0026-895X/88/010074-06$02.00/0

MOLECULAR PHARMACOLOGY, 34:74-79

The Role of Oxidative and Conjugative Pathways in the Activation of 1,2-Dibromo-3-Chloropropane to DNA-Damaging Products in Rat Testicular Cells

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Received December 2, 1987; Accepted April 18, 1988

SUMMARY

The ability of 1,2-dibromo-3-chloropropane (DBCP), several methylated analogs of DBCP and perdeuterated DBCP (DBCP-D_5) to cause DNA damage in isolated testicular cells from rats was measured by the alkaline elution technique. Of the methylated analogs studied, only the C_3-methyl analog was capable of causing significant DNA damage at concentrations of 0–50 μM. In both time- (0–60 min) and concentration- (0–10 μM) dependent experiments, the testicular cell DNA damage caused by the perdeuterated analog of DBCP closely mimicked the damage resulting from DBCP itself. The lack of an isotope effect between DBCP-D_5 and DBCP strongly suggests that metabolism via a cytochrome P-450-dependent pathway is not involved in the DNA-damaging effects of DBCP in rat testicular cells. In contrast, preincubation for 1 hr with diethylmaleate (DEM) inhibited DBCP-induced (10 μM) DNA damage in a concentration-dependent manner (0–500 μM DEM). The decrease in testicular DNA damage was proportional to the decrease in cellular nonprotein sulfhydryl levels. Similarly, it was shown that 1,2-dibromoethane (EDB), a structurally related halogenated alkane, produced DNA damage in isolated testicular cells in both a time- (0–60 min) and concentration- (0–600 μM) dependent fashion. The DNA damage produced by EDB (600 μM) was also inhibited by pretreatment of testicular cells with DEM (1 mM). The testicular genotoxicity induced by EDB is thought to involve its initial conjugation to glutathione and the subsequent formation of a reactive episulfonium ion. The data presented indicate that similar events may be occurring in DBCP-induced DNA damage in rat testicular cells.

The nematocide DBCP is a potent testicular toxicant in humans and several other mammalian species after either repeated or single exposures (1, 2). The earliest report of DBCP-induced testicular toxicity was published in 1961 (2). Histological examination of the testes from animals receiving DBCP by inhalation for 10 weeks revealed that DBCP caused degenerative changes in the seminiferous tubules, with reduction in the total number of sperm cells, increases in the number of Sertoli cells, and the development of abnormal sperm cells (3). DBCP also produced dose-dependent testicular toxicity after single exposures. The acute damage was characterized by architectural disruption of the seminiferous tubules and severe degenerative changes (2). Human testicular toxicity after DBCP exposure was revealed upon examination of workers involved in the manufacturing and formulation of DBCP (1).

This research was supported in part by National Institutes of Health Grant ES02728. J. G. Omichinski was supported by a NATO Postdoctoral Fellowship 1986.

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The mechanism of DBCP-induced testicular necrosis is at present unknown, but it is generally thought that DBCP is activated by either a cytochrome P-450 or a glutathione *S*-transferase pathway to a reactive metabolite (4, 5). Mutagenic activity of DBCP in the *Salmonella* test is mediated by cytochrome P-450 oxidation and DBCP-d_5 is significantly less mutagenic (6). In contrast, DBCP-d_5 was equipotent with DBCP in causing kidney and testicular damage in rats (7, 8). Based on these results, the mechanism by which DBCP produces testicular toxicity seems to be closely related to the mechanism by which it produces nephrotoxicity, which is apparently different from its mechanism of mutagenicity.

