueous medium at 37 °C is 0.2 μM (14). The sources and chromatographic properties are described under "Enzyme Purification." All other chemicals were of the highest purity commercially available.

Enzyme Purification—Fresh bovine lung, obtained from a nearby slaughterhouse, was trimmed of large blood vessels and airways and finely minced before passages through a 1 mm commercial meat grinder. All subsequent steps were performed at 0–4 °C. The mince was washed exhaustively with 10 volumes of 25 mM TEA, pH 7.8, and 0.4 kg of washed mince was homogenized in 3 volumes of 25 mM TEA, pH 7.8, containing 5 mM diethiothreitol (TEA-dithiothreitol) with the aid of a Waring Blender. Following low speed centrifugation (10,000 × g for 20 min), the resulting supernatant was centrifuged at 100,000 × g for 60 min. Guanylate cyclase was removed from the supernatant by batch adsorption onto DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals) as follows. The supernatant was stirred slowly with 400 g of DEAE-Sepharose CL-6B, pre-equilibrated with TEA-dithiothreitol, for 45 min and then the resin was allowed to sediment. The supernatant was discarded and the resin was washed 4 times with 3 volumes of TEA-dithiothreitol. Each wash was performed by slowly stirring the resin in buffer for 15 min. The washed resin was poured into a column (2.5 cm diameter) and packing was achieved by washing the column with TEA-dithiothreitol at a rate of 200 ml/h until the resin bed reached a constant height (80 cm). After washing the resin for 90 min (150 ml/h), 1.3 liters of a linear NaCl gradient (0 to 0.4 M) in TEA-dithiothreitol was passed through the column (100 ml/h). Fractions containing in excess of 75% of maximal enzymatic activity (0.20 to 0.23 pmol/min/mg of protein) revealed single migrating bands. Electrophoresis of the guanylate cyclase fraction, and concentration separately by ultrafiltration (Amicon Corp.; PM-30 membrane) to 13 ml.

Calculation of Kinetic Constants—All guanylate cyclase assays for kinetic analyses were conducted under initial velocity conditions. This implies a maximal conversion of GTP to cyclic GMP of less than 10% and a linear relationship between cyclic GMP formation and time. Kinetic constants were calculated as suggested by Cleland (22). The Kₘ and Kᵢ for metal in excess of metal-GTP were calculated from the data illustrated in Figs. 5 and 6 as follows. From a primary plot of velocity (v) versus [GTP] as a function of [excess metal], a replott of the intercepts or slopes versus [excess metal]¹ yields the Kᵢ or Kᵢᵠ, respectively, for metal. Intercept and slope replots of the data in Figs. 5 and 6 give the Kᵢ and Kᵢᵠ, respectively, for GTP. The inhibition constant (Kᵢ) for hematin, a competitive inhibitor of protoporphyrin IX, was derived from a slope replot of the data in Fig. 9.

RESULTS

Purification and Properties of Guanylate Cyclase—As reported previously (20) the procedures employed to purify guanylate cyclase without loss of heme resulted in homogeneous preparations with high specific activities. Matrex gel blue A, an hydrophobic affinity resin, bound guanylate cyclase tightly and enabled contaminating proteins to be separated by exhaustive washing with pH 7.8 buffer and buffer containing 0.5 M NaCl. Guanylate cyclase eluted with 1–1.3 M NaCl. When employed as the final step, Matrex gel blue A afforded a rapid and convenient means of obtaining stable, purified enzyme for kinetic analyses. The guanylate cyclase activity was determined as described previously (21). The metal concentration present in excess of metal-GTP is therefore uncomplicated, and the metal concentration in the appropriate kinetic plots. Samples in each enzymatic assay were run in duplicate.

Enzyme Purification—Fresh bovine lung, obtained from a nearby slaughterhouse, was trimmed of large blood vessels and airways and finely minced before passages through a 1 mm commercial meat grinder. All subsequent steps were performed at 0–4 °C. The mince was washed exhaustively with 10 volumes of 25 mM TEA, pH 7.8, and 0.4 kg of washed mince was homogenized in 3 volumes of 25 mM TEA, pH 7.8, containing 5 mM diethiothreitol (TEA-dithiothreitol) with the aid of a Waring Blender. Following low speed centrifugation (10,000 × g for 20 min), the resulting supernatant was centrifuged at 100,000 × g for 60 min. Guanylate cyclase was removed from the supernatant by batch adsorption onto DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals) as follows. The supernatant was stirred slowly with 400 g of DEAE-Sepharose CL-6B, pre-equilibrated with TEA-dithiothreitol, for 45 min and then the resin was allowed to sediment. The supernatant was discarded and the resin was washed 4 times with 3 volumes of TEA-dithiothreitol. Each wash was performed by slowly stirring the resin in buffer for 15 min. The washed resin was poured into a column (2.5 cm diameter) and packing was achieved by washing the column with TEA-dithiothreitol at a rate of 200 ml/h until the resin bed reached a constant height (80 cm). After washing the resin for 90 min (150 ml/h), 1.3 liters of a linear NaCl gradient (0 to 0.4 M) in TEA-dithiothreitol was passed through the column (100 ml/h). Fractions containing in excess of 75% of maximal enzymatic activity (0.20 to 0.23 pmol/min/mg of protein) revealed single migrating bands. Electrophoresis of the guanylate cyclase fraction, and concentration separately by ultrafiltration (Amicon Corp.; PM-30 membrane) to 13 ml.

