independent of template binding (Hazra et al., 1984; Ferrin & Mildvan, 1985, 1986). Yet in the polymerization reaction, noncomplementary substrates do not seem to interfere in the addition of complementary substrate. Therefore, a distinct mechanism for the preferential binding of substrates exhibiting complementarity to template nucleotide must exist.

Registry No. Pol I, 9012-90-2; PLP, 54-47-7; L-lysine, 56-87-1; thymidine 5′-triphosphate, 365-08-2.

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Persistent and Heritable Structural Damage Induced in Heterochromatic DNA from Rat Liver by *N*-Nitrosodimethylamine†

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ABSTRACT: Analysis, by benzoylated DEAE-cellulose chromatography, has been made of structural change in eu- and heterochromatic DNA from rat liver following administration of the carcinogen *N*-nitrosodimethylamine (10 mg/kg body weight). Either hepatic DNA was prelabeled with [^{3}H]thymidine administered 2–3 weeks before injection of the carcinogen or the labeled precursor was given during regenerative hyperplasia in rats treated earlier with *N*-nitrosodimethylamine. Following phenol extraction of either whole liver homogenate or nuclease-fractionated eu- and heterochromatin, carcinogen-modified DNA was examined by stepwise or caffeine gradient elution from benzoylated DEAE-cellulose. In whole DNA, nitrosamine-induced single-stranded character was maximal 4–24 h after treatment, declining rapidly thereafter; gradient elution of these DNA preparations also provided short-term evidence of structural change. Following incubation of purified nuclei with micrococcal nuclease, 10–12% of labeled DNA was solubilized (euchromatin) by 1.0 unit of micrococcal nuclease (5 mg of DNA)$^{-1}$ mL^{-1} after 9 min. In prelabeled animals, administration of *N*-nitrosodimethylamine caused a marked fall in the specific radioactivity of solubilized DNA, while that of sedimenting DNA was not affected. Caffeine gradient chromatography suggested short-term nitrosamine-induced structural change in euchromatic DNA, while increased binding of heterochromatic DNA was evident for up to 3 months after carcinogen treatment. Preparations of newly synthesized heterochromatic DNA from animals subjected to hepatectomy up to 2 months after carcinogen treatment provided evidence of heritable structural damage. Carcinogen-induced binding of heterochromatic DNA to benzoylated DEAE-cellulose was indicative of specific structural lesions whose affinity equalled that of single-stranded DNA up to 1.0 kilobase in length. The data suggest that structural lesions in heterochromatin, which may be a consequence of incomplete repair, are preferentially degraded by endogenous nuclease(s).

In mammalian cells, various stages in the excision repair of DNA have been identified (Collins & Johnson, 1984). Best characterized have been those reactions involved in the initial stages (Teebor & Frenkel, 1983). Most work on nitrosamine-induced DNA repair has concerned monitoring the concentration of alkylated bases in DNA (Preussmann & Stewart, 1984), a parameter of the first stage of DNA repair. The distribution of carcinogen adducts within subfractions of DNA generated by digestion of chromatin has provided evi-

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dence of heterogeneous repair processes [for references, see Baranyi-Furlong & Goodman (1984)]. However, in terms of chromatin, evidence concerning intermediate and late stages of DNA repair is lacking. Information regarding these processes necessitates structural analysis of DNA.

Chromatography on benzoylated DEAE-cellulose (BD-cellulose)[1] permits structural analysis of DNA: wholly double-stranded DNA is eluted with NaCl while recovery of DNA containing single-stranded regions requires the addition of caffeine to the salt solution (Strauss, 1981). The amount of caffeine required is proportional to the extent of single strandedness. In our laboratory, DNA isolated from carcinogen-treated animals has been analyzed by stepwise BD-cellulose chromatography (Stewart, 1981). Correlation of these data with the generation and loss of carcinogen adducts from DNA suggests that generation of single-stranded regions in DNA is a consequence of repair (Stewart & Haski, 1984; Stewart et al., 1985). The amount of DNA at this intermediate stage of repair is in dynamic equilibrium between the initial rate of repair (indicated by adduct loss) and the rate at which repair is completed (dependent upon polymerization and ligation). In the present study, we have sought to further examine nitrosamine-induced structural damage to DNA by caffeine gradient elution from BD-cellulose (Haber & Stewart, 1981, 1985). This more sensitive analytical procedure permited structural analysis not only of total DNA but also after fractionation of DNA according to its association with eu- or heterochromatin.

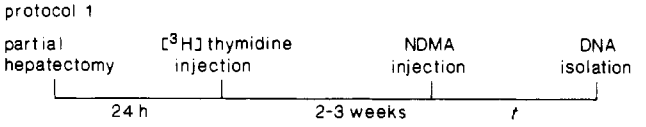
While originally histologically descriptive terms, "euchromatin" and "heterochromatin" can be distinguished by genetic, cytological, structural, biochemical, and functional characteristics [for reviews, see Cartwright et al. (1982) and Reeves (1984)]. Euchromatic DNA (about 10% of the mammalian genome) represents transcriptionally active genes while its heterochromatic counterpart (remaining 90%) is transcriptionally inactive. Central to the investigation of chromatin structure, at all levels of organization, is the use of both exogenous and endogenous nucleases. Exogenous nucleases, mainly micrococcal nuclease and DNase I, are extensively used to probe nucleosomal and chromatin structure (Kornberg, 1977; McGhee & Felsenfeld, 1980; Igo-Kemenes et al., 1982). Most studies of chromatin–carcinogen interrelationships have involved exhaustive, usually DNase I mediated, degradation of chromatin to the single nucleosome level. However, micrococcal nuclease has become the enzyme of choice in the study of transcriptionally active chromatin because at low levels it does not overly degrade DNA (Dimitriadis & Tata, 1980; Kilianska et al. 1982). Tata and Baker (1978) reported euchromatic DNA solubilized as polynucleosomal aggregates of 6–20 covalently linked nucleosomes (1200–6000 bp) following micrococcal nuclease digestion. Nuclease solubilization of euchromatin presumably results from its comparative open structure necessary for transcriptional activity.

The present paper describes structural change in eu- and heterochromatic DNA, rather than in linker and core DNA, following administration of *N*-nitrosodimethylamine (NDMA) to rats. By BD-cellulose chromatography, the conspicuous feature noted was the detection of single-stranded regions in heterochromatic DNA, suggesting incomplete repair. In nitrosamine carcinogenesis specifically, relationships have been established between DNA repair, cell replication, and carcinogenesis by the tumorigenic effect of surgical hepatectomy in carcinogen-treated animals (Grisham et al., 1983). Our investigation included the effects of cell replication on the persistence of such structural damage. The effect of template strand lesions on the structure of newly synthesized DNA was assessed by subjecting animals to partial hepatectomy at intervals after administration of NDMA. The results suggest that administration of the nitrosamine results in the generation of heritable structural lesions in heterochromatic DNA which may be detected for months in exposed animals.

