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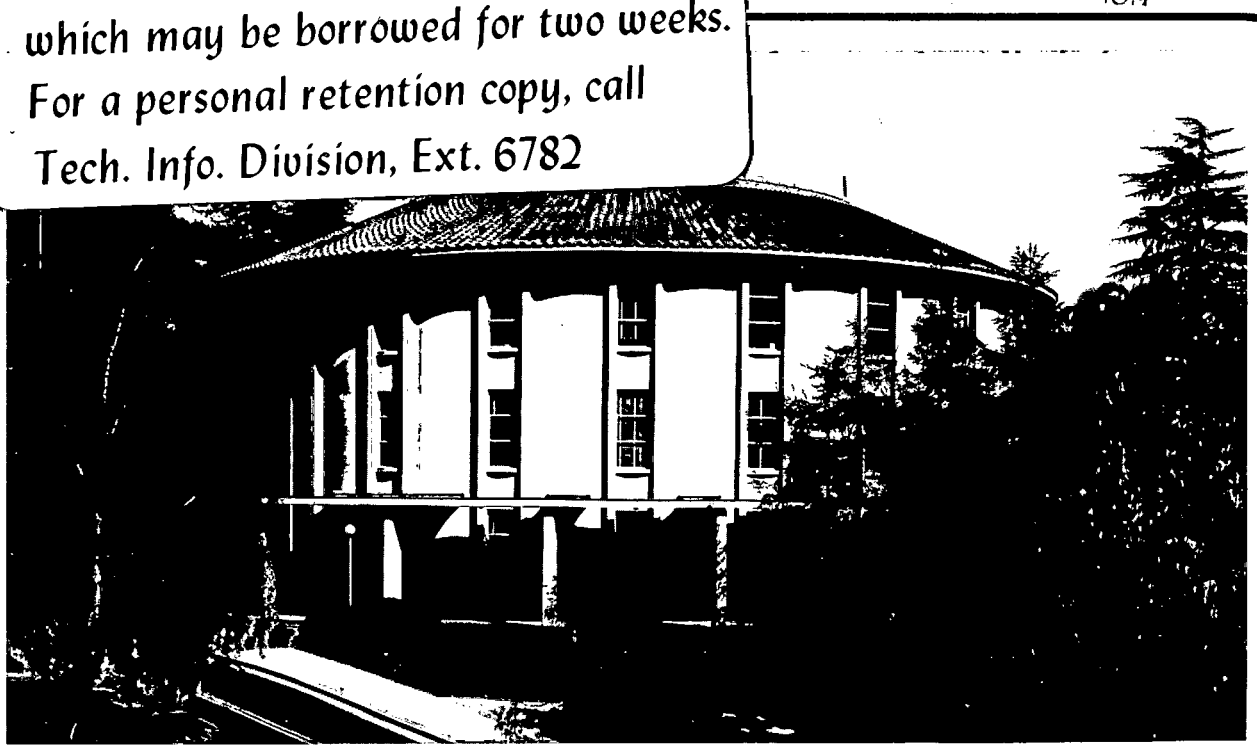

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ALTERATIONS IN SV40 DNA SYNTHESIS CAUSED BY BENZO[a]PYRENE DIOL EPOXIDE

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SUMMARY

The effect of the benzo[a]pyrene derivative (±) r-7, t-8-dihydroxy-t-9, 10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BaP diol epoxide) on the replication of SV40 DNA in TC-7 cells was studied. BaP diol epoxide inhibited replication of the viral DNA in a concentration dependent fashion. Analysis of the replicated forms of DNA in a pulse chase experiment after treatment with BaP diol epoxide indicated that form II sturctures (molecules with gaps in one strand of the DNA) accumulated after 1 hr incubation. At the concentrations of BaP diol epoxide used in these experiments (1.0 ug/ml) the initial number of adducts formed depended upon the form of the SV40 DNA. Form I DNA had twice the number of adducts when compared to form II DNA. Size analysis of the newly synthesized DNA by alkaline sucrose density gradient centrifugation of the accumulated form II structures indicated that even though there were an average of 4 BaP diol epoxides per genome the DNA synthesized was full length or nearly full length. The data is consistent with the replication complex on SV40 DNA being able to read through BaP diol epoxide adducts but being unable to terminate DNA synthesis properly.

