AURINTRICARBOXILIC ACID AS A PROBE FOR THE ANALYSIS OF CHROMATIN STRUCTURE

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Received March 18,1975

SUMMARY: Aurintricarboxylic acid is shown to cause nuclear swelling, disaggregation of chromatin structure and release of histones from chromatin. The nuclear swelling is inhibited by Ca^{++} and Mg^{++}. The potential usefulness of aurintricarboxylic acid as a probe in chromatin studies is suggested.

Aurintricarboxylic acid* has been demonstrated to inhibit the initiation of protein synthesis (1, 2) and the binding of Qβ replicase, E. coli RNA polymerase and lac repressor to their templates (3). From these findings, it was predicted that ATA inhibits the function of most nucleic acid-binding proteins (3). In addition, certain polyanions such as heparin (4, 5, 6), dextran sulfate (6), and polystyrene sulfonate (7, 8) have been reported to induce nuclear swelling or decondensation of chromatin by binding with histone and dissociating it from DNA.

On the basis of these findings, we assumed that ATA may interact with histone and alter nuclear structure. The results reported in this paper clearly indicate that ATA binds to histone and induces nuclear swelling, loosening of chromatin, and release of proteins from chromatin.

In our experiments, ATA-induced nuclear swelling was inhibited by Ca^{++} and Mg^{++}. Although the distinct compartmentation of Ca^{++} and Mg^{++} in nuclei has been proposed (9), the binding site of these cations is not yet clearly understood. Using ATA as a probe, it would be possible to obtain more detailed infor

* abbreviated as ATA

mation about the mode of interaction among histones, divalent cations, and DNA in chromatin. The low molecularity of ATA will be convenient in interpreting experimental results.

MATERIALS AND METHODS

Ammonium salt of ATA was purchased from Nakarai Chemicals, Ltd. and used without further purification. Calf thymus histone Type II was obtained from Sigma.

Calf and rat liver nuclei were isolated by centrifugation in high density sucrose. Isolation and washing media contained 5 mM $MgCl_2$, 25 mM KCl, 10 mM Tris-HCl pH 7.9, 5 mM 2-mercaptoethanol for calf liver and 2 mM $CaCl_2$, 15 mM Tris-HCl pH 7.9 for rat liver respectively.

For electron microscopic observation of ATA-treated nuclei, they were fixed with 3 % glutaraldehyde in 50 mM potassium cacodylate buffer, pH 7.4 containing 0.25 M sucrose for 3 hr, postfixed in 2 % osmium tetroxide for 2 hr, stained with 1 % uranylacetate, dehydrated in a series of ethanol and propylene oxide, and embedded in epon resin. A Sorval MT-2 ultramicrotome with glass knives was used for sectioning. Sections were stained with lead citrate and examined with a Hitachi Type 11-C electron microscope at an accelerating voltage of 75 KV.

The process of nuclear swelling was measured spectrophotometrically according to the method of Kraemer and Coffey (6), except that a wave length of 650 nm instead of 600 nm was used to avoid the absorption of ATA. The temperature was kept at 30°C throughout the reaction.

Nuclei and chromatin preparation were hydrolyzed overnight at 37°C in 0.3 N KOH and the amount of DNA was determined from the absorbance at 265 nm after digestion with 5 % perchloric acid at 95°C for 15 min. The protein released with ATA from chromatin was measured after ether extraction of ATA from the acidified supernatant because ATA reacted with phenol reagent and increased the value. DNA in the supernatant was determined by indol method (15).

EXPERIMENTAL RESULTS AND DISCUSSION

Nuclear swelling and decondensation of chromatin: When ATA was added to the nuclear suspension, an increase in nuclear diameter and loss of density occurred (Fig. 1b). The condensed chromatin around the nuclear membrane disappeared. Electron microscopic observation also showed the homogeneous appearance of whole nuclear material (Figures 1c, 1d). Nucleoli were relatively resistant to ATA, but their structure gradually deteriorated. A higher concentration of ATA destroyed nuclei and caused gellation. In isolated chromatin, ATA extinguished all the condensed structure and left extended fibers (Fig. 1e). The thinnest fiber observed was approximately 30 Å thick.

