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ACTIONS OF BIS(GUANYLHYDRAZONES) ON ISOLATED RAT LIVER MITOCHONDRIA*

JANUSZ Z. BYCZKOWSKI,†‡ WOJCIECH SALAMON,† JAMES P. HARLOS§ and CARL W. PORTER†‖

†Department of Experimental Therapeutics and §Department of Experimental Pathology, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, NY 14263, U.S.A.

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Abstract—The effects of the anticancer bis(guanylhydrazones), methylglyoxal-bis(guanylhydrazone) (MGBG) and 4,4′-diacetyldiphenylurea-bis(guanylhydrazone) (DDUG), on parameters related to the bioenergetic function of isolated rat liver mitochondria were investigated. At concentrations comparable to those attained intracellularly, both bis(guanylhydrazones) significantly inhibited state 4 respiration but had less of an inhibitory effect on state 3 or uncoupled respiration. DDUG was more effective than MGBG, requiring 0.34 mM to achieve a 25 per cent inhibition of respiration as compared to 6.0 mM for MGBG. The inhibition was prevented by potassium cations and was enhanced in mitochondria "de-energized" with valinomycin, a potassium cationophore. This suggested drug competition for potassium-binding sites, possibly membrane phospholipids. Addition of 1.25 mM MGBM or 0.025 mM DDUG to suspended mitochondria caused a rapid aggregation of organelles and an increase in the optical density of the suspension. Pretreatment with either bis(guanylhydrazone) protected mitochondria against non-specific swelling action of 0.0015% Triton X-100, suggesting membrane binding. By electron microscopy, MGBG- or DDUG-treated mitochondria appeared swollen and the spaces between cristae membranes or inner and outer membranes were collapsed, obliterating the outer mitochondrial compartment. The activity of monoamine oxidase A, an outer membrane marker enzyme, was reduced considerably by 4 mM MGBG or 0.075 mM DDUG. Mobility of mitochondria toward the anode in an electrophoretic field was slowed 50 per cent by 2.5 mM MGBG or 0.1 mM DDUG. These data suggest that positively charged bis(guanylhydrazones) neutralize the net negative surface potential of rat liver mitochondria by binding to sites (possibly phospholipids) at the inner mitochondrial membrane. Subsequent interference with cation binding and/or transport results in inhibition of bioenergetic functions.

Several of the bis(guanylhydrazones) are known to have antiproliferative activity [1] and at least one, methylglyoxal-bis(guanylhydrazone) (MGBG),¶ has achieved clinical usefulness originally against leukemias [2] and, more recently, against a variety of solid tumors [3].

Although MGBG is known to be an effective inhibitor of *S*-adenosylmethionine decarboxylase [4, 5], a key enzyme in the biosynthesis of polyamines, recent studies suggest that the antiproliferative activity of the drug may be related to its effects on mitochondria [6, 7]. In L1210 cells, the bis(guanylhydrazones), MGBG or 4,4′-diacetyldiphenylurea-bis(guanylhydrazone) (DDUG), have profound effects on the ultrastructure and function of mitochondria [8–10]. Moreover, temporal studies detailing the onset of various drug effects in MGBG-treated L1210 cells [6] indicate that the mitochondrial effects are unrelated to those involving polyamines and may contribute significantly to the early antiproliferative action of MGBG.

It is of interest, therefore, to define the effects of bis(guanylhydrazones) on mitochondrial function and ultimately to relate this to ultrastructural damage and, more importantly, to inhibition of cell growth. The present study, examining the effects of MGBG and DDUG on bioenergetic-related functions and properties of isolated rat liver mitochondria, represents an initial step in this regard. Although both molecules are positively charged, DDUG differs from MGBG in that it bears two aromatic rings (Fig. 1) and is therefore likely to be more lipophilic than MGBG. Thus, in membranous systems such as mitochondria, the drug should be more effective than MGBG. The present data are consistent with this expectation and with the possibility that mitochondrial function may represent an important target site for bis(guanylhydrazones).

MATERIALS AND METHODS

Rat liver mitochondria were prepared according to the method of Weinbach [11] essentially as

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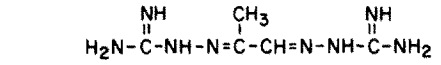
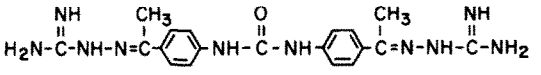
‡ Permanent address: Department of Pharmacology, Medical School in Gdansk, 38 Hibnera str., PL. 80227 Gdansk-6, Poland.

‖ Author to whom all correspondence should be addressed: Dr. Carl W. Porter, Roswell Park Memorial Institute, 666 Elm St., Buffalo, NY 14263, U.S.A.

¶ Abbreviations: MGBG, methylglyoxal-bis(guanylhydrazone); CCCP, carbonyl cyanide-*m*-chlorophenyl hydrazone; DDUG, 4,4′-diacetyldiphenylurea-bis(guanylhydrazone); DNP, 2,4-dinitrophenol; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N,N*-tetra-acetic acid.



MGBG



DDUG

Fig. 1. Structural formulas for the aliphatic bis(guanylhydrazone MGBG and for the aromatic bis(guanylhydrazone) DDUG.

described earlier [12], and the homogeneity of the mitochondrial fraction was confirmed by electron microscopy. Isolated mitochondria were suspended in 0.25 M sucrose (British Drug House Chemical, Ltd., Poole, England) with 10 mM Tris–HCl buffer (pH 7.3) and 1 mM EGTA. Protein concentrations were determined by the method of Lowry *et al.* [13] and samples were adjusted to be equal. The effects of MGBG (Cancer Chemotherapy Center, National Cancer Institute, Bethesda, MD, U.S.A.) or DDUG (CIBA Research Laboratories, Basel, Switzerland) on several parameters relative to mitochondrial bioenergetics were studied.

