Analysis of Methylglyoxal Bis(guanylhydrazone)-induced Alterations of
Hamster Tumor Mitochondria by Correlated Studies of Selective
Rhodamine Binding, Ultrastructural Damage, DNA Replication,
and Reversibility

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ABSTRACT

Because of the renewed importance of methylglyoxal bis-
guanylhydrazone (MGBG) in recent clinical trials for a variety of
cancers, and because the antiproliferative activity of MGBG
cannot be accounted for exclusively by the known inhibition by
the drug of polyamine biosynthesis, but is thought to involve an
alternative action in which the mitochondria are implicated, we
have investigated several new aspects of the nature and revers-
ibility of this mitochondrial damage. Using Rous sarcoma hamster
tumor cells as a model, treatment of monolayer cultures during
exponential growth with 10, 25, and 50 µM MGBG (up to 48 hr)
resulted in dose-dependent (reversible) growth inhibition and
selective ultrastructural damage to the mitochondria (e.g., ex-
treme swelling, loss of cristae and matrix components, and dense
inclusions) in up to 96% of cells, while nuclei appeared normal,
corroborating and extending findings by others in mouse, rat,
and human cells. Mitochondria in 3 to 5% of cells failed to swell,
even at highest drug dosage, but were of unusual structure.
After removal of MGBG, damaged mitochondria in 90 to 95% of
cells recovered near-normal ultrastructure within 1 to 2 days; in
some cells, mitochondrial recovery from severe damage could
be monitored following a lag period of up to 5 days.

The potential-dependent, supravital fluorescent probe rhoda-
mine 123 (RH 123) selectively and relatively uniformly stained
the grossly enlarged mitochondria, strikingly delineating residual
organelle membranes. Quantitative assays of the uptake and
retention of RH 123/108 cells demonstrated the maintenance of
mitochondrial membrane potential in both control and MGBG-
treated cell populations. These data also support the concept of
mitochondrial fusion in MGBG-treated cells.

The fate of mitochondrial DNA (mtDNA) both during (24 to 48
hr) and following (7 hr to 7 days) MGBG treatment was monitored
by ultrastructural, electron autoradiographic, pulse-labeling, gra-
dient centrifugation, restriction cleavage, and electrophoretic
methods. MGBG treatment (50 µM; 7 and 16 hr) selectively
inhibited mtDNA replication (73% at 16 hr) prior to significant
inhibition of nuclear DNA synthesis (19% at 16 hr); the drug
induced structural alterations, without substantial degradation,
of the closed circular (major form) of mtDNA, and cessation of
D-loop strand (7S) initiation within the replication origin. Upon
return to drug-free medium, mtDNA resumed replicative activity,
and mtDNA fibrils appeared to be associated with regenerating
cristae, as assessed by electron microscopy.

The combined results demonstrate mitochondria to be a se-
lective target of MGBG action, and define structural and func-
tional requisites for the reversibility of this drug-induced damage.

INTRODUCTION

MGBG served as a potent agent against acute myelocytic
leukemia in early clinical studies, but its more widespread appli-
cation was limited because of severe toxicity (10, 35). Improved
therapeutic value of MGBG was recently achieved by altered
drug infusion schedules producing more tolerable toxicities (19,
45), and by the use of MGBG in combination with another
inhibitor of polyamine biosynthesis, 2-difluoromethylornithine (13,
41). As a result, MGBG is currently being reevaluated in clinical
trials for a variety of cancers, including solid tumors, tumors of
the central nervous system, leukemias, and lymphomas (19, 43–
45). In addition, pharmacological studies have focused on modes
to understand and attenuate persisting clinical toxicity (14, 37).

Significant aspects of the mechanism of action of MGBG,
however, are unresolved. Two different intracellular sites of
MGBG action have been identified. One is interference with
polyamine biosynthesis by inhibiting S-adenosylmethionine de-
carboxylase, a rate-limiting enzyme in the synthesis of spermi-
dine and spermine (13, 18, 22, 35). Inhibition of nuclear DNA
synthesis (3, 20, 21), which at least in some cells may be related
to the influence of MGBG on polyamine biosynthesis (21, 40),
and growth arrest in G1 (39) have also been observed. There is
increasing evidence, however, that the antiproliferative action of
MGBG cannot be exclusively accounted for by inhibition of
polyamine metabolism (cf. Ref. 35). An alternative site of MGBG
action in proliferating cells appears to be the mitochondria, as
reflected in selective ultrastructural damage (8, 23, 31, 34–36),
impairment of mitochondrial respiration and oxidative phosphi-
rolation (32, 35), reduction of ATP pools (32, 35, 36), and
inhibition of mtDNA replication (9). Early damage to mitochondria,
which appears to precede both the detection of altered polyamine
levels and inhibition of cellular growth and nuclear DNA synthesis
(33), has been implicated in the antiproliferative effects of MGBG
(33, 35). It has also been suggested (18) that the muscle toxicity
of MGBG (45) may be related to the antimitochondrial action of
the drug.