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Kinetic data, double reciprocal plots and replots (with the exception of the data in Fig. 3) were analyzed by least squares linear regression analysis.

Utilizing the data in Fig. 7, a Scatchard plot was constructed in order to obtain estimates of Kᵢ for the guanylate cyclase-protoporphyrin IX complex and the stoichiometry of protoporphyrin IX binding to guanylate cyclase. This analysis requires knowledge of the amounts of protoporphyrin IX unbound and bound to enzyme at each concentration of protoporphyrin IX tested. Since these amounts were unknown, an assumption was made that for a given concentration of enzyme the concentration of enzyme-activator (guanylate cyclase-protoporphyrin IX) complex is proportional to the observed enzyme velocity, and the enzyme is saturated with activator at maximal velocity. The same maximal specific activity was obtained for three different concentrations of guanylate cyclase in the presence of excess protoporphyrin IX. Accordingly, after subtraction of basal activity, velocity at each concentration of activator divided by maximal velocity (6 pmol/min/mg of protein) yields a value equivalent to the fraction of enzyme which exists as enzyme-activator complex (enzyme-bound activator). This value is subtracted from the known total concentration of activator in a given assay sample to yield the concentration of free or unbound activator. These data are then plotted according to Scatchard (23) to yield the Kᵢ (from the slope) and the stoichiometry of binding (x-axis intercept), assuming a molecular weight of bovine lung-soluble guanylate cyclase of 150,000 (24).
guanylate cyclase. The results of a typical guanylate cyclase purification are shown in Table I. The apparent increase in total enzymatic activity after the DEAE-Sepharose CL-6B step was likely due to removal of inhibitory substances (possibly heme or hemoproteins) from the starting soluble fraction. Specific activities of guanylate cyclase (μmol of cGMP/min/mg of protein) were 0.1-0.2 in the presence of 1 mM GTP and 3 mM Mg2+, and 0.3-0.6 with 1 mM GTP and 3 mM Mn2+.

Formation of cyclic GMP was linear for at least 10 min in the presence of either MnGTP or MgGTP (Fig. 1). Protoporphyrin IX (5 μM) activated guanylate cyclase approximately 4- and 26-fold in the presence of MnGTP or MgGTP, respectively, and product formation was linear for at least 10 min (Fig. 1). The rate of product formation was directly proportional to the concentration of guanylate cyclase over the range of 0.12-0.96 μg of enzyme protein (Fig. 2). Bovine serum albumin was not required to stabilize the enzyme and, in fact, inhibited activity in the presence of MgGTP (Fig. 2). In view of the above observations, enzyme reactions were conducted with 0.2-0.4 μg of enzyme protein (0.2 ml reaction volume) and reaction mixtures were incubated at 37 °C for 10 min in the absence of bovine serum albumin. Additional experiments indicated that product formation was linear with time for 90 min in the absence or presence of protoporphyrin IX. However, preincubation of guanylate cyclase at 37 °C in the absence of GTP and/or Mg2+ (or Mn2+) resulted in a steady decline to 60-70% of maximal enzyme activity after 60 min.

Kinetic Properties of Unactivated and Activated Guanylate Cyclase—In the presence of excess Mn2+ the apparent Kₘ for GTP was 0.0 μM and the Vₖₜₜₜ was 0.2 μmol/min/mg of protein for unactivated guanylate cyclase (Fig. 3). Values for both Kₘ and Vₖₜₜₜ were calculated from the linear portions of double reciprocal plots. A deviation resembling substrate activation was observed in the Mn2+-dependent reaction at GTP concentrations in excess of 100 μM. Absolute values for specific activity with 1 mM GTP ranged from 0.3 to 0.6 μmol/min/mg of protein. Saturating concentrations of protoporphyrin IX (5 μM), NO (1 μM), and NO-heme (1 μM) increased the maximal observed specific activity without altering the apparent Kₘ (Fig. 3). In the presence of excess Mg2+ the apparent Kₘ for GTP was 100 μM and the Vₖₜₜₜ was 0.1 μmol/min/mg of protein (Fig. 4, inset). Saturating concentrations of protoporphyrin IX (1 μM), NO (1 μM), NO-heme (0.1 μM), and S-nitroso-N-acetylpenicillamine (0.1 mM) decreased the apparent Kₘ from 100 to 45-55 μM and increased the Vₖₜₜₜ from 0.1 to 2-3 μmol/min/mg of protein (Fig. 4). Depending on the individual preparation of guanylate cyclase tested, specific activities (μmol/min/mg of protein) ranged from 0.1-0.2 (basal activity) and from 2-8 (protoporphyrin IX-activated).

