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Bisphosphonates as Inhibitors of *Trypanosoma cruzi* Hexokinase

*KINETIC AND METABOLIC STUDIES**

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***Trypanosoma cruzi*, the etiologic agent of Chagas disease, has an unusual ATP-dependent hexokinase (TcHK) that is not affected by D-glucose 6-phosphate, but is non-competitively inhibited by inorganic pyrophosphate (PP_i), suggesting a heterotropic modulator effect. In a previous study we identified a novel family of bisphosphonates, metabolically stable analogs of PP_i, which are potent and selective inhibitors of TcHK as well as the proliferation of the clinically relevant intracellular amastigote form of the parasite *in vitro* (Hudock, M. P., Sanz-Rodriguez, C. E., Song, Y., Chan, J. M., Zhang, Y., Odeh, S., Kosztowski, T., Leon-Rossell, A., Concepcion, J. L., Yardley, V., Croft, S. L., Urbina, J. A., and Oldfield, E. (2006) *J. Med. Chem.* 49, 215–223). In this work, we report a detailed kinetic analysis of the effects of three of these bisphosphonates on homogeneous TcHK, as well as on the enzyme in purified intact glycosomes, peroxisome-like organelles that contain most of the glycolytic pathway enzymes in this organism. We also investigated the effects of the same compounds on glucose consumption by intact and digitonin-permeabilized *T. cruzi* epimastigotes, and on the growth of such cells in liver-infusion tryptose medium. The bisphosphonates investigated were several orders of magnitude more active than PP_i as non-competitive or mixed inhibitors of TcHK and blocked the use of glucose by the epimastigotes, inducing a metabolic shift toward the use of amino acids as carbon and energy sources. Furthermore, there was a significant correlation between the IC_{50} values for TcHK inhibition and those for epimastigote growth inhibition for the 12 most potent compounds of this series. Finally, these bisphosphonates did not affect the sterol composition of the treated cells, indicating that they do not act as inhibitors of farnesyl diphosphate synthase. Taken together, our results suggest that these novel bisphosphonates act primarily as specific inhibitors of TcHK and may represent a novel class of selective anti-*T. cruzi* agents.**

Chagas disease remains the major parasitic disease burden in Latin America, despite recent advances in the control of its vectorial and transfusional transmission (1, 2). Specific chemotherapy against its etiological agent, the kinetoplastid parasite *Trypanosoma cruzi*, is unsatisfactory because current drugs have very limited efficacy in the prevalent, chronic phase of the disease, and there are frequent serious side effects (3). Thus, there is an urgent need for safer and more potent drugs to treat this condition, and several rational approaches are being developed, exploiting key biochemical differences between the parasite and its mammalian hosts (3, 4). In this context, it is of note that *T. cruzi*, and several related kinetoplastid protozoa, have several unusual characteristics in their energy metabolism, which differentiates them from other eukaryotes. 1) Most of the glycolytic pathway is compartmentalized in peroxisome-like organelles termed glycosomes (5, 6). 2) The classic, allosteric modulators of the two key regulatory enzymes of the glycolytic pathway in mammalian, fungal, and bacterial organisms, hexokinase and phosphofructokinase, do not affect the kinetoplastid enzymes (7–9). This is associated with the absence of a Pasteur effect in these parasites, in addition to their flexibility in utilizing glucose or amino acids as carbon and energy sources (10–12). 3) Kinetoplastid parasites contain large stores of inorganic pyrophosphate (PP_i) and other short chain polyphosphates, which are by far the most abundant high energy compounds in these cells (13–15).

Although earlier work had shown that *T. cruzi* ATP-dependent hexokinase (TcHK)[2] was not inhibited by its main regulator in vertebrates, D-glucose 6-phosphate (7, 9, 16), more recent studies have shown that this enzyme is inhibited in a non-competitive manner (with respect to ATP) by PP_i, with a K_i of ~500 μM, suggesting that PP_i acts as an heterotropic, allosteric regulator (17). Subsequently, we identified a novel family of metabolically stable PP_i analogs called bisphosphonates, which are potent and selective inhibitors of TcHK, as well as the proliferation of the clinically relevant intracellular amastigote form of the parasite (18). We carried out a QSAR study of 42 compounds and were able to construct pharmacophore and comparative molecular similarity indices analysis (CoMSIA) mod-

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[2] The abbreviations used are: TcHK, *Trypanosoma cruzi* ATP-dependent hexokinase; FPPS, farnesyl diphosphate synthase; LIT, liver infusion tryptose; MIC, minimal inhibitory concentration; PEP, phosphoenolpyruvate.

els for enzyme inhibition (18, 19). However, at that point, no information was available on the mechanism of enzyme inhibition or on the biochemical and physiological effects of these bisphosphonates on the target cells. In the present work, we selected three compounds from the series (Fig. 1), including the two most potent ones, (9-ethyl-9*H*-3-carbazolyl)-aminomethylene-1,1-bisphosphonate (**228**), IC_{50} = 0.81 μM, and (3-bromo-phenyl)-aminomethylene-1,1-bisphosphonate (**302**), IC_{50} = 0.95 μM; and one of intermediate activity (2-(pyridin-4-yl)-1-hydroxyethane-1,1-bisphosphonate (**3**), IC_{50} = 12.7 μM, to carry out a detailed kinetic study of their inhibitory activity against TcHK, as well as to investigate their effects on glucose consumption, energy metabolism, and growth of the extracellular epimastigote form of the parasite. The results show that these compounds are very potent, non-competitive or mixed inhibitors of TcHK that block glucose consumption by intact parasites, leading to a metabolic shift in these cells to the use amino acids as carbon and energy sources. Furthermore, we found that growth inhibition induced by the 12 more potent bisphosphonates of the series was highly correlated with TcHK inhibition. Finally, in agreement with our previous results (18), it was found that the biochemical activity of 228 and 302 was highly selective: they do not affect the parasites sterol composition, which showed that they do not inhibit farnesyl diphosphate synthase (FPPS), a known target of other nitrogen-containing bisphosphonates in eukaryotes, including *T. cruzi* (20–23).

MATERIALS AND METHODS

Parasite—The EP (24) stock of *T. cruzi* was used in this study. Handling of live *T. cruzi* was performed according to established guidelines (25).

T. cruzi Cultivation and Antiproliferative Activities of Bisphosphonates—The epimastigote form of the parasite was cultivated in liver infusion tryptose (LIT) medium (24), supplemented with 10% newborn calf serum (Invitrogen) at 28 °C, with strong agitation (120 rpm). The cultures were initiated at a cell density of 2×10^6 epimastigotes ml^{-1} and cell densities were measured with an electronic particle counter (model ZBI; Coulter Electronics Inc., Hialeah, FL) and by direct counting with a hemocytometer. Cell viability was followed by trypan blue exclusion, using light microscopy. Experimental compounds were added as sterile, 100-fold stocks at a cell density of $1–2 \times 10^7$ epimastigotes ml^{-1}. Growth inhibition was quantified by defining a percent growth factor: % GF = $(GF_{drug}/GF_{control}) \times 100$, where $GF = ((CD)_{96h} - (CD)_{0h})/(CD)_{0h})$, and GF_{drug} is the growth factor in the presence of a given concentration of the drug, $GF_{control}$ is the corresponding value for untreated cells, and $(CD)_{xh}$ are the cell densities of the cultures at *x* hours after the addition of the drug. IC_{50} values were calculated from the % GFs by non-linear correlation with the GraFit software package (Erithacus Software Ltd., Surrey, UK) and statistical analyses were carried out with the JMP 6.0 statistical package (SAS Institute Inc., Cary, NC). Glucose and ammonium concentrations in the growth medium were determined using enzymatic assays, as described (7, 26).

TcHK Isolation, Purification, and Assay—*T. cruzi* hexokinase was purified to homogeneity from exponential phase epimastigotes as described by Caceres *et al.* (17); enzyme activity was determined by using a spectrophotometric assay, coupling its activity to that of D-glucose-6-phosphate dehydrogenase and following the reduction of $NADP^+$ at 340 nm (7, 17); the slope for the change in absorbance as a function of time was constant for at least 5 min and this value was taken to calculate the initial reaction velocities.