Mitochondrial respiration was measured polargraphically [14] using a Clark oxygen electrode at 25°. Oxygen consumption was recorded after addition of 50 μl of mitochondrial suspension containing 3.5 mg of protein into the electrode chamber with 1.5 ml of incubation medium. The composition of the incubation medium varied according to the experimental design and is indicated in the figure legends. In all cases, 5 mM succinate (plus 5 mM glutamate) or 5 mM glutamate served as the substrate. State 3 respiration was induced by addition of 1 μmole of ADP (Sigma Chemical Co., St. Louis, MO, U.S.A.). Uncoupled respiration was achieved with 50 μM dinitrophenol (DNP, Sigma Chemical Co.) or 1 μM carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP, Sigma Chemical Co.). Mitochondria were made permeable to potassium cations with valinomycin (Sigma Chemical Co.) at a final concentration of 3.3 μg/ml.

The effects of bis(guanylhydrazones) on ATP synthesis by isolated rat liver mitochondria were determined. Mitochondria (0.4 mg protein) were suspended in 500 μl of medium containing 15 mM KCl, 50 mM Tris, 1 mM PO_4 and 1 mM $MgSO_4$ at pH 7.3. The organelles were incubated together with 720 nmoles ADP and 5 mM glutamate for 0.5 min at 30° before addition of 10 mM MGBG, 1 mM DDUG or 0.5 μM CCCP. After 2 min, the incubation was halted with an equal volume of cold 12% perchloric acid. The mixture was allowed to sit for 5 min at 4° and then was centrifuged at 9000 *g*. The acid soluble supernatant fluid was removed and neutralized with 1 N KOH and stored at −70°. Ribonucleotide pools were determined in 10 μl aliquots of neutralized supernatant fluid using a high pressure liquid chromatography (h.p.l.c.) system described elsewhere [15].

Monoamine oxidase activity in rat liver mitochondria was measured by the method of Weetman and Sweetman [16] using 5-hydroxytryptamine as substrate. The initial velocity of the enzyme activity was measured in the absence and presence of inhibitor with an oxygen electrode at 25° in medium containing 15 mM KCl, 50 mM Tris–HCl (pH 7.2) and rotenone plus antimycin or KCN. The reaction was initiated by the addition of 5 hydroxytryptamine at final concentrations ranging up to 1 mM.

Changes in optical density of mitochondrial suspensions were followed using a recording spectrophotometer at 540 or 800 nm. A decrease in optical density of a mitochondrial suspension at 540 nm was used as a measure of organelle swelling [17, 18] while an increase at 800 nm [19] was taken as an indication of mitochondrial aggregation and confirmed microscopically. Mitochondria were suspended in media containing various concentrations of salts and/or sucrose and buffered to pH 7.2 with Tris–HCl. Spectrophotometric measurements were taken at room temperature immediately after the addition of 10 μl of mitochondrial suspension (0.7 mg protein) into the medium (final volume, 3.0 ml). The specific compositions of the media are given in the legends of the figures. Subsequent additions of various compounds were made as 10 μl volumes.

Samples of the mitochondrial suspensions were taken for electron microscopy following drug treatment. Aliquots (1.5 ml) of control and drug-treated mitochondria were transferred into 3 ml of 0.1 M phosphate-buffered 3% glutaraldehyde at 4°. After 45 min of fixation, the mitochondrial suspension was centrifuged at 10,000 *g* for 1 min. Fixed mitochondrial pellets were washed overnight in phosphate buffer, postfixed in 1% phosphate-buffered osmium textroxide at 4° for 3 hr, dehydrated in graded alcohol series, and embedded in Epon-Araldite plastic resin. Thin sections (90 nm) were prepared using a Porter–Blum MT-1 ultramicrotome (Sorvall Corp., Norwalk, CT, U.S.A.), stained with uranyl acetate-lead citrate, and examined with a Siemens Elmiskop 101 electron microscope.

For electrophoretic mobility studies, mitochondria (100 μl) were suspended in medium containing 250 mM sucrose, 15 mM KCl, 2 mM succinate and 10 mM Tris–HCl (pH 7.2) at a final volume of 10 ml. Treated and control mitochondria were preincubated for 5 min at 37° before mobility measurements. Electrophoresis was carried out at 37° using a cylindrical tube apparatus essentially as described elsewhere [20]. A constant voltage of 50 V was applied across gray sintered platinum electrodes. A running distance of 10 μm was timed for each particle. Electrode polarity was reversed after each transit and each particle was measured for two transits toward the anode. Transit times were obtained using a recording timer, and a minimum of fifty particles were measured (100 transits). Apparatus parameters were checked using human red blood cells in phosphate-buffered saline ($\mu = -1.38$ μm/sec/V/cm). All mobilities were measured at the proximal stationary level.

RESULT

Drug effects on respiration of rat liver mitochondria. Addition of either MGBG or DDUG to rat

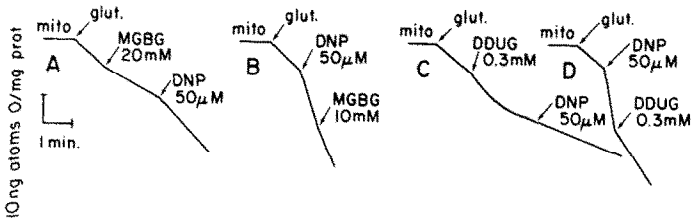


Fig. 2. Effects of MGBG and DDUG on state 4 respiration or intact rat liver mitochondria and on the respiration of mitochondria uncoupled by DNP. The incubation medium contained 50 mM Tris–HCl (pH 7.2) and 15 mM KCl. The substrate was 5 mM glutamate. This and subsequent figures are representative tracings obtained from at least three different mitochondrial preparations.

