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TRYPANOSOMA CRUZI

Surface change characteristics of cultured epimastigotes, trypomastigotes and amastigotes

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SUMMARY

The surface charge on **T. cruzi** parasites of various morphologic types was elucidated by studying their adsorption-elution properties on DEAE-cellulose and their migration characteristics in a particle electrophoresis system. The results indicated that at pH 8 the surfaces of all three forms were negatively charged. Trypomastigotes had less net negative charge than epimastigotes while cell culture amastigotes, when present, showed adsorption-elution properties similar to those of the trypomastigotes. Amastigoids and sphaeromastigotes present in old cell free cultures had the charge characteristics of epimastigotes.

INTRODUCTION

The cell surface plays a vital role in the establishment of any host-parasite relationship. It is the surface of a parasite which is in contact with the host's defense mechanisms. Humoral antibody and complement action as well as phagocytic ingestion all depend on the host's ability to recognize the surface of the parasite as foreign. Separation of parasites from host cells and separation of the various forms of the parasite, one from the other, also depend on surface properties. In the present paper we report results of studies on one aspect of the surface characteristics of **T. cruzi** parasites, their electrical charge and discuss the use of this attribute in separation of the morphologic forms of **T. cruzi.**

MATERIALS AND METHODS

T. cruzi Strain and its Maintenance

T. cruzi Brazil strain was kindly supplied by Dr. Teresa I. Mercado of the National Institute of Health. The strain has been maintained routinely in Senekjie's medium (SENEKJIE[7]) with an overlay of Locke's solution at room temperature. The cultures were transferred every two weeks.

Maintenance of the Cell Lines

HeLa cells were maintained in sterile 16 oz. prescription bottles and were fed Joklik's modified MEM with 10% heat-inactivated calf serum. The cells in each bottle were routinely split when they formed a confluent monolayer of growth.

Powdered Minimum Essential Medium (Joklik-Modified) without calcium chloride (Grand Island Biological Company, Grand Island, New York) was used throughout the experiments. Ten percent of heat-inactivated calf serum (North American Biological Inc., North Miami, Florida) was added to the culture medium before it was used. The pH of the medium was about pH 7.0.

Cultivation of the Trypomastigote Form of T. cruzi — Sixteen oz. prescription bottles containing confluent layers of HeLa cells were infected with approximately 1.6×10^8 **T. cruzi** organisms contained in 2 ml of the liquid phase of 14-day old Senekjie's biphasic culture. Just before infection each bottle was fed with 40 ml fresh culture medium. At least 98% of the organisms in the Senekjie's medium were epimastigotes. The infected cultures were incubated at 32.5°C. They were refed on the third and sixth days of incubation. On the ninth day of incubation the pa-

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rasites suspended in the culture fluid were harvested. A total count was made in a hemocytometer and slides were prepared for later differential counts.

DEAE Cellulose Column Chromatography — Mixtures containing epimastigotes, sphaeromastigotes and metacyclic trypomastigotes were obtained from the liquid phase of cultures. Mixtures containing slender trypomastigotes and epimastigotes and some amastigotes were produced in HeLa cell cultures.

Buffer Solutions — Stock phosphate buffered saline solution (PBS) with a pH of 8 and an ionic strength of 0.322 was prepared by the procedure of LANHAM & GODFREY [4]. Phosphate buffered saline glucose (PBSG) of lower ionic strength (i.e., 0.290) was prepared by mixing four parts of the stock phosphate buffered saline (PBS) with one part of isotonic (5.4%) glucose solution in distilled water. It was assumed that glucose was ionically inert in the buffer solution.

Column Preparation — To remove the fines from the anionic ion exchanger, (Diethylamine cellulose, General Biochemicals, Chagrin Falls, Ohio) it was washed three times by decantation in an ample amount of phosphate buffered saline glucose. After the last wash the pH of the supernatant buffer was lowered to 8.0 with 5% (v/v) solution of phosphoric acid. After lowering the pH, the exchanger was given three more washings with the same buffer. Finally, the pH of the equilibrated slurry of DEAE cellulose was checked again before it was used to pack the column (LANHAM & GODFREY [4]).

