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EFFECTS OF NITROHETEROCYCLIC DRUGS ON MACROMOLECULE SYNTHESIS AND DEGRADATION IN *TRYPANOSOMA CRUZI*

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Abstract—Nifurtimox and benznidazole, two nitroheterocyclic drugs, (a) inhibited DNA, RNA and protein synthesis, (b) stimulated macromolecular degradation, and (c) stimulated unscheduled DNA synthesis in *Trypanosoma cruzi* (Tulahuen strain). Significant differences in the mode of action of these drugs could be established and, in every case, nifurtimox was more active than benznidazole. The inhibition of macromolecular synthesis varied with drug concentration, precursor and incubation time. Nifurtimox effect was time dependent and irreversible. When assayed on macromolecular degradation, nifurtimox was more effective on DNA and protein than on RNA, while benznidazole displayed almost the same activity on DNA, RNA and protein. Labeling of RNA with [^{3}H]uridine in the presence of nifurtimox followed atypical kinetics since, depending on incubation time and drug concentration, RNA degradation prevailed over RNA synthesis.

Nifurtimox† (a 5-nitrofuran derivative) and benznidazole (a 2-nitroimidazole derivative) are the drugs most frequently used for the treatment of Chagas' disease (American trypanosomiasis) [1, 2]. Both inhibit *in vitro* growth of *Trypanosoma cruzi* (the agent of Chagas' disease), nifurtimox being the more effective [3, 4]. Several studies have been performed on the mode of action of these drugs, but knowledge on this subject is still incomplete. The mode of action of trypanocidal drugs on *T. cruzi* is a highly relevant topic for the chemotherapy of Chagas' disease, since, besides its intrinsic interest, it allows one to detect biochemical targets for new drugs, more effective than those used at present. DNA, RNA and protein synthesis are suitable parameters for the assay of therapeutic drugs since suppression of the synthesis of these macromolecules *in vitro* may predict the biological event *in vivo*. DNA, RNA and protein synthesis in living cells can be monitored by measuring the incorporation of labeled thymidine, uridine and L-leucine, respectively, and this procedure has been extensively applied in studies on trypanosomatidae [5–8] and cancer cells [9–11]. These considerations prompted us to compare the effect of nifurtimox and benznidazole on [^{3}H]thymidine, [^{3}H]uridine and L-[^{3}H]leucine incorporation into acid-stable macromolecules of *T. cruzi* culture forms. Complementary information was obtained by assaying the same drugs on macromolecule degradation and unscheduled DNA synthesis. The results obtained show significant differences between the modes of action of nifurtimox and benznidazole.

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† Definitions: nifurtimox, 3-methyl-4-(5′-nitrofurfurylidene-amino)-tetrahydro-4*H*-1,4-thiazone-1,1′-dioxide; and benznidazole, *N*-benzyl-2-nitro-1-imidazole acetamide.

MATERIALS AND METHODS

Organism T. cruzi (Tulahuen strain) was cultured for 3–4 days, at 28°, in a liquid medium made of brain–heart infusion (Difco), 37 g; hemin, 20 mg; fetal bovine serum, 40 ml; and water, to 1 litre [3, 4]. The cells, at exponential phase of growth, were collected by centrifugation and resuspended in fresh warm medium at a concentration of 1×10^6 cells/ml. Krebs–Ringer was used for resuspending the cells when uptake or incorporation of [^{3}H]leucine was determined.

Reagents. These were obtained from the following sources: [*methyl*-^{3}H]thymidine (5 Ci/mmole), [5,6-^{3}H]uridine (40 Ci/mmole) and L-[4,5-^{3}H]leucine (40 Ci/mmole) (henceforth [^{3}H]thymidine, [^{3}H]uridine and L-[^{3}H]leucine respectively) from the Radiochemical Centre, Amersham, U.K.; nifurtimox from Bayer A.G., Leverkusen, Federal Republic of Germany; benznidazole from Hoffmann-La Roche Inc., Basel, Switzerland; and hydroxyurea, caffeine, cycloheximide, phenyloxazolylbenzene (POPOP) and diphenyloxyazole (PPO), from the Sigma Chemical Co., St. Louis, MO, U.S.A. Nifurtimox and benznidazole were dissolved in a 50% (v/v) dimethyl sulfoxide–ethanol mixture. The control samples received the corresponding volume of solvent.

