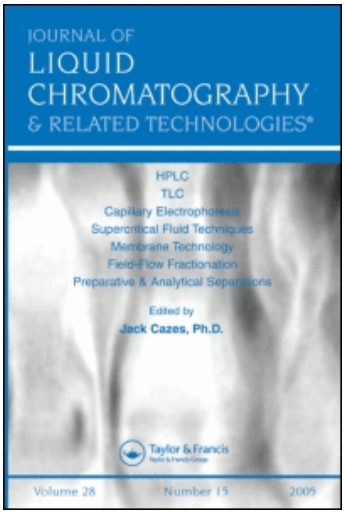

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Countercurrent Chromatography of an Anti-trypanosomal Factor from *Pseudomonas Fluorescens*

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COUNTERCURRENT CHROMATOGRAPHY OF AN ANTI-TRYPANOSOMAL FACTOR FROM *PSEUDOMONAS FLUORESCENS*

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ABSTRACT

Countercurrent chromatography (CCC) employing the Ito multilayer coiled column was an effective procedure for the purification of an anti-trypanosomal factor (ATF-II) from Pseudomonas fluorescens. Two types of anti-trypanosomal activity were observed, i.e., lysis, and paralysis and killing of the parasites without lysis. High performance liquid chromatography (HPLC) was very useful for the differentiation and further purification of these fractions, and bioassays performed with Trypanosoma equiperdum, indicated the characteristic pathologic changes produced by these components. The lipopolysaccharide content of the purified fractions was markedly reduced and indicated an additional advantage of CCC in future studies with experimental infections with T. cruzi.

INTRODUCTION

The use of countercurrent chromatography (CCC) for the purification of natural products has been reported by many investigators [1-5]. High-speed CCC, a type of CCC system developed in recent years [6-8], is a fast and efficient technique for the separation of biologically active components with potential pharmacological properties. In studies of an anti-trypanosomal substance (ATF-II) produced by the bacterial species, Pseudomonas fluorescens [9], we were limited in its purification procedure by the small volumes that could be eluted over silica columns, the only type of column that provided optimal separation of the active fractions [10]. High-speed CCC was considered an excellent procedure for the purification of this substance since it is especially applicable for the fractionation of polar compounds. Furthermore, considerably larger amounts of solutes may be processed with the multilayer coiled column, the separation times are shorter, and the degree of partition efficiency is high. Other factors, such as freedom from adsorptive sample loss, deactivation, and contamination, were also beneficial and high reproducibility and predictability were ensured. In this report we describe the purification of the P. fluorescens factor obtained with this technique and further fractionation of the CCC-derived fractions with high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

The anti-trypanosom l factor was obtained according to Mercado and Colón-Whitt [9]. It was lyophilized and extracted with absolute ethanol. At least 1 gram was extracted in 200 ml of the alcohol with constant stirring at 26°C for 48 hrs. The sample was centrifuged at 164 g for 5 min and the supernatant was concentrated in a rotary evaporator (Brinkmann Rotavapor-R) set at 5. The water bath was maintained at 45°C and when evaporation was completed drying was continued under vacuum for 30 to 60 min. CCC was performed at 26°C with a table-top model of the multilayer coil planet centrifuge (P.C., Inc., Potomac, MD 20854). The revolutional radius was 4 inches and the multilayer coil β values ranged from 0.5 at the internal terminal to 0.8 at the external terminal. The column was prepared from a single piece of 1.6 mm i.d. PTFE tubing (130 m long) and had a total capacity of 280 ml. The flow rate was maintained with a Beckman Accu-Flo pump at a setting of 12 (in slow stroke) and the absorbance was monitored with an LKB Uvicord S at 280 nm. The recorder was an LKB model (6 channel) and a chart speed of 1 cm/20 min was used. Three-ml fractions were collected with an LKB fraction collector. The solvents were evaporated under vacuum with a Savant speed evaporator and the residues weighed. Four to 5 evaporation cycles were needed to remove the NH_4HCO_3 totally. The volatile solvent system was selected from a number of different solvent mixtures on the basis of the partition coefficient of the solutes determined by UV absorbance at 280 mm with a Zeiss