Because DBCP causes testicular DNA damage after *in vitro* exposure as measured by alkaline elution (9), we have studied the ability of several DBCP analogs (Fig. 1) to cause DNA damage in isolated rat testicular cells and compared this with the effects of DBCP. By using specifically deuterated DBCP analogs the involvement of oxidative pathways in DBCP activation was addressed. Specifically methylated DBCP analogs were used to study how larger perturbations of the DBCP

ABBREVIATIONS: DBCP, 1,2-dibromo-3-chloropropane; EDB, ethylene dibromide; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DMSO, dimethyl sulfoxide; DEM, diethylmaleate; NPS, nonprotein sulfhydryl; BSO, buthionine sulfoximine.

structure affected DBCP activation. Further, the effect of lowering testicular cell NPS levels was investigated to reveal an involvement of glutathione conjugate pathways in DBCP-induced DNA damage. The results of similar experiments with 1,2-dibromoethane (EDB), which is known to be activated by a glutathione conjugate mechanism (10, 11), were compared with those with DBCP. Finally, DBCP-induced DNA damage was compared with its plasma membrane-damaging effects to see which of these toxic endpoints could be correlated with *in vivo* toxicity.

Materials and Methods

Chemicals. DBCP, DBCP-d_5 and the methylated DBCP analogs (Fig. 1) were prepared as previously described (6, 12). All analogs tested were ≥98% pure as determined by gas chromatographic analysis. Deuterium content in the deuterated analog was <0.1% $^2H_3$, 10.1% $^2H_4$, and 89.9% $^2H_5$ as determined by mass spectral analysis. 1,2-Dibromoethane (EDB; Gold Label) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and purified by distillation before use. The DBE used in these studies was >99% pure as determined by gas chromatographic analysis. Other chemicals were obtained from the following sources: N-methylnitrosourea and trypsin from Sigma Chemical Company (St. Louis, MO); DMSO from Rathburn Chemicals Limited (Walkerburn, Scotland); DEM from Merck-Schuchardt (Darmstadt, FRG); and collagenase from Worthington Biochemical Company (Freehold, NJ). Other chemicals were analytical grade from commercial suppliers.

Animals. Male Wistar rats weighing 200–250 g were obtained from Bomholdtgård Breeding and Research Centre (Ejby, Denmark). Animals were given food (Ewos R3; Ewos AB, Södertälje, Sweden) and water *ad libitum*.

Preparation of testicular cells. Testicular cells were prepared in each experiment from testes pooled from three or more animals as previously described (7, 9). Seminiferous tubules were incubated in Hanks' HEPES buffer with collagenase (100 U/ml) for 20 min at 33°, trypsin (0.25 mg/ml) was then added, and the tubular suspensions were incubated for an additional 20 min. To remove large fragments and spermatozoa, the suspensions were filtered through a layer of gauze and then through a 0.4-mm nylon mesh. The filtrate was then centrifuged three times at 200 × *g* for 4 min. The pellet from the final centrifugation was resuspended in Hanks' HEPES buffer containing 1% bovine serum albumin and filtered through a 0.25-mm nylon mesh. The final cell suspensions (total yield, ~10^8 cells/rat) contained on average approximately 70% primary and secondary spermatocytes, 15% spermatids, 10% Sertoli cells, and 5% Leydig cells, as determined by microscopic examination of stained cells, which is in accordance with values reported (9). Cell viability was always greater than 95% as measured by trypan blue exclusion (13).

Testicular DNA damage. Testicular cells (4 × 10^6), as a suspension in 2 ml of Hanks' HEPES buffer with 1% albumin, were exposed to appropriate concentrations of the test chemicals at 33°. The test substances were dissolved in DMSO and the final concentration of DMSO in the incubations was 0.25%, v/v, of the medium (DMSO served as the control). After the exposure periods, 2 ml of additional cold buffer was added to each incubation and the resulting suspensions were centrifuged at 200 × *g* for 4 min. The supernatant was discarded, the cell pellet was resuspended in 4 ml fresh cold buffer, and ~1.5 × 10^6 cells of the resulting suspension was used to assess DNA damage. The DNA damage was measured using an automated version of the alkaline elution technique, as previously described (14, 15).