INTRODUCTION

Current concepts of the mechanism(s) of chemical carcinogenesis involve the covalent interaction of the carcinogen with DNA [1-4]. Interest in this particular type of interaction originated from the observation that the phenotypic properties of cancer cells are passed on to progeny cells in a "heritable" fashion implying an alteration in the genome through mutation. In fact, there is a good correlation between the mutagenicity of a compound and its carcinogenicity [5,6] as well as between the ability of a compound to bind covalenty to DNA and its carcinogenic potential [7]. Although the mutation theory of chemical carcinogenesis has been disputed [8-11], it remains a central focus of much of the research into the mechanisms of chemical carcinogenesis. The general flow of this research has been to identify compounds that form covalent adducts with DNA, determine the structure of these adducts and how they alter the DNA secondary structure, and search for specificity of reaction that might relate to carcinogenesis. The emphasis of these studies has been principally on the chemistry of the interaction of carcinogens with DNA. This report describes experiments designed to understand how a chemical carcinogen that binds to DNA alters the ability of that DNA to serve as a template for DNA replication.

Carcinogens inhibit DNA synthesis with an efficiency that correlates with their carcinogenicity [12-14]. The

mechanism(s) by which chemicals cause inhibition of DNA synthesis in higher eucaryotes is not known; however, analogies with UV and X-ray irradiation inhibition of DNA synthesis would suggest that effects may be expected on both initiation of DNA synthesis as well as elongation of already initiated fragments [15-17]. A system which may provide considerable new information as to how chemical carcinogens perturb DNA synthesis is the replication of SV40 virus in african green monkey kidney cells. The replication of SV40 DNA primarily utilizes components synthesized from the host genome [18,19]. Therefore, the effects of carcinogens on the process of replication of SV40 should reflect what these compounds do to cellular DNA synthesis. The advantage of studying carcinogen effects on SV40 DNA replication rather than replication of cellular DNA is that SV40 replicative intermediates can be readily isolated and be used for structural studies.

MATERIALS AND METHODS

1. Cells and virus culture techniques

TC-7 cells, a cell line derived from African green monkey kidney cells by J. Robb, were grown in Dulbecco's Modified Eagle's medium(DME, Grand Island Biological, Grand Island, New York), supplemented with 10% Donor calf serum (Flow Laboratories, McLean, Virginia) on 100 mm plastic tissue culture dishes (Falcon Plastics, Oxnard California). The cell were infected upon reaching confluency with virus twice plaque purified from a stock of the small plaque forming strain SP12 of SV40 originally isolated by J. Robb. Radioactive materials were added as described in the text.

2. Isolation of SV40 DNA

SV40 DNA was isolated from infected cells by a modification of the Hirt procedure [20]. Approximately 10^7 cells were resuspended in 0.8 ml of 25 mM Tris, pH 7.5, 10 mM EDTA, 0.6% SDS and the lysate was mixed gently and incubated at 37° C for 10 min. The solution was then made 1.0 M in NaCl by adding 0.2 ml of 5 M NaCl, mixed gently and stored overnight at 4° C. After centrifugation at 4° C for 60 min at 15,000 rpm in the Beckman SW 50.1 rotor, the supernatant was collected. The SV40 DNA was banded by density equilibrium centrifugation in CsCl (4.2 gm CsCl and 1.0 mg ethidium bromide added to 5.5 ml of DNA solution, centrifuged at 35,000 rpm and 4° C for 48 hrs in the Beckman SW 50.1 rotor)

to separate supercoiled SV40 DNA from nicked DNA. The DNA was then extracted at least five times with salt-saturated butanol to remove ethidium bromide and dialyzed against storage buffer (TE, 10 mM Tris-hydrochloride, pH 7.9, 1 mM EDTA).

