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DNA CLASTOGENIC ACTIVITY OF DIETHYLSTILBESTROL

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Abstract—Incubation of human leukocytes with the synthetic estrogen and known human carcinogen, diethylstilbestrol (DES), for 40 min caused extensive DNA strand breakage (clastogenesis), as measured by a fluorometric assay. The level of DNA clastogenesis was dose dependent above an apparent threshold of 10 μM. Clastogenesis was increased by addition of cysteamine, a reducing agent and hydroxyl radical scavenger, and was blocked by low concentrations of plasma. DES epoxide, a weakly estrogenic derivative, was about one-tenth as potent as a DNA clastogen. Unexpected and paradoxical findings were observed when cells were treated with DES in the presence of a hydrogen peroxide-generating system plus a peroxidase. At the subthreshold concentration of 10 μM DES, the oxidizing system *increased* DNA clastogenicity, yet at 30 μM DES the oxidizing system *decreased* clastogenicity. The addition of superoxide dismutase to the oxidizing system increased clastogenicity at both concentrations of DES. DNA damage was largely blocked by arsenite, *N*-ethylmaleimide, iodoacetamide and bromophenacyl bromide. These experiments provide further indication of the complex nature of reactions involving DES which can lead to DNA damage and which may be relevant to DES-induced carcinogenesis.

The synthetic estrogen diethylstilbestrol (DES)† has been investigated extensively in terms of the mechanism of its carcinogenic action, particularly since the discovery that *in utero* exposure predisposes young women to adenocarcinoma of the vagina [1]. While DES itself has not been shown to react with DNA, several investigators have shown that radiolabeled DES will bind covalently to DNA and/or protein in the presence of oxidizing systems such as iodine [2], various purified and partially-purified peroxidases plus hydrogen peroxide [3, 4], liver microsomes plus NADPH [5] and prostaglandin synthetase plus arachidonate [6, 7]. DES does not possess mutagenic activity for bacteria or mammalian cells in culture [8–11]. However, there is a report that in the presence of strong oxidizing agents it causes mutations in yeast [12] and an unconfirmed report that it converts mouse lymphoma L5178Y cells to the TK-deficient phenotype in the presence of liver extract [13]. In both cases, cell killing was extensive under conditions where these phenotypic changes could be observed. In the presence of an extract with peroxidase activity (but not in its absence), exposure to DES caused unscheduled DNA synthesis (UDS) in rat hepatocytes and in HeLa and Syrian hamster embryo cells [14–16]. Despite its apparent ineffectiveness as a mutagen, either with or without an oxidizing system, DES has been reported to cause neoplastic transformation in Syrian hamster embryo cells [8] and chromosomal abnormalities such as polyploidy in Chinese hamster cells [17], mitotic chromosome nondisjunction in HeLa cells [18] and aneuploidy in mouse embryos, mouse bone marrow and cultured mammalian cells [19–21]. It has been reported to cause SCEs in some systems but not in others [10, 22–24]. Some of the chromosome changes, particularly changes in ploidy, are presumably due to the colchicine-like action of DES [17]. The genotoxic and cytotoxic potential of DES is also of interest in connection with its use in the chemotherapy of prostatic and mammary cancer.

In this report we describe experiments which indicate that a short period of exposure to DES in the absence of serum or plasma is capable of causing DNA strand breaks in human leukocytes and mouse thymocytes. Similar observations about DNA strand breakage have been made recently by Goldenberg and Froese [25] with L5178Y cells and by Douglas *et al.* [26] which CHO cells. Since oxidizing conditions have been reported to "activate" DES *in vitro*, we also studied the effect of a hydrogen peroxide-generating system plus a peroxidase on the DNA clastogenic (strand-breaking) action of DES. Unexpected and paradoxical effects were observed. These oxidizing conditions increased the DNA clastogenic activity of 10 μM DES but decreased the clastogenic activity of 30 μM DES. The addition of superoxide dismutase increased clastogenicity for both concentrations of DES under these oxidizing conditions. These experiments provide additional indication of the potential complexity of DES-cellular interactions and support the possibility that DNA damage may be involved in DES carcinogenicity.

