Structural Defects in Rat Liver Deoxyribonucleic Acid

ENDOGENOUS SINGLE-STRANDED REGIONS IN COMPARISON WITH DAMAGE INDUCED *IN VIVO* BY A CARCINOGEN

By BERNARD W. STEWART, PETER H. T. HUANG and MICHAEL J. BRIAN
School of Pathology, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033, Australia

(Received 30 August 1978)

Rat liver DNA may be separated into two fractions by stepwise elution from benzoylated-DEAE-cellulose with NaCl and caffeine solutions respectively. Other studies using bacterial and yeast DNA suggested that the first fraction contains native DNA, whereas the second may exhibit some degree of single-stranded character. In the present experiments, chromatography of DNA was monitored by labelling *in vivo* with [methyl-³H]thymidine in rats previously subjected to partial hepatectomy. In animals killed up to 1 h after thymidine injection, radioactivity eluted in the second fraction was inversely related to the incorporation time, being greatest when animals were killed 10 min after radioisotope injection. However, for most experiments, animals were allowed to survive 2–4 weeks after surgery before use, analysis being made on non-dividing DNA. Under these conditions, the proportion of caffeine-eluted DNA was decreased by subjecting the preparation to shear, before chromatography. A procedure that resulted in 12% of the recovered radioactivity being eluted with caffeine was adopted for experiments involving comparison of the two DNA fractions. Under these conditions, cross-contamination could be detected by rechromatography, but this did not preclude distinction being made between the two fractions in terms of DNA structure. NaCl-eluted DNA did not bind to nitrocellulose filters. Caffeine-eluted DNA was retained by the filters and released by washing with 3 mM-Tris/HCl, pH 9.4. The DNA fractions did not differ in terms of isopycnic centrifugation in CsCl. The NaCl-eluted fraction migrated as a single band in polyacrylamide gels, and this pattern was not modified by prior digestion with *Neurospora crassa* endonuclease. In contrast, caffeine-eluted DNA contained a minor component having a wide molecular-weight distribution and was subject to limited digestion by the endonuclease. The kinetics of denaturation of NaCl-eluted DNA in the presence of formaldehyde, in common with unfractionated DNA, were consistent with double-stranded structure. The same analysis of caffeine-eluted DNA revealed structural abnormality equivalent to two defects per 10000 base-pairs. The data are consistent with the minor fraction of rat liver DNA, separated by using benzoylated-DEAE-cellulose, containing regions of local denaturation. We previously showed that administration of the hepatocarcinogen dimethylnitrosamine is associated with an increase in the proportion of caffeine-eluted DNA. In terms of most analyses, differences between DNA fractions from nitrosamine-treated rats were similar to differences exhibited by preparations from control animals. However, structural analysis using denaturation kinetics indicated defects in both the NaCl- and caffeine-eluted DNA isolated from nitrosamine-treated rats. The two fractions differed from each other in that caffeine-eluted DNA exhibited a degree of structural damage far greater than that detected in any preparation from control animals. The relationship of this structural damage to alkylation of DNA *in vivo* by the nitrosamine is discussed.

Benzoylated-DEAE-cellulose, together with benzoylated-naphthoylated-DEAE-cellulose, was introduced by Gillam *et al.* (1967) for the fractionation of tRNA. Kiger & Sinsheimer (1969) separated pulse-labelled replicating bacteriophage- λ DNA from completed bacteriophage DNA using benzoylated-naphthoylated-DEAE-cellulose. Since that time benzoylated-naphthoylated-DEAE-

cellulose has been used extensively to fractionate DNA on the basis of secondary structure. Thus, by treating native bacteriophage-T7 DNA with exonuclease III, Schlegel *et al.* (1972) provided further evidence that fractionation was dependent on the presence of single-stranded regions in a preparation of otherwise native DNA. Scuderio & Strauss (1974) used benzoylated-naphthoylated-DEAE-cellulose to

fractionate DNA isolated from mammalian cells shortly after treatment with methyl methanesulphonate. In contrast, chromatographic fractionation of DNA on benzoylated-DEAE-cellulose has been the subject of limited investigation.

Caffin & Mackinlay (1975) eluted two fractions of *Escherichia coli* DNA from benzoylated-DEAE-cellulose. The strongly binding fraction eluted with caffeine differed from the major fraction only in its binding to nitrocellulose filters and not in buoyant-density or chromatographic behaviour on hydroxyapatite. In contrast, completely denatured DNA, also eluted with caffeine from benzoylated-DEAE-cellulose, could be distinguished from native DNA by hydroxyapatite chromatography. Pirro & Feldmann (1975) analysed fractions of *Saccharomyces cerevisiae* DNA separated by chromatography on benzoylated-DEAE-cellulose, the second fraction being eluted with formamide. They demonstrated characteristics including S_1 -nuclease-sensitive binding to nitrocellulose and labelling *in vivo* which suggested that the second fraction of DNA contained single-stranded regions.