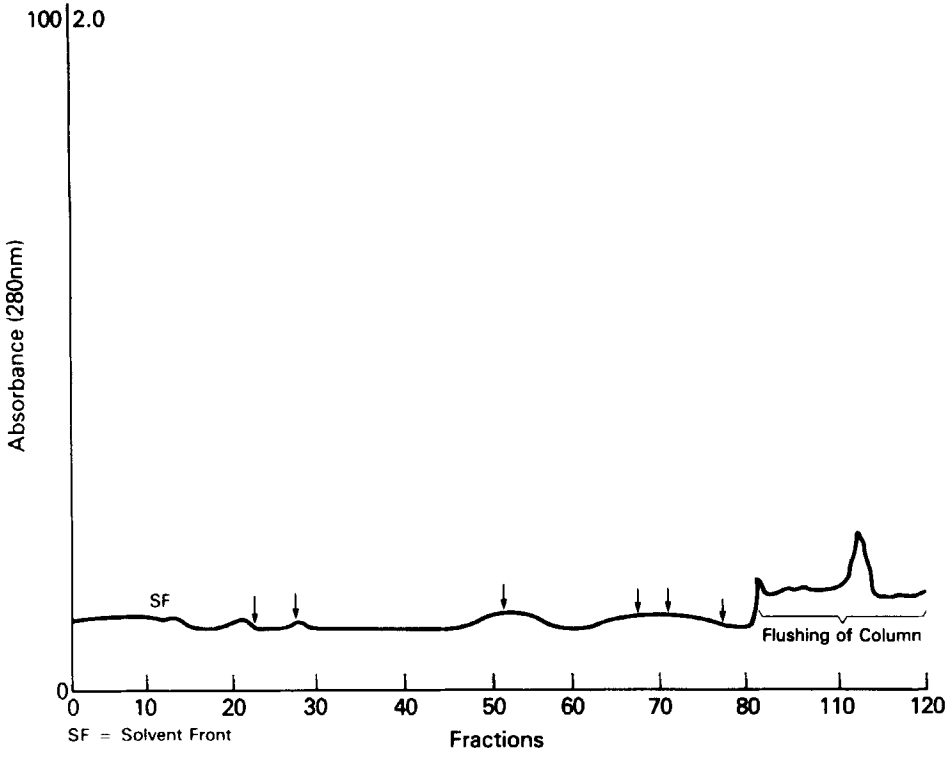
spectrophotometer. Each solvent system was thoroughly equilibrated in a separatory funnel at room temperature and separated before use. A determination of the partition coefficient was made by dissolving a small amount of ATF-II in the solvent system, shaking and after separation of the phases, measuring the concentration of the sample in both phases thus giving the ratio of upper to lower (u/l) phase. For analysis, a solution of the ATF-II was dissolved in an amount of a 1:1 mixture of the lower and upper phases and inserted with a syringe into the solvent pumping line at the column head. Ethyl acetate/n-butanol/water (1:1:2) and n-butanol/methanol/saturated NH_4HCO_3 (7:3:10) were used. In some fractionations the upper and lower phases were used alternatively as the mobile or stationary phase. Fractions which exhibited significant anti-trypanosomal activity were analyzed further with HPLC employing a Millipore-Waters Chromatography System consisting of model 510 solvent delivery system, model 440 UV detector at 280 nm, and a model U6K sample injector. A Waters carbohydrate analysis column (3.9 mm X 30 cm) with an aminopropyl functional group was used. The samples were eluted with 70% acetonitrile:water at a flow rate of 0.2 ml/min.

In order to assess the lytic activity of the purified fractions, bioassays were performed with trypomastigotes of Trypanosoma equiperdum. This parasitic species exhibits the same sensitivity to ATF-II as previously observed with T. cruzi [10]. It was maintained by intraperitoneal blood passaging in 6-week-old male Sprague-Dawley rats every 4 to 5 days. Blood

samples were obtained by cardiac puncture under ether anesthesia. The parasites were isolated from blood according to Lanham and Godfrey [11] using a 6:4 buffer dilution. Bioassays were performed with 0.2 ml of the lyophilized fractions dissolved in phosphate-saline-glucose buffer (PSG, 95 mM Na_2HPO_4, 5 mM NaH_2PO_4, 73 mM NaCl, 1% glucose, pH 8.0) and at least 2.5 X 10^7 trypomastigotes suspended in 0.2 ml of the same buffer. Microscopic examination of the parasite-ATF suspensions were performed after 10 min and 3, 5, and 20 hrs. They were maintained at 4°C for the longer incubation periods. The purified fractions were assayed also for lipopolysaccharide using the Limulus amoebocyte lysate test for endotoxin [12,13]. Protein determinations were made according to Lowry [14]. For electron microscopic examination, the trypanosomal suspensions were pelleted in an Eppendorf microfuge for 10 min, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, washed with buffer, dehydrated with a graded series of ethanols and propylene oxide, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope at an accelerating voltage of 80 KV.

RESULTS

The ethyl acetate/n-butanol/water system elicited the best separation of the lytic fractions when the upper non-aqueous phase was used as the mobile phase. As shown in Fig. 1, many

