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OXIDATIVE MODULATION OF SOLUBLE GUANYLATE CYCLASE BY MANGANESE

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Summary

Homogeneous or partially purified soluble guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2) from rat liver exhibited variable sensitivity to assay pH that was dependent upon buffer composition and the cation cofactor. Enzyme activity with 3 mM Mn^{2+} in excess of Mn^{2+}-GTP was considerably less in Tris buffers above pH 8.0 than in glycine buffer. In the pH range of 6.0—7.6, however, manganese-supported activity was greater in Tris buffers than in imidazole or cacodylate buffers of corresponding pH. The differences in activity seen with various buffers were not apparent when Mg^{2+} was the sole cation cofactor but were dependent upon Mn^{2+} concentrations in excess of Mn^{2+}-GTP. The effects of excess Mn^{2+} on guanylate cyclase varied with assay pH and buffer composition. At pH 7.6 in Tris-HCl buffer, excess Mn^{2+} increased guanylate cyclase activity with an apparent K_a of 0.25 mM and concentrations above 3 mM were slightly inhibitory. At pH 9.0 in Tris-HCl buffer, however, concentrations of excess Mn^{2+} above 0.1 mM were strongly inhibitory. By comparison, in cacodylate (pH 7.6) or glycine (pH 9.0) buffers, high concentrations of excess Mn^{2+} were considerably less inhibitory and the apparent K_a values for excess Mn^{2+} were greater than in Tris-HCl buffer at equivalent pH. The variable effects of Mn^{2+} on enzyme activity as a function of buffer pH and composition were qualitatively similar to its effects on catecholamine oxidation. Furthermore, the inhibition of guanylate cyclase by excess Mn^{2+} was partially prevented by dithiothreitol and the stimulation of enzyme activity by excess cation was completely blocked by the antioxidant hydroquinone. The studies suggest that the apparent requirement and preference of soluble guanylate cyclase for excess Mn^{2+} as cation cofactor, as well as the inhibition of enzyme activity by excess Mn^{2+} may be mediated by oxidative events associated with changes in the oxidation state of the free cation.

Introduction

An increasing number of studies have demonstrated that guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2) may be regulated by redox events [1—8]. This enzyme exists in both soluble and particulate forms and is responsible for the synthesis of cyclic GMP in tissues. A rather unique property of guanylate cyclase, especially the soluble form, is its activation by oxidative compounds. Nitric oxide, fatty acid hydroperoxides, prostaglandin endoperoxides, hydroxyl radical and superoxide anion are among a number of oxidative species that have been shown to stimulate guanylate cyclase activity in cell-free systems as well as to increase cyclic GMP levels in tissues [1—5]. Under the appropriate conditions, antioxidants or sulfhydryl reagents can either block [1—6] or enhance [7,8] enzyme activation. Many questions yet remain unanswered concerning the exact mechanism and physiological significance of guanylate cyclase interaction with oxidative compounds.

Another somewhat unique property of guanylate cyclase is its apparent high specificity for Mn^{2+} as cation cofactor. Although the cyclase can utilize Mg^{2+} as the sole cation cofactor [9,10], maximal activity in the absence of an oxidative activator requires concentrations of Mn^{2+} in excess of Mn^{2+}-GTP [11—13]. The apparent requirement of guanylate cyclase for excess Mn^{2+} far exceeds the low concentrations of Mn^{2+} found in cells [10,11,14]. This problem may be partially resolved by the fact that following activation, Mg^{2+} becomes as or more effective than Mn^{2+} as the sole cation cofactor [10]. Even so, that an enzyme can possess a specific requirement for relatively high concentrations of a divalent cation that virtually does not exist as the free cation in the cell is somewhat curious.

Manganese, being transition metal, has several oxidation states. Although Mn^{2+} is the most stable form under acid or near neutral conditions, Mn^{2+} readily oxidizes to Mn^{4+} or Mn^{7+} in alkali or in the presence of relatively electrophilic compounds [15]. Mn^{4+} and Mn^{7+} are, as one might expect, powerful oxidants. In light of the emerging role of redox in the regulation of guanylate cyclase and the apparent requirement of the enzyme for free Mn^{2+} a study was undertaken to determine if these two properties may possibly be interrelated. The present report describes results which suggest that some of the effects of Mn^{2+} on guanylate cyclase may be attributable to oxidative events.

