PRESERVATION OF DNA STRUCTURE AND CONTENT IN CELL NUCLEI DURING ACID HYDROLYSIS

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Although the Feulgen reaction has been in use for a long time its mechanisms are still largely unexplained [3]. Investigations conducted in the 1970s shed light on some aspects of the kinetics of liberation of aldehyde groups binding Schiff's reagent during acid hydrolysis of DNA [7, 10]. On this basis was formulated the concepts of "chain with stable structure" [11], according to which, as a result of treatment with acid, not only are purine groups detached with liberation of reactable aldehyde groups in the DNA chain, but progressive rupture of sugar-phosphate bonds also takes place, as a result of which shorter and shorter DNA fragments (apurinic acid) are formed and are liberated into the hydrolyzing solution, taking with them some of the liberated or potential aldehyde groups. During cytophotometric determination of DNA in a preparation stained by the Feulgen method its content is thus found to be 10-15% less than in the native preparation [5].

It thus becomes clear that to preserve the largest number of aldehyde groups in apurinic acid the extraction of its depolymerized fragments must be prevented or delayed. This can be done in two ways: either by strengthening (recreating) the bonds between DNA and protein or by creating conditions in the hydrolyzing solution preventing the release of depolymerized apurinic acid fragments into it.

One method of creating additional DNA—protein bonds is by treatment with low concentrations of formalin [9]. To prevent the release of depolymerized DNA fragments into the hydrolyzing solution it has been suggested that the "excluded volume" method [12] be used: high-molecular-weight polymers of polyethylene glycol (PEG) or polyvinyl alcohol are added to the acid solution. The polymer molecules form a net in the solution through which small molecules of detached purines can pass, but the large DNA fragments are held up.

The object of the present investigation was accordingly to verify the above-mentioned methods of fixation of material and the conditions for conduct of acid hydrolysis for quantitative analysis of DNA in nuclei of various types of cells.

EXPERIMENTAL METHOD

Chicken erythrocytes and human leukocytes were used. The films were prepared as described previously [1, 2], and fixed in 96 and 100% ethanol, in Carnoy's fixative, and in FEA (formalin—ethanol—glacial acetic acid, 9:3:1), MFA (methanol—formalin—glacial acetic acid, 17:2:1), and EAF (ethanol—glacial acetic acid—formalin) mixtures. The last fixative was made up on the basis of data showing that treatment of DNP *in vitro* with 2% formalin solution leads to binding of 100% of DNA with histones by cross linkages [6]. To make up the EAF, 2 ml of undiluted formalin was added to 98 ml of a mixture of 96% ethanol and glacial acetic acid (3:1). The fixation time varied from 15 to 150 min. Hydrolysis was carried out in 5 N HCl at 22°C (erythrocytes) and at 37°C (leukocytes) for 1-150 min. For some types of fixation, PEG with a molecular weight of 1500 or 6000 daltons was added to the hydrolyzing solution in a final concentration of 20%. The films were stained with Schiff's reagent (pH 2.2; Diamant fuchsin for chicken erythrocytes, and pH 1.6, Reanal fuchsin for human

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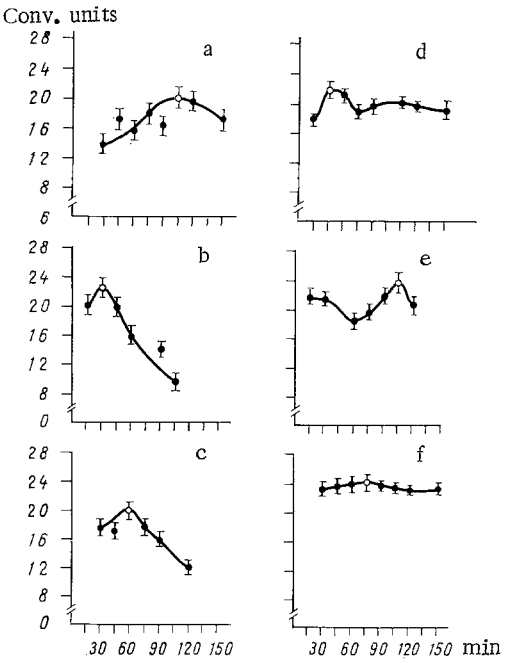