The molecular basis of MGBG-induced mitochondrial effects
is not understood. Although the latter strongly resemble in many
respects mitochondrial toxic effects elicited by etidium bromide
(36, 42), MGBG-induced damage, for reasons unknown, appears

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2 The abbreviations used are: MGBG, methylglyoxal bis(guanylhydrazone);
RSHT, Rous sarcoma virus-induced hamster tumor; RH 123, rhodamine 123;
mtDNA, mitochondrial DNA.
to occur more rapidly and more selectively and is more readily reversible (35, 36). The fate of mtDNA following prolonged MGBG treatment is also obscure. The major (closed circular) form of mtDNA could no longer be detected in L1210 cells exposed to MGBG for 16 hr (9); yet mitochondrial recovery may occur after such treatment. Further studies are needed to better understand the properties of mitochondria-targeted drug effects and their relevant contribution to overall growth inhibition and cytotoxicity; such knowledge may be pertinent in strategies aimed at improving the clinical usefulness of MGBG.

To answer some of these questions, the following experiments were performed, using an integrated structural, functional, and biochemical approach to investigate the nature of mitochondrial MGBG-induced damage, and its reversal in monolayer cultures of hamster tumor cells. Preliminary results have been reported (30).

MATERIALS AND METHODS

Cell Culture and Drug Treatment. The Syrian hamster tumor cell line (RSHT), originally derived from a tumor induced in vivo by Rous sarcoma virus, was obtained from Flow Laboratories (Rockville, MD), and grown in monolayer culture in Eagle’s minimum essential medium with 10% fetal calf serum (26, 28). Normal baby hamster kidney cells (BHK21/C13) and a derived clone transformed in vitro by Rous sarcoma virus (Cv/B1) were also cultured as described (26, 28). The generation time of these cell lines is about 20 to 22 hr. Cells during logarithmic growth were treated with MGBG (Aldrich Chemical Co., Inc., Milwaukee, WI) at varying concentrations of drug (1 to 50 μM) for 5 to 48 hr, and then grown in MGBG-free medium from 0 to 10 days (subcultivated by brief trypsinization as required).

Cell growth was monitored by cell counts (hemocytometer) and protein assays (6, 11); cell viability was assessed by trypan blue dye exclusion tests. Due to rapid clumping of RSHT cells removed from flasks for cell counts, the total protein assay method, as recommended for monolayer cultures (11), gave more reproducible results than did cell counts in multiple samples. To account for possible fluctuations between protein content and cell number, calibration assays were performed (11), yielding a value of 0.42 ± 0.03 (S. E.) mg protein/10⁶ cells in aliquots between 2 and 6 days following plating of control and MGBG-treated cells (media changed daily).

Rhodamine Fluorescence Microscopy. RH 123 (Eastman Organic Chemicals, Rochester, NY) was added from an aqueous stock solution (1 mg/ml) to standard culture medium to a final concentration of 5 to 10 μg/ml for 60 min at 37°. These conditions gave optimal dye uptake in separate test series (2 to 50 μg/ml; 30 to 60 min). Cells maintained on coverslips (with and without MGBG) were stained with RH 123, rinsed in three 5-ml changes of medium (5 min each) (17), and mounted on a live-cell observation chamber. Stained cells were examined in an Olympus Vanox microscope by epifluorescent illumination (530 nm, excitation; 570 nm, emission) using a ×100 Planapochromat objective lens. Both phase-contrast and fluorescent photomicrographs were taken of each selected field, using Kodak Tri-X (400 ASA) film, which was developed with Acufine to obtain a 1000 ASA rating.

Quantitative Rhodamine Fluorescence Measurements. Control and MGBG-treated cells, maintained in culture flasks (25 sq cm; 5 to 5 × 10⁶ cells/dish), and stained with RH 123 (see above), were extensively rinsed with Buffer A (0.15 M NaCl-0.25 mM Tris·HCl, pH 7.4), and extracted with 2 ml n-butyl alcohol (15). The butyl alcohol extract was cleared of cell debris by centrifugation at 10,000 × g. Rhodamine fluorescence (15) was measured in a Farrand fluorometer with filtered light from 410 to 490 nm for excitation and 532 nm for emission. Fluorescence values were corrected (1 to 2% of total) for autofluorescence of unstained cells and nonspecific adherence of RH 123 to plastic culture flasks (15). The cell residue of each flask after butyl alcohol extraction was quantitatively recovered, and its protein content was related to cell counts of duplicate flasks (6, 11) to normalize RH 123 uptake and retention per 10⁶ cells.

Electron Microscopy. Cells grown in Falcon flasks (75 sq cm) were rinsed twice with normal culture medium at 37°, and fixed in situ with 4% glutaraldehyde in Buffer B (0.1 M phosphate buffer, pH 7.4) at 4° for 2 hr. Cells were then gently scraped with a rubber policeman, collected by centrifugation (900 × g for 5 min), suspended in fresh fixative for 2 hr, washed twice in Buffer B, and postfixed with 2% osmium tetroxide in Buffer B for 2 hr (all steps at 4°). Cell pellets, after 2 washes each in Buffers B and C (0.15 M Veronal acetate buffer, pH 7.4; cf. Ref. 38), were given a second postfixation with 0.5% uranyl acetate in Buffer C, pH 6.5, to optimize preservation of membrane structure and mtDNA fibers (cf. Ref. 25), based on the procedure by Kellenberger et al. (See Ref. 38) to preserve DNA fibers in bacterial nucleoids. In some cases, cells were cast in 2% agar in Buffer C (38) prior to the second postfixation. Samples were dehydrated using graded series of ethanol and embedded in Araldite or Epon resins. Ultrathin sections were stained with uranyl acetate and/or lead citrate, and examined in a Siemens Elmiskop la electron microscope. DNA molecules were prepared for electron microscopy as described (26).