liver mitochondria caused significant inhibition of state 4 respiration as measured by oxygen consumption (Fig. 2). MGBG was less effective in this regard, requiring about 17-fold greater concentration than DDUG to cause a 25 per cent inhibition (I_{25}) of respiration (Table 1). By contrast, state 3 respiration was less affected by MGBG and required twice as much drug to achieve I_{25} (Fig. 3, Table 1). Essentially the same effect, but complicated with a concomitant uncoupling action, was observed with DUGG. Similarly, greater amounts of either bis(guanylhydrazone) were required to inhibit respiration of mitochondria uncoupled by DNP (Fig. 2, Table 1). Pretreatment of mitochondria with either drug seemed to decrease the stimulatory effect of DNP on respiration (Fig. 2). In contrast, mitochondria specifically made permeable to potassium by valinomycin actually became more sensitive to respiratory inhibition by MGBG or DDUG. Addition of either drug to such mitochondrial preparations resulted in a dramatic inhibition of oxygen utilization (Figs. 4 and 5).

At *low* drug concentrations, the effects of DDUG and MGBG on state 4 respiration differed. DDUG actually stimulated respiration (Fig. 6), while MGBG did not. This stimulatory effect of low concentrations of DDUG was slightly pH dependent. Relative oxygen uptake increased from 230 per cent at pH 6.8 to 260 per cent at pH 7.6. Stimulation was not apparent in mitochondria aged at 4° for 72 hr in 0.25 M sucrose, and aging actually enhanced the

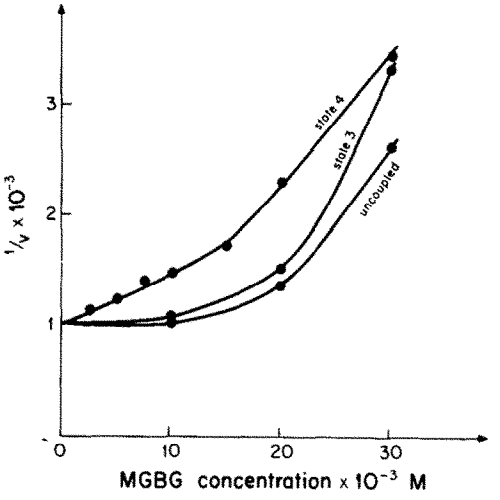


Fig. 3. Dose-dependent effects of MGBG on reciprocal relative oxygen uptake by rat liver mitochondria at state 4, state 3, and uncoupled, with 50 μM DNP with succinate (plus glutamate) as substrate. For other details, see Figs. 2 and 5.

Table 1. Bis(guanylhydrazone) inhibition of respiratory activity of rat liver mitochondria

		Incubation conditions				
Drug	Substrate	Metabolic state*	K^+ conc [mM]	Mg^{2+} conc [mM]	5 mM PO_4^{3-}	I_{25} [mM]
MGBG	Succinate (+glutamate)	4	15	0	−	6.0
		4	15	2.5	−	6.5
		4	15	5.0	+	7.0
		4	110	0	−	11.0
		4	290	0	−	27.0
		U	15	0	−	12.5
		U	15	5.0	+	20.0
		3	15	5.0	+	16.0
	Glutamate	4	15	0	−	5.0
		U	15	0	−	11.0
DDUG	Succinate (+glutamate)	4	15	0	−	0.34
	Glutamate	4	15	0	−	0.32

* State 4, without phosphate acceptor; State 3, in the presence of 0.7 mM ADP; U, uncoupled by the presence of 50 μM DNP.

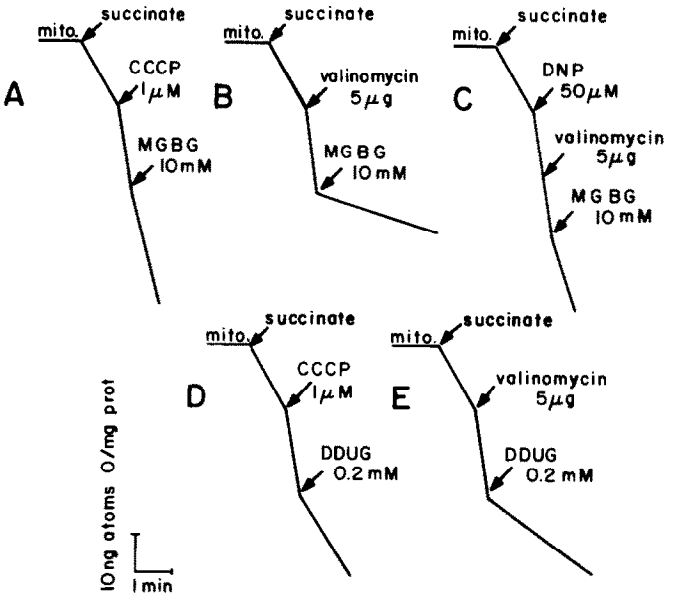


Fig. 4. Effects of MGBG and DDUG on oxygen uptake by rat liver mitochondria de-energized by uncouplers or valinomycin. For experimental details, see Fig. 2.

inhibitory potential of DDUG (Fig. 2) or MGBG (data not shown).

The inhibitory action of MGBG on state 4 respiration was virtually abolished by high potassium levels (Fig. 7) and, less effectively, by magnesium ions. DDUG was found to behave in a similar manner. In the presence of magnesium and phosphate ions, the I_{25} for MGBG increased from 6.0 mM to 7.0 mM for state 4 respiration and from 12.5 to 20 mM for DNP-uncoupled respiration (Table 1).

