Sodium azide activation of guanylate cyclase requires a protein factor that has been partially purified from rat liver. While the activator factor and guanylate cyclase in rat liver supernatant fractions were not separable with gel filtration, they were separable with DEAE-cellulose chromatography. The activator factor was heat-labile and inactivated with bacterial protease and α-chymotrypsin. Its activity was not altered by a variety of other hydrolytic enzymes including trypsin. With gel filtration the protein factor was estimated to be about 200,000 daltons. Polyanalyzamide gel electrophoresis resulted in a single peak of activator that in the presence of NaN₃ increased guanylate cyclase activity assayed with either Mn²⁺ or Mg²⁺. The activator in the absence of NaN₃ also increased guanylate cyclase activity partially purified from liver or cerebral cortex. However, the activation was greater in the presence of NaN₃ and the degree of activation was a function of the concentration of both NaN₃ and activator.

Guanyl-5′-yl imidodiphosphate was 5 to 10% as effective as the substrate GTP for rat liver guanylate cyclase and the relative stimulatory effect of NaN₃, increased guanylate cyclase activity assayed with either Mn²⁺ or Mg²⁺. The activator in the absence of NaN₃ also increased guanylate cyclase activity partially purified from liver or cerebral cortex. However, the activation was greater in the presence of NaN₃ and the degree of activation was a function of the concentration of both NaN₃ and activator.

Studies from a number of laboratories have suggested the guanosine 3′:5′ monophosphate may act as a regulator of some biological processes since various hormones, neurohormones, and other agents can increase cyclic GMP accumulation in tissues (1, 2). Increases in cyclic GMP levels could result from an increased rate of synthesis by guanylate cyclase (EC 4.6.1.2) or decreased hydrolysis by cyclic nucleotide phosphodiesterase (EC 3.1.4.17). In most instances the mechanism by which agents increase cyclic GMP accumulation is unknown. Although several preliminary reports have described activation of guanylate cyclase in cell-free systems by some hormones and neurohormones (3–7), these observations have not been confirmed (8, 9). Possible mechanisms to regulate guanylate cyclase activity have been reviewed recently (10, 11).

We have described the activation of guanylate cyclase from several tissues with sodium azide, hydroxylamine, phenyldrazine, sodium nitrite, nitroglycerin, and sodium nitroprusside (12, 13). Some of these agents also increase cyclic GMP levels in incubations of slices from liver, cerebral cortex, cerebellum (14), tracheal smooth muscle (15), taenia coli, atria (16), and kidney (17). Activation of guanylate cyclase with sodium azide is dependent upon sodium azide concentration, incubation time, and temperature (12, 18). Since the mechanism of activation of guanylate cyclase with sodium azide could provide important clues regarding possible hormonal regulation of the enzyme, the requirements for the activation were studied in greater detail. Purification of guanylate cyclase resulted in loss of the sodium azide response (12, 18, 19). The activation of guanylate cyclase from rat liver and other tissues required the presence of a macromolecular factor (19). Readidion of this protein-activating factor to partially purified enzyme restored the sodium azide response. The purification and properties of this activating factor from rat liver supernatant fractions are described in this report. Some of these observations have been presented in abstract form (20).

MATERIALS AND METHODS
Male Sprague-Dawley rats weighing 100 to 250 g were killed by cervical dislocation. Liver and cerebral cortex were quickly removed and placed in cold 0.25 m sucrose containing 10 mM Tris/HCl buffer (pH 8.0), 1 mM EDTA, and 1 mM dithiothreitol. Tissues were homogenized in 8 to 9 volumes of the same mixture using a glass homogenizer with a Teflon pestle at 4°C. Homogenates were centrifuged at 105,000 × g for 60 min and supernatant fractions were used for the purification of the activating factor and guanylate cyclase.