MATERIALS AND METHODS

Human leukocytes were isolated from freshly drawn blood, collected in 10-ml B-D Vacutainer tubes containing EDTA (3.6 mM) as anticoagulant. Red blood cells were removed by lysis with ammonium chloride (3 vol. of 0.15 N NH_4Cl, 0.01 M

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† Abbreviations: DES, diethylstilbestrol; UDS, unscheduled DNA synthesis; SCE, sister chromatid exchange; FADU, fluorometric analysis of DNA unwinding; SOD, superoxide dismutase; DES epoxide, DES-α, β-oxide; BSS, balanced salt solution; and TPA, tetradecanoylphorbol acetate. AECL no. 8810.

Tris·HCl, pH 7.4, to 1 vol. of blood) at 15° for 10–15 min. Leukocytes were collected by centrifugation (10 min, 0°, 600 *g*) and suspended in 1 vol. of BSS (137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 8.5 mM sodium phosphate, 5 mM glucose, pH 7.4) at 0°. Residual contaminating red blood cells were removed by hypotonic lysis as follows. Two volumes of cold water (0°) was added briskly to the cell suspension which was immediately mixed several times by inversion. After 60 sec, 0.22 vol. of 1.5 M NaCl was added to restore isotonicity; the leukocytes were collected by centrifugation, washed once with BSS and suspended in BSS at 2–3 × 10^6 cells/ml. Where autologous plasma was required, blood samples were centrifuged lightly in their original Vacutainer tubes, 20–40% of the plasma layer was removed, and this was clarified further by centrifugation at 2000 *g* for 10 min at room temperature. The remainder of the blood sample was treated with ammonium chloride as above to isolate leukocytes.

Mouse thymocytes were isolated from 16 to 20 week-old female CD-1 by pressing the thymus through an 80 mesh stainless steel screen as described earlier [27]; the cells were suspended in BSS.

Cell viability was monitored by trypan blue exclusion [28]. Treated cell samples (0.15 ml) were mixed with 0.05 ml of 0.4% trypan blue (Gibco Laboratories) and 0.05 ml of fetal calf serum. After a 5-min incubation at ambient temperature in 1.5-ml polypropylene tubes, the samples were transferred to 0°. Hemocytometer counts of total and stained cells were carried out twice over a period of approximately 2 hr. Under these conditions, the cells appeared to be stable, i.e. the proportion of stained cells did not change significantly between the two counts.

DNA damage analysis was carried out using a fluorometric technique (Fluorometric Analysis of DNA Unwinding, FADU) which measures the rate of unwinding of cellular DNA on exposure to alkaline conditions; the technique has been described in detail previously [29]. In brief, 5 ml of cell suspension (1 to 1.5 × 10^7 cells) was treated with DES or other agents as described in Results. The cells were then washed and lysed with a urea/detergent solution. An alkaline solution was added and DNA strand unwinding was allowed to occur for a 60-min period at 15°. Following this treatment, the samples were neutralized, and the amount of residual double-strand DNA was estimated using a fluorescent dye, ethidium bromide. This allows calculation of the rate of DNA unwinding which, in turn, can be correlated with the number of strand breaks per cell by reference to the effect produced by gamma-rays, assuming that 1 Gy produces 1200 strand breaks/cell [30]. Single-strand breaks, double-strand breaks and alkali-labile lesions are detected but not distinguished by this method.

Diethylstilbestrol and catalase were supplied by the Sigma Chemical Co. DES was used from a stock solution of 3 mM in DMSO. Diethylstilbestrol epoxide (DES-epoxide), prepared as described earlier [31], was a gift from Dr. P. H. Jellinck. Superoxide dismutase (SOD) was obtained as Palosein (Orgotein) from Diagnostic Data Inc., Mountain View, CA.

