Alterations in Thermal Stability of Rat Liver Chromatin and DNA Induced in Vivo by DimethylNitrosamine and DiethylNitrosamine

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ABSTRACT

A study was made of the effects of administration to rats of dimethylnitrosamine and diethylnitrosamine on the transition temperature (Tm) of sheared chromatin and DNA isolated from the liver. The analysis was made by thermal chromatography on hydroxylapatite with the use of DNA prelabeled with [3H]thymidine and following the elution pattern during the operation of a continuous temperature gradient. With a nonnecrogenic dose of dimethylnitrosamine (10 mg/kg), the alterations in chromatin were maximal at 24 hr and disappeared by 3 days. Greatest differences in elution profiles of chromatin after dimethylnitrosamine treatment were observed in the region above 80°. Administration of the carcinogen caused a lowering of the "melting" curve in this region, the displacement from control position being proportional to the dose. The maximum dose (60 mg/kg) displaced the complete chromatin melting curve up to 5° to the lower side. DNA isolated from this chromatin melted 3° less than that from control rats. However, administration of lower doses of dimethylnitrosamine did not affect the melting profile of DNA. The administration of diethylnitrosamine caused a similar type of change. However, the modification was also seen at 50–60°.

INTRODUCTION

It is now well documented that, when studied, virtually every chemical carcinogen, after suitable activation if necessary, interacts with DNA in target organs to produce covalently bound products (19, 35). These products are now being progressively identified for an increasing number of carcinogens. However, the localization of the sites of interaction, the consequences to the structure and function of the DNA and chromatin, and the significance, if any, of such changes in cancer development in the target cells or organs are poorly understood (19, 35).

Since chromatin is considered to represent an important level of organization of cellular DNA and since this organization imposes not only selectivity in the interactions of carcinogens with DNA (6, 15, 24, 25, 27, 28, 32–34), but also functional restrictions upon the expression of information in DNA, it is important to understand more about the effects of carcinogens on chromatin in target organs.

This work is directed toward this problem through the detection of alterations in the physical properties of DNA in purified DNA and chromatin from liver of rats following exposure to the hepatocarcinogens DMN and DENA in vivo. The results, in addition to demonstrating the possible importance of chromatin structure in determining the levels of DNA damage, permit suggestion of some relationship between elimination reactions and the overall DNA repair process.

MATERIALS AND METHODS

Animals. White male Wistar rats weighing 100 g were obtained from Carworth Farms (Rockland County, N. Y.) and fed Purina rat chow. The preparation of the animals by partial hepatectomy and radioactive labeling of hepatic DNA have been described in detail by Cox et al. (7). In brief, after partial hepatectomy, radioactive thymidine ([methyl-3H]thymidine; specific activity, 20 Ci/mmole) was administered every 4 hr beginning at 16 to 17 hr after operation until each animal had received 450 μCi. After a recovery period of at least 2 weeks, the animals were treated with carcinogens. DMN and DENA (Eastman Kodak, Rochester, N. Y.) in aqueous solutions were administered by i.p. injection at 10 a.m. Control animals were left untreated at this point. The animals were allowed food and water until killed by decapitation at various times after treatment, as judged by reference to the rate of metabolism of DMN and DENA (16).

Preparation of Chromatin and DNA. Chromatin was prepared from rat liver using a method based on that described by McConaughy and McCarthy (23). The liver was homogenized in 4 volumes 0.075 M NaCl-0.024 M EDTA (pH 8.0) with 5 strokes of a Teflon motor-driven homogenizer and centrifuged for 10 min at 1000 × g. The nuclear pellet was washed 3 times with the same buffer (25 ml) and twice with 0.05 M Tris-HCl (pH 7.5 at 4°). The nuclei were washed in 4 volumes 0.075 M NaCl-0.024 M EDTA (pH 8.0) with 5 strokes of a Teflon motor-driven homogenizer and centrifuged for 10 min at 1000 × g. The nuclear pellet was washed 3 times with the same buffer (25 ml) and twice with 0.05 M Tris-HCl (pH 7.5 at 4°). The nuclei were washed once in 0.01 M glycine (pH 6.0), centrifuged at 1000 × g, and resuspended in glycine. After standing overnight at 4°, the chromatin was sheared in a VirTis 45 homogenizer at maximum speed (45,000 rpm) for 10 sec, and the preparation was centrifuged at 10,000 × g for 15 min to remove insoluble impurities. Absorbance at 240, 260, and 280 nm of the chromatin solution was measured (22) and DNA (4) and protein (21) content were determined.