High-Resolution Autoradiography of Thymidine Incorporation. RSHT cells in exponential phase, incubated with and without MGBG, were pulse labeled for 30 and 60 min with 50 μCi of [methy1-3H]thymidine/ml of culture medium (New England Nuclear; 50 to 60 Ci/mmol). Cells were processed for electron microscopy as described above. Sections were coated with Ilford L-4 nuclear emulsion, exposed for 6 to 12 weeks, developed in Microdol-X, and stained with a 5 times diluted solution of standard lead citrate (38). Thymidine-labeled Escherichia coli were processed in parallel (38), to serve as an additional methodological control to assess significant localization of silver grains over small profiles of approximate mitochondrial size.

Isolation of Pulse-labeled mtDNA and Nuclear DNA. RSHT cells growing exponentially in roller bottles (850 sq cm) were exposed either to carrier-free P3 (10 μCi/ml) or to [methy1-3H]thymidine (0.2 μCi/ml; specific activity, 51 mCi/mmol) for 2 to 3 cell generations to uniformly label mtDNA and nuclear DNA (27, 29). The cells were then incubated in the presence of 0, 25, and 50 μM MGBG for 7 and 16 hr, and pulse-labeled with [methy1-3H]thymidine (70 μCi/ml; specific activity, 75 Ci/mmol) during the last 40 min of MGBG treatment. After the pulse, the cells were quickly rinsed with 3 changes of ice-cold nonradioactive medium containing 50 μM unlabeled thymidine (to prevent redistribution of pulse-label radioactivity) (27). All subsequent steps were carried out at 4°. The cells, harvested by quick scraping, were gently homogenized in Solution D [0.7% bovine serum albumin-25 mM Tris·HCl (pH 7.4)-2 mM EDTA-30 mM nicotinamide] (6) and adjusted to 0.3 M sucrose. The following modification permitted recovery of at least 60% of the swollen mitochondria from MGBG-treated cells. The nuclear pellet (480 × g) was gently rinsed with the buffered sucrose solution to separate a loose surface layer of additional enlarged mitochondria; the latter suspension was centrifuged again at 320 × g to sediment traces of nuclei. The combined mitochondrial suspensions were pelleted at 17,000 × g. All cell fractionation procedures were monitored by phase-contrast microscopy; a reference cell fractionation, using MGBG-treated cells stained live with RH 123, was also followed by fluorescence illumination.

The procedures for isolation of radiolabeled mtDNA by centrifugation in CsCl-propidium diodide and linear sucrose gradients, cleavage of mtDNA by restriction endonucleases, agarose gel electrophoresis, and quantitative analysis of the distribution of pulse label in full-length mtDNA and in mapped restriction fragments, have been reported (26–29). Nuclear DNA was prepared from the nuclear pellets (24).

RESULTS

Dosage and Growth Effects of MGBG on RSHT Cells in Monolayer Culture

At least 95% of cells, examined by phase microscopy, uniformly displayed mitochondrial damage at 50 μM MGBG (20 hr)
MGBG Effects on Hamster Tumor Mitochondria

(Fig. 1C) as compared to untreated control cells (Fig. 1A), and nearly 70% of cells were affected to a comparable or lesser extent at 25 μM MGBG. In contrast, exposure to 10 μM MGBG for 24 hr, a condition causing about 40% growth inhibition and extensive ultrastructural damage to mitochondria of mouse leukemia L1210 cells (31), revealed little mitochondrial damage in RSHT cells (less than 5% of cells were visibly affected). The degree of structural impairment in RSHT mitochondria correlated well with the extent of growth inhibition observed at these 3 drug concentrations (Chart 1). The inhibition, only slight at 10 μM MGBG, progressively increased at 25 and 50 μM during exposures of up to 48 hr. Except for a 5 to 10% reduction of attached cells after 24 to 48 hr of culture in 50 μM MGBG, no gross cell loss was evident at any time point monitored; cell viability was at least 96% in cells treated with 25 and 50 μM MGBG, and 100% in control cells and in cells exposed to 10 μM MGBG. Following removal of the drug, nearly normal growth rates resumed after an initial lag period during the first day of culture in drug-free medium (Chart 1).

Mitochondria-associated Rhodamine Probe Fluorescence in Living Control and MGBG-treated Cells

Following a 60-min exposure to the cationic potential-dependent fluorescent probe RH 123, globular and short filamentous mitochondria are the only structures stained in untreated living control cells (Fig. 1B). Strikingly, in at least 96% of MGBG-treated cells, fluorescence is associated also with the extensively swollen mitochondria, appearing most intense at their peripheral membranes (Fig. 1D). Several of the enlarged mitochondria show membranous partitions bisecting or trisecting the organelles (Fig. 1, C and D, arrows), and one organelle profile displays no detectable fluorescence (e.g., Fig. 1, C and D, upper cell, above arrow). The origin of the fluorescent dots seen in many abnormal mitochondria near their periphery is not clear; these may be condensed remnant cristae and/or clusters of mitochondria-associated virus-like particles that are harbored by the RSHT cell line (28). The overall fluorescent intensity appears to differ only slightly within a given cell, suggesting that each subcellular population of mitochondria responds nearly uniformly to both MGBG and rhodamine treatments.

