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The surface charge of trypanosomatids*

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ABSTRACT

The surface charge of trypanosomatids was evaluated by means of the binding of cationic particles, as visualized by electron microscopy and by direct measurements of the electrophoretic mobility of cells. The results obtained indicate that most of the trypanosomatids exhibit a negatively charged surface whose value is species specific and varies according to the developmental stages. Sialic acids associated with glycoproteins, glycolipids and phosphate groups are the major components responsible for the net negative surface charge of the trypanosomatids.

Key words: trypanosomatids, cell surface charge, electrophoretic mobility, cytochemistry.

INTRODUCTION

The Trypanosomatidae family comprises a large group of flagellated protozoa separated in 12 genera, which comprise monogenetic (*Angomonas*, *Blastocrithidia*, *Crithidia*, *Herpetomonas*, *Leptomonas*, *Rhynchoidomonas* and *Wallaceina*) and digenetic (*Endotrypanum*, *Leishmania*, *Phytomonas*, *Sauroleishmania* and *Trypanosoma*) parasites with ability to parasitise a very diverse range of hosts, including animals, plants and other protists.

The parasites from the genus *Trypanosoma* are responsible for diseases of great medical and veterinary importance such as Chagas' disease or American Trypanosomiasis, sleeping sickness or African Trypanosomiasis. The several species of the *Leishmania* genus are agents of visceral and cutaneous leishmaniasis.

Species from the genera *Herpetomonas*, *Leptomonas*, *Crithidia* and *Blastocrithidia* are found only in insects. *Phytomonas* spp is pathogenic for plants. The genera *Angomonas*, *Rhynchoidomonas*, *Wallaceina*, *Endotrypanum* and *Sauroleishmania* will not be discussed here because of the lack of data about cell surface charge.

Trypanosomatids have a complex life cycle, exhibiting a number of differentiation stages that appears as a result of transformations involving both structural and physiological changes. Previous studies have shown that the occurrence of differentiated cell stages is usually associated with changes in cell surface components and that changes in the net surface charge can also be found as a result of cell differentiation. Such changes directly influence the parasite-host cell interaction.

SURFACE CHARGE

Different methods have been applied to the study of the electrical properties of cell membranes indicating the presence of two distinct potentials: the

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membrane and the electrokinetic potentials. The first, also denominated trans-membrane potential, is due to the semi-permeable characteristic of the biological membranes and is maintained by an energy-dependent mechanism. The second, also denominated cell surface charge, is due to a complex interaction between the polar groups residing at the cell surface with different ions of the surrounding medium (James 1979, Mehereish 1972, Weiss 1969).

Pro and eukaryotic cells bear a net negative electrical charge which influences the adhesiveness and selective affinity between cells, playing an important role in morphogenesis during the embryonic development (Schaeffer et al. 1973), in the malignancy and ability of the cells to metastasize (Haeffner et al. 1988, Carter et al. 1989) and in parasite-host cell interaction (Meirelles et al. 1984, Araújo-Jorge et al. 1992, Monteiro et al. 1998).

Previous studies indicate that the candidates most likely for anionic sites in the cell membrane are sulfate groups, found in acid mucopolysaccharides, ionized phosphate groups, found in phospholipids, and charged carboxyl groups largely due to the presence of sialic acid and from carboxyl groups from acidic amino acids of proteins (Ambrose 1966, Burry and Wood 1979, Eylar et al. 1962, Seaman and Uhlenbruck 1963, Gasic et al. 1968).

The surface charge of trypanosomatids has been studied by the use of the following techniques: a) passage of cells through diethylaminoethyl (DEAE)-cellulose columns; b) visualization, by electron microscopy, of cationic particles bound to the cell surface; c) determination of cell surface charge by Atomic Force Microscopy associated to surface potential spectroscopy; d) determination of the cellular electrophoretic mobility; and e) use of sialic acid-binding components.

USE OF DEAE-CELLULOSE COLUMNS

The application of anion exchange columns to the study of Trypanosomatids started with the necessity to develop an efficient method to separate trypomastigote forms of trypanosomes from blood cells or from other evolutive forms for biological, chemical or immunological studies (Lanham 1968, 1971, Lanham and Godfrey 1970, Al-Abbassy et al. 1972, Howells and Chiari 1973, Jackson 1975, Goldberg et al. 1976, Kreier et al. 1977, Gutteridge et al. 1978, Villalta and Leon 1979, Alvarenga and Brener 1979, Selden and Baker 1980, Langenbach 1985). On passing infected blood throughout a DEAE-cellulose column the blood cells and platelets, which are very negative, were completely adsorbed while the parasites could be easily eluted (Lanham 1968, 1971, Lanham and Godfrey 1970). A standard technique was established which successfully separated different species of Salivarian trypanosomes, confirming previous studies which showed that the surface of bloodstream forms of *T. brucei* was not very negative. However, it was not possible to separate *T. cruzi* trypomastigotes of the Y strain. According to Lanham (1968), the bloodstream forms of trypanosomes belonging to different species could be arranged in the following order of negative surface *T. cruzi* > *T. lewisi* > *T. vivax* > *T. congolense* > *T. rhodesiense*, *T. gambiense*, *T. brucei* and *T. evansi*.

The slight difference in the surface charge of bloodstream trypomastigotes of *T. cruzi* and blood cells discouraged the use of DEAE-cellulose and to resolve this problem this step was preceded by a differential centrifugation (Gutteridge et al. 1978, Villalta and Leon 1979) or centrifugation in a Ficoll-hypaque gradient (Mercado and Katusha 1979) to remove most of the red cells. Differently from what was described for African trypanosomes the infectivity of bloodstream forms of *T. cruzi* isolated in DEAE-cellulose column appears to be lower than when parasites are isolated by other means (Lanham 1968, Villalta and Leon 1979, Hungerer et al. 1981). Some conditions for purifying *T. cruzi* using a DEAE-cellulose column without affecting the antigenicity and infectivity of the parasites were established (Souza 1982, M.Sc.thesis, Fundação Oswaldo Cruz, Rio de Janeiro). It was found that the efficiency of separation varied according to the strain of *T. cruzi* used and the morphology of trypomastigote forms. It was observed that slender trypomastigotes are more negative than the broad ones (Souza 1982, loc.cit.).

DEAE-cellulose columns have also been used for the purification of *T. cruzi* trypomastigotes from tissue and axenic culture (Al-Abbassy et al. 1972, Goldberg et al. 1976, Kreier et al. 1977, Selden and Baker 1980, Langenbach 1985) and from feces of triatomines (Alvarenga and Brener 1979) where they are mixed with epimastigote forms. As these metacyclic trypomastigotes have a negative surface similar to that of bloodstream trypomastigotes and epimastigotes have a less negative surface it would be expected that during the process of separation the first forms to be eluted should be the epimastigotes. However, trypomastigotes are initially eluted and the epimastigotes remain associated with DEAE-cellulose. It was showed that passage of metacyclic trypomastigotes through a DEAE-cellulose column alters the kinetics of arginine and lysine transport by the plasma membrane (Goldberg et al. 1976) and removes some negatively-charged constituents of the parasite surface as shown by the decrease of the surface charge, determined by cellular electrophoretic mobility (De Souza et al. 1977) and the change in the attachment of parasites to chitosan (Kleffmann et al. 1998).

To avoid the effects of DEAE-cellulose column Pinho and coworkers (1986) developed a method of purification of metacyclic trypomastigotes using glass - wool columns. As it occurs with DEAE - cellulose column, trypomastigotes are the first to be eluted while the epimastigote forms remain adsorbed on the glass wool. Mortality studies in mice showed that the biological activity of the glass wool purified trypomastigotes remained unchanged. No estimation of cell surface charge of these trypomastigotes was reported.

There are no reports about the use of DEAE-cellulose columns to isolate other trypanosomatids or evolutive forms.

ULTRASTRUCTURAL DETECTION OF CELL SURFACE ANIONIC SITES

Electrically charged electron stains may be bound electrostatically by inorganic groups of the cell surface. Two cationic labels have been widely used: the colloidal iron hydroxide (CIH) and the cationized ferritin (CF) (Danon et al. 1972, Gasic et al. 1968). There are some important differences between these two labels: only pre-fixed cells can be labeled with CIH since the positive colloid was used at pH 1.8. Cationized ferritin is a derivative of native ferritin that can be used at physiological pH either in pre-fixed or even in living cells (Danon et al. 1972). Treatments such as methylation and the use of neuraminidase prevent the staining by the CIH indicating that the iron micelles are bound almost exclusively to sialic acid (Gasic et al. 1968). Nowadays, poly-L-lysine-gold probes have also been used as markers for anionic sites (Goode et al. 1992). However, this technique has not been applied to trypanosomatids.

Labeling of *T. brucei* with CIH and CF was described by De Souza (1989) as results of unpublished observation in collaboration with Benchimol, Tetley and Vickerman. According to De Souza (1989), CIH and CF bind to the surface of epimastigote forms obtained from axenic culture. There is no binding of CIH to the surface of bloodstream trypomastigotes. A weak labeling with CF, indicated by a single layer of particles, is only observed on the surface of glutaraldehyde fixed trypomastigotes. No label was seen on parasites, which were incubated in the presence of CF before fixation or after fixation in formaldehyde. The same effect of glutaraldehyde fixation was described for the binding of antibodies against VSG on the surface of *T. brucei* (Masterson et al. 1988). According to Masterson and coworkers (1988) and De Souza (1989), there are at least two explanations of this phenomenon: a) fixation may denature the VSG, producing new epitopes which then should be recognized by antibodies; and b) that glutaraldehyde may induce conformational changes on the surface components of trypomastigote forms, leading to the exposure of cryptic epitopes and acidic amino acids which bind to CF but not to CIH (Masterson et al. 1988, De Souza 1989).

