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Further studies on the cell surface charge of *Trypanosoma cruzi*

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Summary

Trypanosoma cruzi has a negative surface which varies according to the ionic strength and the pH of the solution in which the cells are suspended. At low pH there is a decrease in the negative surface charge with an isoelectric point at pH 2.6 and 3.0 for epimastigote and trypomastigote forms, respectively. Below these pH values the cells have a positive surface charge. At higher pH there is an increase in the surface charge. Glutaraldehyde fixation did not interfere with the electrophoretic mobility (EPM) of the cells. Epimastigote and trypomastigote forms of *T. cruzi* have a characteristic EPM independent of the medium in which the cells were grown, the origin of the trypomastigotes or the strain of the parasite. Trypomastigotes have a higher negative surface charge than epimastigotes. Based on the change in the EPM of the cells treated with neuraminidase, it is concluded that sialic acid is present on the cell surface of *T. cruzi* and that it is the main component responsible for the high negative surface charge of the trypomastigote form. Trypsin treatment also reduces the EPM of *T. cruzi*. Neuraminidase or trypsin-treated parasites recovered their normal EPM when incubated for 4 h in fresh culture medium. This process involves synthesis of protein since it is inhibited by puromycin.

Key words: *Trypanosoma cruzi*; surface charge; sialic acid.

Introduction

The cell surface of eucaryotic cells plays a fundamental role in processes such as cell communication, regulation of the cell growth and differentiation, cell recognition, etc. (Meherish, 1972; Weiss, 1969). Most of the eucaryotic cells

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studied have a net negative surface charge which is determined by electrical charges located on the outer face of cell membrane (Meherish, 1972; James, 1979; Weiss, 1969). Previous studies indicate that the candidates most likely for anionic sites in the cell membrane are sulphate groups found in acid mucopolysaccharides, ionized phosphate groups found in phospholipids and charged carboxyl groups largely due to the presence of sialic acid (Ambrose, 1966; Burry and Wood, 1979; Eylar et al., 1962; Seaman and Uhlenbruck, 1963; Gasic et al., 1968). Evidence has been obtained for the presence of sialic acid on the cell surface of *Trypanosoma cruzi* (Martinez-Palomo et al., 1976; De Souza, 1978; Pereira et al., 1980; Kipnis et al., 1981; De Araújo Jorge and De Souza, 1981, 1984).

One approach to study the cell surface charge is the determination of the cellular electrophoretic mobility (EPM) which is a measure of the movement of a particle in a solution when subjected to an externally applied electric field. Previous studies have shown that differences exist in the EPM according to the evolutionary forms of *T. cruzi* (Hollingshead et al., 1963; De Souza et al., 1977; Kreier et al., 1977).

In the present study we examined the surface charge of epimastigote and trypomastigote forms of two strains of *T. cruzi*. These two strains (Y and CL) have been widely used in studies of host cell-parasite interaction. It has been shown that they present marked differences in their biological behaviour (Brenner, 1973). We also analysed the effect of the ionic strength and the pH of the solution in which the cells were suspended, as well as the effect of trypsin and neuraminidase on the EPM of *T. cruzi*.

Materials and Methods

Parasites. – The Y and CL strains of *Trypanosoma cruzi* were used. Bloodstream trypomastigotes have been maintained in Swiss albino mice by intraperitoneal inoculation. Trypomastigotes were collected on the 7th or 14th day of infection as previously described (Meirelles et al., 1982). They were separated from blood cells by centrifugation in a gradient of metrizamide as described previously (Loures et al., 1980). After isolation, bloodstream forms were washed with medium 199 without serum and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h at room temperature. Tissue culture derived trypomastigotes were obtained from LA9 cells which were infected with bloodstream forms of *T. cruzi* (De Carvalho and De Souza, 1983). Epimastigotes, trypomastigotes and transitional forms from axenic cultures were obtained according to the method of Chiari (1981). Clones of Y and CL strains, were cultivated in LIT medium (Camargo, 1964) at 28 °C for 2 days in order to obtain epimastigote forms. These forms were then transferred to a TSH modified medium (M16). After 4 days of culture $7-8 \times 10^7$ parasites/ml were provided. The advantage of M16 medium was the large rate of differentiation of epimastigotes into trypomastigotes yielding up to 90–95% trypomastigotes. Transitional and trypomastigote forms were obtained from M16 medium after 96 and 168 h of cultivation, respectively. All cells were washed in 0.1 M phosphate buffer and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h at room temperature.