Materials and Methods

Soluble guanylate cyclase was prepared from rat liver homogenates as described [16]. Enzyme purified through chromatography on Sepharose 6B was used in the majority of experiments, however, a prepration of apparently homogeneous guanylate cyclase purified as described [16] was used where indicated. The Sepharose 6B and homogenous enzyme preparations represent a maximum purification of 70—100-fold and 8000—9000-fold, respectively, over the soluble fraction of homogenates [16]. The Sepharose 6B preparations were dialyzed against 20 mM Tris-HCl buffer (pH 7.6) to remove EDTA and dithiothreitol added during purification, and stored at —70°C. Homogeneous preparations were stored similarly in 20 mM Tris-HCl (pH 7.6) but also contained 750

mM sucrose, 1 mM EDTA, and 10 mM dithiothreitol required for stability [16]. Enzyme was thawed immediately prior to use and was not refrozen for use again.

Guanylate cyclase activity was determined essentially as described [8,16] but with some modification. Incubations were in a volume of 100 μl for 10 min at 37°C and contained 50 mM buffer, GTP, $MnCl_2$ or $MgCl_2$, and 0.087—2.1 μg enzyme protein. Buffer composition, pH and the concentrations of GTP and metal were varied as indicated. Theophylline and a nucleoside triphosphate-regenerating system were not required for maximal activity of the enzyme used in these studies and were omitted from the assay system [16]. Cyclic GMP formed was determined by radioimmunoassay [17] with some modification [18]. All values represent means of duplicate incubations from representative experiments. The specific activities reported in this study are as expected for enzymes of similar purity assayed at the protein concentrations indicated under the conditions described [16]. Protein was measured by the method of Lowry et al. [19].

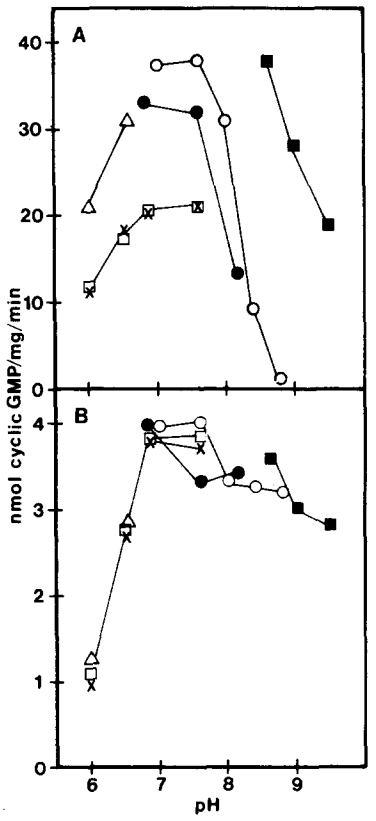
The oxidation of epinephrine to adrenochrome was followed in a reaction (1 ml at 24°C) containing 50 mM buffer (pH and composition varied as indicated) and 0.1 mM epinephrine. Adrenochrome formation was monitored by the increase in absorbance at 297 nm [20] with time. Where indicated, additions to the ongoing reaction were made in vols. of 10 μl followed by rapid mixing.

Preparation of ^{125}I-labelled 2'-O-succinyl cyclic GMP tyrosine methyl ester for the radioimmunoassay was as described [21]. Antibody to cyclic GMP was a gift from Dr. Ferid Murad, University of Virginia. Sprague-Dawley rats were from Zivic Miller Co., Pittsburgh, PA. GTP, Tris-base, Tris-maleate, glycine, imidazole, cacodylic acid, triethanolamine, EDTA, dithiothreitol, hydroquinone, epinephrine, norepinephrine and dopamine were from Sigma Chemical Co. All other chemicals were obtained from Fisher Scientific, Cleveland, OH or as described [16].