Since direct visualization does not quantitatively assess the ability of the 2 cell types to accumulate and retain probe fluorescence, a property believed to be related to the maintenance of mitochondrial transmembrane function (16), the uptake and retention of RH 123 were assayed biochemically. As shown in Chart 2, both parameters were nearly equivalent in control and MGBG-treated cells; fluorescence retention in rhodamine-free medium by MGBG-treated cells closely paralleled (at a slightly higher level) that by control cells. By contrast, the extent of rhodamine uptake and retention by nontransformed hamster BHK21/C13 cells was considerably higher than that by either RSHT tumor cells or by transformed C13/B4 cells (Chart 2), suggesting innate differences in the ability of different cell types to acquire probe fluorescence (cf. Ref. 15). The findings

Chart 1. Growth kinetics of monolayer RSHT cells in the absence and presence of MGBG, and following drug removal. Cells in log phase (~10⁶ cells/25 cm² flask) were treated with different concentrations of MGBG for 24 to 48 hr, then grown in drug-free medium, and fed daily. Cells were assayed in duplicate (11) at each time point (0.42 ± 0.03 mg protein/10⁶ RSHT cells; see "Materials and Methods"). Points, means; bars, S.E. The attached cells at each point shown were at least 96% viable by trypan blue dye exclusion criteria.

Chart 2. Uptake and retention of potential-dependent fluorescent probe RH 123 by exponentially growing hamster tumor cells (RSHT) untreated (Δ, O) and treated with 50 μM MGBG for 20 hr (Δ, φ), and by nontransformed (C) and virus-transformed (φ) baby hamster kidney fibroblasts. Two separate experiments are shown for RSHT cells grown from 2 different frozen cell stocks. Relative fluorescence was assayed quantitatively (see "Materials and Methods"); RH 123 (5 μg/ml) was added to medium for 60 min, then cells were maintained in RH 123-free medium for up to 20 hr.
for RSHT cells corroborate the fluorescence microscopy data (Fig. 1).

Ultrastructural Changes during MGBG Treatment and Recovery from Drug Exposure

The abnormal mitochondrial profiles of RSHT cells treated with 50 \( \mu \text{M} \) MGBG for 20 hr (Fig. 2, B and C), although enlarged to 5 to 10 times the size of control cell profiles (Fig. 2A), were still enclosed by extended inner and outer membranes; occasionally, small remnants of cristae were evident, or a single crista partition traversed the entire width of the swollen organelles, as also seen by phase and fluorescence microscopy (noted above). Electron-dense inclusions were prominent in many mitochondrial profiles (Fig. 2C); similar inclusions have been seen in MGBG-treated rat intestinal crypt cells (34) and in human but not in mouse cells (23), and may be derived from degenerating cristae or matrix components.

Strikingly, a network of fine fibrils of approximately 20 Å unit width, with the ultrastructural characteristics of mtDNA (cf. Ref. 25; see below), was evident in most clear regions of the swollen matrix (Fig. 2B), sometimes connected to residual cristae, peripheral inner membrane, or to clumped electron-dense inclusions (Fig. 2C). In control cells, visualization of these fibrils was limited within the small electron-lucent regions of the mitochondria (not shown). Extensive cytochemical studies of many cell types have shown previously (see review, Ref. 25) that mtDNA, as well as bacterial and other DNA deficient in bound protein, can be visualized in ultrathin sections, either as condensed clumps of fibers if osmium tetroxide is used as a fixative, or, more characteristically, as a fine network of about 20-Å-thick units if uranyl acetate is used in postfixation; these fibrils are also digestible by DNase, and are frequently seen attached to portions of inner membrane. In the present study, these fibrils of MGBG-treated cells, examined both without (not shown) and with uranyl acetate postfixation, paralleled the typical fixing properties of mtDNA documented earlier; such fixation-dependent changes were not evident in the ultrastructural appearance of other mitochondrial components. In situ autoradiographic studies and isolation and visualization of closed circular mtDNA of characteristic size and nucleotide sequence from these MGBG-damaged organelles (see below) further support the conclusion that mtDNA is retained during MGBG treatment.

The described mitochondrial damage has been observed in 75 to 80% of RSHT cells after 20 hr, and in 90 to 95% of cells after 48 hr of treatment with 25 or 50 \( \mu \text{M} \) MGBG. Nonmitochondrial cytoplasmic structures and nuclei did not appear significantly different from those in control cells. A small, perhaps more resistant, subpopulation of cells revealed nearly normal-sized mitochondria of uncommon ring-shaped structure, which were progressively more apparent from 20 to 48 hr of drug treatment (Fig. 2D), but were not seen in untreated control cells.

After 24 hr in drug-free recovery medium (Fig. 3A), both the number and length of cristae increased in the still slightly enlarged organelles; partially formed cristae appeared diffuse, with blistered extensions, in regions closest to the organelle interior (Fig. 3A and D). At high resolution (Fig. 3D), the fine fibrils interpreted to be mtDNA (see above) were clearly visible, extending from the diffuse distal portions of these cristae. Ring-shaped mitochondria remained abundant (Fig. 3B); endoplasmic reticulum membranes frequently completely surrounded the mitochondria and appeared to be continuous with the outer mitochondrial membrane at selected sites, at which endogenous virus-like particles were typically found either outside or inside the mitochondria (30). Remnants of the original electron-dense inclusions could be resolved within some mitochondria as a crystalline pattern or tubular array (Fig. 3C).