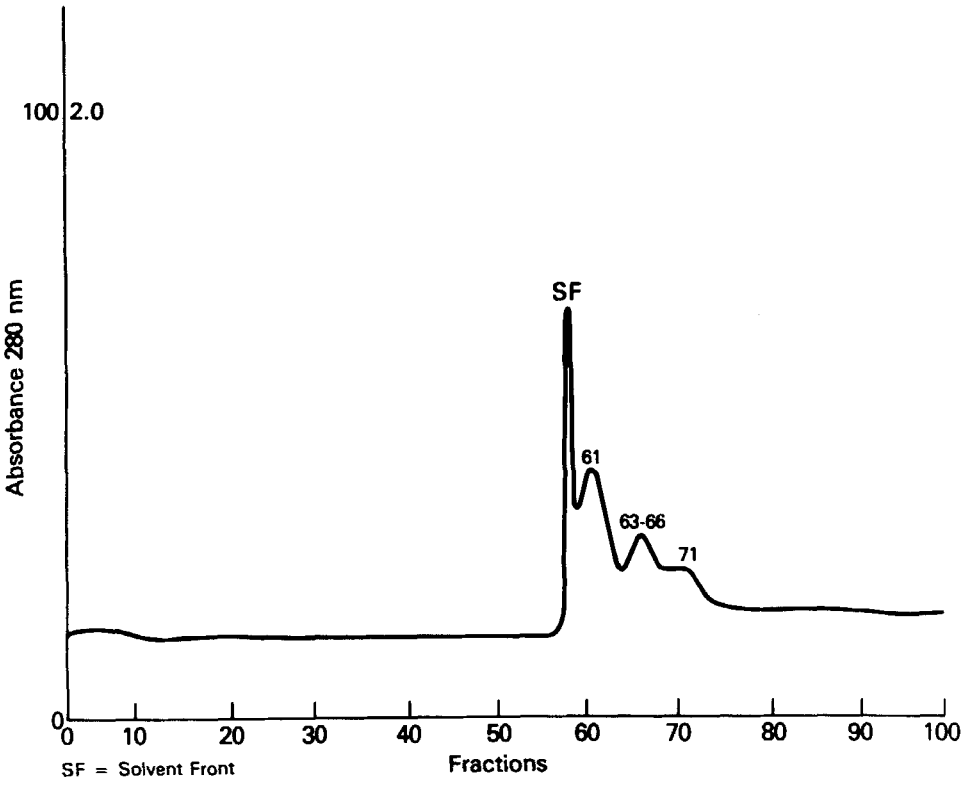


Solvent System: Ethyl acetate:n-butanol:water (1:1:2)
Mobile Phase: Upper non-aqueous phase
Sample: 2.196 g
Flow Rate: 100ml/hr
Revolution: 800 rpm
Retention of
Stationary Phase: 80%
Pressure: Below 20 psi

Fig. 1: Countercurrent chromatography of ethanol-extracted ATF-II. A total of 80 fractions was collected. Twenty two fractions produced parasitic lysis of 60 to 80 percent (arrows). Many produced the lytic effect in minimal concentrations equivalent to 40 to 80 µg of the lyophilized compound.

fractions were eluted which produced considerable lysis, e.g., 22 and 27, 48 to 52, 61 to 67, 72 to 75, and 77 to 80, in amounts of the lyophilized compound ranging from 40 to 464 μg. However, it is curious that this activity was not represented by distinct peaks on the chromatogram but by a series of unresolved peaks that gave the appearance of waves of absorbance comprising the area of anti-trypanosomal activity. Sixty two percent of the sample injected was retained by the column. Thirty three fractions of a total of 59 obtained upon flushing of the column exhibited lytic activity of 60 to 100 percent.

CCC of the ATF-II ethanol extract with the n-butanol/methanol/NH_4HCO_3 system yielded a more distinct separation of the active fractions and fractionation of lytic activity was more rapid and circumscribed. As shown in Fig. 2, elution of active components began and ended within a period of 18 min following the solvent front, i.e., fractions 61 to 66. Fractions 61 and 62, which comprised the first peak on the chromatogram, produced parasitic lysis of 70 to 100 percent at a concentration of 4.3 and 5.6 mg/0.2 ml, respectively, of the lyophilized compound. Eluates 63 to 66 produced paralysis and death of the parasites but not lysis at a concentration of 4.4 to 8.6 mg. The third well-defined peak on the chromatogram (fraction 71) did not exhibit anti-trypanosomal activity. Rechromatography of the paralyzing fraction (Fig. 3) yielded both paralyzing and lytic components. Of a total of 20 fractions collected 3 produced paralysis and 8 induced lysis.



Solvent System: N-butanol:methanol:saturated NH_4HCO_3 aqueous solution (7:3:10)