Liver supernatant fractions were treated with trypsin (1 mg/ml) at 37°C for 60 min and then transferred to an ice bath. With some preparations the trypsin treatment was omitted. Ammonium sulfate was added with constant stirring to give 20% saturation. The resulting precipitate was collected with centrifugation at 10,000 × g for 20 min. Additional ammonium sulfate was added to the supernatant.
fraction to yield 55% saturation. The resulting precipitate was suspended in 10 mM Tris/HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM dithiothreitol and passed through a Sephadex G-25 column (2.5 x 30 cm). The desalted sample was applied to a DEAE-cellulose column (1.5 x 25 cm) equilibrated with 10 mM Tris/HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM dithiothreitol. Columns were washed with the same buffer and eluted with a 0 to 0.3 M NaCl linear gradient containing 10 mM Tris/HCl (pH 7.6), 1 mM EDTA, and 1 mM dithiothreitol. Fractions were tested for activator activity using either partially purified rat cerebral cortex or liver supernatant guanylate cyclase in the presence and absence of 1 mM NaN₃. Fractions containing activator activity were pooled and dialyzed against 10 mM Tris/HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM dithiothreitol and used fresh or stored at -70°.

Samples (40 to 50 μg of protein) of these and other preparations were applied to 5% polyacrylamide gels (0.6 x 8 cm) and electrophoresed for 5 h at 4-6° using 50 mM Tris/glycine buffer (pH 8.0) containing 1 mM EDTA and 1 mM dithiothreitol. Some gels were stained with Coomassie blue R-250 and others were sliced and eluted with 50 mM Tris/HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM dithiothreitol for assay of activator activity.

Guanylate cyclase was partially purified from 105,000 x g supernatant fractions of rat cerebral cortex or liver homogenates. With both tissues, guanylate cyclase was prepared through the DEAE-cellulose chromatography step as described previously (19). Preparations of guanylate cyclase through DEAE-cellulose chromatography were similar to that of the activator described above except that trypsin treatment was omitted and cyclase eluted in later fractions. Sodium azide had no effect with the DEAE-cellulose preparations of guanylate cyclase from cerebral cortex unless the activator was added. Fresh DEAE-cellulose fractions of soluble liver guanylate cyclase had a relatively small (less than 2- to 5-fold) effect with sodium azide that was absent when preparations were stored at -70° for several days. When EDTA and dithiothreitol were omitted from buffers, fresh preparations of liver guanylate cyclase from DEAE-cellulose columns were usually not responsive to NaN₃ (19).

Guanylate cyclase activity was determined as described previously (9, 12, 19). Reaction mixtures contained 50 mM Tris/HCl buffer (pH 7.6), 10 mM theophylline, 15 mM creatine phosphate, 20 μg/ml of creatinine phosphokinase (120 to 135 units/mg), 1 mM GTP, and 4 mM MnCl₂ or 4 mM MgCl₂. Manganese ion was used in most experiments only where designated. Assays for guanylate cyclase activity were acetylated by the procedure described by Harper and Brooker (23) prior to radioimmunoassay. The latter method increased the sensitivity of the assay about 30-fold and permitted the detection of 2 to 5 fmol of cyclic GMP. With the conditions of the guanylate cyclase assay cyclic GMP formed was linear with protein concentration and incubation time and less than 5 to 10% of added cyclic [3H]GMP was degraded by cyclic nucleotide phosphodiesterase. Values reported are means of duplicate or triplicate cyclase incubations of representative experiments.

One unit of the activating factor is defined as the amount of material capable of giving 50% stimulation of guanylate cyclase in the presence of 1 mM NaN₃ under the assay conditions described. With low concentrations of the activator factor there was a linear relationship between the amount of activator factor and the extent of stimulation (Fig. 1). Also, quantification of the activator was independent of the amount of guanylate cyclase used. Protein was estimated by the procedure of Lowry et al. (24).

The molecular size of the activating factor was determined by the procedure described by Reiland (25). A Sepharose 6B column (2.6 x 65 cm) was equilibrated with 10 mM Tris/HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM dithiothreitol. The column was run with reverse flow and the void volume was determined using blue dextran 2000. The column was calibrated using hemoglobin, human γ-globulin, xanthine oxidase, urease, and rabbit thyroglobulin. Xanthine oxidase and urease were assayed by procedures previously described (26). Catalase was assayed by measuring the decrease in absorbance at 240 nm of H₂O₂ (27).