Kinetic parameters for the Mg2+-dependent reaction of guanylate cyclase are given in Table II. The apparent Kₘ for excess Mg2+ was 72 μM, which was calculated from an intercept

**Table I**

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble fraction (105,000 x g superna-tant)*</td>
<td>16,280</td>
<td>651</td>
<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B*</td>
<td>2,000</td>
<td>1,280</td>
<td>0.64</td>
<td>16</td>
</tr>
<tr>
<td>Ultragel Aca-34*</td>
<td>133</td>
<td>798</td>
<td>0.6</td>
<td>150</td>
</tr>
<tr>
<td>Matrex gel blue A</td>
<td>0.85</td>
<td>275</td>
<td>324</td>
<td>8,100</td>
</tr>
<tr>
<td>Eluate 1</td>
<td>0.21</td>
<td>94</td>
<td>446</td>
<td>11,150</td>
</tr>
<tr>
<td>Eluate 2</td>
<td>0.85</td>
<td>275</td>
<td>324</td>
<td>8,100</td>
</tr>
</tbody>
</table>

* Preparation of fractions is described under "Experimental Procedures." All incubations were at 37 °C for 10 min, and reaction mixtures (0.2 ml) contained 40 mM TEA, pH 7.4, 2 mM dithiothreitol, 1 mM GTP, and 3 mM Mn2+.

† Reaction mixtures also contained 0.3 mM 1-methyl-3-isobutylxanthine.
Kinetics of Lung Guanylate Cyclase

A less extensive analysis of the kinetic parameters for the Mn$^{2+}$-dependent reaction of guanylate cyclase was conducted. The apparent $K_m$ and $K_i$ for excess Mn$^{2+}$, in the MnGTP concentration range of 0.1–1 mM, with unactivated guanylate cyclase were approximately 475 and 950 μM, respectively. These values were obtained from intercept and slope replots of the data illustrated in Fig. 6, which were performed as described above for excess Mg$^{2+}$. Protoporphyrin IX markedly decreased both the $K_m$ and $K_i$ for excess Mn$^{2+}$ to 45–55 μM, as determined from replots of the data in Fig. 6 (inset). Thus, in the presence of protoporphyrin IX the $K_m$ and $K_i$ for excess Mn$^{2+}$ were decreased to nearly equivalent values.

**Kinetic Parameters for Protoporphyrin IX**—Initial observations indicated that the $K_m$ for protoporphyrin IX was a function of guanylate cyclase concentration (Fig. 7), under the assay conditions employed. The convergence of maximal specific activities at saturating concentrations of protoporphyrin IX suggests that the protoporphyrin IX-guanylate cyclase complex has a specific activity of 6 μmol/min/mg of protein. Thus, under typical assay conditions, the $K_m$ for protoporphyrin IX was a function of enzyme concentration.

This was attributed to a reduction in the free protoporphyrin IX concentration in solution resulting from increased amounts of enzyme-bound protoporphyrin IX. The data in Fig. 7 were utilized to estimate both the equilibrium dissociation constant of the guanylate cyclase-protoporphyrin IX complex and the stoichiometry of binding by transformation of the data into a form suitable for Scatchard analysis (see “Experimental Procedures”). The inset to Fig. 7 illustrates that a Scatchard replot of the data obtained with 6 nM guanylate cyclase was linear with a calculated $K_D$ of 1.4 nM (from the slope) and a stoichiometry of protoporphyrin IX binding to guanylate cyclase of 0.92 mol/mol of holoenzyme (from the x-axis intercept). Similar replots of data for 3 and 9 nM enzyme yielded values that were identical to those obtained with 6 nM enzyme.

**Kinetic Study of the Binding Interactions of Guanylate Cyclase with Protoporphyrin IX and Hematin**—An experiment was conducted to assess the time required for guanylate cyclase to reach equilibrium with protoporphyrin IX and with hematin. In the presence of 10 μM hematin with or without 1 μM protoporphyrin IX, the rate of product formation became constant at 8–10 min of incubation at 37 °C (Fig. 8). In contrast, the rate of product formation in the presence of 1 μM protoporphyrin IX appeared to remain constant with time during 20 min of incubation. Therefore, hematin, at concentrations that do not inhibit basal activity, and protoporphyrin IX were preincubated with enzyme for 10 min prior to addition of MgGTP. This allowed binding interactions to reach equilibrium so that steady state velocities could be examined. Hematin inhibited protoporphyrin IX activation in a competitive manner (Fig. 9). In the experiment shown in Fig. 9, the

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**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Unactivated</th>
<th>Protoporphyrin IX-activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgGTP</td>
<td>97</td>
<td>172</td>
</tr>
<tr>
<td>MgGTP</td>
<td>72</td>
<td>168</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Unactivated</th>
<th>Protoporphyrin IX-activated</th>
</tr>
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<tbody>
<tr>
<td>MgGTP</td>
<td>97</td>
<td>172</td>
</tr>
<tr>
<td>MgGTP</td>
<td>72</td>
<td>168</td>
</tr>
</tbody>
</table>

---

*1 μM protoporphyrin IX

*Obtained from intercept replots of primary plots; expressed as micromolar.

