Biochemical Pharmacology, Vol. 38, No. 17, pp. 2873–2877, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

EFFECTS OF TRYPANOCIDAL DRUGS ON PROTEIN BIOSYNTHESIS *IN VITRO* AND *IN VIVO* BY *TRYPANOSOMA CRUZI*

NELIDA S. GONZALEZ* and JUAN JOSE CAZZULO

Instituto de Investigaciones Bioquimicas, "Fundacion Campomar", Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1405 Buenos Aires, Argentina

(*Received* 12 *April* 1988; *accepted* 13 *February* 1989)

Abstract—Nifurtimox (NF) and benznidazole (BZ), drugs used in the treatment of Chagas' disease, did not inhibit protein biosynthesis in *in vitro* homologous cell-free systems isolated from *Trypanosoma cruzi* and *Crithidia fasciculata*; nevertheless, their addition to growing cultures caused polyribosomal depolymerization. On the other hand, Berenil®, Antrycide® and suramin, used against African trypanosomiasis, inhibited protein biosynthesis *in vitro* but did not affect ribosomal distribution, probably due to low permeability to the drugs. The results suggest that the inhibition by NF and BZ of protein synthesis, measured as [^{14}C]leucine incorporation by other authors, is indirect, probably through inhibition of nucleic acid synthesis and energy metabolism.

Trypanosoma cruzi is the flagellate that causes the American trypanosomiasis, Chagas' disease. Nifurtimox (NF†) and benznidazole (BZ), nitrofuran and nitroimidazole derivatives respectively, are the drugs used most extensively in the treatment of acute Chagas' disease. Side effects indicate that there is a need for better drugs. The actions of these drugs, as well as other nitroheterocyclic derivatives, probably involve interference with biological oxidations, membrane alterations, and inhibition of macromolecular synthesis [1]. NF is more active than BZ, as shown by the concentrations required for 50% inhibition (I_{50}) of epimastigote growth, 10 and 50 μM respectively [1].

By following the incorporation of radiolabeled leucine into proteins, Polak and Richle [2] and Goijman *et al.* [1] proposed that BZ and NF, respectively, inhibited protein synthesis *in vivo*; the effect of BZ, however, in contrast with that of NF, seemed to be mostly an inhibition of leucine uptake by the parasite [1]. Gugliotta *et al.* [3] proposed that another trypanocidal nitrofuran, SQ-18506, also inhibits protein biosynthesis, although Sims and Gutteridge [4] suggested that the primary site of action of this drug was on nucleic acid synthesis.

None of these studies, however, measured directly the effects of the drugs on the protein-synthesizing machinery *in vitro*, and the results obtained might reflect indirect effects affecting primarily DNA and/or RNA synthesis, energy metabolism or even amino acid uptake.

In the present study we examined the actions of NF and BZ, as well as other drugs used in the treatment of African trypanosomiasis, namely Berenil® (diminazine aceturate, BE), pentamidine (PE), Antrycide® (quinapyramine, ANT) and suramin, on amino acid activation and amino acid polymerization *in vitro* using cell-free systems obtained from *T. cruzi*; *Crithidia fasciculata*, in which protein biosynthesis has been studied to some extent [5], was used for comparative purposes. We also compared these results with the effects of the same drugs *in vivo* through a study of polysomal profiles of lysates obtained from cultures of the parasites collected at the exponential phase of growth.

The results suggest that BZ and NF do not act primarily on protein biosynthesis, the effects *in vivo* being probably mediated by interference with nucleic acid synthesis and energy metabolism.

MATERIALS AND METHODS

Cells

Epimastigotes of *T. cruzi*, Tul 0 stock, were grown in a liquid medium containing brain heart infusion, tryptose, fetal calf serum, glucose and hemin as main constituents [6].

Preparations of the lysates

Cellular extracts for amino acid activation or incorporation into hot trichloroacetic acid (TCA)-insoluble material were obtained from parasites collected at the end of the exponential growth. Cells were washed twice with cold PBS [0.14 M NaCl, 0.01 M sodium phosphate, 3 mM KCl (pH 7.2)] and resuspended in lysis buffer [20 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 5 mM KCl, 3 mM $CaCl_2$, 5 mM 2-mercaptoethanol and 250 mM sucrose] at a ratio of 7 g (wet weight) of cells per 4 ml of buffer. The cells were lysed through three cycles of freezing in liquid N_2, and thawing. All subsequent manipulations were carried out at 0–4°. Nuclei, mitochondria and cellular debris were removed by

* Correspondence: Dr Nelida S. Gonzalez, Instituto de Investigaciones Bioquimicas, "Fundacion Campomar", Universidad de Buenos Aires, Patricias Argentinas 435, (1405) Buenos Aires, Argentina.

