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CYTOTOXICITY OF BENZO [a] PYRENE DIOL EPOXIDE IS ASSOCIATED WITH PREMATURE ARREST OF NASCENT STRAND ELONGATION IN SERUM STIMULATED MONKEY KIDNEY CELLS

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Publication Date

1982-08-01

LBL-14833
Preprint

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CHEMICAL BIODYNAMICS DIVISION

Submitted to the Journal of Biochemistry

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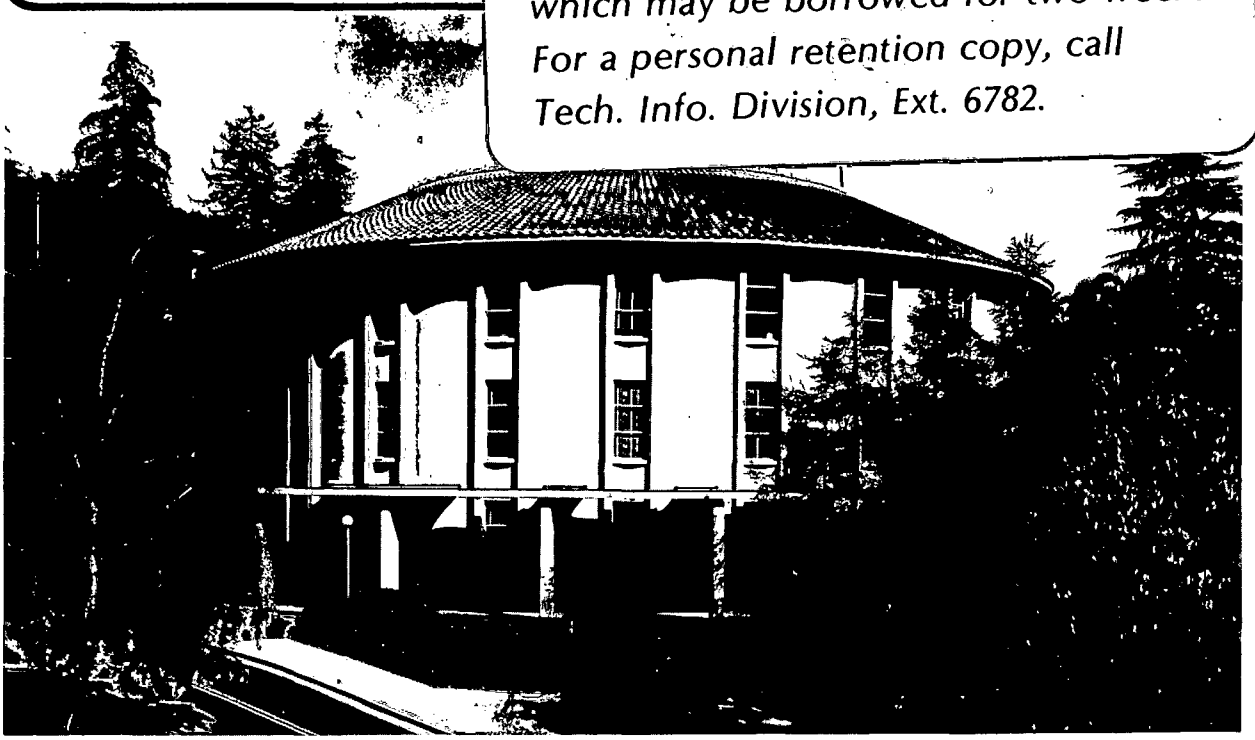
August 1982



Prepared for the U.S. Department of Energy under Contract DE-AC03-76SF00098

DISCLAIMER

TITLE

Cytotoxicity of Benzo[a]pyrene Diol Epoxide is Associated with Premature Arrest of Nascent Strand Elongation in Serum Stimulated Monkey Kidney Cells.

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This work was supported by the Office of Energy Research, Office of Health and Environmental Research, Health Effects Research Division of the U.S. Department of Energy under Contract No. DE-AC03-76SF00098.

SUMMARY

The effects of Benzo[a]pyrene diol epoxide on DNA replication and growth of serum stimulated TC-7 monkey kidney cells was examined by clonal assay, flow cytometry, thymidine incorporation, and size analysis of labelled parental and nascent DNAs. Above 1.7μM, BaP DE was highly cytotoxic whereas below this concentration colony forming efficiency was the same as controls even though DNA adducts and DNA synthesis inhibition could be seen at all concentrations tested. Concentration studies showed a strict linear relationship between DNA binding and BaP DE concentration with no evidence of parental strand nicking over the ranges examined.

Analysis of nascent DNA synthesis showed inhibition of replicon initiation, premature arrest of strand elongation, and inhibition of DNA segment maturation were all contributing factors to the decreased level of DNA synthesis caused by BaP DE. Above 1.7 μM BaP DE arrested strand elongation at subreplicon sizes which correlated with the average interadduct distances estimated from DNA binding studies. Lower concentrations permitted the completion of replicons but inhibited the maturation of these nascent segments. The results of these studies suggest that toxicity results when adduct levels exceed 1 per strand per replicon due to the inability of the replication apparatus to complete replicons.

INTRODUCTION

Benzo[a]pyrene diol epoxide (BaP DE[1])is an ultimate carcinogen whose mutagenicity, cytotoxicity, and ability to induce transformation have been well characterized [1-3]. Although the ability to induce mutations is usually attributed to error prone repair processes, there is presently no established molecular mechanism(s) explaining the S-phase dependent cytotoxic [4] and transformation potentials of compounds such as BaP DE.

Numerous studies have examined the chemical nature and base specificities of DNA adduct formation [5-9]. *In vivo* and *in vitro* binding has been studied in both animal and viral systems [10-12]. In all cases DNA binding was shown to be a linear function of the applied carcinogen concentration. The major adduct formed results from nucleophilic attack of the exocyclic amino group of guanine on the C-10 of the epoxide (more than 90 percent of the adducts) [5]. Smaller amounts of cytosine [10] and adenine [13] species have also been detected. The cytotoxic and mutagenic activities have been found to parallel the chemical activity and base specificity towards guanine [14].

Chromosome replication is known to be perturbed by agents which bind to template DNA. There are good correlations between the carcinogenicity of many chemical agents and their ability to inhibit DNA synthesis [15]. These substances generally reduce the rate of synthesis in two ways. The first is by reducing the rate of replicon initiation [16]. The second effect, usually seen at higher concentrations, is to inhibit strand elongation either by reducing the rate of elongation

[16] or by blocking elongation completely [17].

Several in vitro systems have been developed to examine the nature of elongation arrest on a template of defined sequence [18-19]. Studies using BaP DE [17], AAF [18], and AAAF [19] have utilized these template systems to determine the arrest position of strand elongation in relation to adducted bases. In all cases the data indicate that termination occurs one residue before an adducted base (primarily guanine). Estimates of the degree of read through suggest these adducts pose a firm block to elongation on the time scales used in these experiments [18]. Identical results have been obtained using polymerases from a number of sources [20] strongly suggesting that termination of nascent DNA synthesis by large adducts is a general phenomenon. However the biological effects of this class of lesion remains to be established.

It is not unreasonable to expect that the covalent binding of large adduct carcinogens such as BaP DE to the DNA of replicating cells would interfer with the normal template dependent processes of dividing cells. In addition to inhibiting the initiation and elongation of nascent DNA. alkylation may also perturb or prevent the normal maturation sequence of gene segments. Dose dependent increases in the incidence of SCE have been reported in metabolically proficient cells treated with alkylating agents including BaP [21]. Such interference on the part of DNA adducts may play a role in predisposing cells towards transformation and oncogenic states as well as increasing the incidence of cell death. It was therefore of interest to study the effects of BaP DE on the replication patterns of a model eukaryotic cell system. Richter and Hand [22] have recently examined the characteristics of chromosome

replication in CV-1 monkey kidney cells using autoradiography. Their findings show that this primate cell line possesses the same general patterns of replication seen in other eukaryotic cell types including: rate of fork migration, synchrony of initiation of tandem RU, and bidirectionality of elongation from initiation sites. Therefore monkey kidney cells have been used as a model system in which to characterize the type and effects of BaP DE lesions on DNA replication and cytotoxicity.

EXPERIMENTAL PROCEDURES

CELL CULTURE

The TC-7 line of African green monkey kidney cells is a subclone of CV-1 cells obtained from J. Robb of the University of California at San Diego. Cells were maintained in Dulbecco's modified Eagle's media (DME) supplemented with 10 percent (v/v) donor calf serum (Flow Laboratories). Cell stimulation was achieved by transferring saturation density cultures to a seeding density of 2.5×10^4 cells/cm^2 using DME containing 20 percent (v/v) calf serum. BaP DE (3.3mM in Me_2SO_4) was applied at the indicated concentrations in 2.0ml of media for 60 minutes at 18 hours after transfer. Control dishes were treated with appropriate volumes of Me_2SO_4. After treatment dishes were washed twice with dilute Tris buffer (25 mM Tris, 0.37mM sodium phosphate, 5mM KCl, 137mM NaCl, pH 7.4) and 10ml of fresh media applied to each dish.