Enzymatic treatment. – Axenic cultured epimastigotes and bloodstream trypomastigotes were collected by centrifugation, washed twice in PBS, resuspended in PBS (1×10^6 cells/ml) and then incubated for 30 min at 37 °C in the presence of 0.2 U/ml neuraminidase (Sigma type X) in Tyrode's solution, pH 6.0 or for 5 min at 37 °C in the presence of 500 µg/ml trypsin (Sigma type III) in

Ringer's solution, pH 7.2. Trypsin was inhibited by addition of bovine serum (final concentration, 10%) to the test tube. After enzyme treatment the cells were collected by centrifugation, washed twice in PBS and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer. In some experiments enzyme-treated epimastigotes were reincubated in fresh culture medium and samples were collected after 1, 2 and 4 h, washed with PBS and fixed in glutaraldehyde. In order to determine the influence of a de novo protein synthesis on the results obtained, enzyme-treated cells were reincubated in fresh medium containing 10 $\mu\text{g}/\text{ml}$ of puromycin. Samples were collected as described above.

Microelectrophoretic measurement. – The electrophoretic mobility of the cells was determined in a Zeiss Cytopherometer with a current of 4–6 mA and a final voltage of 100 V. The cell suspension was placed into the chamber and then allowed to equilibrate for 10 min. Measurements were made at a temperature of 25° in a 0.85% sodium chloride solution with an ionic strength of 0.145 $\text{mol} \times \text{dm}^{-3}$, at pH 7.2. We also determined the electrophoretic mobility of living parasites. In this case measurements were made at 4 °C to prevent active movement of the cells. When current was switched on, we measured the time necessary for one cell to travel across two vertical lines, separated by a distance of 16 μm . Then the polarity was reversed and the time was measured again for the cell travelling in the opposite direction. 50–100 cells were measured for each sample analysed. In some experiments the pH of the solution was changed varying from 2.0 to 12.0. The changes in the pH were obtained by adding different amounts of 0.1 M HCl or NaOH to the NaCl solution. Before the measurement the chamber was filled with a solution of appropriate pH and allowed to equilibrate for 20 min. Then the parasite suspension was added to the chamber. In other experiments we changed the ionic strength of the solution in which the cells were suspended, varying from 0.07 to 0.29 $\text{mol} \times \text{dm}^{-3}$.

Calibration of the equipment was made by measuring the electrophoretic mobility of fresh human erythrocytes. Statistical analysis was performed using the T test.

Results

Our observations of the electrophoretic mobility of *Trypanosoma cruzi* indicated that under standard conditions of ionic strength ($I = 0.145 \text{ mol} \times \text{dm}^{-3}$) at pH 7.2 and at 25 °C, cells had a negative surface charge, but the magnitude depended on the form under which the parasite appeared during its life cycle (Table 1). In order to investigate the influence of the fixation on the EPM and to avoid the active movement of living cells, which render EPM measurements difficult, glutaraldehyde fixed cells were used. The fixation had no influence on the EPM of cells and the results were similar to those obtained with living cells at 4 °C. The orientation of the parasites was random during migration towards the positive electrode; some moved with the anterior end towards the cathode, others with the posterior end and others laterally. Epimastigote forms from the Y and the CL strains cultivated in 4 different culture media had basically the same mean EPM (Table 1). Population analysis indicated that the cells in general were very heterogeneous in their EPM. Epimastigotes of the CL strain were more homogeneous than those of the Y strain (Fig. 1). Although the cells were very heterogeneous in their EPM, such heterogeneity was not related to the growth phase of cells, since the mean EPM of cells collected in the lag, in the log or in the stationary phase of growth was essentially the same (not shown).