Columns with a diameter of two centimeters were packed with the equilibrated slurry to a height of 10 cm. Packing of the column was carried out in the conventional way with the addition of successive amounts of the exchanger slurry.

The rate of flow of the eluant was adjusted to 5 ml/minute.

Determination of the Adsorption-elution Characteristics — The total number of all morphologic forms of the parasite in the various mixtures used in these experiments was estimated with the aid of a hemocytometer, and a differential count of each mixture was made on glutaraldehyde fixed Giemsa stained thin films. The protozoal suspensions from the cultures were centrifuged at 5°C at 1300 g for 20 minutes, the supernatant fluids were discarded and the parasites contained in the pellet were resuspended in phosphate buffered saline glucose (PBSG) to a concentration of about 0.5-1 x 10^9 per ml.

PBSG was passed through the column until the meniscus of the fluid was level with the packed DEAE-cellulose. Two ml of the parasite mixture was layered onto the exchanger; additional buffer was then added as the parasite mixture entered the column. The presence of the parasites in the eluate was determined with the aid of a phase contrast microscope; immediately after the parasites were detected in the eluate collection was started. Three hundred ml of the subsequently eluted buffer was collected.

In other experiments column preparation was the same as above. However, after the layering of the parasite sample on the column, a volume of PSG equal to the bed volume was passed through the column before the elution of the organisms was continued with the stock phosphate buffered saline (PS). A total of 100 ml of the buffer eluted after the parasite first appeared in the eluate was collected. The numbers of each morphologic form of **T. cruzi** that entered into each column and those eluted was estimated by first performing a total count which was followed by a differential count of stained smears of the various mixtures.

Electrophoretic Techniques — Electrophoretic mobilities were measured in a Cytophoremeter (Carl Zeiss). The electrophoresis chamber was filled and allowed to equilibrate for 15 minutes with the appropriate pH buffer. The sample to be tested was drained into the chamber. The current was set at 20 milliamps. A stopwatch was used to measure the time one cell took to travel across three vertical lines of the reticule. Then the polarity was reversed and the same cell was measured again traveling in the opposite direction. Twenty to thirty cells were measured this way for each type of parasite at each pH buffer. After each run the buffer was drain-

ed and the chamber washed out with distilled water several times before the next pH buffer was added.

For each cell measured the velocity (μ/sec) was determined using the equation,

$$V = L/T$$

where L equals the distance in μ the cell traveled (one space equaled 24 μ) and T equaled the time in seconds. The average velocity was determined for all the cells measured at that pH.

The field strength of the system (volt/cm) was found with the equation,

$$E = I \times R_{spec}/A$$

where E is the field strength, I the amperage (.002 amps), R_{spec} the specific resistance (ohms), and A the cross sectional area of the chamber (0.98 cm).

The mobility of the cell was determined with the equation,

$$M = V/E$$

where M is the mobility, V the average velocity of the cell, and E is the electric field strength of the system. The mobility of the cell was plotted on a graph against pH and the isoelectric point was determined by examination.

The buffer system used was the Universal Buffer (MARINE BIOLOGICAL LABORATORY) [5]. The buffers were made isotonic by the addition of 0.85% NaCl.

RESULTS

Adsorption-elution Properties of the Various Morphologic Forms of T. cruzi on DEAE-cellulose Columns — The results of three experiments to determine the adsorption-elution properties of the various forms of **T. cruzi** on DEAE-cellulose columns flushed with PBSG at pH 8 and of ionic strength 0.290 are shown in Table I. The data in this figure indicate that most of the epimastigotes were retained in the column and in the three different experiments various percentages of the trypomastigotes were eluted. Nevertheless, the percentage of trypomastigotes recovered in the eluate was relatively low in all three experiments. It ranged from 24% in the second experiment to about 43% in the first. In the first experiment not only was the number of the eluted trypomastigotes higher than in the second, but more epimastigotes were eluted. Because the total number of organisms passed through the column in the first experiment was considerably higher than in the second, it is probable that the capacity of the column was exceeded in the first experiment and that is why more organisms came through.