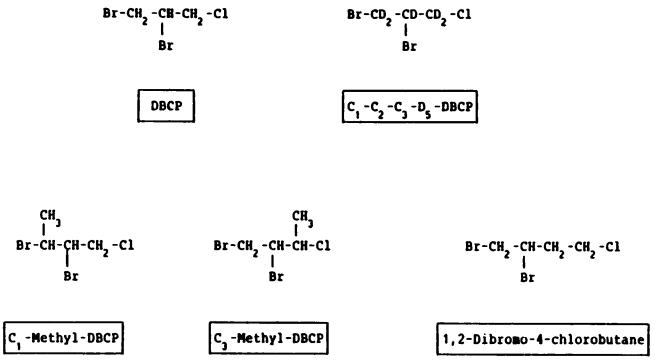

Fig. 1. Structures of DBCP, DBCP-d_5, and methylated DBCP analogs.

The experiments to deplete testicular glutathione levels required that a slight modification was made in the exposure protocol. The testicular cell suspensions were first incubated in the presence of DEM (0.05–1.0 mM) for 60 min at 33° before exposure to the test substances for 30 min at 33°. After exposure to the test compounds, the cells were prepared and analyzed for DNA damage as described above. DEM pretreatment was not cytotoxic to the cells.

Variability between experiments in this study was less than 10% as determined by calculation of the first-order rate constant for the initial phase of the elution (16). DMSO values were 9.2 ± 2.2 hr^{-1} (10 experiments) whereas 10 μM DBCP values were 224.0 ± 14.6 hr^{-1} (10 experiments) after a 60-min incubation time.

Cytotoxicity studies. Testicular cells (2 × 10^6 cells /ml) were incubated for 60 and 180 min at 33° with varying concentrations of the test substances added in DMSO (final DMSO concentration was 0.25%, v/v, of the medium). Cell viability indicative of plasma membrane damage was assessed after the exposure period by the trypan blue exclusion method (13).

Determination of cellular NPS levels. Relative NPS levels were determined using the method of Ellman (17) as follows: The pellet from 4 ml of cell suspension (8 × 10^6 cells) was resuspended in 0.25 ml of 0.2 M phosphate buffer, pH 7.4, precipitated immediately with 0.25 ml of 4% salicylic acid, and centrifuged 15 min at 1500 × *g*. Then, 0.2 ml of the supernatant was added to 1.8 ml of a solution of 1 mM 5,5-dithiobis-(2-nitrobenzoid acid) prepared in 0.2 M phosphate buffer, pH 8.0, and the absorbance at 412 nm was determined.

Mutagenicity studies. Mutagenic activity was assayed using *Salmonella typhimurium* TA100 (18). An overnight culture of tester strain TA100 (0.1 ml, ~ 10^9 bacteria) was incubated at 33° for 2 hr either in the presence or absence of isolated testicular cells (4 × 10^6/ml), in a total volume of 2 ml of Hanks' HEPES buffer containing 1% bovine serum albumin. The test compounds were added as solutions in DMSO (5 μl). After the exposure period, the suspension was centrifuged 15 min at 1500 × *g*. The resulting pellet was resuspended in 0.5 ml of phosphate-buffered saline and plated to determine the number of His^+ revertants. The spontaneous revertants in each experiment were subtracted from the revertants obtained with the test compound.

Results

Effects of methylation of DBCP on alkaline elution of DNA from testicular cells. The testicular DNA-damaging effects of DBCP, C_1-methyl DBCP, C_3-methyl DBCP, and 1,2-dibromo-4-chlorobutane were assessed in isolated testicular cells using the alkaline elution method (14, 15). All three of the analogs were less potent than DBCP in causing testicular DNA damage after 60-min exposures at several concentrations (Fig. 2, A–C). At 10 μM concentrations, only DBCP caused DNA damage that was different from control (DMSO) after a 60-min exposure period (Fig. 2A). Of the three analogs examined, only C_3-methyl DBCP produced a concentration-dependent increase in DNA damage in the alkaline elution assay at the concentrations tested. The relative *in vitro* DNA-damaging potencies of the four compounds tested here correlates with their ability to induce testicular necrosis and testicular DNA damage after single *in vivo* exposures (7).