Table 1. Electrophoretic mobility (EPM) of *T. cruzi*^{1,2}

Developmental stage	Treatment	Source	Strain	EPM ($-\mu\text{m} \times \text{s}^{-1} \times \text{V}^{-1} \times \text{cm}$)	
				Mean	SD
Epimastigote	No	M16 medium	Y	0.65	0.11
Epimastigote	No	M16 medium	CL	0.74	0.11
Epimastigote	No	Defined medium	Y	0.66	0.05
Epimastigote	No	Warren's medium	LY	0.62	0.09
Epimastigote	trypsin ³	Warren's medium	Y	0.58	0.03
Epimastigote	neuraminidase ³	Warren's medium	Y	0.54	0.03
Transitional	No	M16 medium	Y	1.00	0.14
Transitional	No	M16 medium	CL	1.10	0.15
Trypomastigote	No	tissue culture	Y	1.04	0.11
Trypomastigote	No	M16 medium	Y	1.20	0.20
Trypomastigote	No	M16 medium	CL	1.06	0.18
Trypomastigote	No	bloodstream	CL	1.10	0.07
Trypomastigote	No	bloodstream	Y	1.15	0.17
Trypomastigote	trypsin	bloodstream	Y	0.69	0.05
Trypomastigote	neuraminidase	bloodstream	Y	0.55	0.06

¹ Data are from at least 100 cells

² In all instances, $I = 0.145$ at pH 7.2

³ Enzymatic treatment as described in Materials and Methods

Fig. 2 shows the effect of the cell suspension medium pH on the EPM of epimastigote forms. At pH 2.0 epimastigotes had a positive surface charge and migrated towards the negative electrode. Between pH 5.0 and 7.0 the EPM did not vary but increased again at pH above 8.0. Based on the curve shown in Fig. 2 we determined that the isoelectric point of epimastigote forms was at pH 2.6 and that of trypomastigotes at pH 3.0. The effect of the ionic strength of the epimastigotes suspension solution on the EPM can be seen in Table 2. At an ionic strength of $0.07 \text{ mol} \times \text{dm}^{-3}$ the net surface charge of epimastigotes became more negative with a mean EPM of -1.19 . At the ionic strength used in most of the experiments ($I = 0.145 \text{ mol} \times \text{dm}^{-3}$) the mean value was -0.62 . We observed a small variation of EPM at ionic strengths of 0.217 and $0.29 \text{ mol} \times \text{dm}^{-3}$. At these ionic strengths, however, the net surface charge becomes less negative.

We analysed trypomastigote forms of *T. cruzi* from the Y and CL strains obtained from 3 different sources. Fig. 1 and 3 show the population analysis of EPM of trypomastigotes derived from the bloodstream, axenic culture, and tissue culture. As was observed for epimastigotes, trypomastigotes were also very heterogeneous in their EPM. Bloodstream trypomastigotes of the Y strain presented a mean EPM of -1.15 with a distribution much more heterogeneous than that found for the CL strain, where the mean EPM was -1.10 and 82% of