Results

The classical assay for soluble or particulate guanylate cyclase is generally in a Tris-HCl or triethanolamine buffer around pH 7.6 with 2—3 mM Mn^{2+} in excess of saturating Mn^{2+}-GTP [1—13,16,18]. Most studies with crude guanylate cyclase preparations show a broad pH optimum around 7.4—8.6 with somewhat lower activity in the alkaline range [9,13]. The degree of enzyme inhibition seen at alkaline pH varies greatly depending upon the purity of the enzyme preparation. For example, the activity of a highly purified soluble enzyme from liver has been shown to be markedly inhibited at pH values above 8.0 [22], whereas guanylate cyclase activity in the crude soluble fraction of liver homogenates was relatively constant between the pH values 7.4—8.6 [9].

Homogeneous guanylate cyclase with 1 mM Mn^{2+}-GTP as substrate and a 3 mM excess of Mn^{2+} had a narrow pH optimum around 7.6 and was markedly inhibited in the strongly basic amine buffers above pH 8.0 (Fig. 1A). Similar results have been reported by others [22]. Manganese-supported activity was not, however, inhibited to as great an extent at pH values above 8.0 in a gly-



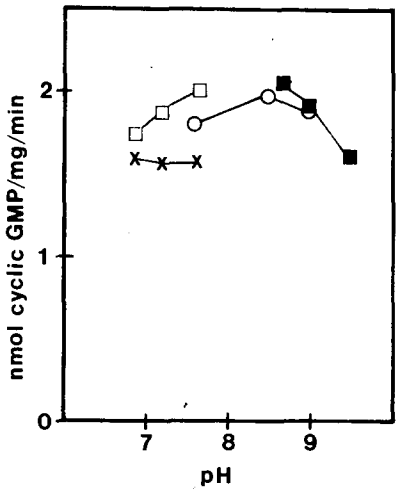


Fig. 1. The effect of pH and buffer composition on purified guanylate cyclase activity. Guanylate cyclase activity was determined as described in Materials and Methods with 0.087 μg purified enzyme and 1 mM GTP in 50 mM buffer at the pH indicated. In panel A the cation was 4 mM $MnCl_2$ and in panel B, 4 mM $MgCl_2$. Buffers were: cacodylate (X———X), imidazole (□———□), Tris-maleate (△———△), Tris-HCl (○———○), triethanolamine (●———●), and glycine (■———■). Assays also contained 0.1 mM EDTA, 1 mM dithiothreitol and 70 mM sucrose derived from the purified enzyme preparation.

Fig. 2. The effect of pH and buffer composition on purified guanylate cyclase activity in the absence of free manganese. Conditions were identical to Fig. 1A except that the concentration of $MnCl_2$ was 1 mM (equal molar with GTP). Buffers were: Cacodylate (X———X), imidazole (□———□), Tris-HCl (○———○) and glycine (■———■).

cine-NaOH buffer. At pH values of 6.0—7.6, Mn^{2+}-supported guanylate cyclase activity was considerably less in imidazole or cacodylate buffers when compared to several Tris buffers (Fig. 1A). The activity with Mg^{2+} as sole action cofactor was relatively unaffected by the buffer composition and was not strongly inhibited at pH values above 8.0 in Tris-HCl, or triethanolamine buffers (Fig. 1B).

The marked inhibition of enzyme activity in the strongly basic amine buffers above pH 8.0 with Mn^{2+} as cofactor, as well as the comparatively greater Mn^{2+} activity seen with these buffers in the lower pH range (6.0—7.6), was due to excess Mn^{2+} cation. When guanylate cyclase was assayed with Mn^{2+}-GTP as substrate in the absence of excess Mn^{2+} (Fig. 2), very little inhibition of activity was observed at pH values above 8.0 in Tris-HCl buffer. Furthermore, the activ-

ity with imidazole and cacodylate buffers was similar to that seen in Tris-HCl. These results suggested that excess Mn^{2+} may have an inhibitory or stimulatory effect on enzyme activity that is dependent upon the buffer composition and pH of the incubation. Similar findings were obtained with enzyme purified through chromatography on Sepharose 6B (data not shown).