RESULTS

DNA strand breakage by DES. DNA strand breaks induced in human leukocytes by a variety of physical and chemical agents can readily be detected by the FADU technique [29]. Because of our interest in the relationship between DNA strand breaks and early events in carcinogenesis [30, 32, 33], we examined the effects of DES (a known carcinogen) on DNA of human white blood cells (WBC). The data of Fig. 1 indicate that this agent was DNA clastogenic when human white blood cells were treated for 40 min at 37° in a balanced salt solution. The effect was not limited to human cells; mouse thymocytes treated under the same conditions showed similar DNA damage (Fig. 1B). In both cases, DNA strand breakage showed a clear threshold effect, with few strand breaks detected at or below 10 μM.

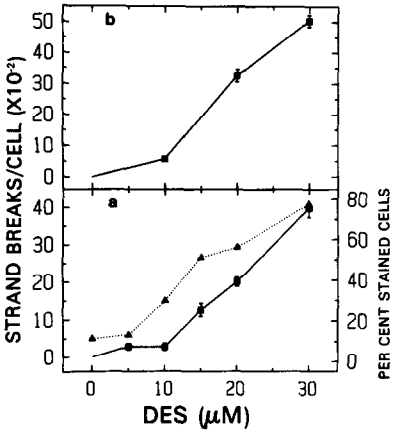

Fig. 1. DNA clastogenesis and cytotoxicity induced in human leukocytes and mouse thymocytes by DES. Cells were suspended in BSS at 2–3 × 10^6/ml and incubated with the indicated concentration of DES for 40 min at 37°. DNA clastogenesis (strand breakage) was then measured by the FADU technique. Cytotoxicity was determined as the percentage of cells which stained with trypan blue. (a) Human leukocytes. (b) Mouse thymocytes. Key: (■) strand breaks; and (▲) stained cells. Error bars: standard error of the mean.

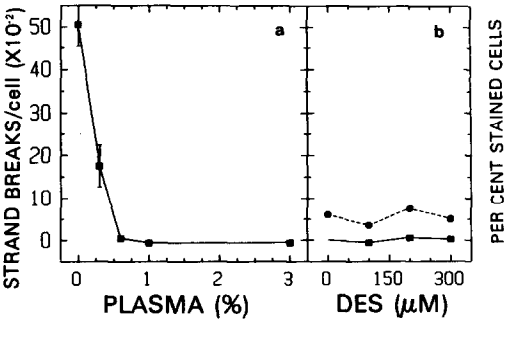

Fig. 2. Inhibition of DES-induced DNA clastogenesis and cytotoxicity by plasma. Human leukocytes were incubated in BSS with DES for 40 min at 37°. (a) DES concentration was 30 μM and the indicated amount of autologous plasma was added. Four analyses were carried out; the S.E.M. is indicated by error bars or is within the symbol. (b) 10% Autologous plasma was used; single experiment. Key: (■) strand breaks; and (●) stained cells.

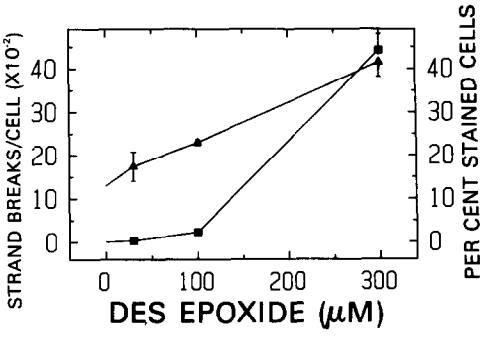

Fig. 3. DNA clastogenesis and cytotoxicity induced in human leukocytes by DES epoxide. Conditions were as in Fig. 1. Average of five experiments. Error bars indicate S.E.M.; where error bars are not shown, they are within the symbols. Key: (■) strand breaks; and (▲) stained cells.