Figures 1A and B are photomicrographs of the parasites from HeLa cell cultures that were placed on the column in experiment 2 and of the parasites in the eluate, respectively. In Fig. 1B some amastigotes are evident. This form was not observed in the mixture before its passage through the column. However, amastigotes constituted approximately 8% of the eluted organisms in both the first and second experiments (Table I). It was probable that the percentage of amastigotes in the original mixture was so low that they were not detected. Passage of a large percentage of the amastigotes and adsorption of most of the epimastigotes may therefore account for the abundance of the amastigotes in the eluate.

The organisms used in the first two experiments were obtained from HeLa cell cultures, while those of the third experiment were from 35-day old Senekjie's biphasic cultures. The results of the third experiment carried out on parasites raised in Senekjie's medium coincide with those of the first and second experiments carried out on parasites raised in HeLa cultures. Figure 2A is a photomicrograph of the organisms from the old Senekjie's culture which were passed though the column in the third experiment. The sphaeromastigotes or amastigoids were retained in the column as were the epimastigotes. Figure 2B is a photomicrograph of the predominately metacyclic trypomastigote population recovered in the eluate.

The data shown in Table II summarizes the results obtained when PS pH 8 of a high ionic strength (i.e., I = 0.362) was used as an eluant. With the high ionic strength buffer the percentage of the eluted trypomastigotes

