

Methylmercury-Chromosome Interactions.

I. Thermal Denaturation of Calf Thymus Chromatin in Presence of CH_3HgOH

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The effect of CH_3HgOH on the thermal stability of calf thymus chromatin in Na_2SO_4 (10 mM Na^+ , pH 6.8) has been studied spectrophotometrically by heat denaturation. From changes observed in the integral and differential melting profiles, gathered *via* absorbance measurements at 260 nm as a function of CH_3HgOH concentration, it is concluded that methylmercury at low concentrations (1–10 μM), and prior to its denaturing the double-helix of the chromosomal DNA, interferes with the binding of DNA by histones H3 and H4 which is followed, at methylmercury concentrations between 10 and 32 μM , also by its interfering with the complexing of DNA by the less basic histones H2A and H2B. DNA denaturation, in absence of the histones, proceeds only at methylmercury concentrations above 32 μM under the experimental conditions given.

Introduction

Organomercurials, in particular methylmercuric derivatives, are genetically highly active compounds that, at sublethal concentration levels, cause polyploidy and aneuploidy in plants and animals [1] while at lethal concentrations extensive chromosome damage, *viz.*, chromosome breakage and dissolution, pycnotic clotting, alteration in stainability and so forth, has been observed [1–4]. Although there exist numerous sites in a cell such as membranes [5], organelles [6], or enzymes [7], that are known to be affected by methylmercury under suitable conditions and that, therefore, could give rise to the cytological alterations mentioned, the opinion has been voiced that, for instance, chromosome breakage emanates from a direct effect of methylmercury on chromosomes rather than through its inhibiting the enzymes involved in nuclear division [1]. The fact that methylmercury can disrupt Watson-Crick hydrogen bonding in double-stranded nucleic acids by forming coordinate bonds with the nitrogen binding sites of the bases [8–10] was offered as example of such a direct chromosomal effect [1]. Further evidence for methylmercury interacting directly with genetic material may be seen in the finding that it inhibits the

growth of a recombination-deficient (*rec*[−]) strain of *Bacillus subtilis* more severely than the growth of a wild-type (*rec*⁺) strain, *i. e.*, that it displays mutagenic activity in *rec*[−] prokaryotic systems [11, 12]. Current interpretations of the mechanism of mutagenesis brought about by chemicals envision the mutagen to react directly with DNA, as the most target-specific component of the genetic material, and not with nuclear enzymes or other intracellular structures.

In view of the fact that in eukaryotes the genetic material consists of protein-DNA complexes, rather than DNA alone, we decided to investigate the effect of methylmercury on chromatin, the diffuse interphase form of the chromosomes of the cell nucleus, in an attempt to see in which way the protein-DNA complexes can serve directly as “target-specific” material. In the following sections results of a heat denaturation study are presented in which the effect of the methylmercury derivative CH_3HgOH on the thermal stability of chromatin was investigated. Methylmercury, apart from its tendency to interact strongly with sulfhydryl groups, has a high affinity for basic nitrogen binding sites [13]. Consequently, its interaction with chromatin, in particular with the histones, should be noticeable *via* heat denaturation provided the interaction leads to changes in the tertiary or secondary structure of the protein-DNA complexes. We are unaware of any studies that have been undertaken along the lines mentioned.

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Experimental

Materials

Calf thymus was purchased from Pel Freez Biologicals, Inc. and stored at -80°C until used. Calf thymus DNA (sodium salt) was a product of Worthington Biochemical Corporation. It was stored at -20°C prior to use. Anhydrous Na_2SO_4 , analytical grade, was purchased from Mallinckrodt. It served as supporting electrolyte since it does not react with methylmercuric hydroxide. CH_3HgOH (97 + %) was purchased from Alfa Products, Ventron Corporation. Sodium cacodylate, cacodylic acid, and disodium ethylenediamine tetraacetate (EDTA) were products of Sigma Chemical Company. Further information concerning the reagents used in this study can be found elsewhere [14].