*Obtained from slope replots of primary plots; expressed as micromolar.

*Expressed as μmol of cGMP/min/mg of protein.
Kinetics of Lung Guanylate Cyclase

**Fig. 5.** Relationship between reaction velocity and concentration of excess Mg$^{2+}$ as a function of GTP in the absence and presence of protoporphyrin IX. Enzymatic reactions were conducted for 10 min at 37°C in the absence or presence (inset) of 1 μM protoporphyrin IX in 40 mM TEA, pH 7.4, containing 2 mM dithiothreitol, 0.24 μg of guanylate cyclase, and from 0.2 to 3 mM excess Mg$^{2+}$ at the indicated MgGTP concentrations. Velocity signifies pmol of cGMP/min/mg of protein. Incubation volumes in the absence or presence of protoporphyrin IX were 0.2 or 1 ml, respectively.

**Fig. 6.** Relationship between reaction velocity and concentration of excess Mn$^{2+}$ as a function of GTP in the absence and presence of protoporphyrin IX. Enzymatic reactions were conducted for 10 min at 37°C in the absence or presence (inset) of 5 pM protoporphyrin IX in 40 mM TEA, pH 7.4, containing 2 mM dithiothreitol, 0.24 μg of guanylate cyclase, and from 0.2 to 3 mM excess Mn$^{2+}$ at the indicated MnGTP concentrations. Velocity signifies pmol of cGMP/min/mg of protein. Incubation volumes in the absence or presence of protoporphyrin IX were 0.2 or 1 ml, respectively.

$K_m$ for protoporphyrin IX was 38 nM and the $V_{max}$ was 8 μmol/min/mg of protein. A replot of slopes against hematin concentration (0–1.5 μM) was linear and yielded a $K_i$ for hematin of 0.35 μM (Fig. 9, inset). These observations indicate that hematin and protoporphyrin IX may interact at, and compete for a common binding site on guanylate cyclase. However, hematin concentrations in excess of 1.5 μM yielded a concave-upward, slope replot (Fig. 10). This deviation from linearity occurred at higher hematin concentrations which inhibited unactivated as well as activated enzyme. The inhibition of unactivated guanylate cyclase by these elevated hematin concentrations was noncompetitive with respect to GTP (20). Micromolar concentrations of hematin also inhibited guanylate cyclase activation by NO, NO-heme, S-nitroso-N-acetylpenicillamine, and nitroprusside (Table III).

**Binding of Protoporphyrin IX to Guanylate Cyclase**—In order to more directly ascertain whether protoporphyrin IX binds to guanylate cyclase, the protocol developed for determining the binding of heme to the enzyme was employed as described previously (19). Guanylate cyclase used in this experiment contained stoichiometric amounts of heme as determined spectrally (20, 25). A solution of 0.2 or 2 μM guanylate cyclase (15 or 150 μg in 0.5 ml of 25 mM TEA, pH 7.8, containing 5 mM dithiothreitol and 30% (v/v) glycerol) was mixed with protoporphyrin IX (final concentration, 5 μM), kept at 0°C for 20 min and applied to a column (0.7 × 15 cm) of Sephadex G-25 previously equilibrated with the above buffer solution. Guanylate cyclase eluted in the void volume. A visible absorption scan (320–700 nm) of the void volume revealed a sharp absorbance peak at 390 nm and distinct shoulders at 540 and 585 nm, which are characteristic of protoporphyrin IX (26). Untreated guanylate cyclase (not reacted with protoporphyrin IX) showed a distinct absorbance at 425 nm due to the presence of heme in the enzyme (20, 25). An estimation of the amount of protoporphyrin IX bound to 2 μM guanylate cyclase indicated a binding stoichiometry of approximately 0.9 mol/mol of holoenzyme. This estimate was made with the assumption that the extinction coefficient of protoporphyrin IX is not appreciably altered after binding to enzyme. This assumption may be valid because the value obtained (0.9) agrees closely with that of 0.92, obtained by Scatchard analysis. Guanylate cyclase reconstituted with protoporphyrin IX was in the maximally activated state and was not further activated by protoporphyrin IX, NO, NO-heme,
Kinetics of Lung Guanylate Cyclase

