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## The influence of hydroxyurea and colchicine on growth and morphology of *Trypanosoma cruzi*

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### Summary

Cultures of *Trypanosoma cruzi* have been exposed to the drugs hydroxyurea and colchicine. We found that hydroxyurea (200  $\mu\text{g/ml}$ ) completely inhibited growth and differentiation of *T. cruzi* Y strain. Colchicine (200  $\mu\text{g/ml}$ ) reduced the growth of *T. cruzi* 30% and stimulated cell differentiation from epimastigotes to trypomastigotes. Furthermore it caused anuclear cells with apparently intact kinetoplasts. The possible use of these anuclear forms in studies on kinetoplast DNA organization and expression is suggested.

*Key words:* *Trypanosoma cruzi*; hydroxyurea; colchicine; differentiation; anuclear cells.

### Introduction

The characteristic feature of the order Kinetoplastida is the presence of a large amount of extranuclear DNA concentrated in the kinetoplast (Alexeieff, 1917). In these cells the kinetoplast DNA (k-DNA) comprises up to 20–25% of the total DNA (Simpson and da Silva, 1971). At present little is known about the role of k-DNA and total DNA in the life cycle of the members of Kinetoplastida.

One approach to elucidate their function is the application of drugs that selectively act on one or the other genome or influence the orderly redistribution of the genomes during cell division and the subsequent morphological and biochemical studies of the alterations produced.

The antimitotic drugs hydroxyurea (HU) and colchicine (CO) seem to be suitable for this type of investigation. It was recently shown by Knight (1976) that HU acts differentially on the nuclear and the k-DNA when applied to

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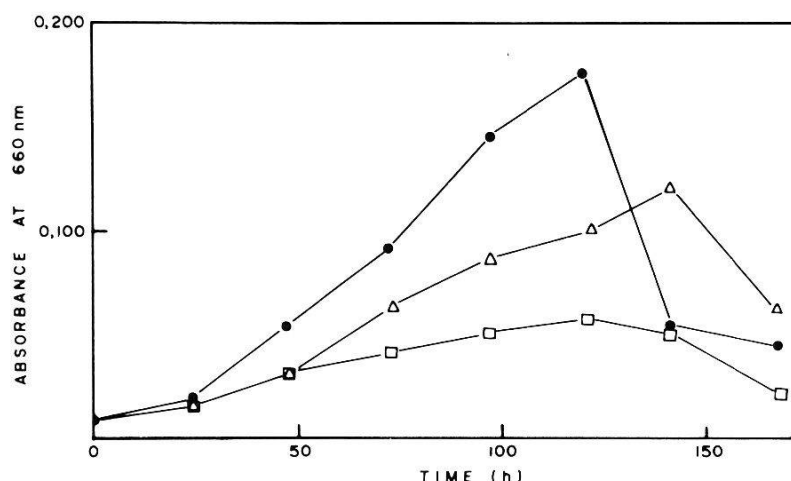


Fig. 1. Growth curve of cultures of *T. cruzi* treated with HU and CO. ●—● = control culture, □—□ = HU-treated culture, △—△ = CO-treated culture.

cultures of *Herpetomonas megaseliae* at a concentration of 200  $\mu\text{g/ml}$ . There is evidence that this drug acts primarily on the nuclear genome by inhibiting DNA synthesis while the k-DNA is not affected as judged by the appearance of multikinetoplast-multiflagellar forms (Knight, 1976). In contrast to HU which affects primary events during cell division, the alkaloid colchicine seems not to affect DNA synthesis but rather to act on microtubules during spindle formation (Lohmander et al., 1976). Because trypanosomes are rich in microtubules and because these structures seem to play an important role during cell division, for instance in the redistribution of the endosome (Brack, 1968; Vickerman and Preston, 1970; De Souza and Meyer, 1974), we exposed cultures of *T. cruzi* to colchicine and hydroxyurea.

The present report describes the influence of these drugs on growth and morphology of *T. cruzi* culture forms.

## Material and methods

*Strain.* — *T. cruzi* strain Y, isolated and described by Silva and Nussenzweig (1953) was obtained from Dr. Isaac Roitman, Department of Cell Biology, University of Brasilia, and used throughout this study.