Relationship between strand breakage and cytotoxicity. Earlier studies have indicated that DES can be very cytotoxic, particularly when clonogenic assays are used [34]. To explore the relationship between strand breakage and "cytotoxicity", the effect of DES on cell viability (measured as trypan blue exclusion) was examined under conditions identical to those used for testing strand breakage. Dye exclusion was used as a measure of viability because (i) it provides an early indication of cytotoxicity as compared to colony-forming ability, and (ii) a clonogenic assay cannot be used since leukocytes are not dividing cells. The data of Fig. 1 indicate that cytotoxicity closely parallels DNA strand breakage as a function of DES concentration, perhaps preceding it slightly. Cell clumping was visible at 30 μM DES, but there was no microscopic indication of gross cell disruption.

Influence of plasma on DES-induced strand breakage and cytotoxicity. Most published reports have indicated that cytotoxicity is observed only at concentrations of DES higher than those used in Fig. 1; one exception is the report of Goldenberg and Froese [34]. In their experiments, treatment of cells with DES was carried out in the absence of serum or plasma, so we explored the possibility that plasma could have a significant protective effect. The data of Fig. 2 indicate that this is indeed the case. The presence of as little as 0.3% autologous serum protected cells against the induction of strand breaks by 30 μM DES (Fig. 2). The presence of 10% autologous plasma protected cells against both the cytotoxic and DNA clastogenic effect of up to 300 μM DES

Table 1. Effect of post-treatment incubation on the level of DES-induced strand breaks*

Donor No.	Age, sex	Initial level of strand breaks ($\times 10^{-2}$)	Final level of strand breaks ($\times 10^{-2}$)	Percent change
1	52,m	47.9	90.7	+189
2	50,m	52.8	74.4	+141
3	29,m	39.6	86.7	+219
4	20,m	36.6	99.2	+271
5	44,m	33.0	55.1	+167
Average		42.0	81.2	+193

* Human WBC were isolated as described in Materials and Methods and incubated at 37° for 40 min with DES (30 μM). A portion of the cell suspension was chilled, washed once with BSS, resuspended in BSS containing 10% autologous plasma, and incubated further for 30 min. The "initial level" of DNA strand breaks refers to cells before the additional 30-min incubation period. Control cells (not exposed to DES) were subjected to similar manipulations. Strand breaks were determined by the FADU technique.

Table 2. Modifiers of DES-induced DNA damage*

		Strand breaks/cell ($\times 10^{-2}$)	Relative amount of damage (%)			
Donor No.	Age, sex	No addition	Cysteamine (20 mM)	KCN (1 mM)	SOD (250 μg/ml)	Catalase (200 μg/ml)
6	43,m	49.6				112.7
7	33,f	31.1				196.5
8	22,m	30.0	215.5	96.4	152.7	155.2
		25.4	184.4			148.0
9	35,m	35.1	190.3	125.8	111.8	72.7
		30.9	163.0			
10	26,m	50.5	164.0	78.5	92.9	91.5
		46.8	151.9		98.3	97.6

* Human WBC were incubated in BSS at 37° for 40 min with DES (30 μM) and the indicated agent. The final concentration of solvent (DMSO) was 1%. Strand breaks were determined by the FADU technique.