† Abbreviations: NF, nifurtimox; BZ, benznidazole; BE, Berenil (diminazine aceturate); PE, pentamidine; ANT, Antrycide (quinapyramine); CH, cycloheximide; and Hepes, *N*-2-hydroxyethylpiperazine.*N'*-2-ethanesulfonic acid.

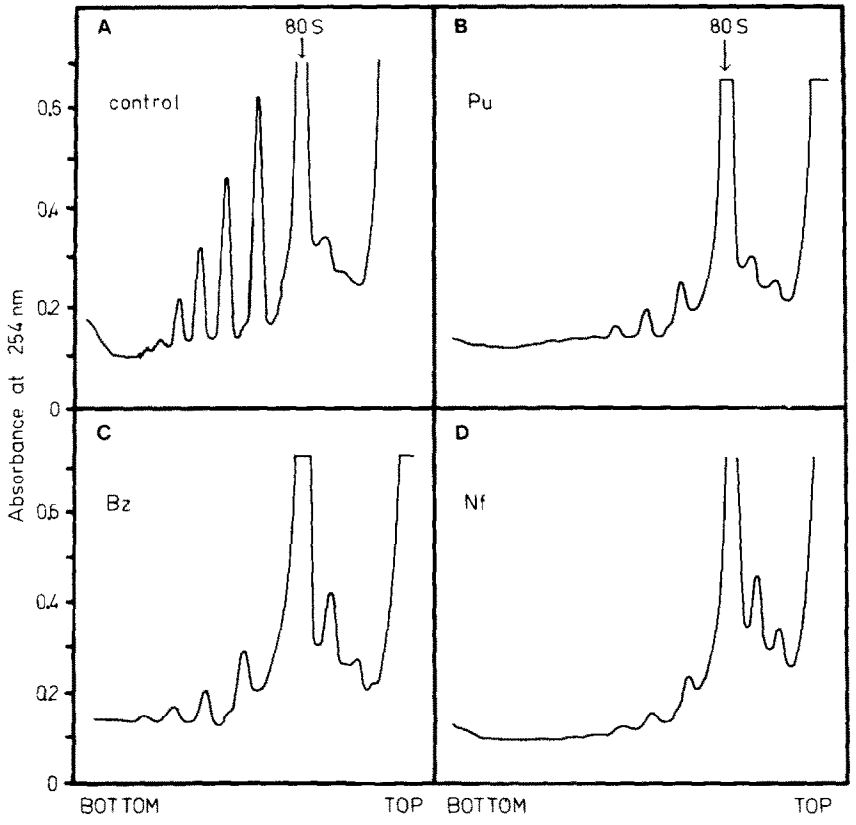

Fig. 1. Polysomal profiles from trypanocidal drug-treated *T. cruzi* epimastigotes, determined as described under Materials and Methods. Abbreviations: Pu, control to which puromycin (50 μg/ml) was added; Bz, benznidazole (200 μM); and Nf, nifurtimox (200 μM).

sedimentation at 10,000 *g* for 20 min. The lysate was spun down at 140,000 *g* for 2 hr and the upper two-thirds of the supernatant fluid was collected, fractionated in small aliquots, and kept at −70° as the source of activating enzymes.

To obtain polysomes with a high degree of polymerization, the parasites were harvested at the middle exponential phase of growth and in the presence of cycloheximide (CH, 50 μg/ml) to block further protein elongation. The presence of the antibiotic at the time of collection is essential for *C. fasciculata*, but it has little effect on the profile of polysomes isolated from *T. cruzi*. After 10 min at 28°, the cells were centrifuged at 4° and washed twice with cold PBS containing CH (50 μg/ml). The cells were resuspended in lysis buffer as before and lysed by exposure to the nonionic detergent Nonidet P-40 at a final concentration of 0.2% (v/v) for 10 min in ice. The homogenate was freed of debris and kinetoplasts by centrifugation at low speed. The supernatant fraction containing polysomes and free ribosomes was layered onto a discontinuous gradient of 2 M and 1.5 M sucrose made up in TMK buffer [10 mM Tris–HCl (pH 7.6), 1 mM magnesium acetate and 100 mM potassium acetate]. The gradient was centrifuged for 2 hr at 150,000 *g*. The supernatant fluid was discarded and the ribosomal pellet carefully resuspended in a small volume of TMK buffer and kept frozen at −70°.