Trypsin, α-chymotrypsin, 5'-nucleotidase, bacterial protease, alkaline phosphatase, ribonuclease, neuraminidase, phospholipase A₁, phospholipase C, beef liver catalase, horseradish peroxidase, xanthine oxidase, urease, cytochrome b₅, human γ-globulin, and Gpp(NH)p were purchased from Sigma Chemical Co. Coomassie blue R-250 was procured from Eastman Kodak. Other reagents were obtained as described previously (9, 12, 18, 19).

RESULTS

We have previously reported that sodium azide activates guanylate cyclase from soluble and particulate fractions of rat liver and kidney and particulate fractions from cerebral cortex and cerebellum (12, 18). Preparations of many other tissues were not affected by sodium azide. While basal guanylate cyclase activity in supernatant and particulate fractions from a variety of tissues is quite stable to storage at -20° or -70°, the effect of sodium azide decreased with storage (not shown). The loss of the sodium azide effect with crude supernatant guanylate from rat liver could be prevented with storage of preparations at -70° with 1 mM dithiothreitol and 1 mM EDTA (not shown). Partial purification of liver supernatant guanylate cyclase also resulted in the loss of the NaN₃ response that could be restored with the addition of the activating factor (18, 19).

The activator factor was resistant to trypsin (see below) whereas guanylate cyclase activity could be completely abolished by treatment with 1 mg/ml of trypsin. This property of the activating factor, i.e. resistance to trypsin, was utilized for its purification. The method of purification of the activating factor is summarized in Table I. The recovery of 158% cannot be readily explained. However, some tissue extracts contain materials that inhibit the sodium azide effect (12) and these are probably removed during purification to account for the high recovery of activity. The elution pattern of the activator on DEAE-cellulose columns is illustrated in Fig. 2. The activator elutes with 50 to 100 mM NaCl and has no guanylate cyclase activity. As reported previously, soluble rat liver guanylate cyclase when applied to similar columns elutes with 200 to 500 mM NaCl (19).

The abbreviation used is: Gpp(NH)p, guanyl-5'-yl imidophosphate.
The activator was purified as described under "Materials and Methods." The activator was prepared through the DEAE-cellulose chromatography as described under "Materials and Methods." The preparation of the activator designated A was prepared with prior trypsin treatment. Preparation B was similar except that trypsin treatment was omitted. Activator was assayed as described in Fig. 2 using either Mn²⁺ or Mg²⁺ as sole cation in cyclase incubations.

The molecular size of the activating factor was estimated to be about 200,000 with gel filtration on a calibrated Sepharose 6B column (Fig. 3). The preparation of the activator designated A was prepared with prior trypsin treatment. Preparation B was similar except that trypsin treatment was omitted. Activator was assayed as described in Fig. 2 using either Mn²⁺ or Mg²⁺ as sole cation in cyclase incubations.

Electrophoresis of the activating factor from the DEAE-cellulose step on polyacrylamide gels yielded a single peak of activity upon elution of gel slices (Fig. 4). The peak of activity was similar when assayed with enzyme, 1 mM NaN₃, and either Mn²⁺ or Mg²⁺ as sole cation. We previously reported that guanylate cyclase from several tissues activated with NaN₃ can effectively use Mn²⁺ or Mg²⁺ as the sole cation (18).

With partially purified guanylate cyclase and the activator a time lag was not observed with activation by the protein factor alone or the activator plus NaN₃ (Fig. 5). We have previously observed that a time lag of several minutes occurred with NaN₃ activation of crude preparations of guanylate cyclase (12). As summarized in Fig. 1 the activating factor increased guanylate cyclase activity in a concentration...
Guanylate Cyclase Activation with Sodium Azide

**Fig. 4.** Electrophoresis of the activator on polyacrylamide gels. The 50 μg of activator prepared through DEAE-cellulose chromatography was applied to a 5% polyacrylamide gel and run for 5 h at 3 mA/tube using 50 mM Tris/glycine buffer (pH 8.0) containing 1 mM dithiothreitol and 1 mM EDTA. The 3-mm segments of the gel were eluted with 50 mM Tris/HCl (pH 7.6) buffer. Eluates were assayed for activator using partially purified rat cerebral cortex guanylate cyclase (11.4 μg of protein) with and without 1 mM NaN₃ and either Mn²⁺ (B) or Mg²⁺ (C) as sole cation. Samples were also assayed for catalase activity (A).