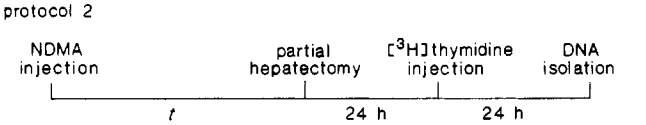
Materials and Methods

Materials. BD-cellulose was purchased from Boehringer-Mannheim (Mannheim, West Germany), [*methyl*-^{3}H]thymidine (20 Ci/mmol) from the Radiochemical Centre (Amersham, U.K.), *N*-nitrosodimethylamine from Sigma Chemical Co. (St. Louis, MO), and micrococcal nuclease (EC 3.1.31.1) from either BoehringerhMannheim or Worthington Biochemical Corp. (Freehold, NJ).

Treatment of Animals. Female Wistar rats were maintained as previously described (Stewart & Haski, 1984). For the purpose of analysis, hepatic DNA was in vivo radiolabeled by subjecting 120-g rats to partial hepatectomy (Higgins & Anderson, 1931) and administering [^{3}H]thymidine (50 μCi) 21 and 29 h after surgery. Animals were then allowed to recover for at least 2 weeks before administration of a single intraperitoneal injection of NDMA at a nonnecrotizing dose of 10 mg/kg body weight (Damajanov, 1973; Den Engelse & Philippus, 1977) in sterile isotonic saline (protocol 1). Control



animals were injected with an equivalent volume of saline by the same route. The effect of nitrosamine treatment on the structure of DNA synthesized subsequently was assessed by subjecting animals to partial hepatectomy at intervals after administration of NDMA. [^{3}H]Thymidine (50 μCi) was then injected 24 h after surgery. Nuclei isolation and enzymic digestion and/or isolation of DNA from crude homogenate was routinely undertaken 24 h after labeling (protocol 2).



Relationships between the two labeling protocols are illustrated above. In either case, the interval *t* was varied in the course of the investigation.

Purification of Rat Liver Nuclei. Nuclei were prepared from rat liver at 4 °C as described by Ward et al. (1985a) based on the methods of Blobel and Potter (1966) and Tata and Baker (1978). Isolated nuclei were counted by using an improved Neubauer hemocytometer, and the DNA concentration was estimated by taking 10^8 nuclei as equivalent to 2 mg of DNA.

Micrococcal Nuclease Digestion and Chromatin Fractionation. The activity of micrococcal nuclease was assayed (Heins et al., 1967) prior to use. Isolated nuclei were digested and fractionated under the same conditions described by Tata and Baker (1978) to generate eu- and heterochromatin from rat liver nuclei. The concentration of nuclei was brought to 5 mg of DNA/mL of digestion buffer (10 mM Tris-HCl, pH 8.0,

[1] Abbreviations: BD-cellulose, benzoylated DEAE-cellulose; NDMA, *N*-nitrosodimethylamine; bp, base pair(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid.

100 mM NaCl, 1.0 mM dithiothreitol, and 8% v/v glycerol). $CaCl_2$ was added (final concentration 0.25 mM) immediately before addition of micrococcal nuclease. Digestion was performed at 29 °C and the level of exogenous nuclease adjusted to 1.0 unit of enzyme (5 mg of nuclear DNA)$^{-1}$ mL^{-1} to solubilize the first 10–12% of radiolabeled DNA (1 unit = change in absorbance of 1.0 OD unit at 260 nm at 37 °C, pH 8.0, using calf thymus DNA as substrate; Heins et al., 1967). The reaction was terminated by addition of ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (final concentration 2 mM) with ice cooling. Digestion products were immediately separated by centrifugation at 1200*g* for 10 min at 4 °C, euchromatin being recovered in the supernatant.

Isolation of DNA. Hepatic DNA was isolated by phenol extraction (Ward et al., 1985a) from either a crude liver homogenate or the nuclease digestion products of purified liver nuclei.

Structural Analysis of Phenol-Extracted DNA by BD-cellulose Chromatography. Following phenol extraction of either whole homogenized DNA or nuclease-fractionated eu- and heterochromatin, carcinogen-modified DNA was examined for repair-related structural change by either stepwise or caffeine gradient elution from BD-cellulose (Haber & Stewart, 1981; Ward et al., 1985a).

Use of phenol extraction and quantification of BD-cellulose data were predicated on a series of studies involving DNA isolation procedures and reproducibility of chromatographic analyses. Habener et al. (1970), using various DNA isolation procedures including phenol extraction, reported that the yield of single-stranded DNA molecules was affected by the method of isolation. In our laboratory, three methods for preparing DNA were assessed with a view to minimizing the structural change attributable to endogenous nucleases. These methods involved (1) phenol extraction (as presently employed), (2) phenol extraction after digestion with proteinase K, and (3) lysis (in the absence of phenol) of nuclei in 8 M urea/1% sodium dodecyl sulfate (Stewart et al., 1986). Following phenol extraction (1), the proportion of sheared (modal length 10 kilobases) rat liver DNA recovered in caffeine solution after stepwise elution from BD-cellulose was 0.067 ± 0.006; other isolation procedures increased this proportion (Stewart et al., 1986).

While the reproducibility of stepwise elution has been assessed at the level of individual fractions (Huang & Stewart, 1976), gradient elution of DNA from BD-cellulose has not been described in the same detail. Wholly single-stranded DNA was eluted from BD-cellulose in proportion (after logarithmic transformation) to the concentration of caffeine (Haber & Stewart, 1981), the relationship between caffeine concentration and base length being consistent to 50 000 nucleotides ($R = 0.969$, $p < 0.0001$). The reproducibility of gradient elution implicit in these data was extended to rat liver DNA by studies in which the DNA, isolated by different procedures (designated 1–3 above), was subjected to both stepwise and gradient elution from BD-cellulose. The study involved DNA isolated from the livers of both control rats and rats injected with 120 mg/kg methyl methanesulfonate 3 h before sacrifice. Irrespective of treatment and isolation procedure, the proportion of caffeine-eluted DNA in a particular preparation was not affected by the chromatographic technique and could be determined equally from stepwise elution results or by summation of radioactivity recovered over 120 fractions during caffeine gradient elution (Table I). Confidence in the reproducibility and consistency of caffeine gradient elution data is further increased by coincidence of peaks in related preparations (Ireland & Stewart, 1986).

