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# INHIBITION OF DIAMINE OXIDASE BY 1,1-[(METHYLETHANEDIYLIDENE)-DINITRILO]-BIS-(3-AMINOGUANIDINE) AND 1,1′-[(METHYLETHANEDIYLIDENE)-DINITRILO]-DIGUANIDINE

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**Abstract**—It was found that two inhibitors currently being used to inhibit spermidine formation in mammalian cells were more potent inhibitors of diamine oxidase than of polyamine synthesis. Eukaryotic *S*-adenosylmethionine decarboxylase is inhibited reversibly by 1,1′-[(methylethanediylidene)-dinitrilo]-diguanidine (MGBG) and irreversibly by 1,1′-[(methylethanediylidene)-dinitrilo]-bis-(3-aminoguanidine) (MBAG). Since this enzyme is essential for the generation of propylamine groups needed to convert putrescine into spermidine, these drugs were used to prevent spermidine synthesis in mammalian cells. It was found that both drugs were potent inhibitors *in vitro* of pig kidney diamine oxidase activity. MGBG appeared to be a non-competitive inhibitor with a $K_i$ of about 0.1 $\mu$M and MBAG was a competitive inhibitor with a $K_i$ of 0.02 $\mu$M. Inhibition by MBAG was partially reversible after prolonged dialysis. The period during which diamine oxidase activity remained suppressed in the rat after treatment with these inhibitors was determined by measuring the conversion of (1,4-$^{14}$C]putrescine to $^{14}CO_2$. A single dose of MGBG (60 mg/kg body wt) inhibited $^{14}CO_2$ production more than 90 per cent for 21 hr and a single dose of MBAG (50 mg/kg body wt) produced a similar inhibition for 72 hr.

There is considerable research interest at present in the function of polyamines [1–3]. One approach to determining the role of these compounds in the cell has been to use specific inhibitors of their biosynthesis. A number of these inhibitors have been described. Most of these inhibit L-ornithine decarboxylase and thus block the production of putrescine [4–8]. Others prevent the decarboxylation of *S*-adenosylmethionine [8–10] and hence the conversion of putrescine into spermidine since decarboxylated *S*-adenosylmethionine is the sole source of propylamine groups for this reaction. The first of the latter inhibitors to be used was the antileukemic drug, 1,1′-[(methylethanediylidene)-dinitrilo]-diguanidine (MGBG) (Fig. 1) which was found to be a potent competitive inhibitor of mammalian *S*-adenosylmethionine decarboxylase [9]. More recently, we have studied a related compound, 1,1′ - [(methylethanediylidene) - dinitrilo] - bis - (3-aminoguanidine) (MBAG) (Fig. 1) which inhibits *S*-adenosylmethionine decarboxylase irreversibly [10]. MGBG has been used in a number of studies on the function of spermidine [11–15], and both MGBG[16, 17] and MBAG[18] have been shown to lead to an increased cellular level of putrescine which may be related to its lack of conversion to spermidine. However, although it has been noted briefly before that MGBG is also an inhibitor of rat thymus diamine oxidase [19], the possible influence of these drugs (which are chemically related to aminoguanidine, a classical inhibitor of diamine oxidase [20]) on diamine oxidase activity has not been considered in any detail. In the present paper, it is shown that both MBAG and MGBG are powerful inhibitors *in vitro* and *in vivo* of diamine oxidase and the consequences of this inhibition on the interpretation of studies of their physiological effects are discussed.

## MATERIALS AND METHODS

MGBG, diaminoguanidine and aminoguanidine were purchased from the Aldrich Chemical Co., Milwaukee, WI. MBAG was synthesized by a published procedure [21]. [1,4-$^{14}$C]putrescine (60