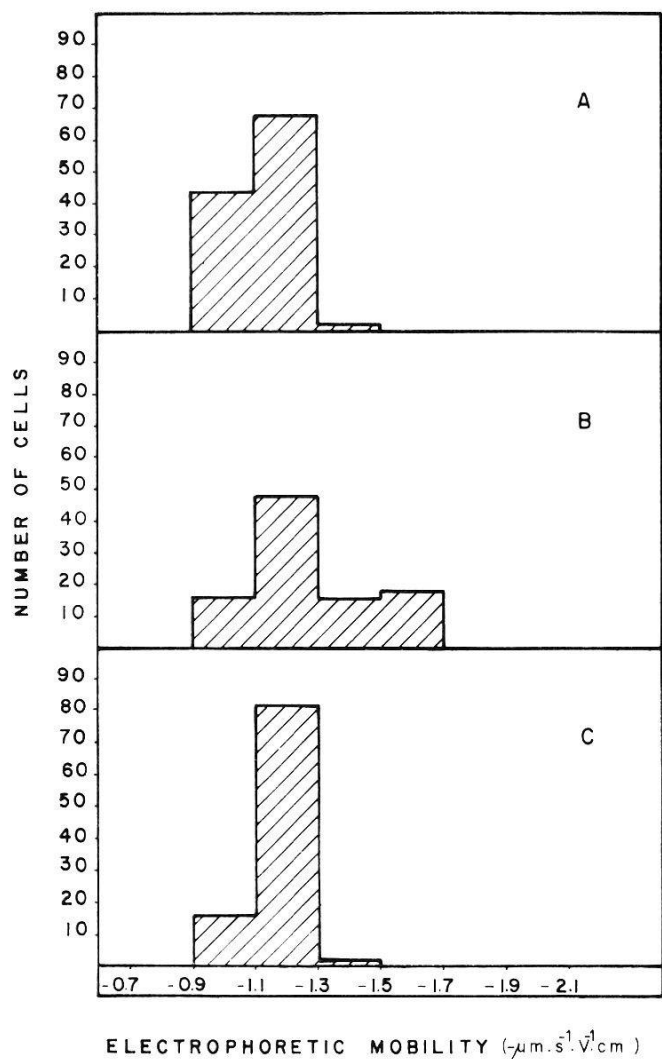


Fig. 1. Distribution of the electrophoretic mobility of different populations of *T. cruzi* obtained from axenic cultures (M16 medium). For all measurements, $I = 0.145$, pH 7.2. A: Epimastigotes, Y strain; B: epimastigotes, CL strain; C: trypomastigotes, Y strain.

the parasites presented an EPM between -1.00 and -1.20 . Axenic culture derived trypomastigotes of the Y and CL strains presented a mean EPM of -1.20 and -1.06 , respectively.

Transitional forms from axenic culture showed a high EPM for both the Y and CL strains, which was similar to that observed for trypomastigotes. Transitional forms of Y and CL strains presented an EPM of $-1.00 \mu\text{m} \times \text{s}^{-1} \times \text{V}^{-1} \times \text{cm}$ and -1.10 , respectively. The distribution of the EPM values was very heterogeneous in both strains. As previously mentioned, these forms were obtained from M16 medium on the 4th day of culture when we could see that about 30% of the cells were epimastigotes, 27% were trypomastigotes and 23% were transitional forms.

Treatment of living cells with either trypsin or neuraminidase reduced the EPM of the epimastigote and trypomastigote forms. Table 1 indicates that neuraminidase was more effective than trypsin in reducing the EPM of the cells