The present paper concerns the fractionation of rat liver DNA on benzoylated-DEAE-cellulose. Comparative analyses have been made of DNA preparations obtained by stepwise elution from benzoylated-DEAE-cellulose to elucidate the molecular basis of fractionation. Experiments were conducted to determine the extent to which concepts based on fractionation of bacterial and yeast DNA are applicable to mammalian DNA. We have previously reported that the proportion of rat liver DNA eluted by caffeine from benzoylated-DEAE-cellulose is increased by prior administration of the hepatocarcinogen dimethylnitrosamine (Huang & Stewart, 1976). In terms of macromolecular structure, distinction is now made between endogenous caffeine-eluted DNA isolated from control animals and caffeine-eluted DNA isolated from animals treated with dimethylnitrosamine. Overall, the data are consistent with fractionation of DNA on benzoylated-DEAE-cellulose being dependent on single-stranded regions and illustrate the considerable degree of structural damage to DNA after exposure to the alkylating carcinogen *in vivo*.

Materials and Methods

Chemicals

Chemicals were purchased as follows: caffeine (B.P.) from Prosana Laboratories, Sydney, Australia; dimethylnitrosamine from Eastman Organic Chemicals, Rochester, NY, U.S.A.; DEAE-cellulose (Whatman DE-22) from Whatman Biochemicals, Springfield Mill, Maidstone, Kent, U.K.; acrylamide, *NN'*-methylenebisacrylamide and *NNN'*-tetra-

methylethylenediamine from Scientific and Research Equipment Co., Sydney, Australia; CsCl from Hawshaw Chemical Co., Solon, OH, U.S.A.; formaldehyde from ICI Australia Ltd., Sydney, Australia; 4,4',5,5'-dibenzo-3,3'-diethyl-9-methylthiacarbocyanine bromide ('Stains-all') from Serva Feinbiochemica, Heidelberg, Germany; and [^3H]-thymidine (20 Ci/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.

Neurospora crassa endonuclease (965 units/mg) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and *Eco*R1 restriction endonuclease and λ -phage DNA were generously provided by Dr. E. Goldring, Division of Plant Industry, C.S.I.R.O., Canberra, Australia.

Animals

Female Wistar rats (120 g) from the University of New South Wales colony, maintained on Allied Rat and Mouse Kubes (Allied Feeds, Sydney, Australia), were subjected to partial hepatectomy by the method of Higgins & Anderson (1931): 23 h after surgery [^3H]thymidine (50 μCi) was administered by intraperitoneal injection. Unless otherwise stated, animals were allowed a minimum recovery period of 2 weeks before being used. Dimethylnitrosamine (10 mg/kg body wt.) was administered in aqueous solution by intraperitoneal injection. Animals were killed by a sharp blow to the head, followed by jugular-vein exsanguination.

Preparation of benzoylated-DEAE-cellulose

Benzoylated-DEAE-cellulose was prepared from a formulation of DEAE-cellulose (DE-22) which has higher chromatographic capacity and separation efficiency compared with the formulation (DE-11) used for this purpose by Gillam *et al.* (1967). This improved formulation of DEAE-cellulose is especially suitable for chromatography in short columns. Preparation of benzoylated-DEAE-cellulose from DE-22 DEAE-cellulose necessitated slight modification of the method described by Gillam *et al.* (1967). The mixture of DEAE-cellulose, pyridine and benzoyl chloride was boiled under reflux for approx. 2½ h rather than 45 min before the appearance of a 'viscous red-brown mass'. No difference was detected in the elution profile of rat liver DNA when benzoylated-DEAE-cellulose prepared from DE-22 or DE-11 was used.

Preparation and chromatography of rat liver DNA

DNA labelled *in vivo* with [^3H]thymidine was isolated as previously described (Huang & Stewart, 1977). Before column chromatography on benzoylated-DEAE-cellulose, DNA was subjected to

shearing either by multiple passages through a 25-gauge needle or by sonication (Huang & Stewart, 1976).

Benzoylated-DEAE-cellulose was stored in 25% (v/v) ethanol in 2M-NaCl at 4°C until required. Immediately before use the resin was washed exhaustively with water (3×500 ml) and finally suspended in 10mM-Tris/HCl/1mM-EDTA, pH 8.0 (TE-buffer), containing 0.3M-NaCl. Two sizes of column were used. Fractionation of 100–200 µg of DNA on 9mm×25mm columns has been previously described (Huang & Stewart, 1977). For fractionation of up to 40 mg of DNA, a 20mm×50mm column of resin was packed and washed exhaustively with TE-buffer containing 0.3M-NaCl. The DNA solution was loaded at a flow rate of 0.5 ml/min and the column eluted with 200–250 ml of TE-buffer containing 1.0M-NaCl, followed by 80–100 ml of 1.8% (w/v) caffeine dissolved in TE-buffer containing 1.0M-NaCl. The NaCl-eluted and caffeine-eluted fractions were dialysed against 10mM-Tris/HCl, pH 8.0 (2 litres), at 4°C for at least 24h, the dialysis tubing having been treated to eliminate deoxyribonuclease activity (Brewer *et al.*, 1974). During dialysis the buffer was changed at 4–6h intervals. The concentration of non-diffusible material was increased by using poly(ethylene glycol) (Smith, 1976) and the amount of DNA determined by the method of Burton (1956).