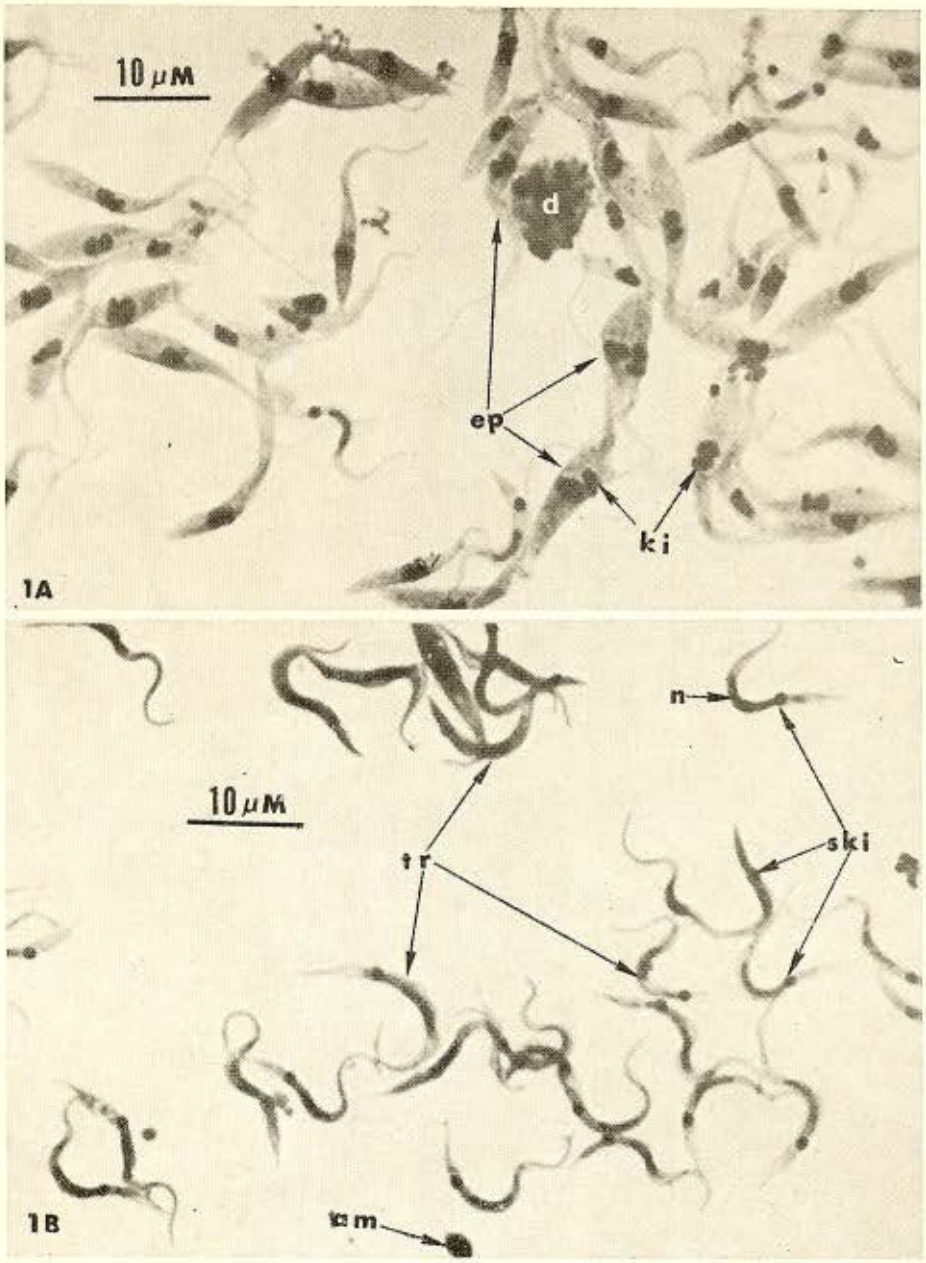

Fig. 1 (A) — A photomicrograph of a Giemsa stained preparation of the epimastigote and trypomastigote mixture produced in HeLa cell cultures which were placed on a DEAE cellulose column in PBSG of pH 8 and ionic strength 0.290. The epimastigote (ep), which is the predominate form, has a wheat grain-shaped kinetoplast (ki) located just in front of the nucleus. 1 (B) is a photomicrograph of a Giemsa stained preparation of the almost pure trypomastigotes recovered in the eluate. The trypomastigotes (tr), which are of the long slender form, have an elongated nucleus (n) and a subterminal posteriorly located spherical kinetoplast (ski). Amastigotes, none of which are present in Fig. A, are seen in B. The cell debris (d) in the original mixture (A) is absent after passage through the column and is not seen in Fig. 1B.