After 5 to 7 days of recovery, 90 to 95% of cells had regained normal or near-normal ultrastructure; many small intramitochondrial vacuoles with lipid-type inclusions prevailed (Fig. 4A). Of special interest is a minor population of cells (about 10% at 5 days and 5% at 7 days of recovery), which had essentially normal ultrastructure, except that mitochondrial recovery was evidently retarded (Fig. 4B). These double membrane-enclosed organelle profiles at high resolution showed abundant mtDNA networks. The viability (judged by trypan blue dye exclusion) of this cell population, identified in attached monolayers by phase microscopy, was indistinguishable (100%) from that of control cells.

In Situ Localization of Thymidine Incorporation in MGBG-treated Cells

Although total mtDNA per cell is only 0.2 to 1% of total nuclear DNA (25), autoradiography (using high isotope levels) is the only method that can show directly whether mtDNA within the MGBG-damaged swollen organelles is capable of replicative activity. The incorporation of a 60-min pulse with \([\text{3H}]\)thymidine was therefore monitored, both during the last hr of a 24- and 48-hr exposure to 50 \( \mu \text{M} \) MGBG, and within 5 hr of recovery in drug-free medium. After 24 hr of exposure to MGBG (Fig. 5B), nuclei of about 20% of cells were labeled (30 to 35% less intensely than in control cells); cytoplasmic areas showed no evidence of \([\text{3H}]\)thymidine incorporation (Fig. 5B), whereas untreated control cells displayed label at the expected level of 1 to 2 silver grains per 2 to 3 small mitochondrial profiles (not shown). At the end of 48-hr exposure to MGBG, only about 3% of nuclei were labeled (at 50% reduced level). Following removal of the drug for 5 hr, however, nuclear DNA synthesis resumed, and many of the still swollen mitochondrial profiles also displayed evidence of \([\text{3H}]\)thymidine incorporation (Fig. 5C and D), especially near cristae or peripheral organelle membranes. This mitochondrial localization of radiolabel is judged significant, since control sections of replicating bacterial profiles processed in parallel revealed almost no random background grains (Fig. 5A). Mitochondrial label was more apparent in cells in which nuclei were unlabeled (probably outside S phase), presumably because a higher level of radiolabel was available for mitochondrial incorporation in the absence of nuclear DNA synthesis; mitochondria of mammalian cells have been shown to synthesize mtDNA continuously throughout the cell cycle (5, 12, 25).

Pulse-Label Analysis of Isolated Topological and Replicative Forms of mtDNA in Control and MGBG-treated Cells

To elucidate the fate of mtDNA species as a result of MGBG treatment, RSHT cells were radiolabeled as described in Materials and Methods. A 40-min \( \text{3H} \) pulse was selected to resolve...
early replicative forms of mtDNA, because it is shorter than the
time required for synthesis of full-length (not yet topologically
matured) mtDNA strands (about 60 min), or for complete repli-
cation of mature circular mtDNA (90 to 120 min) in different
mammalian cells (5, 27). The identification of various topological
and replicative forms of mtDNA in CsCl-dye and linear sucrose
gradients has been well documented (2, 5, 6, 24–29), and the
properties of mtDNA from control RSHT cells (Chart 3A and
Chart 4, A and D) are in close agreement with these results.
After MGBG treatment, however, several findings were of partic-
ular interest. Chart 3 demonstrates that the closed circular
mtDNA (Form I) long-labeled with [14C]thymidine prior to MGBG
treatment, is retained even after a 16-hr exposure to the drug
(Chart 3, B and C). In contrast, the 3H-pulse-labeled mtDNA in
position I (Chart 3A) is progressively reduced and shifted toward
a lighter density region in CsCl-dye gradients (Chart 3, B and C).
In addition, as also observed in L1210 cells (9), the 3H-labeled
dense shoulder of Peak I (Chart 3A), which is the typical position
of the circular (pre-D-loop) replicative intermediate, E-mtDNA (2,
5, 27), is not detected, and 3H radioactivity is moderately reduced
in regions (lm) which contain other replicative intermediates (5,
27) (Chart 3, B and C). On the other hand, Peak II, which consists
largely of nuclear DNA and a small fraction of nicked circular and
linear mtDNA, shows relatively little inhibition of DNA synthesis,
i.e., reduction in 3H/14C labeling. Table 1 quantitates the differ-
tential specific activities of nuclear DNA (from isolated nuclei) and
mtDNA species. MGBG after 24 hr or longer also inhibited
nuclear DNA synthesis to a significant extent (70 to 90%, not
shown).

It was of considerable interest to analyze further the nature of
the abnormally banding pulse-labeled mtDNA MGBG species
(Chart 3), and of the most important results are summarized
below.

Restriction Endonuclease Analysis. The sequence-specific
HindIII and PstI/EcoRI cleavage products of mtDNA I MGBG
(Fig. 6) were typical for mtDNA of Syrian hamster origin (26, 28),
and the ratios of 3H-pulse label to 32P-prelabel in each separated
restriction fragment were nearly equivalent (Table 1). These data
demonstrate that during MGBG treatment 3H-pulse label is ac-
tually incorporated into the interior of at least a fraction of mtDNA
molecules, and is not due to a contaminating nuclear or other
DNA species that copurifies with the mtDNA.