Isolation and Purification of Glycosomes and Assay of TcHK Activity in Situ—Highly purified intact glycosomes were obtained from exponential phase epimastigotes as described (27); latencies, defined as: % latency = 100 × (TcHK activity of glycosomes in 0.2% Triton X-100 − TcHK activity of intact glycosomes/TcHK activity of glycosomes in 0.2% Triton X-100), were typically >95% (Triton X-100 concentration is v/v). Glycosomes were incubated in the presence of digitonin (25 $\mu g\ mg^{-1}$ of protein) for 1 min, centrifuged at 35,000 × *g* for 2 min, suspended in buffer A (sucrose 225 mM, KCl 20 mM, KH_2PO_4 10 mM, Na_2EDTA 5 mM, Tris-HCl 20 mM, $MgCl_2$ 1 mM, pH 7.2) and assayed for hexokinase activity, as described above.

Analysis of Kinetic Data—Initial velocity data were obtained as a function of substrate (S) and inhibitor (I) concentrations, keeping the co-substrate concentration at saturating levels and fitted data to the following equation (28): $1/V = (1 + [I]/K_{ii})/V_m + (1 + [I]/K_{is})K_m/V_m[S]$. Where K_m and V_m are the apparent Michaelis-Menten constant and maximal velocity, respectively, and $K_{ii} = [E'S][I]/[E'SI]$; $K_{is} = [E'][I]/[E']$, where $[E']$ is the concentration of the enzyme in the presence of fixed concentrations of co-substrate and co-factors.

Glucose Consumption by Digitonin-permeabilized Epimastigotes—Exponential phase epimastigotes were collected by centrifugation (4,000 × *g* for 10 min), resuspended in buffer B (Tris-HCl 75 mM, NaCl 140 mM, KCl 11 mM, pH 7.4) at a final cell density of 10^8 cells ml^{-1} and incubated in the presence of digitonin at 30 $\mu g\ mg^{-1}$ protein for 20 min, washed twice in the same buffer, and finally resuspended in buffer B, supplemented with phosphoenolpyruvate (PEP) 6 mM, ADP 6 mM, $NaHCO_3$ 7 mM, L-malate 1 mM. The parasites were preincubated at 28 °C with strong agitation (100 rpm) in the presence of varying concentrations of bisphosphonates for 60 min, and the experiment were started by addition of 3 mM D-glucose; 100-μl samples were taken every 15 min, centrifuged at 35,000 × *g* for 5 min, and the supernatants stored at −80 °C until assayed for D-glucose content, as described above (7, 26).

^{13}C NMR Assay of Glucose Consumption by Intact Epimastigotes—These experiments were carried out as described (28), with minor modifications. Exponential phase epimastigotes were collected by centrifugation and washed in buffer B as described above, resuspended at a cell density of 10^{10} cells ml^{-1} in the same buffer, preincubated with strong agitation (100 rpm) at 28 °C in the presence of 50 μM **228** or **302** for 6 h, and the experiment started by the addition of 10 mM 1-D-[^{13}C]glucose (Sigma); 1-ml samples were collected every 30 min, centrifuged at 35,000 × *g* for 5 min, and the supernatants stored at −80 °C until assayed by ^{13}C NMR. Perchloric acid extracts of the sediments (whole cells) were prepared as described before (14) and also stored at −80 °C. ^{13}C NMR spectra were obtained using a 11.744 tesla Bruker ADVANCE 500 NMR spectrome-

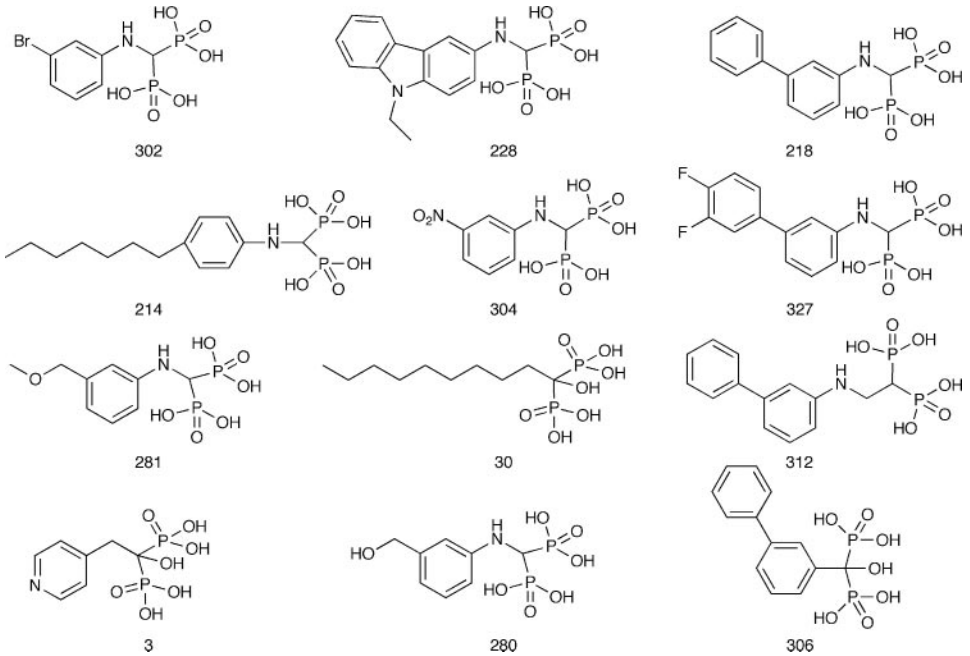

FIGURE 1. **Chemical structures of the bisphosphonates studied in this work.** For the synthesis, characterization, and anti-TcHK activity of these compounds, see Hudock *et al.* (18) and "Results."

ter, which operates at 125.77 MHz for ^{13}C (500.13 MHz for ^{1}H); chemical shifts are reported with respect to external *p*-dioxane (66.5 ppm). ^{1}H decoupled ^{13}C NMR free induction decays were acquired with 6.4 μs (45°) excitation pulses, a 250 ppm (31.447 Hz) spectral width, composite pulse ^{1}H decoupling, and a 2-s recycle delay; 1024 free induction decays were acquired and averaged for each spectrum and processed with a 1-Hz exponential filter before Fourier transformation. Three independent experiments were carried out, with essentially identical results.

Studies of Lipid Composition—For the analysis of the effects of the experimental compounds on epimastigote lipid composition, total lipids from control and drug-treated cells were extracted and fractionated into neutral and polar lipid fractions by silicic acid column chromatography and gas-liquid chromatography (29, 30). The neutral lipid fractions were first analyzed by thin layer chromatography (on Merck 5721 silica gel plates with heptane/isopropyl ether/glacial acetic acid (60:40:4) as developing solvent) and conventional gas-liquid chromatography (isothermal separation in a 4-m glass column packed with 3% OV-1 on Chromosorb 100/200 mesh, with nitrogen as carrier gas at 24 ml min^{-1} and flame ionization detection using a Varian 3700 gas chromatograph). For quantitative analysis and structural assignments, the neutral lipids were separated in a high-resolution capillary column (25 m × 0.20-mm inner diameter Ultra-2 column, 5% phenylmethylsiloxane, 0.33 μm film thickness) using a Hewlett-Packard 6890 Plus gas chromatograph equipped with a HP5973N mass sensitive detector. The lipids were dissolved in chloroform and injected; the column was kept a 50 °C for 1 min, then the temperature was increased to 270 °C at a rate of 25 °C min^{-1} and finally to 300 °C at a rate of 1 °C min^{-1}. The carrier gas (He) flow was kept constant at 0.5 ml min^{-1}. Injector temperature was 250 °C and the detector was kept at 280 °C.

Drugs—The bisphosphonates used in this work (Fig. 1) were synthesized, purified, and characterized as described before (18).