DNA was isolated from sheared chromatin by preparative

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isopyonic centrifugation in CaCl based on a procedure described by Flamm et al. (12). CsCl (12.6 g) was dissolved in chromatin solution (10 ml) and the preparation centrifuged at 2000 × g for 10 min. The density of the solution below the floating protein layer was adjusted to 1.70 g/ml, and 5-ml aliquots overlaid with mineral oil in cellulose nitrate tubes (that had been boiled in 0.5% EDTA solution) were centrifuged in a Beckman Type 40 rotor at 37,000 rpm for 24 hr. The contents were fractionated and the DNA-containing fractions, identified by radioactivity, were pooled and dialyzed overnight against distilled water.

**Thermal Chromatography on Hydroxylapatite.** Chromatin containing approximately 0.4 mg of DNA (total radioactivity, 50 to 70 × 10^3 cpm) in 0.14 M phosphate buffer was adsorbed to 0.55 g of hydroxylapatite (Bio-Gel HTP; Bio-Rad Laboratories, Richmond, Calif.) at 60°. Details of the necessary apparatus are described by Miyazawa and Thomas (26). Each experiment involved a pooled chromatin or DNA preparation from 2 identically treated animals. The temperature of the column was increased uniformly from 60° at a rate of 0.1° per min, and the hydroxylapatite eluted with 0.14 M phosphate buffer (flow rate, 6 ml/hr). Fractions corresponding to a minimum of 1° rise in temperature were collected at 60–100°. Acid-insoluble radioactivity in each fraction was determined (7). All of the radioactivity applied to the column was recovered. The amount of DNA eluted at a particular temperature expressed as a fraction of the total correct to 3 decimal places was used to plot the complete "melting" curve.

**RESULTS**

A study was made of the elution patterns of chromatin and DNA isolated from control animals to establish the limits of resolution of thermal chromatography as used in these experiments. The fraction of radioactivity eluted at a particular temperature during chromatography of a series of control preparations was expressed as a mean ± 1 S.D., and these data were used to generate a control melting curve for chromatin (Chart 1) and DNA (Chart 3). The melting curves of DNA as chromatin and as isolated nucleic acid, and their relationship to one another, were similar to published data (29, 37, 43, 47). In general, the greatest variation in the fraction eluted was observed at temperatures at which most rapid elution occurred: 69–75° in the instance of chromatin and 58–67° for DNA. The resolution between individual points on each of these control curves was sufficient to justify collection of fractions at the rate indicated, 1 per degree rise in temperature.

Prior to a study of chromatin from nitrosamine-treated animals, the effect of modification of certain preparative steps in the isolation procedure on the melting curve was examined. It is known that the T_m of a particular species of DNA, whether determined optically or by thermal chromatography on hydroxylapatite, is dependent upon the ionic strength of the solvent and, under certain conditions, the degree to which the nucleic acid is sheared (2, 22). The elution profile of chromatin varied with the degree to which the preparation was sheared as had been previously reported (11), as well as with the time of storage at 4° prior to chromatography. For example, the temperature of maximal elution shifted from 68 to 73°, with a decrease in shearing time from 50 to 5 sec. Storage at 4° for 48 hr decreased the temperature from 68 to 61° with 30 sec of shearing time. With 10 sec of shearing time, the temperature of maximal elution did not change significantly on allowing the preparation to remain at 4° for 8 hr.

The spectral properties and the DNA and protein content of all chromatin preparations from nitrosamine-treated rats did not differ from the respective results for control chromatin preparations. For example, the DNA content (±S.D.) of control chromatin and of chromatin after the administration of 10 or 60 mg of DMN per kg was 1.18 ± 0.09, 1.21 ± 0.12, and 1.08 ± 0.06, respectively. The protein content was 2.77 ± 0.21, 2.71 ± 0.17, and 2.36 ± 0.26, respectively. Similar observations of lack of change of chromatin composition were reported by Cooper and Itzhaki (5).

Injection of either DMN or DENA altered the chromatin melting curve, as defined by hydroxylapatite chromatography, by displacement of the whole curve or by distortion in particular regions. There was a common finding throughout this investigation: in all instances in which nitrosamine modification of chromatin (or DNA) was observed, the melting curve, either in whole or in part, was displaced toward a lower, and never higher, temperature. The greatest differences in elution profiles of chromatin after administration of DMN were observed in the region above 80°. Thus, after injection of 10 mg of DMN per kg and thermal chromatography on hydroxylapatite, the fraction of radioactivity eluted at each temperature between 60 and 79° was within the range of 1 S.D. of the control result. However, above this temperature the elution profile following administration of DMN was displaced toward lower temperatures and these data are shown in detail in Chart 2. The effect of DMN was dependent on the dose, in that treatment with 30 and 60 mg/kg progressively displaced the melting curve toward lower temperatures. The dose of 60 mg/kg modified the whole curve such that it was almost parallel and 1.5 to 2.0° lower than that of the control in the range 66 to 78°.
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respectively (above which temperature it varied as shown in Chart 2).