Manganese readily changes oxidation states in solution [15] and its effects on catecholamine oxidation are known. An attempt was made, therefore, to study the effects of buffer pH and composition on the redox properties of Mn^{2+} by its effect on catecholamine oxidation. It is well known that epinephrine undergoes spontaneous oxidation in aqueous solutions to adrenochrome that is more rapid at alkaline pH (Fig. 3A and B). The rate of epinephrine oxidation was more rapid in Tris-HCl buffer than in cacodylate or glycine buffers of equivalent pH. The addition of $MnCl_2$ to the reaction markedly accelerated the rate of adrenochrome formation in a dose-dependent manner (Fig. 3A) and this effect was further enhanced at alkaline pH (Fig. 3B). The effect of Mn^{2+} was greatest in Tris-HCl buffer when compared to cacodylate (pH 7.6) or glycine (pH 9.0) buffers. A 10-fold greater concentration of $MgCl_2$ had no effect on adrenochrome formation at pH 7.6 and caused only a modest (100%)

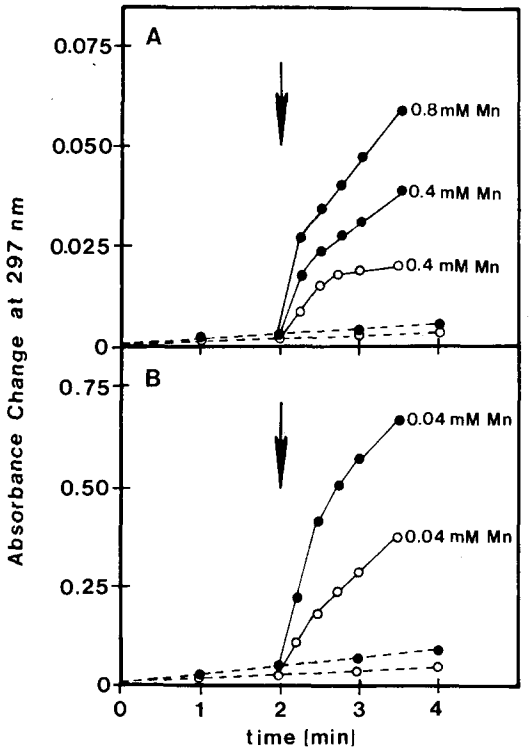


Fig. 3. The effects of manganese on the rate of epinephrine oxidation as a function of buffer pH and composition. The rate of oxidation of 0.1 mM epinephrine to adrenochrome in 50 mM buffer was monitored at 297 nm as described in Materials and Methods. Control rates indicated by the broken lines were at pH 7.6 (panel A) or pH 9.0 (panel B). Buffer composition was Tris-HCl (●———●), cacodylate (○———○) panel A) or glycine (○———○, panel B). At the arrows the concentration of $MnCl_2$ indicated was added to the reaction (solid lines).

increase in rate at pH 9.0 that was independent of buffer composition (not shown). The addition of an equal molar combination of Mn^{2+}-GTP to a reaction was not effective in stimulating adrenochrome formation, however, a stimulatory effect was observed with Mn^{2+} in excess of Mn^{2+}-GTP (not shown). Thus, the enhanced rate of catecholamine oxidation caused by Mn^{2+} was dependent upon excess cation and varied with buffer pH and composition. Since the magnitude of the Mn^{2+} effect on adrenochrome formation and its variability with buffer composition and pH were qualitatively similar to the effects of excess Mn^{2+} on guanylate cyclase activity seen in Figs. 1 and 2, it followed that perhaps redox changes were responsible for the differences in Mn^{2+}-supported enzyme activity.

The remainder of experiments described in this study were carried out with enzyme purified through chromatography on Sepharose 6B due to the extreme liability of homogeneous preparations in the absence of EDTA and high concentrations of dithiothreitol [16,22].

The effects of varying concentrations of excess Mn^{2+} on guanylate cyclase activity at pH 7.6 and 9.0 in different buffers are shown in Fig. 4. At pH 7.6 in Tris-HCl, concentrations of excess Mn^{2+} above 3.2 mM were slightly inhibitory and the apparent K_a of 0.25 mM agrees with that reported by others [11,23]. In cacodylate buffer at pH 7.6, guanylate cyclase activity was less than in Tris-HCl buffer and the apparent K_a for excess Mn^{2+} was slightly increased. Also, excess Mn^{2+} was not inhibitory at the concentrations tested in cacodylate buf-