RESULTS

Kinetics of Inhibition of TcHK by Bisphosphonates—The chemical structures of the 12 most potent bisphosphonate inhibitors of TcHK identified in our previous study (18) are shown in Fig. 1. We have now performed a detailed kinetic study to clarify the inhibition mechanism of three of these compounds (**228**, **302**, and **3**) against the pure enzyme, as well as the enzyme in digitonin-permeabilized but otherwise intact glycosomes, and these results are presented in Figs. 2 and 3 and Tables 1 and 2. Against the pure enzyme (Fig. 2 and Table 1) all three compounds behaved as mixed to non-competitive inhibitors against ATP, as indicated by the similar values of K_{ii} and K_{is}. Against D-glucose, **228** and **302** exhibited competitive behavior (data not shown), but compound **3** was again a non-competitive inhibitor (Table 1). In all cases, the values of the inhibitory constants were 100–1000-fold lower that the K_i values obtained for PP_i in our previous study (500 μM, see Ref. (17)).

TcHK, as well as the five subsequent enzymes of the glycolytic pathway in *T. cruzi* and related organisms, functions *in vivo* in the matrix of the glycosomes, membrane bound peroxisome-like organelles in which protein concentrations can be as high as 350 mg ml^{-1} (5, 6). In an effort to study the activity of this enzyme under more natural conditions, we next investigated its kinetic properties and inhibition by bisphosphonates in highly purified glycosomes, made permeable to small molecules by briefly incubating with digitonin. The use of this plant glycoside detergent to permeabilize the glycosomal membrane was suggested by previous studies from our group, which showed that it was rich in the endogenous sterols of the parasite (31), plus that the glycosome is one the organelles involved in *de novo* sterol biosynthesis in this parasite (27). Preliminary data indicated that incubation of glycosomes in the presence of 25 μg of digitonin/mg of protein for 1 min allowed full expression of TcHK activity with <5% release of the enzyme (or other glycosomal markers, such as phosphoglucose isomerase) from the matrix of the organelle. Because the *in situ* activity of this enzyme had not been reported previously, a detailed kinetic study was carried out; the results (not shown) indicated classical Michaelis-Menten behavior, with K_m values of 463 and 30 μM for ATP and D-glucose, respectively, and a V_m value of 11.5 μmol min^{-1} mg of protein^{-1}. We also investigated the inhibitory effects of PP_i on the *in situ* TcHK activity and found that it was purely non-competitive toward ATP ($K_{ii} = K_{is} = 819$ μM), but mixed toward D-glucose (Table 2). The values of K_m for

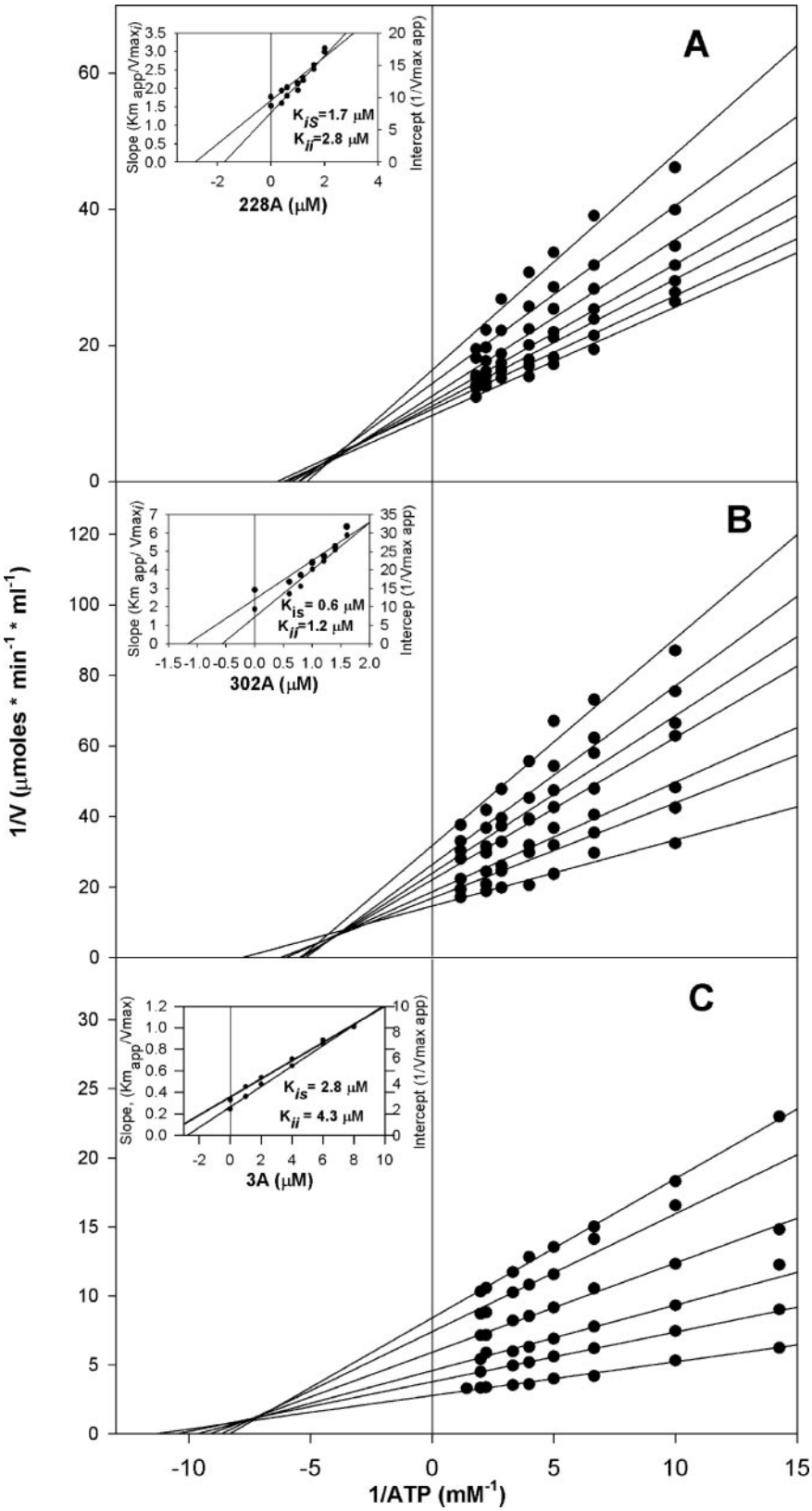

FIGURE 2. **Kinetics of inhibition of homogeneous TcHK by bisphosphonates.** Lineweaver-Burk plots of the effects of 228 (*A*), 302 (*B*), and 3 (*C*) on TcHK, isolated and purified from T. cruzi epimastigotes, as described under "Materials and Methods." The inhibitor concentrations were 0, 0.4, 0.6, 1, 1.2, 1.6, and 2 μM for 228; 0, 0.6, 0.8, 1, 1.2, 1.4, and 1.6 μM for 302; and 0, 1, 2, 3, 4, 6, and 8 for 3. *Inset*, secondary plots of intercepts and slopes *versus* inhibitor concentration, used to calculate K_{ii} and K_{is}, respectively.

ATP and the K_i for PP_i for the glycosomal enzyme were significantly higher than those previously obtained for the pure enzyme (17). When we investigated the kinetics of inhibition of the enzyme in glycosomes (Fig. 3 and Table 2), we found that **228** and **3** were again mixed to non-competitive inhibitors toward ATP, but **302** produced an apparently competitive inhibition; all compounds were non-competitive or mixed inhibitors against D-glucose (data not shown, Table 2). Again, the values of the inhibitory constants for bisphosphonates against this form of the enzyme were 2–3 orders of magnitude lower than were those for PP_i (Table 2).