Fig. 1. Effect of different fixatives and fixation times on DNA–fuchsin content in chicken erythrocytes. a) 96% ethanol; b) 100% ethanol; c) Carnoy's fixative; d) FEA; e) MFA; f) EAF. Empty circles denotes times when additional treatment was carried out. Abscissa, time of fixation of films (in min); ordinate, DNA–fuchsin content at point of optimal hydrolysis (in conventional units).

leukocytes) and mounted in Canada balsam. Photometry of the DNA–fuchsin complex in the nuclei was carried out on an SIM-1 scanning microscope-photometer [4] and on an Opton SMP05 microspectrophotometer, with subsequent data processing on the Wang 720c computer at wavelengths of 546 nm (leukocytes) and 555 nm (erythrocytes). On the basis of the data of these scans a histogram of distribution of the quantity of DNA–fuchsin by optical densities was drawn for each measured object, so that the "DNA loss" in regions with different chromatin packing density could be objectively assessed.

EXPERIMENTAL RESULTS

1. Effect of Different Fixatives on DNP Structure and DNA–Fuchsin Content. Data showing changes in the intensity of binding of Schiff's reagent with DNA of chicken erythrocyte nuclei depending on the duration of action of the different fixatives are given in Fig. 1. The highest intensity of staining and, consequently, the lowest degree of extraction of depolymerized DNA fragments was observed when EAF was used. The stabilizing action of this fixative is also well illustrated by curves of distribution of the measured points with respect to passage through and to the quantity of DNA–fuchsin (Fig. 2). Comparison of the distribution shows that fixation in 100% ethanol and in Carnoy's fluid shifts the histograms into the region of higher values for passage through the net, a decrease in the content of DNA–fuchsin, and a decrease in the fraction of compact chromatin. The erythrocyte nuclei became much smaller, as a result of which an increase in the fraction of optically dense regions of chromatin might be expected. In fact, however, the opposite effect was observed.

The action of 96% ethanol was rather less rigorous, but fixatives containing formalin were much to be preferred, the best of which was EAF. The distinguishing features of its action were independent of fixation time (for other fixatives the optimal duration of fixation of the films had to be selected; Fig. 1) and good preservation of the native form of the nuclei, structure of the chromatin, and content of DNA–fuchsin.

Similar results also were obtained when human leukocytes were tested. Analysis of the DNA hydrolysis curves for these cells in the case of fixation with 96% ethanol (60 min) and EAF (90 min) showed that fixation with EAF not only increased the DNA–fuchsin content at the

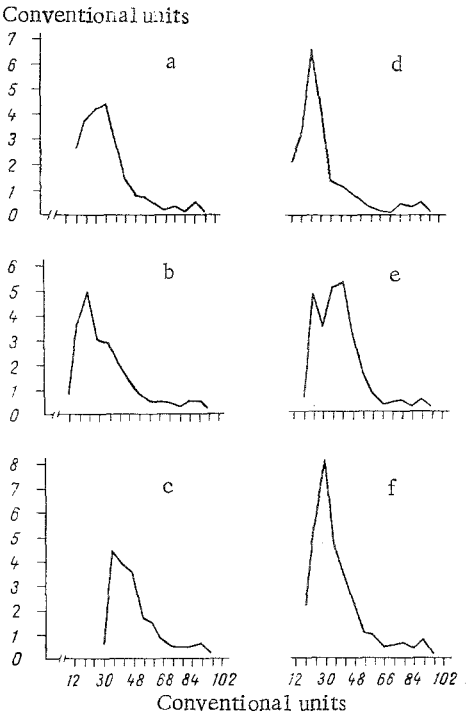

Fig. 2. Distribution of data obtained by scanning chicken erythrocyte nuclei with respect to passage through the molecular sieve (abscissa, conventional units) and to DNA–fuchsin content in each passage class (ordinate, convensional units). Abscissa, passage of DNA–fuchsin (in conventional units); ordinate, DNA–fuchsin content in each passage class (in conventional units). Remainder of legend as to Fig. 1.