Analysis of DNA fractions from benzoylated-DEAE-cellulose chromatography

Sedimentation velocity. Analytical ultracentrifugation at 44000 rev./min and 20°C was carried out essentially as described by Vinograd *et al.* (1963): rotor type AnD and boundary-forming centrepiece together with Schlieren counter-balance were used. After benzoylated-DEAE-cellulose chromatography, NaCl-eluted DNA dissolved in TE-buffer (1–2 µg/µl, total volume 20 µl) was layered on 0.5 ml of TE-buffer containing 1M-NaCl. During ultracentrifugation, photographs were taken at 4 min intervals with a 10s shutter opening. Sedimentation coefficient was calculated by using the formula derived by Vinograd *et al.* (1963).

Kinetics of denaturation in formaldehyde. Formaldehyde was purified by saturation of 100 ml with a mixture of CaCO₃ and activated charcoal (1:1, w/w) for at least 4h. The mixture was filtered (Whatman no. 1 paper) and the clear solution kept at –18°C for 24h to precipitate polymer, which was later removed by filtration. The concentration of purified formaldehyde was determined by titration against 1M-HCl in the presence of previously neutralized 1M-Na₂SO₃ (Walker, 1964).

Formaldehyde (0.1%, v/v; 0.1 ml) was added to a solution of DNA (150 µg) in 0.026M-NaCl/0.0026M-sodium citrate, pH 7.0 (final volume 2.14 ml). The

mixture was incubated at 62°C for 40 min and 2.5% (v/v) formaldehyde (2.4 ml at 62°C) containing 0.006M-triethanolamine/0.047M-NaCl, pH 8.8, was rapidly added. The reaction mixture was transferred to a quartz cuvette with lid supported in a constant-temperature cell housing maintained at 65±0.5°C, and the A_{260} determined at 2 or 4 min intervals. Data were calculated by using the formulae derived by Trifonov *et al.* (1968), by which:

$$\frac{D_{\max.} - D}{D_{\max.} - D_{\min.}} = \theta$$

where D = absorbance, $D_{\min.}$ = the initial absorbance, $D_{\max.}$ = absorbance at the end of the reaction, and θ = degree of spirality, and:

$$I = \rho v + 2cv$$

where I = initial rate of uncoiling of DNA, ρ = number of embryos formed per unit time, v = number of nucleotide pairs, c = concentration of defects, and v = number of nucleotide pairs separated per unit time.

Nitrocellulose binding. The degree of retention by nitrocellulose filters of the two DNA fractions was determined. In the course of this experiment some DNA samples were denatured by incubation in a water bath at 100°C for 10 min before rapid cooling in ice. Nitrocellulose filters (25 mm diameter, 0.45 µm pore size; from Millipore Corp., Bedford, MA, U.S.A.) were soaked in 0.9M-NaCl/0.09M-sodium citrate, pH 7.0, and washed with 10 ml of this solution before loading DNA (100 µg) dissolved in the same solution. The filters were washed as described by Caffin & Mackinlay (1975). Radioactivity retained was determined by dissolving the filters in 2-ethoxyethanol (2 ml) and adding scintillation fluid (Cox *et al.*, 1973).

Isopycnic centrifugation. Solid CsCl was added to solutions of DNA to a final density of 1.70 g/ml (Flamm *et al.*, 1969) and portions (5 ml), overlaid with mineral oil, were centrifuged at 89590g in a Beckman type 40 rotor for 48 h. The gradients were fractionated and acid-insoluble radioactivity was determined.

Digestion with *Neurospora crassa* endonuclease. DNA was digested with this endonuclease based on the method described by Linn & Lehman (1965). The reaction mixture (total volume 355 µl) contained 140 µg of DNA, 2.4 µg of enzyme and 6mM-MgCl₂, 60mM-NaCl, 4mM-mercaptoethanol and 6mM-Tris/HCl, pH 8.0. During incubation at 37°C, samples (50 µl) were withdrawn after 0, 5, 10, 20 and 30 min, immediately cooled to 0°C and added to 0.2M-EDTA (20 µl). After addition of 8M-urea containing 30mM-diethylbarbituric acid/3mM-Tris/HCl, pH 7.0 (30 µl), the samples were incubated at 65°C for 10 min and cooled to room temperature before electrophoresis.

Polyacrylamide-gel electrophoresis. Gel slabs (6.6cm×7.0cm×3mm) containing a linear gradient of 6–25% polyacrylamide together with 3–8M-urea to stabilize the gradient during polymerization were prepared. The gel buffer was 0.125M-HCl/0.376M-Tris/HCl, pH8.9, containing 0.58mg of *NNN'*-tetramethylethylenediamine/ml (Akroyd, 1967). The gradient was generated by using a mixer for which the low-concentration solution contained 2.44% acrylamide, 0.06% *NN'*-methylenebisacrylamide and 3M-urea. The solution was degassed for 15 min and immediately before use *NNN'*-tetramethylethylenediamine and ammonium persulphate (final concentrations 2µg/ml and 50µg/ml) were added. The high-concentration solution was 30% acrylamide/0.83% *NN'*-methylenebisacrylamide/8M-urea containing 10µg of Bromophenol Blue/ml, degassed for 30 min and supplemented with *NNN'*-tetramethylethylenediamine and ammonium persulphate (final concentrations 1µg/ml and 30µg/ml) immediately before use. Techniques described by Jeppesen (1974) were used during mixing of the solutions to ensure generation of a linear gradient. Electrophoresis, in a Gradipore Four-Cell system (Scientific and Research Equipment Co., Sydney, Australia) was performed at room temperature (22°C) with 30mm-diethylbarbituric acid/3.3mm-Tris/HCl, pH7.0, as electrode buffer (Philippsen & Zachau, 1972) for 4 h at 120 V constant.