Velocity Sedimentation Analysis. The 3H-pulse-labeled
mtDNA I control sedimented mainly as a 37S peak (Chart 4A); after
denaturation-renaturation, which releases the nonco-
valently attached 7S D-loop strands (which mark the
origin of heavy-strand replication) (5, 26, 27), a prominent 7S
peak is evident (Chart 4D); long-term 14C label was not signifi-
cantly retained in 7S mtDNA, due to the relatively high turnover
rate of the 7S species (2, 5, 27). In contrast, after 7 and 16 hr of
MGBG treatment, the sedimentation properties of both 14C-long-
labeled and 3H-pulse-labeled mtDNAs MGBG were altered, but
in a differential manner (Chart 4, B, C, E, and F); the former
banded at 26 to 27S, rather than at 37S, as the control species,
and the latter was shifted toward a ~30S position, first as a split
peak at 7 hr MGBG (Chart 4B), then as a reduced broad single
peak (Chart 4C). Strikingly, after brief denaturation-renaturation

\[
\begin{array}{ccc}
\text{Treatment} & \text{3H (cpm/µg mtDNA)} & \text{Nuclear DNA} \\
\text{Control} & 3814 & 100 & 100 \\
\text{MGBG (7 hr)} & 2850 & 81 & 76 \\
\text{MGBG (16 hr)} & 950 & 27 & 58 \\
\end{array}
\]

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<td>13.0</td>
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* Ratio of 40-min 3H-pulse-label to 14C-long-label (3 days), calculated as percentage of control ratios. MGBG (50 µM) included in pulse. Duplicate analyses agreed within ±5%.
* Ratio of 60-min 3H-pulse-label to 32P-long-label (3 days) x 10. MGBG (50 µM) included in pulse. Ratios from untreated control cells were 4 times higher (±10%).

b Size of fragments in kilobase pairs (kb); genome size is taken as 15.7 kb (see Ref. 26 for restriction map of Syrian hamster mtDNA, applicable to RSHT). The electrophoresis patterns from which the 4 largest fragments were analyzed are shown in Fig. 6.

d Fragment contains Os, the D-loop replication origin of heavy-strand mtDNA.

Table 1 Effect of MGBG on specific activities of circular mtDNA, nuclear DNA, and restriction fragments of mtDNA

<table>
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<th>Treatment</th>
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<th>Nuclear DNA</th>
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<tr>
<td>Control</td>
<td>3814</td>
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<td>MGBG (7 hr)</td>
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<td>76</td>
</tr>
<tr>
<td>MGBG (16 hr)</td>
<td>950</td>
<td>27</td>
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M. M. K. A/ass confirmed directly the virtual absence of electrophoretically de
control cells; £, 7 hr MGBG; £, 16 hr MGBG. See text for details.
control cells; B, 7 hr MGBG; C, 16 hr MGBG. D to F, mtDNA sedimented after brief
sedimented under native conditions from the following RSHT cells: A, untreated
lyzed by sucrose velocity sedimentation (for isolation, see Chart 3). A to C, mtDNA
velocity sedimentation (Chart 4) contained at least 95% circular
rated into 15 to 20S extended strands) at the time the effects of
progress of replication past the 7S D-loop stage (label incorpo
in vitro 3’ end-labeling of 7S mtDNA with [32P]cordycepin
in the 7S region in either MGBG sample, and the 3H and 14C
peaks were coincident. Further analysis of these mtDNA samples by in vitro 3’ end-labeling of 7S mtDNA with [32P]cordycepin confirmed directly the virtual absence of electrophoretically detectable multiple (cf. Ref. 5) D-loop strands in mtDNA from
MGBG became manifested.
Electron Microscopic Analysis. mtDNA samples used for
velocity sedimentation (Chart 4) contained at least 95% circular
molecules in all control and MGBG mtDNAs (not shown), confirming
the relative lack of degradation of circular genome-sized (~16
kiobase pairs) mtDNA in MGBG-treated cells. The molecules
after drug treatment, however, appeared less supercoiled than
did control mtDNA. All 3 mtDNAs contained approximately 15%
(of total number of molecules scored) complex catenated forms
(2 to 4 linked monomers) (cf. Ref. 25), which under the sedimentation
tions used (Chart 4), are positioned at the bottom
and in Fractions 1 of the gradients.