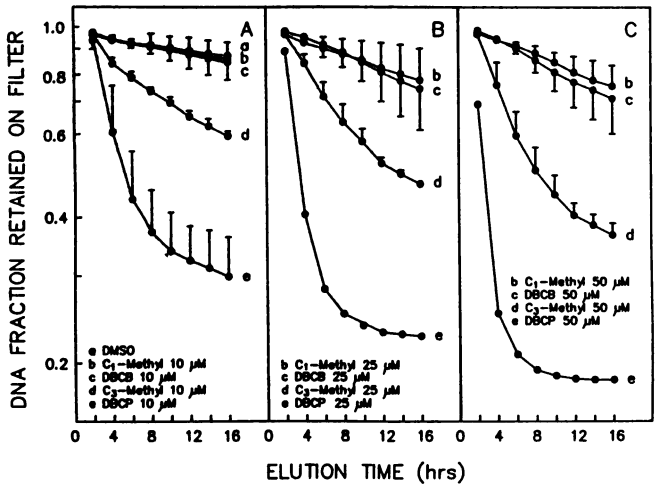

Fig. 2. Testicular DNA damage caused by DBCP and methylated DBCP analogs as measured by alkaline elution. Isolated testicular cells from rats were exposed to 10 μM (A), 25 μM (B), and 50 μM (C) concentrations of the test substances for 60 min. Values are means ± standard deviations of three experiments or means of two using cells pooled from three or more animals.

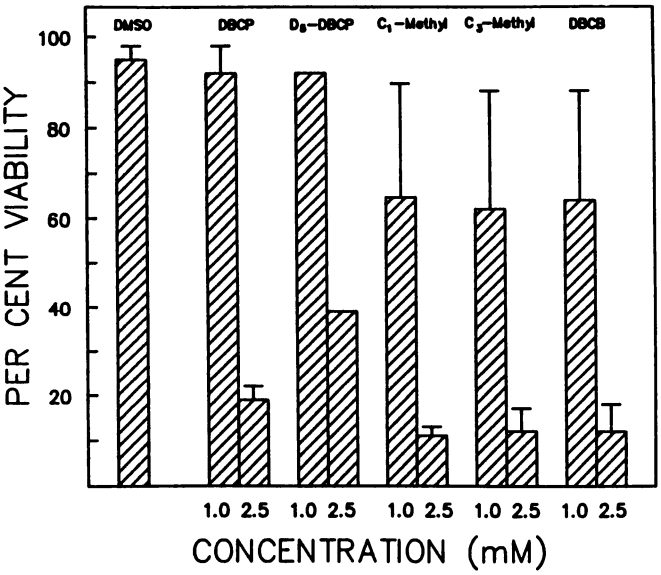

Fig. 3. Effect of various concentrations of DBCP and DBCP analogs on cell viability (trypan blue exclusion) of testicular cells isolated from rats. Values are means ± standard deviations of three experiments or means of two using cells pooled from three or more animals.