3. Agarose Gel Electrophoresis.

Aliquots of DNA from the Hirt supernatants were analyzed by electrophoresis on 1.4% agarose gels at 50 volts for 10 hrs at room temperature. Radioactivity was quantified by soaking the gel slices in Aquasol-2 (Packard Instrument Comp., Inc., Downers Grove, Ill.) until the counts were no longer released from the gel.

4. Alkaline Sucrose Density Sedimentation.

Samples of the Hirt supernatants were prepared for sedimentation by extracting 2 times with buffered phenol, precipitating with 3 volumes of ethanol, and resuspending in 10 mM Tris, 1 mM EDTA buffer, pH 8.0. SV40 form II marker DNA (32P labeled) was included in the sample. Alkaline sedimentation analysis was performed on 5-20% sucrose gradients containing 0.7 M NaCl, 0.3 M NaOH for 6 hrs at 50,000 rpm in an SW 50.1 rotor at 4° C. Fractions were collected from the bottom of the tube, precipitated with excess 10% TCA and collected on filters for counting.

5. BaP Diol Epoxide

The diol epoxide derivative of benzo[a]pyrene used in these experiments was the (±) r-7, t-8-dihydroxy-t-9, 10-oxy-7,8,9,10- tetrahydrobenzo[a]pyrene (BaP diol epoxide). This compound and its tritiated derivative were supplied by Dr. K. Straub. Some experiments were done with BaP diol epoxide obtained from the ITT Research Institute (Chicago, Ill.).

RESULTS

1. Inhibition of SV40 Replication

To investigate the effect of BaP diol epoxide on SV40 DNA replication, the compound was added at different concentrations to TC-7 cells productively infected with SV40. At various times after the addition, the cultures were pulsed with 3H-thymidine to label the DNA being synthesized. The SV40 DNA was separated from cellular DNA by Hirt extraction and the amount of 3H-thymidine incorporated into viral DNA quantified. Figure 1 shows that shortly after the addition of BaP diol epoxide there was an increase over controls of the amount of Hirt extractible newly synthesized DNA. As time progressed after treatment, the amount of DNA synthesis dropped below the control and by 60 min inhibition was quite evident. Essentially similar results were seen when the cellular DNA in the Hirt pellet was analyzed (data not presented).

2. Agarose Electrophoretic Analysis of Replicative Intermediates

Agarose gel electrophoresis has been used to separate replicative intermediates of both SV40 and polyoma virus [21,22]. The technique resolves newly initiated DNA from DNA later in the replication cycle as well as from the termination complex. When agarose gel electrophoresis was used to follow the change in distribution of replicative inter-

mediates caused by BaP diol epoxide the data in Figure 2 were obtained. Electrophoresis resolved form I DNA which migrated approximately 5 cm into the gel from form II DNA which electrophoresed to 1.5 cm. Other replicative forms were spread throughout the gel from the top to the form I. In this experiment, the 3H-thymidine was added at the same time as the BaP diol epoxide and then either the cells were harvested after 15 min, or chased for 60 min with unlabeled thymidine. After 15 min treatment there was little change in the distribution of replicative intermediates (compare Fig. 2a and 2b). However, when the replicating DNA was chased for 60 min there was evidence of a build-up of replicative intermediates that correspond to the form II (Fig. 2d). This form of the replicative intermediate was also observed to accumulate when Hirt extracted BaP diol epoxide DNA is analyzed by CsCl-ethidium bromide equilibrium centrifugation (data not shown).