The effect of increasing the concentration of ATA on nuclear swelling is shown in Figure 2a. The net swelling at 10 sec after addition of ATA could be regarded as the approximate initial rate of swelling reaction. When plotted

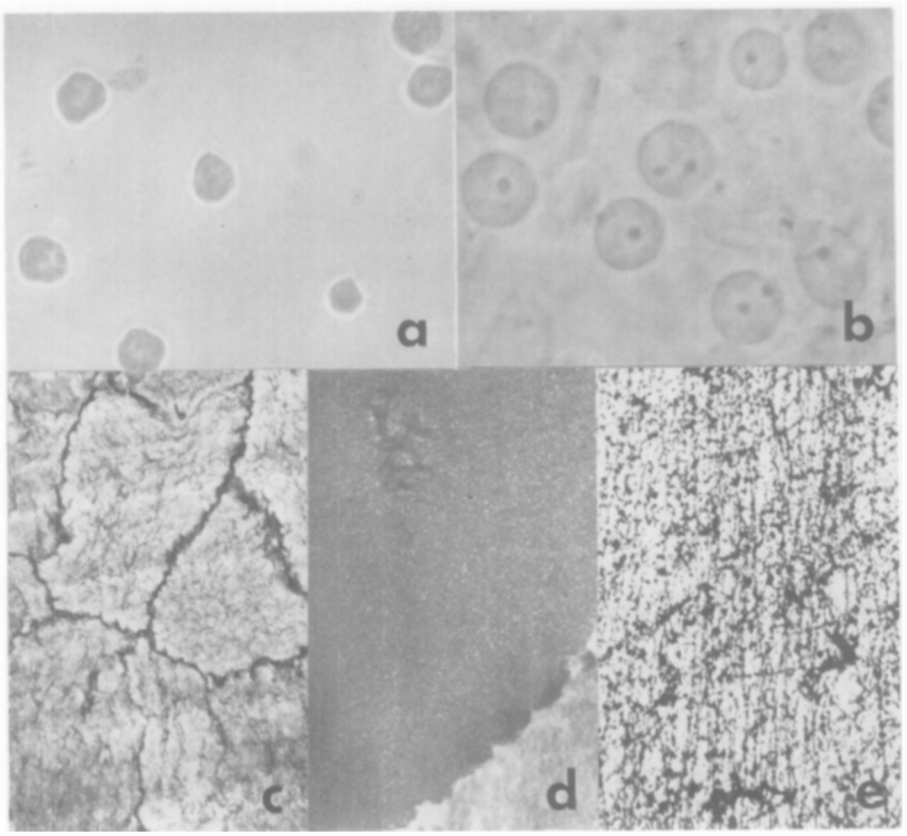

FIGURE 1. Microscopic observation of ATA-treated nuclei and chromatin. Nuclei isolated from rat liver (0.3 mg DNA) were incubated at 20°C with 1 mM ATA (0.5 μmoles in amount) in the medium containing 0.3 M sucrose, 2 mM $CaCl_2$, 10 mM Tris-HCl pH 7.9 and fixed after 30 min (see under "methods").

(a), (b) Phase contrast micrographs of the fixed nuclei; control (a) and treated (b). Material released from nuclei is seen in the background. Magnification. X 1,760

(c) An electron micrograph of the treated nuclei. The nuclei turned adhesive one another after the treatment. Note the dense substance along the nuclear membrane. X 10,500

(d) A higher magnification of the treated nucleus showing the homogeneous and reticular chromatin. There are breaks of the nuclear membrane. X 16,000

(e) An electron micrograph of isolated chromatin treated with ATA. Rat liver chromatin containing 0.8 mg DNA (prepared as in Table 2 and dispersed in 5 mM Tris-HCl pH 7.9) was incubated with 2 mM ATA (2 μmoles in amount) for 5 min at 30°C, and stained with 1 % uranyl acetate. X 7,800

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double reciprocally (Fig. 2b), dots were fitted by two lines. The swelling process, therefore, seems to have two distinct phases with regard to ATA concentration (temporarily referred to as phase 1 and phase 2 for lower and

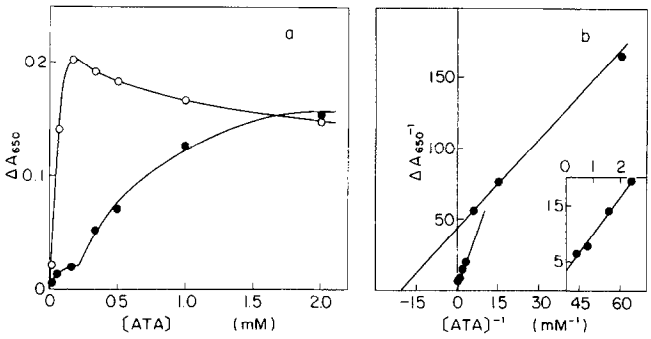

FIGURE 2. Swelling of nuclei spectrophotometrically monitored. Calf liver nuclei had been kept at 0°C before use; 0.1 ml of nuclear suspension (73 μg DNA) was added to 2.9 ml of suspension medium (0.25 M sucrose, 20 mM Tris-HCl pH 7.9, 25 mM KCl, 5 mM $MgCl_2$) equilibriated at 30°C. The swelling reaction was started by the addition of ATA.