Both CIH and CF bind to the surface of epimastigote, amastigote and trypomastigote forms of *Trypanosoma cruzi* (Figs. 1a-1c) (Martinez-Palomo et al. 1976, De Souza et al. 1977, Carvalho et al. 1985, Souto-Padrón and De Souza 1986). The

extent of the binding is represented by the number of layers of particles on the surface of the different evolutive forms and could be arranged in the following decreasing order: bloodstream trypomastigotes > cell culture trypomastigotes > metacyclic trypomastigotes > amastigotes > epimastigotes. In epimastigotes both cationic particles appeared uniformly distributed over the cell surface and the flagellum, in the flagellar pocket and in the region between the flagellum and the cell body (Martinez-Palomo et al. 1976). In bloodstream trypomastigotes and amastigotes, CIH and CF particles were seen distributed homogeneously throughout the cell surface and flagellar membranes. However, no cationic particles were seen associated with the small portion of the flagellum which remains inside the flagellar pocket, with the membrane which lines the flagellar pocket or in the region of adhesion between cell body and flagellar membranes (Figs. 1a-1b). A special array of integral proteins is involved in the adhesion of the flagellum to the cell body impairing the diffusion of such cationic labels to the flagellar pocket (De Souza et al. 1977, Carvalho et al. 1985).

Treatment of *T. cruzi* with neuraminidase or trypsin completely abolishes the binding of CIH particles to the parasites surface and almost completely abolishes the binding of CF particles. This suggests that the cationic particles bind to sialoglycoproteins exposed on the surface of amastigote, epimastiogote and trypomastigote forms (Carvalho et al. 1985, Souto-Padrón and De Souza 1986).

There are few studies concerning the cytochemical localization of negative surface charge on trypanosomes of rodents (Dwyer 1975, Dwyer and D'Alessandro 1976a). Large quantities of cationized ferritin were bound to the cell body and flagellar membranes of *T. lewisi* and *T. musculi* bloodstream forms (Dwyer 1975, Dwyer and D'Alessandro 1976a). As described for trypomastigotes of *T. cruzi*, no particles were seen in the region of adhesion between the cell body and the flagellum and in the flagellar pocket (Dwyer 1975, Dwyer and D'Alessandro 1976a). Trypsin treated trypomastigotes from *T. lewisi* and *T. musculi* had 3 and 4 times less ferritin bound / $100\mu^2$ of cell surface area than intact bloodstream forms, respectively. Culture forms of *T. lewisi* presented 3 times less cationic particles than bloodstream forms and the labeling was observed on the cell and flagellar membranes, as well as in the region of adhesion between cell body and the flagellum and in the flagellar pocket (Dwyer 1975).

Promastigote and amastigote forms of some species of *Leishmania* also presented negative surface charge detected by cationic particles (Mühlpfordt 1975, Pimenta and De Souza 1983, Ayesta et al. 1985, Saraiva et al. 1989).

Cationized ferritin binds to the surface of promastigotes of *L. donovani*, *L. tropica* and *L. braziliensis* (Mühlpfordt 1975, Dwyer 1977, Ayesta et al. 1985). In *L. braziliensis* CF binding was used to identify promastigotes, which belong to pathogenic and non-pathogenic strains. Only pathogenic promastigotes were strongly agglutinated by CF and presented an abundant CF surface labeling (Ayesta et al. 1985). Cationized ferritin and colloidal iron hydroxide bind to the surface of amastigote and promastigote forms of *L. mexicana amazonensis* (Pimenta and De Souza 1983). An intense labeling with both CF and CIH was observed on the surface of all evolutive forms analysed. There are no differences in the amount of cationic particles bound to the surface of promastigotes which had been passed 5 or 176 times in axenic culture, thus suggesting that in the case of the strain of *L. mexicana amazonensis* studied there is no relationship between cell surface charge and its ability to induce lesion in hamsters. In the case of amastigotes, labeling was more intense when the membrane of the endocytic vacuole surrounded amastigotes isolated from lesions in hamsters. Treatment with neuraminidase did not interfere with the binding of cationic particles to the cell surface (Pimenta and De Souza 1983). Treatment of promastigotes of *L. mexicana amazonensis* with trypsin, alkaline phosphatase and phospholipase C, but not with neuraminidase, reduced the CF binding (Silva Filho et al. 1990).

Both cationic particles bind to the surface of different species of *Phytomonas* (Esteves et al. 1988, Vommaro et al. 1989). Treatments of the par-

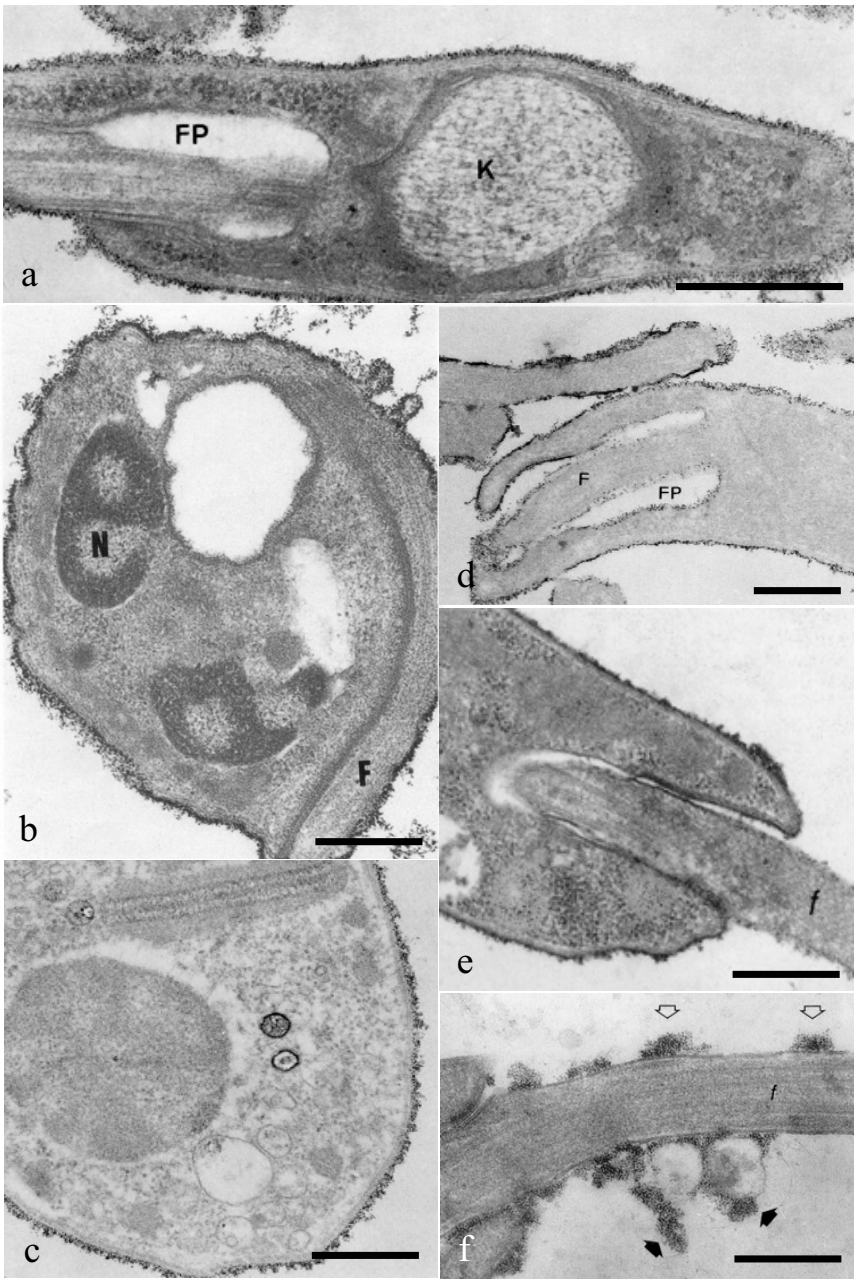

Fig. 1 – Binding of cationic particles to the surface of *Trypanosoma cruzi* (a-c) and *Leishmania mexicana amazonensis* (d-f). (a) Bloodstream form of *T. cruzi* incubated in the presence of cationized ferritin (CF) and (b) colloidal iron hydroxide (CIH). An intense labeling is observed on the cell surface. The flagellar pocket is unlabeled. (c) Amastigote form incubated in the presence of CF. Pre-fixed promastigote forms of *L. m. amazonensis* incubated in the presence of CIH (d) and CF. A uniform distribution of cationic particles is observed on both flagellar and cell body membranes. (e) Living promastigotes incubated in the presence of CF showed a non-uniform distribution of particles. CF patching (open arrows) and shedding (filled arrows) are observed. F or f, flagellum; FP, flagellar pocket; K, kinetoplast. Bars = 0.5μm. (After De Souza et al. 1977, Pimenta and De Souza 1983, Carvalho et al. 1985, Souto-Padrón and De Souza 1986, Saraiva et al. 1989).

asites with neuraminidase reduced the binding of CF and CIH particles only on the surface of *Phytomonas davidi* (Esteves et al. 1988).