DNA SYNTHESIS ASSAY

DNA synthesis was measured by incorporation of [^{3}H] dThd into trichloroacetic acid precipitable material. Media was reduced to 3.0ml and 10μCi [^{3}H] dThd (30Ci/mmole) applied for 30 minutes. Labelling was terminated by washing the dishes 4 times with dilute Tris buffer and harvesting the cells by trypsinization. Cell numbers were determined using a ZB1 Coulter counter and incorporated label was quantified by precipitation onto nitrocellulose filters.

FLOW CYTOMETRY

Cells for FCM analysis were harvested by trypsinization, pelletted, and fixed in 2.0ml of 25 percent (v/v) ethanol which was 15mM in $MgCl_2$. Cells were prepared for analysis as previously described [22]. The proportion of cells in G_1, S, and G_2+M was calculated by computer analysis of the FCM histograms.

LABELLING AND SIZE ANALYSIS OF PARENTAL AND NACENT DNAS

Parental DNA was labelled by growing stimulated cells in the presence of [^{14}C] dThd (0.02μCi/ml) for 48 hours, changing the media and allowing the cells to grow to confluency. Nascent DNA of replicating cells was pulse labelled by applying 100μCi [^{3}H] dThd in 2.0ml of media for 5 minutes, washing the cells twice with cold, dilute Tris buffer and applying 10ml of fresh media.

Size analysis of labelled DNAs was performed using the alkaline sucrose gradient method of Studier [23] with changes as described by Kowalski and Cheevers [24]. Sucrose gradients (15-45 percent (w/v)) with cell lysates were centrifuged at 80,450g, 25^{o}C, for 9 hours using an SW27 rotor. Gradients were collected in 10 drop fractions from the bottom and aliquots analyzed for labelled DNA by liquid scintillation counting. Aliquots of fractions were analyzed for acid precipitable label using nitrocellulose filters as described earlier. [^{14}C] labelled SV40 form I (53S) and form II (16+18S) DNAs were used as sedimentation markers.

CYTOTOXICITY ASSAY OF BaP DE

Cytotoxicity of BaP DE was measured from the cloning efficiencies of serum stimulated cells replated immediately after treatment with the indicated BaP DE concentrations. Colonies containing 50 cells or more were tabulated after staining with crystal violet.

DETERMINATION OF BaP DE BINDING TO PARENTAL DNA

Cells prelabelled with [^{14}C] dThd were transferred with serum stimulation as previously described. Sets of 5 100mm dishes were treated with the indicated concentrations of [^{3}H] BaP DE (1.2Ci/mmole) 18 hours after stimulation and the DNA purified by standard methods. Noncovalently bound species were removed by repeated extraction of the purified DNA with ethyl acetate. Aliquots of the purified DNAs were oxidized to determine the [^{3}H] and [^{14}C] contents of each sample. Final DNA concentrations were determined from the UV spectra of the final preparations.

RESULTS

CYTOTOXICITY OF BaP DE

BaP and other PAH have been shown to possess a high degree of toxicity in cells capable of metabolizing these compounds [25]. Landolph et al. [4] have shown that cell division is required for expression of cytotoxicity of both BaP and its ultimate carcinogenic form-BaP DE. Although there are many possible modes of action by which BaP DE can exert its toxic effects, the requirement for cell division and the documented inhibition of DNA synthesis by BaP [26] and its metobolites [27] suggest the possibility of a replication requirement. The concentration dependence of BaP DE cytotoxicity on serum stimulated TC-7 monkey kidney cells was therefore determined in order to better evaluate this possibility. The results of the clonal toxicity assay shown in Fig. 1 indicate a severe toxic response above 1.7μM BaP DE. Below this level little or no toxicity was detectable. Cloning experiments performed using different BaP DE stocks never reduced the surviving fraction at lower doses below 0.7 although the fraction of surviving colonies at doses above 1.7μM BaP DE was frequently less than 0.05. This variation may be attributable to partial hydrolysis of the epoxide during storage to form the corresponding tetraol. The latter compound is known to be significantly less toxic than BaP DE [4].

ALTERATIONS OF THE TC-7 CELL CYCLE BY BaP DE TREATMENT

BaP DE has been shown to covalently bind to all classes of macromolecules *in vivo* [28]. Disruption of transcription and replication resulting from alkylation of template DNA would be reflected in perturbations of the cell cycle times characteristic of the cell type. Fig. 2 shows the FCM histogram sequence obtained from normal, serum stimulated TC-7 cultures and parallel cultures treated with 1.7 μM BaP DE 18 hours after stimulation. Carcinogen treatment clearly altered the normal stimulation pattern of this cell line resulting in a decreased rate of genome duplication and an accumulation of early S phase cells (Fig. 2: 28 hours). By 24 hours a significant fraction of control cells (31 percent) had completed DNA synthesis and entered the G_2+M phase of the cell cycle whereas only 16 percent of the population was in this cell cycle compartment in cultures exposed to 1.7μM BaP DE 5 hours earlier. Independent measurement of DNA synthesis by thymidine incorporation (Fig. 3) showed reductions in the rate of DNA synthesis for all concentrations of BaP DE suggesting that the decreased rate of cell cycling reflects reductions in the rate of chromosome replication.

The accumulation of early S phase cells seen at 28 hours for treated cells was never observed in controls and shows that initiation of genome duplication by committed cells is not prevented by the carcinogen treatment. FCM experiments performed on stimulated cell populations blocked by colcemid (0.2 μg/ml) were used to determine if carcinogen treatment produced alterations in the percentage of cycling cells. The maximum cycling population found in the primary stimulation waves was 70 percent for both treated and control cells (Table 1).

Therefore the accumulation of early S population after BaP DE treatment does not result from the recruitment of noncycling population and probably reflects synchronization which occurs at the G_1/S boundary due to the decreased rate of DNA synthesis. Kinetic modelling studies have shown that such an inhibition can account for the observed S phase accumulation [29].

Although BaP DE treatment increases the time required for genome duplication (20 hours for treated cells vs 8 to 10 hours for controls) this concentration does not prevent completion of DNA replication (Fig. 2: 40 and 46 hours). Correlated with the accumulation of the 4C DNA content peak of treated cells was an increase in the mitotic index of the cultures 45-55 hours after stimulation. Since there is no measurable difference in the position of the 4C DNA peak of treated and control cells, it may be concluded that cells treated with this concentration of BaP DE are able to complete the duplication of their chromosomes after a prolonged DNA synthetic period and that at least some fraction of these cells begin mitosis. Further, since treated cell populations were observed to increase in size with time, albeit at a slower rate, after BaP DE (Table 1) some percentage of the mitotic events must be complete.

BaP DE TREATMENT INTERFERS WITH THE NORMAL ELONGATION AND MATURATION OF TC7 REPLICATION UNITS

The alterations of the TC-7 cell cycle after BaP DE treatment indicate that S phase is the most seriously affected of the cell cycle compartments. Bartholomew et al. has reported similar findings using PAH

metabolizing mouse liver cells treated with BaP [30]. Corroborating this interpretation, the data of Fig. 3 show significant quantitative decreases in the rate of dThd incorporation with increasing doses of BaP DE. These decreases reflect composite reductions in the rates of RU initiation, elongation, and termination which result from alkylation. In order to determine the precise effect of carcinogen treatment on the synthesis and ligation (maturation) of nascent DNA in treated cells alkaline sedimentation profiles of pulse-chased cultures have been analyzed. Chasing pulse labelled cells allows the depletion of label in internal thymidine pools through incorporation into elongating RUs. The effects of BaP DE treatment on the ligation of completed units can be observed as the labelled nascent DNAs mature to bulk cellular DNA which sediments considerably faster than SV40 form I supercoiled marker DNA (53S).

Nascent DNA of treated and control cells was labelled with 5 minute pulses of [^{3}H] dThd, chased for various times, and the size distributions of labelled, elongating DNA analyzed by alkaline sucrose gradient analysis. Fig. 4 shows a typical pulse-chase sequence obtained from control and BaP DE treated (1.7 μM) cells. Block 'a' shows the size profiles obtained from cells harvested immediately after pulse labelling. In agreement with the incorporation results of Fig. 3, the experimental profile contained only 60 percent of the total counts found in that of controls even though the percentage of S phase cells in both cultures was indistinquishable (Fig. 2: 19 hours). There was a marked absence of label sedimenting in fractions 24-36 (MW= 1.2- 5.3 x 10^7) of the treated cell profile of Fig. 4a. All label in this region of the control profiles was acid precipitable and represents label incorporated

onto the 3' ends of elongating strands which initiated prior to the pulse [31].