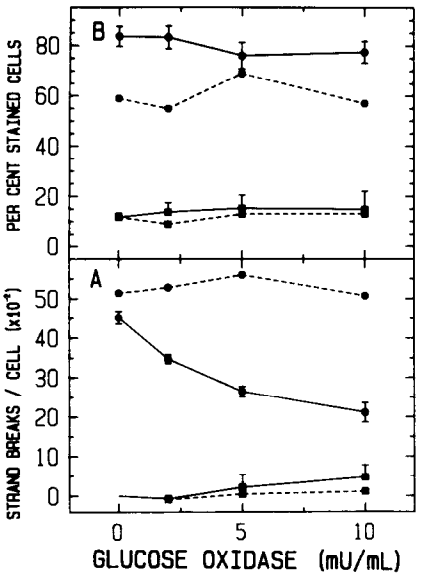

Fig. 4. Effect of oxidizing conditions on (A) DNA clastogenesis and (B) cytotoxicity induced in human leukocytes by DES. Cells were incubated with horseradish peroxidase (2 μg/ml) and indicated concentration of glucose oxidase. Other conditions were as in Fig. 1. Key: (●) DES at 30 μM (■) no DES added; (———) no catalase; and (---) catalase at 50 μg/ml. Error bars: S.E.M. of six experiments.

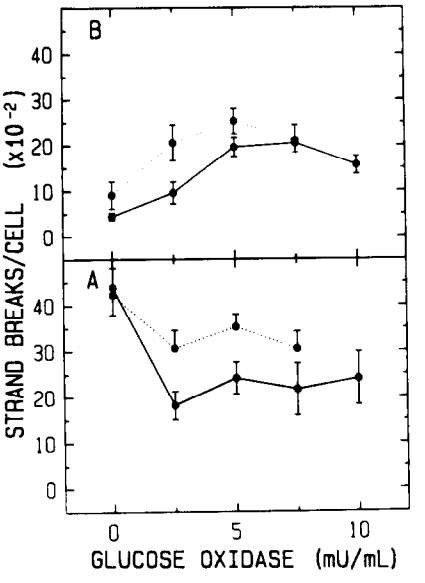

Fig. 5. Effect of oxidizing conditions and SOD on DNA clastogenesis induced in human leukocytes by high and low levels of DES. Cells were incubated with horseradish peroxidase (2 μg/ml) and indicated concentration of glucose oxidase. Other conditions were as in Fig. 1. (A) DES at 30 μg/ml. (B) DES at 10 μg/ml. Key: (———) no SOD; and (·····) 10 μg/ml SOD added. Error bars: S.E.M. of three experiments.

Table 3. Inhibitors of DES-induced DNA damage*

Agent tested (no. of donors)	Strand breaks/cell (× 10^{-2}) DES alone	DES + agent	Percent inhibition
Arsenite (3)			
0.2 mM	47.8	24.5	48.7
1.0 mM	50.9	12.9	74.7
2.0 mM	48.4 ± 0.6 (2)	14.3 ± 1.1 (2)	70.6
3.0 mM	50.9	12.2	76.0
4.0 mM	47.8	10.8	77.4
N-Ethylmaleimide (2)			
5 μM	49.4 ± 1.5 (2)	25.9 ± 13.6	47.6
7.5 μM	50.9	25.2	50.5
10 μM	49.4 ± 1.5 (2)	10.6 ± 1.1 (2)	78.5
Iodoacetamide (1)			
100 μM	47.8	18.7	60.9
200 μM	47.8	10.9	77.2
Disulfiram (5)			
5 μM	52.6 ± 1.7 (3)	20.5 ± 13.5 (3)	61.0
10 μM	47.8 ± 6.4 (6)	15.8 ± 6.5 (6)	66.9
50 μM	42.9	13.7 ± 1.5 (2)	68.0
p-Bromophenacyl bromide (5)			
5 μM	43.8 ± 10.6 (6)	15.0 ± 7.4 (5)	65.7
10 μM	48.7 ± 1.8 (2)	11.2	77.0

* Conditions were as in Table 2. In all cases, the level of DNA strand break damage due to the agent alone was less than 20% of the damage due to DES alone, and is subtracted from the DES + agent level. Percent inhibition is therefore a measure of inhibition by the agent of DES-induced damage. Values shown are either single observations, average ± range (for duplicates) or average ± S.D. (for three more observations). Because some samples were used to test more than one agent, in some cases the "DES alone" results are given more than once for clarity.