After removal from the glass cassette, the gel slabs were fixed in ice-cold 50% (v/v) methanol for at least 1 h with three changes during that time. The gels were stained overnight in the dark with a solution of 5µg of 'Stains-all'/ml in 25% (v/v) methanol/5% (v/v) formamide/0.01M-Tris/HCl, pH8.0. Gels were photographed by using a yellow filter Y₂, to permit identification of DNA bands stained blue.

Results

We have previously reported that the fractionation by benzoylated-DEAE-cellulose chromatography of portions of the same DNA sample is reproducible within ±0.3% (Huang & Stewart, 1976). The chromatographic behaviour of rat liver DNA in these experiments was monitored by labelling *in vivo* with [³H]thymidine. Fractionation of DNA by benzoylated-DEAE-cellulose could be varied by extreme manipulation of the labelling procedure. DNA isolated 1 h after administration of [³H]thymidine to partially hepatectomized rats exhibited an elution profile only slightly different from that obtained by using DNA isolated from animals allowed to survive for a further 2–4 weeks, as used in most experiments. The proportion of radioactivity eluted with caffeine increased from 12 to 16% with this decrease in labelling time. A further decrease in the interval between injection of thymidine and isolation of DNA increased the proportion of caffeine-eluted DNA to

a point where radioactivity was approximately equally distributed between the two fractions (Fig. 1).

To elucidate the basis of fractionation of rat liver DNA by benzoylated-DEAE-cellulose chromatography, close examination was made of those parameters that determined the distribution of DNA between the fractions. With respect to preparation of DNA for chromatography, the parameter that most markedly affected the fractionation was shear. The proportion of NaCl-eluted DNA could be adjusted by variation of the duration of sonication and/or of hydrodynamic shear breakage. Progressive increase in the percentage of NaCl-eluted DNA as more vigorous shearing procedures were used (Table 1) strongly suggested that structural features causing binding of caffeine-eluted DNA constituted a minor

Fig. 1. Modification of the chromatographic behaviour of DNA by variation of the procedure for labelling *in vivo*. Rat liver DNA was isolated, sheared and chromatographed in benzoylated-DEAE-cellulose as previously described (Huang & Stewart, 1976), the column being eluted with 1.0M-NaCl after collection of fraction 3 and caffeine solution after fraction 10. In these experiments [³H]thymidine was administered to rats subjected to partial hepatectomy 23 h previously. Progressive increase in the interval from administration of radioisotope to death of the animal from 10 min (●) through 30 min (○) to 60 min (▲) caused a decrease in the proportion of radioactivity eluted with caffeine. The individual elution profiles are typical results from at least two experiments in each case and are contrasted with a mean result (----) obtained from a large number of animals allowed to survive for at least 2 weeks after administration of [³H]thymidine (Huang & Stewart, 1977).

fraction of the total DNA. The molecular size of DNA sheared by six passages through a 25-gauge needle was determined by velocity sedimentation. DNA, previously sheared and eluted from benzoylated-DEAE-cellulose with NaCl, appeared during analytical centrifugation as a single broad band which sedimented at a rate corresponding to 17S (Fig. 2). A similar study of caffeine-eluted DNA was complicated by heterogeneity of fragment size. The composition of caffeine-eluted DNA in terms of the molecular size distribution was subsequently determined by electrophoresis.

In all the following experiments DNA was isolated from rats labelled 2-4 weeks previously and sheared by six passages through a 25-gauge needle. Under these conditions 88.1% of the radioactivity recovered was eluted with NaCl, the remaining 11.9% being eluted with caffeine solution. There was no difference between the densities of these two DNA preparations as determined by equilibrium centrifugation in CsCl. On rechromatography, 53% of the NaCl-eluted radioactivity was again eluted with 1.0M-NaCl, and 73% of caffeine-eluted radioactivity once more required caffeine for elution from benzoylated-DEAE-cellulose (Fig. 3).

Polyacrylamide-slab-gel electrophoresis was used to determine the composition and molecular weight of DNA fractions and to assess the effect on these preparations of *Neurospora crassa* endonuclease. The major proportion of both NaCl-eluted and caffeine-eluted DNA, whether analysed before or after digestion with endonuclease, was invariably located in a single band (Fig. 4) which penetrated the gel to the same extent as the 7180-base fragment of an *Eco*R1 digest of λ -phage DNA. For both NaCl-eluted and caffeine-eluted DNA, progression of digestion with endonuclease was associated with a slight decrease in the staining intensity of this main band fraction. Caffeine-eluted DNA was distinguished from NaCl-eluted DNA by the presence of DNA fragments of both greater and lesser molecular

Fig. 2. Sedimentation of NaCl-eluted DNA after benzoylated-DEAE-cellulose chromatography
Analysis by ultracentrifugation was made of DNA isolated from control animals, sheared by six passages through a 25-gauge needle and eluted from benzoylated-DEAE-cellulose with 1.0M-NaCl. The distance in cm of the sedimenting sample from the axis of rotation (x) was determined by using densitometer tracings of photographs taken during centrifugation.