(a) ATA concentration dependency. The decrease in the absorbance at 650 nm at 10 seconds (●—●) and 5 minutes (o—o) after the addition of ATA was plotted against the ATA concentration.

(b) A double reciprocal plot of the decrease of absorbance in 10 seconds and the concentration of ATA. The high concentration region is shown in the inset.

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higher concentration regions of ATA). The intercepts on the abscissa yield two constants, K_1=0.047 mM and K_2=2.0 mM for phase 1 and phase 2 respectively. The application of the Michaelis-Menten type equation to the kinetics of nuclear swelling is only a tentative one, since the implication of ΔA_{650} could not be estimated; however, we can regard K_1 as reflecting the affinity of ATA to a binding site in chromatin. The decrease in absorbance at 5 minutes is also shown in Figure 2a. Since the absorbance reaches an almost constant state after 5 minutes, the net swelling at this time is practically a maximal swelling. Obviously, the concentration of ATA that is required for completion of phase 1 is enough to cause the end point of swelling. If this concentration is converted into number of moles per system and compared with that of the phosphate groups of DNA (Table 1), similar ratios are obtained both with calf liver and rat liver nuclei under somewhat different conditions. Since about half of the phosphate groups of DNA are combined with the basic groups of histone (10), this consideration leads to the suggestion that ATA binds to

	ATA (μmole)	DNA (μg)	DNA-P (μmole)	ATA/DNA-P
Calf liver nuclei	0.66	73	0.24	2.75
Rat liver nuclei	0.90	101	0.34	2.65

TABLE 1. Comparison of ATA amount required for completion of phase 1 and the DNA phosphate added to the system. The same procedure as in Fig. 2 was performed for rat liver nuclei except that the medium for swelling was 0.25 M sucrose, 15 mM Tris-HCl pH 7.9, 2 mM $CaCl_2$.

those basic groups of histone and that the ATA-histone complex formed in phase 1 contains a comparable number of ATA molecules to basic groups of histone. The complex formed in phase 2 seems to have higher ATA/histone ratio.

The ATA-induced nuclear swelling was inhibited by Ca^{++} and Mg^{++} (Fig. 3). Calcium ion completely inhibited both initial rate and final level of swelling at 26 μmoles per system (8.6 mM in concentration). In the case of Mg^{++}, a higher concentration was required for complete inhibition. Our preliminary experiments indicate that the inhibition was not overcome by excess ATA, therefore the conceivable manner of inhibition is not a competitive one. Divalent cations are known to play an important role in maintaining condensed

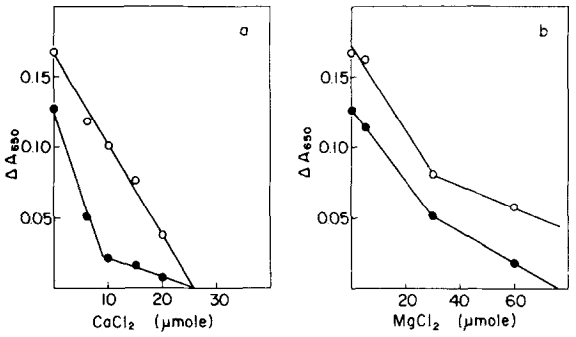

FIGURE 3. Inhibition of ATA-induced nuclear swelling by Ca^{++} and Mg^{++}. Indicated amount of Ca^{++} (a) or Mg^{++} (b) had been added before addition of ATA. Almost no change in absorbance was observed at this time. The decrease in the absorbance at 650 nm at 10 sec (●—●) and 5 min (o—o) after 1 mM ATA was added is plotted against the added amount of cations. Other conditions were the same as in Figure 2.

state of chromatin (11). Since ATA combines with Ca^{++} and Mg^{++} (12, 13), it is quite possible that the swelling process is a result of the deprivation of these cations by ATA. The primary action of ATA does not seem to be based on its chelating potency, however, because if it were, the inhibition by Ca^{++} and Mg^{++} would be competitive. Moreover, EDTA can induce the nuclear swelling; however, when ATA was added to nuclei maximally swollen by EDTA, additional swelling was induced. Reversing the order of addition gave the same result (data not shown).

