INCORPORATION OF NUCLEOTIDES INTO NUCLEI OF FIXED CELLS BY DNA POLYMERASE

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ABSTRACT

The enzyme calf thymus polymerase requires denatured or single-stranded DNA as a primer for DNA synthesis and is inactive on native DNA preparations. The enzyme and tritium-labeled deoxyribonucleoside triphosphates were incubated with alcohol-fixed and Carnoy-fixed tissue preparations to see if primer DNA could be found in several types of cells undergoing DNA synthesis. In all cases, low-pH controls were prepared for comparison. Priming activity was not found in nuclei that had been fixed in alcohol. Priming activity was found in cell nuclei that had been fixed with an acid fixative or had been treated at a low pH prior to treatment with the enzyme reaction mixture.

INTRODUCTION

The enzyme calf thymus DNA polymerase requires denatured or single-stranded DNA as a primer for DNA synthesis and is inactive on native DNA preparations (1). We have used this property to examine the cytological location of priming and/or nonpriming DNA by allowing the enzyme and tritiated substrates to react with suitably fixed preparations prior to radioautography.

MATERIALS AND METHODS

Tissues used in this study were isolated nuclei or squashed preparations of the ciliate protozoan *Euplotes*, HeLa cells grown in culture, squashed preparations of the alga *Nitella*, and smeared preparations of mouse blood. The cells were fixed in (*a*) modified Carnoy's fluid (three parts glacial acetic acid and two parts 35% alcohol), (*b*) in the case of the blood smear, dried prior to fixation in alcohol, or preferably (*c*) 70% alcohol.

The preparations were routinely placed in absolute alcohol after fixation and taken through an alcohol series to water. One-half of the preparations were treated with 0.01 N HCl for 30 min at room temperature to denature the DNA.

The cells were taken back through the alcohol series and dried. Small glass rings were placed around the cells on the slide and held in place with paraffin to provide a well. One-tenth of a milliliter of a reaction mixture, containing 20 μg calf thymus DNA polymerase (2), 0.1 mM deoxyribonucleoside triphosphates of cytosine, thymine, adenine and guanine, 40 mM potassium phosphate buffer, pH 7.0, and 8 mM $MgCl_2$, was placed on the tissue in the well. The well was covered to prevent evaporation. The deoxycytidine triphosphate was labeled with tritium (34 $\mu c/\mu M$) in these experiments. The reaction mixture was incubated with the cells for 2 to 2½ hr at 37°C.

The enzyme and substrate were then rinsed away with 0.9% NaCl, and the slides were processed through 5% trichloroacetic acid, soaked in cold 5% trichloroacetic acid for 30 min, and then placed in 70% alcohol for 16 hr at 0°C.