Table I: BD-cellulose Chromatography of Hepatic DNA from Control and Methyl Methanesulfonate Treated Rats: Comparison of Stepwise and Gradient Elution

isolation procedure[a]	caffeine-eluted DNA: proportion by stepwise elution	caffeine-eluted DNA: proportion by gradient elution
(1) immediate phenol extraction		
control	0.070	0.069
+methyl methanesulfonate	0.168	0.167
(2) proteinase K		
control	0.091	0.083
+methyl methanesulfonate	0.230	0.273
(3) cell lysis		
control	0.138	0.174
+methyl methanesulfonate	0.220	0.230

[a] For details of isolation procedures, see Stewart et al. (1986).

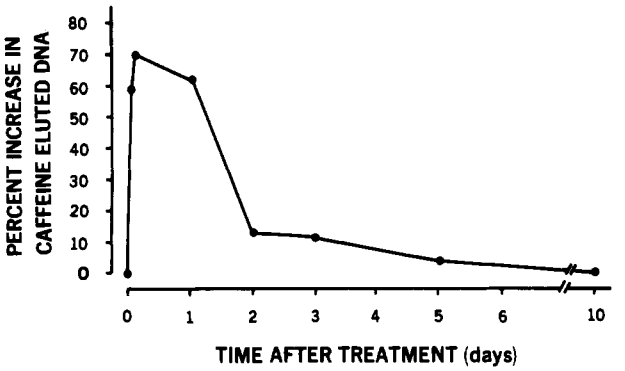


FIGURE 1: Generation and persistence of single-stranded regions in rat liver DNA as determined by caffeine elution from BD-cellulose in preparations isolated from rats treated with 10 mg/kg body weight NDMA (same dose applies to all other figures). Levels of caffeine-eluted DNA are expressed as a percentage of the caffeine-eluted DNA fraction from uninjected animals.

Results

In the course of this investigation, comparison has been made between structural change evident in DNA obtained by immediate phenol extraction of crude liver homogenate ("whole DNA") and DNA obtained by extraction of eu- or heterochromatin. In preparation of whole rat liver DNA, a higher proportion was bound to BD-cellulose in the presence of 1.0 M NaCl following injection of animals with NDMA (10 mg/kg body weight). Stepwise chromatography indicated a 70% increase in the proportion of DNA eluted with caffeine 4 h after NDMA injection. The amount of DNA exhibiting single-stranded character rapidly declined by 48 h and was not detectable after 10 days (Figure 1). These DNA preparations were also subject to caffeine gradient elution from BD-cellulose. DNA isolated within an hour of NDMA administration could be distinguished from control preparations by an increased proportion of DNA having affinity similar to that of single-stranded polynucleotides from 100 to 3000 residues (Figure 2). Four hours after carcinogen treatment, DNA in the caffeine fraction was eluted as two separate peaks, fractions 25–40 and fractions 40–60, corresponding to single-stranded regions of 120–400 bases and 440–2000 bases, respectively. Chromatograms of whole DNA isolated 24 h after injection of NDMA were marginally different from control, while by 5 days no consistent treatment-related effect could be discerned.

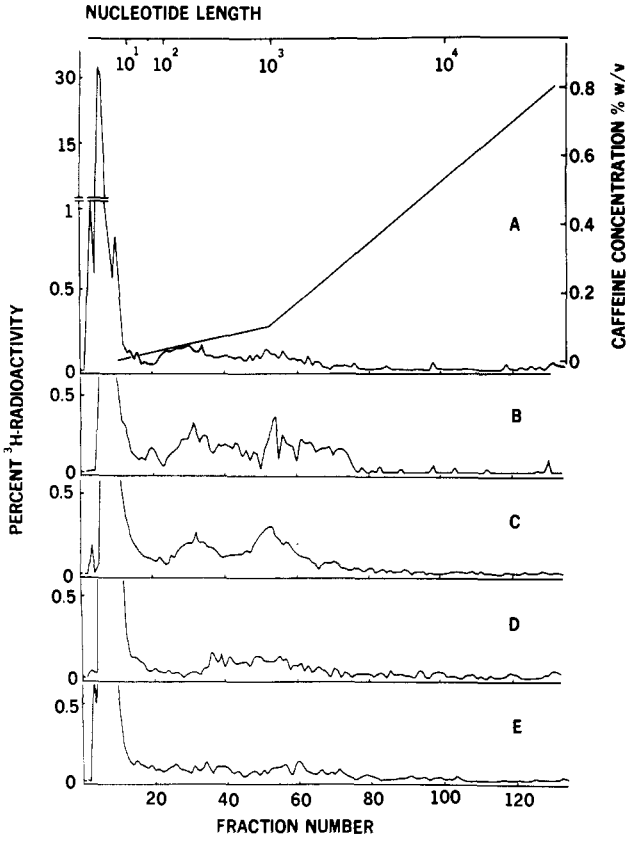


FIGURE 2: Caffeine gradient elution from BD-cellulose of hepatic DNA isolated from NDMA-treated rats. A chromatogram obtained by using DNA isolated from untreated animals (A) is contrasted with that from animals killed 1 h (B), 4 h (C), 24 h (D), and 5 days (E) following injection of the carcinogen. Following adsorption to BD-cellulose in 0.3 M NaCl (fractions 1–5), and recovery of double-stranded DNA in 1.0 M NaCl (fractions 6–10), DNA containing single-stranded regions was eluted with a biphasic 0–0.8% caffeine gradient as indicated in the top panel. These conditions apply to these and caffeine gradient chromatograms in other figures. The affinity of single-stranded DNA for BD-cellulose under the present conditions (Haber & Stewart, 1981) is indicated at the top of the figure. In these and all similar experiments, radioactivity per chromatogram was (2–5) $\times 10^4$ dpm (except for Figure 5E), and each chromatogram is typical of at least two results from different animals.

Following incubation of purified nuclei with micrococcal nuclease, euchromatin was separated from heterochromatin by low-speed centrifugation. Between 10% and 12% of the total labeled nuclear DNA was categorized euchromatin as a result of solubilization using 1.0 unit of enzyme (5 mg of nuclear DNA^{-1} mL^{-1} after 9 min. Endogenous nuclease activity in the course of the incubation accounted for solubilization of about 2% of the total DNA. Administration of NDMA to rats differentially affected the specific radioactivity of DNA in the eu- and heterochromatin fractions. Within 48 h of NDMA injection, there was a marked fall in the amount of solubilized radioactivity. The proportion of DNA solubilized by micrococcal digestion, as judged by absorbance, was unchanged by in vivo exposure to NDMA with the exception of preparations isolated an hour after carcinogen treatment. In contrast to the loss of radioactivity from euchromatic DNA, no NDMA-induced change was observed in the specific radioactivity of DNA which sedimented after digestion (Figure 3).