Drug effects on ATP synthesis by rat liver mitochondria. At concentrations comparable to those required for inhibition of state 3 respiration (Table 1), both MGBG and DDUG interfered substantially with the synthesis of ATP by isolated mitochondria in the presence of exogenously added ADP and

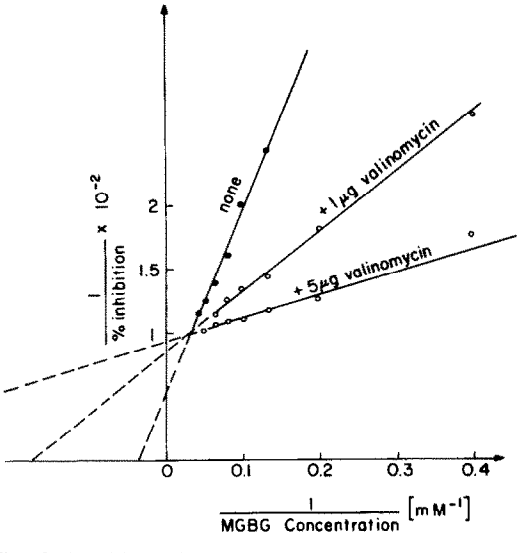


Fig. 5. Double reciprocal plot of MGBG inhibition of respiratory activity of rat liver mitochondria using succinate (+glutamate) as substrate in the presence of two different concentrations of valinomycin. For experimental details, see Fig. 2. Per cent inhibition was calculated as 100 minus the relative respiratory activity. The latter was determined as oxygen consumption in the presence of MGBG at given concentrations divided by oxygen consumption before MGBG addition, multiplied by 100.

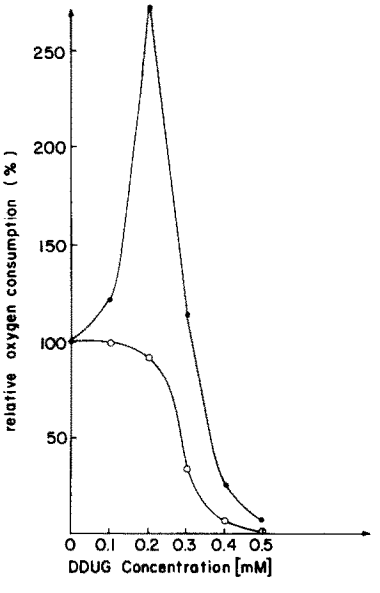


Fig. 6. Dose-dependent effect of DDUG on oxygen uptake by energized rat liver mitochondria (●) and mitochondria aged 72 hr (○) using succinate (+glutamate) as substrate. Relative oxygen consumption was calculated as specific respiratory activity in the presence of inhibitor at the given concentrations divided by specific respiratory activity before inhibitor, then multiplied by 100. For experimental details, see Fig. 2.

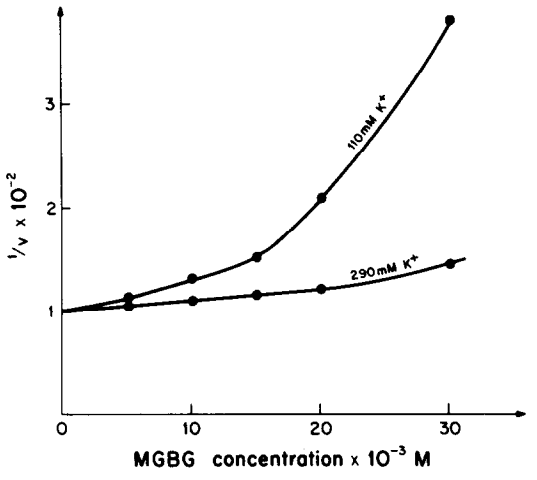


Fig. 7. Dose-dependent effects of MGBG on reciprocal relative oxygen uptake by rat liver mitochondria at state 4 in the presence of different concentrations of potassium. The incubation medium contained 110 mM KCl and 20 mM Tris–HCl (pH 7.2) or 290 mM KCl and 5 mM Tris–HCl (pH 7.2). The substrate was 5 mM succinate (plus 5 mM glutamate). Calculations were made as in Figs. 3 and 5.

phosphate ions. Following a 2-min incubation the ATP:ADP ratio dropped 31 per cent with 10 mM MGBG and 42 per cent with 1 mM DDUG (Table 2). A comparable drop was observed with 0.5 μM CCCP. As with inhibition of respiration, DDUG was about 10-fold more effective than MGBG in inhibiting ATP synthesis.

Drug effects on monoamine oxidase activity in rat liver mitochondria. The initial velocity of oxidation of 5-hydroxytryptamine was determined using the oxygen electrode as a measure for monoamine oxidase A, a marker enzyme for the outer mitochondrial membrane. Both bis(guanylhydrazones) effectively inhibited the enzyme activity (Fig. 8). At 1 mM concentrations of 5-hydroxytryptamine, the I_{25} values for MGBG and DDUG were 4 and 0.075 mM respectively.

Drug effects on optical density of rat liver mitochondria suspensions. Addition of MGBG or DDUG to rat liver mitochondria increased the optical density of the suspension by spectrophotometric measurements (Fig. 9). With either drug, the effect was dose dependent as shown for MGBG in Fig. 9, using untreated or Triton X-100-swollen mitochondria. As with inhibition of respiration, DDUG was more effective than MGBG in producing increases in optical density. In mitochondria pretreated with either drug, the swelling effect of sublytic concentrations of Triton X-100 were attenuated in low potassium concentrations (Fig. 10). Changes in optical density by MGBG or DDUG were even more impressive in mitochondria that were pre-swollen by valinomycin in the presence of potassium and the protonophone, CCCP (Fig. 11).

The extent and velocity of drug-induced increases in optical density were decreased in the presence of certain cations in the medium. In particular, potassium suppressed increases in optical density caused by MGBG but enhanced swelling initiated by sublytic concentrations of Triton X-100 (Fig. 10). Comparable increases in osmolarity using sucrose had no measurable effect on MGBG- or DDUG-induced changes in optical density (data not shown).