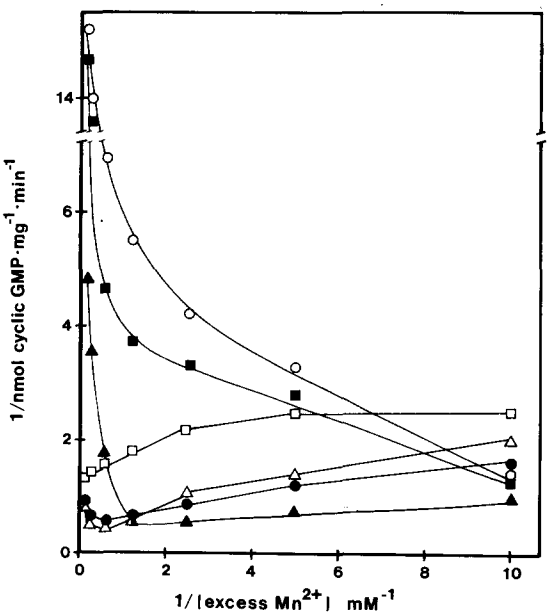


Fig. 4. The effects of excess manganese on guanylate cyclase activity in different buffers at pH 7.6 and 9.0. Guanylate cyclase activity was determined as described in Materials and Methods with 2.1 μg of a Sepharose 6B enzyme preparation in 50 mM buffer containing 1 mM GTP and 1 mM $MnCl_2$. Buffer pH and composition were: Tris-HCl, pH 7.6 (●——●); Tris-HCl, pH 9.0 (○——○); cacodylate, pH 7.6 (□——□); glycine, pH 9.0 (△——△). Some assays with Tris-HCl, pH 9.0 also contained 1 mM (■——■) or 10 mM (▲——▲) dithiothreitol.

fer. At pH 9.0 in Tris-HCl buffer, the apparent K_a for excess Mn^{2+} was less than 0.08 mM (data not shown) and concentrations of excess Mn^{2+} above 0.1 mM were strongly inhibitory. In glycine buffer at pH 9.0, however, excess Mn^{2+} was considerably less inhibitory and the apparent K_a was increased. It has been shown that excessive oxidation of guanylate cyclase by nitric oxide or other oxidants leads to enzyme inhibition that can be blocked by dithiothreitol [8]. Dithiothreitol prevented some of the inhibition by excess Mn^{2+} at pH 9.0 in Tris-HCl buffer in a dose-dependent manner (Fig. 4). The slight inhibition by Mn^{2+} at pH 7.6, was completely blocked by 1 mM dithiothreitol (data not shown). Enzyme activity in Tris at pH 9.0 in the absence of excess Mn^{2+} was increased from 189 pmol cyclic GMP/mg per min to 290 pmol cyclic GMP/mg per min, and to 400 pmol cyclic GMP/mg per min by 1 and 10 mM dithiothreitol, respectively. This has also been observed at pH 7.6 [26]. Dithiothreitol increased the apparent K_a for excess Mn^{2+} at pH 9.0 in Tris-HCl buffer, however, this effect is difficult to interpret due to the concomitant activation of enzyme activity by the sulfhydryl reagent.

The variable effects of excess Mn^{2+} on guanylate cyclase with different buffers and pH, its qualitative similarity to the effects of Mn^{2+} on catecholamine oxidation and the effects of dithiothreitol on Mn^{2+} inhibition, suggested that inhibition and perhaps activation of guanylate cyclase by Mn^{2+} were due to oxidative events. This hypothesis is further strengthened by the fact that both the stimulatory and inhibitory effects of free Mn^{2+} were completely blocked by the antioxidant hydroquinone (Fig. 5).