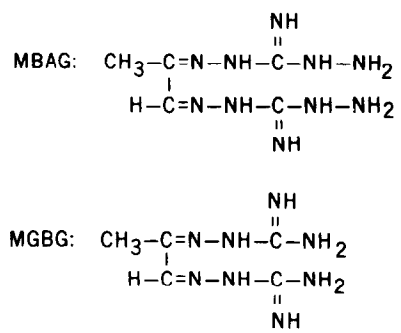


Fig. 1. Structures of MGBG and MBAG. The correct chemical name for MGBG is 1,1′-[(methylethanediylidene)-dinitrilo]-diguanidine but the compound is frequently referred to as methylglyoxal bis(guanylhydrazone). MBAG is 1,1′-[(methylethanediylidene)-dinitrilo]-bis-(3-aminoguanidine).

mCi/m-mole) was purchased from New England Nuclear, Boston, MS, and was diluted with unlabeled putrescine to an appropriate specific activity before use. All other biochemical reagents were products of the Sigma Chemical Co., St. Louis, MO. Pig kidney diamine oxidase was purified to a specific activity of 1.6 units/mg of protein by the method of Mondovi *et al.* [22] starting from crude enzyme purchased from Sigma, St. Louis, MO. The assay medium had a final volume of 3 ml and a pH of 7.5. It contained 0.01 to 0.03 units of enzyme, 0.1 M Na phosphate buffer, and the concentrations of (1,4-$^{14}$C]putrescine and inhibitors indicated in the legends. After incubation at 37° for 30 min, or the time indicated, the reaction was stopped and the amount of putrescine which had been oxidized was determined by extraction into toluene [23]. The conversion of 620,000 dis/min to a toluene extractable form represented the conversion of 1 μmole substrate. One unit of enzyme activity corresponded to the ability to oxidize 1 μmole putrescine/hr at 37° at saturating substrate concentrations.

For measurement *in vivo* of diamine oxidase activity male Sprague–Dawley strain rats weighing 200–250 g (Charles River Breeding Laboratories, Wilmington, MA) were used. The inhibitors were administered by intraperitoneal injection of a solution in 0.5 ml of 0.9% (w/v) NaCl. The control rats received 0.5 ml of 0.9% NaCl. At various times later the rats were given a single intraperitoneal injection of 20 mg of [1,4-$^{14}$C]putrescine (1 μCi) dissolved in about 0.3 ml of 0.9% (w/v) NaCl. Each rat was then placed in an enclosed chamber through which a constant stream of air was pumped. The air leaving the chamber bubbled through three wash bottles each containing 150 ml of 1 N NaOH to trap the exhaled $^{14}CO_2$. A 1-ml sample from each bottle was counted after addition of 4 ml water and 10 ml LSC-Cocktail Formula 947 (New England Nuclear Corp., Boston, MA). The percentage of the administered dose of [$^{14}$C]putrescine which was converted to $^{14}CO_2$ was then determined. In order to check that the trapped radioactivity did, in fact, represent $Na_2{}^{14}CO_3$, samples of 5 ml were transferred to a small conical flask fitted with a rubber cap carrying a plastic well containing 0.3 ml of 1 N hyamine hydroxide in methanol. The sample was then acidified by injection of 2 ml of 5 N $H_2SO_4$ through the cap and the flask shaken for 30 min. The plastic well and its contents were then assayed for radioactivity after addition of 10 ml toluene containing 6.0 g "Omnifluor" (NEN)/liter. It was found that more than 95 per cent of the label was displaced by acidification and trapped in the hyamine. Authentic $Na_2{}^{14}CO_3$ gave similar results and thus the 5 per cent of the label not accounted for was probably due to the lack of complete absorption by the hyamine.

Radioactivity was determined using a Beckman LS3133T liquid scintillation counter. Efficiency, which was determined by use of an internal standard, was about 60 per cent for $^{14}$C in the enzyme assays and 50 per cent for the aqueous, alkaline samples.

## RESULTS

Under the conditions used for the assay of pig kidney diamine oxidase, the reaction was linear with time of incubation for at least 1 hr (Fig. 2). The addition of either MGBG or MBAG at a concentration of 0.05 μM reduced the rate of putrescine oxidation but the rate was still linear with time (Fig. 2). MBAG was the more powerful inhibitor and as shown in Fig. 2 was, in fact, more active on a molar basis than diaminoguanidine. This rules out the possibility that small traces of diaminoguanidine, which is an intermediate in the synthesis of MBAG, might be responsible for the inhibition.