Effects of Bisphosphonates on Glucose Consumption by Digitonin-permeabilized and Intact T. cruzi Epimastigotes—Next, we investigated the effect of bisphosphonates on the glucose consumption by whole, digitonin-permeabilized *T. cruzi* epimastigotes. Previous work had shown that incubation of these cells with digitonin at 30 μg mg^{-1} protein for 20 min render their plasma membranes permeable to both low and high molecular weight compounds, but no glycosomal proteins were released (27); similar conditions have been used in the same cells to characterize the *in situ* activities of other organelles, such as mitochondria (32) and acidocalcisomes (15, 33, 34). Our results here showed that the presence of PEP, ADP, and $NaHCO_3$ (possibly a source of CO_2) as well as L-malate were required to sustain glucose consumption by the permeabilized cells (see "Materials and Methods"). Also, to observe the full effects of bisphosphonates, it was necessary to preincubate the permeabilized cells with these compounds for at least 60 min, most likely to permit penetration of these highly charged compounds into the glycosomal matrix. The results obtained (Fig. 4) showed that the bisphosphonates induced a dose-dependent inhibition of glucose consumption, with IC_{50} values for these effects (0.62, 1.2, and 10.3 μM for **228**, **302**, and **3**, respectively) closely matching the corresponding IC_{50} values for inhibition of the purified enzyme (Fig. 2 and Ref. 18).

We also studied the effects of the three bisphosphonates on glucose consumption and metabolism by intact epimastigotes, using ^{13}C NMR spectroscopy at 125 MHz as described previously (28, 35), with the aim of elucidating the chemical nature of the metabolic products under the different experimental conditions. In agreement with these earlier studies, consumption of 1-D-[^{13}C]glucose by control cells, as determined by the reduction of the intensity of the integrated intensities of resonance at 96.3 and 92.4 ppm (corresponding to the β and α anomeric C1 of D-glucose) in the incubation medium was accompanied by the appearance of resonance at 33.9, 22.3, and 16.3 ppm, corresponding to 2-[^{13}C]succinate, 2-[^{13}C]pyruvate, and 2-L-[^{13}C]alanine, respectively, the known end products of aerobic or anaerobic fermentation of glucose by these cells[3] (10–12, 28, 35, 36), see Fig. 5*A*. Cells pretreated (for 6 h) with 50 μM **228** or **302** (Fig. 5, *B* and *C*) consumed glucose and excreted succinate, pyruvate, and alanine at slower rates than control cells, but the nature and relative proportion of the end products were not modified. Moreover, no accumulation of glycolytic intermediates or new end products could be detected from ^{13}C NMR spectra of perchloric acid extracts of treated cells at any of the experimental times (data not shown). Taken together, these facts strongly suggested that the observed reduction of the glycolytic flux occurred at the level of hexokinase.

Effects of Bisphosphonates on Growth and Use of Energy Sources by T. cruzi Epimastigotes in LIT Medium—Having established the effects of bisphosphonates on TcHK activity and glycolysis, we next investigated the effects of these compounds on the proliferation of epimastigotes in LIT medium, in addition to the cellular response to the drug-induced blockade of glucose consumption. **228** and **302** induced a dose-dependent inhibition of growth (Fig. 6) with IC_{50} values of 6.4 and 7.0 μM, respectively, and minimal inhibitory concentration (MIC) of 20 μM for both compounds. Similar effects were observed for 3 but, consistent with its lower potency as an enzyme and glycolytic inhibitor, the IC_{50} and MIC values were significantly higher (40.6 and 200 μM, respectively; data not shown). We also sought to probe the metabolic response of the cells in culture to the presence of these glycolytic inhibitors. Figs. 7 and 8 show that, as reported previously by several groups (10–12), control (non-treated) exponential phase *T. cruzi* epimastigotes rapidly depleted glucose present in the medium with a concomitant reduction in its pH, a result of the production of succinate and pyruvate end products. However, as the end of the exponential phase approached and the glucose content of the medium was significantly reduced, the pH began to rise again, due to production of ammonia, a result of the oxidative breakdown of amino acids (10). Cells grown in the presence of 15 μM **228** (Fig. 7) or **302** (Fig. 8) had a significant reduction in proliferation (Figs. 7*A* and 8*A*), associated with a marked reduction of glucose consumption, which stopped completely after 96 (**228**, Fig. 7*B*) or 120 h (**302**, Fig. 8*B*) contact with the inhibitor. However, in these cells the increase in the pH of the medium began earlier, and the levels of ammonia excreted into the medium were significantly higher than those in control cultures (Figs. 7, *B* and *C*, and 8, *B* and *C*).

Correlation between TcHK and Growth Inhibition Induced by Bisphosphonates—Although growth inhibition induced by the three bisphosphonates characterized in the previous sections was associated with a blockade of glucose consumption and correlated with the potency of the compounds as TcHK inhibitors, we sought to obtain further confirmation for a causal relationship of these events by investigating the growth inhibitory activity of the 12 most potent compounds identified as TcHK inhibitors in our previous studies (18), see Fig. 1. The IC_{50} values of these bisphosphonates as TcHK inhibitors vary 43-fold (range 0.81–35 μM, see Table 2 of Ref. 18) and we have now found that their growth inhibitory activity is indeed highly correlated with their effect on pure TcHK (Fig. 9), with a correlation coefficient of 0.951, $p < 0.0001$.

Effects of Bisphosphonates on Parasite Sterol Content—In previous work, we showed that growth inhibition of *T. cruzi* and related parasites by nitrogen-containing bisphosphonates (such as risedronate) was associated with a profound depletion of the endogenous sterol levels of the parasite (37, 38), due to a specific inhibition of a key enzyme of the isoprenoid biosynthesis pathway, FPPS (20–23). It was therefore of interest to investigate here if growth inhibition induced by these novel nitro-

[3] Under our experimental conditions the cells are expected to be at very low oxygen levels.