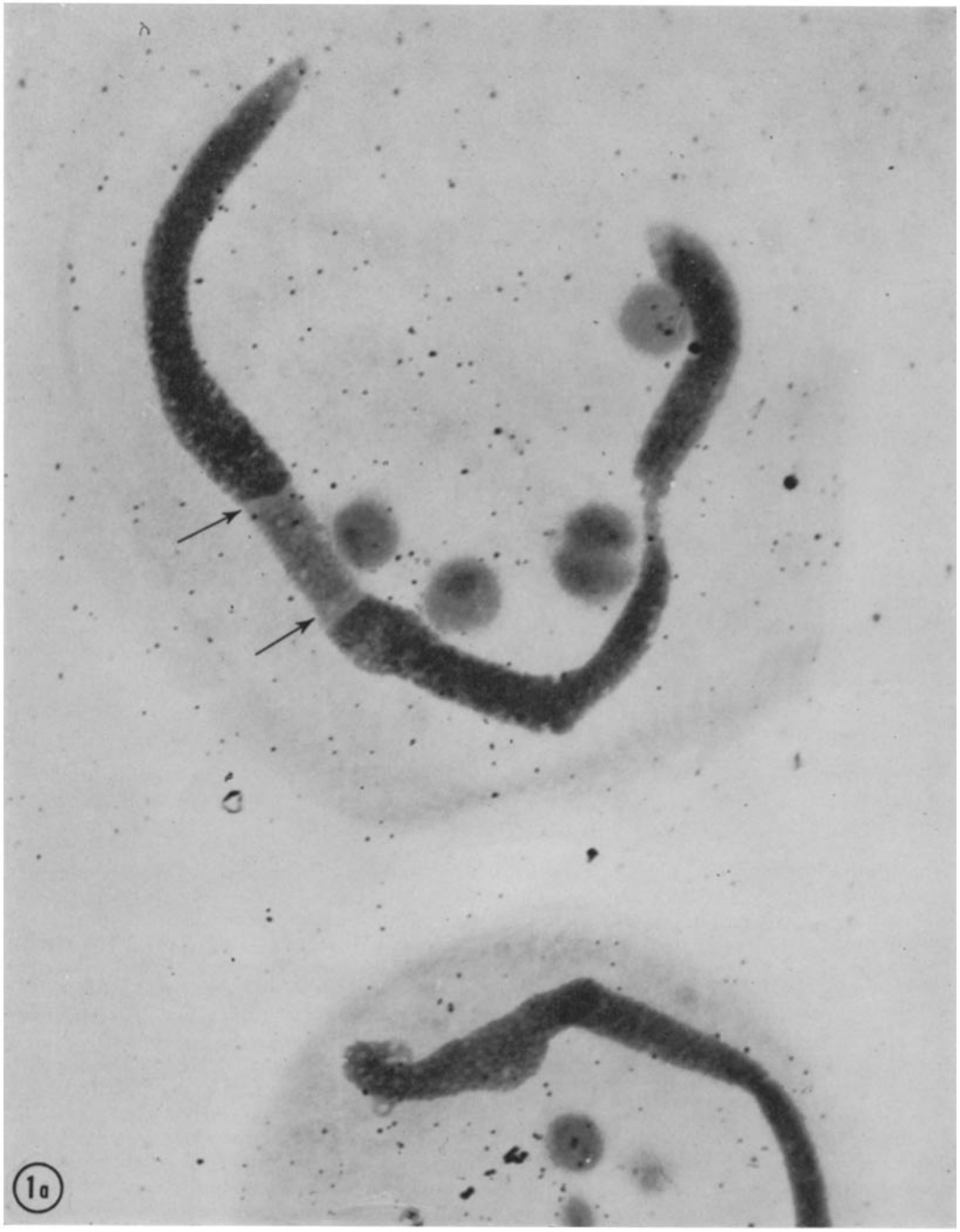

FIGURE 1 *a* Alcohol squash preparation of *Euplotes* having no acid treatment prior to incubation with enzyme reaction mixture. Replication of the DNA proceeds from both ends of the nucleus toward the center at the replication bands (arrows). × ca. 600.