(Fig. 2b). Complete protection against strand breakage by 30 μM DES was also afforded by purified bovine albumin (fraction V from Sigma) at concentrations down to 0.5 mg/ml (data not shown).

Cytotoxicity and DNA clastogenicity of DES epoxide. DES epoxide is a chemically reactive metabolite of DES which has been shown to be about ten times more potent than DES in producing SCEs in human fibroblasts [24]. On the other hand, DES-epoxide is 1/10 as potent as DES when tested for its estrogenic activity [31]. The data of Fig. 3 indicate that it is also about 1/10 as potent as DES in terms of its cytotoxic and DNA clastogenic activity. As in the case of DES, there appeared to be a threshold (100 μM) at or below which no DNA damage was observed; cytotoxicity was evident at slightly lower concentrations than DNA clastogenicity.

Repair of DES-induced strand breaks. To provide some indication as to whether cellular DNA repair enzyme systems could act on DES-induced strand breaks, the effect of post-treatment incubation was examined. Leukocytes were incubated for 40 min with 30 μM DES and then washed and incubated for a further 30 min in the presence of plasma to inhibit the action of extracellular DES (see above). The results of experiments with five different donors are shown in Table 1. Rather than decrease the level of damage as might be expected if repair processes could act on the breaks, incubation served only to increase the number of observed DNA strand breaks.

Investigation of the mechanism of action of DES as a DNA clastogen. Tumor promoting agents such as tetradecanoyl phorbol acetate (TPA) can cause DNA strand breaks by a mechanism involving oxygen free radicals, and a number of agents have been shown to modify the level of DNA damage [30, 32, 33]. Some of these were tested for their ability to alter the level of DES-induced damage (Table 2). Cysteamine, which blocks TPA-induced damage, *increased* the level of DES-induced damage. Cyanide, which increased TPA-induced damage, had little effect. Interestingly, catalase and SOD (which decrease TPA-induced damage) appeared to *increase* DES-induced damage in leukocytes from some donors but not from others.

The observation that catalase and SOD increased the damaging action of DES in cells from some donors coupled with the earlier observations of other workers that DES is susceptible to oxidizing conditions prompted us to examine the effect of hydrogen peroxide plus peroxidase on DES activity in our system. Different amounts of glucose oxidase were used to vary the flux of hydrogen peroxide; horseradish peroxidase was the source of peroxidase. Their effects on the cytotoxic and DNA clastogenic activity of 30 μm DES are shown in Fig. 4. Sufficiently low levels of hydrogen peroxide were chosen so as to cause little DNA damage in the absence of DES. The results shown in Fig. 4a clearly indicate that the addition of increasing amounts of glucose oxidase and a constant amount of peroxidase to 30 μM DES had a marked protective effect on cellular DNA in terms of strand breaks. That hydrogen peroxide was involved is indicated by the obliteration of this protective effect by catalase. At the same time, there was no significant protection afforded by glucose oxidase plus peroxidase against the cytotoxic effect of DES (Fig. 4B); the addition of catalase had a slight protective effect. The experiment of Fig. 4A may explain why increased levels of DNA damage were seen when catalase was included in some cases in Table 2 (donors 7 and 8). Catalase was presumably effective due to the presence of endogenous hydrogen peroxide and myeloperoxidase resulting from inadvertent release from granulocytes during cell isolation.

The experiment of Fig. 4A was repeated with another group of donors and essentially the same results were obtained, viz. the oxidizing system decreased DNA clastogenicity by 30 μM DES (Fig. 5A). However, an unexpected result was obtained when 10 μM DES was tested. As shown in Fig. 5B, DNA clastogenicity was *increased* by the oxidizing system. As predicted by the observations of Table 2, the addition of SOD increased DNA clastogenicity by 30 μM DES in the presence of the oxidizing system, and also further increased clastogenicity by 10 μM DES under some conditions.