Precursor incorporation into macromolecules. [^{3}H]-Thymidine, [^{3}H]uridine and L-[^{3}H]leucine were assayed simultaneously on the same batch of epimastigotes. Aliquots of *T. cruzi* epimastigotes (10^6/ml) in culture medium were given drug and the ^{3}H-labeled precursor (2 μCi/ml). Epimastigote samples were incubated in a New Brunswick Gyratory Water Bath, model 45, at 37°. After incubation for the time stated, 1-ml duplicate samples were withdrawn and

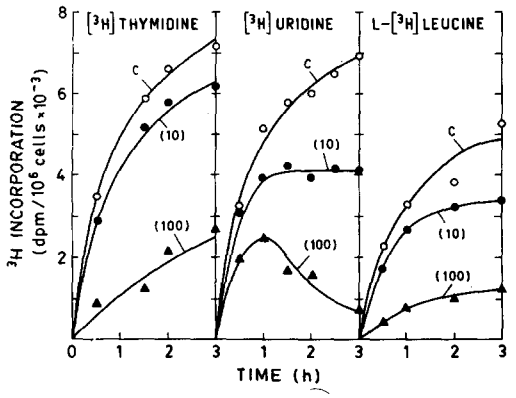

Fig. 1. Effect of nifurtimox on ^{3}H-labeled precursor incorporation into macromolecules. Samples contained *T. cruzi* epimastigotes at the exponential phase of growth (10^6 cells/ml), precursor (2 μCi/ml) and nifurtimox (μM) as indicated by the figures in parentheses; C indicates the control sample. Incubation time was as indicated on the abscissa; other experimental conditions were as described under Materials and Methods. The points represent the mean from duplicate samples of the same epimastigote suspension. The difference between the experimental values and the mean value was less than 5%.

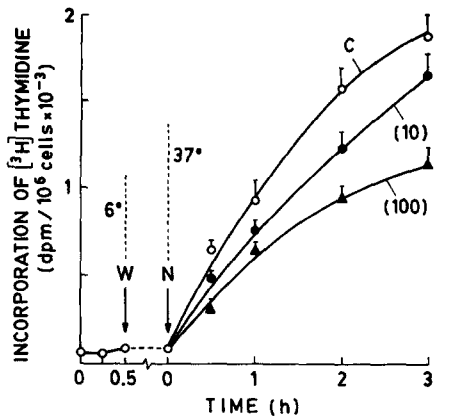

Fig. 2. Effect of nifurtimox on [^{3}H]thymidine incorporation into macromolecules in *T. cruzi* epimastigotes preincubated at 6° with [^{3}H]thymidine and then incubated at 37° in fresh medium with nifurtimox, without precursor. W and N indicate times at which epimastigotes were washed free of precursor, and at which nifurtimox was added, respectively. Nifurtimox concentration (μM) is indicated by the figures in parentheses. Other conditions were as described in the legend of Fig. 1 and the text. Values are the mean from three different epimastigote suspensions; the bars over the points represent the S.D.

filtered immediately on 0.45 μm pore-size Metricel filters. Samples for measuring L-[^{3}H]leucine incorporation into protein were heated to 100° for 10 min, before filtering, to eliminate *t*RNA complexes. The cells trapped on the filters were washed twice with 8 ml of ice-cold 9% (w/v) NaCl, once with 8 ml of ice-cold 10% (w/v) trichloroacetic acid and once with 8 ml of 5% (w/v) trichloroacetic acid. Filters were dried and placed in vials containing 10 ml of scintillation liquid (2.5 g PPO and 0.05 g POPOP in 1 litre toluene). Radioactivity was measured in a Tracor 2000 Analytical System working at 30–35% efficiency. Each experiment was repeated three times.