A 30-min incubation period was chosen for more detailed studies of the kinetics of inhibition of activity by MGBG and MBAG (Figs. 3 and 4). As shown in Fig. 3, MGBG appeared to be a non-competitive inhibitor having a $K_i$ of 0.1 μM. This value is somewhat lower than that reported by Hölttä *et al.* [17] who used a crude enzyme preparation from rat thymus but confirms their conclusion that MGBG is a potent non-competitive inhibitor of diamine oxidase. As shown in Fig. 4, MBAG was apparently a competitive inhibitor of the enzyme having a $K_i$ of 0.02 μM. It is possible from this data that MBAG might actually be a substrate for the enzyme but this appears unlikely since we were unable to detect any hydrogen peroxide after incubation of the enzyme with MBAG in the absence of the substrate. Such incubation led to a loss of enzyme activity which was only partially restored with extensive dialysis. In part, this may be due to the very high affinity of the enzyme for MBAG as indicated by the low $K_i$, but the difficulty in reversing the inhibition suggests that MBAG may react

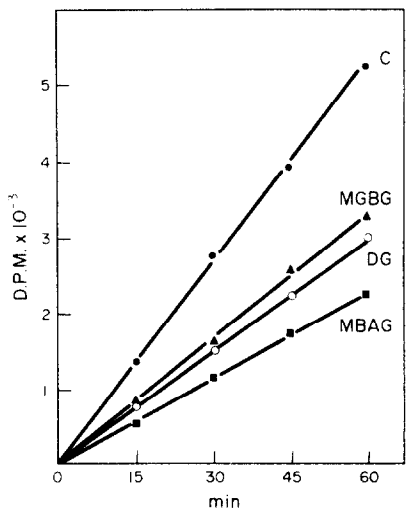


Fig. 2. Time course of diamine oxidase reaction. The assay medium contained 0.03 units of pig kidney diamine oxidase, 0.1 mM [1,4-$^{14}$C]putrescine and the inhibitors as follows: (●) no inhibitor; (▲) 0.05 μM MGBG; (○) 0.05 μM diaminoguanidine (DG); and (■) 0.05 μM MBAG. After incubation at 37° for the time shown, the radioactivity converted to material soluble in toluene was determined as shown. The release of 620 dis/min represents the oxidation of 1 nmole putrescine.

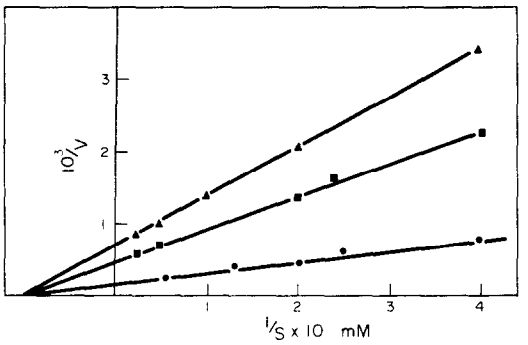


Fig. 3. Kinetics of inhibition of diamine oxidase by MGBG. The assays were incubated at 37° for 30 min with 0.15 units of enzyme and substrate concentrations of 0.025 to 0.4 mM [1,4-$^{14}$C]putrescine. The amount of radioactivity converted to a form soluble in toluene was then determined and the dis/min were used as the velocity of reaction, $V$. (As in Fig. 2, the conversion of 620 dis/min represented the oxidation of 1 nmole putrescine.) The reciprocal of $V$ ($\times 10^3$) was plotted against the reciprocal of the substrate concentration. Results are shown for no addition (●) and in the presence of 0.2 $\mu$M (■) and 0.4 $\mu$M (▲) MGBG.