There was no change in the melting curve of rat liver DNA as a result of administration of DMN in doses up to and at 30 mg/kg. All points defining the melting curve of DNA isolated after injection of 10 mg of DMN per kg lay within the range of 1 S.D. of the control, as did the corresponding results with 30 mg/kg. For clarity, only a single elution profile from this group of experiments is shown in Chart 3. After a dose of 60 mg/kg was used, the melting curve was displaced uniformly toward lower temperatures (Chart 3). In the Wistar rat, 30 mg of DMN per kg is 50% lethal dose (17) and 60 mg/kg is a 90% lethal dose (B. W. Stewart, unpublished work), respectively.

Injection of 100 or 200 mg of DENA per kg modified the chromatin melting profile. Variation between control and DENA-treated preparations was observed in the range from 63 to 67°, with most distortion observed after administration of the higher dose of DENA (Chart 4). Above this temperature both curves gradually approached the control curve to come within the range of 1 S.D. at 75°. Above 80° the curves again varied from the control in a pattern similar to that observed after DMN treatment (Chart 2). The comparative changes in Tm with different doses of DMN and DENA are summarized in Chart 5. When DNA from DENA-treated rats was examined, only the highest dose caused any change from the control pattern. After injection of 200 mg/kg, the result was similar to that recorded after 60 mg of DMN per kg (Chart 3). The 50% lethal dose of DENA in the rat is 250 mg/kg (17) and doses of 140 mg/kg cause extensive centrilobular necrosis (17, 40).

The effect of increasing survival time on DMN-induced changes in chromatin melting profile was examined. Such experiments were confined to animals inoculated with a nonnecrotizing dose (1) of DMN (10 mg/kg). Twenty-four hr after treatment, the chromatin melting profile was unchanged from the 4-hr result, with discrepancy from control being confined to the region 80-100° (Chart 6). During the ensuing 24-hr interval, there was substantial change in the thermal stability of chromatin such that 48 hr after administration of the carcinogen the melting profile was only slightly different from that of the control. This difference was no longer apparent by 72 hr, at which time the control and treated curves were indistinguishable.

**DISCUSSION**

Alterations in the thermal stability of DNA modified in vitro with chemicals of biological significance (14, 36, 45, 46), including alkylating agents (36, 45), have been re-
in such DNA is a result of the limited resolving power of the assay, rather than a fundamental difference between the *in vivo* and *in vitro* situations.

Interpretation of studies on the transition temperature of alkylated DNA is complicated by thermal depurination. Alkylation of DNA causes destabilization that results (at neutral pH) in the hydrolysis of 7-alkylguanine and the formation of apurinic acid (19, 20, 42). The apurinic sites are in turn subject to hydrolysis to yield DNA with single-strand breaks (19, 35, 41). Strauss and Hill (39), in an extensive study of this reaction sequence with ethylating and methylating agents, established that the rate of depurination is dependent on temperature and the stability of apurinic sites is dependent on the buffer in which the DNA is kept. Measurements of the rate of degradation of DNA (methylated *in vitro* with dimethyl sulfate) in 0.1 M phosphate buffer solution indicated 10% of the total was rendered acid soluble after 3 hr at 70°, whereas the result in Tris buffer was 70% acid soluble (39). It is apparent that induction of single-strand breaks at apurinic sites on DNA during thermal chromatography in phosphate buffer is a much less common event than loss of 7-alkylguanine from the DNA chain. DNA alkylated *in vivo* contains a relatively small proportion of modified bases (30) compared with the product alkylated *in vitro* (39), and the small changes in thermal stability observed in this study are consistent with depurination during chromatography having a minimal effect on this parameter. Strauss and Hill (39) also concluded that, with respect to thermal depurination, there were no differences between methylating and ethylating agents. Qualitative differences recorded between DMN and DENA would therefore appear to depend on *in vivo* differences.