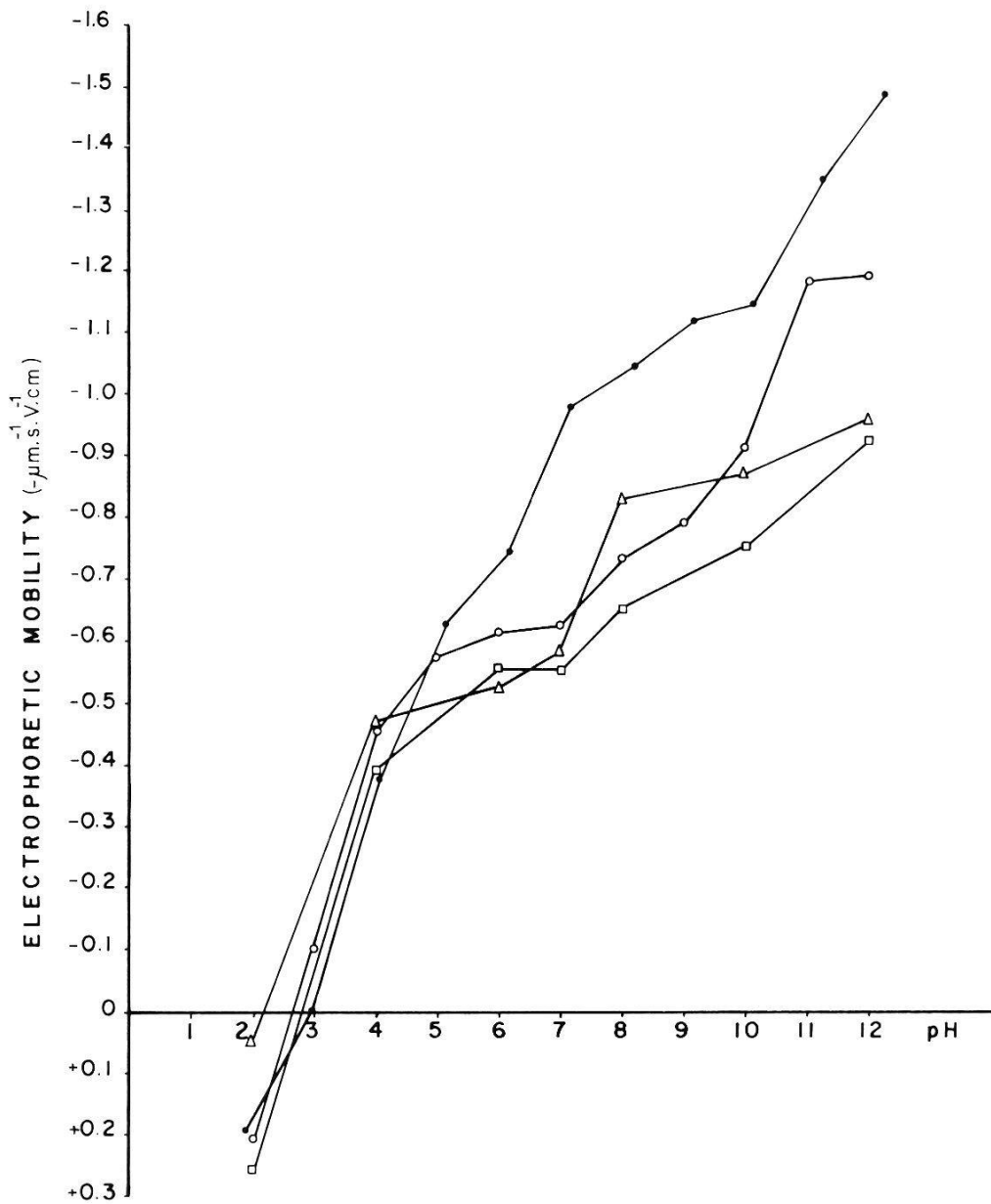


Fig. 2. Influence of the pH of the solution in which the cell are suspended on the electrophoretic mobility of control (○---○), trypsin (△---△) or neuraminidase-treated (□---□) epimastigotes and on axenic derived trypomastigotes (●---●) of *T. cruzi*.

Table 2. Effect of the ionic strength of the solution in which the cells were suspended on the electrophoretic mobility (EPM) of epimastigotes of *T. cruzi*

Ionic strength $\text{mol} \times \text{dm}^{-3}$	EPM $(-\mu\text{m} \times \text{s}^{-1} \times \text{V}^{-1} \times \text{cm})$	
	Mean	SD
0.072	-1.19	± 0.16
0.145	-0.65	± 0.08
0.217	-0.50	± 0.10
0.29	-0.50	$+0.07$

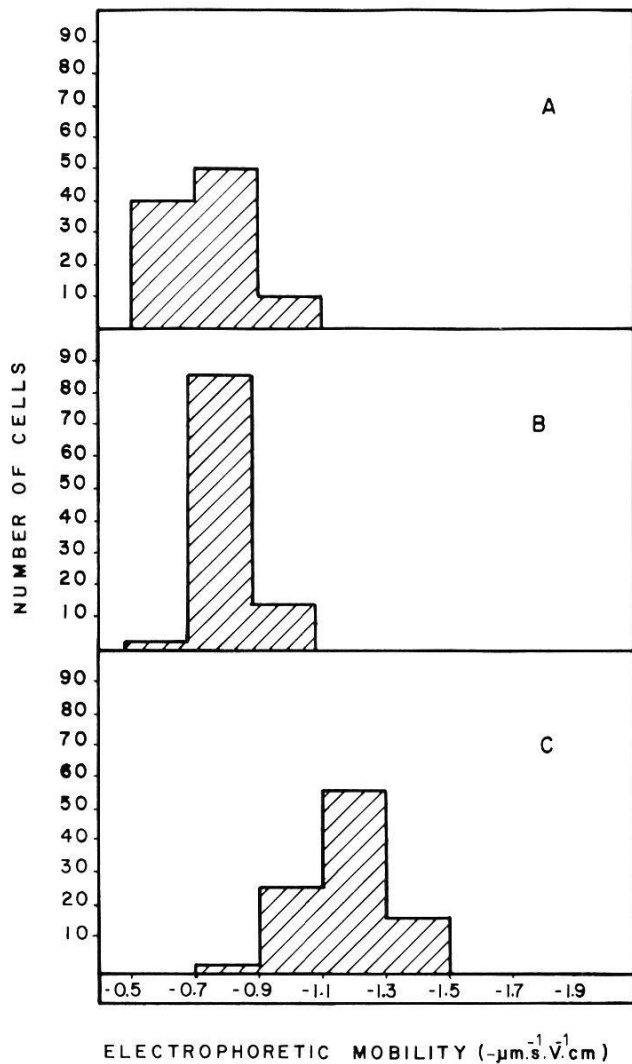


Fig. 3. Distribution of the electrophoretic mobility of different populations of trypomastigotes of *T. cruzi*. For all measurements, $I = 0.145$, pH 7.2. A: trypomastigotes from the Y strain purified from the supernatant of LA9 cells previously infected with bloodstream forms; B: bloodstream trypomastigotes from the Y strain; C: bloodstream trypomastigotes from the CL strain.