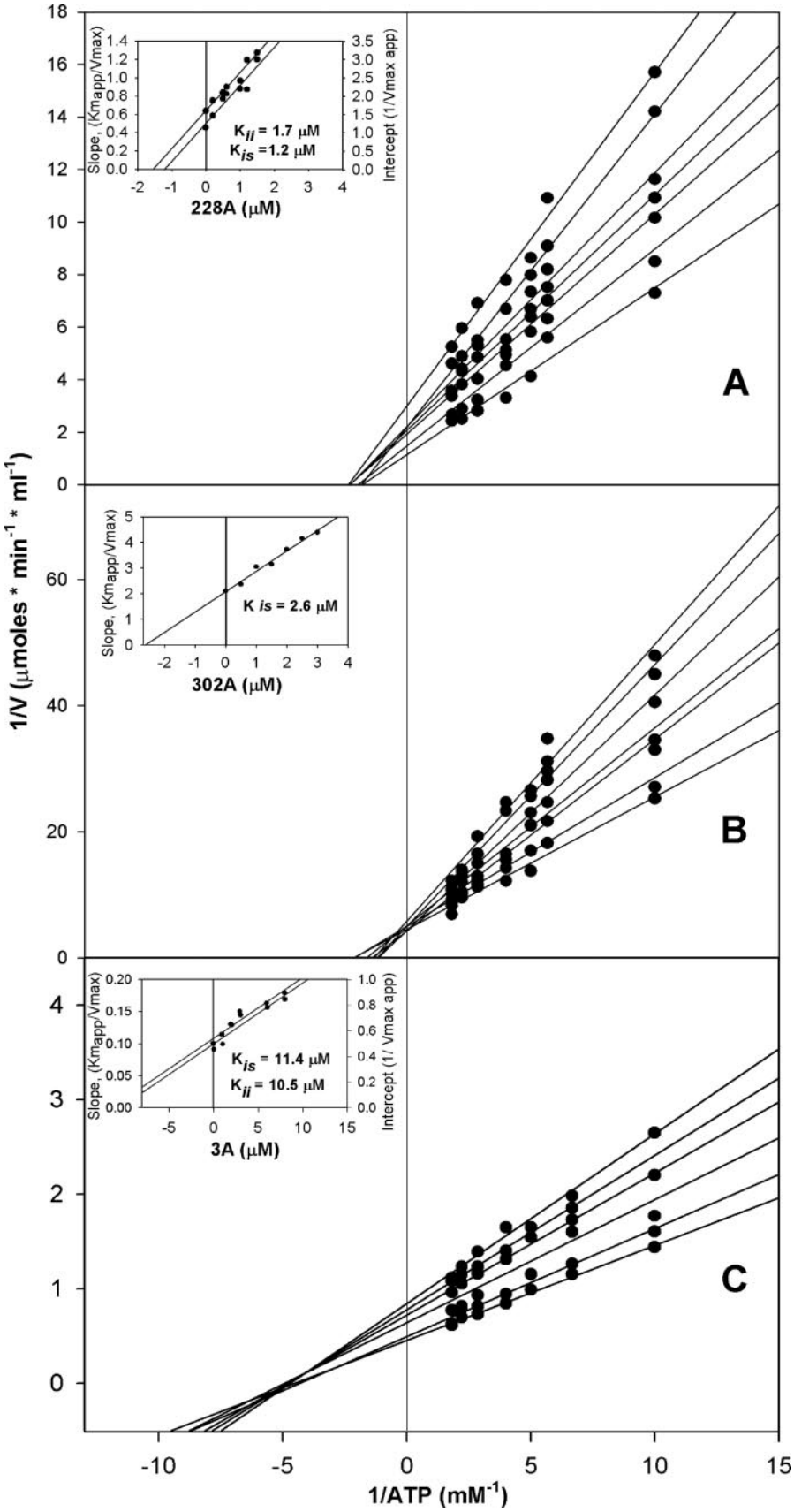

FIGURE 3. **Kinetics of inhibition of *in situ* glycosomal TcHK by bisphosphonates.** Lineweaver-Burk plots of the effects of 228 (*A*), 302 (*B*), and 3 (*C*) on glycosomal TcHK, isolated and purified from *T. cruzi* epimastigotes as described under "Materials and Methods." The inhibitor concentrations were 0, 0.2, 0.5, 0.6, 1, 1.2, and 1.5 μM for 228; 0, 0.5, 1, 1.5, 2, 2.5, and 3 μM for 302; and 0, 1, 2, 3, 6, and 8 for 3. *Inset*, secondary plots of intercepts and slopes *versus* inhibitor concentrations, used to calculate K_{ii} and K_{is}, respectively.

TABLE 1
Kinetics of inhibition of pure *T. cruzi* hexokinase by bisphosphonates

Inhibitor/substrate	K_{ii}[a]	K_{is}[a]	Mechanism
228/ATP[b]	2.8	1.7	Mixed
228/D-glucose[c]		0.5	Competitive
302/ATP[b]	1.2	0.6	Mixed
302/D-glucose[c]		0.4	Competitive
3/ATP[b]	4.3	2.8	Mixed
3/D-glucose[c]	6.0	3.3	Non-competitive

[a] Inhibition constants given in micromolar.
[b] In the presence of 2 mM D-glucose and 3 mM $MgCl_2$.
[c] In the presence of 1 mM ATP and 3 mM $MgCl_2$.

TABLE 2
Kinetics of inhibition of glycosomal *T. cruzi* hexokinase *in situ* by bisphosphonates

Inhibitor/substrate	K_{ii}[a]	K_{is}[a]	Mechanism
228/ATP[b]	1.7	1.2	Non-competitive
228/D-glucose[c]	4.3	2.4	Non-competitive
302/ATP[b]		2.6	Competitive
302/D-glucose[c]	10.6	2.9	Mixed
3/ATP[b]	10.5	11.4	Non-competitive
3/D-glucose[c]	43.9	3.4	Mixed
PP_i/ATP[b]	819	819	Non-competitive
PP_i/D-glucose[c]	3,040	1,720	Mixed

[a] Inhibition constants given in micromolar.
[b] In the presence of 2 mM D-glucose and 3 mM $MgCl_2$.
[c] In the presence of 1 mM ATP and 3 mM $MgCl_2$.

gen-containing bisphosphonates (18) was also associated to any extent with sterol depletion. Table 3 presents the sterol composition of epimastigotes grown for 120 h in the absence or presence of the MICs of **228** (20 μM), **302** (20 μM), or **3** (100 μM). It can be seen that for the first two compounds the sterol composition of the treated cells was indistinguishable from that of control cells, consistent with their weak inhibitory activity against an expressed FPPS (17), whereas for compound **3** there was a potent and dose-dependent reduction of the content of endogenous sterols, which accounted for less than 7% at the MIC.

DISCUSSION

The activity of most hexokinases in eukaryotic organisms is under potent feedback regulation by the product of the reaction, D-glucose 6-phosphate, or derived metabolites such as D-glucose 1,6-diphosphate (16). The unusual inhibitory effects of PP_i on TcHK, a strictly ATP-dependent kinase, is probably associated with the existence of large levels of PP_i and other short chain polyphosphates in these cells, mostly concentrated in acidocalcisomes (15), and the presence of active PP_i-dependent metabolic processes in all Kinetoplastida and Apicomplexan protozoa (15, 39–41). The effects of PP_i may be effected through their interaction with a regulatory site distinct from the catalytic one (heterotropic allosterism), as indicated by the non-competitive character of its effects on both the pure soluble enzyme as well as the enzyme *in situ* (as measured in digitonin-permeabilized but otherwise intact glycosomes). Similarly, the novel bisphosphonates seem to be interacting with site(s) in the enzyme distinct from the active site, as indicated by the mixed to non-competitive inhibition kinetics (Figs. 2 and 3, Tables 1 and 2). Furthermore, these synthetic "PP_i analogs" are 100 to 1000-fold more potent than is the putative physiological effector, PP_i.

To the best of our knowledge, these results represent the first detailed characterization of a glycolytic enzyme activity in the glycosomal matrix. The results indicate that, although TcHK displays classical Michaelis-Menten kinetics *in situ*, the apparent affinities for both ATP and PP_i as substrate and effector, respectively, are low, an observation that seems likely to be related to the expected high levels of such compounds inside glycosomes. Likewise, some qualitative and quantitative differences were observed in the inhibition kinetics by bisphosphonates of the pure soluble enzyme and the enzyme associated with the matrix of the glycosome (Figs. 2 and 3, Tables 1 and 2), which could reflect differences in the catalytic properties of the