Cytochemical studies on the surface charge of monogenetic trypanosomatids were made in different species of the genera *Crithidia* and *Herpetomonas*. *Crithidia deanei* is known to harbor endosymbiotic bacterium-like organism that is integrated into the physiology of the host cell (McGhee and Cosgrove 1980). Although symbiote-bearing and symbiote-free strains of *C. deanei* presented a negative cell surface charge due to the presence of sialic acid, no significant binding of CIH was observed and binding of CF to the surface of both strains was not observed (Oda et al. 1984). Binding of CF particles was observed on the cell surface of *Crithidia luciliae* and of both wild and drug-resistant mutant strains of *Crithidia fasciculata*. Enzymatic treatment with phospholipase C and neuraminidase showed that phosphate groups and sialic acid significantly contributed to the negative surface charge (Motta et al. 1991, Matta et al. 1992).

Cationic particles bind to the surface of *Herpetomonas samuelpessoai* (Soares et al. 1988), *Herpetomonas muscarum muscarum* (Lopes et al. 1989) and *Herpetomonas megaseliae* (Fiorini et al. 1991). In these studies parasites were incubated in the presence of substances which induce cell differentiation since it has been observed that the change in form is either accompanied by or preceded by changes in the cell surface composition. Dimethylsulphoxide (DMSO) treated *H. samuelpessoai* presented a more intense labeling compared with untreated cells (Soares et al. 1988). Differentiation of *H. muscarum muscarum* induced by propranolol caused a significant increase in the negative surface charge. In cells incubated in the presence of the drug the CF particles were not uniformly distributed on the cell body and flagellar membranes as described for control cells (Lopes et al. 1989). Previous results on *H. megaseliae* showed that lipopolysaccharides (LPS) trigger the process of cell differentiation from promastigotes to both para and opisthomastigote forms and affect the composition of membrane-associated polysaccharides (Fiorini et al. 1985). LPS treatment caused a marked decrease in binding of CF, CIH and Sendai virus particles to the cell surface (Fiorini et al. 1991).

More recently the Atomic Force Microscopy (AFM) was introduced in basic studies in biology and medicine. The AFM is based on the principle of scanning the surface of a sample with an atomically sharp tip of silicon on a very soft cantilever. AFM records the repulsive forces generated by the overlap of the electron cloud at the silicon tip with the electron clouds of surface atoms. According to the intensity of the repulsive forces the distance between the tip and the specimen varies. The force of deflection can be converted to a surface image of the specimen, using a computer. AFM resolution is limited only by the fineness of the silicon tip and not by any wavelength (Binning et al. 1986). Biological substances are resolved nearly atomic detail. The major advantage of AFM for biological research is that it can be used for imaging in solution. Atomic force microscopy associated to surface potential spectroscopy (SPS) was introduced on the study of cell surface charge (Aikawa et al. 1996,1997). SPS analysis uses a Si probe coated with CoCr, in order to obtain a high electronic conductivity of the tip surface, associated to a resonance frequency of about 70-90 kHz. This methodology provided the possibility to measure net surface charges on specific domains of cell membranes (Yokoyama and Inoue 1994).

According Akaki et al. (2001) the flagellum of amastigote forms of *T. cruzi* and amastigotes and promastigotes of *L. amazonensis* presented a positive charge of about +340, +90 and +70 mV, respectively. Promastigotes of *L. amazonensis* presented several positively charged spots on the anterior and posterior regions of the cell body while trypomastigote forms of *T. cruzi* did not show distinct charge differences over their entire surface. They also showed that the positive charged area on the surface of the invasive forms of these parasites makes the initial contact with host cells facilitating the binding of the invasive parasite to negatively charged host cell.

CELLULAR ELECTROPHORETIC MOBILITY

When a charged particle is suspended in an ionic medium, the ionic cloud of opposite charge that is attracted by the particle reduces the surface potential. The resultant potential, i.e. the sum of the potential on the surface of the particle and the potential of the ions in the neighborhood of the particle, is the Zeta potential (ζ) (Fig. 2). The ζ potential is evaluated by the mobility of cells under the influence of an electric current of known intensity and it appears to depend entirely on the composition of the suspending medium and is independent of the size, shape or the nature of the particle itself. The technique which measures the ζ potential was named cell electrophoresis or electrophoretic mobility. The mobility (V) of the cells and particles is expressed as μm.sec^{-1}.V^{-1}.cm, i.e. as velocity per unit field strength, and was converted into ζ potential by use of Helmoltz-Smoluchowski equation $\zeta = (4\pi\eta/\mathrm{D})\mathrm{V}$ where η and D are the viscosity and the dielectric constants of the suspending medium, respectively.

Several instruments were developed for determining the electrostatic charge (zeta potential) of small solid particles dispersed in water. In some of them, particles or cells are observed with a microscope. It is common to refer to this method as microelectrophoresis. Particles to be measured are placed in an electrophoresis chamber consisting of two electrode compartments and a connecting chamber, which can be cylindrical or rectangular. A voltage is applied between the two electrodes producing a uniform electric field in the connecting chamber. Cells respond by moving toward one or the other electrode according to the sign of their charges. The speed of a particle or cell is directly proportional to the magnitude of the surface charge or Zeta potential. Measurements must be made in the so-called **stationary planes** where the electrophoretic mobility of the particles measured is not affected by the circulation of the liquid inside the chamber. In some instruments the measuring chamber is in the interior of a temperature-controlled chamber connected to the circulating thermostat.

There are different kinds of microelectrophoresis apparatus. The common feature of all conventional instruments has been the measurement of one particle at a time. The velocity of the particles is measured by timing them over a given number of graticule squares in the eyepiece with the aid of a stopwatch, and at a given potential gradient. The electrophoretic mobility is expressed as μm.s^{-1}.V^{-1}.cm where μm = the distance covered during the measurement by the particle the particle under study, s = time (sec) required by the particle to cover the distance μm, V = the final voltage and cm = the distance between the two electrodes. This method allows the direct observation of particles displacement and the analysis of individual values of the electrophoretic mobility. In such conventional analysis time of measurement is relatively large inducing to errors such as: a) convection currents caused by heating of the sample; b) contamination of the sample by diffusion of electrode reactions products; c) flocculation of particles; d) polarization of the electrodes and others (Figs. 3a-b).

To avoid these errors a new technique for microelectrophoretic measurements was developed. In this technique the operator simultaneously observes and measures many particles with the same precision as they were measured at once. The result is a faster generation of data with fewer time-dependent errors. The microscope used to view the particles has a sharp depth focus and once the microscope is focused on the stationary layer, particles above or below are out of focus and therefore not visible. An appropriated shaped laser beam provides a sheet of illumination only in the focal plane of the microscope. This method is denominated rotating prism method. The prism is located inside the microscope interposed between the objective lens and the eyepiece. It is mounted on a galvanometer which causes the prism repeatedly to rotate few degrees and then flips back to start another cycle. This technique makes moving particles appear stationary. A zeta potential measurement is made by adjusting the prism control until the apparent motion caused by the prism exactly cancels the particle velocity caused by the applied field. At this point, particles appear stationary in the same focal plane.

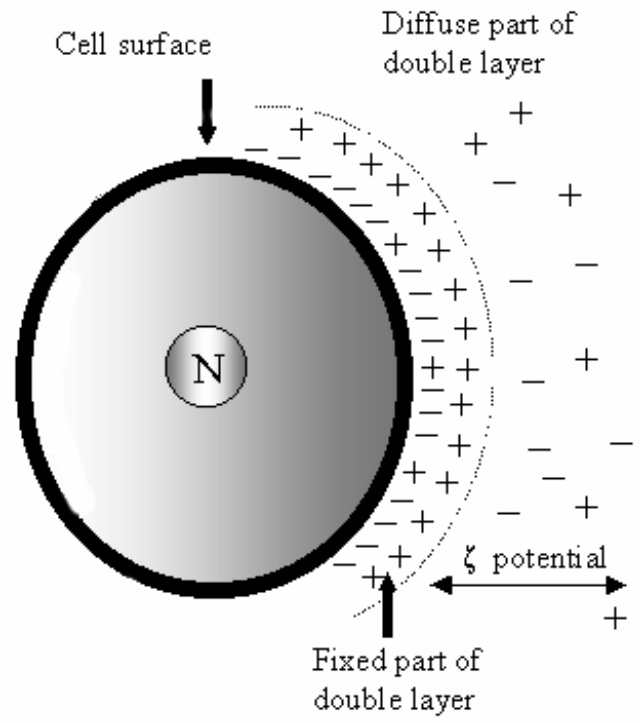

Fig. 2 – - The surface charge of a particle or cell influences the distribution of nearby ions in the polar medium. Ions of opposite charge (counter-ions) are attracted towards the surface and ions of like charge (co-ions) are repelled away from the surface. This leads to the formation of an electric double layer made up of the charged surface and a neutralizing excess of counter-ions over co-ions distributed in a diffuse manner in the polar medium. The electric double layer can be regarded generally as consisting of two regions: an inner or fixed region which may include adsorbed ions and a diffuse region in which ions are distributed according to the influence of electrical forces and random thermal motion. Electrokinetic or ζ (zeta) potential is measured on an imaginary plane (Stern plane) between the fixed part of double layer and the electrolyte solution (diffuse part of double layer).