DNA extracted from nuclease-fractionated chromatin, isolated at various times after treatment of rats with NDMA, was subjected to BD-cellulose chromatography. Initial studies, involving stepwise elution of control preparations of eu- and heterochromatin-associated DNA, revealed that purification

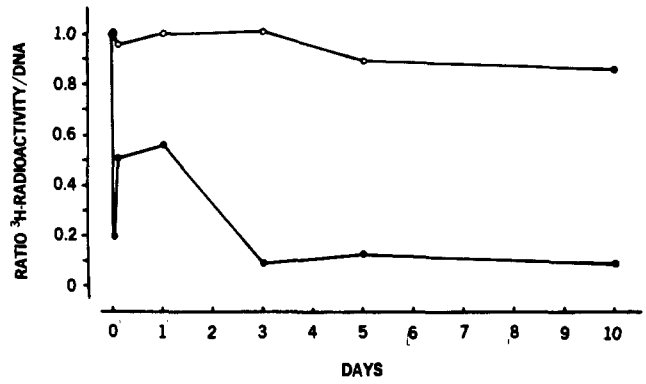


FIGURE 3: Relative persistence of radioactivity, derived from preincorporated [^{3}H]thymidine (i.e., Protocol 1; see Materials and Methods), in preparations of euchromatin (●) and heterochromatin (○) following injection of animals with NDMA. At times up to 10 days after NDMA treatment, isolated liver nuclei were fractionated into eu- and heterochromatin by mild micrococcal nuclease digestion. After extraction of DNA from the respective fractions, its specific activity was determined by absorbance and scintillation counting. This ratio was normalized to 1.0 for the respective control preparations, relative to which the nitrosamine-induced changes are expressed.

of nuclei resulted in approximately half the isolated DNA being recovered from BD-cellulose during caffeine elution, the respective proportions being 0.433 ± 0.026 and 0.540 ± 0.022. This increased binding to BD-cellulose (relative to preparations of whole DNA) was subsequently characterized by caffeine gradient elution of the respective preparations. In chromatograms of heterochromatic DNA from control rats (Figure 4A), the principal feature was a broad peak (fractions 50–80) indicating DNA binding to BD-cellulose with the affinity of single-stranded DNA from 1000 to 5600 bases. This peak proved characteristic of the micrococcal nuclease digestion procedure and was identifiable in other chromatograms. Thus, euchromatic control profiles (Figure 5A) were similar to those derived from heterochromatic DNA (Figure 4A) in that a considerable proportion of DNA containing single-stranded regions was eluted between fractions 50 and 80, presumptively a consequence of structural change generated in the course of nuclei isolation and related procedures. This feature, and in particular the sharp increase at fraction 50, could also be discerned in chromatograms from NDMA-treated rats (Figure 4A–F and Figure 7E–H).

The high proportion of caffeine-eluted DNA, recorded after purification of nuclei, was further increased by administration of NDMA only in the case of heterochromatin-associated DNA. Assayed up to 10 days after nitrosamine treatment, the proportion of caffeine-eluted DNA from heterochromatin preparations increased to a maximum of 0.713. No NDMA-induced increase was observed in the proportion of caffeine-eluted DNA isolated from euchromatin. Much greater insight into NDMA-induced structural change was obtained following comparison of the caffeine gradient chromatograms. In response to NDMA treatment, an increased amount of heterochromatic DNA was eluted at caffeine concentrations (fractions 20–60) similar to those at which increase was noted in unfractionated DNA preparations (Figure 2B,C) between 1 and 4 h following administration of the carcinogen (Figure 4B,C). Preparations of DNA from heterochromatin isolated 5 days to 1 month after NDMA (Figure 4E–G) were similarly characterized by recovery of radioactivity early in the caffeine gradient. By 3 months, the chromatograms could not readily be distinguished from appropriate controls, although increased recovery of radioactivity early in the gradient could be demonstrated (Table II).