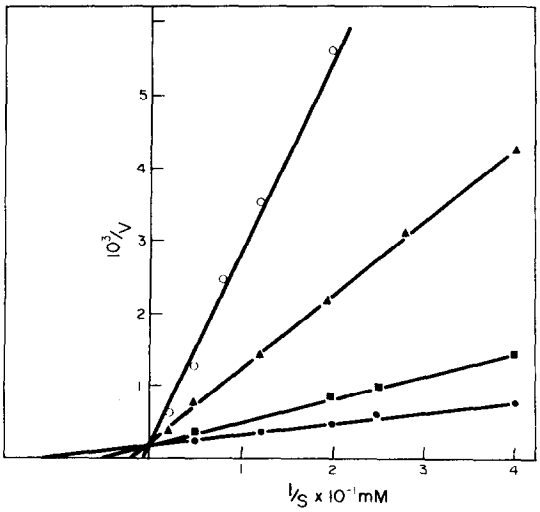


Fig. 4. Kinetics of inhibition of diamine oxidase by MBAG. The assays were incubated at 37° for 30 min as in Fig. 3. Results are shown for no addition (●) and in the presence of 0.02 $\mu$M (■), 0.1 $\mu$M (▲) and 0.2 $\mu$M (○) MBAG.

with the enzyme as a carbonyl reagent in a manner similar to that of aminoguanidine [20].

The length of time during which diamine oxidase activity was inhibited in the rat after administration of these drugs was investigated by measurement of their effects on the conversion of [1,4-$^{14}$C]putrescine into $^{14}CO_2$. Both in rats [24] and mice [25] a substantial proportion of exogenous [1,4-$^{14}$C]putrescine is excreted as $^{14}CO_2$. The administration of 50 mg aminoguanidine/kg body weight 2–8 hr before putrescine completely prevented this $^{14}CO_2$ production (Fig. 5). A similar inhibition occurs in mice [25]. This finding and other experiments in which putrescine metabolites have been examined support the hypothesis that diamine oxidase plays an important role in putrescine catabolism in some organs [25] even though the diamine can be degraded in other ways in tissues such as brain [26].

In the present experiments, aminoguanidine (50 mg/kg body wt) given 24 hr prior to the labeled putrescine still inhibited $^{14}CO_2$ production by about 90 per cent, but by 46 hr after the inhibitor treatment putrescine degradation was only slightly slower than in control rats (Fig. 5). A substantial inhibition of $^{14}CO_2$ production was produced by administration of 60 mg MGBG/kg body wt 2 or 18 hr before the putrescine (Fig. 6). The inhibitory effect was beginning to wear off 21 hr after MGBG injection and by 24 hr there was only a slight effect on the rate of putrescine oxidation. Thus, MGBG is an effective

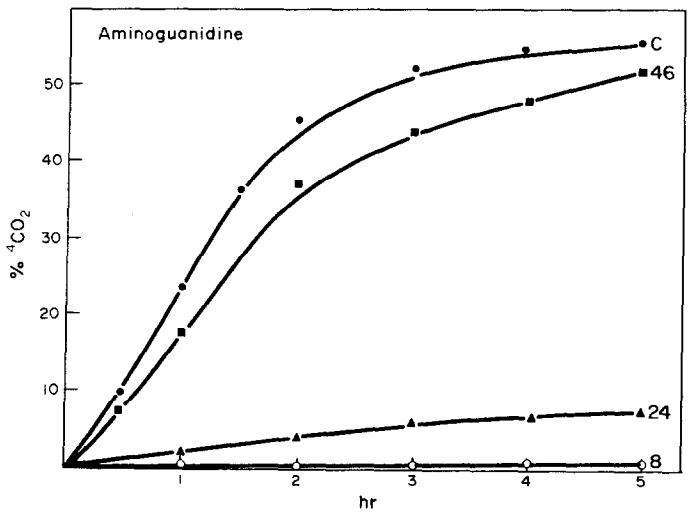


Fig. 5. Effect of aminoguanidine on conversion of [1,4-$^{14}$C]putrescine into $^{14}CO_2$. Rats were given a single intraperitoneal injection of 50 mg aminoguanidine chloride/kg body wt in 0.9% NaCl at 8 (○), 24 (▲) or 46 (■) hr before injection of [1,4-$^{14}$C]putrescine (20 mg/kg; 1 $\mu$Ci). Control rats (●) received 0.9% NaCl alone 8 hr before the [$^{14}$C]putrescine. The expired air was bubbled through wash bottles containing 1 N NaOH to trap the expired $^{14}CO_2$ and, at intervals as shown, samples were counted. The cumulative exhalation of $^{14}CO_2$ is shown as a percentage of the $^{14}$C-label administered.