Effect of drugs on macromolecular turnover and degradation. Epimastigotes were preincubated for 3 hr in culture medium supplemented with [^{3}H]thymidine, [^{3}H]uridine or L-[^{3}H]leucine (0.5 to 1.0 μCi/ml), to label DNA, RNA and protein respectively. The cells were collected by centrifugation, washed once, and resuspended in fresh medium, at a concentration of 10^6 cells/ml. Nifurtimox and benznidazole were added as indicated under Results, and the incubation mixture was supplemented with fresh medium to 1 ml. Drug-treated and control epimastigotes were further incubated in a New Brunswick Gyratory Water Bath, for 3 hr at 37°. Samples were taken at 1-hr intervals and filtered through 0.45 μm pore-size Metricel filters; the filters were washed with 10% and 5% (v/v) trichloroacetic acid and, after drying, radioactivity was counted as described above. Half-life ($t_{0.5}$) of labeled pools was calculated from the equation $2.3 \log (A_t/A_0) = -k \cdot t$ where A_t and A_0 are the relative radioactivity values in epimastigotes at times t and 0, respectively (A_0 = 100%), and $-k$ is the slope of the corresponding straight line. For $A_t = 0.5\ A_0$, $0.69/k = t_{0.5}$.

Assay of unscheduled DNA synthesis. Epimastigotes at the exponential phase of growth (phase S; 10^6/ml) were incubated in culture medium with 10 mM hydroxyurea and 0.5 mM cycloheximide for 16 hr (a control experiment showed 89% inhibition of thymidine incorporation). [^{3}H]Thymidine (1 μC/ml) and trypanocide agents were then added and,

Table 1. Effect of nifurtimox on the incorporation of ^{3}H-labeled precursors into *T. cruzi* macromolecules*

Nifurtimox (μM)	Macromolecule precursor (%)		
	[^{3}H]Thymidine	[^{3}H]Uridine	L-[^{3}H]Leucine
0	100 ± 6	100 ± 10	100 ± 5
10	87 ± 6†	58 ± 8	65 ± 5
100	38 ± 4	8 ± 3	22 ± 5

* Experimental conditions were as in Fig. 1; time of incubation, 3 hr. Results represent average values for duplicate samples from three experiments and are expressed as percentages of control ± S.D. of the mean. Radioactivity values in control samples were approximately as in Fig. 1. Student's *t*-test of nifurtimox values yielded $P < 0.05$ except where indicated by †.

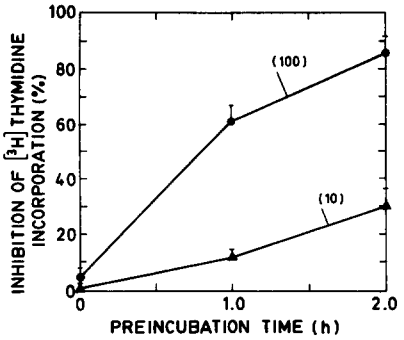

Fig. 3. Irreversibility of nifurtimox inhibition of DNA synthesis. To the *T. cruzi* epimastigotes suspended in culture medium (10^6/ml), nifurtimox was added as indicated by the figures in parentheses (μM). After incubation for the time indicated on the abscissa, control and nifurtimox-treated epimastigotes were washed, resuspended in fresh medium to the original concentration and assayed for [^{3}H]-thymidine incorporation, for 1 hr. Other conditions were as described in the legend of Fig. 1 and the text. The first preincubation time was less than 10 sec ("0-time sample"). Values are the mean from three different epimastigote suspensions; the bar over the points represent the S.D.