Table 1. Effect of shear on the fractionation of DNA by benzoylated-DEAE-cellulose

DNA was sheared by sonication and/or by passing the solution through a 25-gauge needle before chromatography as described in the Materials and Methods section.

Shear	Percentage of DNA in caffeine eluate
None	48.00
Sonicated, 2 x 30s	17.63
Sonicated, 3 x 30s	6.76
5 x 25-gauge needle	14.10
6 x 25-gauge needle	12.06
Sonicated 2 x 30s and 4 x 25-gauge needle	5.57

weight than the main band. These minor fractions, particularly those of relatively high molecular weight, were lost in the course of incubation in the presence of endonuclease. After 30min digestion it was barely possible to distinguish any discrete bands from a background stain corresponding to molecular size from 2000 to 28 base-pairs. When this experiment was repeated with DNA isolated from a rat treated with 10mg/kg body wt. 4h before death a similar result was obtained (Fig. 5). Thus incubation of the NaCl-eluted fraction in the presence of *Neurospora crassa* endonuclease was not associated with the appearance of minor fractions or with loss of the main band. The caffeine-eluted fraction of nitrosamine-exposed DNA was made up of a principal species of approx. 7000 base-pairs which persisted throughout the incubation with endonuclease. The digestion was accompanied by loss in staining

Fig. 3. *Rechromatography, on benzoylated-DEAE-cellulose, of (a) NaCl- and (b) caffeine-eluted DNA fractions* DNA isolated from control animals was initially chromatographed on benzoylated-DEAE-cellulose. Appropriate pooled fractions were dialysed against 0.01 M-Tris/HCl, pH 8.0, for 24 h and the respective preparations rechromatographed as described (Huang & Stewart, 1977). Columns were eluted with NaCl and caffeine solutions as described in the legend to Fig. 1.

Fig. 4. *Electrophoretic analysis of DNA previously chromatographed on benzoylated-DEAE-cellulose and digested with N. crassa endonuclease*

DNA from control animals was fractionated and the two preparations, after separate incubation in the presence of endonuclease, were subjected to electrophoresis on polyacrylamide as described in the Materials and Methods section. NaCl-eluted DNA is shown on the left of the gel (channels 1-6) and caffeine-eluted DNA on the right (channels 7-12). Samples were removed from the digestion reaction immediately before addition of the enzyme (channels 1 and 7). Further samples, removed 5, 10, 15, 20 and 30 min after initiation of the reaction, were placed respectively on the gel to indicate increasing digestion from left to right. The direction of migration is downwards toward the positive electrode.

Fig. 5. *Electrophoretic analysis of dimethylnitrosamine-treated DNA after chromatography on benzoylated-DEAE-cellulose and digestion with N. crassa endonuclease* DNA was isolated from rat liver 4 h after administration of 10 mg of dimethylnitrosamine/kg body wt. Other details are as given in the legend to Fig. 4.

intensity of the minor fractions, particularly those of high molecular weight.

Nitrocellulose filters bind single-stranded DNA so that it is eluted with buffers of high pH and low ionic strength: double-stranded DNA is not retained. If subjected to prior heat denaturation, both NaCl-

and caffeine-eluted DNA were completely bound to nitrocellulose filters (Table 2). In the absence of denaturation procedures, 1% or less of the NaCl-eluted DNA was retained by the filters. In contrast, approx. 50% of radioactivity eluted from benzoylated-DEAE-cellulose in the presence of caffeine was retained until the filters were washed with 0.003M-Tris/HCl, pH9.4.

The kinetics of DNA denaturation in the presence of formaldehyde were monitored as a further method of determining secondary structure. The results of these experiments are summarized in Table 3. DNA from control animals eluted from benzoylated-DEAE-cellulose by caffeine exhibited approx. 4% less hyperchromicity than did either unfractionated or NaCl-eluted DNA. Correspondingly, the short time required for complete denaturation of caffeine-eluted DNA is consistent with a structural difference between it and the NaCl-eluted fraction. The kinetics of denaturation of unfractionated DNA, when analysed by the method of Trifonov *et al.* (1967, 1968), were consistent with the uncoiling of double-stranded DNA (Fig. 6). A similar result was obtained

by using NaCl-eluted DNA. For both these preparations the function *I* (Trifonov *et al.*, 1967), defined in terms of the initial rate of uncoiling of DNA partially digested with deoxyribonuclease I, was equal to 0, the result expected for double-helical DNA having no internal defects. Denaturation kinetics distinguished caffeine-eluted DNA from unfractionated and NaCl-eluted DNA. Displacement of the 'melting' curve (Fig. 7) generated an intercept on the ordinate corresponding to less than two defects per 10^4 base-pairs.

The hyperchromicity of NaCl-eluted DNA from nitrosamine-treated animals was 49%, compared with 28% exhibited by corresponding control preparations (Table 3). Increase in absorbance by DNA during denaturation in formaldehyde solution is due both to strand separation and also to hydroxymethylation of amino as well as imino groups (McGhee & von Hippel, 1977; Malinina *et al.*, 1977). The effect on these reactions of methylation of substrate DNA has not been studied. Thus the implication from the present studies of an increase in hyperchromicity of DNA in formaldehyde solution

Table 2. *Binding of DNA to nitrocellulose filters*

DNA was isolated from control rats or rats treated 4h previously with 10mg of dimethylnitrosamine/kg body wt., sheared by six passages through a 25-gauge needle and chromatographed on benzoylated-DEAE-cellulose. Binding of the various DNA preparations to nitrocellulose filters was assayed as described in the Materials and Methods section. Results in parentheses indicate the fraction of radioactivity retained when DNA preparations were subjected to thermal denaturation before application to the filters.