Calf thymus DNA solutions for heat denaturation, containing a constant level of buffer (5 mM cacodylic acid-sodium cacodylate mixture of pH 6.8), but varied amounts of Na_2SO_4 and CH_3HgOH , were prepared as described previously [15]. A modification was introduced insofar in that CH_3HgOH was added to the experimental solutions *via* dialysis and not directly, for instance, by means of a micropipet, as done in the past [8, 15]. Chromatin (soluble) was isolated from calf thymus by following, by and large, the techniques employed by Panyim and Chalkley [16]. All glassware and centrifuge tubes were rinsed with 20 mM EDTA, pH 8, prior to use and all isolation steps were carried out at 5°C . One major alteration was introduced by performing the isolation in presence of 1 mM phenylmethylsulfonylfluoride (PMSF) in order to inhibit endogenous proteases. PMSF was removed in the end by exhaustive dialysis of the soluble chromatin against 10 mM Tris-HCl buffer of pH 8. The nucleohistone thus prepared yielded DNA-to-RNA mass ratios above 25 and its protein-to-DNA mass ratio amounted to 2. Absorbance readings at 320 nm were about 0.01 to 0.02 of the readings measured at 260 nm indicating negligible quantities of aggregated material. Acid extraction with sulfuric acid [17] yielded the histone fractions H1, H2A, H2B, H3, and H4 that could not be distinguished electrophoretically from commercially available histone preparations. Gel electrophoresis was performed by using the acetic acid-urea-polyacrylamide gel system of Panyim and Chalkley [16].

For heat denaturation, stock nucleohistone, contained in 10 mM Tris-HCl, pH 8, was dialyzed first

against three changes each of solutions constant in buffer (5 mM cacodylic acid-sodium cacodylate, pH 6.8), but varied in CH_3HgOH and Na_2SO_4 , and sedimented for 25 min at 13 000 *g* to remove any aggregated material that might have formed during dialysis. All operations were performed at 5°C .

The experimental solutions are referred to in terms of pNa and pM. $\text{pNa} \equiv -\log [\text{Na}^+]$, as brought into the system by Na_2SO_4 and buffer, and $\text{pM} \equiv -\log [\text{CH}_3\text{HgOH}]_{\text{added}}$, with $[\text{CH}_3\text{HgOH}]$ representing the stoichiometric CH_3HgOH concentrations of the solutions. Although experiments were performed at the salt levels pNa 2.0, 1.5, 1.0, and 0.0 best results were obtained in the pNa 2.0 medium since here chromatin displayed the highest degree of solubility at all levels of CH_3HgOH . This was not the case at $\text{pNa} < 1.5$ where precipitation took place at moderate and elevated levels of CH_3HgOH . For this reason, only the pNa 2.0 data are shown.

Methods

Heat denaturation was performed as outlined in detail elsewhere [18]. A Gilford Model 2000 spectrophotometer was used. Temperatures were measured with a Hewlett-Packard Model 2801A quartz thermometer. The digital temperature readings were converted into analog outputs (Hewlett-Packard Model 580A D/A converter) and registered with the help of the Mosely Model 80A strip-chart recorder. The heating chamber of the Gilford spectrophotometer was modified to accommodate the thermometer probe and probe cord. The probe was placed in a cuvette containing the appropriate pNa medium. Spectral and temperature changes were recorded in 10–15 sec intervals.

Results of the measurements are presented in terms of hyperchromicity H^{260} defined, in the usual way, as the normalized absorbance increase at 260 nm after treatment of the sample with heat. Denaturation profiles are given in integral ($H^{260} = f(t)$) and differential ($[dH^{260}/dt] = f(t)$) form, with (*t*) being the temperature, whereby the slope $[dH^{260}/dt]$ was evaluated according to Li and Bonner [19] for each temperature. The temperature at which a differential melting profile assumes maximum values (thermalite peak) is called the melting temperature T_m of that peak. DNA and chromatin concentrations were determined by using the molar absorptivities of 6600 and 6760 (1/mol DNA·P·cm at 260 nm), respectively. For melting, samples with 0.8–1.6 A^{260} units were employed.

Results

Calf thymus DNA

The absorbance of chromatin in the ultraviolet is determined almost exclusively by the DNA it contains, with proteins contributing only to a minor degree. Hence, if one wishes to study the effects of a chemical on the physicochemical properties of chromatin *via* UV absorbance measurements, it is prudent to investigate its effect also on the constituent DNA alone in order to have a suitable reference. We used calf thymus DNA as denaturation "standard", *i. e.*, we employed it as calf thymus "chromatin" void of all histone proteins. We will report on its interaction with methylmercury first.

Heating native calf thymus DNA at pNa 2.0 and at the CH₃HgOH concentrations given produces the denaturation curves shown in Fig. 1 (inset). The solid curve, denoted as curve (1), consists actually of melting curves obtained at methylmercury levels of pM ∞ (control), 6.0, 5.5, and 5.0, respectively, showing that CH₃HgOH at concentrations up to 10 μM (pM 5.0) does not affect the secondary structure of DNA prior to heat denaturation. A slight methylmercury effect is noted at 32 μM CH₃HgOH (pM 4.5), involving the low-temperature end of the integral melting curve, without, however, affecting the melting temperature(s) to any extent (*cf.*, Table I). At methylmercury levels of pM < 4.5, calf thymus

DNA becomes increasingly denatured by CH₃HgOH prior to heating. This is noted by the lowering of the maximum (limiting) value of H^{260} and by the shifting of the melting curves towards lower temperatures. In fact, at pM 3.0, methylmercury-induced denaturation took place prior to denaturation by heat: only the typical heating curve of denatured DNA, signaling base-unstacking of randomly-coiled single-strands, is obtained [8].