For studying the polysomal profile of lysates obtained from cultures of parasites grown in the presence or absence of different trypanocidal drugs, 10-ml aliquots of cultures containing 35×10^6 parasites per ml were treated with the drugs following incubation at 28° in a rotatory shaker. After 2 hr for *C. fasciculata*, or 2 and 4 for *T. cruzi*, CH (50 μg/ml) was added and growth continued for 15 min at the same temperature. Cultures were cooled down in ice, centrifuged at low speed, and the cells were washed twice with cold PBS containing CH. The cells were lysed in the presence of nonionic detergent as described above. The homogenates were layered on top of a 15–45% linear sucrose gradient in TMK buffer and centrifuged in a Beckman SW-50.1 rotor at 45,000 rpm during 45 min. Polysomal profiles were analyzed through an ISCO analyzer by monitoring the absorbance at 254 nm.

The cell-free system

Assay of amino acid activation. The incubation mixture contained in a final volume of 50 μl: 40 mM Hepes (pH 7.5), 13 mM creatine phosphate, 50 μg of creatine phosphokinase, 2 mM dithiothreitol, a 0.04 mM concentration of a mixture of nineteen unlabeled amino acids minus methionine, 20 mM potassium acetate, 3 mM CTP, 11 mM ATP, 0.3 mM GTP, 0.3 mg of tRNA from wheat germ, 0.5 mM spermidine, 10 mM magnesium acetate, 1 μCi of

Table 1. Lack of effect of nifurtimox and benznidazole on protein synthesis *in vitro* by cell-free preparations of *Crithidia fasciculata* and *Trypanosoma cruzi*

Expt	Organism	Radioactivity (cpm)		
		Control	NF	BZ
1	*C. fasciculata*	37,760 ± 2,448 (3)	38,290 ± 1.163 (3)	38,270 ± 821 (3)
2	*T. cruzi*	1,922 ± 292 (5)	—	1,785 ± 514 (5)
3	*T. cruzi*	6,499 ± 826 (3)	5,000 ± 736 (3)	—

Protein biosynthesis was assayed as described under Materials and Methods, with the addition of 10 mM ascorbic acid and, where indicated, 400 μM NF or BZ. The results are means ± SD; the numbers of determinations are given in parentheses. The values shown were not statistically significant in the ANOVA test ($P > 0.05$). Ascorbate *per se* did not affect significantly protein synthesis.

[^{35}S]methionine and lysate. After incubation at 37° for 15 min, the mixtures were diluted with 1 ml of water containing bovine serum albumin and unlabeled methionine as carriers. The macromolecules were precipitated with 0.5 ml of cold 25% (w/v) TCA. After standing at 4° for 30 min, the precipitates were filtered on glass fiber filters, washed with cold 8% TCA, ethanol, and the radioactivity was estimated in a scintillation counter after drying.

Assay for protein biosynthesis. The cell-free system contained in a total volume of 50 μl: 20 mM Hepes (pH 7.5), 13 mM creatine phosphate, 100 μg of creatine phosphokinase, 2 mM dithiothreitol, a 0.04 mM concentration of an unlabeled amino acids mixture without methionine, 60 mM potassium acetate, 2 mM ATP, 0.18 mM GTP, 0.1 mg of tRNA from rat liver or wheat germ, 0.3 mM spermidine, 3 mM magnesium acetate for *T. cruzi* or 4 mM for *C. fasciculata*, 1 to 1.5 units of absorbance at 260 nm of polysomal fraction, 1 μCi of [^{35}S]methionine and lysate. The incubations were carried out at 37° during 15 min and stopped by addition of 0.5 ml of 25% (w/v) TCA. After heating at 90° for 15 min in the presence of albumin and unlabeled methionine, the precipitates were processed and counted as described above.

Chemicals

NF and BZ, donated by Dr J. A. Castro, CEITOX, Buenos Aires, Argentina, were dissolved in dimethyl sulfoxide (DMSO). BE, suramin, PE and ANT, given by Dr G. C. Hill, then at the Colorado State University, Fort Collins, CO, were dissolved in water.CH and puromycin, purchased from Sigma Chemical Co. (St Louis, MO), were also dissolved in water.

