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Effects of fixatives on high order chromatin structure: a calorimetric study

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Abstract

Differential scanning calorimetry has been carried out on native calf thymus cells in order to investigate, by a non-invasive tool, the effects of the most common fixatives at the level of higher order chromatin structure.

Therefore the effects of 2% glutaraldehyde in saline buffer and ethanol acetic acid 3:1 v/v mixture have been analysed: the first one is known to induce cross-linking between the various cell constituents, and the second one induces dehydration.

Looking at the thermal profiles the disappearance of all four transitions and the appearance of a new single broad transition at 390 K are the most remarkable effects following the glutaraldehyde fixation of thymocytes. In contrast ethanol/acetic acid causes a reduction of the protein melting at 345 K, a loss of the 375 K transition and a shift of the 360 K transition. These data are explained in terms of opposite dramatic chromatin structure changes.

INTRODUCTION

Glutaraldehyde and ethanol are the most used fixatives in cytological studies and the mechanisms of their action at a chemical level are well known. Glutaraldehyde actually is known to bring about a cross-linking of some cell constituents (proteins in particular) by reaction with their aminic and hydroxyl groups. Ethanol instead gives rise to water removal from the cells: the resulting cellular coarctation is partially offset using a mixture of ethanol and acetic acid in the proportion 3:1 v/v. Although the effects of these fixatives at the level of supermolecular complexes (chromatin) have been studied for a long time [1–3], a complete understanding is still lacking.

Previous papers [4–7] have shown the existence of at least four thermal transitions in nuclei and cells isolated from rat liver. These transitions have been related to the denaturation of different macromolecules (namely

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proteins, DNA and RNA). In particular the 345 K transition has been related to protein melting, the 365 K transition to DNA organized in a nucleosomal filament (tertiary structure) and the 375 K transition to DNA organized in a higher order structure (quaternary structure) [10,11].

For this reason DSC has been used as a non-invasive biophysical tool to investigate the structural changes induced by the different fixatives on the higher order chromatin structure.

MATERIALS AND METHODS

Cell isolation

Intact cells were isolated from calf thymus by the following procedure: small pieces of thymus were homogenized in homogenization buffer (0.8% NaCl dissolved in 0.1 M tris-HCl pH 7.2) and filtered through a steel mesh. After the first centrifugation at $300 \times g$ for 4 min the resulting pellet was washed in the same buffer and then in Dulbecco's phosphate buffered saline (PBS) to which 2 mM EDTA and 2 mM $MgCl_2$ had been added. All these procedures were of course carried out at 4°C to preserve the cellular organization.

Cell fixation

After washing in PBS the cellular pellet was fixed overnight in fixation solutions (2% glutaraldehyde or 3:1 v/v ethanol:acetic acid) and then it was centrifuged at $300 \times g$ for 4 min and repeatedly washed in PBS buffer.

Prior to the calorimetric measurement the sample was further centrifuged (30 min at $9000 \times g$) in order to reach an optimal consistency (namely a larger sample concentration) to yield a good signal-to-noise ratio.

Differential scanning calorimetry

DSC experiments were performed on a Perkin-Elmer DSC-2C instrument with 75 μl aluminium capsules, in a temperature range between 310 and 410 K as previously described [4].

In order to improve the reproducibility and sensitivity of measurements, instead of a standard data recovery signal (i.e. a chart recorder) an electronically interfaced computerized system was used, offering greater possibilities of acquisition, background subtraction and data display [8].

In order to recover even very weak signals, the thermal scanning of the sample was performed at a low rate (5 K min^{-1}) high sensitivity (0.1 mcal s^{-1}) and high sample size (about 60 mg per capsule). The corresponding baselines were obtained by a new thermal scanning of the denatured sample and were subtracted from the corresponding thermograms.

Total proteins / DNA determination

After the thermal scanning for every capsule, the total amounts of DNA and proteins were calculated by spectrophotometric methods: the Monroe method was used for DNA and the Hartree method for proteins.

RESULTS AND DISCUSSION

Figure 1 shows the calorimetric profile of unfixed thymocytes: the four transitions described above for the hepatocytes are also evident for these cells. When we compare the thermal profiles of calf thymocytes and the nuclei from G0 rat hepatocytes, an enhancement of the 375 K transition with respect to the 365 K one should be noted in agreement with the more closely packed genome characteristic of the thymus cells.