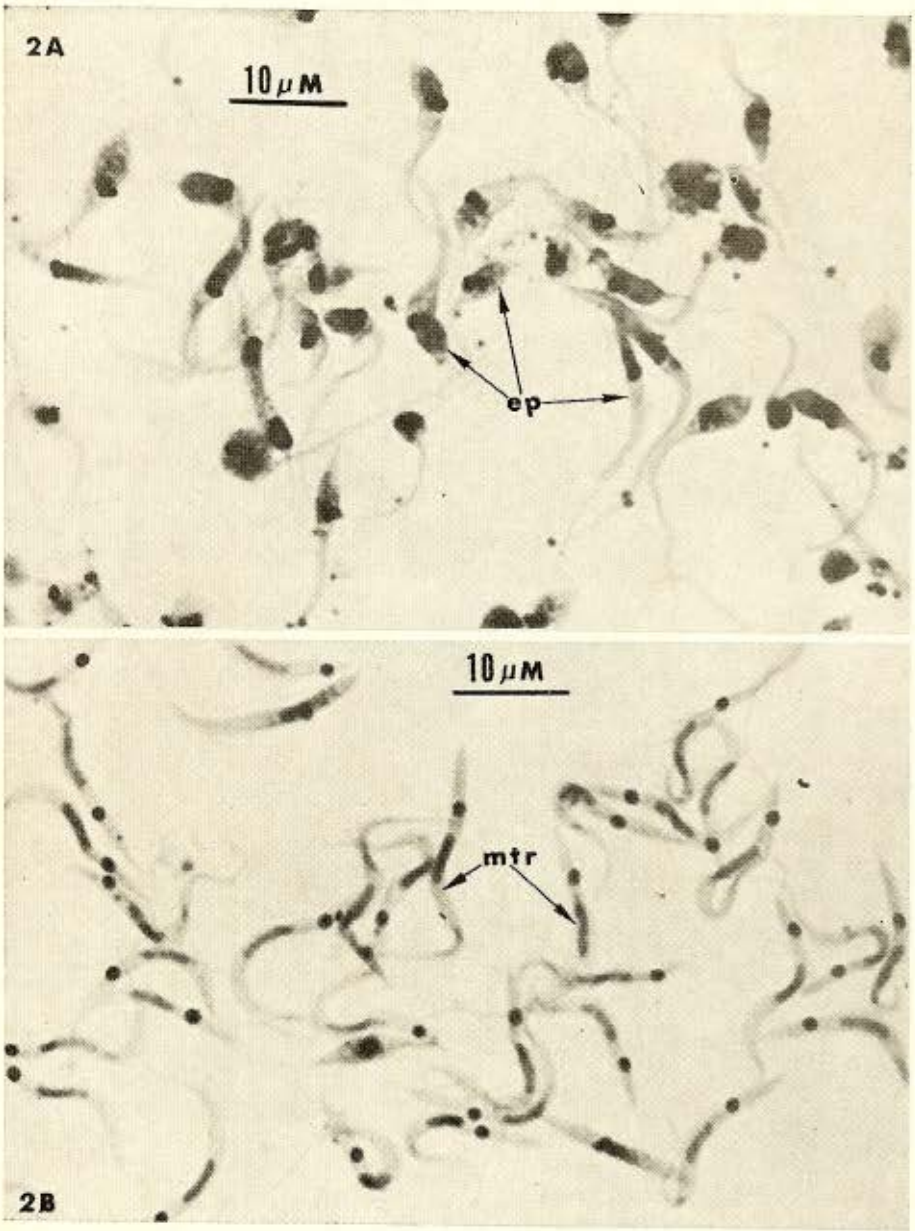

Fig. 2 — A photomicrograph of the culture forms of **Trypanosoma cruzi** placed on DEAE-cellulose column with PBSG of pH 8 and ionic strength of 0.290. (A) Photomicrograph of a Giemsa stained preparation from a 35-day old Senekjie's culture. Epimastigotes (ep), and sphaeromastigotes are the most abundant forms. (B) Photomicrograph of the predominantly metacyclic trypomastigote population (mtr) obtained after passage through the column. The similarity of morphology of this form to the slender form of trypomastigote produced in cell culture (Fig. 1B) is apparent.

was greater and the rate of their elution was higher than with the low strength (i.e., I = 0.290) (Table I) buffer. The degree to which the yield was greater with the higher ionic strength buffer is apparent when the data in Tables I and II are compared. With the lower strength buffer 24% to 43% of the trypomastigotes were recovered in 300 ml of the eluate whereas 43% to 100% were eluted by 100 ml of the buffer of the higher ionic strength. Therefore the latter buffer is the better eluant for the isolation of trypomastigotes from mixtures containing epimastigotes and trypomastigotes. Separation can often be accomplished with one passage if the percentage of the trypomastigotes in the starting mixture is high. In some cases, however, when the starting percentage is low two passages may be necessary to obtain trypomastigotes free of epimastigotes. This was demonstrated in experiments 3 and 4 of Table II. In the original mixture passed through the column in experiment 3, the percentage of the trypomastigotes was 12%; it increased to 66% in the eluate. When the organisms recovered in experiment 3 were passed through another column (experiment 4), the percentage of the trypomastigotes in the eluate was 94%.

The parasites used in the first two experiments summarized in Table II were obtained from cell cultures, while those in the third and fourth experiments were obtained from cell free Senekjie's medium cultures. The trypomastigotes in both parasite preparations

TABLE I

Adsorption elution characteristics of various forms of **T. cruzi** on a 2 x 10 cm. DEAE-cellulose column with PBSG pH 8 and ionic strength 0.290. Trypomastigotes and amastigotes from cell cultures (expt. 1 and 2), and metacyclic trypomastigotes from culture media (expt. 3) pass through the column while epimastigotes are retained. The trypomastigotes yields range from 24% to 43%.

			Experiment 1	2	3
Entering Column	Number Millions		1488	1090	1280
	Morphologic Form %	Trypo	23	20	12
		Epi	77	80	88
		Ama	1	1	—
Leaving Column	Number Millions	Trypo	147	52	44
		Epi	38.2	1.2	8.3
		Ama	16.1	4.6	—
	Morphologic Form %	Trypo	73	90	84
		Epi	19	2	16
		Ama	8	8	—
	Recovery or Yield %	Trypo	43	24	29
		Epi	3.3	0.13	0.47
		Ama	- - -	- - -	—

— None Present
- - - Undetermined

passed through the column while the epimastigotes were retained. Thus, the similarity of adsorption-elution characteristics of epimastigotes and trypomastigotes from cell cultures and those from cultures in cell-free Senekjie's medium observed in the first set of experiments (Table I) was confirmed in the second set (Table II).