Either bis(guanylhydrazone) caused marked increases in optical density in mitochondria pre-swollen spontaneously by hypotonic potassium nitrate but had only a slight effect when added before swelling had taken place. Figure 12 illustrates this effect, using DDUG.

Drug effects on mitochondrial morphology. Light microscopic observation of wet mount preparations of control and drug-treated mitochondrial suspensions revealed that both MGBG and DDUG induced a rapid aggregation of individual mitochondria into clusters of five to thirty organelles. The effect was dose dependent and DDUG was more effective in producing it. It was found to correlate with the increases in optical density shown in Figs. 9–11, but other factors may be involved.

In electron micrographs of untreated mitochondria the various membranes of the mitochondria were easily distinguished (Fig. 13). At state 4, there was a space between the inner and outer mitochondrial membranes, and intercristal spaces were obvious. Following a brief treatment with MGBG or DDUG, the space between the inner and outer membranes was no longer apparent and the intercristal space collapsed, giving the appearance of a single membrane (Fig. 13).

Drug effects on electrophoretic mobility of rat liver mitochondria. Treatment of rat liver mitochondria with MGBG or DDUG rapidly caused a significant decrease in the electrophoretic mobility of the particles toward the anode (Table 3). This effect occurs separately from organelle aggregation since it is dependent on surface charge density rather than size of the particle. Presumably, then, particle slowing

Table 2. Bis(guanylhydrazone) effects of ATP synthesis by isolated rat liver mitochondria

Treatment	ADP*	ATP*	ATP/ADP	Per cent of control
None	5.04	1.30	0.26	100
MGBG (10 mM)	6.94	1.24	0.18	69
DDUG (1 mM)	6.62	1.02	0.15	58
CCCP (0.5 μM)	5.97	0.93	0.15	58

* nmoles per 10 ml of neutralized perchloric acid extract. Based on the average of two experiments.

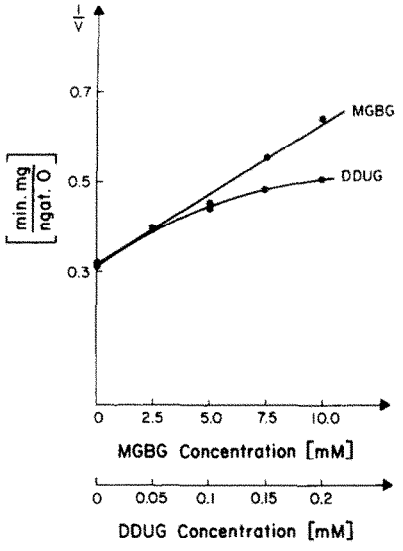


Fig. 8. Dose-dependent effects of MGBG and DDUG on reciprocal initial velocity of monoamine oxidase-catalyzed oxidative deamination of 5-hydroxytryptamine by rat liver mitochondria. The incubation medium contained 50 mM Tris–HCl (pH 7.2), 10 mM KCl and 1 mM 5-hydroxytryptamine as a substrate. Endogenous oxidation was inhibited by 4 μg rotenone plus 4 μg antimycin A.

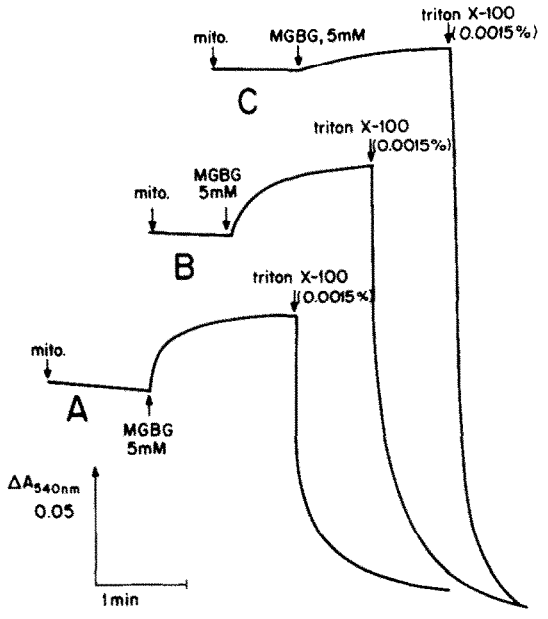


Fig. 10. Effect of potassium concentration on MGBG-induced increases in optical density of rat liver mitochondria in 110 mM potassium chloride and 20 mM Tris–HCl (A), 200 mM potassium chloride and 10 mM Tris-HCl (B), and 290 mM potassium chloride and 1 mM Tris–HCl (C) as measured spectrophotomerically at 540 nm.

occurred as a consequence of negative charge neutralization at the particle surface (surface potential). Accordingly, discharge of the transmembrane potential of mitochondria with the uncoupling agent, DNP, failed to change the electrophoretic mobility (Table 3).

DISCUSSION

Presently, MGBG is probably best known for its effects on polyamine metabolism. In particular, MGBG is a potent competitive inhibitor of *S*-adenosylmethionine decarboxylase, a critical enzyme in the biosynthesis of spermidine and spermine. It has become apparent, however, that the early antiproliferative effects of MGBG cannot be attributed to drug-induced depletion of polyamines alone [7, 21–23]. Thus, the mitochondrion is being considered as an alternative site of drug action and possibly the site with the greater potential for growth inhibition. In cells treated with MGBG, intracellular ATP pools are reduced markedly [15, 24, 25] acetate incorporation into lipid decreases [24], mitochondrial pyruvate utilization drops rapidly [6], and biosynthesis of mitochondrial DNA is inhibited [25]. Following these effects, but prior to detectable inhibition of cell growth, the ultrastructure of mitochondria is distorted markedly, while that of other cellular organelles is unaffected [8–10]. As is apparent from these studies, the function and structure of mitochondria in the context of the intact cell are compromised markedly by MGBG. The present data provide insight into the possible molecular basis for these effects.