Mobile Phase: Lower aqueous phase

Sample: 175.2 mg

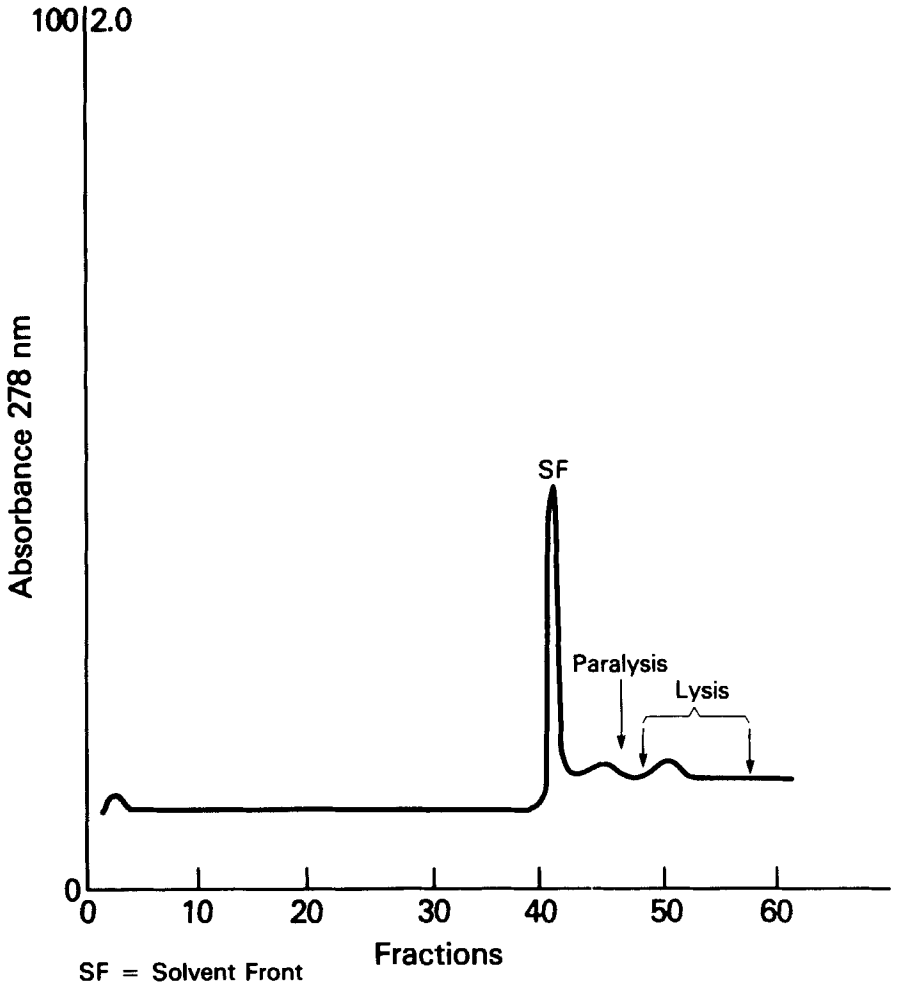
Revolution: 900 rpm

Flow Rate: 60 ml/hr

Retention of
Stationary Phase: 40%

Pressure: 25 psi

Fig. 2: Countercurrent chromatography of ethanol-extracted ATF-II. In contrast to the ethyl acetate/n-butanol/water system, absorbance peaks were distinct and the active fractions were eluted within a shorter period of time.



Solvent System: N-butanol:methanol:saturated NH_4HCO_3 aqueous solution (7:3:10)
Mobile Phase: Lower aqueous phase
Sample: 58.6 mg (combined fractions 63-66)
Revolution: 900 rpm
Flow Rate: 60 ml/hr
Retention: 53.5%
Pressure: 50 psi

Fig. 3: Rechromatography of combined paralyzing fractions 63 to 66 shown in Figure 2. Both paralyzing and lytic components were eluted.

High performance liquid chromatography was very effective for differentiating the paralyzing from the lytic components. As shown in Fig. 4, during the fractionation of sample 61 many components were eluted which exhibited significant lytic activity. Thirteen of 22 fractions collected produced parasitic lysis of 50 to 80 percent (arrows). The largest amounts of the lytic compounds recovered corresponded to the high peaks on the chromatogram, i.e., 2.6 and 3.9 mg for the first and second peaks, respectively. Paralysis without lysis was not observed and many peaks which represented lysis occurred at close elution intervals. HPLC of the paralyzing fraction (Fig. 5) yielded many well-defined peaks although the amounts of the lyophilized compound obtained were considerably smaller than those eluted from fraction 61 described above. The highest amount, 1.5 mg, was obtained 26 minutes after injection (arrow). As was observed in the rechromatography of eluates 63 to 66 mentioned above, HPLC also yielded both paralyzing and lytic fractions. Lysis of 50 to 80 percent was produced by fractions eluted after 13, 17, 23, 24, and 32 min. Clearly the lytic activity corresponded to the large peaks on the chromatogram. Twenty nine of the remaining 34 fractions, showed mainly reduction of movement of 50 percent of the parasites, e.g., fraction 26, to marked inactivation without killing (fractions 28 to 31).

Electron micrographs of control, untreated trypomastigotes of T. equiperdum disclosed clearly discernible organelles (Fig. 6). However, treatment with the lytic fraction produced marked

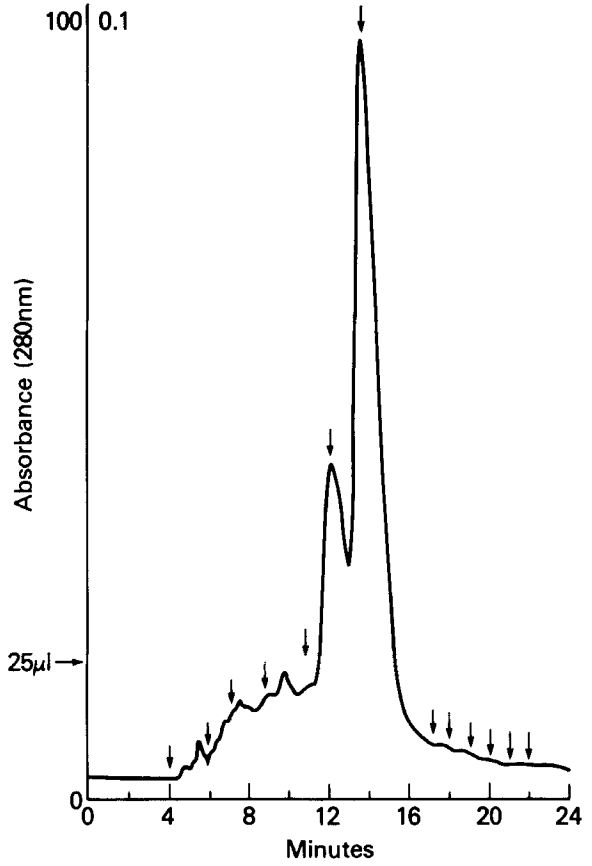


Fig. 4: High performance liquid chromatography of lytic fraction 61 shown in figure 2. Two main peaks comprised by fractions 11 and 12 and 13, 14, and 15, produced lysis but many small peaks (arrows) were chromatographed also. The sample injected into the column was equivalent to 16.3 µg protein in 860 µg of the lyophilized compound.