The complete chase sequence proceeding from the profiles of Fig. 4a shows that carcinogen exposure interferes with the normal elongation of active RUs. Both treated and untreated cells showed a rapid conversion of slower sedimenting label to higher molecular weight DNA between 15 and 30 minutes after pulse labelling, but the size of the accumulated DNA was decreased after BaP DE treatment (Fig. 4c). Control cells accumulated labelled strands into a band which was well separated from the top of the gradient and appeared to contain multiple components. The molecular weight of this band was calculated to range from 1 to 2 x 10^7. By 45 minutes all the label of control cells had chased into DNA with a molecular weight corresponding to the fastest component found in Fig. 4c. Because of the long residence time of label in this class of nascent DNA, the average single strand molecular weight of the TC7 RU was estimated from the position of this component and found to be 2.3 x 10^7. This value is in excellent agreement with values obtained by autoradiography for the CV-1 line of monkey kidney cells [21].

Cells which were exposed to 1.7 μM BaP DE initially accumulated label into DNA with a molecular weight of 5.7 x 10^6 (17kb: Fig. 4c). By 45 minutes the size of the labelled DNA increased to 1.2 x 10^7 36 kb: Fig. 4d) but was still significantly smaller than that found for untreated cells (70 kb). No further increase was observed in cells treated with BaP DE whereas some labelled DNA of control cells began to mature to forms sedimenting faster than the SV40 53S marker DNA during this time (Fig. 4f). These results show that one mode of replication inhibition by BaP DE is the premature arrest of nascent strand

elongation.

The persistence of the elongation block imposed by BaP DE, and its effect on the ligation (maturation) of the nascent DNA was evaluated by chasing pulse-labelled cells for time periods exceeding the 46 hour timepoint of Fig. 2. The sedimentation profile of cells treated with 1.7 μM BaP DE and pulse-chased for 30 hours is shown in Fig. 5c. Profiles obtained from untreated cells chased for 4 and 8 hours after labelling are shown in blocks a and b for comparison. Both the 4 and 8 hour profiles of control cells have a large bulk DNA component (DNA sedimenting faster than an average RU) which extends over the bottom third of the centrifuge tubes. In contrast, the profile of the BaP DE treated cells shows no labelled bulk DNA component even though 100 percent of the cells which were in S phase at the time of pulse labelling had completed genome replication (Fig. 2: 46 hours). The maturation of nascent DNA synthesized after BaP DE treatment is inhibited. It therefore appears that ligation of adjacent RUs in TC-7 cells occurs as a rate limiting step which happens relatively late in S phase or not at all when cells are treated with BaP DE.

BaP DE DECREASES THE RATE OF RU INITIATION IN TREATED CELLS

If the sole action of the carcinogen was to reduce the uptake and/or phosphorylation of the [^{3}H]dThd, the specific activity of the labelled nascent DNA would be less and the profiles of Fig. 4a should differ by some constant proportion. Fig. 6 shows the data of the carcinogen treated cells (Fig. 4a) replotted as percent of the control according to the recommendations of Kaufmann *et al.* (see legend) [32].

Pulse labelled cells which have been pretreated with BaP DE do not give an alkaline sedimentation profile which is a constant fraction of the control profile. The data of Fig. 6 show a definite absence of higher molecular weight forms and an accumulation of intermediate sized nascent DNA with a mean molecular weight of 5.7×10^6 in excellent agreement with the data of Fig. 4c. There is also a deficiency of lower molecular weight forms (fractions 44-54) in the treated cells. This deficiency is diagnostic of a decreased rate of RU initiation [33]. Thus at least part of the DNA synthesis inhibition seen in Fig. 3 is due to a decreased rate of initiation of TC-7 RUs. Similar decreases in initiation rate have been found in replicating cells treated with X-rays [33], UV light [32], a number of known carcinogens and alkylating agents [34], and recently with TPA [35]. However, whereas the target size for initation inhibition by X-ray and UV light is on the order of 5 RUs or more, that of alkylating agents such as aflatoxin B1 appears to be at the level of a single RU [34].

BaP DE DOES NOT PREVENT THE INITIATION OF NEW REPLICATION UNITS

The decreased rate of RU initiation shown by the data of Fig. 6 may reflect total arrest of RU initiation as a result of carcinogen treatment or may be due to inhibition of only a fraction of the potentially active RUs. To distinquish between these two possibilities the [^{3}H] dThd pulse was delayed for increasing periods of time after BaP DE treatment to determine whether treated cells were able to circumvent the effects of the carcinogen treatment. Since the FCM cell cycle distributions change dramatically with time, a comparison such as that

of Fig. 6 is not possible because differences in the total number of S phase cells or their distributions would possess intrinsic differences in the rate of RU initiation. However it was still possible to compare the effects of BaP DE on elongation and maturation. Delaying the pulse for any period up to 20 hours after treatment produced a kinetic sequence for treated cells which was identical to that of Fig. 4. Label was primarily incorporated into nascent strands which elongated to the termination size characteristic for 1.7 μM BaP DE (MW=1.2 x 10^7 showing that new RUs were continually initiating and that all RUs contained adducted regions at this concentration.

RELATIONSHIP OF DNA BINDING TO NASCENT DNA TERMINATION SIZE

If arrest of nascent strand elongation results from blockage of advancing replication complexes by adducted template regions as has been found *in vitro* for AAF [18] and BaP DE [17], then increasing the BaP DE concentration should increase the adduct density of parental DNA and decrease the observed termination size. Figs. 7a and b show the results of concentration studies of termination size and adduct formation respectively. The data of Fig. 7b show that binding of [^{3}H] BaP DE to DNA of stimulated TC-7 cells increased linearly with concentration over the entire range examined in agreement with earlier *in vivo* studies [36]. At 1.7 μM BaP DE the average binding level was one adduct per 18.5 kb (single strand MW = 6.2 x 10^6).

The maximum size attained by the nascent DNA in treated cells did not show the same linear relationship over the entire concentration range. The termination size showed a linear though inverse relationship

with the applied concentration above 1 μM. In this region elongation arrested at sizes consistently twice the average interadduct distance shown in Fig. 7b. A concentration of 1.7 μM BaP DE resulted in a maximum nascent strand size of 1.2×10^7 compared to an average interadduct distance (reported as single strand MW) of 6.2×10^6. This is the expected relationship if binding to cellular DNA is random both in spatial distribution along the contour length of the parental DNA as well as between the two parental strands and if an adduct only arrests nascent DNA elongation on the strand to which it is attached. Thus for 1.7 μM BaP DE the interadduct distance on either strand would be 37 kb (1.2×10^7 single strand MW) in excellent agreement with the observations made in these studies.

Below 3.3 μM BaP DE the termination size showed a reduced concentration dependence (Fig. 7a). The change in this dependence can be explained if maturation (ligation) is a separate event which does not necessarily occur when adjacent growing forks meet or if BaP DE adducts inhibit maturation (ligation) of adjacent RUs. Under these conditions decreased adduct levels would increase the maximum size attained by nascent strands until the level of 1 adduct per strand per RU. Below this level of binding two classes of RU would be formed: those containing an adduct and producing nascent DNA slightly smaller than an average RU, and unadducted RUs which would produce full length nascent DNA. Thus at lower concentrations the labelled band obtained by alkaline gradient analysis would be composed of two closely spaced bands. As the BaP DE concentration was decreased the proportion of full length labelled strands would increase shifting the mode of the composite peak to higher apparent molecular weights. The chase time used

for the data of Fig. 7a (90 minutes) is sufficient for the completion of RU elongation but too short for a significant amount of maturation to take place (see Fig. 5e control profile). Thus the concentration dependence of Fig. 7a probably reflects the differential sensitivity of at least two processes to DNA alkylation by BaP DE- elongation and ligation.

INTEGRITY OF TEMPLATE DNA

The integrity of [^{14}C] labelled parental DNA has been checked by alkaline sucrose gradient analysis after BaP DE treatment. No evidence of nicking was observed after 4 hour treatment with BaP DE concentrations below 17 μM. Some nicking and degradation was apparent with concentrations above 8.0 μM if cells were incubated for 8 hours after treatment but the degree of degradation was insufficient to account for the uniformity and extent of premature termination seen in the delayed pulse experiments. Therefore it is unlikely that the observed termination is caused by nicking or depurination (alkali labile sites) of template DNA particularly at the lower doses and shorter times used in these studies.