The third method most used in the determination of cell surface charges was the free flow electrophoresis. The basic principle of free-flow electrophoresis is that the mixture of cells to be separated is injected in a fine stream into a solution which is flowing perpendicular to the lines of force of an electric field. Electrically charged particles are deflected from the direction of flow at an angle determined by a combination of the flow velocity and the electrophoretic mobility of the particle. Thus cells with different electrophoretic mobility move in different directions, and can be collected separately in a fraction collector after leaving the separation chamber (Fig. 3c).

The first report about the determination of cell surface charge in trypanosomatids was made by Broom and coworkers (1936). They incubated parasites and erythrocytes during 30 minutes in normal saline plus glucose and observed that negatively charged trypanosomes remained free, whereas positively charged parasites were attached to one or more erythrocytes (Broom et al. 1936). Later,

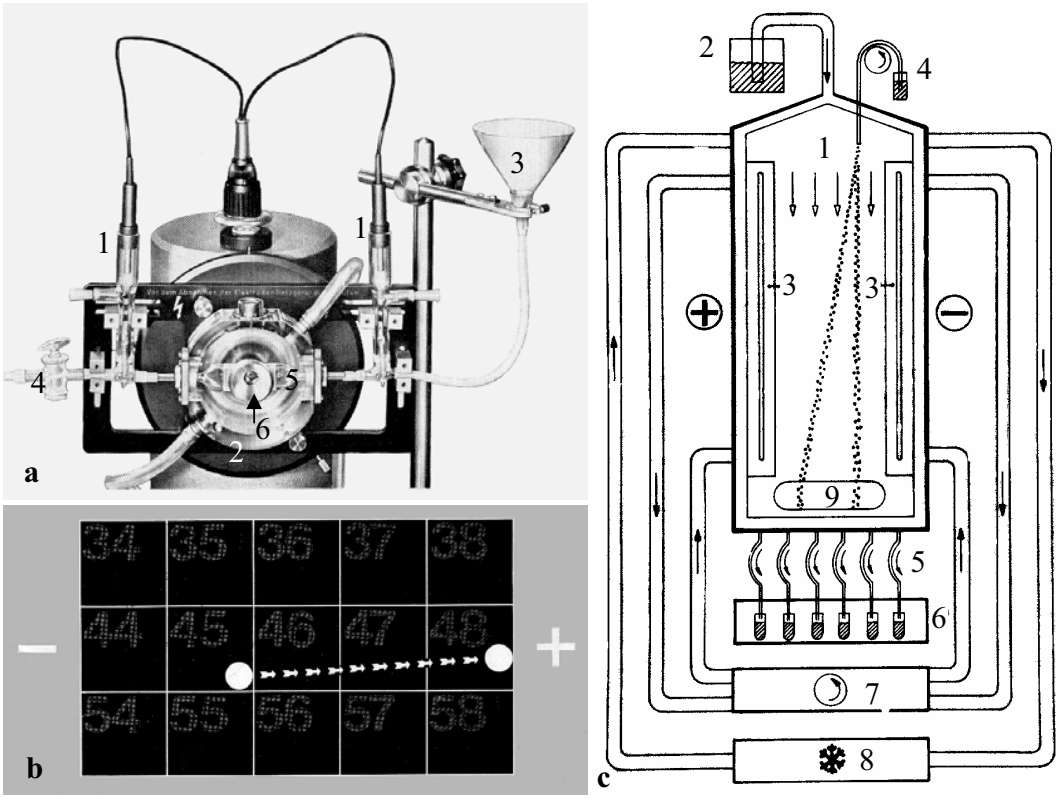

Fig. 3 – Principle and construction diagrams of microelectrophoresis (a-b) and free-flow electrophoresis. Fig. 3a – 1) Electrode compartment in electrical contact with the measuring chamber via a membrane; 2) Temperature controlled chamber connected to the circulating thermostat; 3) Funnel-cells in normal saline solution were introduced into the measuring chamber through plastic tubes. The liquid stream is controlled by a cock (4); 5) Measuring chamber; 6) Viewing window-place where the front lens of the microscope objective had to be connected to the temperature-controlled chamber. Fig. 3b – One of the focusing eyepieces of the light microscope contains a special reticule which serves as micrometer. The time it takes a cell edge to pass two or more whole micrometer intervals is measured with the aid of a stopwatch. Fig. 3c – 1) Separation chamber; 2) Chamber buffer; 3) Electrode compartment in electrical contact with the separation area via a membrane; 4) Sample pump; 5) Multitube pump; 6) Cooled fraction container with vials for sample collection; 7) Electrode buffer circulation; 8) Electronically regulated cooling system. 9) Optical window for analytical measurements.

Hollingshead and coworkers (1963) using the same methodology showed that bloodstream forms of *T. lewisi*, *T. cruzi* and *T. rhodesiense* did not attach to normal erythrocytes whereas under the same conditions bloodstream forms of *T. equinum*, *T. vivax* and *T. congolense* were completely covered by erythrocytes. In the same study Hollingshead and coworkers (1963) determined the electrophoretic mobility (EPM) of some trypanosome species and observed that the random orientation of trypanosomes is unaffected by the electric field, suggesting that there can be no gross localization of charge at either tip of the trypanosomatids, as previously described for spermatozoa (Lopes et al. 1987). Bloodstream forms of *T. rhodesiense*, the causative organism of human Sleeping Sickness, presented a mean electrophoretic mobility of about -0.1μm.s^{-1}.V^{-1}.cm while culture forms are more negative with a mean EPM of -0.91μm.s^{-1}.V^{-1}.cm (Table I). More recently Souto-Padrón and coworkers (1990) analyz-

ing the EPM of *T. brucei*, the causative agent of Nagana in livestock and which is non infective to man, showed that bloodstream forms presented a mean electrophoretic mobility of -0.68μm.s^{-1}.V^{-1}.cm while epimastigotes presented a mean EPM of -0.83μm.s^{-1}.V^{-1}.cm (Table I). An important observation is that the mean electrophoretic values for bloodstream forms of both species are less negative than those observed for epimastigote forms. It is important to mention that according to Broom and coworkers (1936) bloodstream form of *T. gambiense*, another specie of parasite causing human Sleeping Sickness, also presented positive or slight negative surface charge, as mentioned for *T. rhodesiense*.

T. brucei and *T. rhodesiense* belong to the subgenus *Trypanozoon* which is the most homogeneous group of Salivarian trypanosomes, represented by species which are morphologically indistinguishable but differing in biological features. The possibility to identify small differences on surface charge suggests that cell electrophoresis, under controlled conditions, would help to distinguish morphologically identical trypanosomatids.

Trypomastigote forms of *T. equinum*, another member of the Salivarian trypanosomes, had an electrophoretic mobility which varied from 0 to $+0.4\mu$m.s^{-1}.V^{-1}.cm. The cell surface charge increases in magnitude as the parasite was kept in culture medium for 1h at room temperature, reaching a mean EPM value of -0.95μm.s^{-1}.V^{-1}.cm. This change in surface charge accompanies loss of motility and death. The same behavior was described for bloodstream forms of *T. rhodesiense*. According to Hollingshead and coworkers (1963), the loss of surface component may be responsible for the change with time to a more negative electrophoretic mobility.

Cell surface charge of *Trypanosoma cruzi* has been analyzed in more detail (De Souza et al. 1977, Kreier et al. 1977, Souto-Padrón et al. 1984, 1990, Carvalho et al. 1985, Souto-Padrón and De Souza 1985, Bonaldo et al. 1988). The electrophoretic mobility analysis of the different developmental stages of *T. cruzi* showed that each evolutive form has a characteristic mean EPM which does not vary in the different strains or isolates of the parasite, is independent of the medium in which the cells were grown and do not change after glutaraldehyde fixation (Table II) (Souto-Padrón et al. 1990). Epimastigotes present the smallest negative surface, with a mean EPM of -0.65μm.s^{-1}.V^{-1}.cm. Amastigotes obtained from cell culture infected with parasites of the Y or CL strains, or isolated from the spleen of mice infected with the Y strain of the parasite, present similar mean EPM, which is of about -0.85μm.s^{-1}.V^{-1}.cm (Carvalho et al. 1985). Trypomastigotes present a mean EPM of -1.15μm.s^{-1}.V^{-1}.cm which is the most negative surface charge compared with the other evolutive forms. As observed for the epimastigote and amastigote forms, the mean EPM of trypomastigotes obtained from the bloodstream of infected mice, from the supernatant of cell cultures, from axenic culture in complex or defined media does not differ significantly (Souto-Padrón et al. 1984, 1990, Bonaldo et al. 1988). Some results are not in agreement with those reported by Kreier and coworkers (1977) and Murray and coworkers (1982). Using free-flow electrophoresis, Murray and coworkers (1982) found that amastigotes have the lowest charge, epimastigote the highest and the charge of trypomastigotes law in between. Kreier and coworkers (1977) reported that trypomastigotes had less negative charge than epimastigotes and that amastigotes had the same charge as epimastigote. The results obtained by Kreier and coworkers (1977) and Murray and coworkers (1982) do not explain the difficulties found for the separation of erythrocytes and bloodstream forms using DEAE-cellulose column, which is probably due to the ionic strength of the solution in which the parasites were suspended, as will be discussed below.