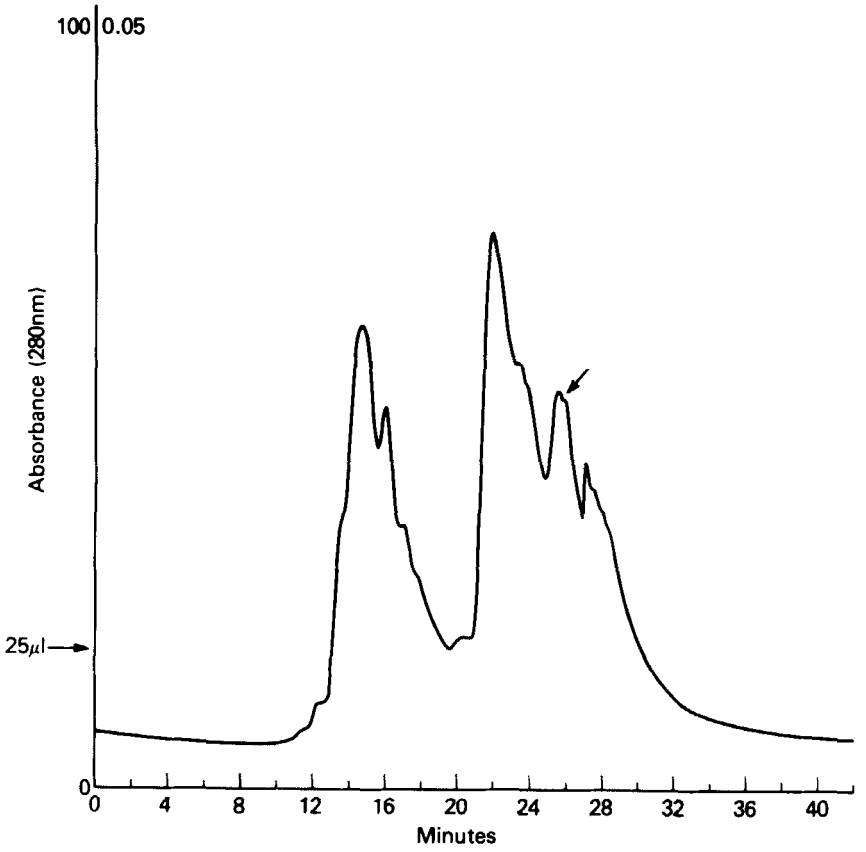


Fig. 5: High performance liquid chromatography of paralyzing fraction shown in Figure 2. The sample injected into the column was equivalent to 13.3 µg protein in 1.48 mg of the lyophilized compound.

cytological changes. The parasites became extremely swollen and the cytoplasm became vacuolated, giving the parasites the appearance of ghosts (Fig. 7). By contrast, the most common change produced by the paralyzing fraction (Fig. 8) was a detachment of cytoplasmic areas subjacent to the plasma membrane

(a) and a separation of the external nuclear membrane which produced an expansion of the perinuclear space (b).

As was observed in fractions eluted with HPLC [10], the lipopolysaccharide (LPS) content of the CCC-derived fractions was markedly reduced. Amounts of 0.125 and 0.25 endotoxin units (EU)/ml were obtained. Unpurified ATF-II contained 1.25×10^3 ng/ml LPS. Ten to 40 EU elicit a pyrogenic reaction in the rabbit test [15].

DISCUSSION

Countercurrent chromatography with the high-speed multilayer coil was a reliable technique for the purification of the ethanol-extracted ATF-II. It was particularly effective for the fractionation of large amounts of this substance (e.g., 2.1 g) which could be resolved in 1 to 5 hrs. Of the solvent systems used in this study, the n-butanol/methanol/NH_4HCO_3 system produced the fastest and most distinct separation of the fractions, although the retention capability of the stationary phase was lower than that observed with the ethyl acetate/n-butanol/water system, i.e., 47 vs 80 percent. With the latter the elution profile was characterized by waves of lytic activity rather than by distinct peaks. However, results obtained with HPLC of the CCC-derived fraction 61 (Fig. 2) suggested that the waves of lytic activity did not represent single components eluted over a wide area but, rather, many unresolved components which differed only slightly in chemical structure, as described in our recent report [10]. The