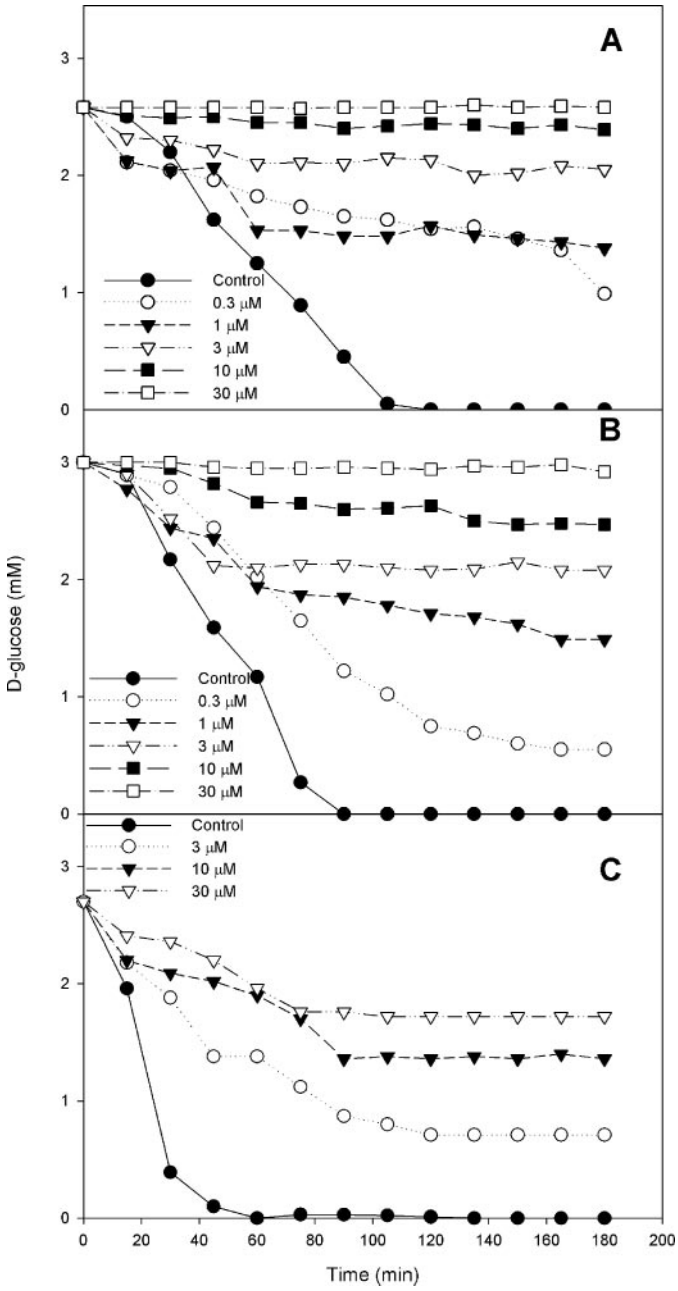

FIGURE 4. **Effects of bisphosphonates on glucose consumption by digitonin-permeabilized *T. cruzi* epimastigotes.** Epimastigotes were digitonin-permeabilized and preincubated for 60 min in the presence of the indicated concentrations of 228 (*A*), 302 (*B*), and 3 (*C*). Details of the protocols for digitonin permeabilization of the cells and glucose consumption assays are given under "Materials and Methods."

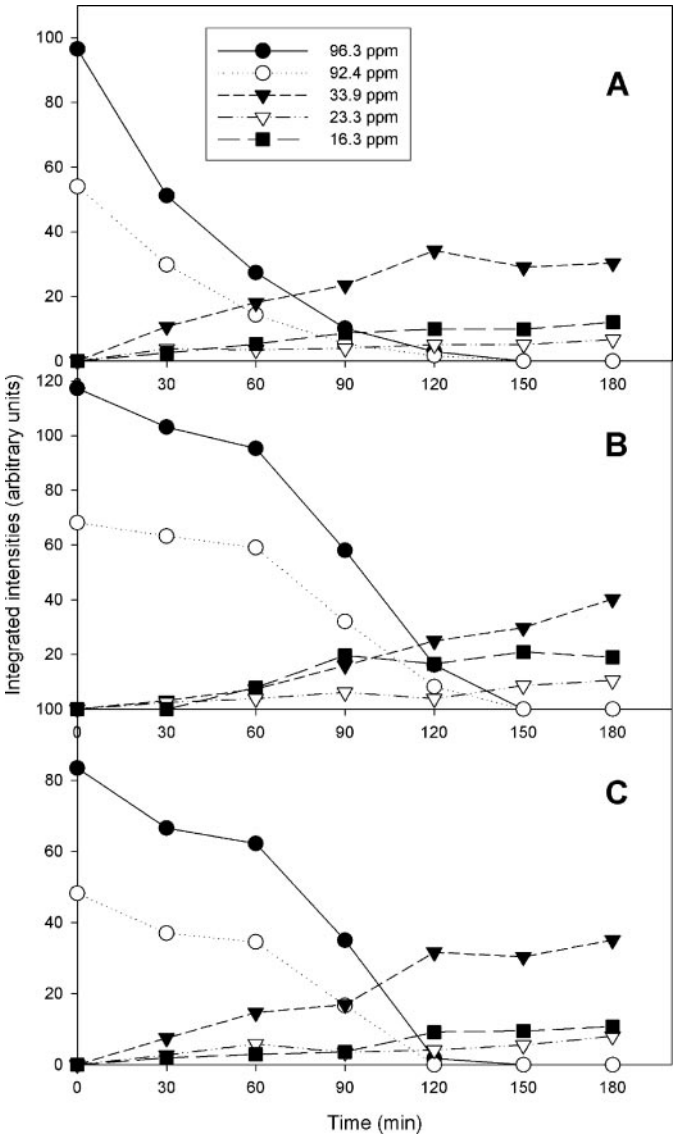

FIGURE 5. **Effects of bisphosphonates on D-[^{13}C]glucose consumption by intact *T. cruzi* epimastigotes followed by ^{13}C NMR spectroscopy.** The intensity of resonance at 96.3, 92.4, 33.9, 22.3, and 16.3 ppm, associated to the β and α anomeric C1 of D-glucose, 2-[^{13}C]succinate, 2-[^{13}C]pyruvate, and 2-L-[^{13}C]alanine, respectively, are plotted as a function of time for cells preincubated in the absence (*A*) or presence of 50 μM 228 (*B*) or 302 (*C*) for 6 h. For details, see "Materials and Methods."

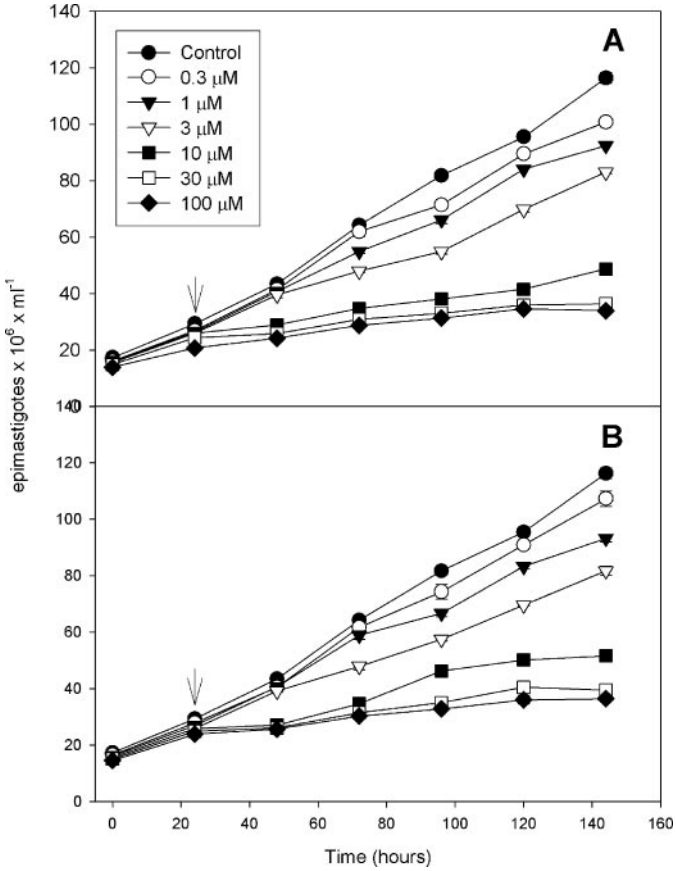

FIGURE 6. **Effects of bisphosphonates on proliferation of *T. cruzi* epimastigotes in LIT medium.** Epimastigotes were cultured in liver infusion tryptose medium at 28 °C, with agitation in the presence of the indicated concentrations of 228 (*A*) and 302 (*B*), as described under "Materials and Methods." *Arrows* indicate the time of addition of the experimental compounds. Experiments were carried out in triplicate and each bar represents 1 S.D.

enzyme related to the molecular environment of the protein in the two situations.