Measurements of the mean EPM of *T. cruzi* maintained in axenic culture under conditions which stimulate the process of transformation of epimastigote into trypomastigote forms indicate that during this process there was a gradual increase in the negative surface charge of the parasites (De Souza et al. 1977, Souto-Padrón et al. 1984, Bonaldo et

TABLE I

Mean electrophoretic mobility of trypanosomatids.

Species	Strain/ Isolate	Developmental form	Mean EPM (μm.s^{-1}.V^{-1}.cm)	References
T. brucei		Epimastigote	–0.83	Souto-Padrón et al. (1990)
		Trypomastigote[a]	–0.68	
T. cruzi	Y	Epimastigote	–0.65	Souto-Padrón et al. (1984)
		Trypomastigote[b]	–1.15	Carvalho et al. (1985)
		Amastigote	–0.85	
T. conorhini		Epimastigote	–1.73	Souto-Padrón et al. (1990)
T. dionisii		Epimastigote	–1.05	Souto-Padrón et al. (1990)
		Trypomastigote[c]	–1.90	
T. equinum		Trypomastigote	+0.40	Hollingshead et al. (1963)
T. lewisi		Trypomastigote	–0.61	Hollingshead et al. (1963)
T. myoti	1	Epimastigote	–1.03	Souto-Padrón et al. (1990)
	3	Epimastigote	–1.03	
	4	Epimastigote	–1.06	
	5	Epimastigote	–1.14	
T. rangeli		Epimastigote	–0.62	Souto-Padrón et al. (1990)
T. rhodesiense		Epimastigote	–0.91	Hollingshead et al. (1963)
		Trypomastigote[d]	–0.10	
T. vespertilionis		Epimastigote	–0.61	Souto-Padrón et al. (1990)
Subgenus	M5	Epimastigote	–0.57	Pinto et al. (1996)
Schizotrypanum[e]	M29	Epimastigote	–0.56	
Leishmania	Josefa	Amastigote[f]	–1.58	Pimenta and
mexicana		Amastigote[g]	–1.14	De Souza (1983)
amazonensis		Promastigote[h]	–1.14	
		Promastigote[i]	–1.49	
		Promastigote[j]	–1.47	
Phytomonas davidi			–0.98	Esteves et al. (1988)
Phytomonas sp*	Isolate 1		–1.22	
	Isolate 2		–1.09	Vommaro et al. (1989)
	Isolate 3		–1.08	
	Isolate 4		–1.03	
Herpetomonas samuelpessoai		Promastigote	–0.76	Soares et al. (1988)
Herpetomonas muscarum muscarum		Promastigote	–0.69	Lopes et al. (1989)
Herpetomonas megaseliae		Promastigote	–0.98	Fiorini et al. (1991)
Herpetomonas roitmani		Promastigote	–0.73	Faria-e-Silva et al. (1999)
		Opisthomorph	–1.20	

TABLE I (continuation)

Species	Strain / Isolate	Developmental form	Mean EPM (μm.s^{-1}.V^{-1}.cm)	References
Crithidia deanei	Symbiote free	Choanomastigote	–0.99	Oda et al. (1984)
	Symbiote bearing	Choanomastigote	–0.85	
Crithidia luciliae		Choanomastigote	–0.80	Motta et al. (1991)
Crithidia fasciculata		Choanomastigote	–0.97	Matta et al. (1992)

[a]Bloodstream trypomastigotes isolated using DEAE cellulose column; [b]Bloodstream trypomastigotes isolated by differential centrifugation; [c]Trypomastigotes obtained in axenic culture medium and isolated using DEAE-cellulose column; [d]Bloodstream trypomastigotes; [e]Trypanosomes of the subgenus *Schizotrypanum* isolated from the bat *Phyllostomus hastatus*; [f]Amastigote forms surrounded by the membrane of the endocytic vacuole; [g]amastigote forms without the membrane of endocytic vacuole; [h]Promastigote forms in the 1st passage in axenic culture; [i]Promastigote forms in the 5th passage in axenic culture; [j]Promastigote forms in the 176th passage in axenic culture; *Isolate 1, from *Euphorbia hyssopifolia*; Isolate 2, from *Euphorbia pinea*; Isolate 3, from *Euphorbia characias*; Isolate 4, from *Manihot esculenta*.

al. 1988). Transitional forms, which have a rod-like kinetoplast localized beside the cell nucleus, presented a mean EPM similar to the trypomastigote forms indicating that during metacyclogenesis, morphological changes are preceded by alterations in some physiological characteristics (Bonaldo et al. 1988).

The pH of the solution in which the parasites are suspended interferes directly on the mean EPM indicating that the surface of *T. cruzi* 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. At low pH values the negative charge decrease and below a certain value which corresponds to the isoelectrophoretic point, the surface become positive. The isoelectrophoretic point is around 2.0 for amastigotes and epimastigotes, and around 3.0 for trypomastigotes (Souto-Padrón et al. 1984, Carvalho et al. 1985).

The ionic strength of the solution in which the parasites are suspended also interferes with the mean EPM. In a solution with an ionic strength of 0.072 mol.dm^{-3} epimastigotes have a mean EPM of -1.19μm.s^{-1}.V^{-1}.cm, a value similar to that of trypomastigote forms suspended in a solution with an ionic strength of 0.145 mol.dm^{-3}.

Cell electrophoresis, in association with treatment of parasites with cationized ferritin, enzymes, lectins and inhibitors of protein synthesis and protein glycosylation, was used to analyze the nature of surface components which contribute to the net negative cell surface charge (Meirelles et al. 1984, Souto-Padrón et al. 1984, Carvalho et al. 1985, Souto-Padrón and De Souza 1985, 1986). Binding of cationized ferritin to the surface of trypomastigote forms reduced the mean EPM by about 53% while the same treatment did not interfere with the mean surface charge of epimastigote forms (Meirelles et al. 1984).

Incubation of *T. cruzi* in the presence of neuraminidase from *Clostridium perfringens*, under conditions which do not interfere with cell viability, suggested that sialic acid residues exposed on the parasite surface and sensitive to the enzyme, account for about 18, 37 and 50% of the negative surface charge of epimastigote, amastigote and trypomastigote forms, respectively. It was observed, however, that surface residues of sialic acid of epimastigote forms were much more susceptible to the neuraminidase from *Vibrio cholerae* than to the neuraminidase from *C. perfringens*. Differences in susceptibility of sialic acid residues to distinct neuraminidases were previously described by Tamura and coworkers (1982) and Seaman and Heard (1960) on erythrocytes.

TABLE II

Mean electrophoretic mobility of *Trypanosoma cruzi.**

Developmental stage	Source	Origin	EPM[g] ($-\mu$m.s^{-1}.V^{-1}.cm)
Epimastigote	M 16 medium	Y strain	0.65 ± 0.11
Epimastigote	M 16 medium	CL strain	0.74 ± 0.11
Epimastigote	Warrem medium	Y strain	0.62 ± 0.09
Epimastigote	TAU medium	Dm28c clone	0.64 ± 0.02
Epimastigote	TAU 3AAG medium	Dm 28c clone	0.66 ± 0.05
Amastigote	Spleen	Y strain	0.86 ± 0.20
Amastigote	J774 G8 cells	Y strain	0.85 ± 0.02
Amastigote	J774 G8 cells	CL strain	0.86 ± 0.10
Amastigote	LA9 cells	Y strain	0.82 ± 0.12
Trypomastigote	M 16 medium	Y strain	1.20 ± 0.20
Trypomastigote	M 16 medium	CL strain	1.10 ± 0.15
Trypomastigote	Tissue culture	Y strain	1.04 ± 0.11
Trypomastigote	Tissue culture	CL strain	1.04 ± 0.05
Trypomastigote	Bloodstream	Y strain	1.15 ± 0.17
Trypomastigote	Bloodstream	CL strain	1.10 ± 0.07
Trypomastigote	M 16 medium	1001 stock[a]	1.08 ± 0.07
Trypomastigote	M 16 medium	1042 stock[a]	1.09 ± 0.07
Trypomastigote	M 16 medium	1048 stock[a]	1.11 ± 0.07
Trypomastigote	M 16 medium	A1-DN clone[b]	1.09 ± 0.06
Trypomastigote	M 16 medium	A1-7 clone[b]	1.09 ± 0.05
Trypomastigote	M 16 medium	147 isolate[c]	1.10 ± 0.06
Trypomastigote	M 16 medium	271 isolate[c]	1.06 ± 0.07
Trypomastigote	M 16 medium	181 isolate[d]	1.06 ± 0.06
Trypomastigote	M 16 medium	143 isolate[d]	1.11 ± 0.05
Trypomastigote	M 16 medium	231 isolate[d]	1.08 ± 0.06
Trypomastigote	M 16 medium	254 isolate[d]	1.07 ± 0.05
Trypomastigote	M 16 medium	1049 isolate[e]	1.10 ± 0.07
Trypomastigote	M 16 medium	Reis[f]	1.09 ± 0.05
Trypomastigote	M 16 medium	Silvio[f]	1.08 ± 0.07
Trypomastigote	M 16 medium	Noel[f]	1.09 ± 0.06

For details see Souto-Padrón et al. 1984, Carvalho et al. 1985, Bonaldo et al. 1988, Souto-Padrón et al. 1990. [a]Isolated from the opossum *Didelphis albiventris*; [b]Isolated from a chronic patient and which belong all to zymodeme A; [c]Isolated from a chronic patient and which belong all to zymodeme B; [d]Isolated from a chronic patient and which belong all to zymodeme C; [e]Isolated from triatomine *Panstrongylus megistus*; [f]Isolated from patients with the acute phase of Chagas' disease; [g]Mean EPM plus standard deviation.