3. Analysis of Adducts on SV40 DNA

The BaP diol epoxide adducts on the various forms of SV40 DNA were determined by reacting productively infected cells with 3H-BaP diol epoxide, harvesting the SV40 DNA by Hirt extraction, and analyzing the molecules by agarose gel electrophoresis. The SV40 DNA on the gel was quantified by integrating the fluorescence of the ethidium bromide stained gel. The area was related to DNA mass by comparison with a stained standard of known specific activity in 14C-

thymidine. The BaP diol epoxide in the SV40 DNA bands was measured by counting the 3H in the individual bands. Table 1 shows the average number of adducts determined in this fashion from SV40 DNA after 15 min treatment with BaP diol epoxide and after 75 min incubation. After a 15 min treatment with 2.0 ug/ml BaP diol epoxide, the average number of adducts per form I DNA was 9.9 per genome; whereas, the form II DNA had 4.2 adducts per genome. After an additional hour incubation, the number of adducts in the form I DNA had increased only slightly, but the form II DNA contained twice the number of adducts. Presumably, this increase in the number of adducts is due in part to further reaction of the BaP diol epoxide as well as the conversion of the form I molecules with higher adduct levels to form II through replication. The level of adducts reported in Table 1 was shown not to cleave the parental 14C-thymidine labeled DNA(data not shown).

4. Size Analysis of Newly Synthesized DNA

The number of adducts formed on the SV40 DNA under the conditions described in Figure 2 would allow for an average of one adduct per 2476 to 1040 base pairs. If the adducts act as complete stops to replication, then form II structures would not accumulate unless the replication complex were able to bypass adducts. The replication apparatus may replicate through the adduct, or it may skip the adduct leaving gaps in one or both of the newly replicated strand.

To investigate this possibility, the accumulated form II was harvested and the size of the DNA synthesized after BaP diol epoxide treatment was compared to untreated termination complex by alkaline sucrose sedimentation analysis. The termination complex of untreated control contains a parental circular single strand DNA and a linear almost full length complementary single strand [23]. Figure 3 presents the alkaline sucrose density gradients of form II populations isolated from BaP diol epoxide treated (1.0 ug/ml) replicating SV40 and untreated controls. Panel "a" contains the data from the analysis of the form II population isolated from replicating SV40 after pulse labeling with 3H-thymidine and chasing for 60 min. This population contains an 18s circular SV40 single strand labeled with thymidine as well as a single strand 16s molecule. The form II molecules presumably arise from form I molecules that were labeled during the initial pulse and cleaved randomly during the subsequent work-up. In panel "b" the data is from SV40 replicating as described for "a" except that the cells were treated with BaP diol epoxide at the time of labeling with 3H-thymidine. When BaP diol epoxide was present during DNA synthesis, the SV40 replication system progressed to the 16s linear single strand, but very little 18s circular SV40 DNA was formed. Also, the majority of the newly replicated DNA was full length or nearly full length. There was very little tailing from the 16s material towards the low s values. Such tailing would indicate that gaps were being formed at the loca-

tion of the adducts. These data indicate that the BaP diol epoxide adducts on the DNA inhibit the conversion of the single strand DNA into circular forms, but do not act as complete blocks to the replication apparatus, or leave gaps opposite all adducts. When the form II from BaP diol epoxide treated replication systems was treated with eco R1 restriction enzyme to map the position of the gap in the single strand molecule, the resulting gap was found to be randomly distributed around the molecule in contrast to the gap in control termination complexes (data not presented).

DISCUSSION

The use of replicating viruses to study the effect of DNA binding compounds has primarily been with bacteriophages [24-27]. With BaP derivatives these studies have shown that compounds that bind covalently to the template block the replication of both DNA and RNA containing genomes. Recently, Chang et al. [28] reported that BaP derivatives, one of which was the BaP diol epoxide, could inhibit SV40 replication in CV-1 monkey cells and that viruses produced in the presence of BaP diol epoxide had reduced infectivity. The study reported here deals with the form of the SV40 replicative intermediate that accumulates in the presence of BaP diol epoxide.