1.5 E-Prot. IX (pmol)

0.1 0.2 0.3 0.4 0.5 0.6

1/Protoporphyrin IX (nM-1)

FIG. 7. Guanylate cyclase activation by protoporphyrin IX as a function of enzyme concentration. Enzymatic reactions (0.2 ml) were conducted for 10 min at 37 °C in 40 mM TEA, pH 7.4, containing 2 mM dithiothreitol, 1 mM GTP, 3 mM Mg²⁺, three different concentrations of guanylate cyclase (0.09 µg, 3 nM; 0.18 µg, 6 nM; 0.27 µg, 9 nM) and the indicated concentrations of protoporphyrin IX. Velocity signifies µmol of cGMP/min/mg of protein. The inset represents a Scatchard type replot of the data obtained with 6 nM guanylate cyclase (see "Experimental Procedures"). E, guanylate cyclase; Prot. IX, protoporphyrin IX.

Fig. 8. Time course for cyclic GMP formation in the presence of protoporphyrin IX and/or hematin. Enzymatic reactions (0.2 ml) were conducted for up to 20 min at 37 °C in the presence of 10 µM hematin (lower) or 1 µM protoporphyrin IX (upper) or both (C). Reactions were in 40 mM TEA, pH 7.4, containing 2 mM dithiothreitol, 1 mM GTP, 1 mM Mg²⁺ and 0.24 µg of guanylate cyclase.

and responsiveness to protoporphyrin IX, NO, and related agents.

Differences Between Protoporphyrin IX and NO on Guanylate Cyclase Activation—The data in Table V reveal some differences between protoporphyrin IX and NO-related agents with respect to guanylate cyclase activation. Two types of compound were tested. One type, represented by CN⁻ and CO, readily bind to heme iron. The second type, represented by phenazine methosulfate, FMN, and riboflavin, are oxidizing agents. The data in Table V reveal some differences between protoporphyrin IX and NO-related agents with respect to guanylate cyclase activation. Two types of compound were tested. One type, represented by CN⁻ and CO, readily bind to heme iron. The second type, represented by phenazine methosulfate, FMN, and riboflavin, are oxidizing agents.

TABLE III

Inhibitory effect of hematin on unactivated and activated guanylate cyclase

<table>
<thead>
<tr>
<th>Additions</th>
<th>Guanylate cyclase activity at hematin concentration (µmol cGMP/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0.13</td>
</tr>
<tr>
<td>Protoporphyrin IX, 0.1 µM</td>
<td>4.6</td>
</tr>
<tr>
<td>NO, 0.5 µM</td>
<td>6.0</td>
</tr>
<tr>
<td>NO-heme, 0.1 µM</td>
<td>6.2</td>
</tr>
<tr>
<td>S-Nitroso-N-acetylpenicillamine, 0.1 mM</td>
<td>5.6</td>
</tr>
<tr>
<td>Nitroprusside, 0.1 mM</td>
<td>1.5</td>
</tr>
</tbody>
</table>

*Hematin was added prior to incubation and activators were added at incubation immediately after enzyme. Reaction mixtures (0.2 ml) contained 40 mM TEA, pH 7.4, 2 mM dithiothreitol, 1 mM GTP, 3 mM Mg²⁺, and 0.24 µg of enzyme and were incubated at 37 °C for 10 min.
agents. None of the agents tested altered basal or protoporphyrin IX-activated guanylate cyclase (Table V). In contrast, CN− and the three oxidants tested markedly inhibited enzyme activation by NO, NO-heme, nitroprusside, and S-nitroso-N-acetylpenicillamine. CO, however, failed to inhibit enzyme activation by any agent (not shown).

The procedures for guanylate cyclase purification reported previously (20) and described in detail here, afford a rapid and relatively simple means of purifying the soluble enzyme from bovine lung. The data in the present study show that the naturally occurring immediate precursor to heme, protoporphyrin IX, is a potent activator of soluble guanylate cyclase purified from bovine lung. On the other hand, hematin inhibits both basal guanylate cyclase activity and enzyme activation by protoporphyrin IX, NO-heme, NO, and nitroso compounds. As recently reported (20), guanylate cyclase purified as described contains heme and is markedly activated by NO. Recently, Gerzer et al. (24) also reported the purification of heme-containing guanylate cyclase which is activated by nitroprusside. This is in contrast to other reports showing that purified guanylate cyclase was only partially activated by NO (27, 28), thus implying a loss of heme during enzyme purification. Observations from this laboratory indicate that bovine lung soluble guanylate cyclase can be purified to homogeneity also in a form which is heme-deficient (29). The latter enzyme requires addition of 0.1–1 μM heme in order to observe activation by NO, nitroprusside, or S-nitroso-N-acetylpenicillamine. NO-heme, however, markedly activates heme-deficient guanylate cyclase. On the other hand, heme-containing guanylate cyclase is markedly activated by NO and nitroso compounds (20, 24). These observations suggest strongly that guanylate cyclase activation by NO requires heme, as was originally proposed by Craven and DeRubertis (16).