*Culture growth.* — One half ml of starter culture (approximately  $2 \times 10^7$  cells) in Liver-Infusion-Tryptose (LIT) liquid medium (Camargo, 1964) was added to 5 ml of the same medium and incubated at 28° C in an incubator-shaker (New Brunswick Scientific Co., Inc., New York) for 170 h. Colchicine (Fisher Scientific Company, New York) and hydroxyurea (Sigma, St. Louis, Mo.), respectively, sterilized by Millipore filtration, were added to the culture medium immediately before inoculation with the starter culture to a final concentration of 200  $\mu\text{g/ml}$ . In the case of CO this particular concentration was chosen because pilot-experiments with 50, 100, 200 and 400  $\mu\text{g/ml}$  revealed that at 200  $\mu\text{g/ml}$  an optimal differentiation from epi- to trypomastigote forms could be observed. With HU too the same concentration range (50, 100, 200 and 400  $\mu\text{g/ml}$ ) was tested; we chose 200  $\mu\text{g/ml}$  in order to be able to compare our results with those obtained by Knight (1976) with *H. megaseliae*. The growth of the culture was monitored by measuring the absorbance at 660 nm in a "Spekol" colorimeter (Zeiss, Jena) or by determining the cell-number in a coulter-counter. Each experiment was done triplicate.

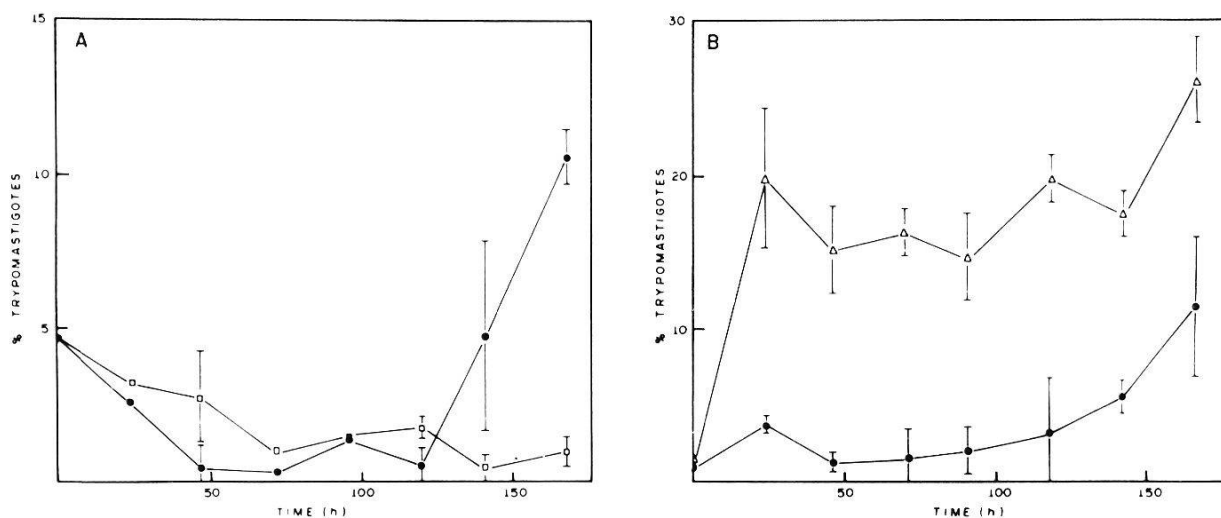


Fig. 2. Differentiation in cultures of *T. cruzi* (% trypomastigotes). 2 A: HU-treated culture, 2 B: CO-treated culture, ●—● = control culture, □—□ = HU-treated culture, △—△ = CO-treated culture. Vertical bars represent standard error.

*Light microscopic examination.* — At the times indicated in the graphs aliquots were withdrawn from the cultures, smears were prepared and after fixation with methanol, stained by the Giemsa- or Feulgen technique (Gabe, 1968). The morphology of 200 forms per slide was examined at a magnification of  $1000\times$ .