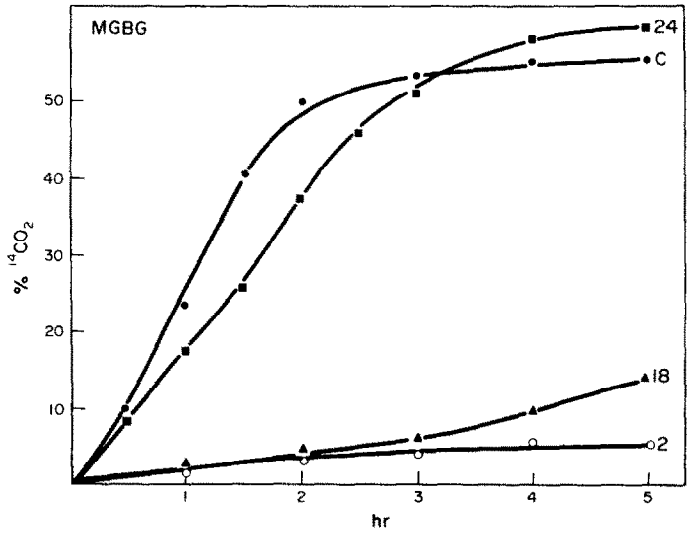


Fig. 6. Effects of MGBG on conversion of [1,4-$^{14}$C]putrescine into $^{14}CO_2$. Rats were given a single intraperitoneal injection of 60 mg MGBG/kg body wt at 2 (○), 18 (▲) and 24 (■) hr prior to administration of the labeled putrescine. Other details were as in Fig. 5.

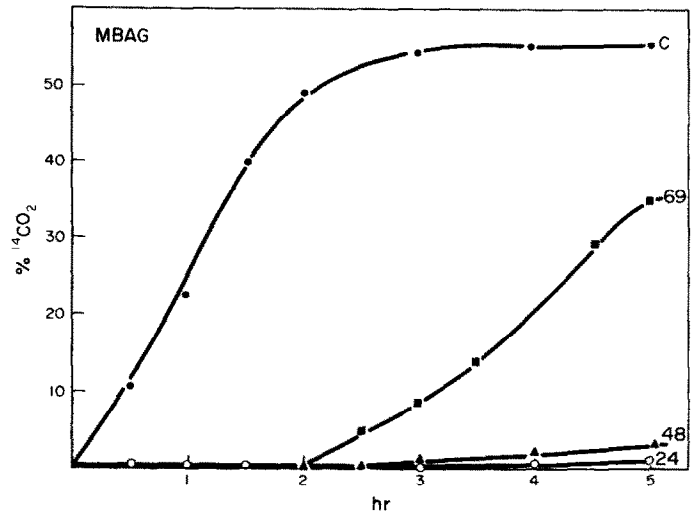


Fig. 7. Effect of MBAG on conversion of [1,4-$^{14}$C]putrescine into $^{14}CO_2$. Rats were given a single intraperitoneal injection of 50 mg MBAG/kg body wt at 24 (○), 48 (▲) and 69 (■) hr prior to administration of labeled putrescine. Other details were as in Fig. 5.

inhibitor of putrescine catabolism for about 20 hr after a single dose. MBAG was even more effective in this respect, as shown in Fig. 7. At 48 hr after administration of a single dose of 50 mg/kg body wt, conversion of putrescine into $^{14}CO_2$ was still completely inhibited. Restoration of normal metabolism began only at about 71 hr after administration of the drug. At equimolar doses MBAG was, therefore, more effective in blocking putrescine oxidation than was aminoguanidine.