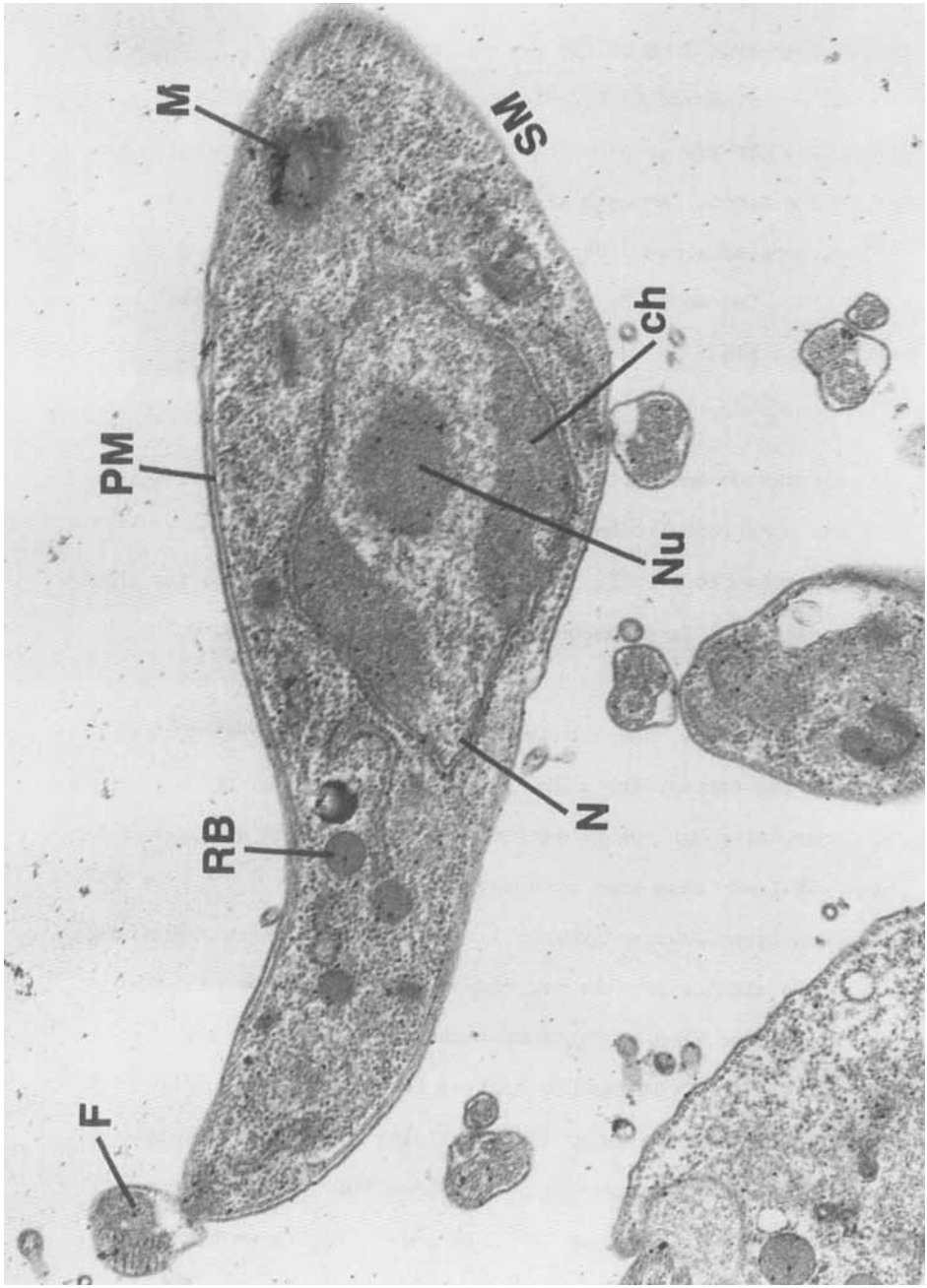


Fig. 6: Ultrastructure of untreated trypomastigotes of Trypanosoma equiperdum (akinetoplastic strain) after incubation in phosphate-saline-glucose buffer (pH 8.0) at 4°C for 20 hr. Organelles clearly discernible include plasma membrane (PM), nucleus (N), nucleolus (Nu), mitochondrion (M), chromatin (Ch), flagellum (F), subpellicular microtubules (SM) and unidentified round bodies (RB) X 36,000.