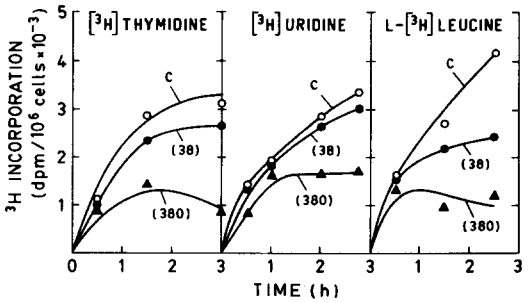

Fig. 4. Effect of benznidazole on precursor incorporation into macromolecules. Experimental conditions were as described in the legend of Fig. 1, except for the drug and the epimastigote sample. Typical experiment.

after 5 hr of incubation at 37° [^{3}H]thymidine incorporation was measured following the standard procedure.

RESULTS

Effect of nifurtimox on precursor incorporation into macromolecules. In good agreement with previous observations with other strains of *T. cruzi* [5–7, 12, 13], the Tulahuen strain incorporated [^{3}H]-thymidine, [^{3}H]uridine and L-[^{3}H]leucine into acid-stable macromolecules. Figure 1 shows the result of a typical experiment. The incorporation rate declined with time, as occurs with epimastigotes approaching the stationary state [12]. Nifurtimox inhibited precursor incorporation at concentrations effective on the parasite growth [3, 14]. The inhibition varied with the drug concentration, precursor and incubation time. The kinetics of [^{3}H]thymidine and L-[^{3}H]leucine incorporation in the presence of nifurtimox were similar and resembled the corresponding control. However, with [^{3}H]uridine, the kinetics were atypical since, after the first hour of incubation with 10 μM nifurtimox, the nucleotide incorporation apparently ceased, and with 100 μM nifurtimox, RNA radioactivity decreased below the first hour level, thus originating a characteristic peak in the curve. Table 1 summarizes the results obtained with three different samples of epimastigotes. After 3 hr of incubation, precursor incorporation was inhibited significantly, [^{3}H]thymidine incorporation being less affected than that of [^{3}H]uridine or L-[^{3}H]leucine, especially with 10 μM nifurtimox.

To establish unequivocally whether the effect of nifurtimox on [^{3}H]thymidine incorporation reflected the inhibition of DNA synthesis and was not due only to inhibition of precursor uptake, the procedure of Plagemann [15] was adopted. This procedure enables one to bypass the inhibition of the precursor transport by measuring the incorporation of the [^{3}H]-thymidine that was present in the cells when nifurtimox was added. Accordingly, epimastigotes (10^6/ml) were preincubated in medium containing [^{3}H]-thymidine (2 μC/ml) for 30 min at 6° (under these conditions, the nucleotide was taken up by the cells, but was not incorporated into DNA). After preincubation, the cells were quickly washed, resuspended in the original volume of fresh medium and reincubated at 37°, without [^{3}H]thymidine, as indicated in Fig. 2. It is to be seen that after 3 hr of incubation, 10 μM (or 100 μM) nifurtimox inhibited [^{3}H]thymidine incorporation by 13% (or 41%). The effect of 100 μM nifurtimox was somewhat lower than the corresponding value in Table 1, thus showing the contribution of [^{3}H]thymidine transport (or phosphorylation) inhibition to the overall effect of nifurtimox.

The kinetics of nifurtimox inhibition in Fig. 1 suggest a time-dependent effect. To confirm this

Table 2. Effect of benznidazole on the incorporation of ^{3}H-labeled precursors into *T. cruzi* macromolecules*

Benznidazole (μM)	Macromolecule precursor (%)		
	[^{3}H]Thymidine	[^{3}H]Uridine	L-[^{3}H]Leucine
0	100 ± 5	100 ± 5	100 ± 5
38	85 ± 6	89 ± 7†	57 ± 6
380	25 ± 4	50 ± 6	28 ± 5

* Experimental conditions were as in Fig. 4; time of incubation, 2.5 to 3 hr. Results are expressed as in Table 1. Radioactivity values in control samples were approximately as in Fig. 4. Student's *t*-test of benznidazole values yielded $P < 0.05$, except where indicated by †.