Our studies indicate that the presence of a DNA adduct does not stop elongation of newly synthesized DNA, but that the adduct does act to inhibit complete termination of the replicative intermediate. The gap in the newly synthesized DNA is not located in one section of the molecule as is the case for the normal termination complex [23]. Whether one of the adducts is located within the gap is not known; however, experiments to ligate the gaps have not been successful(data not presented). Incomplete termination and gap filling may be due to altered topological features of the molecule caused by adducts on the DNA. We have recently reported that large topological alterations do occur in naked DNA when only a few adducts are present [29]. Whether

this phenomenon occurs with the 2 to 5 adducts per molecule formed in culture in these experiments is not known.

Sarasin and Hanawalt [30] have used SV40 replication to study the effect of UV induced thymine dimers on DNA replication. Their observations indicated that the newly synthesized DNA did eventually elongate beyond the inter-dimer distance. These results and ours reported here are in contrast to the in vitro studies reported by Moore and Strauss [31] in which both thymine dimers and N-acetoxy-2-acetyl aminofluorene residues were shown to block the replication apparatus. However, Yamaura et al. [32] found that BaP diol epoxide adducts on synthetic homopolymers of both deoxy and ribonucleic acids did not alter the size of the DNA copies.

We found no evidence that the BaP diol epoxide inhibited initiation of replication directly. Of course, inhibition of termination as described above will indirectly inhibit initiation since it has been shown that molecules that have been involved with replication are more likely to be those molecules that are initiated [33].

The effect of BaP diol epoxide on termination of SV40 DNA synthesis may explain some of the observations concerning carcinogen inhibition of cellular DNA synthesis. The reduced rate of DNA synthesis, seen as carcinogen-treated cells progress further into S [34], may be caused by the inability to terminate replicon synthesis and allow the maturation into domains. These maturation events are known

to predominate in the later parts of S.

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Table 1. BaP Diol Epoxide Adducts on Replicating SV40 DNA.

Time (min)	Adducts per SV40 Form I	Form II
15	9.9	4.2
75	11.4	9.6

TC-7 cells infected with SV40 as described in MATERIALS AND METHODS were treated for the indicated times with 2.0 ug/ml ^{3}H-BaP diol epoxide (1.23 Ci/mmole) at the peak of DNA replication. The DNA was harvested by Hirt extraction and analyzed by agarose gel electrophoresis. The quantities of DNA and the amount of BaP diol epoxide in the DNA was quantified as described in the RESULTS.

FIGURE LEGENDS

Figure 1. Inhibition of SV40 DNA Synthesis by BaP Diol Epoxide.

SV40-infected TC-7 cells were treated with (A) 0 ug, (B) 0.1 ug, (C) 0.3 ug, and (D) 1.0 ug BaP diol epoxide per ml of medium. At varying times after treatment, 3H-thymidine was added at 50 uCi/ml for 10 min. Cells were washed with cold isotonic buffer and extracted according to Hirt (20). Aliquots of the Hirt supernatant were precipitated with 10% TCA, collected on filters, washed, and counted.

Figure 2. Pulse-chase of SV40 Replicative Intermediates Made After BaP Diol Epoxide Treatment.

At 30 hr postinfection, cells were labeled for 10 min with medium containing 100 uCi/ml 3H-thymidine and either DMSO (A), or 1.0 ug/ml BaP diol epoxide (B) and harvested immediately using the method of Hirt(20). Other cells were labeled and treated, and then chased for 60 min with medium containing 100 ug/ml unlabeled thymidine with either DMSO (C), or 1.0 ug/ml BaP diol epoxide (D) before harvesting. Aliquots of the Hirt supernatant were analyzed by agarose gel electrophoresis on 1.5% agarose at 50 volts for 10 hr at 18°C. Radioactivity was quantified by soaking the gel slices in counting fluid until the counts eluted remained constant.

Figure 3. Alkaline Sucrose Density Centrifugation of SV40 Form II Molecules that Accumulate in the Presence of BaP Diol Epoxide.