## DISCUSSION

It must be noted that the conversion *in vivo* of [1,4-$^{14}$C]putrescine to $^{14}CO_2$ involves several steps. It is quite possible that MBAG and MGBG could be inhibiting the production of $^{14}CO_2$ at one of these steps in addition to any effect on diamine oxidase. However, the strong inhibitory action *in vitro* of these drugs on diamine oxidase and the fact that diamine oxidase activity was greatly reduced in tissue extracts from rats treated with MGBG or MBAG* suggest that this is not likely to be correct, although the possibility cannot be entirely ruled out.

The present experiments fully confirm a previous report that MGBG is a potent inhibitor *in vitro* of mammalian diamine oxidase [17] and extend the data to show that MGBG treatment inhibits putrescine degradation *in vivo* for a considerable period. It is probable that most of the oxidation of exogenous putrescine measured in these experiments is due to the action of diamine oxidase in the gastrointestinal tract since activity there is very high in the rat [20, 27]. However, the much smaller but readily detectable amounts of activity in rat liver and kidney were completely lost when extracts were assayed 15 hr after treatment with MGBG* so that it appears that loss of activity is widespread. Al-

* A. E. Pegg, unpublished observations.

though MGBG is a potent inhibitor of mammalian S-adenosylmethionine decarboxylase, the effects on this enzyme rapidly wear off due to the stabilization of the enzyme against normal degradation [28, 29] and spermidine synthesis in rat liver and kidney is inhibited completely for only 8 hr after a dose comparable to that used in the present study [16]. Therefore, MGBG is a more potent and long-lasting inhibitor of diamine oxidase than of S-adenosylmethionine decarboxylase in such experiments.

We have found that MBAG has a much longer lasting inhibitory effect on S-adenosylmethionine decarboxylase than does MGBG, producing substantial inhibition for up to 48 hr after a single dose; but even with high doses of MBAG, activity could be reduced only to 15–20 per cent of control values [18]. The complete loss of diamine oxidase activity for up to 72 hr, which was found in the present experiments to result from MBAG treatment, indicates that this compound also is a more potent inhibitor of diamine oxidase than of S-adenosylmethionine decarboxylase.

Studies from this laboratory [10, 16] and others [8, 9, 12, 17] have indicated that MGBG and MBAG are potent inhibitors of eukaryotic S-adenosylmethionine decarboxylase. These drugs have been used successfully as inhibitors of spermidine synthesis [8, 11–15, 30]; columns of MGBG linked to Sepharose provide a useful affinity absorbant for purifying S-adenoxylmethionine decarboxylase [19]. The present studies show that the inhibitors are not as specific as was previously believed. As emphasized in a recent review [8], all experiments using such inhibitors must be interpreted cautiously and in the light of appropriate controls. The inhibition of diamine oxidase by these drugs must be taken into account when attempting to interpret physiological changes brought about by them [11–15, 30–32]. Although diamine oxidase is highly active on putrescine and may play a role in controlling putrescine levels [1–3, 26], the enzyme also acts on other substrates of physiological importance, such as histamine [1, 20], and some preparations of diamine oxidase also attack the higher polyamines, spermidine and spermine [2, 33]. Thus, MBAG and MGBG may raise histamine levels and the blocking of the degradation of putrescine and polyamines may more than compensate for the inhibition of S-adenosylmethionine decarboxylase. The extent to which these side effects are important in cell culture experiments is not yet known but they should not be ignored. Since aminoguanidine [20] is an inhibitor of diamine oxidase of a potency comparable to that of MGBG and MBAG, but is inhibitory to S-adenosylmethionine decarboxylase only at concentrations greater than 1 mM [9], this drug might be used as a control in determining that the effects of MGBG or MBAG are not due simply to inhibition of diamine oxidase activity.

Finally, we have confirmed that rat liver S-adenosylmethionine decarboxylase, purified by affinity chromatography on MGBG-Sepharose [19], is not contaminated with diamine oxidase,* but the possibility that this enzyme might be co-purified by the use of affinity absorbants for other tissues or species should not be overlooked.

* A. E. Pegg, unpublished observations.

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