optimal hydrolysis time, but also elevated the "plateau" of the hydrolysis curve (Fig. 3). The rather slower rate of depurinization of DNA in the case of fixation with EAF will also be noted, for flattening out of the hydrolysis curve on the plateau was delayed by 4-6 min. The course of the curves for nuclei with diffuse chromatin (monocytes) and with compact chromatin (lymphocytes) virtually coincided for the two types of fixation.

2. Effect of PEG on Course of DNA Hydrolysis. Comparison of hydrolysis curves for human leukocytes fixed with 96% ethanol shows that addition of PEG to the hydrolyzing solution gave effects of three types (Fig. 3b). First, the content of the DNA–fuchsin complex at the end of the first minute of hydrolysis was increased by 60% for PEG 1500 and by 32% for PEG 6000 compared with the control. These results confirm the presence of partly depolymerized DNA, arising through the action of the fixatives and quickly extracted during the first minutes of hydrolysis [7, 8]. Second, the maximal DNA–fuchsin content at the peak of the hydrolysis curve was increased (by 25% after addition of PEG 1500 and by 5% after addition of PEG 6000). It is interesting to note that in a previous investigation of leukocytes the writers obtained a 42% increase in the DNA–fuchsin content at the peak of the hydrolysis curve after addition of PEG 6000 to the hydrolyzing solution [2]. In that case 96% ethanol also was used for fixation, but only for 30 min and not 60 min as in the present study. This difference is in good agreement with the relationship mentioned above between the intensity of staining and the fixation time (Fig. 1a): during a short period of fixation with 96% ethanol considerable destabilization of DNP probably takes place. Third, addition of PEG lengthened the "plateau" of the hydrolysis curve, and this effect was most marked when PEG 6000 was used. In the case of fixation with EAF addition of PEG 6000 to the hydrolyzing solution had practically no effect on the content of stained aldehyde groups either at the beginning of the curve or at its peak; the duration of the "plateau" under these circumstances likewise was unchanged. This is further evidence that detection of aldehyde groups in DNA is most complete after fixation with EAF.

The best fixative for use in the Feulgen reaction from the point of view of preservation of structure and integrity of chromatin and also of preventing depolymerization and extraction

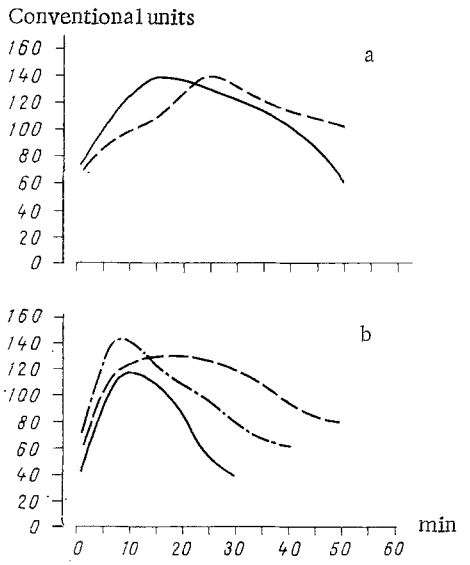

Fig. 3. DNA hydrolysis curves for human leukocytes in control (continuous lines) and after addition of 20% PEG 6000 (broken lines) and PEG 1500 (line of dots and dashes) to hydrolyzing solution. a) Fixation with EAF, 90 min; b) with 96% ethanol, 60 min. Abscissa, hydrolysis time (in min); ordinate, DNA–fuchsin content (in conventional units).

of DNA is thus EAF. When alcohol fixation is used, 20% PEG must be added to the hydrolyzing solution to reduce extraction of apurinic acid fragments formed as a result of depolymerization in the course of fixation and actual hydrolysis.

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