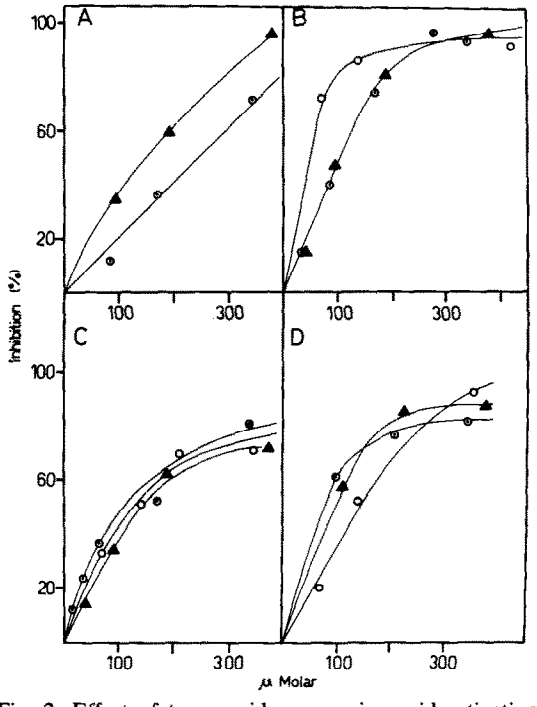

Fig. 2. Effect of trypanocides on amino acid activation (panels A and C) and amino acid polymerization (panels B and D) by cell-free systems isolated from *T. cruzi* (A and B) or *C. fasciculata* (C and D). Experimental conditions were described in Materials and Methods. Key: (○) Bereníl®; (▲) Antrycide®; (○) and suramin. Control values were 38,550 cpm (A), 10,510 cpm (B), 141,270 cpm (C) and 88,270 cpm (D).

RESULTS

Sucrose gradient analysis of *T. cruzi* lysates obtained from cultures incubated with 200 μM NF or BZ for 4 hr at 28° are shown in Fig. 1. Dissociation of polysomes due to ribosomal run-off was observed (panels C and D), giving a profile similar to that obtained with puromycin, which causes polysomal disruption by premature termination of growing polypeptide chains (B). Panel A shows the polysomal profile of the control without drugs, which had most of the ribosomes sedimenting as polysomal aggregates in the heavy zone of the gradient. NF was able to cause an almost complete polysomal dissociation after a 2-hr incubation, whereas in this case BZ showed little difference from the control (not shown); this greater effectiveness of NF was also observed with *C. fasciculata*, which showed a similar

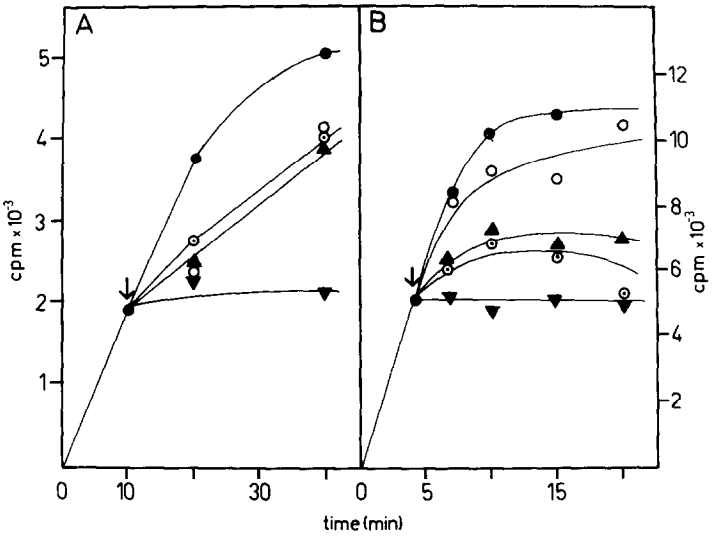

Fig. 3. Effect of the addition of trypanocides or cycloheximide to protein-synthesizing cell-free systems from *T. cruzi* (A) or *C. fasciculata* (B). After the systems had been synthesizing protein for 10 min (A) or 4 min (B), BE (⊙), ANT (▲), suramin (○) or CH (▼) was added (arrow), incubations were continued, and aliquots were taken at the times indicated on the abscissa. The closed circles (●) represent the control.

behavior after a 2-hr incubation with the drugs (data not shown).