The form II peak fractions from the CsCl-EtBr gradient from DMSO and BaP diol epoxide treated DNA were extracted 4 times with butanol and dialyzed against 10 mM Tris, pH 7.8 1 mM EDTA. Analysis by alkaline sucrose gradient sedimentation was on 5 to 20% sucrose gradients with 0.7 M NaCl, 0.3 M NaOH, 2.5 mM EDTA, 0.015% Sarkosyl centrifuged for 6 hr at 50,000 rpm in an SW50.1 rotor at 4°C. Fractions were collected from the bottom of the tube, precipitated with excess 10% TCA and collected on filters for counting. The arrows indicate the peak positions of an internal 32P SV40 form II marker included in each gradient.

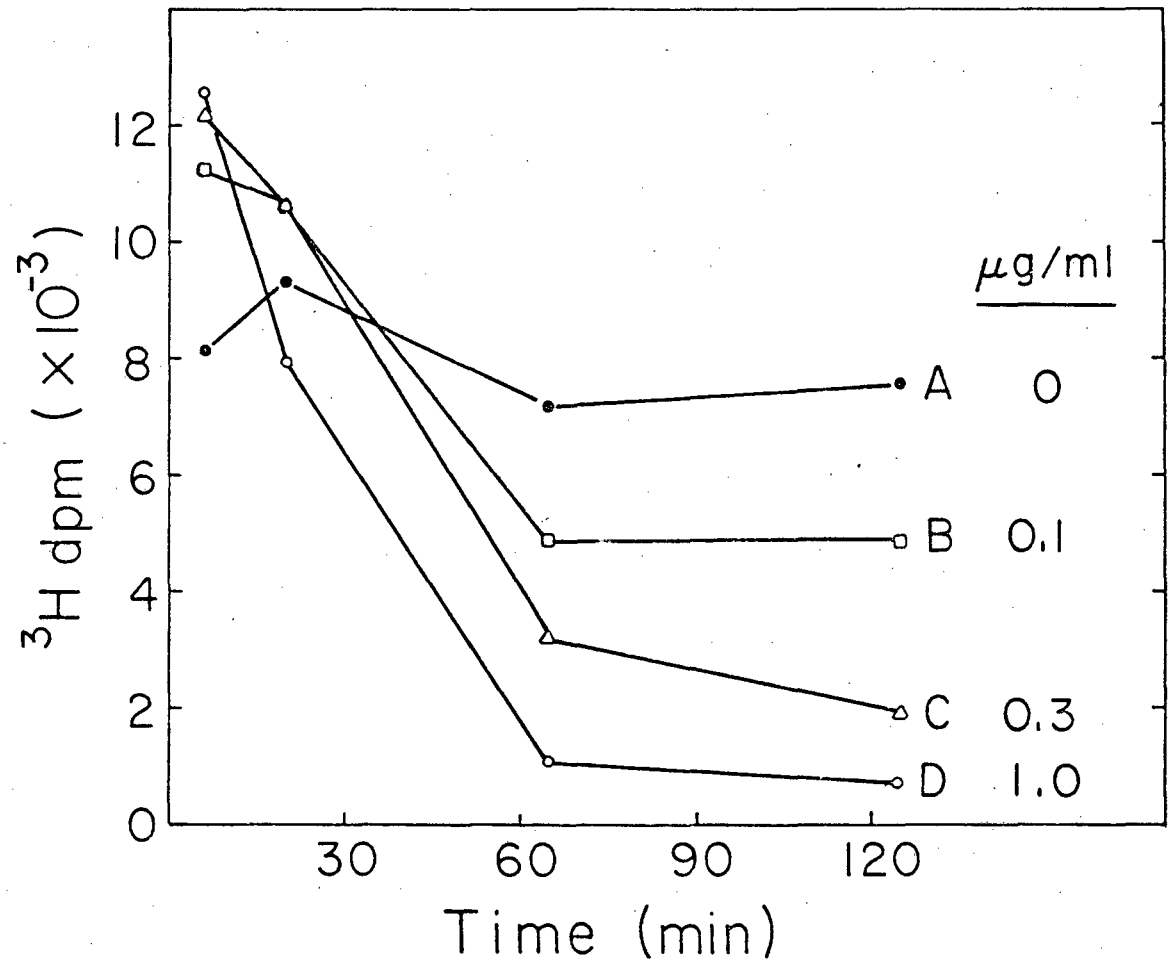

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Figure 1.