DISCUSSION

The decreased rate of DNA synthesis (Fig. 3) found after BaP DE treatment has been shown to be due to decreases in the rate of RU initiation (Fig. 6) as well as premature arrest of elongating nascent DNA segments (Fig. 4) and an inhibition of maturation of these segments (Fig. 5). Whereas the the exact nature of the effect on RU initiation remains to be clarified, the latter two effects appear to result from blockage of advancing replication complexes by BaP DE adducts. The results presented are consistent with a model for chromosome replication in which the maturation of completed RUs is a kinetically separable event which occurs some time after the completion of RU elongation and which is inhibited by the presence of DNA adducts between adjacent replication origins. At low adduct levels (one per strand per RU) elongation proceeds to a size expected for complete or near complete nascent strands but maturation (ligation) is inhibited by the template adducts resulting in the generation of 'nicked' daughter strands. Little or no toxicity is seen with these concentrations. BaP DE concentrations sufficient to introduce multiple adducts within RUs result in the premature termination of nascent strand elongation at subreplicon sizes generating 'gapped' daughter strands and a high level of toxicity.

The coincidence in the threshold concentrations for termination size and cytoxicity is particularly striking and suggests a possible molecular basis for the growth dependent toxic effects of BaP DE in

stimulated cells. Carcinogen treatment is only mildly cytotoxic below the threshold concentration (1.5-3 μM). In this range nascent strand elongation proceeds to an upper limit equal to the estimated size of the monkey kidney cell RU [21]. The results of the long chase studies show that such cells are able to complete chromosome replication but are unable to generate fully mature DNA prior to mitosis. Thus these levels of BaP DE treatment result in large numbers of 'nicked' chromosomes entering mitosis (Fig. 8). The lack of toxicity seen with these lower doses suggests that the presence of nicks during chromosome condensation and mitosis is a relatively tolerable lesion. However the greater number of 'free ends' resulting from the interference of DNA adducts with the process of ligation could make these chromosomes more susceptable to homologous as well as possible nonhomologous exchanges during mitosis thus explaining the increase in SCE observed in BaP treated cells by Craig-Holmes and Shaw [37] and others [3]. Above the threshold concentration multiple adducts between adjacent initiation sites would arrest strand elongation at subreplicon sizes resulting in progressive increases in the quantity of 'gapped' daughter DNA (see Fig. 8). The latter could result in cell division dependent toxicity through increases in the incidence of nondisjunction (due to incomplete separation of unreplicated regions) or deletion of critical gene segments necessary for maintainance of homeostasis after cell division. It is also possible that the higher adduct levels may contribute to increases in cytotoxicity by interfering with the production of functional mRNA. However the absence of toxicity in saturation density cultures treated with high levels of BaP DE reported by Landolph et al. [4] (and data not shown) argues against this possibility.

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FOOTNOTES

[1]Abbreviations: AAF, N-acetylaminofluorene; AAAF, N-acetoxy-2-acetyl aminofluorene; BaP DE, Benzo[a]pyrene-7,8-dihydrodiol-9-10-oxide; DME, Dulbecco's modified Eagle's medium; FCM, flow cytometry; RU, replication unit; SCE, sister chromatid exchange

ACKNOWLEDGMENT

This work was supported by the Office of Energy Research, Office of Health and Environmental Research, Health Effects Research Division of the U.S. Department of Energy under Contract No. DE-AC03-76SF00098.

The authors wish to thank Dr. Melvin Calvin for many helpful discussions concerning multiple aspects of this work and Dr. Joe Gray for discussions and criticisms concerning the flow cytometry analysis used for those experiments.

FIGURE LEGENDS

Figure 1: Cytotoxicity of BaP DE

Serum stimulated TC-7 cells were treated with the indicated concentrations of BaP DE at 18 hours after transfer as described. Immediately after treatment cells were harvested by trypsinization and replated at seeding densities of 250, 500, and 1000 cells per 100 mm dish. Toxicity was measured as reduction in the clonal efficiency of treated cells (colonies containing more than 50 cells). Control cells had a mean cloning efficiency of 28+4 percent. Values shown are the average of 4 dishes and have been normalized to control values.

Figure 2: Effects of BaP DE Treatment on the Cell Cycle of Serum Stimulated TC-7 Cells

Cells were transferred with serum stimulation as previously described. BaP DE (1.7 μM) was applied for 60 minutes at 18 hours after transfer. Dishes were harvested at the indicated times and cells fixed, stained, and analyzed by flow cytometry (see experimental procedures). Blocks 1-8: Me_2SO_4 controls, Blocks 9-16: BaP DE treated cultures.

Figure 3: Rate of DNA Synthesis after BaP DE Treatment

Saturation density TC-7 cells were serum stimulated and treated with BaP DE as described. Values shown are the average of the acid precipitable counts of replicate plates pulse labelled with 10 μCi of [^{3}H] dThd for 30 minutes immediately after BaP DE treatment. All values have been normalized with respect to the controls.

Figure 4: Alterations in the Elongation Patterns of Nascent DNA in BaP DE Treated TC-7 Cells

Cells were stimulated and treated with BaP DE (final concentration=1.7μM) as described. Immediately after treatment all dishes were pulse labelled with 100μCi [^{3}H] dThd (30 Ci/mmole) for 5 minutes, washed twice with buffer, and 10ml of fresh media (20 percent serum) applied per dish. Dishes were harvested after a) 0, b)15, c)30, d)45, e)60, and f)90 minutes of incubation. 2 x 10^5 Cells were applied to the top of preformed gradients in lysis buffer (0.25N NaOH, 0.5M NaCl, 0.1% Sarcosyl) and lysed for 8 hours at 25°C. Tubes were centrifuged in an SW27 rotor at 82,500g for 9 hours at 25°C. Gradients were collected in 10 drop fractions from the bottom and aliquots analyzed as described under methods. Aliquots were taken from the control samples in block d for a separate analysis. The positions of [^{14}C] SV40 form I (53S) and form II (16+18S) marker DNAs are shown for reference. Molecular weights of the labelled bands were calculated

relative to these markers using the equations of Studier (see text for reference). (O-O: Me_2SO_4 control, O-O: 1.7 M BaP DE).

Figure 5: Inhibition of Nascent DNA Maturation by BaP DE

Standard stimulation protocol was used. Cells were treated with either Me_2SO_4 (a and b) or 1.7μM BaP DE (c). Alkaline gradient analysis was performed as described in Fig. 4. Vertical marker bars are identical to those of Figure 4. (a) Me_2SO_4 control : 4 hour chase, b)Me_2SO_4 control: 8 hour chase, c)1.7μM BaP DE: 30 hour chase).

Figure 6: Inhibition of RU Initiation by BaP DE Treatment

The profile of the carcinogen treated cells in Figure 4a has been represented such that the label in each collection tube is plotted as a fraction of the label found in the corresponding control tube. Vertical bars show the positions of SV40 marker DNAs.

Figure 7: BaP DE Concentrations Studies of a) Nascent DNA Termination Size and b) Adduct Formation

Cells were transferred and stimulated as for the other experiments. Dishes to be used for DNA size analysis were treated with the indicated concentrations of BaP DE for 60 minutes at 18 hours after transfer. Dishes were then pulse labelled for 5 minutes as described in Figure 4 and chased for 90 minutes before harvesting and alkaline sucrose gradient analysis. The apparent molecular weights of the labelled

bands were calculated relative to SV40 markers as described earlier. The verification that labelled strands had terminated by 90 minutes was done using time course studies like those represented in Figure 4.

Cells to be used for binding analysis were prelabelled with [^{14}C] dThd as outlined in the methods section. These cells were stimulated in the standard manner, treated with [^{3}H] BaP DE (1.2Ci/mmole) for 60 minutes at 18 hours after transfer and the DNA purified and analyzed as described in the experimental procedures.

Figure 8: Model of BaP DE Action on the Replication of Eukaryotic Chromosomes See text for description.

TABLES

TABLE 1: EFFECT OF BaP DE ON THE GROWTH RATE AND CYCLING FRACTION OF SERUM SIMULATED MONKEY KIDNEY CELLS

Serum stimulated cells were treated with the indicated concentrations of BaP DE at 18 hours after transfer and blocked with colcemid (2 g per dish). Dishes were harvested at regular intervals after treatment and analyzed by FCM. The fraction of the cell populations present in the non-G_1 compartments was determined by computer analysis of the FCM data. The values reported are the maximum values seen during the primary stimulation wave. The doubling times were determined from parallel unblocked cultures harvested at daily intervals.