All forms whose kinetoplasts were posterior to the nucleus were considered to be trypomastigotes, forms with lateral or anterior kinetoplasts were counted as epimastigotes.

*Electron microscopy.* — Cells were harvested from the 90-h cultures (10 ml) by centrifuging at 300 g for 10 min. The cells were then resuspended in 3% (v/v) glutaraldehyde in 0.05 M cacodylate buffer, pH: 7.2 for 1–2 h at  $4^{\circ}\text{C}$ . After rinsing in buffer solution and postfixation in 1% (w/v)  $\text{OsO}_4$ , the material was dehydrated in acetone and embedded in Epon. Thin sections were stained with uranylacetate and lead citrate and examined in a Zeiss EM 9 electron microscope.

## Results

### Growth

The growth pattern of cultures of *T. cruzi* in LIT medium is shown in Fig. 1. The HU-treated cells grew only slightly as indicated by the small increase of the absorbance at 660 nm (Fig. 1). However, when measuring the actual cell number present by means of a coulter-counter, it was found that the cell number never increased.

On the other hand, in cultures exposed to colchicine, the cells multiplied as judged from the pronounced increase in absorbance (Fig. 1) and the presence of dividing forms in smears.

### Differentiation

While control cultures contained about 10% trypomastigotes after 150 h of incubation, HU-treated cultures did not show any significant appearance of these forms (Fig. 2 A). In cultures exposed to colchicine the percentage of trypo-