More detailed information is contained in Fig. 2 (inset) where we have presented the denaturation profiles in derivative form. It is known that calf thymus DNA exhibits a minimum of three melting bands (thermalites) that can be seen spectrophotometrically [20] or by calorimetry [21], provided the data are plotted in differential form to improve resolution. In the salt medium of this study, these three bands are located at $T_m(\text{I}) = 65.5^\circ\text{C}$, $T_m(\text{II}) = 69.2^\circ\text{C}$ (shoulder), and $T_m(\text{III}) = 74.3^\circ\text{C}$ (*cf.*, inset of Fig. 2 and Table I). It is of interest to note that CH₃HgOH at pM 4.0 resolves the shoulder into a well-defined peak. However, at pM 3.5, only two recognizable bands remain. Since methylmercury has a high affinity for (A + T)-rich nucleic acids [8], it is reasonable to assume that the remaining thermalites represent blocks (or satellites) with a high (G + C) content. No fine-structure remains in the melting curves of calf thymus DNA at pM 3.0 or below: the curves meander around 0.2 [d H^{260}/dt]

Table I. Effect of CH₃HgOH on the heat denaturation characteristics of calf thymus DNA and calf thymus chromatin in Na₂SO₄ at pNa 2.0.

pM	$T_m(\text{I})$ [°C]	$T_m(\text{II})$ [°C]	$T_m(\text{III})$ [°C]	$T_m(\text{IV})$ [°C]	H_{max}^{260}	Remarks
DNA:						% Denatured ^a
∞–5.0	65.5	69.2	74.3	—	0.39	0
4.5	64.5	69.2	74.3	—	0.39	0
4.0	58.3	64.0	71.0	—	0.36	10
3.5	—	56.6	67.0	—	0.24	42
3.0	—	—	—	—	0.16	100
Chromatin:						% Labilized ^b
∞	54.5	64.5	73.5	82.5	0.23	0
6.0	54.5	64.5	—	82.5	0.20	7
5.0	—	60.5	75.5	82.5	0.18	43
4.5	—	60.5	~ 70 (75)	~ 82	0.16	58
4.0	—	65.0	~ 73	~ 82	0.14	65 ^c
3.5	—	65.0	~ 72	~ 72 (83)	0.13	75 ^c
3.0	—	—	—	—	0.06	—

^a Prior to melting. Evaluated through integration of the area under the thermalites.

^b Decrease of the area under thermalites III and IV in comparison to the area existing at pM ∞. This area, in turn, comprises 78% of the total melting area.

^c Not corrected for possible DNA denaturation by CH₃HgOH.

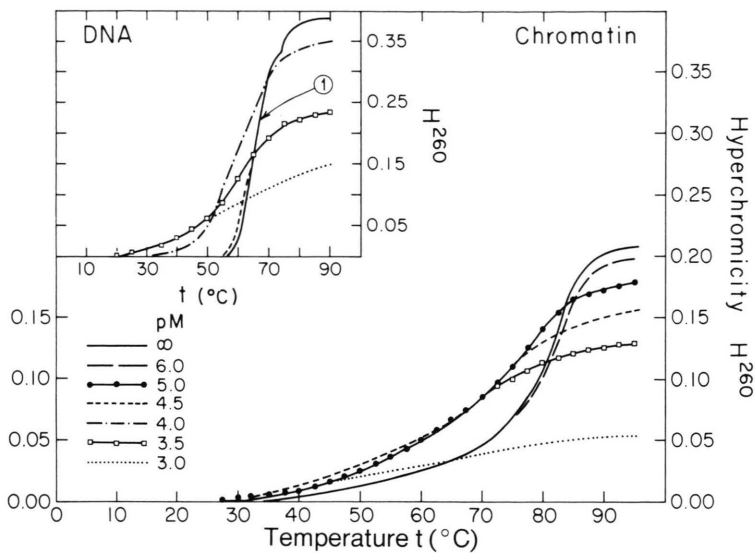


Fig. 1. Integral melting curves of calf thymus chromatin and calf thymus DNA (inset) as a function of pM. $pM \equiv -\log [CH_3HgOH]_{added}$. Solvent: Na_2SO_4 (10mM Na^+ , pH 6.8). The curve marked (1) comprises melting profiles obtained at $pM \infty$, 6.0, 5.5, and 5.0, respectively.