DISCUSSION

Advantage was taken of the recent discovery that the fluores
cent laser dye RH 123 may be used as a selective supravital
mitochondrial probe (16, 17). The uptake and retention of RH
123 and other permeant cationic probes have been shown to
reflect mitochondrial transmembrane potential (inside negative)
that is maintained by functional mitochondria (15, 16); dead cells
do not take up these probes (7, 16, 17). These techniques have
provided a useful new tool to discriminate functionally altered
living cells in studies of cell cycle (12), lymphocyte stimulation
(7), oncogenic viral transformation (15, 17), and effects of drugs,
such as colchicine (15) and inhibitors of metabolic functions (16).
The present combined probe fluorescence studies (Fig. 1; Chart
two) support the conclusion that the mitochondrial transmembrane
potential after MGBG treatment is maintained in the structurally
damaged organelles at approximately equivalent magnitude per
cell, which may be an important requisite for the ready and rather
uniform recovery responses of most mitochondria in cells re
turned to drug-free medium.
The exact mechanism of RH 123 selectivity toward mitochondria
is not understood (7, 15–17). From the present studies (Fig.
1, C and D), it appears that the dye binds to the abnormally
swollen organelles primarily at the mitochondrial membranes, a
distinction that cannot be made readily in most normal mitochondria
due to their much smaller size. Mitochondrial probe fluorescence has been shown to dissipate under a variety of
conditions, using inhibitors of electron transport (e.g., cyanide,
antimycin A, and rotenone), proton ionophores (e.g., 2,4-dinitro
phenol), and anaerobiosis (16). The occasional absence or loss of
fluorescence associated with an individual swollen organelle
(Fig. 1, C and D) may therefore reflect localized damage to the
mitochondrial membranes and/or energy metabolism; it cannot
be excluded, however, that such a profile represents a vacuol
ated structure of nonmitochondrial origin.
The application of fluorescence techniques as used here may
be of future value to other investigations, especially those dealing
with cellular responses to new mutagens, carcinogens, and
chemotherapeutic agents, since these methods can supplement
conventional more general cell viability tests by providing specific
information relevant to energy requirements (cf. Ref. 16) and the
control of energy metabolism.
The mitochondria-selective ultrastructural changes in MGBG-
treated hamster tumor cells are in remarkable agreement with
observations of other types of rodent and human cells treated
with MGBG (23, 31, 34, 36); the combined results strongly
emphasize the general occurrence and possible significance of
this antimitochondrial action of MGBG. In addition, the present
results more directly corroborate the suggestion (cf. Ref. 35)
that mitochondria may fuse in MGBG-treated cells. Visually, a
smaller number of mitochondria appear to be present in MGBG-
treated (Fig. 1, C and D) than in untreated (Fig. 1, A and B) cells,
and some heterogeneity in mitochondrial size is also evident in
the drug-treated cells; yet quantitatively (Chart 2), roughly equi
alent levels of rhodamine fluorescent probe are bound to the 2
main cell types. Mitochondrial fusion, common in some cell types
under various conditions (25), may therefore contribute to the
apparent reduced number and the large size of the abnormal
organelles. The partitions remaining in some mitochondria (Fig.
1, C and D) may be related to such fusion; alternatively, there
may represent division membranes (cf. Ref. 25) in mitochondria
arrested during MGBG treatment while in the process of repli
cation.
The persistence of mitochondrial profiles of near-normal size
but uncommon structure (Fig. 2D) even after extensive (48-hr)
treatment with 50 mM MGBG may reflect a minor, relatively drug
resistant, population of RSHT cells. This cell line, as many other
uncloned in vivo tumor-derived cell populations, would be ex
pected to exhibit some cell heterogeneity. Resistance to the
effects of MGBG can indeed be selected for in cultured cells (35,
46), and we have obtained RSHT derivatives that have grown

Chart 4. Effects of MGBG treatment on radioactivity profiles of mtDNA I ana
lysized by sucrose velocity sedimentation (for isolation, see Chart 3). A to C, mtDNA
sedimented under native conditions from the following RSHT cells: A, untreated
control cells; £, 7 hr MGBG; £, 16 hr MGBG. D to F, mtDNA sedimented after brief
denaturation-renaturation to release 7S D-loop strands if present: D, untreated
control cells; E, 7 hr MGBG; F, 16 hr MGBG. See text for details.
normally for many cell generations in the presence of 20 μM MGBG.4

The presence of mtDNA networks (e.g., Fig. 2, B and C) has not been previously reported in electron micrographs of MGBG-treated cells (23, 34–36), in which no uranyl acetate postfixation was used, thus mtDNA would be expected to appear clumped (see “Results”) and more difficult to distinguish from products of degeneration within the organelles. The persistence of this DNA (although structurally altered, see below) constitutes another important parameter that seems essential to the rapid restoration of mitochondrial structure and functions after MGBG is removed. Since mtDNA is known to code for the biosynthesis of specific components of the electron transport chain (certain subunits of ATPase, cytochrome oxidase, and cytochrome b(561)) (1), Fig. 3D may uniquely demonstrate sites of mtDNA-directed synthetic activity, as cristae membranes are rebuilt after release from MGBG inhibition. Consequent restoration of oxidative energy metabolism of the cell might be expected to contribute significantly to the resumption of cell proliferation.

The conclusion that MGBG treatment inhibits selectively mtDNA synthesis prior to significant inhibitory effects on nuclear DNA (Table 1; Charts 3 and 4) is in good agreement with findings in L1210 mouse leukemia cells (9), although the effective drug doses and times of treatment used differed in the 2 cell types. The fate of Peak I mtDNA (the major form of mtDNA) was not known from previous studies of L1210 cells, however, since this DNA species was virtually absent at 16 hr of MGBG (10 μM) treatment, a result attributed either to the loss of swollen mitochondria during isolation, or to degradation of mtDNA (9). In the present studies, procedures were adapted to maximize the yields both of drug-damaged mitochondria and of closed circular DNA derived from these organelles. In addition, the data (Chart 4) provided the first direct evidence for the MGBG-induced inhibition of 7S D-loop strand synthesis in mtDNA. This effect, although implied previously (9), was not confirmed by isolation of this single-stranded DNA species. The major function of D-loop strand may be to serve as primers in the novel mtDNA displacement replication mechanism, and/or in a role related to DNA-protein interaction and transcriptional control (cf. Ref. 5).