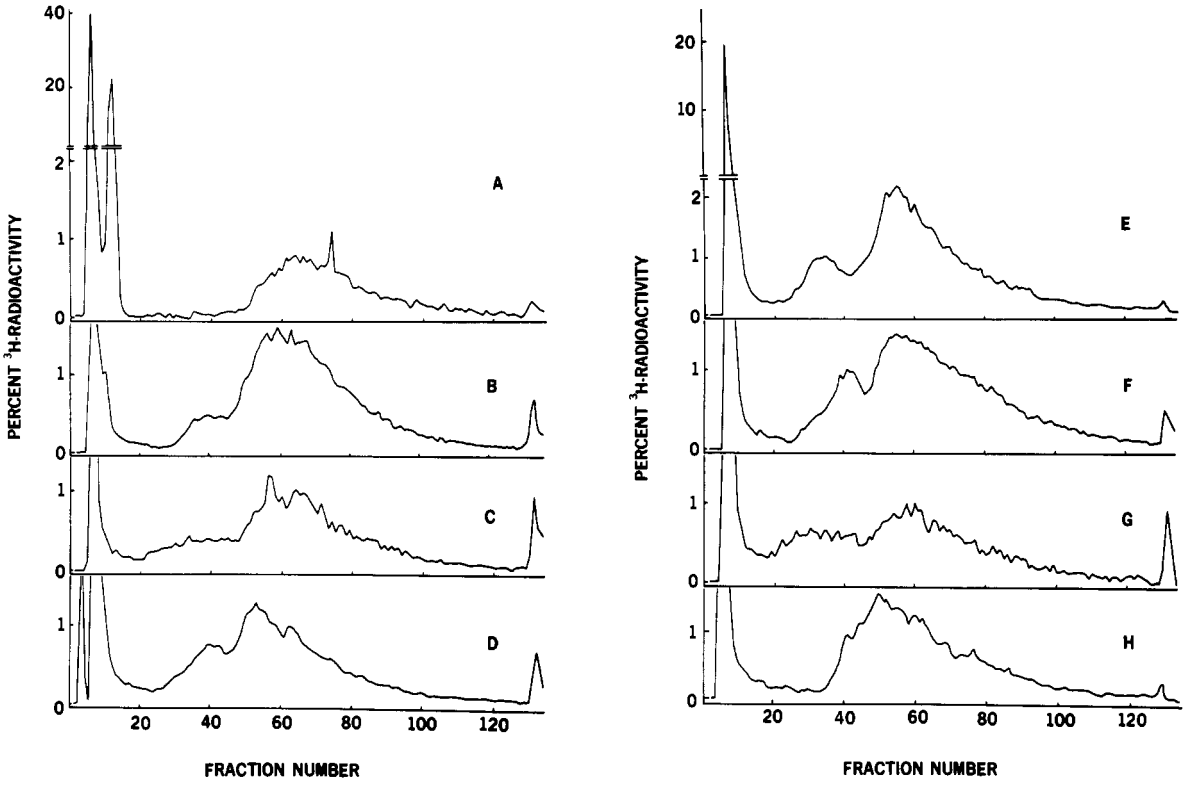


FIGURE 4: Effect of treatment with NDMA on the structure of DNA extracted from rat liver heterochromatin as determined by caffeine gradient elution from BD-cellulose. Chromatin fractionation was achieved by mild micrococcal nuclease digestion as described under Materials and Methods. DNA from control rats was analyzed (A) as well as that isolated from NDMA-treated animals killed 1 h (B), 4 h (C), 24 h (D), 5 d (E), 10 d (F), 1 month (G), and 3 months (H) after injection of the carcinogen. Details regarding the chromatography are given in the legend to Figure 2.

Table II: NDMA-Induced Variation in the Amount of Heterochromatic DNA Recovered in Fractions 20–60 following Caffeine Gradient Elution from BD-cellulose

treatment	time	rel amount of caffeine-eluted DNA in fractions 20–60[a]
saline	4 h	1.0
	1 month	1.1
	3 months	2.8
NDMA	1 h	24.8
	4 h	20.2
	24 h	4.3
	3 days	5.4
	5 days	5.1
	10 days	4.9
	1 month	4.2
	3 months	3.5

[a] Typical chromatograms are shown in Figure 4, which includes relevant experimental details. Following BD-cellulose chromatography of heterochromatic DNA from NDMA- and saline-treated animals, the proportion of radioactivity recovered in fractions 20–60 inclusive was calculated and expressed relative to that recovered in preparations isolated 4 h after administration of saline as control.

In chromatograms of DNA isolated from euchromatin, some slight effect of NDMA administration was apparent within 4 h (Figure 5B,C). A chromatogram of different character, having a distinctive peak at fraction 60 (binding affinity equivalent to ssDNA, 2.0 kilobases), was obtained by using euchromatic DNA from rats killed 24 h after NDMA injection. Chromatograms of euchromatic DNA isolated at longer times following NDMA injection were difficult to interpet because of the reduced specific activity of euchromatic DNA. Granted this limitation, a preparation isolated 3 days after carcinogen treatment (Figure 5E) appeared similar to control. However, a higher proportion of structurally compromised DNA was still eluted in the vicinity of fraction 60.

Using preparations from respective control animals, gradient chromatograms of DNA labeled during regenerative hyperplasia and isolated 24 h later (Figure 6A) could not be distinguished from those obtained from animals allowed to survive several weeks after surgery and labeling (Figure 2A). In either case, a minimal amount of DNA was recovered during caffeine elution. Chromatograms generated by using whole DNA isolated from NDMA-treated animals which had been subjected to partial hepatectomy from 4 h to 10 days after carcinogen treatment were not different from the appropriate noncarcinogen-treated control preparations similarly labeled 24 h after surgery and isolated a further 24 h later (Figure 6).

The impression that an incorporation period of 24 h permitted complete maturation of DNA was further confirmed by using heterochromatic DNA. Rats treated with [^{3}H]thymidine neonatally were allowed to reach 180 g, subjected to hepatectomy, and labeled with [^{14}C]thymidine during restorative hyperplasia. When heterochromatin was isolated 24 h after administration of [^{14}C]thymidine, the same caffeine gradient chromatograms were generated from isotopes respectively contained in the parental and daughter strands (Figure 7A). Further experiments involved single isotope labeling of animals 24 h after hepatectomy and sacrifice 24 h later. An effect on the heterochromatic chromatogram was apparent after animals receiving NDMA were subjected to hepatectomy 1 h after carcinogen administration (Figure 7B). Most distinctive changes in the structure of newly replicated DNA were observed if the interval between carcinogen treatment and surgery was extended to between 4 and 24 h. Under these conditions, the chromatograms were characterized by sharp peaks between fractions 50 and 80 (Figure 7C,D). Analysis of newly replicated heterochromatin DNA from rats exposed up to 2 months previously to NDMA resulted in

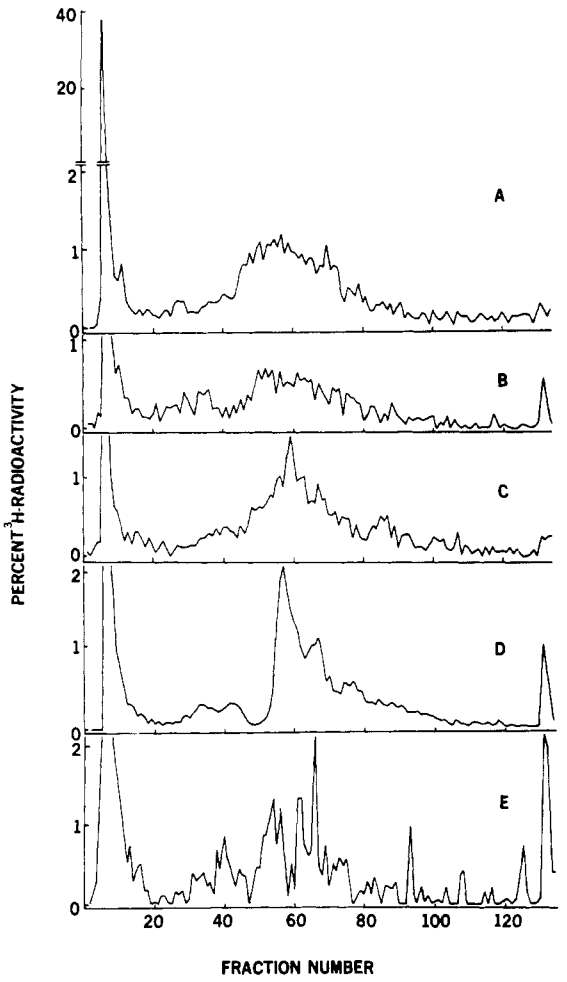


FIGURE 5: Effect of treatment with NDMA on the structure of DNA extracted from rat liver micrococcal nuclease derived euchromatin as determined by caffeine gradient elution from BD-cellulose. DNA from control rats was analyzed (A) as well as that isolated from NDMA-treated animals killed 1 h (B), 4 h (C), 24 h (D), and 3 days (E) after injection of the carcinogen. Radioactivity in chromatogram E was restricted to approximately 5000 dpm due to NDMA-induced loss from this solubilized fraction (see Figure 3).