Release of proteins from ATA-treated chromatin: Isolated chromatin was treated with various concentrations of ATA and centrifuged. Table 2 shows the amount of protein and DNA released, as well as the DNA/protein ratio. The amount of protein released increased proportionately to the ATA concentration. The almost constant and low value of DNA/protein ratio indicates that the proteins in the supernatant were those dissociated from chromatin by ATA, not those from nonsedimenting chromatin. SDS-gel electrophoresis of the released proteins is shown in Figure 4. Proteins seen both in the control gel and other

Treatment	Protein released (% total)	DNA released (% total)	$\frac{\text{DNA}}{\text{Protein}}$
Control, no ATA	3.9	0.7	0.08
ATA, 0.025 mM	5.6	1.1	0.08
ATA, 0.2 mM	13.6	2.0	0.07
ATA, 0.7 mM	13.2	2.2	0.07
ATA, 2.0 mM	14.4	3.2	0.10
EDTA, 5.0 mM	3.3	0	0

TABLE 2. Release of protein from ATA-treated chromatin. Rat liver nuclei were washed several times with 0.075 M NaCl, 0.024 M EDTA, washed twice with 10 mM Tris-HCl pH 8.0, then dispersed in deionized water. DNA/protein ratio of this chromatin preparation was 1 : 2.3. ATA and EDTA in the indicated concentrations were added to tubes containing 2 ml of chromatin (0.62 mg DNA, 1.42 mg protein) in 5 mM Tris-HCl pH 7.9. The tubes were incubated at 30°C for 5 min, cooled to 0°C, and spun at 35,000 rpm in a Hitachi rotor RP 65TA for 15 hr. The supernatant was transferred and the protein and DNA amount was determined.

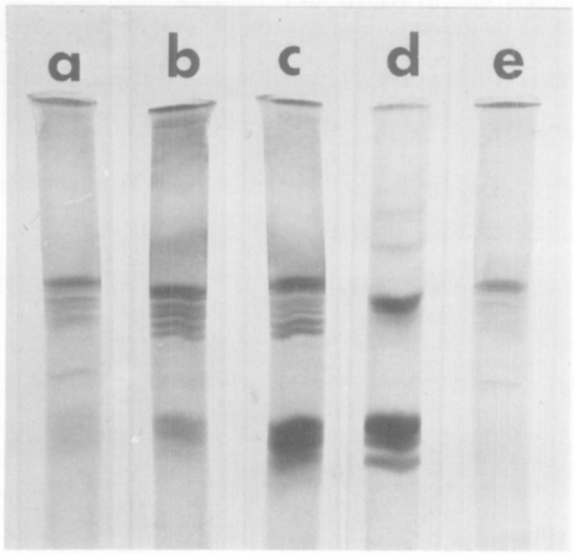

FIGURE 4. SDS-gel electrophoresis of released protein. Supernatants (see Table 2) were dialysed overnight against 1 % SDS, 1 % 2-mercaptoethanol, 0.01 M Tris-acetate buffer pH 8.4 and analysed by 10 % polyacrylamide gel electrophoresis in the presence of 0.1 % SDS. The treatment of loaded samples were (a) no treatment (control) (b) ATA, 0.2 mM (c) ATA, 2.0 mM (d) calf thymus histone, 20 μg, as a reference (e) EDTA, 5.0 mM. The same volumes (0.2 ml) were applied to all columns except (d).

gels seem to be nonhistone proteins released during incubation and not related to ATA action. Most of the proteins released by ATA are histones. An ATA concentration higher than 0.2 mM is required to dissociate histone f2a-1. There seems to be no order of preference for extraction among the other histones. If it is assumed that all of the released proteins were histones, one may calculate that 30 % of the histone in the original chromatin was released. It is not clear whether the rest of the histones remain bound with DNA or form a sedimentable aggregate. No histone proteins were released by EDTA, as shown in Table 2.

Binding of ATA to histone: When calf thymus histone (0.8 mg/ml) was incubated with 1.5 mM ATA at 20°C for 4 hr, in the presence of 0.15 M NaCl and 10 mM Tris-HCl pH 7.9, most histone was precipitated with ATA. This colored aggregate was retained on a Whatman GF/C filter and the ATA bound to the

histone measured 0.73 μmole/mg. The nature of ATA-histone complex is under investigation in this laboratory. According to our unpublished data, ATA does not bind with DNA.

As compared with a polymeric polyanion, such as polystyrene sulfonate, ATA could be referred to as a monomeric or small molecular polyanion. Two or three carboxyl groups of ATA molecule are dissociable in a neutral solution. Nuclear swelling induced by ATA is apparently similar to that induced by polymeric polyanions but differs in that a molecular weight higher than 5,000 to 6,000 is necessary for the action of polymeric types of polyanion (14). Some difference in the mechanism of action between these two types of polyanion may exist. Eriochrome cyanine R, which has a similar structure to ATA and the capacity to chelate aluminum, failed to swell nuclei. This suggests that charge distance and/or steric factor are important for the action. The effect of ATA on nucleic acid synthesis of eukaryotic cells will be published elsewhere.

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