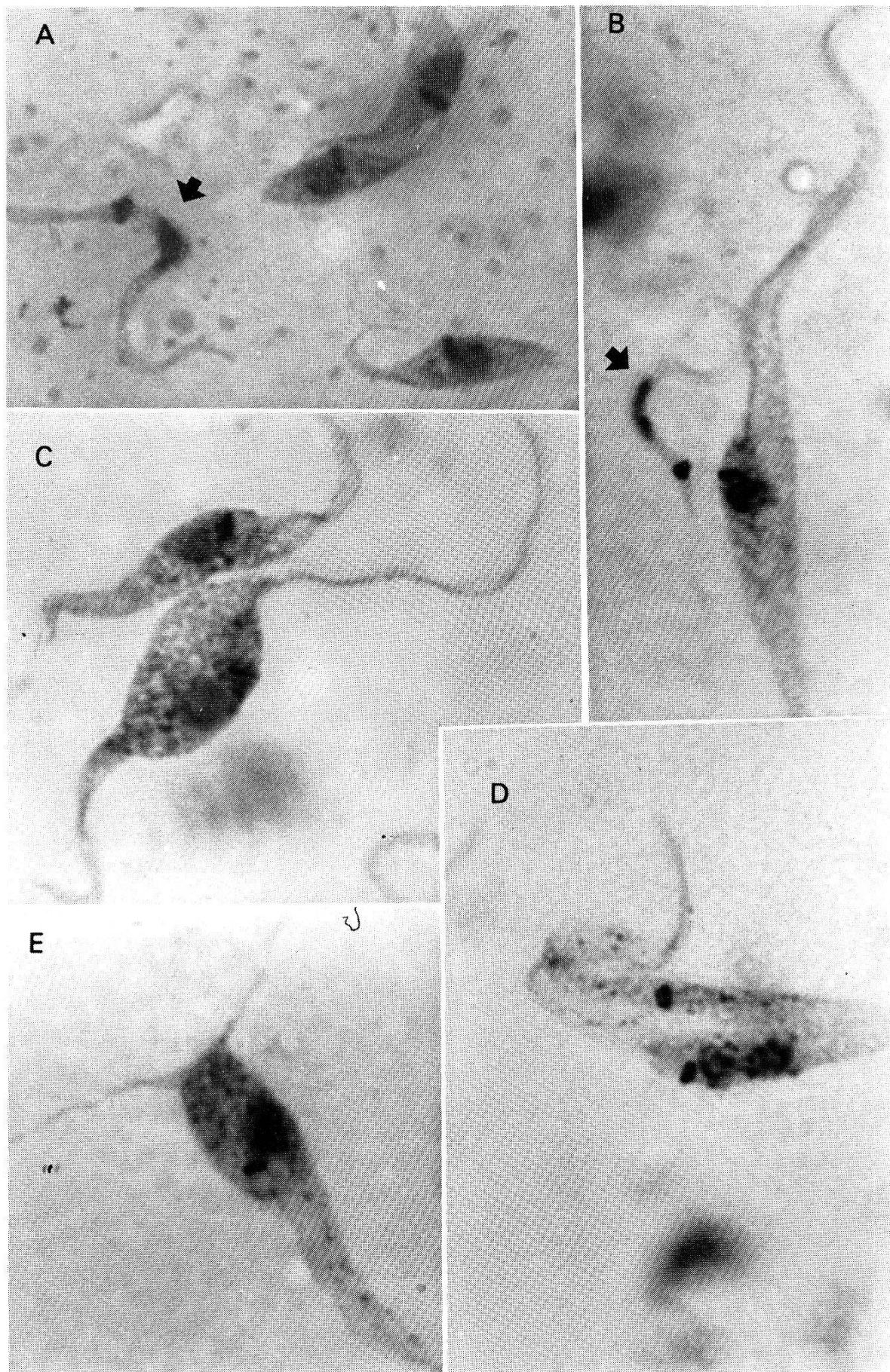


Fig. 3. Light micrographs of *T. cruzi* cultures treated with HU. Giemsa stained, magnification  $\times 1700$ . Fig. 3 A: control. Arrows point to trypanomastigote forms.