In Fig. 2 the calorimetric profile of ethanol/acetic acid fixed thymocytes is shown. When we compare this thermogram with that of the unfixed thymocytes, the dramatic disappearance of the 375 K transition is the most remarkable effect along with a shift of the 360 K transition to a lower temperature (358 K) and a reduction of the 345 K transition. The first effect can be explained by a loss of chromatin higher order structure following ethanol/acetic acid fixation, whereas the second one reflects a reduced melting enthalpy at 345 K related to chromosomal protein removal after ethanol/acetic acid fixation (see also Table 1). These results match the well known effects of this fixative on proteins (precipitation) and DNA (denaturation).

Figure 3 shows the calorimetric profile of 2% glutaraldehyde fixed thymocytes: the disappearance of both the transitions at 365 and 375 K is

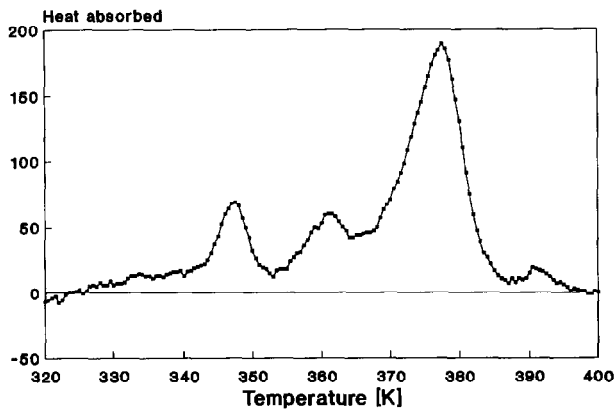

Fig. 1. Heat capacity versus temperature profiles of unfixed calf thymocytes which have been isolated as described in the section Materials and Methods.

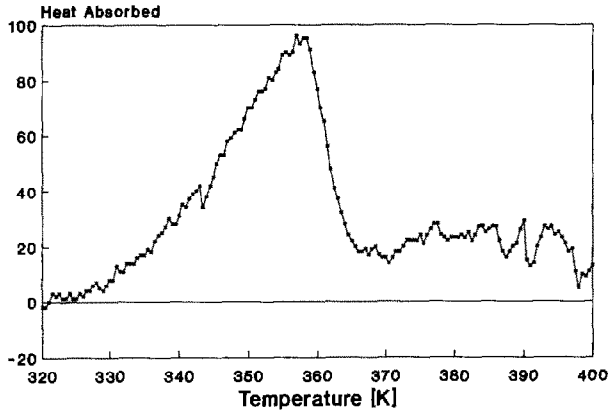

Fig. 2. Effect of ethanol/acetic acid (3:1 v/v) fixation on heat capacity profiles of calf thymocytes.

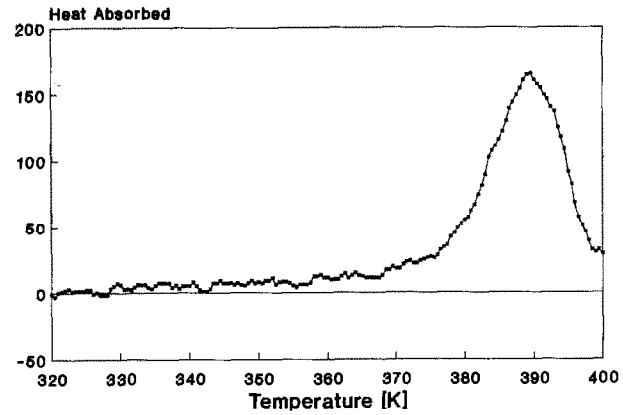

Fig. 3. Effect of 2% glutaraldehyde fixation on heat capacity profiles of calf thymocytes.

accompained by the appearance of a new single broad transition at 390 K. No other transition becomes apparent upon extending the thermal scanning up to 510 K (data not shown). This effect can be ascribed to a

TABLE 1

Ratio between protein and DNA measured for each sample after thermal scanning as described in the section Materials and Methods

Thymocytes	Protein/DNA ratio
Unfixed	3.1
Ethanol/acetic acid fixed	1.7
Glutaraldehyde fixed	3.0

different level of chromatin superfolding appearing after glutaraldehyde fixation and giving rise to a new kind of superstructure. Alternatively glutaraldehyde could cause the lock-in of chromatin–DNA in the native tertiary and quaternary structure, with a subsequent shift towards higher melting temperature. The above results are compatible with recent observations recently obtained on protein solutions after fixation [9].

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