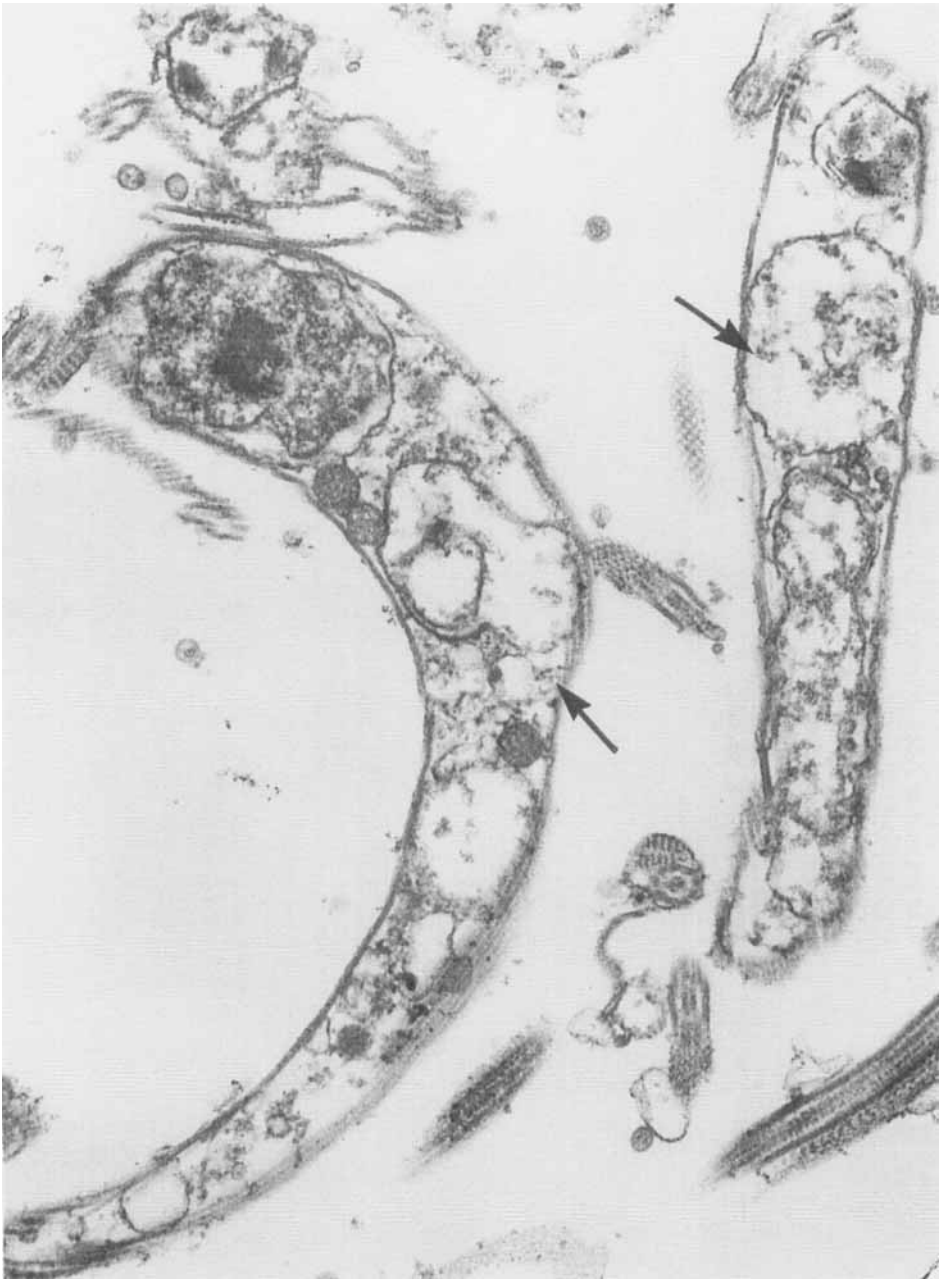


Fig. 7: Ultrastructure of trypomastigotes of Trypanosoma equiperdum (akinetoplastic strain) after treatment with lytic fraction 61 for 20 hr at 4°C. As the bioassay period increased the cytoplasm became extremely vacuolated giving the parasites the appearance of ghosts (arrows). Uniformity of the lytic effect indicates significant purification of the sample. X 36,000.