Mutagenicity and cytotoxicity studies. In an attempt to assess the significance of *in vitro* alkaline elution as a correlate to other DBCP toxic effects, cytotoxicity and mutagenicity experiments were conducted with isolated testicular cells exposed to DBCP. Cytotoxicity (as determined by trypan blue exclusion) after DBCP exposure was not observed until DBCP concentrations exceeded 1 mM (Fig. 3). Similar results were also obtained in experiments with the perdeuterated as well as the methylated analogs of DBCP (Fig. 3). Based on these studies there appears to be a rather poor correlation between the *in vitro* cytotoxicity of these analogs and their *in vivo* testicular effects (7). In mutagenicity experiments with *S. typhimurium* TA 100, DBCP failed to induce a mutagenic response above background levels when tested with several concentrations, either in the presence or absence of isolated testicular cells (Fig. 4). At 2.5 mM concentrations DBCP became directly cytotoxic to the bacterial cells. In contrast, EDB was mutagenic both in the absence and presence of the testicular cells. From these results, it appears that mutagenicity assays also are a poor model to study mechanisms of DBCP-mediated testicular necrosis and/or DNA damage after exposure *in vivo* to a single acute dose of DBCP. In contrast, the results of alkaline elution assays correlate well with the toxicity data *in vivo*.

Effect of perdeuteration of DBCP on alkaline elution of DNA from testicular cells. To study the role of cytochrome P-450 oxidative metabolism in the *in vitro* testicular DNA-damaging effects of DBCP, DBCP-d_5 was tested in the alkaline elution system and its effects were compared with those of the parent compound. The perdeuterated analog caused DNA damage in isolated testicular cells equivalent to that caused by DBCP after 60-min exposures at several concentrations (Fig. 5A). The 10 μm concentration of DBCP and DBCP-d_5 were also tested at various time points (0–60 min) to further evaluate possible differential effects. The degree of DNA damage caused by a 10 μM DBCP-d_5 was similar to that caused by 10 μM DBCP at all time points (Fig. 5B). Thus, at all concentrations and time points tested, there was no apparent isotope effect on the testicular DNA-damaging effects of DBCP. Furthermore, addition of the cytochrome P-450 inhibitor metyrapone (1 mM) failed to significantly alter the DNA-damaging effects of DBCP (10 μM at 30 min) (data not shown; no positive control is known to exist for this inhibitor in this system so its effectiveness cannot be totally evaluated). It therefore seems that cytochrome P-450 oxidative metabolism may serve little or no role as an activation pathway in the *in vitro* testicular DNA-damaging effects of DBCP. These results are in agreement with the testicular necrosis and testicular DNA damage after single *in vivo* exposures to DBCP and DBCP-D_5 (7).

Effect of DEM pretreatment on the testicular DNA damage induced by DBCP. The role of glutathione conjugative metabolism in the *in vitro* testicular DNA-damaging effects of DBCP was examined by pretreating the cells with several different concentrations of DEM (0–1 mM) for 60 min in order to alter intracellular NPS concentrations. The DEM

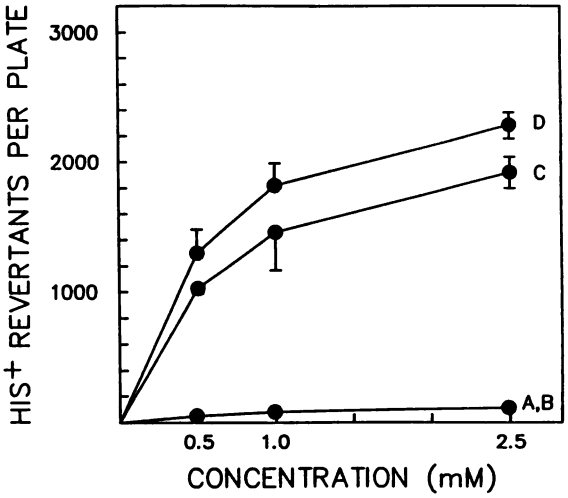

Fig. 4. Concentration-dependent mutagenicity of DBCP (*A*, *B*) and EDB (*C*, *D*) incubated either in the presence (*A*, *C*) or absence (*B*, *D*) of testicular cells isolated from rats. Values are means ± standard deviations of three or more plates of cells pooled from three or more animals.