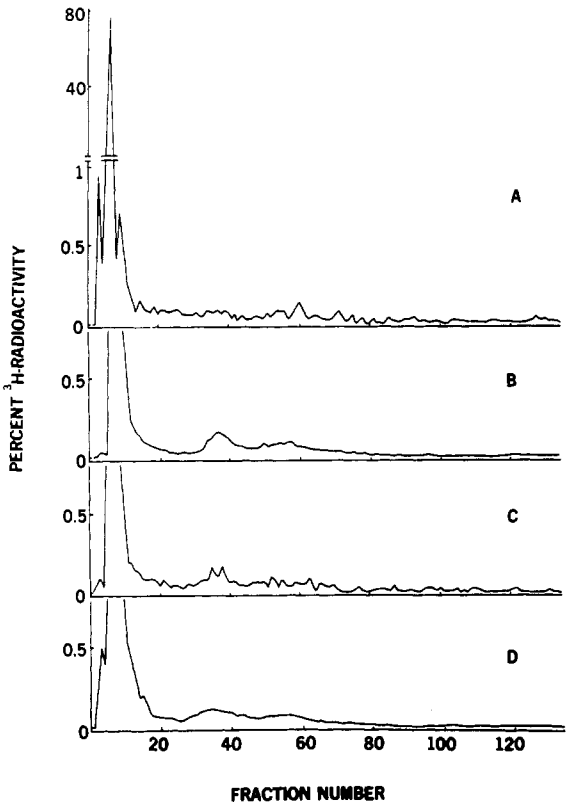


FIGURE 6: Analysis, by caffeine gradient elution from BD-cellulose, of DNA isolated 24 h after administration of [^{3}H]thymidine to rats subjected to partial hepatectomy 24 h earlier (Protocol 2; see Materials and Methods). Animals undergoing surgery were either untreated controls (A) or had received NDMA 4 (B), 24 h (C), or 10 days (D) beforehand. Details regarding chromatography are given in the legend to Figure 2.

chromatograms with two broad peaks. In common with control preparations, radioactivity was recovered in a broad peak from fractions 50–80. On the other hand, administration of the nitrosamine was correlated with recovery of DNA earlier in the gradient (fractions 25–50). Though most conspicuous when hepatectomy was performed 10 days after NDMA injection, the effect was still evident when this interval was extended to 2 months (Figure 7E–H). Certain precise features were common to these four chromatograms. In the region of fractions 25–50, all appeared to be composites of peaks at fractions 30, 35–36, and 40. Moreover, despite high recovery during fractions 50–80, a local maximum at fractions 57–58 was almost invariably observed.

Discussion

In preparations of whole DNA, repair-associated structural change was short-lived, being evident by stepwise chromatography 4–24 h after treatment (Figure 1) but less conspicuous after gradient analysis (Figure 2). Our studies on carcinogen-induced binding of DNA to BD-cellulose suggest that such structural change is attributable to single-stranded regions which mark an intermediate stage in the repair process (Stewart, 1981; Stewart & Haski, 1984). This structural configuration is transient, being detectable only during asynchrony between the initial and final stages of DNA repair (Stewart et al., 1985). Such asynchrony, at least when produced by inhibitors, may be associated with an increase in the length of "patches" and/or an increased number of initiated repair patches (Cleaver, 1985; Ireland & Stewart, 1986). Accordingly, when BD-cellulose binding occurs, the length of single-stranded regions at individual repair sites may well be larger in size or number than repair patches synthesized during efficient operation of the repair process. In the present case, evidence of DNA binding to BD-cellulose with the same affinity as single-stranded polynucleotides up to 2000 residues is suggestive of multiple binding sites within individual DNA fragments which typically are 10 kilobases in length (Ward et al., 1985a) and initially contain approximately 15 N-alkylated bases, principally 7-methylguanine (Den Engelse et al., 1986). Regardless of the localized configuration giving rise to the double peak (Figure 2C), similar chromatograms are observed within hours of administration to rats of methyl methanesulfonate (Stewart et al., 1986) or *N*-nitrosodiethylamine (B. W. Stewart, unpublished results). Such chromatograms are readily distinguished from those consequent upon hepatic necrosis. The latter were characterized by sharp multiple peaks, increasing progressively in height from fraction 15 to fraction 65 (Haber & Stewart, 1985).

Distinctive structural change in DNA (Figures 1 and 2) and the most marked decrease in the specific activity of euchromatin (constituting approximately 10% of the total DNA) occurred within 4 h of NDMA injection (Figure 3), coinciding with rapid excision repair of N-alkylated bases. Den Engelse et al. (1986) have determined the half-lives of all alkylated bases in hepatic DNA after administration to rats of 10 mg/kg body weight NDMA. Substrates for excision repair, N-al-