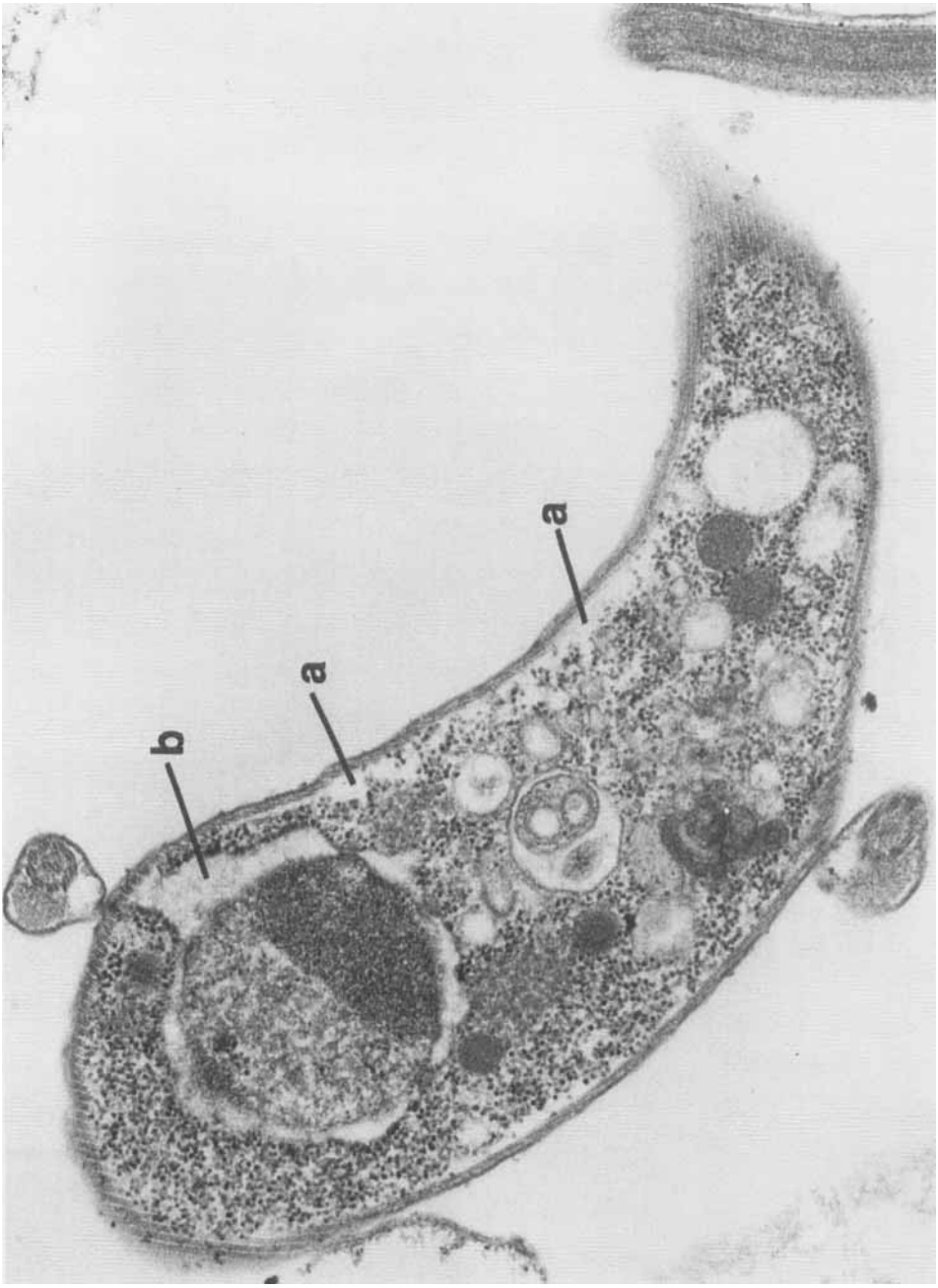


Fig. 8: Ultrastructure of trypomastigotes of Trypanosoma equiperdum after treatment with the paralyzing fraction. Note detachment of cytoplasmic areas subjacent to the plasma membrane (a) and separation of the external nuclear membrane which produced and expansion of the perinuclear space (b). X 36,000.

occurrence of multiple components was also indicated by the fact that some of the fractions within the active areas produced lysis at minimal concentrations of the solute, e.g., 80 μg, whereas others produced a comparable effect at significantly higher concentrations (e.g., 4.3 mg).

One of the aspects of CCC which was particularly useful in this work was the advantage of eluting with either the aqueous or non-aqueous phase of the solvent system and of recovering the sample remaining in the stationary phase by flushing the column at the end of the elution. For example, it aided us in disclosing the hydrophilic and hydrophobic properties of the ATF. When using the ethyl acetate/n-butanol/water system and eluting with the non-aqueous phase, the largest portion of the injected sample was retained in the column. When using the non-aqueous phase as the stationary phase, the largest part of the sample was eluted immediately after the solvent front. The best separation of lytic components was obtained when the aqueous phase was used as the stationary phase. Chemical analyses [16] employing ether extraction disclosed the occurrence of several fatty acids in the unpurified ATF-II, indicating the hydrophobicity of this compound, and in current studies of CCC- and HPLC-derived fractions [17] we have obtained mannose, arginine, and lysine indicating their hydrophilic nature.

It was of interest that fractionation with CCC yielded such a distinct separation of lytic and paralyzing components. We had observed occasionally [18] with HPLC of the ATF-II ethanol

extracts that some fractions produced essentially a paralyzing effect of the parasites but the findings in this study were more consistent, e.g., fractions 61 and 62 produced a distinct lytic effect, but fractions 63 to 66 produced paralysis and killing without lysis. Although rechromatography of the paralyzing fraction did not yield paralyzing components exclusively, it was clear from the HPLC analysis that this fraction differed from that of the lytic compound. Furthermore, ultrastructural studies of the parasites treated with amounts of this fraction comparable to those that produced the lytic effect revealed changes in the membrane but the parasitic structure remained essentially intact. Interestingly, these effects were identical with those observed 15 minutes after the initiation of treatment with unpurified ATF-II (Sephadex eluate, not extracted with ethanol) described in a previous report [19]. We questioned whether this compound served to prepare the membrane for the penetration of the lytic factor, but further study is required before more definite conclusions can be given.

As observed in a previous study employing HPLC [10], the LPS content of the ATF-II ethanol extracts was reduced significantly in the CCC-purified fractions. This is an advantage, since bacterial products used chemotherapeutically in the USA must contain little or no pyrogenic matter.

Although extensive research has been carried out in the chemotherapy of the protozoan parasite, T. cruzi, the causative agent of Chagas' disease [20-22], we still do not have an

effective, non-toxic chemotherapeutic agent to cure this lethal parasitic disease. In view of the antibiotic activity exhibited by the fluorescent pseudomonads [23,24] the results in this study indicate their potential usefulness as anti-trypanosomal agents. High-speed CCC used together with HPLC provided an excellent system for the purification of this Pseudomonas secondary metabolite and will be useful in future studies with experimental infections of T. cruzi.

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