[BaP DE] μM	Cycling Fraction	%C	Doubling Time Hours	Relative Doubling Time
0.0	0.66	100	42	1.0
0.17	0.68	105	40	1.1
0.48	0.70	111	42	1.0
0.83	0.68	105	41	1.0
1.7	0.67	103	92	0.45

Figure 1:

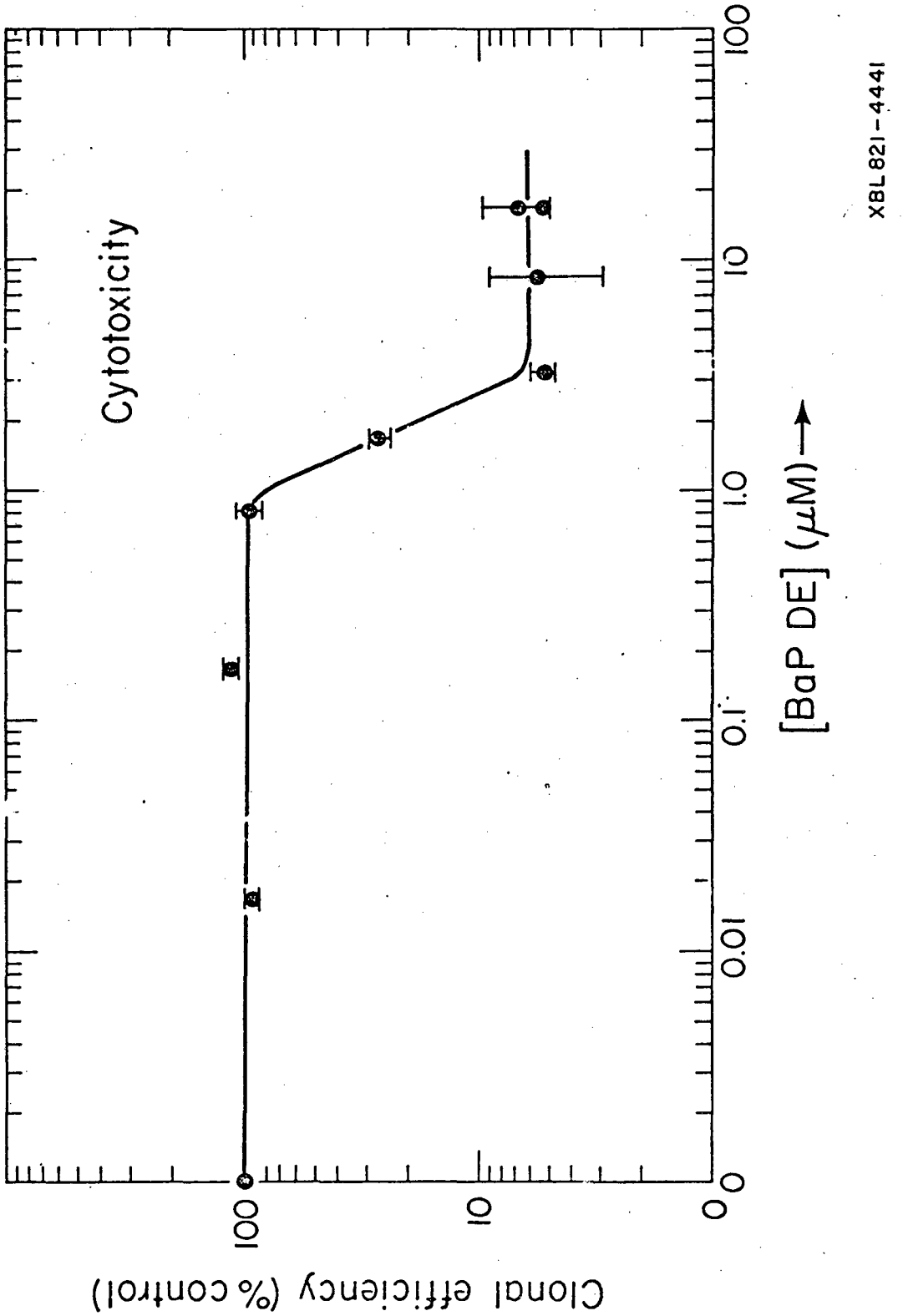
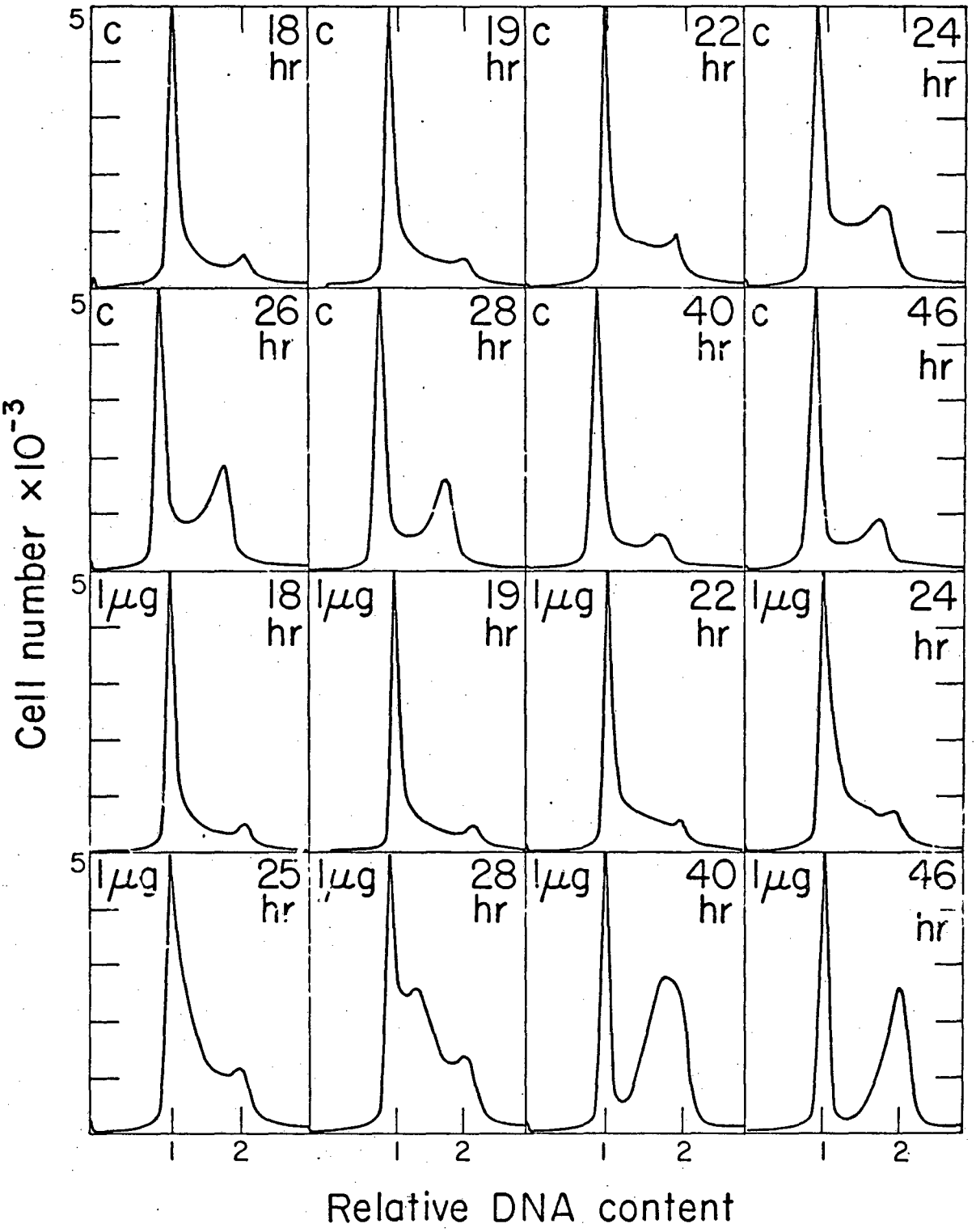

Cytotoxicity
Clonal efficiency (% control)
100
10
0
0
0.01
0.1
1.0
10
100
[BaP DE] (μM)
XBL 821-4441

Figure 2:



XBL 8112-12076

Figure 3:

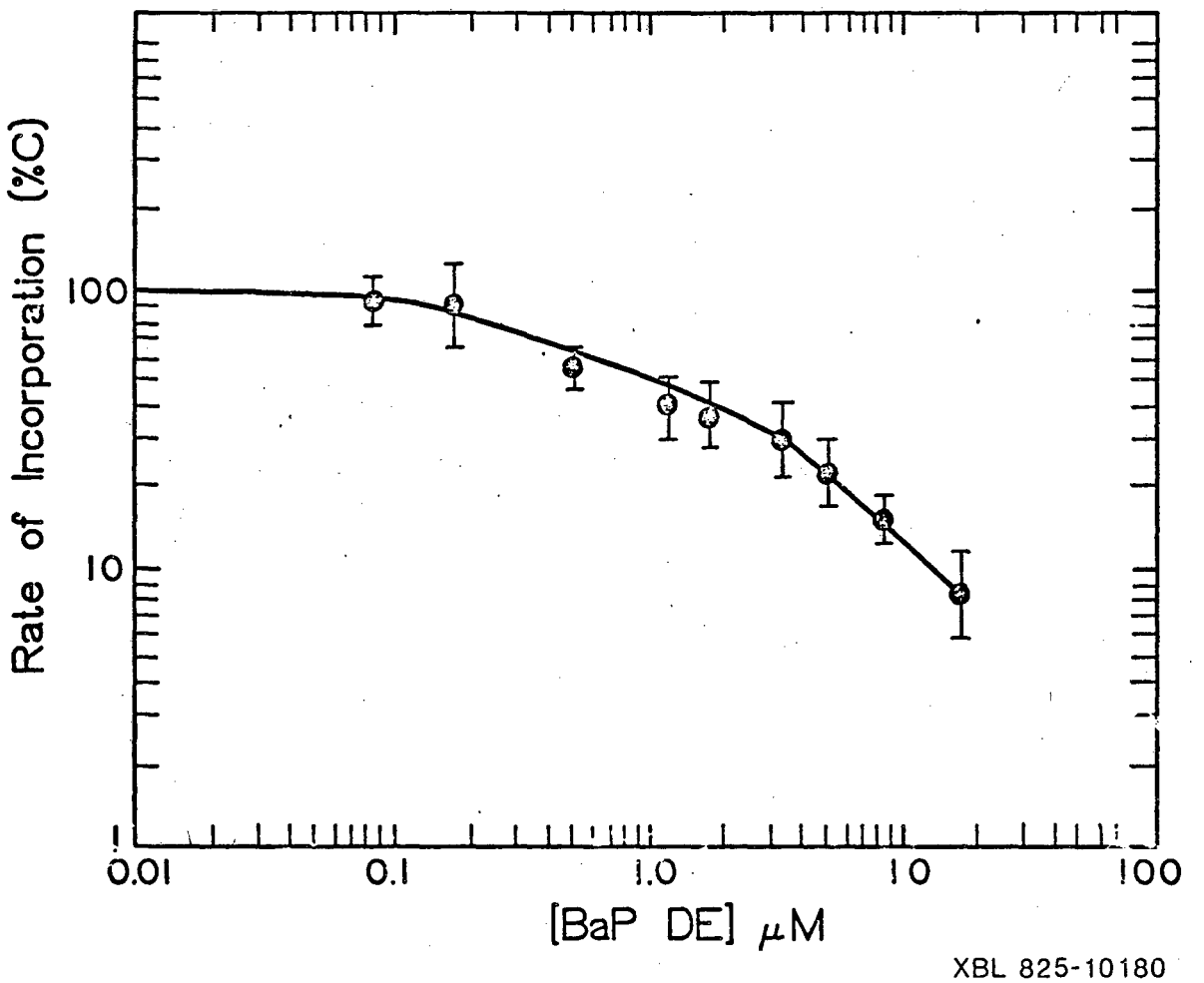

Rate of Incorporation (%C)
100
10
1
0.01
0.1
1.0
10
100
[BaP DE] μM
XBL 825-10180

Figure 4:

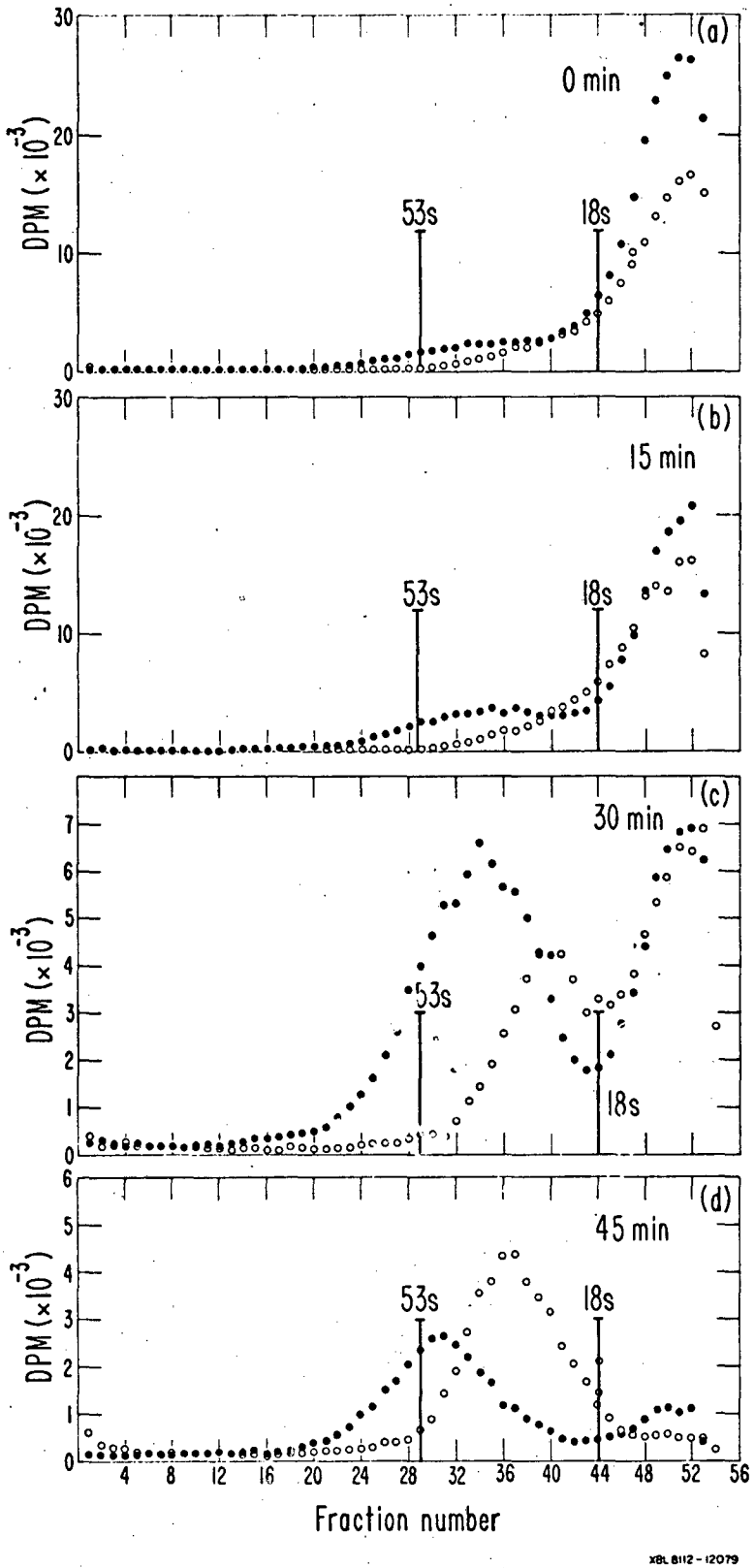
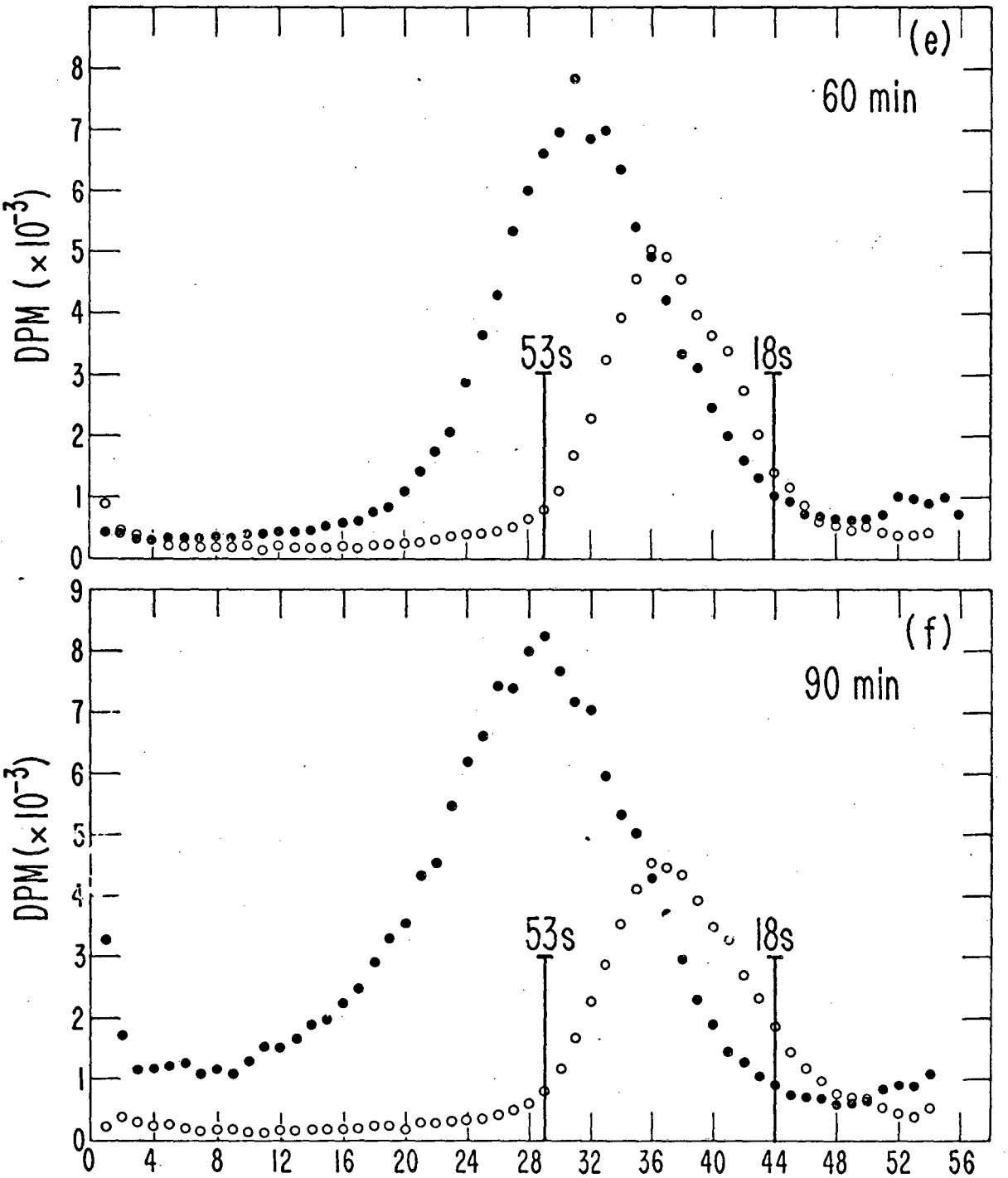