Both guanylate and adenylate cyclase appear to display a metal requirement in excess of the metal-nucleoside triphosphate complex (30–32). The regulatory mechanisms of the cyclases appear to be closely related to the metal cofactor and the binding affinity for uncomplexed metal. It is well known that basal guanylate cyclase activity is 5- to 10-fold greater in the presence of Mn2+ than Mg2+ at comparable metal concentrations. Previous studies on guanylate cyclase and its regulation by NO and related agents suggest that a marked lowering of the free Mn2+ requirement occurs as a result of enzyme activation (2, 4, 16, 33). Studies with Mg2+, however, do not appear to display a marked lowering of this free metal requirement (4, 16). Similar observations and interpretations were made in the present study with soluble guanylate cyclase purified from bovine lung. The effects of protoporphyrin IX on kinetic parameters of guanylate cyclase activation were very similar to those displayed by NO, NO-heme, and S-nitroso-N-acetylpenicillamine. All activators markedly increased the Vmax and decreased the Km for GTP to a small extent in the presence of excess Mg2+. Interestingly, in the presence of excess Mn2+, all activators only moderately in-

<table>
<thead>
<tr>
<th>Additionsa</th>
<th>Frozen/thawed enzyme</th>
<th>Protoporphyrin IX-reconstituted enzyme</th>
<th>Frozen/thawed protoporphyrin IX-reconstituted enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.10</td>
<td>0.11</td>
<td>4.2</td>
</tr>
<tr>
<td>Prottoporphyrin IX</td>
<td>1 μM</td>
<td>3.2</td>
<td>3.8</td>
</tr>
<tr>
<td>NO, 1 μM</td>
<td>3.0</td>
<td>3.8</td>
<td>4.0</td>
</tr>
<tr>
<td>NO-heme, 0.1 μM</td>
<td>4.4</td>
<td>4.8</td>
<td>3.8</td>
</tr>
<tr>
<td>S-Nitroso-N-acetylpenicillamine, 0.1 μM</td>
<td>4.2</td>
<td>4.8</td>
<td>4.2</td>
</tr>
</tbody>
</table>

* Activators were added at incubation immediately after enzyme. Reaction mixtures (0.2 ml) contained 40 mM TEA, pH 7.4, 2 mM dithiothreitol, 0.3 mM GTP, 3 mM MgCl2, and 0.32 μg of enzyme and were incubated at 37 °C for 10 min.

* Enzyme refers to a stock solution of 16 μg of guanylate cyclase in 0.2 ml, which was diluted 10-fold just prior to assay.

* Diluted enzyme (as in b) was refrozen at −60 °C, and thawed for assay 24 h later.

* The same procedure described in Footnote c was employed.

Table IV

**Effects of freeze-thaw on responsiveness of guanylate cyclase and protoporphyrin IX-reconstituted guanylate cyclase to various activators**

Table V

**Differences in the effects of cyanide and oxidizing agents on guanylate cyclase activity by protoporphyrin IX as compared with NO and related agents**

Additionsb | Basal activity | 1 μM Prottoporphyrin IX | 1 μM NO | 0.1 μM NO-heme | 0.1 mM S-nitroso-N-acetylpenicillamine | 0.1 mM Nitroprusside |
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.10c</td>
<td>2.8</td>
<td>4.6</td>
<td>4.8</td>
<td>4.2</td>
<td>1.3</td>
</tr>
<tr>
<td>KCN</td>
<td></td>
<td>0.69</td>
<td>2.4</td>
<td>3.0</td>
<td>4.0</td>
<td>2.8</td>
</tr>
<tr>
<td>1 mM</td>
<td></td>
<td>0.11</td>
<td>3.0</td>
<td>0.58</td>
<td>2.2</td>
<td>1.0</td>
</tr>
<tr>
<td>5 mM</td>
<td></td>
<td>0.11</td>
<td>2.6</td>
<td>0.54</td>
<td>0.56</td>
<td>0.19</td>
</tr>
<tr>
<td>Pheazine methosulfate, 0.1 mM</td>
<td>0.11</td>
<td>2.4</td>
<td>0.36</td>
<td>0.40</td>
<td>0.12</td>
<td>0.11</td>
</tr>
<tr>
<td>FMN, 0.1 mM</td>
<td></td>
<td>0.11</td>
<td>3.2</td>
<td>0.28</td>
<td>0.38</td>
<td>0.13</td>
</tr>
<tr>
<td>Riboflavin, 0.1 mM</td>
<td>0.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Additions of KCN or oxidizing agents were made just prior to warming reaction mixtures at 37 °C. Activators were added at incubation immediately after enzyme. Reaction mixtures (0.2 ml) contained 40 mM TEA, pH 7.4, 2 mM dithiothreitol, 0.3 mM GTP, 3 mM MgCl2, and 0.28 μg of enzyme and were incubated at 37 °C for 10 min.