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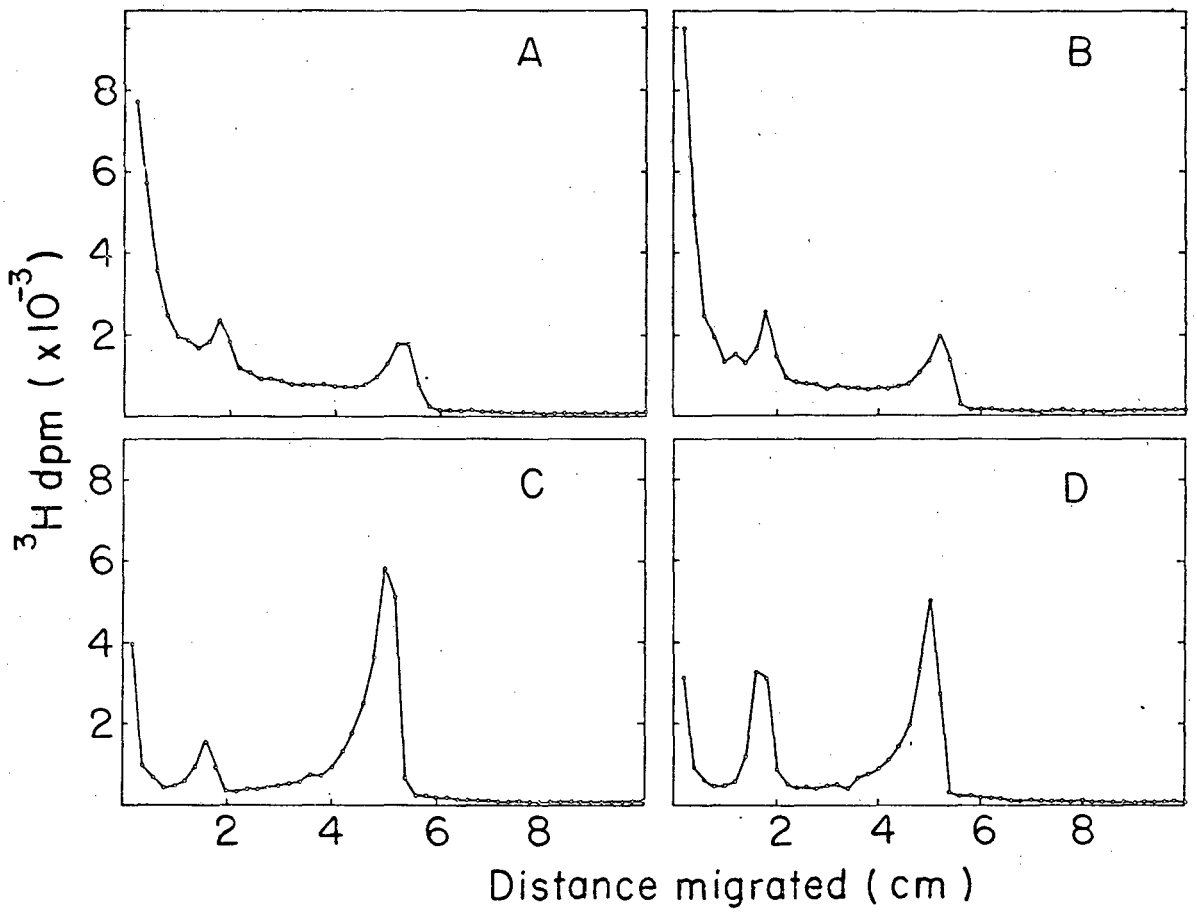