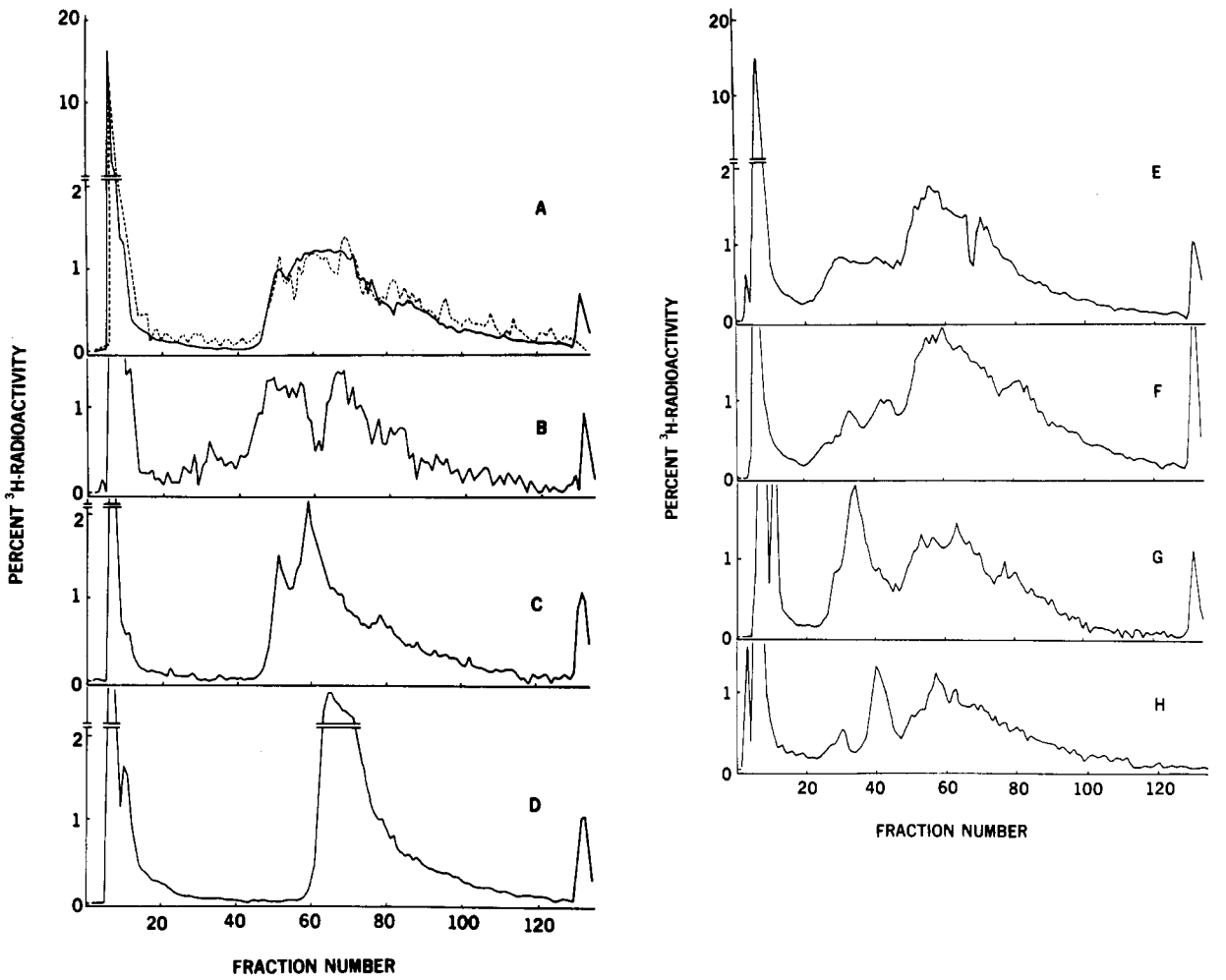


FIGURE 7: Evidence of changes in BD-cellulose binding characteristics in newly synthesized heterochromatic DNA as a consequence of administration of NDMA to rats which were later subjected to partial hepatectomy. Chromatin fractionation was achieved by digestion of isolated nuclei with micrococcal nuclease as described under Materials and Methods. Animals undergoing surgery included controls (A) which were labeled by administration of [^{3}H]thymidine (50 μCi) on a daily basis for the first week of life (···) and with [^{14}C]thymidine 24 h after surgery. Carcinogen-treated animals were subjected to partial hepatectomy 1 h (B), 4 h (C), 24 h (D), 3 days (E), 5 days (F), 10 days (G), and 2 months (H) are administration of NDMA and received [^{3}H]thymidine 24 h after the operation as described in the legend to Figure 6.

kylated purines, are lost from 2 to 10 times faster 2–24 h after NDMA than during tne ensuing 28 days; no such difference in velocity is evident for loss of O^6-methylguanine. The implication that rapid loss of preincorporated radiolabeled thymidine is caused by preferential repair of transcribed DNA is consistent with observations made by Bodell (1977). He noted that in mammalian cells exposed to methyl methanesulfonate, incorporated [^{3}H]thymidine was solubilized early (the first 5 min) in micrococcal digestion of the isolated nuclei. Despite the loss of in vivo label, the proportion of caffeine-eluted DNA could be determined in preparations of eu- and heterochromatic DNA. As methyl methanesulfonate increased the proportion of caffeine-eluted DNA, although different isolation procedures gave rise to variation in the control levels (Table I; Stewart et al., 1986), so treatment with NDMA resulted in an increased binding of chromatin-associated DNA to BD-cellulose, reflecting the result obtained with whole DNA. That the NDMA-induced increase in this parameter was restricted to heterochromatin suggests that excision repair of euchromatin is rate limited by catalysis of the initial stages and is efficiently completed. The failure of NDMA treatment to affect the specific radioactivity of heterochromatin (Figure 3), and the implicit low level of repair activity within this fraction, is consistent with numerous reports of low adduct concentrations in nuclease-resistant DNA fractionated as chromatin (Berkowitz & Silk, 1981; Walker et al., 1979; Metzger et al., 1976; Schwartz et al., 1981). Recently, Ryan et al. (1986), using micrococcal nuclease to fractionate hepatic chromatin (100 units/mg of DNA), have found that regions of active chromatin tend to be methylated more readily than bulk chromatin with respect to formation of both O^6-methylguanine and N-methylpurines after administration of 2 mg/kg body weight NDMA to rats. Repair of O^6-methylguanine proceded more rapidly from active chromatin. We obtained similar results after monitoring O^6-methylguanine levels in the present chromatin preparations (Ward et al., 1985b; unpublished results). However, Ryan et al. (1986) also recorded relatively uniform rates of loss of 7-methylguanine and 3-methyladenine from various chromatin fractions. In considering differential repair of chromatin subfractions, it may be necessary to make distinctions between rates of adduct loss and rates of structural change.

Chromatograms of hetero- and euchromatic DNA (Figures 4A and 5A) isolated from control animals were distinguished from those of unfractionated DNA (Figure 2A) by the former each having a broad peak extending from between fractions 43 and 50 until approximately fraction 80. This feature was seen in similar analyses of unfractionated rat liver DNA extracted after initial isolation of nuclei in the presence of Mg^{2+} (Ward et al., 1985a) and is attributable to digestion by endogenous nucleases. Confidence that endogenous nucleases, rather than micrococcal nuclease, are responsible is increased

by the fact that such degradation was common to both chromatin subfractions rather than being prevalent in the solubilized portion. The occurrence of such damage did not preclude examining nitrosamine-induced effects in the respective DNA preparations. Moreover, the occurrence of a shoulder which varied only between fractions 49 and 51 [Figures 4 and 7; also see Ward et al. (1985a)] provided an internal control and an indicator of reproducibility.