(a)
0 min
53s
18s
(b)
15 min
53s
18s
(c)
30 min
53s
18s
(d)
45 min
53s
18s
DPM (×10⁻³)
Fraction number
XBL 8112-12079

Figure 4:



XBL 8112-12080

Figure 5:

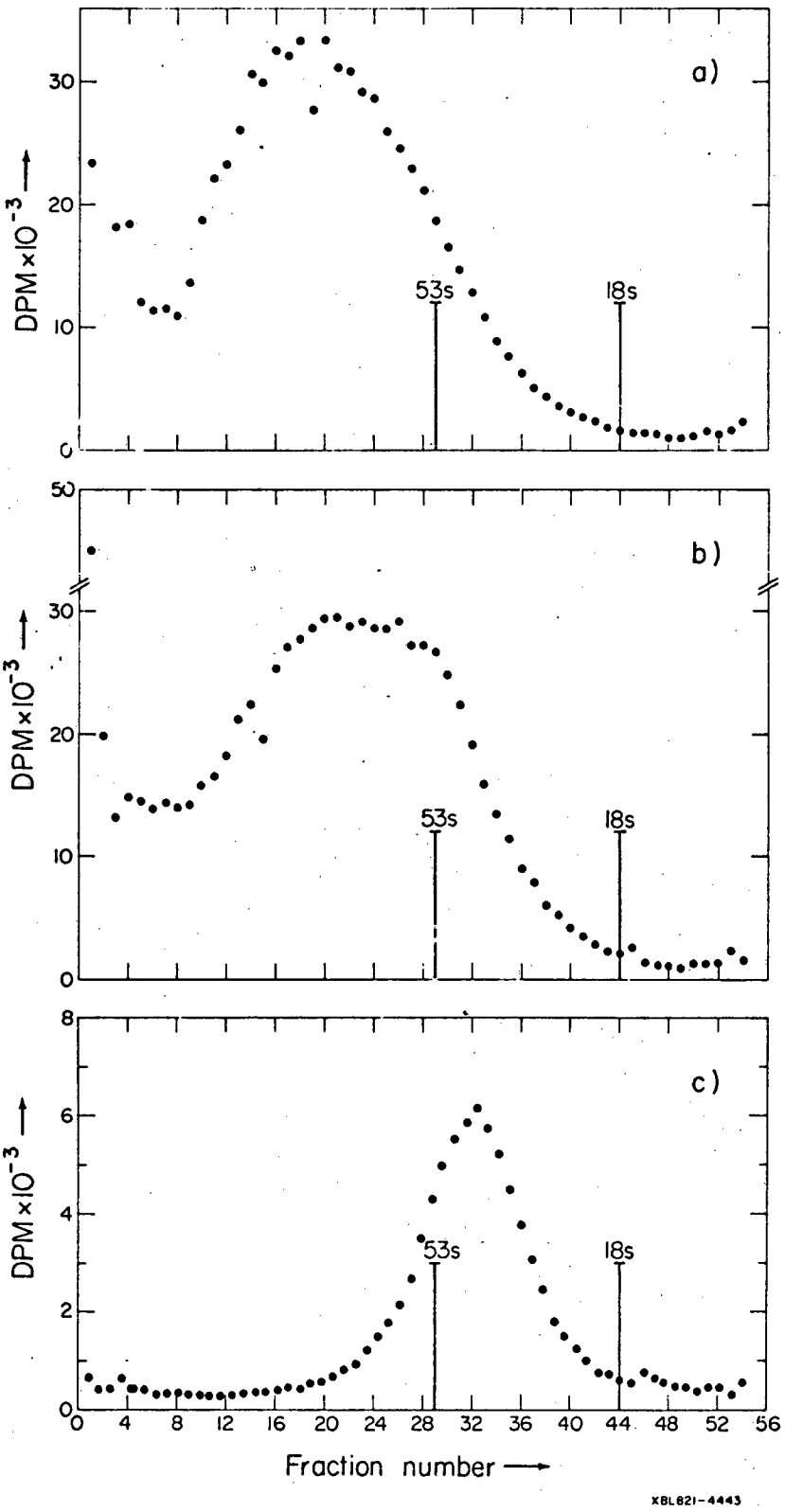


Figure 6:

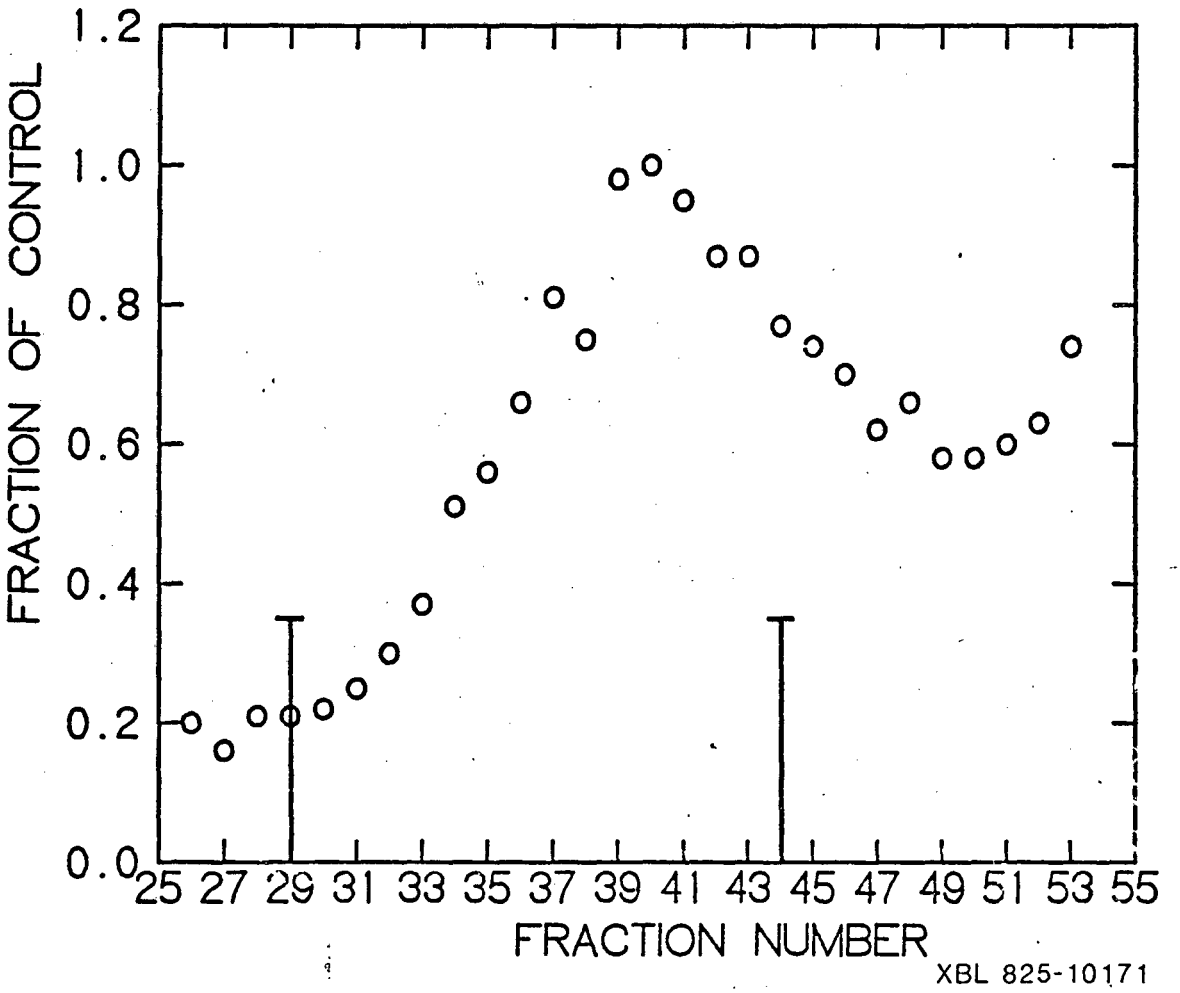

FRACTION OF CONTROL
1.2
1.0
0.8
0.6
0.4
0.2
0.0
25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55
FRACTION NUMBER
XBL 825-10171