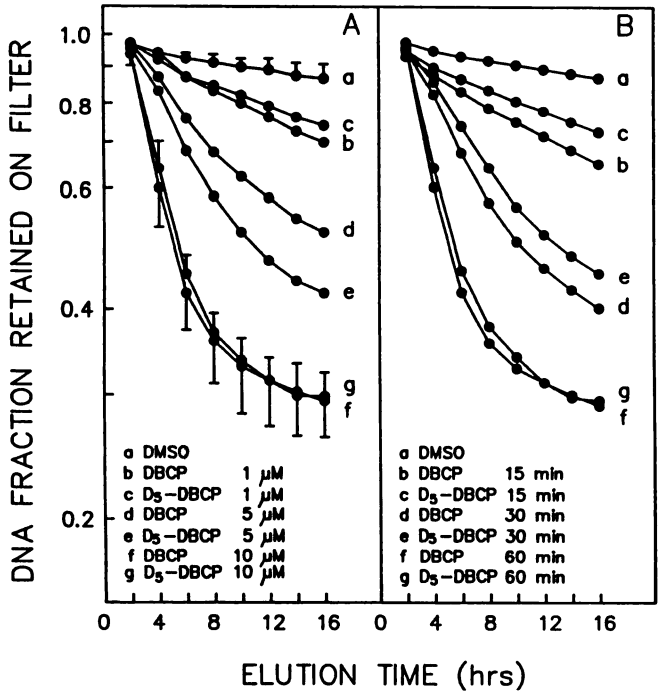

Fig. 5. Effect of concentration (A) and time (B) on the testicular DNA damage induced by DBCP and DBCP-d_5 as measured by alkaline elution. In the concentration-dependent experiments (A), the cells were exposed to the test substances for 60 min, whereas in the time-dependent experiments (B) 10 μM concentration of test substances were used.Values are means ± standard deviations of three experiments or means of two using cells pooled from three or more animals.

pretreatments (60 min) deceased the DNA-damaging actions of DBCP (10 μM at 30 min) in a concentration-dependent (0–1 mM) fashion (Fig. 6A). The alteration of the DNA damage was proportional to the ability of DEM to deplete NPS levels in isolated testicular cells (Fig. 6B). In separate experiments, EDB was found to cause DNA damage in the isolated testicular cells in a concentration-dependent (0–600 μM) and a time-dependent (0–60 min) manner. It could be demonstrated that DEM (1 mM) blocked the DNA damage produced by EDB (600 μM at 30 min; Fig 6C), but it increased the DNA-damaging effects of *N*-methyl-nitrosourea (100 μM at 30 min; Fig 6D). The results after DEM pretreatment were the same whether the exposure to the test agents were done in the presence or absence of DEM (data not shown). Further attempts to deplete NPS levels with BSO were unsuccessful.

Discussion

DBCP is one of several vicinally dihalogenated compounds reported to produce adverse effects in the testis after either single or repeated exposures. The mechanism(s) by which DBCP induces its toxic effects in the testis are unknown. Because DBCP is mutagenic to bacteria after activation by cytochrome P-450-dependent metabolism (6, 19, 20), it has been postulated that a similar pathway is involved in DBCP testicular toxicity (3, 4, 21). This pathway is supported by the fact that 3-chloro-1,2-propaneoxide (epichlorohydrin) and 3-chloropropanediol (α-chlorohydrin), two postulated oxidative metabolites of DBCP, produce testicular lesions similar to those observed after DBCP exposure (21). Recent *in vivo* metabolic studies indicate that these metabolites are insignificant in DBCP-induced toxicity *in vivo* and alternatively, it has been suggested that DBCP activation occurs via a glutathione *S*-transferase-initiated pathway (5). It was proposed that formation of an intermediate episulfonium ion subsequent to an initial glutathione conjugation reaction may result in direct alkylation of target molecules (5, 22). However, the role of glutathione-dependent metabolism in any DBCP-related toxicity has yet to be clearly documented, as has been previously demonstrated with other vicinally dihalogenated compounds such as EDB (10, 11) and dichloroethane (23, 24).