Treatment of *T. cruzi* with trypsin reduces the mean EPM of epimastigote, amastigote and trypomastigote forms by 11, 32 and 35%, respectively, suggesting that sialoglycoproteins contribute to the surface charge of *T. cruzi*. Neuraminidase and trypsin-treated parasites recover their normal mean EPM when incubated in fresh culture medium for 2 and 4h, respectively. Addition of puromycin (an inhibitor of protein synthesis) and tunicamycin (an inhibitor of the process of *N*-glycosilation of proteins) to the culture medium partially inhibited the recovery of the normal mean EPM of trypsin- or

neuraminidase-treated parasites (Souto-Padrón et al. 1984, Souto-Padrón and De Souza 1985, 1986). From these results it was suggested that in trypomastigotes of *T. cruzi* about 37% of the sialic acid residues exposed on the surface are associated with proteins, mainly to *N*-glycosylated proteins, and about 63% with glycolipids. Interestingly, the incubation of trypomastigote forms in the presence of the lectin *Limulus polyphemus* (LPA), which binds to the 4-hydroxyl and carboxyl groups of glycosidically linked sialic acid (Schauer 1982) decreased by about 50% the negative surface charge of trypomastigote forms which is very similar to that obtained when trypomastigote forms were incubated in the presence of cationized ferritin (Meirelles et al. 1984) or neuraminidase (Souto-Padrón et al. 1984, Souto-Padrón and De Souza 1985). More recently, it was observed that LPA not only binds to sialic acids but also to galNAc and glcNAc residues.

Trypanosomatids are unable to synthesize sialic acids. The acquisition of sialic acid is due by a sialic acid metabolizing enzyme named *trans*-sialidase (Colli 1993, Cross and Takle 1993, Schenkman et al. 1994, Frasch 2000). This enzyme is found in epimastigote and trypomastigote forms and catalyzes the transfer of sialic acid from host glycoconjugates to a terminal Galβ of an appropriate molecule on the parasite surface (Colli 1993, Schenkman et al. 1994). The major acceptor of sialic acid of the cell surface *trans*-sialidase is GPI-anchored glycoproteins rich in threonine, serine and proline. Sialic acid is incorporated into O-linked oligosaccharides via *N*-acetylglucosamine and is called mucin-like molecules or TcMUC (Previato et al. 1994). TcMUC resembled the mammalian mucins and were firstly described by Alves and Colli in 1975. There are some evidences that sialic acid residues present on the surface of trypomastigote forms of *T. cruzi* play important roles on the invasion of host cell, on the circulation of parasites in the extracellular matrix or bloodstream and on the resistance of parasite lysis by the alternative pathway of complement (Kipnis et al. 1981, Araújo-Jorge and De Souza 1984, Schenkman and Eichinger 1993). Besides the epimastigote and trypomastigote forms of *T. cruzi trans*-sialidase is also found in insect forms of *T. brucei* (Pontes de Carvalho et al. 1993, Montagna et al. 2002) and *Endotrypanum* (Medina-Acosta et al. 1994).

Treatment of epimastigote and trypomastigote forms with phospholipase C was only effective after previous treatment with trypsin. According to Souto-Padrón and De Souza (1985), phospholipase sensitive phosphate groups account for 17% of the negative surface charge.

Trypanosomes of the subgenus *Schizotrypanum* are morphologically almost indistinguishable from each other and those from bats have cosmopolitan distribution. Parasites isolated from bats are able to infect vertebrate cells where they reproduce as amastigotes, with an intracellular behavior characteristic of members of the *Schizotrypanum* sub-genus. In Latin America *Trypanosoma (Schizotrypanum) cruzi* can be found in several mammalian orders, including bats (Pinto and Costa Bento 1986). Thus, in this region it is of considerable importance to public health to determine whether isolates from bats are distinct from *T. cruzi*. The data presented in Table I show that it is possible, using the electrophoretic mobility, to distinguish *T. cruzi* from other members of the sub-genus *Schizotrypanum*, such as *T. dionisii*, *T. myoti* and M5 and M29 stocks isolated from the bat *P. hastatus* in Minas Gerais, Brazil (Souto-Padrón et al. 1990, Pinto et al. 1996). In the case of *T. dionisii* differences could be observed both in epimastigote and tripomastigote forms. Trypomastigote forms of *T. dionisii* have a very negative surface charge, with a mean EPM of $-1.90\mu\text{m.s}^{-1}.\text{V}^{-1}.\text{cm}$. It was not possible to distinguish epimastigotes of *T. cruzi* and *T. vespertilionis*, since they present a similar mean EPM. The same was observed for epimastigotes of *T. dionisii* and *T. myoti* (Souto-Padrón et al. 1990). In spite of the presence of several common antigens in these trypanosomes at least one antigen of *T. myoti*, *T. vespertilionis* and *T. dionisii* was distinct from those of the other species (Bower and Woo 1982).

Trypanosoma rangeli is one of the most interesting mammalian trypanosomes. *T. rangeli* and *T. cruzi* are morphologically similar (Vallejo et al.

1988), occur in the same mammalian hosts and have common Triatomine vectors. As the second known American trypanosome of man, it is of medical importance since mixed infections may occur in both vertebrate and invertebrate hosts (Hoare 1972). Albeit infection by *T. rangeli* is apparently harmless for the human host, the parasite induces a humoral response with cross-reacting antibodies against *T. cruzi*, causing misleading serodiagnosis of Chagas' disease. It was not possible to distinguish epimastigote forms of *T. cruzi* and *T. rangeli* using cell electrophoresis since they present a similar mean EPM (Table I). However, they can be distinguished using other methods such as sialic acid content (Schottelius 1984), the presence of neuraminidase in the supernatant of cultures (Schottelius 1987) and PCR assays for the amplification of a specific DNA sequence for *T. rangeli* (Vargas et al. 2000).

There are few data about the electrophoretic mobility of trypanosomatids parasite of rodents (Hollingshead et al. 1963, Souto-Padrón et al. 1990). Trypomastigote forms of *T. conorhini* presented a high negative surface charge with a mean EPM of -1.73μm.s^{-1}.V^{-1}.cm while epimastigote forms of *T. lewisi* presented a mean EPM of -0.61μm.s^{-1}.V^{-1}.cm (Table I).

Among the several *Leishmania* species only *Leishmania mexicana amazonensis* was analyzed by cell electrophoresis (Pimenta and De Souza 1983, Saraiva et al. 1989, Silva Filho et al. 1990). All evolutive forms of *L. mexicana amazonensis* studied exhibit negatively charged surfaces. Amastigotes isolated just after homogenization of lesion experimentally induced in hamsters, and which is surrounded by a membrane from the endocytic vacuole of the macrophage, present a very high negative surface charge, with a mean EPM of -1.58μm.s^{-1}.V^{-1}.cm. When this parasite is incubated for 4h in a culture medium, the endocytic membrane that surrounds the parasites is eliminated and the cell surface becomes less negative, with a mean EPM of -1.14μm.s^{-1}.V^{-1}.cm. When amastigote forms obtained from lesions were incubated in axenic medium and maintained at 25°C they gradually transform into promastigote forms. Freshly transformed promastigotes, which have been obtained in the first 48h of culture, and those passed 5-7 times through this medium, were considered infective since they are able to induce lesions when incubated into hamsters. Those promastigotes, which have been transferred 175 or more times in the same axenic medium, are considered noninfective promastigotes (Saraiva et al. 1986).

Promastigotes in the first passage *in vitro* had a mean EPM of -1.14μm.s^{-1}.V^{-1}.cm. Gradually the cell surface charge of promastigote forms became more negative and after five passages parasites presented a surface charge, which varied from -1.10 to -1.79μm.s^{-1}.V^{-1}.cm with mean EPM of -1.49μm.s^{-1}.V^{-1}.cm (Table I). No infective promastigotes presented a mean EPM of -1.47μm.s^{-1}.V^{-1}.cm. indicating that there is an increase in the negativity of the cell surface during amastigote-promastigote transformation and that there is no relationship between cell surface charge of promastigote forms and its ability to induce lesions in hamsters (Pimenta and De Souza 1983). Interestingly, promastigotes of *L. mexicana amazonensis* that had undergone more than 450 passages *in vitro* presented a mean EPM varying between 1.14 and 1.22μm.s^{-1}.V^{-1}.cm (Saraiva et al. 1989, Silva Filho et al. 1990).

Electrophoretic mobility of promastigote and amastigote forms of *L. mexicana amazonensis* varies according to the pH of the solution in which the cells are suspended, indicating the presence of both negatively and positively charged cell surface dissociating groups (Pimenta and De Souza 1983).