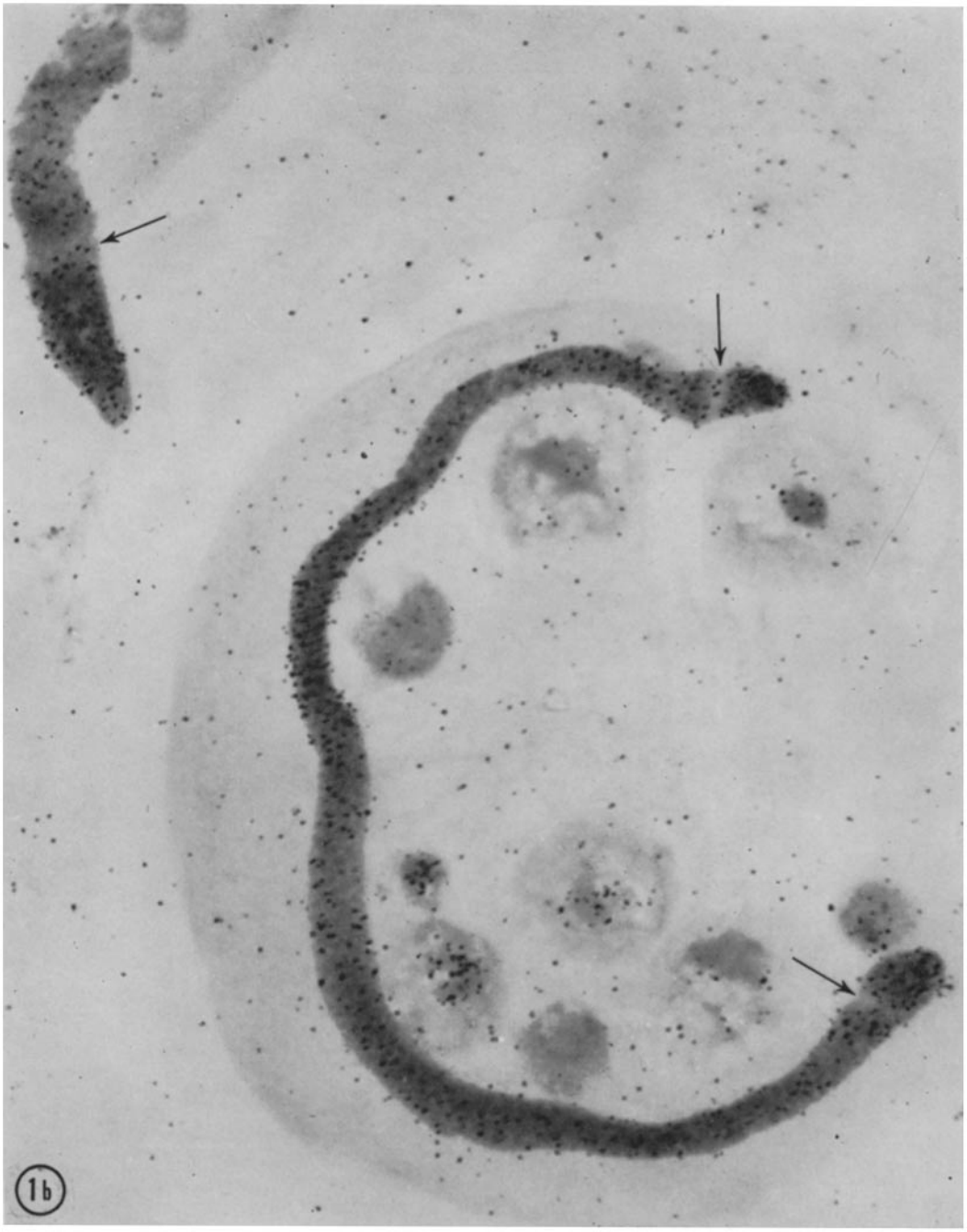

FIGURE 1 *b* Alcohol squash preparation of *Euplotes* denatured in 0.01 N HCl prior to treatment with enzyme reaction mixture. The other nuclei present in the *Euplotes* are of *Tetrahymena* that were fed to the *Euplotes*. × ca. 600.