The influence of inhibitors of arachidonic acid metabolism was also tested. Addition of 100 μM indomethacin (an inhibitor of cyclooxygenase), 10–30 μM eicosatetraynoic acid (ETYA) (an inhibitor of both cyclooxygenase and lipoxygenase) or 3 μM nordihydroguaiaretic acid (NDGA) (inhibitor of lipoxygenase) had no effect on either the level of DNA damage or the number of cells which stained with trypan blue (data not shown).

A variety of other agents have been studied by us in connection with their ability to inhibit the DNA strand break damage associated with the TPA-induced respiratory burst of phagocytic leukocytes ([30, 33] and H. C. Birnboim, unpublished results). Superoxide anion is the principal effector of the damage in this case, but its mechanism of action is unknown. Although there was no *a priori* reason to suspect that DES-induced clastogenesis proceeds through similar or common mechanisms, some agents which are inhibitory for TPA-induced damage were examined in this system. The results are shown in Table 3. Five different chemicals were tested and all proved to be effective as inhibitors of DES-induced strand breakage.

DISCUSSION

The results presented in this paper indicate that exposure of mammalian cells to DES can cause DNA strand break damage both in mouse thymocytes and in human leukocytes. Similar DNA damage has also been reported for CHO cells [26], L5178Y cells [25] and rat hepatocytes [35] treated with DES for short periods of time (1 hr or less) in a defined medium in the absence of serum or plasma. We additionally observed that cytotoxicity, measured by trypan blue staining, increased as a function of DES concentration, roughly in parallel with DNA strand breakage. In fact, the threshold for cytotoxicity appeared to be slightly lower than that for DNA clastogenicity (Fig. 1a). The addition of plasma was shown to have a marked protective effect on cells, even at very low levels (Fig. 2). At 10%, a concentration commonly

employed in cell culture medium, plasma protected human leukocytes against very high doses (up to 300 μM) of DES, both in terms of cytotoxicity and DNA clastogenicity. The protective effect was likely due to the well-known lipid-binding properties of blood proteins since protection was also afforded by as little as 0.5 mg/ml of purified bovine serum albumin. Whatever the mechanism of this protection, awareness of this property could be important in proper interpretation of experiments in different systems where different sera or proteinaceous cellular extracts are utilized.

Recently, Sina *et al.* [36] have published experiments in which isolated rat hepatocytes were exposed to DES for 3 hr. Our results are in agreement with theirs for 30 μM DES: no cytotoxicity or DNA damage was observed in the presence of 10% serum or plasma. However, they reported that 300 μM DES did cause DNA strand breaks, but obtained conflicting results using two different tests of cytotoxicity. Trypan blue staining indicated no loss of viability but release of glutamate-oxaloacetate transaminase from cells into the medium indicated cytotoxicity. At a DES concentration of 3 mM, both tests of cytotoxicity were in agreement. Under our experimental conditions (human leukocytes, 10% autologous serum, 40-min exposure), neither trypan blue staining nor DNA strand breaks were observed with 300 μM DES. The difference is likely to be due to the use of different cell types, different exposure times, or other experimental variables. It is not likely to be due to differences in the analytical techniques used since strand breaks were seen by Goldenberg and Froese [25] using an alkaline elution technique very similar to that used by Sina *et al.* We employed an alkaline unwinding technique which uses a related principle [29], and Douglas *et al.* [26] used sedimentation in alkaline sucrose gradients.