Figure 7:

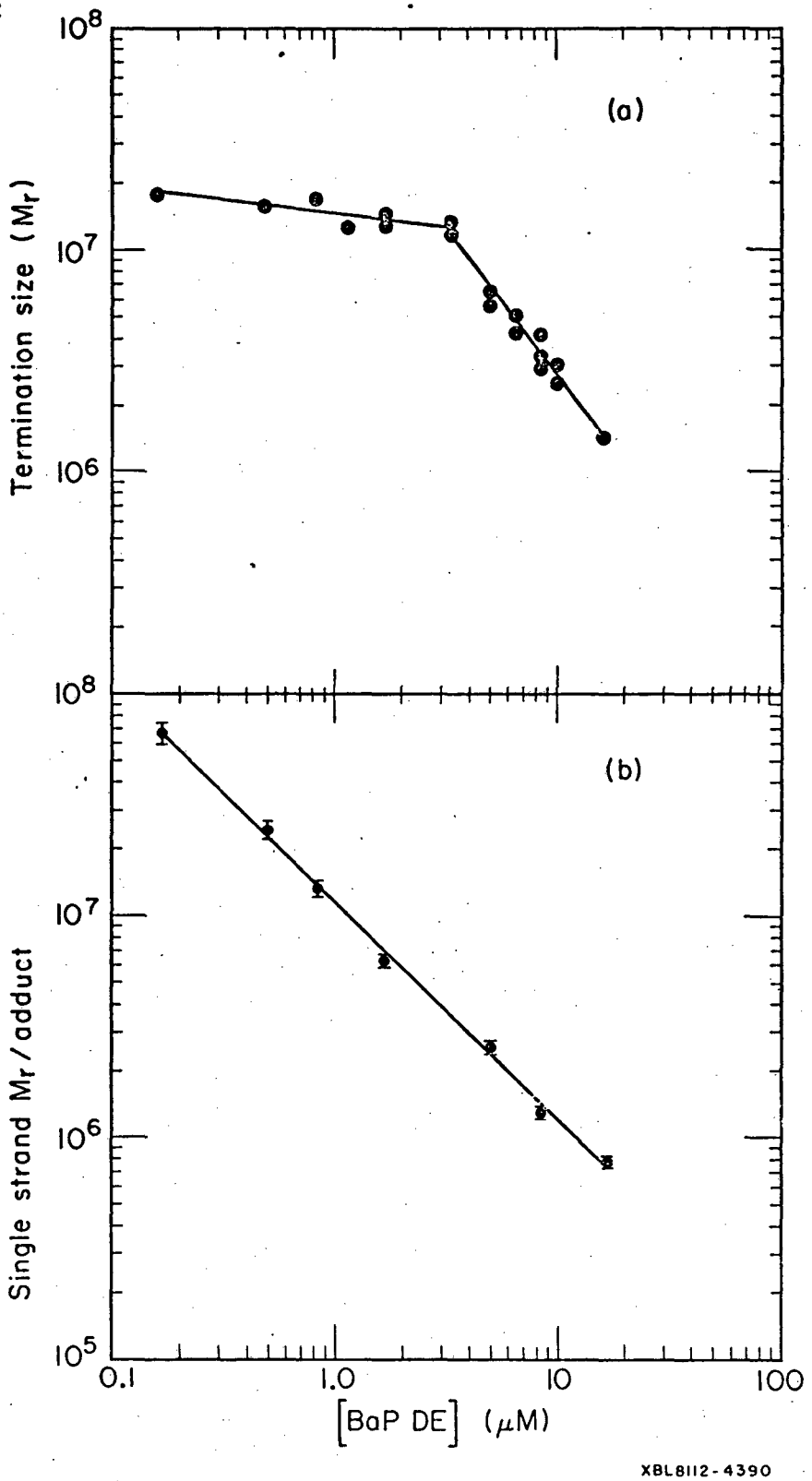
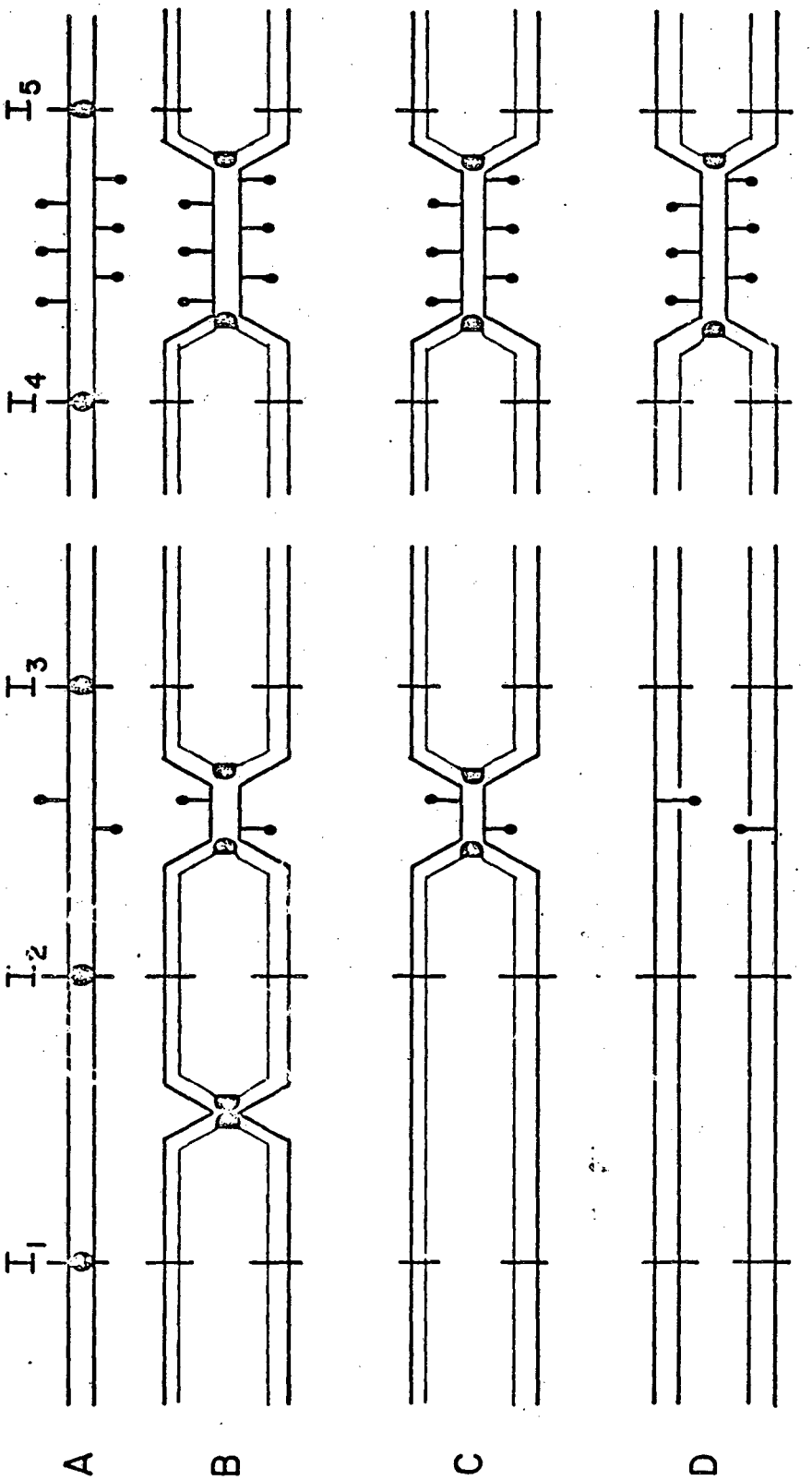

(a)
(b)
Termination size (M_r)
Single strand M_r / adduct
10^8
10^7
10^6
10^5
0.1
1.0
10
100
[BaP DE] (μM)
XBL8112-4390

Figure 8:


I5
I4
I3
I2
I1
A
B
C
D
• =BaP DE adduct
I =origin of replication
XBL 825-10181

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