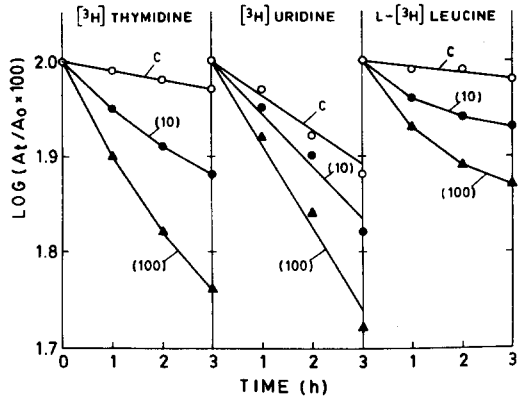

Fig. 5. Effect of nifurtimox on the kinetics of macromolecular degradation. *T. cruzi* epimastigotes, prelabeled with precursor as indicated in the figure, were washed free of precursor, resuspended in fresh medium, and reincubated with nifurtimox for the time indicated on the abscissa. C indicates control samples; nifurtimox concentration (μM) is indicated by the figures in parentheses. The 2.3 factor is included in $-k$. Other experimental conditions as described under Materials and Methods. The points represent the mean of duplicate samples of the same epimastigote suspension. The difference between the experimental value and the mean value was less than 5%. Typical experiment.

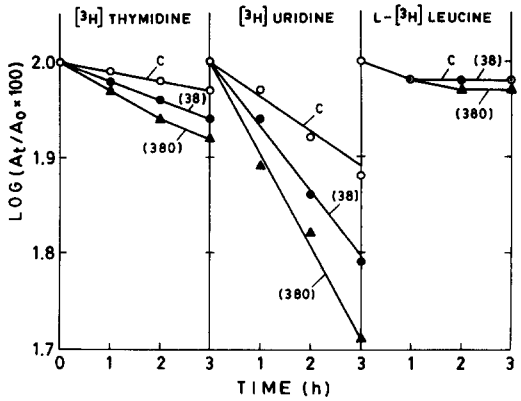

Fig. 6. Effect of benznidazole on the kinetics of macromolecular degradation. Experimental conditions were as described in the legend of Fig. 5, except for the drug and the epimastigote suspension.

hypothesis, *T. cruzi* epimastigotes were incubated with nifurtimox for the times indicated in Fig. 3, washed free of inhibitor, and then tested for [^{3}H]-thymidine incorporation in fresh culture medium. The results presented show that, after a 1-min incubation with 100 μM nifurtimox, the inhibition of DNA synthesis was limited (6%) but after a 1- and 2-hr incubation, it rose to 62 and 86% respectively. Similar results were obtained with 10 μM nifurtimox, at lower levels of inhibition.

Effect of benznidazole on precursor incorporation into macromolecules. The drug concentrations were selected on the basis of the experimental protocol of Polak and Richle [5]. A comparison of results in Figs. 1 and 4 shows that, when assayed on the same strain of *T. cruzi*, benznidazole was significantly less effective than nifurtimox. Benznidazole inhibition of precursor incorporation depended also on time of incubation and drug concentration. Thus, (a) at fixed benznidazole concentration, the effect was much greater after 2–3 hr of incubation than after 30 min (Fig. 4); (b) 38 μM benznidazole inhibited selectively protein synthesis, while 380 μM benznidazole inhibited all macromolecular synthesis, but especially DNA and protein synthesis (Table 2).