DNA preparation	Radioactivity retained (%) after washing with	
	0.9M-NaCl/0.09M-sodium citrate	0.003M-Tris/HCl, pH9.4
Control		
NaCl-eluted	0.8 (91.6)	0 (1.5)
Caffeine-eluted	48.5 (87.2)	13.0 (1.4)
Dimethylnitrosamine-treated		
NaCl-eluted	1.07	0.2
Caffeine-eluted	46.0	0.8

Table 3. *Effect of benzoylated-DEAE-cellulose chromatography on the thermal stability of DNA from control and dimethylnitrosamine-treated rats*

DNA preparations were obtained as described in the legend to Table 2. Denaturation of DNA in the presence of formaldehyde was monitored spectrophotometrically and the data were analysed by using equations derived by Trifonov *et al.* (1968) as described in the Materials and Methods section.

DNA preparation	Time for complete denaturation (min)	Hyperchromic effect (%)	$10^{-4} \times$ Initial rate of uncoiling (min^{-1})	$10^{-4} \times$ Rate constant of uncoiling (min^{-2})
Control				
Unfractionated	40	29.08	0	2.3
NaCl-eluted	26	27.78	0	4.7
Caffeine-eluted	18	23.19	26	5.7
Dimethylnitrosamine-treated				
Unfractionated	18	30.44	95	3.75
NaCl-eluted	18	49.15	95	3.75
Caffeine-eluted	8	16.13	57.5	53.75

Fig. 6. Denaturation kinetics of rat liver DNA in formaldehyde solution

The rate of change of absorbance is expressed in terms of the function θ as defined in the Materials and Methods section.

after methylation of the macromolecule cannot be assessed. The caffeine-eluted fraction of DNA from nitrosamine-treated animals exhibited much less hyperchromicity than did the respective control preparation, compatible with a greater degree of structural damage. The proportion of caffeine-eluted DNA in unfractionated preparations from nitrosamine-treated animals is more than 50% greater than in preparations of DNA from control animals (Huang & Stewart, 1977). The hyperchromicity of unfractionated DNA is presumably a composite expression of that of the NaCl- and caffeine-eluted fractions. Though this value for DNA from nitrosamine-treated animals was quantitatively similar to that of unfractionated control DNA, kinetic analyses reveal that this equivalence is not indicative of similar double-helical structure. Unlike the controls, structural defects are evident in all preparations. Measurements made during denaturation of both unfractionated and NaCl-eluted DNA defined the same line which, by the method of Trifonov *et al.* (1967), corresponded to 20 defects per 10^4 base-pairs (Fig. 8). Caffeine-eluted DNA from nitrosamine-treated animals exhibited properties sufficiently distorted from double-helical behaviour as to preclude calculation of defects. This difference in structure is imme-

Fig. 7. Denaturation kinetics of DNA preparations obtained after fractionation on benzoylated-DEAE-cellulose. DNA was isolated from control animals and subjected to chromatography as described in the Materials and Methods section. The denaturation of (○) NaCl- and (●) caffeine-eluted fractions is expressed in terms of the function θ derived by Trifonov *et al.* (1968).

diately obvious from the extremely short interval required for complete denaturation (Fig. 8) and reflected in the rate constant for despiralization, which was an order of magnitude greater than that determined for all other preparations (Table 3).

Discussion

Data reported in this paper are compatible with those of Caffin & Mackinlay (1975) and Pirro & Feldmann (1975) in supporting the conclusion that fractionation of DNA on benzoylated-DEAE-cellulose is largely dependent on the macromolecular structure of the nucleic acid. Detailed comparison of present findings with these earlier results obtained by using *E. coli* and *S. cerevisiae* define limitations in the application of this chromatographic technique to the fractionation of mammalian DNA. Such limitations are due to the complex structure of mammalian DNA compared with yeast and bacterial DNA. The present data, as were the findings of Caffin & Mackinlay (1975) and Pirro & Feldmann (1975), are discussed below in terms of the second fraction of DNA being distinguished by properties which relate to single-stranded character. The results permit description of a possible structural basis of this single-stranded character in

Fig. 8. Effect of dimethylnitrosamine treatment on denaturation kinetics of DNA

Rats were injected with 10mg of dimethylnitrosamine/kg body wt. and hepatic DNA was isolated as described. A portion of the DNA was subjected to chromatography on benzoylated-DEAE-cellulose. Absorbance data from unfractionated DNA (○) and DNA eluted from benzoylated-DEAE-cellulose with NaCl (●) defined the same line. Denaturation of the DNA fraction eluted with caffeine (□) was complete within 8 min.

rat liver DNA and its relationship to perturbation of structure by methylation *in vivo* after administration of dimethylnitrosamine.