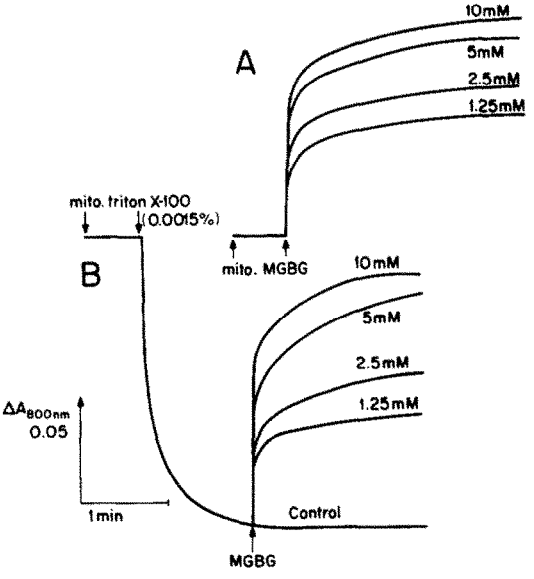


Fig. 9. Dose-dependent effects of MGBG on the optical density at 800 nm of mitochondria (A) and on mitochondria pre-swollen with 0.0015% Triton X-100 (B). Mitochondria were suspended in 250 mM sucrose and 10 mM Tris–HCl.

At millimolar concentrations, both the aliphatic bis(guanylhydrazone), MGBG, and the aromatic derivative, DDUG, inhibited respiration of isolated rat liver mitochondria. This inhibiton was greatest with mitochondria at respiratory state 4 as opposed to those at state 3 or those "de-energized" by uncoupling agents (Figs. 2 and 3). DDUG differed in its inhibitory action by being more effective than MGBG and, at low concentrations, actually stimulated oxygen uptake by intact mitochondria (Fig. 6). Since this latter effect was enhanced in slightly

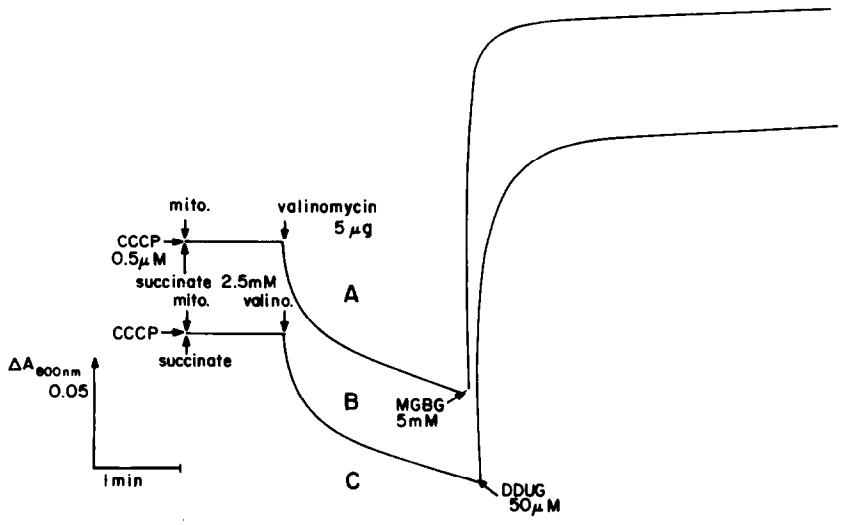


Fig. 11. Effects of MGBG and DDUG on optical density of rat liver mitochondria swollen by valinomycin in 100 mM potassium nitrate (+20 mM Tris–HCl, pH 7.2) as measured spectrophotometrically at 800 nm.

alkaline medium, it is possible that a non-protonated form of DDUG might have uncoupling properties.

The inhibition of mitochondrial respiration by the bis(guanylhydrazones) was accompanied by decreased energy conservation in the organelles. Under conditions of state 3 respiration, mitochondria treated with either MGBG or DDUG displayed a decreased ability to synthesize ATP (Table 2). As with respiratory inhibition, DDUG was more effective than MGBG in producing this effect. The finding corroborates previous reports [15, 24] describing a significant diminution of ATP pools in whole cells treated with MGBG. Given the complexities of intracellular adenylate pool fluctuations, however, the effect could be attributed only indirectly to drug action on mitochondrial function at that time.

One explanation for the differential effect of bis(guanylhydrazones) on mitochondria according to their metabolic state is based on the chemiosmotic theory for oxidative phosphorylation [26, 27]. In the absence of phosphate acceptor (state 4), mitochondria generate a significant electrochemical gradient across the inner mitochondrial membrane (transmembrane potential) and are referred to as being "energized". MGBG and DDUG, being cations under physiological conditions, would be elctrophoretically attracted to the inner mitochondrial membrane by the negative potential at its interior. This interpretation is supported by the non-linear curve for dose-dependent inhibition by MGBG on reciprocal relative oxygen uptake with "energized" (state 4) mitochondria (Fig. 3). Classical linear curves could only be obtained with sonicated mitochondria where transmembrane potential was disrupted (data not shown). Similar selective binding characteristics have also been noted for other cationic compounds. Rhodamine dyes, which are positively charged at physiologic pH, are able to stain mitochondria specifically, whereas uncharged rhodamines and the negatively charged dye, fluorescein, do not [28].