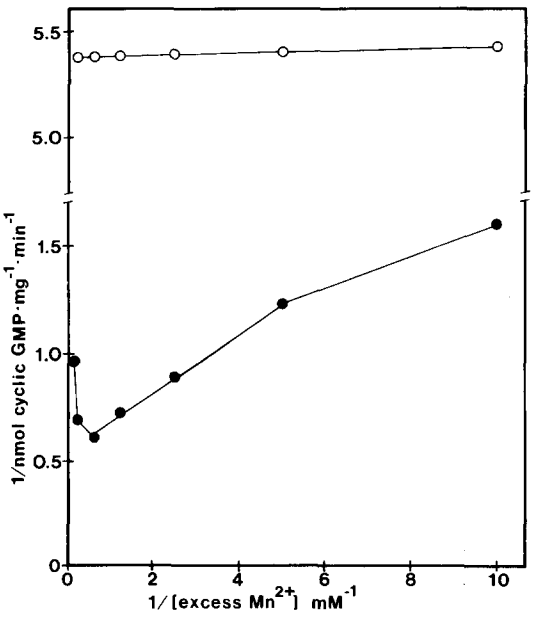


Fig. 5. The effects of hydroquinone on the requirement of guanylate cyclase for excess manganese. Guanylate cyclase activity was determined as described in Materials and Methods with 2.1 μg of a Sepharose 6B enzyme preparation in 50 mM Tris-HCl buffer, pH 7.6 containing 1 mM $MnCl_2$, 1 mM GTP and the concentration of free Mn^{2+} indicated in the absence (●———●) or presence (○———○) of 1 mM hydroquinone.

TABLE I

THE EFFECTS OF HYDROQUINONE AND CATECHOLAMINES ON THE REQUIREMENT OF GUANYLATE CYCLASE FOR EXCESS METAL CATION

Guanylate cyclase activity was determined as described in Materials and Methods with 1.05 μg Sepharose 6B enzyme protein in 50 mM Tris-HCl buffer (pH 7.6) with either Mn^{2+} or Mg^{2+} as cation cofactor. The assay also contained 1 mM Mn^{2+}-GTP or Mg^{2+}-GTP and the concentration of excess metal and/or the addition indicated.

Addition (1 mM)	pmol cyclic GMP/mg per min			
	Mn^{2+}-GTP		Mg^{2+}-GTP	
	3 mM excess Mn^{2+}	No excess Mn^{2+}	3 mM excess Mg^{2+}	No excess Mg^{2+}
None	1415	133	343	129
Hydroquinone	129	126	259	126
Dopamine	157	126	259	126
Norepinephrine	223	126	265	127
Epinephrine	298	126	265	127

The ability of antioxidants to block the stimulation of guanylate cyclase by Mn^{2+} was specific for excess Mn^{2+} and was dependent upon their ability to become oxidized. Table I shows the effectiveness of hydroquinone, dopamine, norepinephrine, and epinephrine in blocking the stimulation of guanylate cyclase by excess Mn^{2+}. All these agents blocked, to varying degrees, the activity in the presence of excess Mn^{2+} but had little effect on Mg^{2+}-supported activity or that activity determined in the absence of excess metal. Hydroquinone is by far the most potent antioxidant of the group. The magnitude of effectiveness of the catecholamines (dopamine > norepinephrine > epinephrine) corresponded to their ability to undergo oxidation in aqueous solution [20]. It is unlikely that inhibition of the Mn^{2+} effect by antioxidants was due to chelation of excess cation since the concentration of excess Mn^{2+} in these experiments was 3-fold greater than that of the antioxidant and these agents had little effect on the activity with Mg^{2+} as cofactor. The slight inhibition by these agents of Mg^{2+}-supported activity or the activity in the absence of free metal, supports the contention that enzyme purification may result in partial enzyme activation [16].

Discussion

Guanylate cyclase is an enzyme that, under the appropriate conditions, is activated by changes in its redox state. Studies with apparently homogeneous soluble guanylate cyclase have indicated that its activity can be altered by many of the agents known to be effective in crude systems, suggesting a direct interaction with, or modification of the enzyme as a possible mechanism for activation by nitric oxide, hydroxyl radical and other agents [8,16,27]. The studies described here suggest that managanese ion may also alter guanylate cyclase activity by an oxidative process.