The testicular DNA damage produced by DBCP and several methylated analogs *in vitro* correlated with their ability to induce testicular necrosis after exposure to DBCP *in vivo* (7). DBCP was clearly the most potent analog *in vitro* and, of the methylated compounds tested, only the C_3-methyl analog caused DNA damage. *In vivo*, only the C_3-methyl analog produced testicular necrosis after a 340 μmol/kg dose, but it was significantly less toxic than DBCP (7). On the basis of these results, it seems that the *in vitro* alkaline elution system is a good model for testicular DNA damage and cell necrosis *in vivo*. This indicates that DBCP testicular toxicity could be initiated by metabolic events that occur entirely within the testis and that transport of metabolites from other organs (e.g., liver, kidney) would not be necessary for DBCP to produce adverse effects in the testes. It would also be tempting to hypothesize that DNA damage may be an initiating event in DBCP-induced cell death *in vivo*. However, to substantiate both of these hypotheses will require further investigations.

In the present studies, the relative contributions of oxidative and conjugative pathways of metabolism in the DNA-damaging effects of DBCP were investigated in isolated testicular cells from rats. The lack of an apparent isotope effect with DBCP-D_5 indicates that a pathway that involves cytochrome P-450-mediated oxidation is not a likely route of activation to the testicular DNA damage induced by DBCP. On the other hand, pretreatment with DEM (0–500 μM) to deplete testicular cell glutathione levels resulted in an attenuation of the DNA-damaging effects of DBCP in a manner directly proportional to the decrease in glutathione levels. At the 500 μM concentration of DEM, the DNA damage caused by 10 μM DBCP was completely abolished. It could therefore be postulated that conjugation of DBCP to glutathione and subsequent formation of a reactive episulfonium ion is the major pathway that causes DNA damage after exposure of isolated testicular cells from rats to DBCP. Such a mechanism has been proposed and tested to explain some of the genotoxic effects observed with the structurally related dibrominated alkane EDB in rat spermatocytes (24). The role of glutathione activation is further supported by the fact that DEM pretreatment was also able to inhibit the DNA-damaging effects of EDB. The differences in potency between DBCP and EDB in this system may be the direct result of their relative rates of metabolism by testicular glutathione-*S*-transferases. It has been reported that release of bromide is approximately 10 times higher from DBCP than from EDB when these compounds are incubated with rat testicular cytosols in the presence of glutathione (25). However, these results must be interpreted with caution because the effects of DEM on the cell are not entirely specific towards glutathione. Attempts to deplete glutathione with more specific agents such as BSO were unsuccessful, possibly due either to lack of uptake by the cells or due to a slow glutathione turnover.