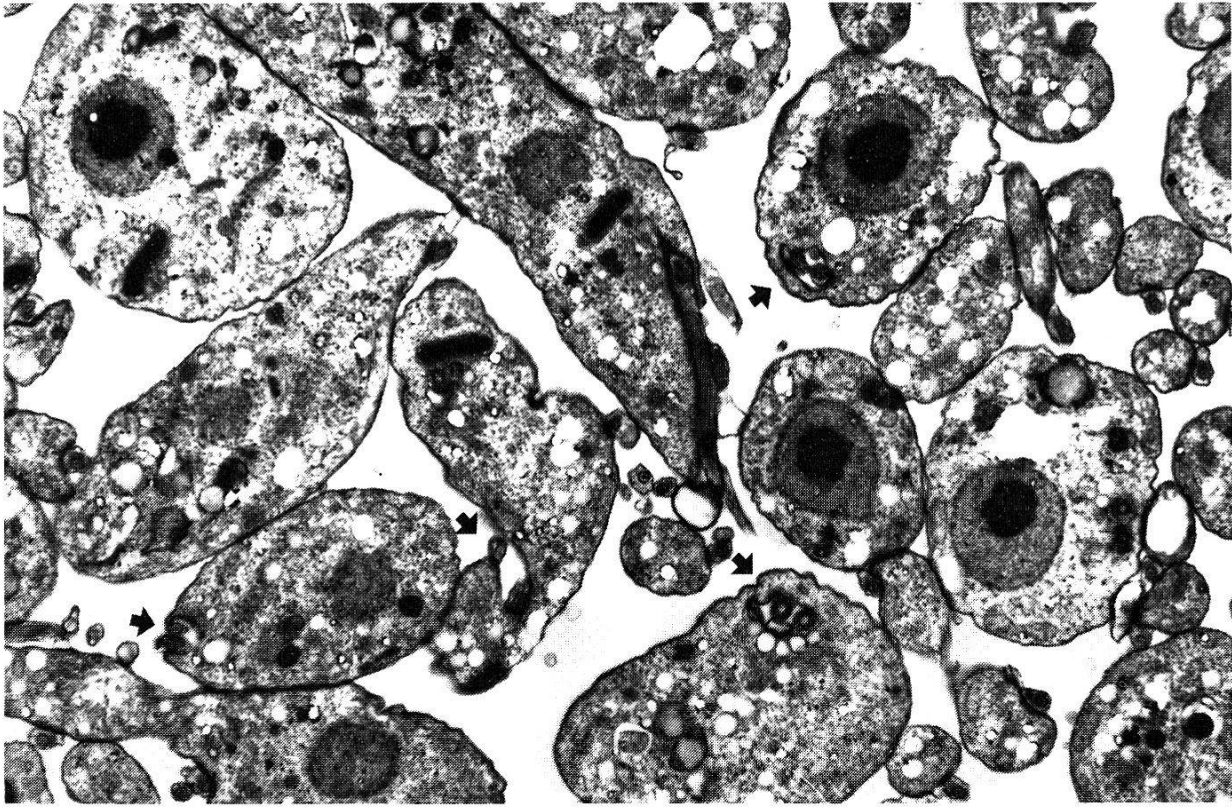


Fig. 4. Electron micrographs of *T. cruzi* treated with HU. Arrows point to two flagella surrounded by one flagellar pocket. Nuclei show no condensed material on the inner nuclear membrane. Magnification  $\times 7400$ .

mastigotes reached 20% one day after inoculation and increased with time to 25–30% (Fig. 2 B).

### *Morphological observations*

*HU-treated cultures.* – The size of the epimastigote forms increased greatly in cultures exposed to HU while trypomastigote forms seemed to be unaffected by the drug (Fig. 3 B–E).

Another alteration caused by HU was the appearance of “rostrum”-like structures at the posterior end of the cells. Very often these structures were present as doublets (Fig. 3 E).

With the light microscope we never detected forms with more than one kinetoplast or more than one flagellum except in extremely rare cases of cells in division where two flagella could be seen (Fig. 3 D). However, the examination of the material with the electron microscope revealed a substantial number of cells with two flagella (Fig. 4, arrows).

Furthermore, the electron-dense masses of chromatin material which were found in close contact with the inner nuclear membrane of normal cells, were missing in HU-treated cells (Fig. 4).

*CO-treated cells.* – Apart from epi- and trypomastigote forms that apparently seemed to be normal as judged from their morphology, “unusual”