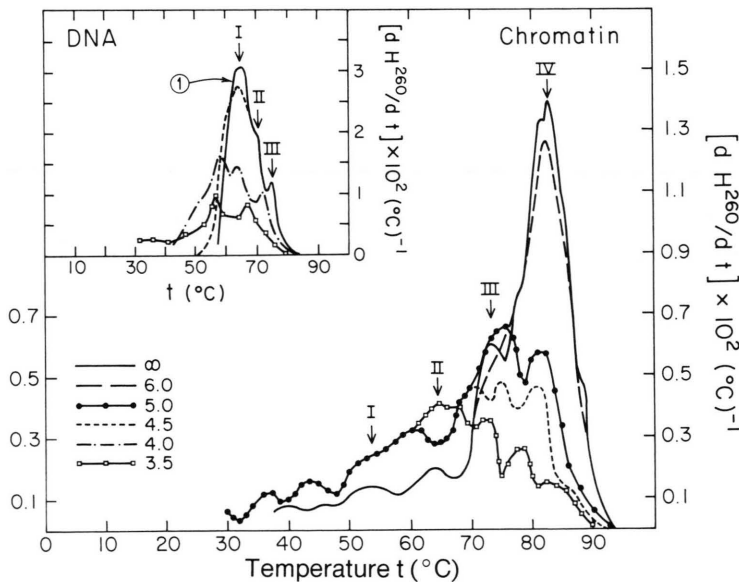


Fig. 2. Differential melting curves of calf thymus chromatin and calf thymus DNA (inset). The experimental conditions are those of Fig. 1, with the curve marked (1) comprising again denaturation profiles obtained at $pM \infty$, 6.0, 5.5, and 5.0, respectively. The meaning of the melting bands (thermalites) I, II, III, and IV is explained in the text.

$\times 10^2$ demonstrating, as already mentioned, that methylmercury-induced denaturation took place prior to heating (not shown in Fig. 2).

Calf thymus chromatin

The heat denaturation curves of chromatin at pNa 2.0 are displayed in integral and differential form in Figs 1 and 2 (main part), respectively. As has been known for some time, the presence of the basic histones, carrying positive charges near physiological pH, imparts a greatly increased thermal

stability on the constituent DNA due to the dramatically decreased negative charge density along the phosphate backbone. Under the experimental conditions of this study (pNa 2.0, pH 6.8), and in absence of CH_3HgOH , the increase in thermal DNA stability (main melting bands) amounts to about $17^\circ C$ (*cf.*, Table I).

Increasing methylmercury concentrations first and foremost lower the limiting H^{260} values of chromatin as can be seen from Fig. 1. This occurs already at levels (pM 6.0–4.5) that have no or only a marginal effect on the secondary structure of calf thymus

DNA alone. At $pM < 6.0$, the denaturation curves of chromatin are also shifted towards lower temperatures. The shift is most dramatic between $pM\ 5.5-5.0$ but levels off thereafter (the $pM\ 5.5$ curve is not shown; it runs between the $pM\ 6.0$ and 5.0 curves).

The differential melting profile obtained for calf thymus chromatin in absence of CH_3HgOH is virtually indistinguishable from the profile presented by Li and collaborators [22]. They find a major melting band at $T_m(IV) = 82^\circ C$ and a minor one at $T_m(III) = 72^\circ C$, and two inflections appear to exist near $66^\circ C$ and $57^\circ C$, respectively (heating was done in $0.25\ mM$ EDTA, $pH\ 8$). Our data are given in Table I. As can be seen, the $pM\ \infty$ data are in excellent agreement with Li *et al.*'s values [22].

Fig. 2 shows that the primary effect of CH_3HgOH on the fine-structure of the chromatin denaturation profile consists of dramatically lowering the size of the melting band IV (*viz.*, $pM\ \infty - 4.5$) while leaving the size of thermalite III intact in this organomercurial concentration range. At $pM\ 5.0$, a peak appears at $60.5^\circ C$ which then moves over to $65^\circ C$ at the pM values 4.0 (not shown) and 3.5 (shown) (peak II). Incidentally, the $pM\ 6.0$ curve cannot be distinguished from the $pM\ \infty$ curve at temperatures up to $70^\circ C$ as the $pM\ 4.5$ curve cannot be distinguished from the $pM\ 5.0$ curve. Lastly, the $pM\ 3.0$ curve has also here no characteristic features indicating that $1\ mM\ CH_3HgOH$ sufficed to denature chromatin already at room temperatures as it sufficed to denature calf thymus DNA alone (not shown in Fig. 2).