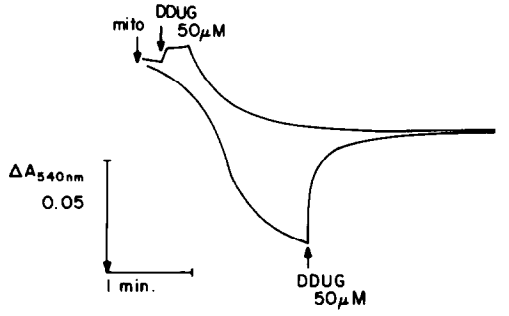


Fig. 12. Effects of DDUG on optical density of rat liver mitochondria in 50 mM potassium nitrate (+20 mM Tris–HCl, pH 7.2) as measured spectrophotometrically at 540 nm.

Once attracted to the mitochondrial periphery by the transmembrane potential, the bis(guanylhydrazones) are likely to bind anionic sites available at the organelle surface. It has already been demonstrated by Hakala [29] that DDUG, which is a lipophilic molecule, interacts with phospholipids excepting those containing choline. Although no similar evidence is available for MGBG, its inability to inhibit respiration as effectively as DDUG may correlate with its molecular structure (Fig. 1). MGBG is an aliphatic molecule lacking the phenyl rings of DDUG and probably interacts less with membrane lipids. A comparison of the effective doses of MGBG and DDUG on mitochondria (Table 4) supports this conclusion. Interestingly, the relative effectiveness of MGBG and DDUG for inhibiting growth parallels their effectiveness in affecting various parameters relevant to mitochondria.

The effects of MGBG or DDUG on respiration of isolated mitochondria were prevented or reversed by higher concentrations of potassium (Fig. 7), suggesting that potassium and bis(guanylhydrazones) compete for the same binding sites at the inner mitochondria membrane. Depletion of endogenous potassium by aging mitochondria enhanced inhibition (Fig. 6), as opposed to uncoupling of oxidative phosphorylation with DNP or CCCP. It is believed

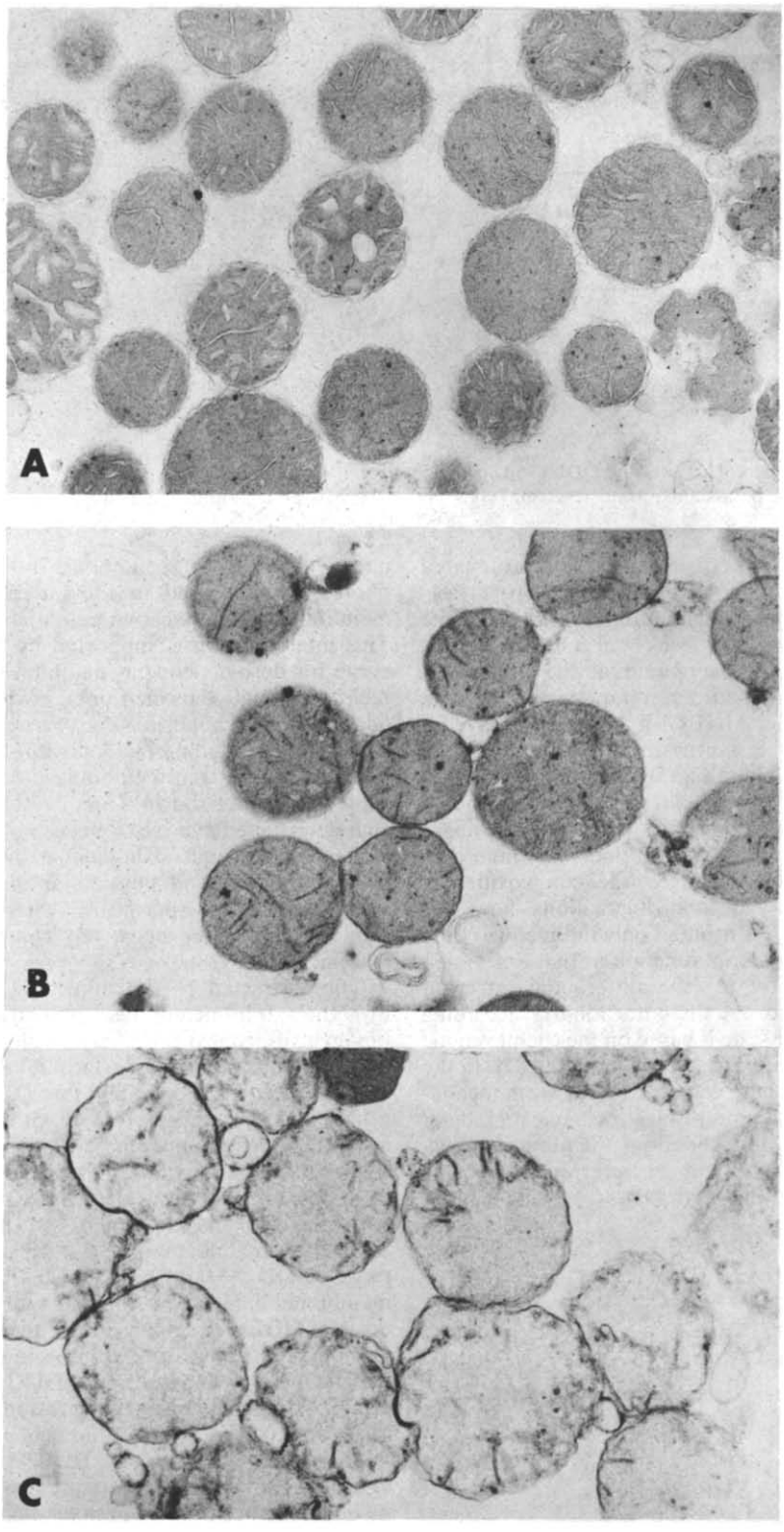


Fig. 13. Electron micrographs of untreated rat liver mitochondria (A) and mitochondria treated with 20 mM MGBG for 3 min (B), or with 0.4 mM DDUG for 3 min (C) (magnifications are: A, ×28,560; B, ×28,560; and C, ×27,200).