Effect of drugs on macromolecule degradation. T. cruzi macromolecules are in a dynamic state resulting from the balance of biosynthesis and degradation. To establish whether nifurtimox and benznidazole stimulated the latter process, the drugs were assayed on epimastigotes previously labeled with [^{3}H]thymidine, [^{3}H]uridine or L-[^{3}H]leucine, and henceforth chased with the medium non-radioactive precursors. Figure 5 shows the results with nifurtimox. Radioactivity in control epimastigotes decayed in accordance with single first-order kinetics, with apparent half-lives of about 7 hr for RNA, 30 hr for DNA and 75 hr for protein. It should be noted that DNA and protein values represented duplication, rather than turnover rates since epimastigotes were still at the exponential phase of growth. On the other hand, RNA half-life was significantly shorter than that of typical ribosomal RNA [16] and, accordingly, it may represent molecules turning over at a relative fast rate, such as mRNA [17]. Addition of nifurtimox increased the decay of macromolecule radioactivity

Table 3. Effects of trypanocidal drugs on macromolecular degradation in *T. cruzi**

Drug	Conc (μM)	Half-life ($t_{0.5}$ in hr) DNA	RNA	Protein
Nifurtimox	0	28.6 ± 1.0	7.5 ± 0.8	75.0 ± 5.0
	10	7.0 ± 1.2 (4.1)†	5.1 ± 0.8 (1.5)†	10.7 ± 1.0 (7.0)
	100	3.3 ± 1.2 (8.7)†	3.2 ± 1.0 (2.3)†	5.4 ± 1.0 (14)
Benznidazole	0	36.1 ± 1.6	8.1 ± 0.7	53.2 ± 1.6
	38	16.1 ± 1.6 (2.2)	4.3 ± 0.8 (1.9)	36.9 ± 1.6 (1.4)
	380	12.4 ± 1.6 (2.9)	3.1 ± 0.8 (2.6)	37.1 ± 1.6 (1.4)

* Experimental conditions were as described in Figs. 5 and 6; $t_{0.5}$ represents the average values for duplicate samples from two experiments. Student's *t*-test of nifurtimox or benznidazole values yielded $P < 0.05$. Values in parentheses: $t_{0.5}$ control cells/$t_{0.5}$ drug-treated cells.

† Calculated with 2-hr incubation values.

Table 4. Effects of nifurtimox and benznidazole on unscheduled synthesis of DNA in *Trypanosoma cruzi**

Drug (μM)	[^{3}H] Thymidine incorporation (dpm/10^6 cells)	Relative increase as compared with the control
None	170 ± 8†	
Nifurtimox (100)	1527 ± 83	9-fold
Benznidazole (380)	457 ± 23	3-fold

* Experimental conditions were as described under Materials and Methods.

† Values are the average of triplicate experiments; mean ± S.E.

(Fig. 5) and a similar, though lesser, action was exerted by benznidazole (Fig. 6). The effects of the drugs were concentration dependent and demonstrated macromolecule degradation rather than dilution by net synthesis, since this was not stimulated by either nifurtimox or benznidazole (Figs. 1 and 4; Tables 1 and 2). Nifurtimox effect was maximal on protein and minimal on RNA, while benznidazole stimulated especially DNA and RNA degradation (Table 3).

Effect of drugs on "unscheduled DNA synthesis". Unscheduled DNA synthesis can be used to measure "repair replication" and "excision repair" in eukaryotic cells, since the newly synthesized DNA can serve as template for normal DNA [18–20]. Monitoring the non-S-phase incorporation of [^{3}H]-thymidine into DNA enabled us to assay cellular DNA repair, but its measurement required the inhibition of DNA normal (semiconservative) replication. Several inhibitors of normal replication were examined for their action in *T. cruzi* and, as a result of these assays, 10 mM hydroxyurea plus 0.3 mM cycloheximide was selected. The results in Table 4 shows that nifurtimox and benznidazole significantly stimulated unscheduled DNA synthesis, nifurtimox being the more effective.