**Fig. 5.** Time course of guanylate cyclase activation with activator and NaN₃. Partially purified rat cerebral cortex guanylate cyclase (4.2 μg of protein) was incubated without and with 2.2 μg of the activator preparation for the times indicated without a preincubation period. Some incubations contained 1 mM NaN₃.

**Fig. 6.** Effect of the activating factor on partially purified guanylate cyclase from rat cerebral cortex and liver. Soluble guanylate cyclases from rat cerebral cortex (A) and liver (B) were prepared with DEAE-cellulose chromatography as described. Cerebral cortex (4.2 μg of protein) and liver (6.5 μg of protein) were incubated with the activator at the concentrations indicated without (○) and with (●) 1 mM NaN₃. Incubations were conducted with either 4 mM Mn²⁺ (---) or Mg²⁺ (—).
activation. This is also in contrast to studies with soluble liver guanylate cyclase in which the apparent $K_m$ for GTP is increased from 35 to 113 $\mu$M with NaN₃ activation (12). The pH optimum for partially purified rat cerebral cortex guanylate cyclase was broad with maximal activity between 7.3 to 8.0 with either Mn²⁺ or Mg²⁺ as sole cation. The effects of NaN₃ and activator were also greatest in this pH range (not shown).

ATP inhibits basal guanylate cyclase activity in crude preparations from various tissues (9, 28-30). ATP inhibited partially purified soluble cerebral cortex enzyme from rat (Fig. 8). The degree of inhibition was similar when activity was increased with the protein factor or the protein factor plus NaN₃. ATP was included in incubations at the concentrations indicated. Cyclic GMP formed in the absence of ATP was 138.7 (Δ), 179.0 (○), and 611.4 (●) pmol/mg of protein/min, respectively.

NaN₃ activation is not due to a phosphorylation or dephosphorylation mechanism. However, additional studies are required.

As summarized in Table III a variety of agents and conditions altered NaN₃ activation of guanylate cyclase in crude rat liver supernatant fractions. Several compounds with sulphydryl groups increased the effect of NaN₃ as did FeCl₃. The effect of NaN₃ was decreased with H₂O₂ and KCN without effects on basal activity. CuCl₂ decreased basal activity without altering the relative stimulatory effect of NaN₃ (4.3-fold). The effect of azide was markedly decreased when incubations were carried out in a nitrogen atmosphere (Table III). The effects of cysteine and CN⁻ were reported previously (12).

![Fig. 7. Effect of NaN₃ and activator concentration on guanylate cyclase activation. Partially purified rat cerebral cortex guanylate cyclase (11.4 µg of protein) was assayed with 2.1 (●), 0.21 (○), 0.042 (Δ), and 0.021 (□) µg of activator protein in the presence of various concentrations of NaN₃.]

![Fig. 8. Inhibition by ATP. Partially purified cerebral cortex guanylate cyclase (4.2 µg of protein) was incubated alone (△), with 2.1 µg of activator (○), and with both activator and 1 mM NaN₃ (●). ATP was included in incubations at the concentrations indicated. Cyclic GMP formed in the absence of ATP was 138.7 (△), 179.0 (○), and 611.4 (●) pmol/mg of protein/min, respectively.

### Table III

**Effects of NaN₃ and other agents on soluble liver guanylate cyclase**

| Condition and concentration | Cyclic GMP formed pmol/mg protein/min | Ratio NaN₃/− NaN₃ |
|-----------------------------|---------------------------------------|-----------------|
| Experiment 1                |                                       |                 |
| Control                     | 66.2                                  | 260.9           | 3.9  |
| Cysteine, 1 mM              | 63.9                                  | 612.4           | 9.6  |
| Glutathione, 1 mM           | 60.3                                  | 408.5           | 6.8  |
| Dithiothreitol, 1 mM        | 62.6                                  | 440.9           | 7.0  |
| H₂O₂, 1 mM                  | 64.4                                  | 199.9           | 3.1  |
| H₂O₂, 10 mM                 | 64.4                                  | 86.6            | 1.3  |
| KCN, 1 mM                   | 63.6                                  | 83.2            | 1.3  |
| CuCl₂, 0.1 mM               | 5.8                                   | 25.2            | 4.3  |
| FeCl₃, 0.1 mM               | 81.5                                  | 313.7           | 3.9  |
| FeCl₃, 1 mM                 | 66.1                                  | 460.3           | 7.2  |
| Experiment 2                |                                       |                 |
| Room air                    | 70.3                                  | 1385.5          | 19.7 |
| N₂ atmosphere               | 71.6                                  | 152.8           | 2.1  |
| 95% O₂ + 5% CO₂             | 57.2                                  | 1437.7          | 25.1 |