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Figure 2

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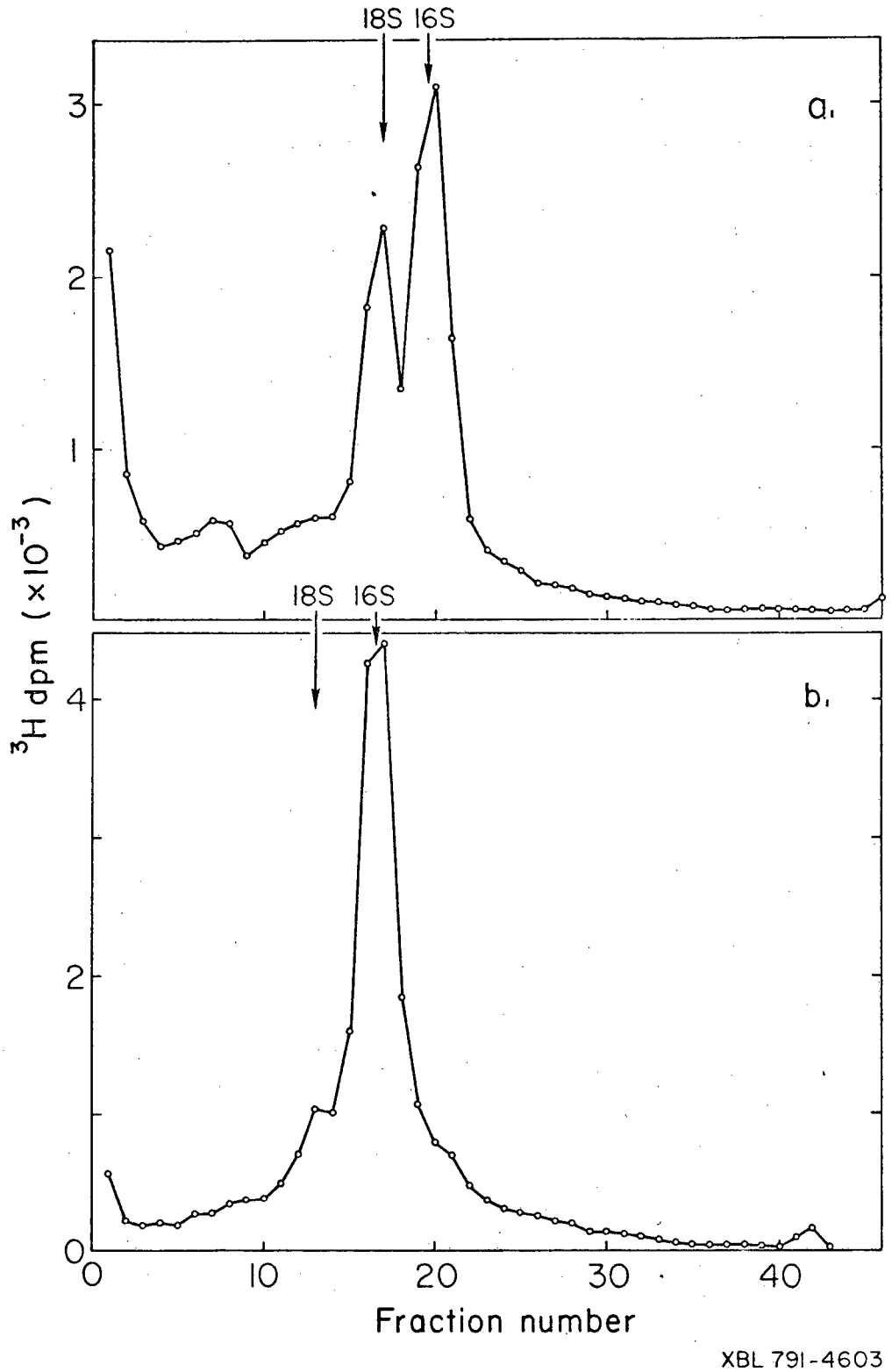

Figure 3

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