DISCUSSION

Previous studies on nitrofuran inhibition of macromolecule biosynthesis in *T. cruzi* produced rather contradictory results. Thus, using the 5-nitrofuran SQ 18506, Gugliotta *et al.* [13] observed inhibition of protein and RNA formation but DNA was not affected. On the other hand, Sims and Gutteridge [6] obtained inhibition of DNA and RNA formation by the same drug. Our observations with nifurtimox are in good agreement with those of Sims and Gutteridge [6], although DNA synthesis was less inhibited by nifurtimox than protein synthesis. As regards benznidazole, the results in Fig. 4 and Table 2 fit in well with those of Polak and Richle [5], except for the inhibition of DNA synthesis. It should be noted that the results quoted were obtained with different strains of *T. cruzi* (Sonia [6, 7], Y [5], "Brazilian" [13] and Tulahuen 0 [present study]) which means that the different responses to the drugs assayed might reflect specific strain characteristics. If this were true, the study of the response of *T. cruzi* strains to trypanocidal agents would be of practical importance for the treatment of Chagas' disease.

Comparison of nifurtimox and benznidazole effects in Tables 1 and 2 with the growth-inhibiting activity of the same drugs [3, 4] shows that 10 μM nifurtimox and 38 μM benznidazole produced half-maximal inhibition of growth and almost the same inhibition of protein synthesis. On the other hand, at concentrations exceeding several-fold those required for maximal effect on growth [3, 4], nifurtimox and benznidazole produced partial inhibition of precursor incorporation (Tables 1 and 2). A possible explanation of this discrepancy is that in growth-inhibition experiments the drugs were allowed to act on the parasite for several days, thus producing time-dependent, irreversible effects (Fig. 3). Interestingly enough, the serum concentration of nifurtimox after administration of a single therapeutic dose of nifurtimox (5 mg/kg) to humans is in the range of 10–20 μM [21], in good agreement with nifurtimox concentrations affecting macromolecules in *T. cruzi.*

Comparison of nifurtimox and benznidazole modes of action on *T. cruzi* shows significant differences. Thus, (a) correlation of inhibitory effects and drug concentration values (Table 1 and 2) indicates that, on a molar basis, nifurtimox was always the more active; (b) the same occurred in regard to stimulation of macromolecular degradation (Table 3) and unscheduled DNA synthesis (Table 4); (c) when assayed on macromolecular degradation, nifurtimox was more active on DNA and protein, while benznidazole did not exert such selective effects, at least to the same extent (Table 3); and (d) incorporation of [^{3}H]uridine in the presence of nifurtimox displayed kinetics apparently resulting from the combination of decreased synthesis and increased degradation of RNA, an effect not observed with benznidazole (Figs. 1 and 4). The different effects of nifurtimox and benznidazole might depend on the characteristic midpoint potentials of these drugs, since the electron affinity of the nitro group is of overwhelming importance in controlling the chemical reactions and biological effects of nitroaryl compounds [22–25]. Nevertheless, the differences noted may also be attributed to oxygen radicals produced by nifurtimox redox cycling in *T. cruzi.* The role of these radicals is supported by (a) the effect of nifurtimox on O_2^- and H_2O_2 generation in the parasite [3]; (b) the similar kinetics of [^{3}H]uridine incoporation in the presence of other "active oxygen" generators, such as β-lapachone [26]: and (c) *T. cruzi* deficiency in enzymatic defenses against oxygen radicals [27, 28]. This latter argument is weakened, however, by the

fact that *Crithidia fasciculata*, an organism possessing catalase, is sensitive to nifurtimox [29]. In addition to the postulated mechanisms of action, the nitroheterocyclic drugs may inhibit specific enzymes (e.g. DNA and RNA polymerase [7]), may interact with DNA [7, 30] and may be reduced to cytotoxic compounds, such as the hydroxylamine derivative [25]. The observations of Agosin [31] with benznidazole are, however, at variance with this latter possibility.

The stimulation of unscheduled DNA synthesis by nifurtimox and benznidazole fits in well with (a) the ability of these drugs to induce strand breaks in the parasite DNAs [32]; (b) *T. cruzi* DNA breakage by the 5-nitrofuran SQ 18506 [7], and (c) the mutagenic action of nifurtimox and benznidazole in organisms such as *Salmonella tiphymurium, Escherichia coli* and *Klebsiella pneumoniae* [33, 34].

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