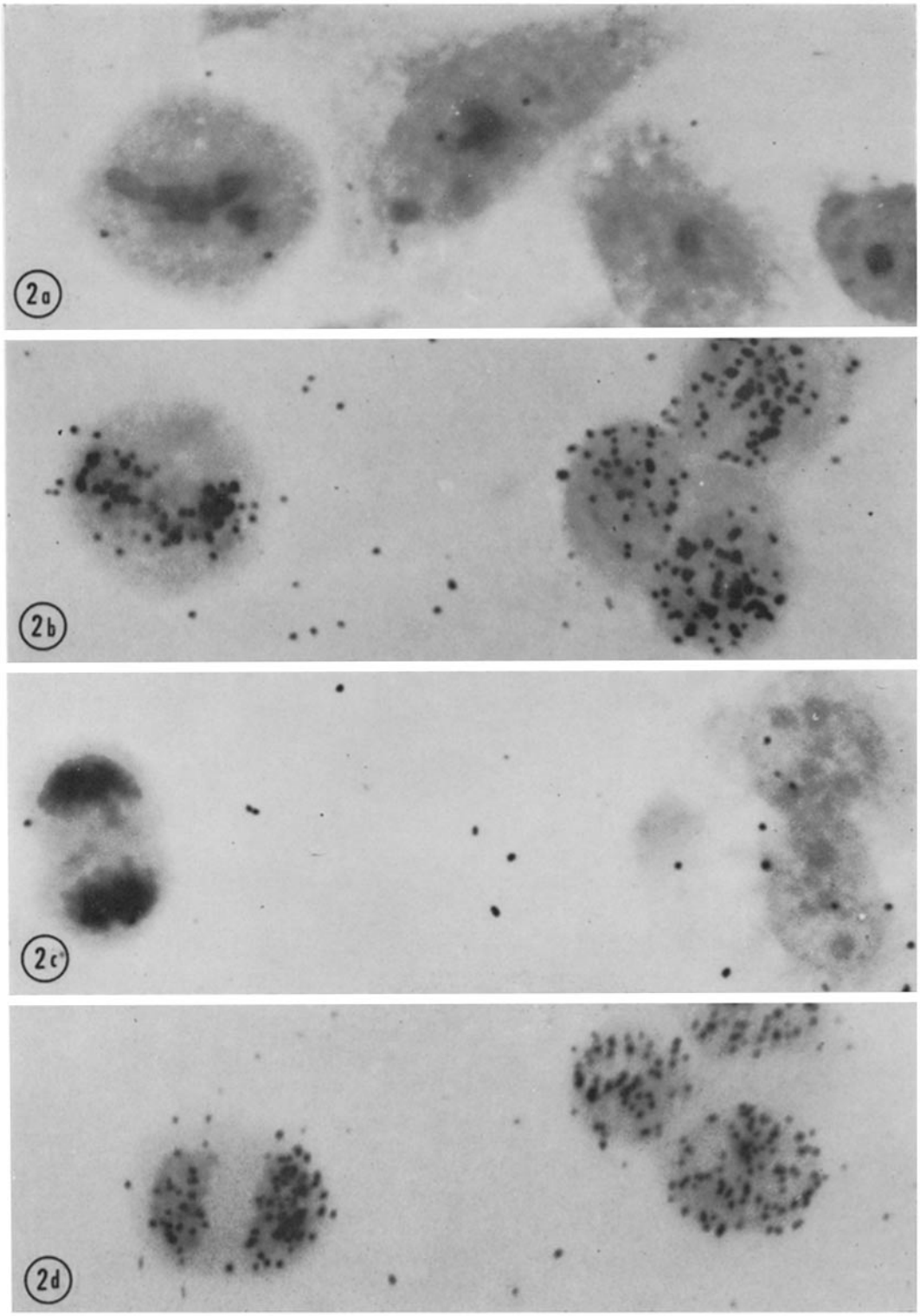

FIGURE 2 HeLa strain cells fixed in ethyl alcohol and exposed to DNA polymerase reaction mixture. Fig. 2 *a*, Without prior denaturation in HCl. Note metaphase figure. Fig. 2 *b*, After denaturation in HCl. Note metaphase figure. Fig. 2 *c*, Without prior denaturation in HCl. Note anaphase figure. Fig. 2 *d*, After denaturation in HCl. Note anaphase figure. × ca. 1000.

The slides were then placed for a few minutes in 95% alcohol, absolute alcohol, and then in xylene to dissociate the glass ring. After the paraffin had dissolved, the slides were placed again in absolute alcohol and air-dried.

The slides were dipped in emulsion (Kodak NTB2) and stored in sealed boxes at room temperature, with no especial precautions for humidity. Test slides were developed at weekly intervals and the tissues examined.

RESULTS

Euplotes

The macronucleus of *Euplotes* has morphologically distinct bands that make it possible to define microscopically the exact area of DNA synthesis (3, 7). DNA synthesis proceeds from the two ends of the nucleus toward the center. The regions of active DNA synthesis can be seen in Fig. 1 as light bands (arrows). In alcohol squashes, nucleotides were not incorporated into any region of the *Euplotes* macronucleus (Fig. 1 *a*) unless the alcohol squashes were placed in 0.01 N HCl prior to treatment with the enzyme reaction mixture (Fig. 1 *b*). It was not possible to observe incorporation of nucleotides into the band region prior to acid denaturation, although DNA synthesis was in progress at the time of fixation. *Tetrahymena* was regularly fed to the *Euplotes*. *Tetrahymena* nuclei can be seen inside the *Euplotes* in Fig. 1 *a* and *b*. The