Other compounds used against African trypanosomiasis, but ineffective against Chagas' disease, namely BE, PE, ANT and suramin, were also tested at the same concentration (except for suramin, tested at 370 μM). The ribosomal profiles of the lysates were identical to those of the control.

These results might suggest an effect of NF and BZ on protein biosynthesis; however, when incorporation of radiolabeled methionine *in vitro* by cell-free protein systems from both *T. cruzi* and *C. fasciculata* was tested, no effects of the drugs were found, even at concentrations as high as 800 μM (data not shown). Since NF is thought to act through the generation of oxygen-free radicals [1], the experiments were repeated in the presence of ascorbic acid, which is known to promote free radical generation from nitrohetero cyclic compounds [7]. Table 1 shows that no significant inhibition was observed, either by NF or BZ in the presence of 10 mM ascorbic acid. Under these conditions there was oxygen consumption, as measured in an oxygraph (1.66 and 6.71 nmol O_2/min, in the absence and presence of 400 μM NF, respectively). This indicates formation of nitro anion radicals by reduction with ascorbate and superoxide anion radical production.

Figure 2 shows the effects of increasing concentrations of BE, ANT and suramin on homologous systems of protein synthesis isolated from *T. cruzi* (panels A and B) and *C. fasciculata* (panels C and D). BE and ANT were inhibitors of both amino acid activation and amino acid incorporation into hot TCA-insoluble material; the latter process was inhibited by lower concentrations of the drugs. Maximal inhibition was attained at about 200 μM; in the case of amino acid incorporation for both organisms, PE was little effective, 300 μM being required for 25% inhibition of amino acid polymerization (not shown).

The effects of suramin were contradictory and showed some variation in different preparations. As previously shown by Chesters [8] with *C. oncopelti*, low concentrations (up to 40 μM) of suramin slightly stimulated amino acid incorporation into protein by preparations of *C. fasciculata*. Higher concentrations caused the inhibition shown in Fig. 2D. In the case of *T. cruzi*, the activation was not found, and suramin was the most effective of the inhibitors of amino acid incorporation into protein tested. However, suramin did not inhibit amino acid activation by *T. cruzi* except after keeping the lysates at −70° for about a month (not shown). When BE, ANT and suramin were added to incubation mixtures using the *T. cruzi* and *C. fasciculata* systems, in which protein synthesis had been going on for 10 or 4 min, respectively, amino acid incorporation was inhibited to some extent, whereas CH, a well-known inhibitor of elongation, stopped immediately further incorporation of label into protein in both systems (Fig. 3). This suggests that, if the inhibition by trypanocidal drugs is related to the elongation step, they are weaker inhibitors than CH.

DISCUSSION

Polysomal profiles obtained by sucrose gradient analysis of lysates of both *T. cruzi* and *C. fasciculata* treated with NF or BZ *in vivo* showed dissociation of polysomes due to ribosomal run-off in both cases; NF was more effective, in good agreement with its greater effectiveness as an inhibitor of growth and macromolecular synthesis in *T. cruzi* [1]. Gugliotta *et al.* [3] have reported a similar effect of another nitrofuran drug, SQ-18506, on *T. cruzi*. Although the alterations of the ribosomal pattern obtained

after treatment with NF, BZ or puromycin were similar, this does not demonstrate that all these drugs are mechanistically identical.

The lack of effect of BE, PE, ANT and suramin on the polysomal profiles agrees well with their ineffectiveness on both *C. fasciculata* [9] and *T. cruzi* [10]. When incorporation of radiolabeled methionine *in vitro* by cell-free protein synthesis systems from both trypanosomatids was tested, no effect of NF or BZ was found, up to concentrations as high as 800 μM (80-fold higher than the I_{50} values for NF [17], even in the presence of ascorbate as an oxygen free-radical-generating system. This negative result contrasts with those obtained *in vivo* by Goijman *et al.* [1] with NF, since 49 and 72% inhibition of L-[^{3}H]leucine incorporation into protein was attained at 10 and 100 μM respectively. The latter authors suggested that this inhibition was not due to inhibition of leucine uptake [1], but they did not rule out the possibility of an indirect effect due to energy shortage, caused by NF-induced mitochondrial membrane damage. The inhibition of [^{14}C]leucine incorporation by BZ found by Polak and Richle [2], 70% at 100 μM, was probably due to inhibition of amino acid uptake [1].