Under certain conditions Mn^{2+} readily oxidizes to Mn^{4+} or Mn^{7+} which are powerful oxidants. The stimulation of epinephrine oxidation by Mn^{2+} is probably by such a mechanism since it is greater at alkaline pH, a condition which strongly favors Mn^{4+} or Mn^{7+} formation [15]. The magnitude of stimulation of epinephrine oxidation by Mn^{2+} varies with buffer pH and composition in a manner that is qualitatively similar to the effects of Mn^{2+} on guanylate cyclase. Manganese is apparently a better oxidant in Tris buffer than in glycine or cacodylate at equivalent pH as evidenced by its effects on catecholamine oxidation. The effects of Mn^{2+} on guanylate cyclase were similarly more pronounced in Tris buffer than in either glycine or cacodylate at equivalent pH. These findings suggest that the variable effects of Mn^{2+} on guanylate cyclase activity seen with different buffers may be attributed in part to its oxidant properties.

The modulation of guanylate cyclase by manganese cation is apparently a biphasic process. It has been shown that while low concentrations of oxidants stimulate guanylate cyclase, higher concentrations can inhibit enzyme activity [8,10,24]. In this sense too, manganese behaves like an oxidant since low concentrations stimulate the cyclase whereas high concentrations are inhibitory.

As guanylate cyclase is purified from the soluble fraction of homogenates, the bell-shaped dose response curve for oxidative activation is shifted markedly to the left [8]. The increased sensitivity to agents such as nitric oxide with enzyme purification probably results from the removal of heme, small molecules and other proteins that can serve as sinks or scavengers of the activating species and prevent excessive oxidation of the enzyme [7,8]. With purification, guanylate cyclase also becomes increasingly sensitive to inhibition by Mn^{2+} at pH values above 8.0 in Tris buffers. The activity of the crude soluble liver enzyme is not significantly different at pH 7.6 or 8.6 in Tris-HCl buffer [9], however, partially purified or homogeneous enzyme is markedly inhibited in Tris buffers above pH 8.0 (Fig. 1 and Ref. 22). The increased sensitivity to pH and buffer composition with enzyme purification may likewise be related to manganese cation induced oxidation and the removal of oxidative sinks or scavengers from the preparation during purification [7,8].

The inhibition of guanylate cyclase by excess manganese cation was partially prevented by dithiothreitol. High concentrations of thiols have been shown to block activation or inhibition of guanylate cyclase by nitric oxide [8], *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [2] and fatty acid hydroperoxides [3]. The failure of dithiothreitol to completely prevent the inhibition by Mn^{2+} in Tris buffer at pH 9.0 cannot be explained at this time. It may, however, be related to the ability of dithiothreitol itself to become an oxidant under certain conditions due to formation of the free radical thiol intermediate [28]. The interaction of thiols with guanylate cyclase is complex and further studies are needed in this regard.

The stimulation of guanylate cyclase by excess Mn^{2+} was blocked by antioxidants such as hydroquinone. Hydroquinone and epinephrine have been shown to block guanylate cyclase activation by hydroxyl radical [4]. These findings are in agreement with the idea that guanylate cyclase activation by excess manganese cation as well as inhibition, may be by an oxidative mechanism. An analysis of the specific sites on the cyclase that are modified by various redox events must be carried out with highly purified enzyme. Such

studies have thus far been hampered by the lability of homogeneous preparations in the absence of high concentrations of sulfhydryl reagents, sucrose and glycerol [16,22,23].

It is unlikely that the effects of excess Mn^{2+} reported here were due to heavy metal contaminants of the $MnCl_2$ since lot analysis of heavy metals (Fisher Scientific) showed less than 1 ppm contamination. Furthermore, heavy metal contamination of the $MgCl_2$ used in this study was identical to that found in the $MnCl_2$, however, Mg^{2+} showed none of the oxidative properties that Mn^{2+} displayed.

The studies described in this report suggest that manganese cation may modulate soluble guanylate cyclase activity by a process involving oxidation. Two important considerations that arise are: first, that guanylate cyclase may not possess a specific preference for Mn^{2+} as has been thought and second, that activity in the presence of excess Mn^{2+} may not represent true basal enzyme activity. This later consideration may partially explain the observation that while basal cyclic GMP levels in tissues are many times lower than basal cyclic AMP levels, basal activity of guanylate cyclase measured in cell homogenates with Mn^{2+} as cofactor is many times greater than basal adenylate cyclase activity [25]. Another important question that arises is whether manganese-supported activity represents a partially activated enzyme or is a distinct species. Studies directed at these questions are currently underway in this laboratory.

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