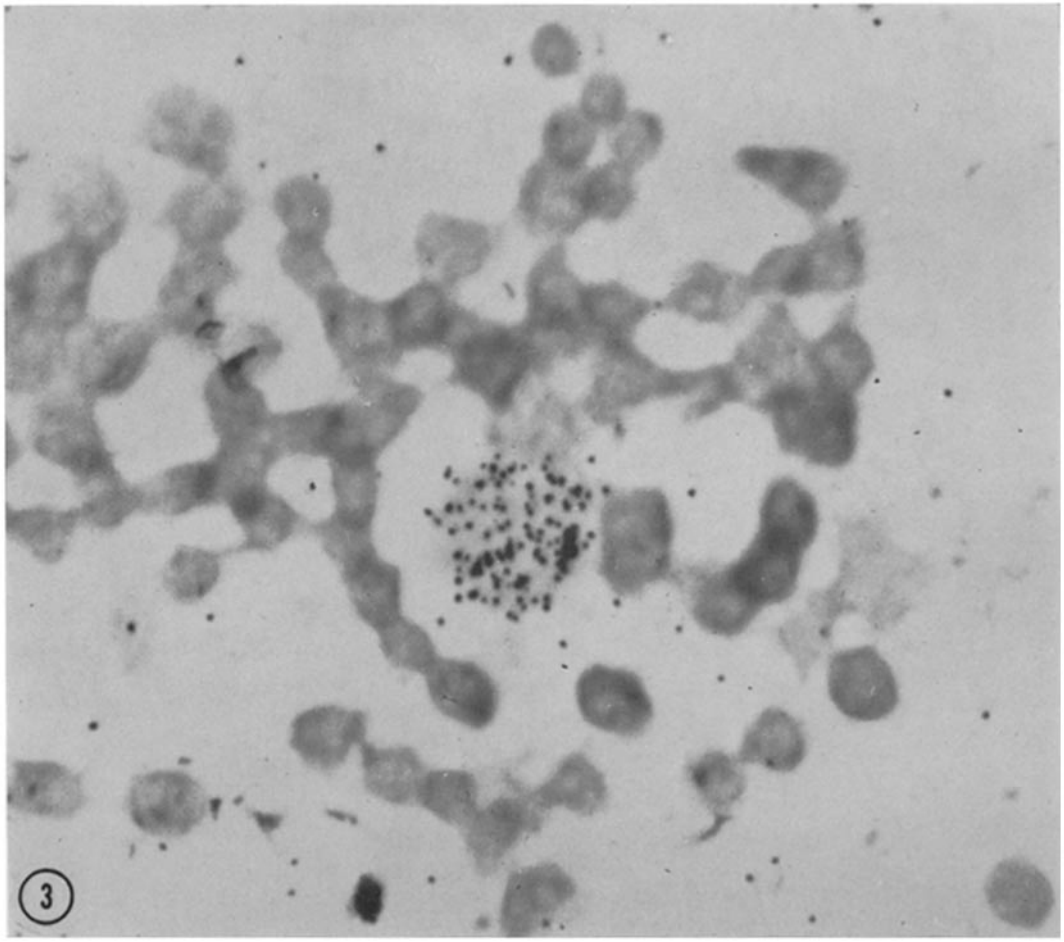

FIGURE 3 Squash preparation of *Nitella* treated with acid prior to incubation with the enzyme reaction mixture. The nucleus is labeled, the chloroplasts are not. × ca. 1000.

Euplotes cytoplasm had not denatured the *Tetrahymena* DNA. When *Euplotes* were fixed in the modified Carnoy's solution, nucleotides were incorporated without further acid denaturation.

HeLa Cells

Nuclei of actively growing HeLa strain cells fixed in alcohol show no incorporation of nucleotides (Fig. 2 *a* and *c*). When treated with acid before being placed in the reaction mixture, the cell nuclei do incorporate nucleotides (Fig. 2*b* and *d*). Chromosomes of cells in metaphase and anaphase also incorporate the nucleotides after denaturation.