The inhibitory effects of bisphosphonates on the catalytic activity of isolated or glycosome-bound TcHK quantitatively matched their effects on glucose consumption by digitonin-permeabilized whole *T. cruzi* epimastigotes (Fig. 4), strongly suggesting a cause-effect relationship between these two observations. This conclusion was further supported by the results of the ^{13}C NMR studies of D-[^{13}C]glucose metabolism by intact epimastigotes, preincubated in the absence or presence of bisphosphonates (Fig. 5). These results showed that the rate of glucose breakdown by treated cells was slower than that of control cells and there was a corresponding reduction in the rate of appearance of end products (succinate, pyruvate, and alanine, see Refs. 9–11, 17, 27, 35, and 36), but no accumulation of intracellular ^{13}C-labeled glycolytic intermediates or new end products was observed, consistent with a blockade of the glycolytic flux at the very first step of the pathway, hexokinase. In digitonin-permeabilized epimastigotes, the effects of bisphosphonates on glycolysis could only be observed if cells were previously incubated for at least 60 min with the inhibitors, indicating that the glycosomal membrane (31) was probably intact, acting as a strong permeability barrier to these highly charged molecules. Consistent with this interpretation, digitonin-permeabilized epimastigotes prepared under similar conditions have previously been used to characterize the *in situ* activities of other organelles, such as mitochondria (32) and acidocalcisomes (15, 33, 34). In preliminary experiments it was found that to maintain the glycolytic flux in digitonin-permeabilized cells, it was necessary to include PEP, $NaHCO_3$, L-malate, and ADP in the incubation medium. We found that 2 mol of PEP were consumed for each mole of glucose, clearly suggesting that it was used, together with the CO_2 generated from $NaHCO_3$, to maintain the redox balance of the glycosome by the combined action of glycosomal phosphoenolpyruvate carboxykinase and malate dehydrogenase (10–12). L-Malate was required in relatively small amounts (1 mM) to start the process, probably by providing an initial, partially reduced state of the glycosomal matrix. ADP was also found to be essential for glucose consumption in

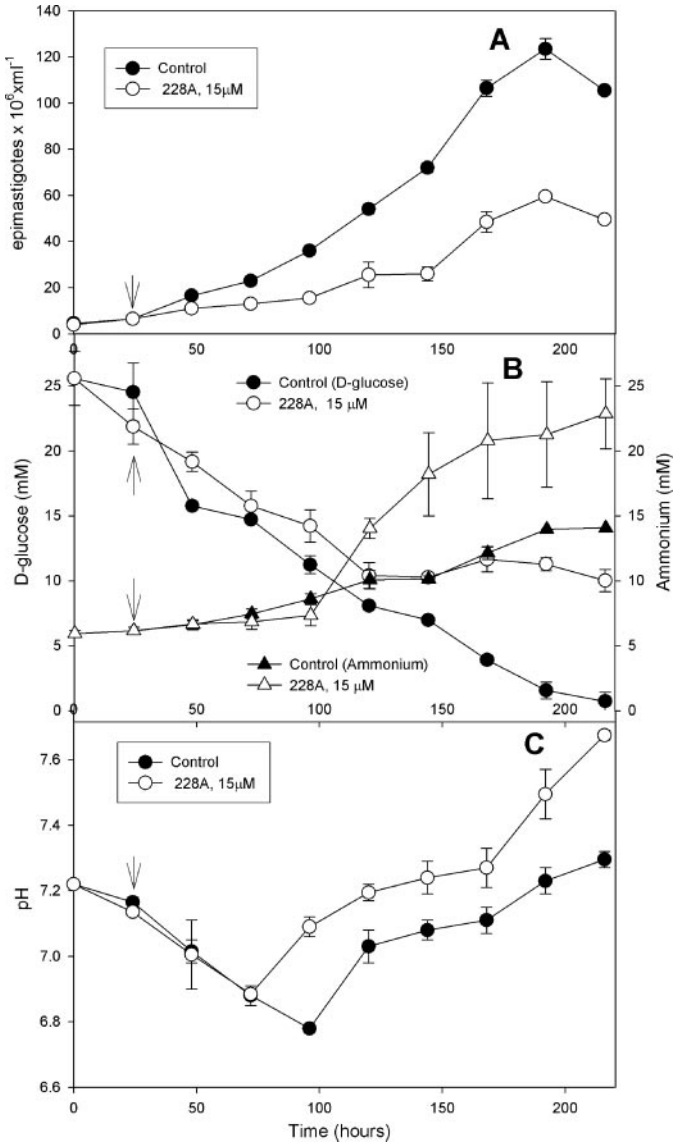

FIGURE 7. **Effects of 228 on proliferation and use of energy sources by *T. cruzi* epimastigotes in LIT medium.** Epimastigotes were cultured in LIT medium at 28 °C, with agitation in the absence (*closed symbols*) or presence (*open symbols*) of 15 μM 228. *A*, proliferation; *B*, medium glucose (*circles*) or ammonium (*triangles*) concentrations; and *C*, medium pH. Details are given under "Materials and Methods." *Arrows* indicate the time of addition of the experimental compounds. Experiments were carried out in triplicate and each bar represents 1 S.D.

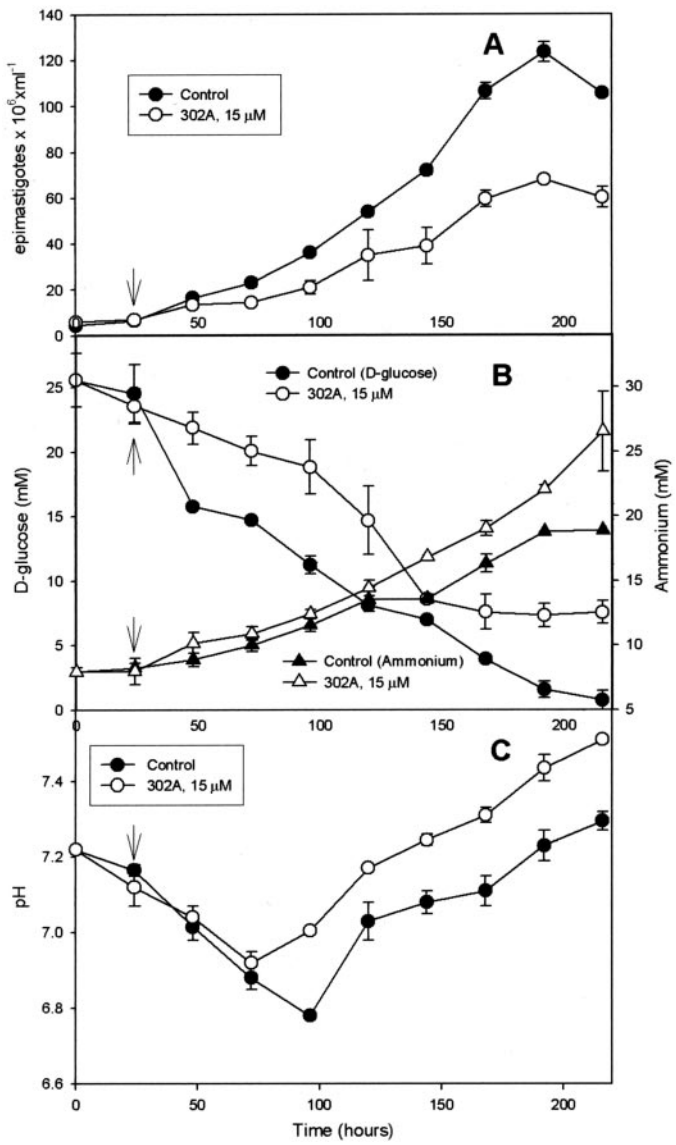

FIGURE 8. **Effects of 302 on proliferation and use of energy sources by *T. cruzi* epimastigotes in LIT medium.** Epimastigotes were cultured in LIT medium at 28 °C, with agitation in the absence (*closed symbols*) or presence (*open symbols*) of 15 μM 302. *A*, proliferation; *B*, medium glucose (*circles*) or ammonium (*triangles*) concentrations; and *C*, medium pH. Details are given under "Materials and Methods." *Arrows* indicate the time of addition of the experimental compounds. Experiments were carried out in triplicate and each bar represents 1 S.D.

this system, probably to sustain substrate level phosphorylation and efflux of ATP from the glycosomes. ATP efflux from glycosomes would be consistent with the fact that in all stages of *T. cruzi* and *Leishmania* spp., as well as in procyclic *T. brucei* trypomastigotes, there should be a net production of ATP in these organelles due to the activity of phosphoenolpyruvate carboxykinase (9–11) and pyruvate phosphate dikinase (see Ref. 36). Support for this idea comes from a recent comparative analysis of the proteome of glycosomes from bloodstream and procyclic stages of *T. brucei*, which indicated the presence of an ATP/ADP exchanger in the membranes of the latter, but not in the former, stage of the parasite (42).