at pH 7.0. Neuraminidase-treated epimastigotes presented a mean EPM of -0.54 while trypsin-treated cells presented a mobility of $-0.58 \mu\text{m} \times \text{s}^{-1} \times \text{V}^{-1} \times \text{cm}$. The effect of enzymes on the EPM of epimastigotes was statistically significant. Enzyme-treated cells recovered their normal EPM when incubated in fresh culture medium. Neuraminidase-treated cells recovered their EPM after 2 h in fresh medium and trypsin-treated cells after 4 h. Recovery of the EPM of enzyme-treated cells was blocked by addition of $10 \mu\text{g}/\text{ml}$ puromycin to the culture medium (Fig. 4). Fig. 2 shows the EPM of neuraminidase and trypsin-treated cells at different pH. Both enzymatic treatments changed the isoelectric point of epimastigotes. Neuraminidase-treated cells presented an isoelectric point at pH 2.4 and trypsin-treated cells at pH 2.1. It is important to notice that all the neuraminidase-treated cells had a net positive surface charge at pH 2.0 and migrated towards the negative electrode.

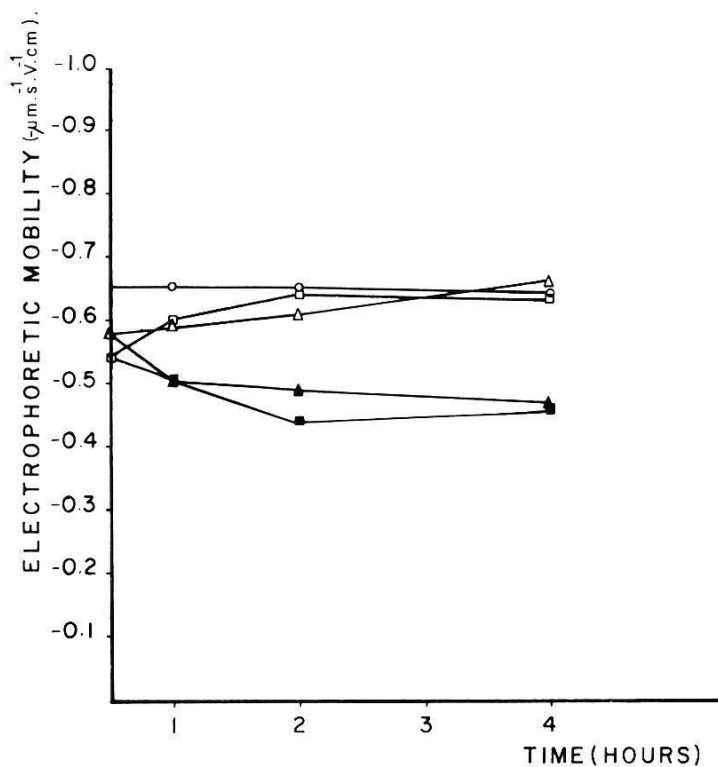


Fig. 4. Effect of treatment of epimastigotes of *T. cruzi* with neuraminidase or trypsin on the electrophoretic mobility. After enzyme treatment the cells were incubated in fresh culture medium and samples were taken out after 1, 2, 3 or 4 h and the electrophoretic mobility of the cells was determined. In some experiments puromycin was added to the fresh culture medium. Control (○---○); neuraminidase-treated parasites incubated in fresh culture medium containing (■---■), not containing (□---□) puromycin; trypsin-treated parasites incubated in fresh culture medium containing (▲---▲), not containing (△---△) puromycin.

Treatment of bloodstream trypomastigotes with trypsin or neuraminidase reduced their EPM by 40 and 50%, respectively.