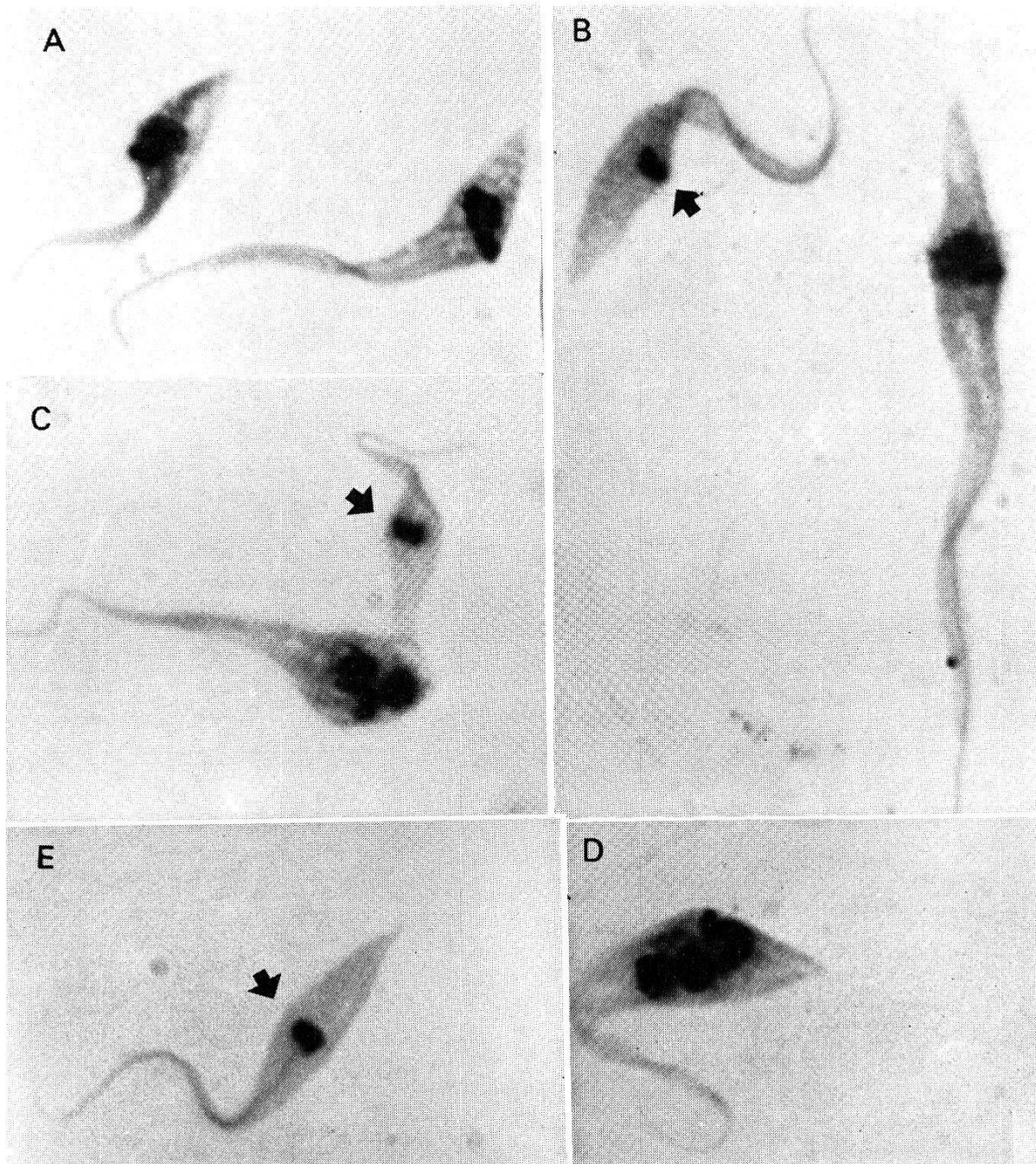


Fig. 5. Light micrographs of CO-treated cultures of *T. cruzi*. Giemsa stained. Magnification  $\times 1700$ . Fig. 5 A: control. Arrows point to anuclear forms.

forms were frequently observed after treatment of cultures with CO (Fig. 5 B–E). Contrary to the forms observed after exposure to HU, CO-exposed cells were not significantly larger than control cells. Of particular interest was that many cells failed to show any Feulgen-positive material with the exception of the kinetoplast (Fig. 5 B, C, E, arrows). After 150 h incubation in LIT, these forms made up to 25% of all cells present (Fig. 6). After 150 h, about 40% of the cells were epimastigotes, 25% trypomastigotes and 10% of the cells were either in the process of division or were polyploid.

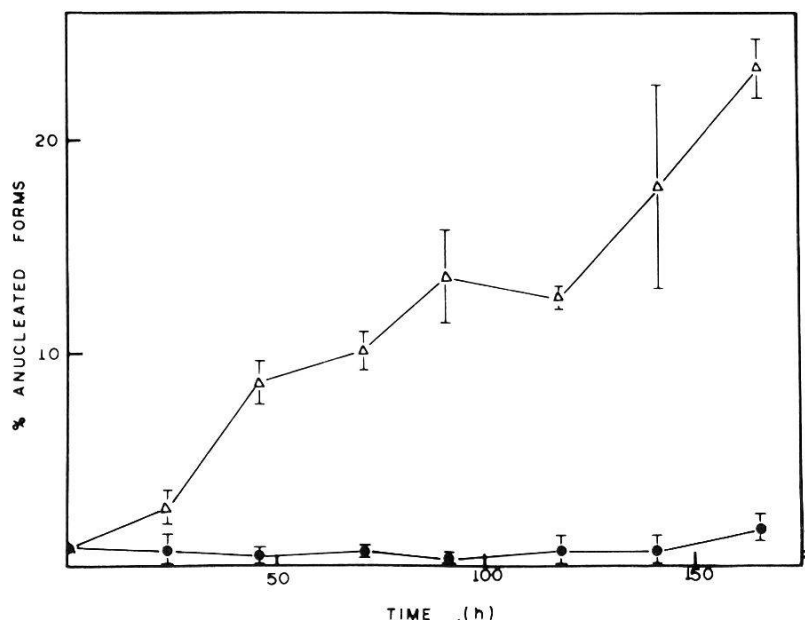


Fig. 6. Kinetics of appearance of anuclear forms (%) in CO-treated cultures of *T. cruzi*. ●—● = control culture, △—△ = CO-treated culture. Vertical bars represent standard error.