Caffin & Mackinlay (1975) determined that the proportion of *E. coli* DNA eluted with caffeine was decreased if preparations were subjected to hydrodynamic shear before chromatography. The approximately equal distribution of DNA between the two fractions when an unsheared preparation was chromatographed (Table 1) is comparable with the finding by Pirro & Feldmann (1975), who recorded 45–33% of DNA from mouse liver, salmon, testes or calf thymus eluted in the second fraction. That only a minor fraction of rat liver DNA is bound to benzoylated-DEAE-cellulose in the presence of NaCl is indicated by the progressive decrease in the caffeine-eluted fraction after shear of the sample. Moreover, it seemed most unlikely that the smallest fragment size could be identified with single-stranded DNA, since hydroxyapatite chromatography had failed to separate a minor single-stranded fraction from such preparations (Stewart, 1976), and Caffin & Mackinlay (1975), using the same analysis, found no evidence of a fraction in caffeine-eluted DNA which co-chromatographed with denatured DNA. The data are compatible with the size of the lesion

binding to benzoylated-DEAE-cellulose being small relative to the smallest fragment size after shearing of the DNA.

Despite vast differences in structural complexity, *E. coli* (Caffin & Mackinlay, 1975) and rat liver (Table 2) DNA fractions were similar in their binding to nitrocellulose, both results indicating single-stranded character confined to the caffeine-eluted fraction. The relative structural complexity of rat liver DNA seems likely to be responsible for high cross-contamination of DNA fractions (Fig. 3) when compared with the data from yeast DNA. Pirro & Feldmann (1975) reported a separation subject to 2% cross-contamination. Analogous experiments using rat liver DNA, although revealing a less definitive separation, indicate that binding to benzoylated-DEAE-cellulose is nevertheless a stable property of the DNA and not the result of a chance separation of equivalent DNA species.

Structural data for the two fractions were provided by digestion with *N. crassa* endonuclease and subsequent electrophoresis (Figs. 4 and 5). DNA was denatured before electrophoresis and renaturation prevented by the presence of urea in the gel (Burgoyne *et al.*, 1974; Hutton, 1977). Migration of the non-digested DNA was consistent with the fragment size of the sheared preparation being approx. 7000 base-pairs. By virtue of the specificity of the *N. crassa* endonuclease, which, unlike the S1 nuclease of *Aspergillus oryzae*, does not degrade native DNA (Schwartz, 1975; Linn & Lehman, 1965), the absence of minor fragments from digested preparations of NaCl-eluted DNA is consistent with the double-stranded character of this fraction (Caffin & Mackinlay, 1975; Pirro & Feldmann, 1975).

The caffeine-eluted fraction includes a minor component able to be digested by the endonuclease with the production of high-molecular-weight species, which are then further degraded in the course of incubation, that is DNA fragments having large single-stranded regions. However, the major proportion of caffeine-eluted DNA is composed of molecules of uniform molecular weight unaffected during the course of incubation. It is concluded that the principal lesion responsible for DNA binding to benzoylated-DEAE-cellulose and presumably for the single-stranded character of this fraction in other assay systems is relatively small and either not degraded by the endonuclease or invariably located in the terminal regions of the fragments such that its digestion makes no appreciable difference to the size of persisting DNA. With minor variations this description is also applicable to DNA isolated from nitrosamine-treated rats.

Distinction between these possible structural defects as the major molecular lesion in caffeine-eluted DNA is possible on the basis of denaturation kinetics. The imperfect unwinding pattern of

caffeine-eluted DNA (Fig. 7) in contrast with unfractionated and NaCl-eluted DNA implies a structural defect confined to a minor fraction of rat liver DNA. A value greater than zero for the initial rate of uncoiling (Table 3) is indicative of defects being distributed between the terminal regions of caffeine-eluted fragments. The presence of two defects per 10^4 base-pairs corresponds to 1.4 per fragment bound to benzoylated-DEAE-cellulose.

The occurrence of single-stranded DNA sequences in replicative intermediates has been extensively reported (Painter & Schaefer, 1969; Habener *et al.*, 1970; Hoffman & Collins, 1976). Chromatography of DNA on benzoylated-naphthoylated-DEAE-cellulose was used to separate such intermediates from native DNA (Scudiero *et al.*, 1975; Henson, 1978). The high proportion of radioactivity eluted in the second fraction of DNA isolated 10 min after labelling *in vivo* and the progressive chase of this radioactivity into the NaCl-eluted fraction (Fig. 1) are consistent with the ability of benzoylated-DEAE-cellulose being able to discriminate between replicative intermediates and native DNA. This is significant, since all analyses of mammalian DNA by using benzoylated-DEAE-cellulose or benzoylated-naphthoylated-DEAE-cellulose have been made with DNA isolated from cells in culture. Such cell populations invariably contain a high proportion of replicating DNA, in contrast with the very low proliferative activity in adult rat liver. There is therefore little relevance of these previous studies to the biological significance of that minor proportion of rat liver DNA bound to benzoylated-DEAE-cellulose in the presence of NaCl which persists for weeks after labelling *in vivo*. This fraction may be prepared by elution of benzoylated-DEAE-cellulose with formamide rather than caffeine, a procedure which obviates any requirement for labelling *in vivo* (R. Haski & B. W. Stewart, unpublished work).