* Each value is the mean of duplicate determinations from 3–4 separate experiments.
creased the \( V_{\text{max}} \) and failed to alter the \( K_m \) for GTP. Increasing the concentration of Mn\(^{2+}\) in the presence of constant GTP resulted in greater basal enzymatic activity but less enzyme activation. These observations with Mn\(^{2+}\) should be interpreted with caution because the elevated basal activity in the presence of MnGTP plus excess Mn\(^{2+}\) may be at least in part attributed to a direct substrate activation of the enzyme. Similar observations were made recently with partially purified hepatic guanylate cyclase (18). Protoporphyrin IX resembled NO and NO-heme also in their effects on initial velocity kinetics of uncomplexed Mg\(^{2+}\) and Mn\(^{2+}\). These enzyme activators decreased the apparent \( K_m \) and \( V_{\text{max}} \) for excess Mg\(^{2+}\) and Mn\(^{2+}\) to a common value of approximately 40 \( \mu \)M. Comparison with unactivated enzyme reveals that activation increases the \( V_{\text{max}} \) and eliminates the influence of GTP on the apparent \( K_m \) for free metal. The kinetic data indicate that the activated state of guanylate cyclase behaves in an identical manner regardless of whether the activator is protoporphyrin IX, NO, or NO-heme.

The mechanism of guanylate cyclase regulation by the porphyrin binding interaction associated with alterations in the kinetic parameters is not yet understood. However, lowering of the affinity for free metal and/or an increase in the maximal velocity may be mechanisms associated with the regulation of soluble guanylate cyclase. Stabilization of the configuration of the enzyme in the transition state of the catalytic reaction, which manifests itself as changes in these kinetic parameters, could be a mechanism of enzyme regulation. In the presence of Mg\(^{2+}\), protoporphyrin IX and NO-heme markedly increase the maximal velocity but increase the apparent binding affinity of enzyme for free Mg\(^{2+}\) to a lesser extent. Therefore, an increase in the maximal velocity may be more significant than an increase in the free metal binding affinity in the expression of guanylate cyclase activation. On the other hand, in the presence of Mn\(^{2+}\), the activators markedly increase the apparent binding affinity of enzyme for free Mn\(^{2+}\) but increase the maximal velocity to a much lesser extent. Thus, the former may be more important than the latter as a mechanism of regulation of guanylate cyclase. However, the latter interpretation is questionable because large concentrations of GTP and free Mn\(^{2+}\), both of which are unphysiological, cause apparent substrate activation of guanylate cyclase.

Guanylate cyclase displays a high affinity binding interaction with protoporphyrin IX. This is suggested by the observations that the guanylate cyclase-protoporphyrin IX complex did not dissociate during gel filtration or dialysis. High affinity binding is further supported by the low equilibrium dissociation constant (\( K_d = 1.4 \) nM) of the guanylate cyclase-protoporphyrin IX complex. The latter was calculated from a Scatchard plot of the data in Fig. 7. A Scatchard (23) analysis of the data was possible because protoporphyrin IX is an enzyme activator, rather than an enzyme substrate, and its binding can be treated as an equilibrium interaction as follows:

\[
E + \text{Prot. IX} \rightleftharpoons E - \text{Prot. IX} \quad \text{with rate constants} \quad k_1, \quad k_{-1}
\]

\[
K_d = k_{-1}/k_1 = [E][\text{Prot. IX}]/[E - \text{Prot. IX}]
\]

where \( E \) and Prot. IX signify guanylate cyclase and protoporphyrin IX, respectively. This equilibrium binding assumption is supported by the observations that the reaction was linear with time, or the velocity was a steady state rate measurement. Further, only one binding interaction was apparent because of the linearity of the double reciprocal plots of velocity \textit{versus} protoporphyrin IX concentration with identical \( K_d \) values for the different enzyme concentrations (Fig. 7), and the competitive inhibition by hematin (Fig. 9). An estimate from the Scatchard plot of the stoichiometry of binding of protoporphyrin IX to guanylate cyclase (0.92 mol/mol of holoenzyme), was in very close agreement with that (0.9 mol/mol of holoenzyme) estimated by spectral analysis. Moreover, these values are consistent with the recent observations that guanylate cyclase purified from bovine lung contained 1 mol of ferroprotoporphyrin IX (heme)/mol of enzyme (25). The latter observations and the competitive inhibition of protoporphyrin IX by hematin are consistent with a possible physiological role for the latter porphyrins in the regulation of guanylate cyclase.