Discussion

The various chromatin melting bands (*cf.*, Fig. 2) that can be observed in any suitable, methylmercury-free medium have been assigned. The assignment, based on the shape of the differential melting profiles produced by partially salt-extracted chromatin, by reconstituted nucleohistones, or by chromatin treated with proteases [19, 22–24], is as follows: band IV corresponds to the melting of DNA regions bound by the very basic arginine-rich histones H3 and H4. Band III is due to the melting of DNA regions complexed by the lysine-rich H2A and H2B histones while the minor inflections, *viz.*, peaks II and I, are believed to result from the melting of DNA regions bound by non-histone proteins or to represent short, free DNA gaps between histone-bound regions. Since the so-called

“core” histones H2A, H2B, H3, and H4 can be dissociated from chromatin by, for instance, high NaCl concentrations, resulting in the decrease in size of the thermalites IV and III, we conclude from the changes observed with chromatin in presence of CH_3HgOH , but at the constant counterion level of $0.01\ M\ Na^+$ and at the constant pH of 6.8 , that the primary action of the organomercurial consists of interfering with (H3)(H4)-DNA interactions. This takes place at methylmercury concentrations that are too low to destabilize the DNA double-helix as can be clearly seen from the melting profiles of the DNA alone. At pM values smaller than 5.0 , the stability of the less basic histone-DNA complexes, involving histones H2A and H2B and, in our case, presumably also H1, is also affected, resulting in the decrease (and splitting up?) of the area pertaining to band III. Whether the reaction of CH_3HgOH with chromatin, represented symbolically, for instance, by



with H_2N^+ = standing for the protonated imino nitrogen of arginine, leads to complete histone dissociation, as occurs in salt or acid extraction, or results merely in the labilization and possible rearrangement of the protein-DNA complexes must be left undecided at this point in time. The composition of the salt solvent employed, a compromise between the requirement of not having anions present that react strongly with CH_3Hg^+ and maintaining ionic strength conditions that minimize aggregation, is such that a clear-cut answer cannot be provided. The emergence of the melting band II at $65^\circ C$ would be in agreement with the formation of “naked” DNA regions under our experimental conditions. However, onset of DNA denaturation (*cf.*, inset of Fig. 2) tends to obscure this. Attempts to monitor histone dissociation from chromatin subsequent to methylmercury treatment by column chromatography on Sephadex G-200 or Sepharose 4B were unsuccessful, due to secondary reactions of the treated chromatin with the bed material, as was diafiltration, due to concentration polarization effects taking place at the membrane during pressure filtration.

By integrating the areas under the melting bands III and IV, Li *et al.* [22] concluded that 79% of all DNA base pairs in calf thymus chromatin are complexed by histones. We find 78% in absence of CH_3HgOH . This value decreases with increasing

CH₃HgOH concentration as can be seen from Table I. We have expressed the decrease as % labilized, rather than dissociated, in view of the uncertainty existing with respect to the precise nature of the chromatin-methylmercury complexes. It is, of course, intriguing to infer from the alterations observed in the differential chromatin melting profiles at low CH₃HgOH concentrations that the organomercurial affects first and foremost the stability of the chromatin subunits termed nucleosomes by, for instance, abolishing the superhelical arrangement of the chromosomal DNA around the outside of the histone octamer "core", represented symbolically, for instance, as [(H2A)₂(H2B)₂(H3)₂(H4)₂] (*cf.*, [25] and references cited therein). As is known, in order for duplex DNA to assume the higher-order structure (supercoiling) seen in chromatin, histones H3 and H4 must be present and must be unperturbed in their interactions with the DNA. Thus, it is conceivable that methylmercury-treated chromatin does not give rise to the "beads" seen in electronmicroscopy. These "beads" result from the periodic arrangement of the "core" histones along the nucleosomal DNA [26–29]. Future research, utilizing nu-

clease-treated chromatin preparations, will be concerned with clarifying this matter.

In conclusion, the results presented above show clearly that chromatin is affected structurally by methylmercury already at concentrations that are too low to destabilize the DNA helix. These structural alterations might very well be the primary events that result in the chromosome damages observed *in vivo* as well as *in vitro* [1–4]. In view of the fact that histones H3 and H4 not only have been most strictly conserved in amino acid sequence during evolution [30, 31] but also are essential to the formation of nucleosomes [25], the finding that methylmercury affects their association with DNA in a most pronounced manner could be the first clue to the mechanism of the genetic activity of organomercurials at the molecular level.

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