The presence of a minor fraction of mammalian DNA in single-stranded form which is not associated with replication has been suggested (Tapiero *et al.*, 1972; Amalric *et al.*, 1973). Such a subfraction has been typically isolated by hydroxyapatite chromatography of DNA exposed to possible local denaturation (56–60°C) in the course of preparation. Leibovitch, Tapiero and their colleagues have presented evidence that such single-stranded DNA is associated with transcription (Tapiero *et al.*, 1976; Leibovitch *et al.*, 1977; Leibovitch & Harel, 1978). DNA helices must undergo localized strand separation in order to permit the onset of RNA synthesis (Frenster, 1976). During transcription approx. 20 or less nucleotides of each nascent RNA chain are protected from nuclease digestion (Kumar & Krakow, 1975). At the same time the completed RNA chain is displaced from the template DNA, which would otherwise form an exact hybrid (Richardson, 1975; Bick *et al.*, 1972).

The available data are compatible with the hypothesis that regions of local denaturation associated with transcription are bound to benzoylated-DEAE-cellulose in the presence of 1.0M-NaCl and hence characterize the caffeine-eluted fraction of DNA. The adequacy of short single-stranded regions to permit binding of DNA to benzoylated-DEAE-cellulose is compatible with the findings of Karran *et al.* (1977) concerning adherence to benzoylated-naphthoylated-DEAE-cellulose of DNA from X-irradiated human lymphoid cells. They considered adherence of such DNA, thought to be isolated before enlargement of single-strand breaks to 'gaps', was due to the immediate effects of irradiation.

The basis of fractionation of DNA by benzoylated-DEAE-cellulose chromatography is not known conclusively. The available data are consistent with earlier studies (Caffin & Mackinlay, 1975; Pirro & Feldmann, 1975), minor differences being attributable to the structural complexity of mammalian DNA compared with bacterial and yeast DNA. It remains to be determined whether the basis of fractionation may be expressed in terms of primary structure. In the first instance, separation based simply on (G+C)- or (A+T)-rich sequences seems unlikely. No difference was observed during density-gradient centrifugation of NaCl- and caffeine-eluted DNA. In experiments in which purine composition of the respective fractions was determined (Huang & Stewart, 1978), no difference in composition was observed. If binding of rat liver DNA to benzoylated-DEAE-cellulose is related to transcription, differences in the content of repetitive and of unique sequences in the two fractions might be expected.

Compared with the endogenous structural defects in double-helical structure, evident in DNA from control animals, varying degrees of destabilization were induced by methylation *in vivo* after administration of dimethylnitrosamine. Being restricted to DNA isolated 4 h after administration of the carcinogen, the present experiments are concerned with structural change induced by methylation rather than the effects of repair processes (Pegg, 1977). Denaturation kinetics (Fig. 8) indicate structural defects in NaCl-eluted DNA from dimethylnitrosamine-treated animals. These defects do not cause binding to benzoylated-DEAE-cellulose and are not substrates for *N. crassa* endonuclease. This structural change may be attributed to the presence of alkylated bases in the DNA. For at least O^6 -methylguanine and 7-methylguanine, the concentration is similar in both DNA fractions 4 h after treatment (Huang & Stewart, 1978). Parameters other than the overall concentration of methylated bases may account for the extreme structural instability exhibited by caffeine-eluted DNA from dimethylnitrosamine-treated rats.

The short interval required for complete denaturation of caffeine-eluted DNA (Fig. 8) is indicative of

extensive disruption of structure compared with the respective control preparation. Evidence of local denaturation has been obtained after reaction *in vitro* of DNA with acetylaminofluorene, methylenedimethanesulphonate and u.v. light (Fuchs, 1975; Poppit & Fox, 1975; Vorlickova & Palecek, 1978). Since only a minor fraction of caffeine-eluted DNA was digested with endonuclease (Fig. 5), it is unlikely that long tracts of single-stranded DNA constitute the principal structural defect. Rather the data suggest regions of local denaturation possibly occurring at critical intervals within each DNA fragment. The failure of these single-stranded regions to be substrates for *N. crassa* endonuclease is consistent with the limited specificity exhibited by this enzyme. Kato & Frazer (1973) observed a low degree of single-strand scission of u.v.-light-irradiated DNA produced by *N. crassa* endonuclease compared with the number of thymine dimers present. Sleight & Grigg (1974) noted limited digestion of phleomycin-thymine complexes in DNA except in the presence of caffeine, which was thought to increase the degree of local denaturation at these sites.

Thus the use of benzoyleated-DEAE-cellulose permits definition of degrees of structural damage in rat liver DNA isolated immediately after administration of dimethylnitrosamine. Structural defects described in the present study are probably the direct consequences of alkylation rather than the products of hepatic glycosylases and endonucleases which are involved in subsequent repair processes (Pegg, 1977; Roberts, 1978). A variety of carcinogen-induced structural defects in DNA considered together with the range of alkylated bases thus far detected (Pegg, 1977) is illustrative of one aspect of the problem of relating DNA damage and its repair to tumour induction.

This research was made possible by grants from the National Health and Medical Research Council of Australia and the New South Wales State Cancer Council. We are grateful to Dr. A. G. Mackinlay of the School of Biochemistry at this University for expert advice on analytical ultracentrifugation and to Mrs. Virginia Black for skilled technical assistance.

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