## Discussion

It has been shown recently that hydroxyurea stimulates the differentiation from pro- to opisthomastigotes in cultures of *H. megaseliae* (Knight, 1976). This differentiation apparently takes place while the DNA synthesis of the nucleus is blocked whereas the synthesis of k-DNA seems not to be affected by this drug.

In *T. cruzi* the mechanisms which govern its complex differentiation pattern during its life cycle in the invertebrate and the vertebrate host as well as in cultures are not clearly understood. The exposure of *T. cruzi* cultures to HU could, therefore, help our understanding of cell differentiation.

We found that none of the effects reported to occur in HU-treated cultures of *H. megaseliae* could be detected in *T. cruzi*. Whereas with *H. megaseliae* it is thought that at least the cells that have already entered G2-phase at the time the drug is administered are capable of another round of division (Knight, 1976), *T. cruzi* cells lose their capability of division almost totally after exposure to HU as shown by determining the actual cell-number with a coulter-counter. The small increase of absorbance at 660 nm shown in Fig. 1 is therefore most probably due to the increase of the cell volumes due to the action of the drug. In contrast to *H. megaseliae*, the effect of HU in *T. cruzi* at the concentration used is not limited to the nuclear DNA but probably affects the k-DNA as well; in no case we have been able to detect multikinoplast forms. It seems, however, that some remote signs of the cells readiness to divide are still manifest on the level of the flagella. It has been reported that multiplication in trypanosomes starts with the appearance of a new basal body followed by the formation of a new flagellum and by the subsequent synchronous division of the nucleus and the kinetoplast (Hoare, 1972).



Though light microscopy failed to reveal any multiflagellar cells in smears of HU-treated *T. cruzi*, the electron microscopic examination of these cells shows that in many cases division is at least initiated, as judged from the presence of cells where two flagella are surrounded by one flagellar pocket. This finding suggests that the initiation of the division of the mastigote system in *T. cruzi* begins independently from the division of either the nuclear or the kinetoplast genome but that for a successful formation of two separate flagella, the division of the kinetoplast- or the nuclear genome is necessary. As in *H. megaseliae*, multiflagellar-multikinetoplast forms appear after inhibition of the nuclear genome by HU, the k-DNA seems to play an important role for the formation of the flagella in these organisms.

Though there is a difference in sensitivity to HU between *H. megaseliae* and *T. cruzi* with respect to the kinetoplast, the mechanism of action of this drug seems to be basically the same in both species by inhibiting the replication of the DNA, probably due to the inhibition of dATP and dGTP synthesis (Hamlin and Pardee, 1976).

On the other hand, CO seems to act on a different level as cultures treated with this drug still show considerable dividing activity. After CO-treatment the population often attains up to 70% of the values obtained in control cultures, however, the shape of the growth curve shown in Fig. 1 suggests that growth and/or division are slowed down by CO as the treated cultures reach their population peak only after about 140 h. In contrast to HU where we could not observe any enhanced differentiation, CO seems to provoke an increase of trypomastigote forms from about 4% in the controls to 20% after the first 24 h exposure to the drug. Of particular interest is the presence of up to 25% anuclear cells after CO-treatment. The appearance of these forms together with cells showing polyploidy suggests that the structures responsible for the correct distribution of the daughter nuclei at the end of cell division may be a site of action of CO.

Though data on the precise mechanisms that regulate the distribution of organelles during cell division are missing, one might speculate on the basis of our findings, that the microtubules are involved in this process, as it has been shown by Lohmander et al. (1976) that colchicine acts on these structures.

The appearance of anuclear forms in CO-treated cultures of *T. cruzi* favours such a hypothesis. Since these anuclear forms still possess a kinetoplast, the mass production of such forms and the subsequent isolation of their DNA and RNA components might offer a powerful tool for studies concerned with the role of the kinetoplast in the life cycle of the trypanosomes. Efforts to quantitatively isolate these forms as well as to characterize their nucleic acid population are currently under way in our laboratory.

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