Guanylate cyclase containing stoichiometric amounts of heme appeared to become fully reconstituted with protoporphyrin IX, as estimated both by spectral and Scatchard analysis. Whether or not the heme was displaced by protoporphyrin IX was difficult to ascertain unambiguously. However, the linearity of the double reciprocal plots of velocity \textit{versus} protoporphyrin IX concentration at different hematin concentrations, as well as the competitive inhibition by hematin of enzyme activation by protoporphyrin IX, suggest that displacement of hematin by protoporphyrin IX from a common binding site on guanylate cyclase is possible. Protoporphyrin IX-reconstituted guanylate cyclase was in the maximally activated state and could not be further activated by protoporphyrin IX or NO-containing substances. Interestingly, whereas unactivated enzyme could be frozen and thawed without apparent alteration in responsiveness to activation, protoporphyrin IX-reconstituted enzyme lost responsiveness to activation after similar treatment. These observations suggest that a marked change occurs in the protoporphyrin IX-enzyme interaction during freezing and thawing. The reasons for these differences are unknown, and studies are in progress to better understand these observations.

Notable differences between protoporphyrin IX and NO or related agents were apparent in this study. CN\(^{-}\) and several oxidizing agents inhibited guanylate cyclase activation by NO, NO-heme, and nitroso compounds without affecting either enzyme activation by protoporphyrin IX or basal enzymatic activity. The mechanisms by which CN\(^{-}\) and oxidants inhibit guanylate cyclase activation by NO compounds are unknown. However, CN\(^{-}\) could inhibit activation by competing with NO for heme iron since both are well known to bind heme iron. Such an interaction would inhibit guanylate cyclase activation by NO since enzyme activation by NO appears to require heme (16, 17). This interpretation is supported by the recent observations of Gerzer \textit{et al.} (25) that NO shifted the absorption spectrum of heme-containing guanylate cyclase and that CN\(^{-}\) inhibited enzyme activation by nitroprusside. Thus, an inhibitory effect of CN\(^{-}\) on either guanylate cyclase activation by protoporphyrin IX, or basal enzymatic activity would not be expected. Oxidizing agents such as phenazine methosulfate, FMN, and riboflavin may oxidize NO or NO-heme to higher oxides of nitrogen, such as NO_2, which do not activate guanylate cyclase (15, 34). Alternatively, oxidants may convert heme to ferri-protoporphyrin IX and thereby liberate NO from the heme complex (35). Previous reports indicate that other oxidants such as methylene blue and ferricyanide likewise inhibit guanylate cyclase activation by NO and nitroso compounds without affecting basal enzymatic activity (34). The finding that CO failed to alter enzyme activation by NO and NO-heme, although CO readily binds heme iron may be explained by the much higher binding affinity of heme iron for NO (36).

The nitrogen oxide-containing activators of guanylate cyclase are also potent vasodilators and inhibitors of platelet...
aggregation (6-8, 10-15). These agents could conceivably function in a complex regulatory system normally modulated by oxygen tension. The fact that oxygen, as well as NO, binds to certain hemoproteins supports the possibility that oxygen could modulate guanylate cyclase activity. Previous studies showed that molecular oxygen rapidly but reversibly inactivated guanylate cyclase (19). Inactivation was prevented or reversed by diethiothreitol. This enzyme inactivation may occur as a result of the binding of oxygen to the heme group associated with guanylate cyclase (25). On the other hand, oxygen radicals may serve to activate guanylate cyclase (37). Thus, if oxygen does play a modulatory role in cyclic GMP formation it is likely to be complex and possibly dependent on other intracellular metabolic events involving calcium, arachidonic acid, and free radical reactions.

In many respects, the activation of guanylate cyclase by protoporphyrin IX closely resembled that by NO-heme, NO, and nitroso compounds. Very similar effects on kinetic parameters of enzyme activation were observed with all activators. In addition, micromolar concentrations of hematin inhibited enzyme activation not only by protoporphyrin IX but also by NO-heme, NO, and nitroso compounds. These observations suggest that a common mechanism may be involved in the activation of guanylate cyclase by each of these compounds. Recent EPR studies indicate that the iron in NO-heme, unlike the iron in heme, protrudes from the plane of the porphyrin ring (38-40). Such an alteration in the structure of heme brought about by NO may result in the development of a protoporphyrin IX-like binding interaction with guanylate cyclase, possibly by the loss of coordination of the heme iron to an amino acid at the porphyrin binding site on guanylate cyclase. It is plausible that the generation of modified porphyrins, which possess a protoporphyrin IX-binding interaction with guanylate cyclase, resulting from chemical reactions of heme or hemoproteins with NO or other agents may be a unifying mechanism for the activation of guanylate cyclase.

The data presented in this study, together with the knowledge that protoporphyrin IX and heme occur naturally in mammalian tissues, suggest that both substances could function biologically to regulate guanylate cyclase activity and tissue cyclic GMP levels.

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REFERENCES