The nature of components that contribute to the surface properties of both amastigote and promastigote forms of *L. mexicana amazonensis* was investigated by the use of cationized ferritin and enzymes such as trypsin, neuraminidase, alkaline phosphatase and phospholipase C (Pimenta and De Souza 1983, Saraiva et al. 1989, Silva Filho et al. 1990). Incubation of amastigote and promastigote forms in the presence of cationized ferritin renders their surfaces less negative (Table III). Neuraminidase isolated from *C. perfringens* reduced the EPM of infective promastigotes by about 46% (Ta-

ble III). Neuraminidase treated promastigotes recover their mean EPM when incubated for 8h in fresh medium by a process that is inhibited by puromicin. Neuraminidase from *V. cholerae* did not alter the EPM of any evolutive form analyzed. Trypsin reduced in about 28 and 22% the cell surface charge of virulent and avirulent promastigotes, respectively. Treatment of promastigotes with alkaline phosphatase (AlkPase) and phospholipase C (PPase) showed the importance of phospholipids as one of the surface anionic species in *L. mexicana amazonensis* (Silva Filho et al. 1990). Alkaline phosphatase treatment reduced by about 30% the cell surface charge of avirulent promastigotes (Table III). Phospholipase C from *Bacillus cereus* significantly reduced the cell surface charge of both virulent and avirulent promastigotes. This effect can be explained by the selective action of phospholipase C from *Bacillus cereus* on Lipophosphoglycan (LPG), the major surface glycoconjugate of promastigote forms of all analyzed *Leishmania* species that has a highly anionic nature and is phosphatydilinositol-anchored (Handman and Goding 1985, Turco 1988).

Parasites of the genus *Phytomonas* are responsible for important diseases in plants of economical interest such as coconut (Parthasarathy and Slobbe 1978), oil palm (Slobbe et al. 1978) and cassava (Kitajima et al. 1986). They are transmitted to the plants through the saliva of phytophagous insects. Most of the parasites occur in plants and insects as promastigote forms presenting a very twisted cell body.

Phytomonas davidi promastigotes, which were isolated from the milkweed plant and have been maintained for several years in axenic medium, present a negative cell surface charge with a mean EPM of -0.99μm.s^{-1}.V^{-1}.cm. (Table I) (Esteves et al. 1988). Another four isolates were also obtained from milkweed plants such as *Euphorbia hyssopifolia*, *Euphorbia pinea*, *Euphorbia characias* and *Manihot esculenta* (Dollet et al. 1982, McGhee and Postell 1976, Attias and De Souza 1986, Vainstein and Roitman 1986, Vainstein et al. 1987). All of them presented negative cell surface charge with a mean EPM varying from -1.03 to -1.22μm.s^{-1}.V^{-1}.cm (Table I). Despite biochemical and ultrastructural evidences of the presence of sialic acid on the surface of all analyzed isolates of *Phytomonas*, treatment with neuraminidase only slightly reduced the mean EPM of *P. davidi* and did not reduce the mean EPM of the isolates from *E. hyssopifolia*, *E. pinea*, *E. characias* and *M. esculenta* (Esteves et al. 1988, Vommaro et al. 1989).

In addition to *Phytomonas* it was shown that both plants and phytophagous insects can harbor parasites from genera *Crithidia*, *Herpetomonas* and *Leptomonas*. Since some of them share indistinguishable promastigote forms, various approaches have been proposed to classify plant trypanosomatids such as the production of monoclonal antibodies, the analysis of enzymatic activities and the use of molecular markers (Camargo 1999). In this context the determination of cell electrophoresis mobility associated to enzymatic treatments would be useful to help the identification of those parasites.

The genus *Crithidia* comprises parasites of insects that were originally characterized by the presence of amastigote and choanomastigote forms in their life cycle (Hoare and Wallace 1966). Cell surface charge of three species of the genus *Crithidia* was analyzed by determining the cellular electrophoretic mobility, ultrastructural cytochemistry, thin layer and gas liquid chromatography, lectin agglutinations assays and enzymatic treatments. *Crithidia deanei*, *C. fasciculata* and *C. luciliae* have a net negative cell surface charge (Table I) (Oda et al. 1984, Motta et al. 1991, Matta et al. 1992). Treatment of these strains with neuraminidase from *C. perfringens* only reduced significantly the EPM of *C. deanei*. Enzyme treatment of *C. fasciculata* and *C. luciliae* showed that phosphate groups, but not sialic acid, contribute to the negative surface charge of these parasites (Motta et al. 1991).

Crithidia deanei belongs to a restrict group of trypanosomatids presenting bacterium endosymbionts in the cytoplasm, which divide synchronously with the host cell (De Souza and Motta 1999). This group also includes *C. oncopleti* (Newton and Horne 1957), *C. desouzai* (Fiorini et al. 1989), *Blast-*

TABLE III

Effect of cationized ferritin and enzymatic treatments on the mean electrophoretic mobility of *Leishmania mexicana amazonensis.**

Evolutive form	Treatment	Percentual change
Amastigote[a]	None	
	Nanase (*C. perfringens*)	–50.0
Amastigote[b]	None	
	Cationized Ferritin	–11.6
	Nanase (*C. perfringens*)	–3.0
Virulent Promastigote[c]	None	
	Cationized Ferritin	–11.6
	Nanase (*C. perfringens*)	–46.0
	Nanase (*V. cholerae*)	–3.3
	Trypsin	–27.6
	AlkPase	–21.7
	PPase (*C. perfringens*)	–13.8
	PPase (*Bacillus cereus*)	–32.2
Avirulent Promastigote[d]	None	
	Cationized Ferritin	–26.3
	Nanase (*C. perfringens*)	–26.1
	Nanase (*V. cholerae*)	–
	Trypsin	–22.1
	AlkPase	–31.2
	PPase (*C. perfringens*)	–8.3
	PPase (*Bacillus cereus*)	–22.3

*For details see Pimenta and De Souza 1983, Saraiva et al. 1989, Silva Filho et al. 1990. [a]Amastigote forms surrounded by the membrane of the endocytic vacuole; [b]Amastigote forms without the membrane of the endocytic vacuole; [c]Obtained by in vitro culture of skin biopsies from hamster lesions; [d]Promastigote forms which had passed 500 times in axenic cultures.

ocrithidia culicis (Novy et al. 1907), *Herpetomonas roitmani* (Faria-e-Silva et al. 1991) and *Trypanosoma cobitis* (Lewis and Ball 1980). The possibility of elimination of the endosymbiont by the use of antibiotics has increased the interest in the study of endosymbiont-harboring species. The available data indicate that the presence of the endosymbiont induces morphological changes, as the lack of paraflagellar structure located in the flagellum (Frey-müller and Camargo 1981) interferes with the metabolism of the trypanosomatid (Newton 1957, Mundim et al. 1974, Alfieri and Camargo 1982, Salzman et al. 1985, De Souza and Motta 1999), diminishes the secretion of proteolytic enzymes (d'Avila-Levy et al. 2001), interferes with surface properties of the protozoan such as exposition of carbohydrate residues (Dwyer and Chang 1976, Esteves et al. 1982, McLaughlin and Cain 1985, Faria e Silva et al. 1994) and on the surface charge (Oda et al. 1984). Symbiont-free *C. deanei* presents a mean EPM of -0.99μm.s^{-1}.V^{-1}.cm, which is 15% more negative than the symbiont-free strain. Treatment of *C. deanei* with neuraminidase reduced in about 45% the EPM of the protozoan, irrespective of the presence of the endosymbiote (Oda et al. 1984).

Parasites of the genus *Herpetomonas* have been used to analyze changes on cell surface during the process of differentiation *in vitro*, since the

composition of the plasma membrane and the surface coat are of primary importance in cell response to environmental stimuli and in the interaction of parasites with their hosts (Soares et al. 1988, Lopes et al. 1989, Fiorini et al. 1991, Faria-e-Silva et al. 1999). Besides these aspects, *Herpetomonas* sp presents four developmental stages (promastigotes, paramastigotes, opisthomastigotes and opisthomorphs), are non pathogenic for men and show close antigenic similarities to *Trypanosoma cruzi* (Souza and Roitman 1971, Souza et al. 1974).

In most species of the genus *Herpetomonas*, cultures in the logarithmic phase of growth (48h of cultivation) show almost exclusively undifferentiated promastigote forms. Promastigotes of *H samuelpessoai*, *H. muscarum muscarum*, *H. megaseliae* and *H. roitmani* display a negative surface charge, with mean EPM varying, according to the species analyzed (Table I). Treatment of *Herpetomonas* with 2-deoxy-D-glucose (Angluster et al. 1977), concanavalin-A (ConA) (Souza et al. 1980), dimethylsulfoxide (DMSO) (Castellanos et al. 1981), propranolol (Lopes et al. 1983) and lipopolysaccharide (LPS) (Fiorini et al. 1985) triggers the process of cell differentiation from promastigote to para- and opisthomastigote forms, affecting the composition of membrane-associated polysaccharides (Alviano et al. 1981, Soares et al. 1984), cell surface charge (Soares et al. 1988, Lopes et al. 1989) and cell respiration (Fiorini et al. 1985). Dimethiylsulfoxide and propranolol caused a significant increase in the net negative surface charge of both *H. samuelpessoai* and *H. muscarum muscarum* due to a markedly increase in the number of *N*-acetylneuraminic acid residues per cell (Soares et al. 1988, Lopes et al. 1989). However, LPS treatment of *H. megaseliae* caused a marked decrease in the negative cell surface altering the nature and number of sialic acid residues. Only *N*-acetylneuraminic acid can be detected on untreated flagellates whereas *N*-acetyl and *N*-glycolylneuraminic acid are found on the parasites exposed to LPS (Fiorini et al. 1991). Incubation of DMSO-, propranolol- and LPS-treated and untreated *Herpetomonas* with neuraminidase from *C. perfringens* resulted in decrease of their mobility to the negative pole (Table IV).