Nitella

A squashed preparation of an algal cell, *Nitella*, is shown in Fig. 3. It was treated with acid prior to

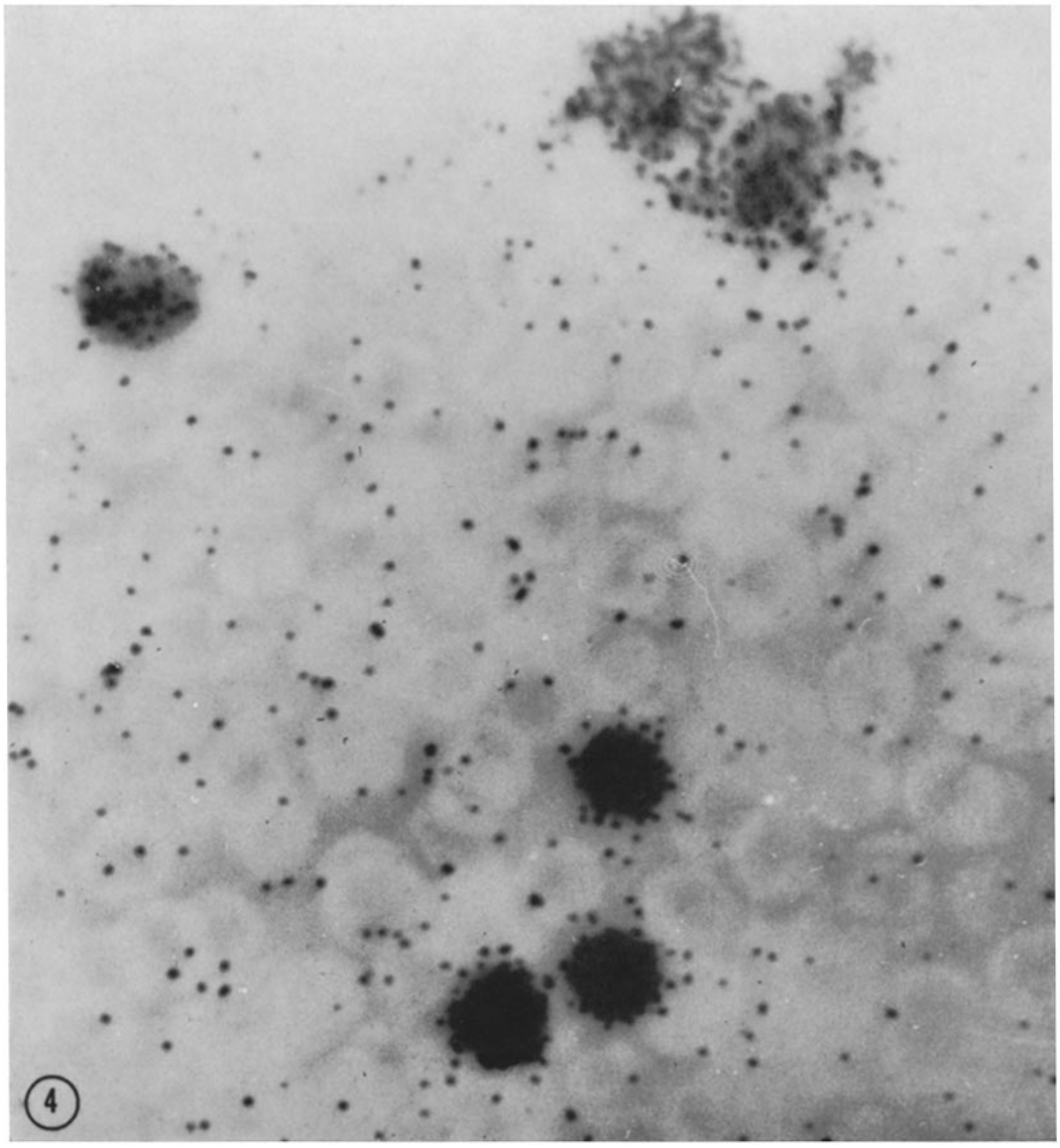

FIGURE 4 Smeared mouse blood preparation treated with acid prior to incubation with enzyme reaction mixture. The white blood cells are labeled, the red blood cells are not. × ca. 1000.

incubation with the enzyme reaction mixture. It is clear that denatured DNA is present in the nucleus. It has been claimed that some chloroplasts contain DNA (Iwamura, reference 4; cf. Prescott, reference 6), but none was detectable in chloroplasts of this organism by this method. Perhaps precursors of higher specific activity would allow detection of chloroplast DNA.