### Table IV

**Effect of activator, catalase, peroxidase, and cytochrome b₅ on guanylate cyclase activation by NaN₃**

Partially purified rat cerebral cortex guanylate cyclase (11.4 µg of protein, Experiment A) and partially purified rat liver enzyme (6.5 µg of protein, Experiments B and C) were assayed with activator, catalase, horseradish peroxidase, and cytochrome b₅ in the absence and presence of 1 mM NaN₃ as described under "Materials and Methods."

| Addition                  | Cyclic GMP formed mg protein/min | NaN₃/− NaN₃ |
|---------------------------|---------------------------------|------------|
| Experiment A              |                                  |            |
| None                      | 60.3                            | 54.0       |
| Activator (5.6 µg)        | 79.5                            | 161.7      |
| Catalase (20 µg)          | 84.0                            | 252.0      |
| Peroxidase (20 µg)        | 60.5                            | 133.2      |
| Experiment B              |                                  |            |
| None                      | 474.7                           | 540.1      |
| Activator (5.6 µg)        | 474.0                           | 1584.0     |
| Catalase (10 µg)          | 408.2                           | 1873.0     |
| Experiment C              |                                  |            |
| None                      | 436.5                           | 440.0      |
| Peroxidase (20 µg)        | 446.0                           | 960.0*     |
| cytochrome b₅ (20 µg)     | 816.8                           | 1235.0     |

* 10 mM NaN₃.
These studies suggested that an oxidative-reductive process was required for the NaN₃ effect. The addition of either beef liver catalase, horseradish peroxidase, or cytochrome b₅ to partially purified guanylate cyclase could replace the requirement for the activator factor for NaN₃ activation (Table IV). Xanthine oxidase or superoxide dismutase did not alter guanylate cyclase activity in the absence or presence of 1 mM NaN₃. Gel electrophoresis of beef liver catalase preparations revealed several protein bands. Two peaks of catalase activity were obtained and fractions from both of these regions could activate guanylate cyclase in the presence of NaN₃ (Fig. 9). DEAE-cellulose fractions of the activator also contained catalase activity. With gel electrophoresis of these preparations catalase and activator activities were obtained in similar regions from gels (Fig. 4).

**Discussion**

A variety of agents such as choline esters, histamine, serotonin, catecholamines, prostaglandin E₂, and others are capable of increasing cyclic GMP levels in intact tissues (1, 2). Since most of these agents do not increase cyclic GMP accumulation in cell-free systems, the mechanism(s) for these effects are not known. Possible mechanisms to regulate guanylate cyclase activity and cyclic GMP accumulation have recently been reviewed (10, 11). Sodium azide, hydroxylamine, sodium nitrite, nitroglycerin, and sodium nitroprusside can increase cyclic GMP accumulation in intact tissue (14–17) and guanylate cyclase in cell-free preparations (12, 13, 18, 19). Recently, nitrosamines have also been reported to produce effects similar to these agents in liver preparations (31). The mechanism(s) in which these agents increase guanylate cyclase activity could provide some clues about hormonal regulation of the enzyme. However, these agents, unlike most hormonal effects on cyclic GMP accumulation in intact tissue do not require Ca²⁺ in the external medium (14, 15, 17, 32). Thus, the ultimate mechanisms may prove to be quite different.