In the second set of experiments as in the first it was also observed that the amastigotes present in the parasite preparations produced in the cell cultures moved with the trypomastigotes. There were aflagellated parasites present in the parasite preparations produced in Senekjie's medium, but these sphaeromastigotes and amastigoids present in the parasite preparations produced in Senekjie's medium behaved as epimastigotes not amastigotes.

To confirm that the behavior of the trypomastigotes and epimastigotes in the column was determined by the parasites charge characteristics and not by some other attribute of the parasites which could have interacted with the resin, the electrophoretic mobility of **T. cruzi** epimastigotes and trypomastigotes was measured on a cytophoremeter.

Figure 3 is a pH-mobility curve of glutaraldehyde-fixed epimastigotes of **T. cruzi.** The isoelectric points of the epimastigotes in various runs ranged around 2.5, thus epimastigotes of **T. cruzi** are negatively charged even at fairly acid pH's.

TABLE II

Adsorption elution characteristics of various forms of **T. cruzi** on a 2 x 10 cm. DEAE-cellulose column with PBS pH 8 and ionic strength 0.362. Trypomastigotes from cell cultures, metacyclic trypomastigotes from culture media and amastigotes pass through the column while epimastigotes are retained. The trypomastigote yields range from 43% to 100%.

			Experiment 1	2	3	4
Entering Column	Number Millions		96	1984	1288	145
	Morphologic Form %	Trypo	88	60	12	66
		Epi	—	33	88	34
		Ama	12	7	—	—
Leaving Column	Number Millions	Trypo	61	1190	96	64
		Epi	—	30	51	4.1
		Ama	6.1	101	—	—
	Morphologic Form %	Trypo	91	90	66	94
		Epi	—	2	34	6
		Ama	9	8	—	—
	Recovery or Yield %	Trypo	71	100	43	66
		Epi	—	4.6	4.4	4.3
		Ama	53	73	—	—

— None Present

Figure 4 is a pH-mobility curve of glutaraldehyde-fixed trypomastigotes of **T. cruzi** from cultures in HeLa cells. The mobility of trypomastigotes (Fig. 4) does not vary much with pH. At pH 7 and above, the epimastigotes have an electrophoretic mobility which is twice as great as that of trypomastigotes, thus at physiological pH's the epimastigotes have a stronger net negative charge (i.e. theta potential) than do the trypomastigotes. These results are in agreement with the results of the column work, as epimastigotes adhered to DEAE-cellulose columns at pH 8.0, and the trypomastigotes went through the column, thus confirming the relationship between behavior on the column and the charge characteristics of the various morphologic forms of the parasites.