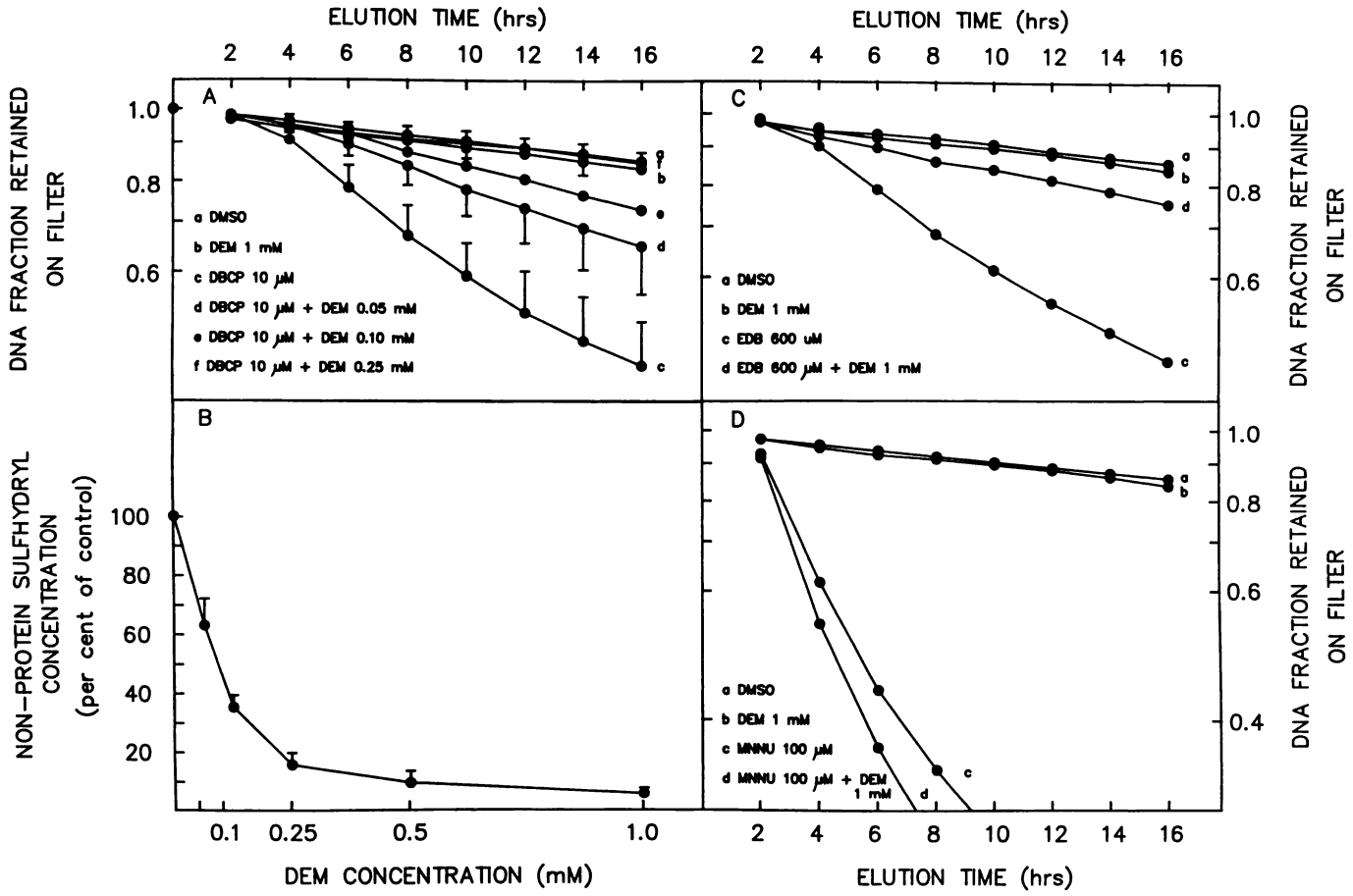

Fig. 6. Effect of DEM on DBCP- (A), EDB- (C), and *N*-methylnitrosourea (*MNNU*)- (D) induced DNA damage and glutathione concentration (B) in isolated testicular cells. Values are means ± standard deviations of three experiments or means of two using cells pooled from three animals.

However, both DEM and BSO have been shown to inhibit DBCP-induced DNA damage in isolated hepatocytes (26).

In summary, this study has provided evidence that DBCP can be directly activated in testicular cells to products capable of causing DNA damage. The ability of several methylated analogs of DBCP to cause DNA damage correlated with their ability to cause testicular necrosis *in vivo*. Depletion of cellular NPS levels with DEM decreased the ability of DBCP to cause DNA damage whereas deuterium substitution for hydrogen in DBCP had little or no effect. Therefore, a role for glutathione conjugation with the subsequent formation of an episulfonium ion must be strongly considered in the pathogenesis of testicular DNA damage and cell death caused by DBCP.

Acknowledgments

We appreciate the technical assistance of Ms. Bente Trygg and Ms. Kirsti Bekkedal, the graphical work performed by Ms. Lise Timm Haug and the typing of Ms. Elisabeth Parr.

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