Discussion

Our results indicate that the electrophoretic mobility of *T. cruzi* strains cultivated in different media is a characteristic of the species and, therefore, can be a useful parameter to classify this parasite. We measured the EPM of bloodstream, tissue culture-derived and axenic culture-derived trypomastigotes of the Y and CL strains of *T. cruzi*. Our observations show that all trypomastigotes tested have basically the same EPM which differs from that observed for epimastigotes, a fact which supports the idea that each developmental stage has a typical EPM which characterizes the *T. cruzi* species.

Analysing a large number of cells it was seen that there were variations in the cellular surface charge within a given population. This heterogeneity probably reflects differences among individual cells and is not related to the phase of growth of the culture since cells collected at the lag-, log-, or stationary phases had the same mean EPM. We cannot exclude the possibility that some parasites

secrete proteases (Rangel et al., 1981) or neuraminidases (Pereira, 1980) into the liquid phase of the culture which would remove surface components of some cells.

Our results are not in agreement with those reported by others which observed that epimastigotes have a higher negative surface charge than trypomastigotes (Kreier et al., 1977; Murray et al., 1982). It is quite possible that these differences are due to the ionic strength of the solution in which the parasites were suspended. While we used an ionic strength of 0.145, these authors used ionic strengths varying from 0.290 to $0.362 \text{ mol} \times \text{dm}^{-3}$. The results obtained by them do not explain the difficulties found for the separation of erythrocytes and bloodstream forms using DEAE cellulose column (Lanham and Godfrey, 1970; Villalta and Leon, 1979).

Kreier et al. (1977) stated that the pH of the solution in which trypomastigotes are suspended does not interfere with their EPM. Contrary to these findings we demonstrate, by keeping the ionic strength constant but varying the pH, that the cell surface of both, epimastigotes and trypomastigotes, must contain positively and negatively charged dissociating groups. At higher pH values the negative charge increases, probably due to the increase in the dissociation of the carboxyl groups, reaching a plateau between pH 5 and 8 where there is an equilibrium between COO^- and NH_3^+ . At pH above 9 the negative charge increases since in this condition the proton is lost and the charge is due to COO^- alone.

We observed that treatment of *T. cruzi* with trypsin reduces the EPM of epimastigotes and trypomastigotes by about 11 and 40%, respectively. This effect might be the consequence of the removal of cell surface proteins since the normal EPM of the cells can be recovered, when the enzyme-treated cells are incubated in a fresh culture medium. Addition of puromycin inhibits recovery. Previous studies have shown that treatment of trypomastigotes with trypsin interferes with some cell surface properties of *T. cruzi* (Nogueira et al., 1980; Andrews et al., 1982; Kipnis et al., 1981; De Araújo Jorge and De Souza, 1984).

Our observations indicate that sialic acid is an important component of the cell surface of *T. cruzi*, mainly in the trypomastigote form. Treatment of the parasites with neuraminidase, under conditions which do not interfere with cell viability, reduced their EPM by about 18% for epimastigote and 50% for trypomastigote forms. The sialic acid containing sites reappeared on the cell surface when neuraminidase-treated cells were incubated in fresh culture medium. The observation that puromycin added to the fresh culture medium blocked the recovery of the neuraminidase-treated cells indicates that sialic acid-containing components localized on the cell surface of *T. cruzi* did not originate from the culture medium but were synthesized by the protozoan. The reduction in the EPM observed when the parasites were treated with neuraminidase was higher for trypomastigote than for epimastigote forms suggesting that there are more sialic acid residues exposed on the cell surface of trypomastigotes than in epi-

mastigotes. However, we can not exclude the possibility that some sialic acid exists on the cell surface of epimastigotes which might be masked in some way by other substances, avoiding the interaction with neuraminidase or that it is insensitive to the used neuraminidase from *Clostridium perfringens*. It was observed that neuraminidase treatment of bloodstream trypomastigotes increases their uptake by macrophages markedly (De Araújo Jorge and De Souza, 1984). It is possible that trypomastigotes with a high negative surface charge are not ingested by macrophages and only those with a low charge would be ingested. This idea is supported by the results obtained when the EPM of a population of bloodstream trypomastigotes was determined. It was seen that about 8% of the trypomastigotes of the Y strain have an EPM of $-9.90 \mu\text{m} \times \text{s}^{-1} \times \text{V}^{-1} \text{ cm}$, and this percentage corresponds to that of parasites which are usually ingested by macrophages after a interaction period of 2 h (De Araújo Jorge and De Souza, 1984).

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