Strand separation in DNA as chromatin is dependent not only upon the base composition of the DNA, but also upon the nature of the nucleoprotein complex (13, 23). It has been suggested (13, 23) that early-melting chromatin contains DNA that is transcribed *in vivo* and that is less tightly complexed than DNA in chromatin that melts at high temperatures. In a study of liver chromatin from rats treated with DMN, Cooper and Itzhaki (5) recorded electric birefringence measurements which suggested that methylation may cause some localized loosening of the DNA protein complex. The present data are compatible with this concept in terms of the observed modification of melting in the high temperature range (Chart 2), along with no change in chromatin melting at lower temperatures (presumably "uncomplexed" DNA) or in the melting profile of DNA itself (Chart 3). Only when the dose of DMN reached 60 mg/kg was the DNA melting profile displaced toward lower temperature which, in turn, suggests alteration of the stability of DNA *per se* as the principle cause of a similar complete change in chromatin melting at this dose level (Charts 2 and 5). In this respect DMN was similar in effect to DENA, in that a displacement of both DNA and chromatin melting curves over their entire range followed injection of either carcinogen at the physiologically similar highest levels used. Also in common with the DMN result, lower doses of DENA modified chromatin melting behavior but not that of isolated DNA. Numerous differences between DMN and DENA, both biochemical and biological, are discussed by Swann and Magee (41). The present findings of qualitative

**Nitrosamine-induced Changes in Chromatin in Vivo**

**Chart 6. Disappearance of DMN-induced change in rat liver chromatin melting profiles.** Rats treated with a nonnecrotizing dose of DMN (10 mg/kg) were allowed to survive for 24, 48, and 72 hr. Chromatin was analyzed by thermal chromatography as described in "Materials and Methods," and the thickened intervals indicate temperature ranges at which the curves following injection of DMN and DENA at the doses shown on the chart. The thickened intervals indicate temperature ranges at which the curves following injection of DMN and DENA at the doses shown on the chart. The present data are compatible with this concept in terms of the observed modification of melting in the high temperature range (Chart 2), along with no change in chromatin melting at lower temperatures (presumably "uncomplexed" DNA) or in the melting profile of DNA itself (Chart 3). Only when the dose of DMN reached 60 mg/kg was the DNA melting profile displaced toward lower temperature which, in turn, suggests alteration of the stability of DNA *per se* as the principle cause of a similar complete change in chromatin melting at this dose level (Charts 2 and 5). In this respect DMN was similar in effect to DENA, in that a displacement of both DNA and chromatin melting curves over their entire range followed injection of either carcinogen at the physiologically similar highest levels used. Also in common with the DMN result, lower doses of DENA modified chromatin melting behavior but not that of isolated DNA. Numerous differences between DMN and DENA, both biochemical and biological, are discussed by Swann and Magee (41). The present findings of qualitative
differences between DMN- and DENA-treated chromatin are not likely to be due to artifacts, as already noted (38), and hence provide additional evidence of difference between these closely related chemicals.

The level of alkylation of rat liver DNA by DMN in vivo is proportional to the dose (8). A similar relationship is now established between dose and changes in the macromolecular structure of DNA following injection of this nitrosamine by studies of velocity sedimentation alkaline sucrose gradients (10) and chromatography on benzoylated DEAE-cellulose. Observation of a relationship between the dose of DMN and the distortion of melting profile (Chart 2) suggests that this distortion is a primary effect of treatment, and extends the level of organization at which DMN is known to modify genetic material in vivo. It should be pointed out that the smallest dose of DMN used in this study, 10 mg/kg, is carcinogenic when given as a single administration to rats after partial hepatectomy (9).

The respective time courses for elimination of methylation products (8, 30, 31), DNA structural change (10), and thermal stability of chromatin (Chart 6) following injection of DMN suggest that these activities all reflect various aspects of the "repair" phenomenon. For each of these activities, return to control values occurred over a period of not less than 3 days, the most rapid period of change being between 1 and 2 days after treatment. Failure to detect abnormalities in chromatin melting more than 3 days after the injection of 10 mg of DMN per kg is further evidence of the sensitivity of the velocity sedimentation analysis of DNA developed by Cox et al. (7), through which alterations in DNA are apparent 14 days after treatment (10). On the other hand, the present experiments, in which variation in the properties of chromatin are demonstrable in the absence of analogous changes in DNA, imply that the nature of DMN-induced changes to genetic material cannot be fully described in terms of the chemistry of DNA alone. It is possible that analysis of such changes and their functional significance will necessitate consideration, among other parameters, of the interaction between the RNA and protein molecules, which along with DNA constitute chromatin. The need for more detailed studies of the effects of carcinogenic chemicals, at levels of organization of the nucleus more complex than that of DNA alone, is further supported by the clear-cut indication for the nonrandom nature of the interaction of several carcinogens with DNA in chromatin (6, 15, 24, 25, 27, 28, 32–34).

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