BE, ANT and suramin, on the other hand, were effective inhibitors of both amino acid activation and amino acid incorporation into hot TCA-insoluble material. The results obtained with *C. fasciculata* agree well with those of Kahan *et al.* [5], except that PE, which in their case was the most effective inhibitor of leucyl-tRNA synthetase, showed only little effect on amino acid activation in the present study. It should be pointed out that different amino acids (Leu and Met) were used in both cases, and therefore this discrepancy may be due to different sensitivities to PE of both aminoacyl-tRNA synthetases. Moreover, when amino acid polymerization was measured, the order of effectiveness of ANT, suramin and PE was the same for Kahan *et al.* [5] and the present study.

Our results suggest that the inhibition of protein biosynthesis by BE, ANT and suramin probably can be explained by a sum of effects on amino acid activation and initiation of polymerization; an effect on elongation, weaker than that of CH, and an effect on termination, however, cannot be excluded. Although we were not able to work with African trypanosomes and cell-free systems derived from them, the inhibition of protein biosynthesis *in vitro* by BE, ANT and suramin, and to a lesser extent, by PE, in the *T. cruzi* and *C. fasciculata* systems suggests that this inhibition may be an important component of the trypanocidal action of these drugs on *T. brucei*; an effect of ANT on this process has been described [11].

The present results suggest that direct inhibition of protein biosynthesis is not the reason for the trypanocidal action of NF and BZ on *T. cruzi*. The effects observed by measuring labeled leucine uptake [1, 2] or by the study of polysomal profiles in the case of SQ-18506 [3] would be indirect, mediated through both inhibition of nucleic acid synthesis and disruption of energy metabolism, essential for an energy consuming process as protein synthesis.

Acknowledgements—We wish to thank Mrs Berta Franke de Cazzulo for her help in obtaining *T. cruzi* and *C. fasciculata* cultures, and Dr I. D. Algranati for helpful discussions. The authors are members of the Research Career of the Consejo Nacional de Investigaciones Cientificas y Técnicas de la Republica Argentina (CONICET).

REFERENCES

1. Goijman SG, Frasch ACC and Stoppani AOM, Efectos diferentes del nifurtimox y el benznidazol sobre la biosintesis de DNA, RNA y proteinas en *Trypanosoma cruzi*. *Medicina (Buenos Aires)* **44**: 261–270, 1984.
2. Polak A and Richle R, Mode of action of the 2-nitroimidazole derivative benznidazole. *Ann Trop Med Parasitol* **72**: 45–54, 1978.
3. Gugliotta JL, Tanowitz HB, Wittner M and Soeiro R, *Trypanosoma cruzi*: inhibition of protein synthesis by nitrofuran SQ-18506. *Exp Parasitol* **49**: 216–224, 1980.
4. Sims P and Gutteridge WE, Mode of action of a 5-nitrofuran drug (SQ 18506) against *Trypanosoma cruzi*. *Int J Parasitol* **9**: 61–67, 1978.
5. Kahan D, Zahalsky AC and Hutner SH, Protein synthesis in cell-free preparations of *Crithidia fasciculata*. *J Protozool* **15**: 385–390, 1968.
6. Cazzulo JJ, Franke de Cazzulo BM, Engel JC and Cannata JJB, End products and enzyme levels of aerobic glucose fermentation in trypanosomatids. *Mol Biochem Parasitol* **16**: 329–343, 1985.
7. Rao DNR, Harman L, Motten A, Schreiber J and Mason RP, Generation of radical anions of nitrofurantoin, misonidazole and metronidazole by ascorbate. *Arch Biochem Biophys* **225**: 419–427, 1987.
8. Chesters JK, Protein synthesis by cell-free extracts of *Crithidia oncopelti*. *Biochim Biophys Acta* **114**: 385–397, 1966.
9. Bacchi CJ, Lambros C, Goldberg B, Hutner SH and de Carvalho GDF, Susceptibility of an insect *Leptomonas* and *Crithidia fasciculata* to several established antitrypanosomatid agents. *Antimicrob Agents Chemother* **6**: 785–790, 1974.
10. Gutteridge WE, Chemotherapy of Chagas' disease: the present situation. *Trop Dis Bull* **73**: 699–705, 1976.
11. Gutteridge WE and Coombs GH, *Biochemistry of Parasitic Protozoa* p. 139. Macmillan Press, London, 1977.