A peculiar metabolic characteristic of all stages of the life cycle of *T. cruzi* and the different species of *Leishmania* parasites, as well as the procyclic (insect form) stages of *Trypanosoma brucei*, is the so called "aerobic fermentation" of glucose, which this carbohydrate is rapidly consumed and converted in large amounts to mono- and dicarboxylic acids (pyruvate, succinate, malate), even in the presence of oxygen and despite the fact that such cells have a fully functional Krebs cycle and phosphorylating electron transport chain (10–12). Several hypotheses have been advanced to explain this phenomenon, among them the requirement of the combined action of glycosomal phosphoenolpyruvate carboxykinase and malate dehydrogenase to maintain the glycosome redox balance (11, 12); malate is probably converted to succinate by a reversion of the Krebs cycle (11, 12) and/or the activity of glycosomal and mitochondrial fumarate reductase (42–44), to generate extra reducing

power. However, it has also been proposed that both glycosomal and mitochondrial phosphoenolpyruvate carboxykinases and malate dehydrogenases are involved in the decarboxylation of oxaloacetate, generated from the oxidation of amino acids through the Krebs cycle, with the production of phosphoenolpyruvate, which can re-enter the cycle to complete oxidative breakdown (10, 26, 28, 45). The results shown in Figs. 7 and 8 show that control epimastigotes rapidly take up glucose from the growth medium, which is then acidified as a result of the excretion of mono- and dicarboxylic acids (see Fig. 5). However, when glucose approaches depletion, a metabolic shift to the use of amino acids to sustain proliferation takes place, as indicated by the accumulation of ammonia in the medium, and its concomitant alkalinization. In cultures carried out in the presence of relatively low bisphosphonate concentrations there was a marked inhibition of cell proliferation, associated with a reduction of glucose consumption, and the increase in the ammonia content and pH of the medium began earlier, strongly suggesting that under the drug pressure, epimastigotes shift to amino acid catabolism to (partially) restore growth (Figs. 7 and 8). Although the experimental data indicated that growth inhibition induced by these bisphosphonates was associated with a blockade of glucose consumption, which in turn correlated with their activity against TcHK, there were some quantitative discrepancies in the time and extent of these effects, which may suggest that permeability barriers, non-linear metabolic effects, or other cellular targets could be involved in the antiproliferative action of these compounds. Looking for evidence to support a causal relationship between bisphosphonate-induced TcHK inhibition and growth arrest, we determined the antiproliferative activities of the 12 bisphosphonates presented in Fig. 1, whose anti-TcHK activities were characterized in a previous study (18), and found that there was indeed a highly significant statistical correlation for the two effects (Fig. 9). This finding leads strong support to the notion that a primary mechanism of action of these compounds as anti-*T. cruzi* agents is hexokinase inhibition, which may be due to energy depletion resulting from impaired glycolysis or to an essential requirement of phosphorylated carbohydrates for the survival of the parasite.

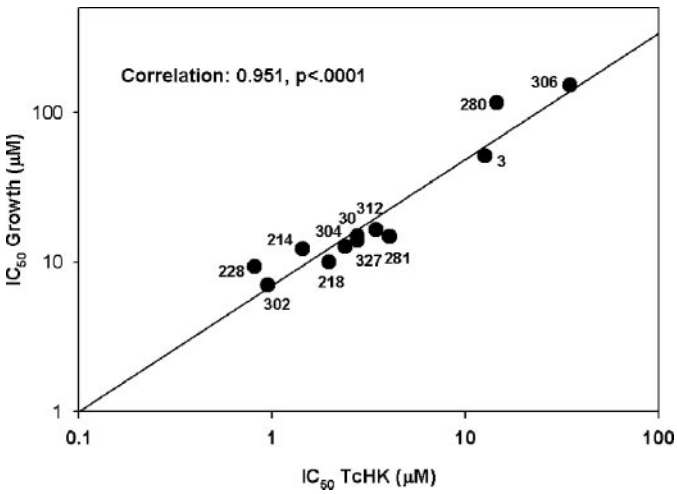

FIGURE 9. **Correlation of the TcHK inhibitory activity and growth inhibition of *T. cruzi* epimastigotes by aromatic aminomethylene bisphosphonates.** The IC_{50} values for TcHK inhibition were taken from Hudok *et al.* (18), whereas those for growth inhibition of the epimastigote were obtained as described under "Materials and Methods." A highly significant correlation is observed.

Previous work has shown that other nitrogen-containing bisphosphonates, such as pamidronate and risedronate, are potent *in vitro* and *in vivo* anti-*T. cruzi* agents and that growth arrest is associated with the depletion of endogenous sterols, a consequence of FPPS inhibition (14, 37, 38, 47). To test if this mechanism also contributes to growth arrest induced by the potent TcHK bisphosphonate inhibitors investigated here, we analyzed the sterol content of epimastigotes grown in the presence of the MICs of **228**, **302**, or **3** for 120 h, at which time the cultures were in full growth arrest. The results (Table 3) indicate that growth arrest induced by the first two compounds was not associated with endogenous sterol depletion. Such results are fully consistent with our previous findings that bisphosphonates with potent anti-TcHK activity were poor inhibitors of FPPS and that good FPPS inhibitors were poor TcHK inhibitors (18). In the case of **3**, we previously found that this bisphosphonate was a more potent inhibitor of *T. cruzi* FPPS than of TcHK (~1 *versus* 13 μM) (18) and, consistently, there was an almost complete depletion of the endogenous sterols of the parasite at the MIC (Table 3).

Taken together, the results we have presented here show that aromatic aminomethylene bisphosphonates (in particular compounds **228** and **302**) have potent and selective antiproliferative *T. cruzi* activity, primarily acting as non-competitive or

TABLE 3

Free sterols and precursors present in *T. cruzi* epimastigotes (EP stock) grown in the presence or absence of bisphosphonates 228, 302, and 3

Sterols were extracted from *T. cruzi* epimastigotes cultured in LIT medium for 120 h in the presence or absence of the indicated concentrations of bisphosphonates (see Fig. 1); they were separated from polar lipids by silicic acid column chromatography and analyzed by quantitative capillary gas-liquid chromatography and mass spectrometry, as described under "Materials and Methods." Results are expressed as mass percent.

Name	Control	228 (20 μM)	302 (20 μM)	3	
				50 μM	100 μM
Exogenous					
Cholesterol	18.3	18.8	21.3	70.9	93.3
Endogenous					
24-Methyl-5,7,22-cholesta-trien-3β-ol (ergosterol)	33.2	23.4	31.0	ND[a]	ND
24-Ethyl-5,7,22-cholesta-trien-3β-ol	15.6	19.3	18.3	14.6	ND
Ergosta-5,7–24(24′)-trien-3β-ol	8.2	3.8	6.0	ND	ND
Ergosta-5,7-dien-3β-ol	18.5	26.3	18.0	ND	ND
Ergosta-7,24(24^1)-dien-3β-ol	2.8	ND	2.0	ND	ND
24-Ethyl-5,7-cholesta-dien-3β-ol	3.4	8.4	3.4	14.5	6.7

[a] ND, not detected.

mixed inhibitors of TcHK. The detailed inhibition mechanism and molecular interactions of the compounds with the enzyme remain to be elucidated, but it is tempting to suggest that they could be related to that of PP_i. The metabolic effects of the compounds on the clinically relevant intracellular amastigote form remains to be investigated, but the limited available data show that they are even more potent against this form of the parasite. Because the bisphosphonates investigated here have little or no effect on the growth of a human (tumor) cell line or *Dictyostelium discoideum* (18), our results suggest that further development of this class TcHK inhibitors could lead to a novel class of selective anti-parasitic agents, similar to the development of small molecule *human* glucokinase activators as novel anti-diabetic agents (46, 48, 49).

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