Table 3. Effects of bis(guanylhydrazones) in the electrophoretic mobility of isolated rat liver mitochondria

Treatment	Mobility* (μm/sec/V/cm)	Percent slowing
None	−1.37 ± 0.02	0
DNP (50 μM)	−1.31 ± 0.02	4.4
MGBG (2.5 mM)	−0.69 ± 0.01	53.3
DDUG (0.1 mM)	−0.58 ± 0.01	57.7

* Based on a minimum of 100 transit measurements per sample.

[30] that prior to transport into the mitochondrion, potassium cations bind temporarily at a fixed superficial site such as phospholipids which may serve as cation exchangers [31]. Thus, as a consequence of drug binding to the inner mitochondrial membrane, there is a neutralization of anionic binding sites for potassium and a resultant dissipation of local potassium ion concentrations which are critical for mitochondrial respiration.

Support for this interpretation is available in the experiments using valinomycin, a specific cationophore for potassium [32]. Valinomycin induces an influx of potassium into the mitochondria in exchange for protons, causing a disappearance of potassium from the surface of the inner mitochondrial membrane. This action is consistent with the enhanced inhibition of respiration by MGBG and DDUG in the presence of valinomycin (Fig. 5). Competition for membrane binding sites by either drug is decreased in low concentrations of potassium cations.

Magnesium cations prevented or partially reversed the effects of MGBG or DDUG (Table 1). This interaction between magnesium and MGBG is similar to findings of Haas and Davidoff [33], who observed competition between magnesium and phenethyl-biguanide in mitochondria, possibly at the level of cation carriers in the membrane. Increasing evidence indicates that magnesium and potassium cations share the same receptor sites and/or carrier at the inner mitochondrial membranes [34, 35]. The binding of MGBG or DDUG in the vicinity of this carrier may interfere with its transport functions.

At sublytic concentrations, the non-ionic detergent, Triton X-100, caused swelling of mitochondria by non-specifically increasing the permeability of mitochondrial membranes (Figs. 9 and 10). Such mitochondria were more susceptible to bis(guanylhydrazone)-induced aggregation, suggesting that both agents are indeed acting at the mitochondrial membranes. As with potassium protection against drug inhibition of respiration, the membrane aggregation effects with Triton-treated mitochondria were also antagonized by the presence of higher concentrations of potassium. Further support for drug-sharing of potassium binding sites or interference with transport is apparent in studies with mitochondria swollen with hypotonic potassium (Fig. 12) or valinomycin (Fig. 11), which underwent rapid increases in optical density in the presence of MGBG or DDUG.

Electrophoretic mobility data indicate that either drug produced a marked slowing effect (Table 3). Since disruption of the transmembrane potential with the uncoupling agent, DNP, had no effect on mobility, it seems that the slowing is a consequence of bis(guanylhydrazone) binding at the particle surface and neutralizing the net negative charge (surface potential). As discussed above, this probably involves membrane phospholipids which are thought to account for the membrane surface potential and hence mobility characteristics. This neutralization phenomenon may be responsible for the collapse of intramembranous spaces seen ultrastrucurally (Fig. 13), as has been described by Stoner *et al.* [19] in heart mitochondria treated with inorganic cations. According to this interpretation, positively-charged multivalent cations may cause mitochondrial membrane spaces to collapse by binding at the membrane outer surface, decreasing the net negative charge, and allowing the opposing surfaces to come together as a result of Van der Waals attraction forces.

The decrease in activity of the outer membrane enzyme marker, monoamine oxidase A, with drug treatment may well be related to a collapse of the inner and outer membranes or to the aggregation of mitochondria. Alternatively it may be due to biochemical events involving the outer membrane alone since it has been shown that monoamine oxidase activity is dependent on the lipid composition of that membrane [36]. In either case, the data indicate that the bis(guanylhydrazone) effects are not confirmed to the inner mitochondrial membrane.

Whether these results with isolated mitochondria accurately reflect the events that occur in an intact cell exposed to MGBG or DDUG is uncertain. The concentrations of drug required for growth inhibition (5–10 μM for MGBG and less than 1 μM for DDUG) are considerably lower than those employed here. Cells, however, are known to effectively concentrate

Table 4. Summary of the effective concentrations of bis(guanylhydrazones) on isolated rat liver mitochondria and cells

Parameter	Drug conc MGBG	Drug conc DDUG
Increase in optical density (aggregation)	1.25 mM	0.025 mM
50% Slowing in electrophoretic mobility	2.50 mM	0.100 mM
I_{25} of monoamine oxidase	4.00 mM	0.075 mM
I_{25} of state 4 respiration	6.00 mM	0.340 mM
50% Growth inhibition of Sarcoma S-180 cells*	6.0 μM	0.8 μM

* Data from Ref. 29.

MGBG by a carrier mechanism that is shared with the polyamine, spermidine [7, 37]. In fact, Mandel and Flintoff [38] estimated that Chinese hamster ovary cells treated for 24 hr with 20 μM MGBG attain intracellular drug levels approaching 2.5 mM. Similarly, Seppanen *et al.* [39] have found that cells can concentrate MGBG some 600- to 1500-fold so that, following exposure to 5–10 μM drug, the internal concentration might be 4–6 mM. A minimum intracellular concentration of 0.5 to 1 mM is required for growth inhibition to occur [39]. DDUG is also accumulated intracellularly by cells, and concentration gradients up to 800-fold have been reported [29]. These figures give no indication of regional drug concentrations in specific cellular compartments such as mitochondria where, due to preferential sequestration, the drug levels might be even higher. For example, Hakala [29] has noted that 30 per cent of the total intracellular DDUG accumulates in the mitochondrial fraction. Finally, it should be noted that many of the drug effects observed with intact cells (i.e. growth inhibition, polyamine pool changes, and ultrastructural damage [6]) require hours of drug exposure, whereas those assessed here are almost immediate.

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