Some features of chromatograms of whole DNA from nitrosamine-treated rats could be related to chromatograms of chromatin-associated DNA obtained after micrococcal digestion of nuclei. Caffeine gradient analysis of heterochromatic DNA indicated that the immediate effect of NDMA was to increase the amount of DNA recovered prior to fraction 45, a result partially reminiscent of that with whole DNA (Figure 2B,C). On the other hand, analysis of euchromatin-derived DNA revealed a sharp peak at fraction 58 consequent upon administration of the carcinogen 24 h earlier (Figure 5D). Such a peak was not evident in whole DNA or easily discerned in heterochromatin labeled prior to alkylation. A similar peak was, however, evident in heterochromatin labeled by administration of thymidine 24 h before death using animals which had been subjected to partial hepatectomy 4 h after treatment with NDMA. The structural lesion giving rise to this peak was thus evident in DNA stimulated to replicate immediately after alkylation. This particular result seems more likely associated with inhibited movement of the replication fork, perhaps as suggested by Scudiero and Strauss (1974), rather than with repair.

Distinguishable from structural lesions immediately associated with repair or replication were features of chromatograms from DNA isolated months, rather than days, after injection of NDMA. Data were restricted to heterochromatin-associated DNA, since sufficient radioactivity for gradient analysis did not persist in euchromatic DNA. The structural defect apparent in chromatograms of DNA from heterochromatin isolated 5 days to 1 month after NDMA was partially reversible, being almost lost by 3 months (Figure 4). However, chromatographic evidence of nitrosamine treatment could be evoked by subjecting such animals to hepatectomy (Figure 7) long after the carcinogen had been metabolized (Swann & Magee, 1968) and immediate effects of alkylation had passed (Stewart & Magee, 1971). As in the case of DNA labeled before NDMA and isolated 1 week to 1 month later, chromatograms of heterochromatic DNA contained two peaks. The later-eluting broad peak (fractions 50–80) is attributable to endogenous nuclease. Radioactivity in fractions 20–50 is correlated with earlier administration of the nitrosamine and presumably due in some way to replication of DNA on an alkylation-damaged template.

Evidence of NDMA-induced changes in newly replicated DNA fractionated as chromatin (Figure 7) in the absence of structural change being apparent in preparations of unfractionated DNA (Figure 6) implies that structural defects present in such DNA are detected as a consequence of the chromatin isolation and fractionation procedure. As noted, isolation of nuclei under the present experimental conditions results in single-strand degradation by endogenous nuclease(s) (Ward et al., 1985a). This activity has not been well characterized, but examination by Fraser and his colleagues (Fraser et al., 1976; Chow & Fraser, 1983) of an extranuclear endo–exonuclease of *Neurospora crassa* has revealed an enzyme that senses single-strand-like regions in superhelical DNA. A similar enzyme is located in the nuclei, bound to chromatin and the nuclear matrix, and is responsive to DNA damaging agents (M. J. Fraser, personal communication). Comparison of the effect of isolation procedures on preservation of alkylation-induced structural damage in DNA implied that repair sites were preferential substrates for nuclease(s) in rat liver nuclei (Stewart et al., 1986). Thus, minor repair-associated structural lesions may, by nuclease activity, give rise to single-stranded regions permitting detection through binding to BD-cellulose. However, while nuclease activity may facilitate detection of structural lesions in DNA, the observations which have been made are internally consistent. This consistency, which includes patterns of variation with time of treatment-induced changes (Figures 1 and 4) and detection of related structural change independent of the DNA labeling procedure (Figures 4 and 7), virtually precludes random artifactual degradation as accounting for the effects observed.

The present findings suggest the persistence of minor lesions in nontranscribed DNA (Figure 4E–G). Association of BD-cellulose binding with intermediate stages of DNA repair (Stewart, 1981) suggests that alkylation damage in heterochromatic DNA may be incompletely repaired. Incomplete filling of repair gaps or failure of ligation may result in lesions (gaps of approximately five nucleotides or less) which would not bind to BD-cellulose but could give rise to binding after nuclease attack during isolation of nuclei. At least two classes of damage in template strand DNA, namely, persistent alkylated bases or sites of incomplete repair, are capable of causing discontinuities or distortions in newly synthesized DNA. Such structural defects, in turn, may be sites for nuclease attack (Columbano et al., 1980). Distinctive peaks in relevant chromatograms (Figure 7F–H) are suggestive of specific lesions in newly synthesized DNA which seem to be more conspicuous than any in DNA originally exposed to the carcinogen. Since the secondary structure of DNA now appears to play a critical role in gene expression (Elgin, 1981), it is not difficult to envisage relationships between various causes of change in secondary structure outlined above and the role of cell replication in nitrosamine carcinogenesis.

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Identification of Methionine-110 as the Residue Covalently Modified in the Electrophilic Inactivation of D-Amino-acid Oxidase by *O*-(2,4-Dinitrophenyl)hydroxylamine†

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ABSTRACT: The reaction of *O*-(2,4-dinitrophenyl)hydroxylamine with D-amino-acid oxidase leads to complete inactivation which can be protected against by the competitive inhibitor benzoate [D'Silva, C., Williams, C. H., Jr., & Massey, V. (1986) *Biochemistry 25*, 5602–5608]. The residue modified has been identified as methionine-110. Differential high-performance liquid chromatography mapping of tryptic digests of D-amino-acid oxidase modified in the absence and presence of benzoate allows the isolation of a single methionine-containing tryptic peptide corresponding to residues 100–115 and referred to as T_6-T_7. In unmodified enzyme, the bond involving Arg-108 is readily cleaved and T_6 and T_7 are isolated. Brief treatment of peptide T_6-T_7 with carboxypeptidase Y released residues 112–115, and the residual peptide was isolated in good yield. Further treatment of this peptide (residues 100–111) with carboxypeptidase Y released Val and an unknown amino acid that comigrated with synthetically prepared *S*-aminomethionine sulfonium salt. The unknown compound and *S*-aminomethionine break down to methionine on treatment with dithiothreitol.

D-Amino-acid oxidase (EC 1.4.3.3) is a flavoprotein that has been the target of numerous mechanistic and structural investigations aimed at understanding the roles that specific amino acid residues play in its catalysis. In an effort to identify these amino acid residues, a number of different chemical modifications have been reported (Williams et al., 1984). Recently we reported the use of *O*-(2,4-dinitrophenyl)-hydroxylamine (DNPHA)[1] in the covalent modification of pig kidney D-amino-acid oxidase (D'Silva et al., 1985, 1986).

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[1] Abbreviations: CPY, carboxypeptidase Y; DNPHA, *O*-(2,4-dinitrophenyl)hydroxylamine; FAD, flavin adenine dinucleotide; HPLC, high-performance liquid chromatography; PTC, phenylthiocarbamoyl; TFA, trifluoroacetic acid.

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