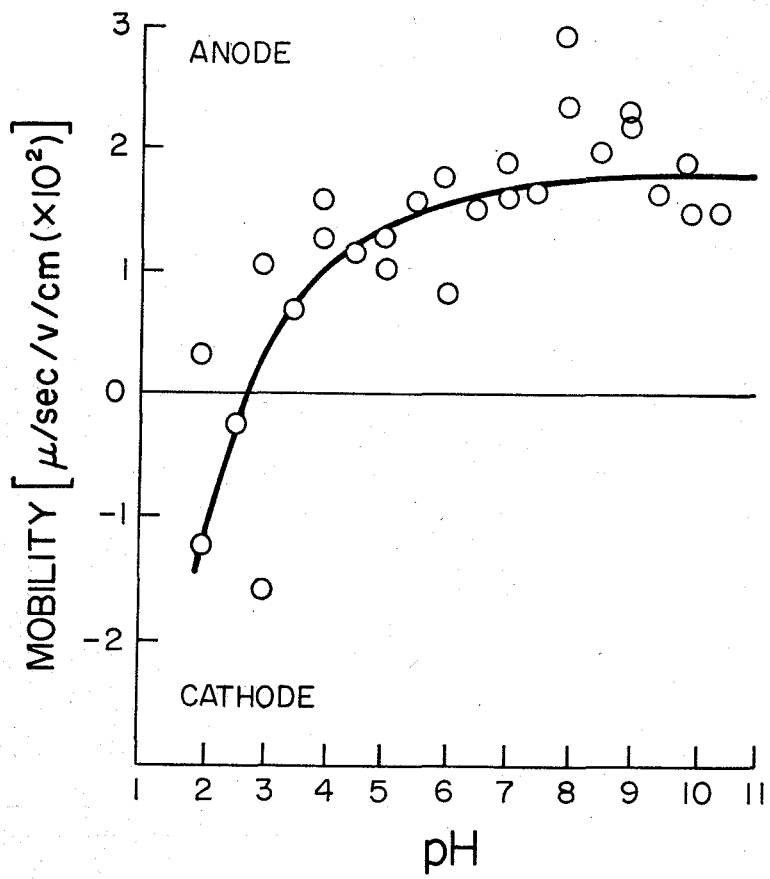

Fig. 3 — The relationship between pH and mobility of glutaraldehyde-fixed epimastigotes of the Brazil strain of **T. cruzi**. The epimastigotes have an isoelectric point of about 2.5.

DISCUSSION

The nature of the surface charge of the various morphologic forms of **T. cruzi** was the subject of several publications. BROOM et al. [2], by using a fairly simple technique noted that the blood form of **T. cruzi** carried a net negative surface charge. Broom and his coresearchers' technique involved the addition of 2 μl of infected blood to 0.3 ml of saline consisting of 1 volume of normal saline and 9 volumes of 4% (w/v) glucose solution.

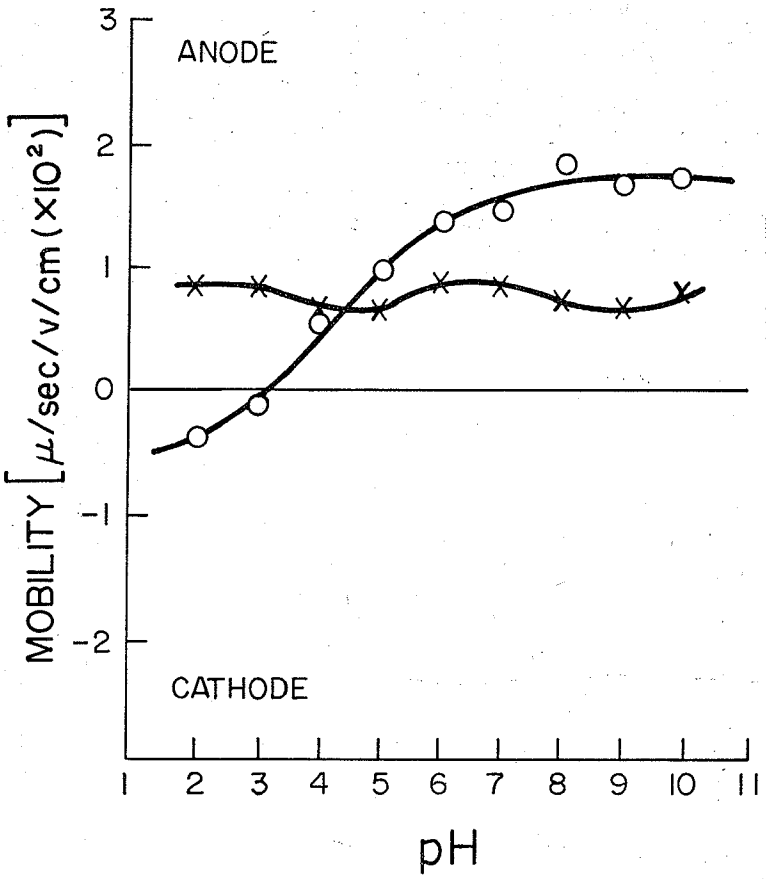

Fig. 4 — The relationship between pH and mobility of glutaraldehyde-fixed trypomastigotes of **T. cruzi** (X) and of rat erythrocytes (O). The trypomastigotes have a low mobility which does not vary much with pH and no isoelectric point in the ranges tested.