The altered topology of mtDNA molecules in MGBG-treated cells (Charts 3 and 4) may be a reflection in part of an increased sensitivity of closed circular DNA molecules to subsequent nicking to open circular forms, or (more likely), of induced changes in helical (or other) conformation, which is known to affect the buoyant and sedimentation properties of closed circular DNA (2, 5, 27). Whether MGBG directly interacts in some way with the circular mtDNA in situ is not known, and deserves further studies. Ethidium bromide has been shown to intercalate into mtDNA in vivo, thus altering the topology (superhelical density) (24). However, previous work with native calf thymus DNA (cf. Ref. 35) showed that MGBG and other aliphatic derivatives bind poorly to DNA, as compared to aromatic derivatives. It has been speculated (35) that spermidine (or related compound), which has a stabilizing action on some types of nuclear DNA in vivo, may also have a significant role in the maintenance of in vivo structure and function of mtDNA, especially since the latter, like prokaryotic DNA, is relatively devoid of bound accessory proteins (25). Elucidation of the mechanism and genetic consequence of MGBG-induced mtDNA alterations, and a possible organelle-sequestered and separately regulated polyamine involvement (4, 35), promise to be of particular value in further investigations of mammalian cells damaged by this antitumor drug.

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Fig. 1. Mitochondria-specific staining with the potential-dependent fluorescent probe, RH 123, in living hamster tumor cells (RSHT). A, phase micrograph of control RSHT cell; mitochondria (M) are small and relatively inconspicuous. B, rhodamine fluorescence image of the same cells as in A; note (above horizontal arrow) the lack of fluorescent staining of other cell organelles compared to mitochondria. C, phase micrograph of RSHT cell treated with 50 µM MGBG for 20 hr; mitochondrial internal organization of cristae and matrix is mostly lost; numerous extensively swollen mitochondrial profiles are visible; arrows, groups of mitochondria containing one or 2 membranous partitions. D, rhodamine fluorescence image of the same cells as in C; note (above horizontal arrow) the lack of fluorescent staining of other cell organelles.

Fig. 2. Electron micrographs of hamster tumor cells exposed to 50 µM MGBG. A, untreated RSHT cell; mitochondria (M) are small and relatively inconspicuous. B, RSHT cell treated with MGBG for 20 hr; mitochondrial internal organization of cristae and matrix is mostly lost; numerous extensively swollen mitochondrial profiles are still enclosed by double membranes (see C). C, enlarged view of a mitochondrion in RSHT cell treated with MGBG for 48 hr, showing surrounding double membrane, electron-dense inclusions, and networks of mtDNA fibrils (e.g., arrow) also evident in some profiles in B) within the partially disrupted matrix. × 75,000. D, mitochondria in RSHT cell treated with MGBG for 48 hr; ring-shaped mitochondrial profiles appear relatively resistant to the MGBG-induced effects portrayed in B and C; virus-like particle is seen in upper left; × 55,000, N, nucleus.

Fig. 3. Ultrastructural changes in mitochondria during recovery of MGBG-treated (48 hr) RSHT cells in drug-free medium (24 hr). A and B, swelling of mitochondria is reduced, and many display irregular shapes; a few cristae have formed, which tend to be diffuse or blistered in some portions. A, × 45,000; B, × 41,000. C, high-resolution pattern of intramitochondrial electron-dense inclusions, seen before their dissolution after the first day of cell culture in recovery medium; portion of inner and outer mitochondrial membrane at right; × 153,000. D, enlarged view of mitochondrial revealing mtDNA strands (arrow) connected with cristae at regions that appear relatively translucent (perhaps in the process of regeneration); × 66,000.

Fig. 4. Differences in ultrastructure of mitochondria after culture of MGBG-treated RSHT cells (50 µM; 48 hr) in drug-free medium (7 days). A, examples of near-normal appearance of mitochondria in the majority of cells. × 24,000. B, mitochondrial recovery retarded in some cells (see test); sparse cristae have mostly circular arrangement; after 9 days in drug-free medium (not shown) these cristae are more abundant. × 24,000.

Fig. 5. Electron autoradiographic evidence of [3H]thymidine incorporation into RSHT cells following treatment with MGBG (50 µM; 24 hr). A, methodological control using [3H]thymidine-labeled growing E. coli (see test); × 9,500. B, labeled nucleus of RSHT cell given a 60-min pulse of [3H]thymidine 5 hr after MGBG treatment. × 10,000. C and D, labeled silver grains over mitochondria (same experiment as B) is most pronounced in cells in which nuclei are outside DNA synthetic phase. N, nucleus; C, × 12,000; D, × 28,000.

Fig. 6. Electrophoretic patterns of isolated circular mtDNA from damaged mitochondria of [32P]-labeled RSHT cells treated with 50 µM MGBG for 48 hr. mtDNA digested with (a) HindIII, and (b) PstI + EcoRI restriction endonucleases; kb, reference scale for molecular sizes in kilobase pairs. Patterns match restriction map data for other Syrian hamster mtDNAs (26, 28).
MGBG Effects on Hamster Tumor Mitochondria

3A

3B

3C

3D
Analysis of Methylglyoxal Bis(guanylhydrazone)-induced Alterations of Hamster Tumor Mitochondria by Correlated Studies of Selective Rhodamine Binding, Ultrastructural Damage, DNA Replication, and Reversibility

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