Mouse Blood

Mouse blood was smeared and dried before fixation in alcohol. As can be seen from Fig. 4, acid treatment denatures DNA in white blood cells. The red blood cells can be seen in the figure as light circular structures.

DISCUSSION

The method described appears to be a sensitive means for detection of single-stranded or denatured DNA in situ in fixed cells and tissues. The nature of the test requires that care be taken with tissue preparation to avoid denaturation of native DNA prior to assay. In particular, acid fixatives should be avoided if one is looking for single-stranded DNA. When visualization of total DNA is required, the acid treatment (e.g., 0.01 N HCl) or acid fixation should be used. Occasionally it was observed that, when tissues were fixed by drying, the DNA behaved as if denatured. We presume that this is an artifact caused by deoxyribonuclease action during drying and complete fixation.

When the tissue preparations are treated with acid prior to treatment with the enzyme reaction mixture, then the technique is an accurate means of detection of DNA that was formerly double-stranded; that is, total DNA.

Using this procedure we were unable to demonstrate the presence of single-stranded primer DNA in nuclei of *Euplotes*, where DNA is known to be synthesized in the replication bands. Tests were also made on specimens digested with trypsin, ribonuclease, and pepsin, and single-stranded primer DNA was never detected without DNA denaturation.

Also, it was not possible to demonstrate primer in growing HeLa cells fixed in alcohol. In this tissue the period of synthesis of DNA lasts about 40% of the entire cycle in HeLa cells, and roughly 40% of the cells in Fig. 2 could be expected to be engaged in DNA synthesis. If single-stranded DNA exists in these experiments, it is clear that we will need to increase the sensitivity by having high specific activity in all four deoxyribonucleoside triphosphates.

We estimate that if 5% of the total DNA had been in a single-stranded condition the cells synthesizing DNA could have exhibited the presence of the primer. This estimate represents a maximum sensitivity of the method, based on an overestimate of the amount of DNA denatured by acid treatment. If it is assumed that 100 % of the DNA in these cell nuclei is denatured by acid treatment and not renatured, and that the HeLa cells have approximately 100 grains above each nucleus, then 5 grains above 40 % of the nuclei in the alcohol-fixed preparations would have raised the radioactivity above the background level. The potential sensitivity of the method may be better than stated, because 0.01 N HCl probably does not denature all the DNA in a tissue preparation, and proximity of denatured polynucleotide strands in a fixed preparation may allow extensive renaturation.

Finally, the denaturation of DNA by acid fixation probably explains many of the difficulties experienced by histologists who use differential staining techniques employing several different dyes for detection of RNA and DNA. Single-stranded DNA fluoresces like RNA after acridine orange treatment (5) and stains like RNA after methyl green and pyronin treatment (von Borstel, unpublished). If pH of fixation is improperly controlled, stainability of nuclei and cytoplasms may vary from cell to cell, tissue to tissue, and batch to batch.

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REFERENCES

1. BOLLUM, F. J., Thermal conversion of nonpriming deoxyribonucleic acid to primer, *J. Biol. Chem.*, 1959, **234**, 2733.
2. BOLLUM, F. J., Calf thymus polymerase, *J. Biol. Chem.*, 1960, **235**, 2399.
3. GALL, J. G., Macronuclear duplication in the ciliated protozoan *Euplotes*, *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 295.
4. IWAMURA, T., Distribution of nucleic acids among

subcellular fractions of *Chlorella*, *Biochim. et Biophysica Acta*, 1960, **42**, 161.

5. MAYOR, H. D., and HILL, N. O., Acridine orange staining of a single-stranded DNA bacteriophage, *Virology*, 1961, **14**, 264.
6. PRESCOTT, D. M., Cellular sites of RNA synthesis, *Progr. Nucleic Acid Research and Mol. Biol.*, 1964, **3**, 33.
7. PRESCOTT, D. M., and KIMBALL, R. F., Relation between RNA, DNA, and protein synthesis in the replicating nucleus of *Euplotes*, *Proc. Nat. Acad. Sc.*, 1961, **47**, 686.