Microscopic examination 30 minutes later showed that positively charged trypanosomes adhered firmly to the negatively charged red blood cells. Negatively charged trypanosomes, on the other hand, remained free. Later HOLLINGSHEAD et al. [3], using the same technique confirmed the earlier findings of Broom and his collaborators. AL-ABBASSY et al [1], have shown that culture trypomastigotes could be separated from a mixture containing this form and epimastigotes using the anion-exchanger DEAE-cellulose. These investigators' findings suggest that there is a difference between the surface charge of the two forms. The results presented in this study have shown that with phosphate buffered saline pH 8 and I = 0.290 most of the epimastigotes were adsorbed to the exchanger whereas various but relatively small percentages of the trypomastigotes were eluted. This indicates that the outer surfaces of both these forms under these conditions are negatively charged and therefore the organisms were adsorbed to the anionic exchanger. However, it seems that the trypomastigotes are less negatively charged than the epimastigotes since high percentages of the former were eluted when the ionic strength of the eluate was increased to 0.362. In contrast, the more negatively charged epimastigotes were still adsorbed to the DEAE cellulose. The small number of epimastigotes which passed through the column with the higher ionic strength buffer were

probably able to elute themselves from the exchanger by their physical activity. Differences in the surface charges of the various individuals of this form should, however, also be considered.

LANHAM & GODFREY [4] have shown that most salivarian trypanosomes were eluted from DEAE-cellulose with a buffer of a much lower ionic strength than that used in this work to elute cell culture trypomastigotes. This indicates that cell culture trypomastigotes of **T. cruzi** have a net charge which is more negative than that of any of the salivarian trypomastigotes.

The similarity in the adsorption elution properties of the cell culture trypomastigotes to those of the metacyclic trypomastigotes that develop during the stationary phase of Senekjie's culture indicates that the two forms have about the same charge on their outer surface. Amastigotes pass through the column with the trypomastigotes and thus also have a low surface charge. Sphaeromastigotes and amastigoides which developed in cell-free cultures while morphologically approaching amastigotes, were in charge characteristics still epimastigotes.

The chemical nature of the surface charge bearing structures on the epimastigotes and the trypomastigotes of **T. cruzi** has not yet been studied. The previous work from this laboratory (AL-ABBASSY et al. [1]), was perhaps the first demonstration that there was such a difference. On the other hand, the difference between the surface charge of the culture and the blood form of the salivarian trypanosomes has been known for some time (BROOM et al.[2]; HOLLINGSHEAD et al.[3]). VICKERMAN [8] has attributed the absence of the negative surface charge in the blood stream and metacyclic forms of **Trypanosoma rhodesiense,** the causative agent of sleeping sickness, to the presence of the surface coat. The surface coat, which is an additional pellicular layer outside the limiting membrane, has been considered to only be present in the metacyclic and blood form trypanosomes. It has been demonstrated, however, by freeze-cleaving to be present on the epimastigote form of **T. cruzi** also (SEED et al. [6]), but as a thin layer which is commonly lost in preparation for thin sectioning. Perhaps the thinness of the layer in the epimastigotes of **T. cruzi** prevents it from blocking the charge completely. It is not known if amastigotes, which also are only slightly charged, have a surface coat.

RESUMO

Trypanosoma cruzi. **Características de modificações de superfície em epimastigotas cultivadas** in vitro

As cargas elétricas de superfície das diferentes formas do **T. cruzi** foram elucidadas através do estudo das propriedades de absorção e eluição em DEAE celulose e das características de migração em um sistema de eletroforese de partículas.

Os resultados indicaram que a pH 8 a superfície das três diferentes formas morfológicas estavam carregadas negativamente. As formas tripomastigota, tinham uma carga negativa menor do que a das formas epimastigota, enquanto que as amastigotas obtidas de cultura de tecido, quando presentes, apresentaram características de absorção e eluição similares àquelas encontradas no tripomastigota. As formas amastigóides e esferomastigota, de meio de cultura livre de células, apresentaram características de cargas das formas epimastigota.

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