More recently Faria-e-Silva and coworkers (1991) isolated a new *Herpetomonas* species, *H. roitmani*. This parasite was isolated as opisthomorph forms, which proliferate in culture unlike other *Herpetomonas* species (Faria-e-Silva et al. 1996). Promastigote and opisthomorph forms of *H. roitmani* display a net negative charge of about -0.73 and $-1.20 \mu m.s^{-1}.V^{-1}.cm$, respectively. Neuraminidase and phospholipase C treatments significantly reduced the surface charge of both evolutive forms (Table V). The authors concluded that sialic acid is an important component of the cell surface of *H. roitmani*, which is probably added to surface glycoconjugates during the process of promastigote-opisthomorph transformation (Faria-e-Silva et al. 1999).

SIALIC ACID-BINDING COMPONENTS

Concomitantly with ultrastructural cytochemistry and electrophoretic mobility studies, lectins were also used to identify sialic acids on the surface of trypanosomatids. Wheat germ (WGA), *Limulus polyphemus* (LPA) and *Limax flavus* (LFA) agglutinins were the lectins of choice, and the presence of sialic acid was evaluated by the following approaches: a) quantitative and qualitative analysis of cell agglutination; b) localization of binding sites by fluorescence microscopy and flow cytometry; c) localization of binding sites by electron microscopy; d) quantitative determination of cell surface binding sites using ^{3}H-, ^{125}I- and ^{131}I-labeled lectins; and e) use of lectins for purification of glycoconjugates containing sialic acids by affinity chromatography. Agglutination with Sendai virus was also used to evaluate the presence of sialic acid residues. Since WGA also binds specifically to *N*-acetylglucosamine residues, identification of sialic acid using this lectin has to be evaluated before and after sialidase treatment.

WGA do not bind or agglutinate bloodstream forms of *T. brucei* (Steiger 1975), *T. equiperdum* (Jackson 1977) and procyclic form of *T. simiae* (Mutharia and Pearson 1987). Bloodstream forms

TABLE IV

Effect of drugs which induce cell differentiation on the electrophoretic mobility of *Herpetomonas* sp.*

System	EPM ± SD[a] (μm.s^{-1}.V^{-1}.cm)	Percentual change[b]
H. samuelpessoai (untreated)	-0.76 ± 0.03	
H. samuelpessoai (untreated) + neuraminidase	-0.51 ± 0.02	-32
H. samuelpessoai (DMSO treated)	-1.42 ± 0.05	
H. samuelpessoai (DMSO treated) + neuraminidase	-1.02 ± 0.10	-28
H. m. muscarum (untreated)	-0.69 ± 0.16	
H. m. muscarum (untreated) + neuraminidase	-0.18 ± 0.01	-75
H. m. muscarum (propranolol treated)	-1.09 ± 0.19	
H. m. muscarum (propranolol treated) + neuraminidase	-0.18 ± 0.06	-84
H. megaseliae (untreated)	-0.98 ± 0.09	
H. megaseliae (untreated) + neuraminidase	-0.53 ± 0.02	-34
H. megaseliae (LPS treated)	-0.63 ± 0.07	
H. megaseliae (LPS treated) + neuraminidase	-0.35 ± 0.05	-36

*For details see Soares et al. 1988, Lopes et al. 1989, Fiorini et al. 1991. [a]Standard deviation; [b]mean EPM of control – mean EPM of neuraminidase treated cell / mean EPM of control $\times 100$.

TABLE V

Effect of enzyme treatment on the surface charge of promastigote and opisthomorph forms of *Herpetomonas roitmani.**

Evolutive form	Treatment	Percentual change[a]
Promastigote	None	
	Neuraminidase	–24
	Phospholipase C	–33
	Trypsin	–34
Opisthomorph	None	
	Neuraminidase	–39
	Phospholipase C	–41
	Trypsin	–33

*For details see Faria-e-Silva et al. 1999. [a]mean EPM of control – mean EPM of neuraminidase treated cell / mean EPM of control $\times 100$.

of *T. musculi* (Dwyer and D'Alessandro 1976b) and *T. congolense* (Jackson et al. 1978, Rauthenberg et al. 1980) and procyclics of *T. brucei brucei*, *T. brucei rhodesiense* and *T. congolense* (Mutharia and Pearson 1987) were agglutinated in the presence of WGA. However, in all these studies there are no indications of treatment of the parasites with neuraminidase.

Epimastigote forms of *T. cruzi* were agglutinated with low concentrations of WGA, presenting 3×10^6 WGA-binding sites per cell (Pereira et al. 1980, Katzin and Colli 1983, Andrade et al. 1991). Such binding was inhibited by sialic acid containing proteins and also by treatment of parasites with neuraminidase (Pereira et al. 1980). Bloodstream and cell culture derived trypomastigotes also agglutinated with WGA albeit at a higher concentration (Pereira et al. 1980). According to Katzin and Colli (1983), trypomastigote forms presented two WGA

receptors with distinct affinities and capacities. Tissue culture and bloodstream trypomastigotes presented 1.2×10^6 and 2.3×10^6 WGA-binding sites, respectively (Katzin and Colli 1983). Although amastigote forms of *T. cruzi* presented the highest number of WGA binding sites, 4.6×10^6, no agglutination has been observed (Pereira et al. 1980, Katzin and Colli 1983). *Limulus polyphemus* agglutinin and LFA also bind to the surface of epimastigote and trypomastigote forms of *T. cruzi* as showed by lectin agglutination assays (Pereira et al. 1980), fluorescence microscopy (Souto-Padrón and De Souza 1985) and ultrastructural cytochemistry using gold-labeled lectins (Bourguignon et al. 1998).

Infective and non-infective promastigotes of *Leishmania mexicana amazonensis* were agglutinated by LPA confirming the electrophoretic mobility data (Saraiva et al. 1986).

Promastigote forms of *Phytomonas davidi* presented sialic acid of the *N*-acetyl-neuraminic acid type identified by paper and gas-liquid chromatography. However, agglutination of promastigotes in the presence of LPA became only possible after brief trypsinization. This observation suggests that sialic acid residues were masked so that after trypsin they became exposed or that they were associated with glycolipids, which would be covered by glycoproteins (Esteves et al. 1988).

Lectin-induced agglutination was used to analyze the presence of surface exposed carbohydrates in *Crithidia fasciculata* and *Crithidia luciliae*. Both species were not agglutinated, even in high concentrations, by the lectins LPA and LFA (Motta et al. 1991).

Presence of sialic acid on the surface of promastigote (PRO) and opisthomorph (OPM) of *Herpetomonas roitmani* were analyzed using FITC-labeled LPA and flow cytometry. The FITC signal for LPA was stronger in OPM than in PRO forms. Prior incubation of cells in the presence of neuraminidase from *Clostridium perfringens* reduced the fluorescence intensity in OPM but not in PRO forms. Thin-layer chromatography analysis of *H. roitmani* showed the presence of *N*-acetyl-neuraminic acid (Faria-e-Silva et al. 1999).

Presence of sialic acid residues on the surface of *H. samuelpessoai* (Esteves et al. 1988), *H. muscarum muscarum* (Lopes et al. 1989) and *H. megaseliae* (Fiorini et al. 1991) was evaluated by agglutination with Sendai virus, visualization of virus particles on the surface of parasites by scanning electron microscopy and thin-layer chromatography.

Analysis of net surface charge of trypanosomatids determined by cell electrophoresis and ultrastructural cytochemistry has provided evidences for a net negative surface charge. The surface charge is species specific and varies according to the developmental stages opening the possibility of their isolation. Association of the previous techniques with trypsin and neuraminidase treatments indicated that sialic acids significantly contributed to the negative surface charge and that they are associated with glycoproteins and glycolipids. The use of sialic acid-binding lectins corroborated these findings. Phospholipase treatment also indicated the importance of phosphate-containing molecules on the net surface charge of trypanosomatids. Contribution of sulfate groups has not been determined yet.

Nowadays, new possibilities have been opened to the study of cell surface charges. New lectins that selectively recognize different types and linkages of sialic acid could be useful as: a) markers in the evaluation of the biosynthesis of sialylated glycoconjugates; and b) to understand the effects of distinct neuraminidases on cell surface charge of trypanosomatids.

The use of gold labeled lectins, enzymes or antibodies in association with label fracture technique, high-resolution scanning microscopy and AFM will be useful to visualize, with high resolution, the topographical distribution of the different cell surface charged components as well as the participation of such molecules in the process of interaction between parasites and host cells. More recently the still powerful AFM was improved by the development of an antibody-coated microcantilever as a potential imuno-based biosensor.

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RESUMO

A carga de superfície de tripanosomatídeos foi avaliada através da ligação de partículas catiônicas, visualizadas por microscopia eletrônica e por medida direta da mobilidade eletroforética celular. Os resultados obtidos indicam que a grande maioria dos tripanosomatídeos apresenta carga de superfície negativa cujo valor é espécie específico e que varia com o estágio evolutivo. Resíduos de ácido siálico associados a glicoproteínas e glicolipídios assim como grupamentos fosfato, são os principais responsáveis pela carga negativa da superfície de tripanosomatideos.

Palavras-chave: tripanosomatídeos, carga da superfície celular, morfologia eletroforética, citoquímica.

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