It is pertinent to question the relevance of DNA strand breakage in carcinogenesis in the face of demonstrable cytotoxicity. Sina *et al.* [36] attempted to answer this by examining ninety-one chemical compounds for their ability to cause DNA strand breaks and simultaneously to cause cytotoxicity. Of these, sixteen compounds caused DNA damage only at high (>30%) cytotoxic concentrations and twelve of these were weak carcinogens. They classified DES as a strong carcinogen which could cause strand breaks with low (<30%) cytotoxicity. This would appear to differ from our observation that (in the absence of plasma) cytotoxicity and strand breaks are closely linked. The significance of strand breaks in carcinogenesis by DES will depend on whether or not DNA damage occurs only in "dead" cells. That is, if a target cell is first killed by DES and DNA damage is secondary, then the DNA damage is unlikely to be of significance. On the other hand, if a given cell can suffer strand breaks while receiving sublethal damage due to an attack on another (possibly membrane) target, then DNA damage may be related to carcinogenesis. Tsutsui *et al.* [21] have argued that aneuploidy induction by DES in Syrian hamster embryo cells is likely the most important chromosome change contributing to cell transformation. Aneuploidy but not chromosome aberrations correlated well with cell transformation, and cells in mitosis were more sensitive than at other cell cycle stages.

Our experiments have provided indication of the complex reactions involving DES which can occur in relation to DNA clastogenesis. DES epoxide is probably not an important intermediate in clastogenesis (Fig. 3), although it is reported to be very potent in producing SCEs in human fibroblasts [24]. Cysteamine increased the level of DES-induced clastogenesis (Table 2); this could be due to an effect either on the oxidation/reduction state of DES or on the target cells. Lack of inhibition by cysteamine suggests that superoxide anion and/or hydrogen peroxide are not the species responsible for DES-induced damage since this concentration of cysteamine blocks DNA clastogenesis in TPA-stimulated cells [30] and in cells exposed to hydrogen peroxide [33]. The addition of superoxide dismutase increased the level of damage in the presence of a peroxidizing system (Fig. 5). However, this does not necessarily indicate a direct role for superoxide anion; rather, SOD may influence the concentration of a semiquinone-like intermediate, as has been reported for a series of quinones [37]. The compounds tested in Table 3 provide an indication that metabolic processes are involved in the action of DES as a DNA clastogen but no definite active intermediate can be identified or deduced. The intracellular target for arsenite is usually considered to be lipoic acid (cofactor for decarboxylation of pyruvate and other α-keto carboxylic acids), but other thiols may also react. Iodoacetamide and *N*-ethylmaleimide are well known thiol-reactive chemicals which can react either with low molecular weight thiols such as glutathione or with protein sulfhydryls; they can also react with histidine and methionine. Disulfiram is a potent inhibitor, reacting possibly with thiol groups preferentially. It is known to inhibit dehydrogenases and oxidases [38], and has been shown to be an inhibitor of DNA damage by etoposide (VP-16) [39]. Bromophenacyl bromide reacts with histidine residues in proteins and inhibits phospholipase A_2 [40]; it also inhibits superoxide anion production by human neutrophils [41].

Both Goldenberg and Froese [25] and Bradley and Taylor [35] have presented evidence that DES produces double-strand breaks in DNA. These would be detected but not distinguished from single-strand breaks in our FADU assay. Our evidence that the strand breaks appear not to be repaired during a 30-min period of post-treatment incubation (Table 1) is probably consistent with their observation. Bradley and Taylor have speculated that lysosomal acid deoxyribonuclease (which produces double-strand breaks in DNA) is responsible. The hog spleen acid deoxyribonuclease is not known to be sensitive to sulfhydryl reagents [42], but is inactivated by tryptophan and methionine/histidine specific agents [43]. If Bradley and Taylor are correct, then a histidine residue in a lysomal deoxyribonuclease may be the target of some of the clastogenesis inhibitors we have studied.

We have argued earlier that DNA strand breaks are a significant event in terms of tumor promotion in mouse skin while mutational events are important in initiation [33]. Could a similar model apply to

carcinogenesis by DES, which appears to be a complete carcinogen? Our observation that low doses of DES show increased clastogenicity in the presence of peroxidase (whereas higher doses are inhibited) may be relevant in this regard, since target organs for DES-induced carcinogenesis may have higher levels of peroxidase [44]. The available evidence is at least compatible with such a model for carcinogenesis since DES seems capable of causing both clastogenic and base damage to DNA.

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