NaN₃ can increase guanylate cyclase activity in soluble and particulate preparations from rat liver and kidney and particulate preparations of cerebral cortex and cerebellum (12, 18). The absence of an effect in heart, lung, and other tissue preparations may be due to the absence of the activator factor, the presence of a protein inhibitor, or both (11, 12). Addition of the macromolecular activator factor to azide-nonresponsive guanylate cyclase preparations permits NaN₃ activation to occur (18, 19). The activator factor from rat liver was partially purified and characterized. It has a molecular weight of about 200,000, is heat-labile, and is inactivated with bacterial protease and α-chymotrypsin. However, it was resistant to trypsin treatment and other hydrolases under the conditions used. In our earlier report the azide effect was decreased by trypsin treatment of lower concentrations of crude rat liver preparations (12). The activator protein alone increased liver and cerebral cortex guanylate cyclase activity when either Mn²⁺ or Mg²⁺ was used as sole cation. In the presence of NaN₃, greater stimulatory effects were observed. The effects of both the activator and NaN₃ were concentration-dependent and were not altered by the concentration of guanylate cyclase in incubations. The concentration of NaN₃ required for half-maximal activation of partially purified rat cerebral cortex granulate cyclase was 0.2 to 1.5 μM depending upon the concentration of activator. This value is similar to that observed with activator and partially purified liver guanylate cyclase of 1 μM (18) and much less than the Kₘ of 40 μM in crude rat liver supernatant fractions (12). Unlike effects in crude liver guanylate cyclase preparations no time lag was observed with NaN₃ activation of partially purified preparations.

ATP inhibited both basal and NaN₃-activated cerebral cortex guanylate cyclase to a similar degree. Although Gpp(NH)p is a poor substrate for liver guanylate cyclase, the relative stimulatory effect of NaN₃ was not altered. NaF did not modify the NaN₃ effect. These studies suggest that the mechanism of NaN₃ activation does not involve a phosphate transfer. The effects of several agents indicated that an oxidative-reductive process was required for NaN₃ activation of guanylate cyclase. With crude liver supernatant fractions sodium azide activation was not observed in a nitrogen atmosphere, enhanced with sulfhydryl agents, enhanced with Fe³⁺ and inhibited by cyanide and H₂O₂ (Table III). Sino azide and cyanide can interact with catalase (33), this enzyme was added to incubations and found to replace the requirement for the activator factor. Both catalase activity and the activator activity migrated to similar areas when catalase or activator preparations were applied to polyacrylamide gel electrophoresis. Cata-
lase is also similar in size to the activator. These studies indicate that the activator factor is probably catalase. However, additional studies with further purification of samples are needed. During the preparation of this manuscript Miki et al. (34) also reported that catalase addition to guanylate cyclase preparations permitted NaN₃ activation. Addition of peroxidase or cytochrome b₅ also results in NaN₃ activation. Xanthine oxidase and superoxide dismutase were not effective. Thus, several materials can satisfy the requirement for an activator factor. The ability of NaN₃ to activate fresh but not stored DEAE-cellulose preparations of liver guanylate cyclase suggests that another labile activator factor exists in liver that co-chromatographs with guanylate cyclase.

NaN₃ activation of guanylate cyclase results in some alterations in the properties of guanylate cyclase (12, 18, 35). With NaN₃ activation the specificity for cation cofactor and nucleotide substrate are altered. The activated enzyme can effectively utilize Mg²⁺ as well as Mn²⁺, is no longer activated by Ca²⁺ (18), and can catalyze the formation of cyclic AMP from ATP (35). The latter may explain the mechanism for ATP inhibition.

The mechanism(s) of azide activation of guanylate cyclase is not known. Catalase can convert azide to very reactive materials such as nitric oxide (33, 36). Perhaps this or another reactive material can interact with guanylate cyclase and lead to its activation. Nitric oxide activates crude and partially purified guanylate cyclase from several tissues (13). This hypothesis receives additional support from the stimulatory effects of other agents on guanylate cyclase such as hydroxylamine, phenylhydrazine, NaN₃, activation the specificity for cation cofactor and nucleotide substrate are altered. The activated enzyme can effectively utilize Mg²⁺ as well as Mn²⁺, is no longer activated by Ca²⁺ (18), and can catalyze the formation of cyclic AMP from ATP (35). The latter may explain the mechanism for ATP inhibition.

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Purification and properties of a protein required for sodium azide activation of guanylate cyclase.
C K Mittal, H Kimura and F Murad

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