Biochemical Pharmacology, Vol. 45, No. 12, pp. 2381–2387, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

INHIBITION OF PHOSPHATIDYLCHOLINE BIOSYNTHESIS AND CELL PROLIFERATION IN *TRYPANOSOMA CRUZI* BY AJOENE, AN ANTIPLATELET COMPOUND ISOLATED FROM GARLIC

JULIO A. URBINA,*† EDGAR MARCHAN,* KEYLA LAZARDI‡
GONZALO VISBAL,* RAFAEL APITZ-CASTRO,§ FRANCISCO GIL,‖ TANIA AGUIRRE,¶
MARTA M. PIRAS¶ and ROMANO PIRAS¶

*Laboratorio de Química Biológica, ‡Laboratorio de Química de los Metales de Transición, §Laboratorio de Trombosis Experimental, and ‖Centro de Microbiología y Biología Celular, Instituto Venezolano de Investigaciones Científicas, Caracas 1020A; and ¶Laboratorio de Investigaciones, Centro Medico-Docente La Trinidad, Caracas 1060, Venezuela

(*Received* 30 *October* 1992; *accepted* 29 *March* 1993)

Abstract—Ajoene [(*E*,*Z*)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide], a potent antiplatelet compound derived from garlic, inhibits the proliferation of both epimastigotes and amastigotes of *Trypanosoma cruzi*, the causative agent of Chagas' disease. The growth of the epimastigote form was immediately arrested by 80 μM ajoene, while 100 μM induced cell lysis in 24 hr. In the amastigote form proliferating inside VERO cells, 40 μM ajoene was sufficient to eradicate the parasite from the host cells in 96 hr. Growth inhibition of the epimastigotes was accompanied by a gross alteration of the phospholipid composition of the treated cells in which phosphatidylcholine (PC), the major phospholipid class present in control cells, dropped to the least abundant phospholipid in cells treated with 60 μM ajoene for 96 hr, while its immediate precursor, phosphatidylethanolamine (PE), became the predominant species; this was correlated with a marked drop in the incorporation of [^{14}C-U]acetate in PC and a corresponding increase in PE. Concomitant with the change in the phospholipid headgroup composition of the cells, the fatty acids esterified to this lipid fraction underwent a dramatic alteration due to the increase in the content of saturated fatty acids and a marked reduction in the content of linoleic (18:2) acid, which is the predominant fatty acid in control cells. We also found that ajoene inhibited the *de novo* synthesis of neutral lipids and, in particular, of sterols in the epimastigotes, but the resultant changes in the sterol composition were not sufficient to explain the antiproliferative effects of the drug. Electron-microscopy showed a concentration-dependent alteration of intracellular membranous structures, particularly the mitochondrion and endoplasmatic reticulum. The results suggest that one important factor associated with the antiproliferative effects of ajoene against *T. cruzi* is its specific alteration of the phospholipid composition of these cells.

Ajoene [(*E*,*Z*)-4,5,9-trithiadodeca-1,6,11-triene 9-oxide], a compound isolated from garlic, is enzymatically derived from alliin, a cysteine derivative stored in garlic bulbs (Fig. 1 and Refs. 1–5), and exhibits a potent inhibitory action against platelet aggregation [1–6]. The mechanism(s) of this reversible activity is still under investigation, but *in vitro* it involves the blockade of the release reaction induced by all known agonists and the agonist-induced exposure of fibrinogen receptors; its locus of action is not shared by any other known antiplatelet compound [1, 4–6]. Garlic-derived compounds, including ajoene, have also been shown to display *in vitro* and *in vivo* antifungal and antibacterial activities, but the mechanism of action of these effects remains obscure as in many cases crude garlic extracts were used [7–11]. In this paper we show that synthetic ajoene is a potent antiproliferative agent against both epimastigotes and amastigotes of *Trypanosoma (Schizotrypanum) cruzi*, the causative agent of Chagas' disease. We also found that although ajoene inhibits both polar and neutral lipid biosynthesis in the epimastigotes at the concentration range that blocks cell growth, the antiproliferative effects of the compound are more readily explained by its specific effect on the biosynthesis of the major phospholipid fraction of the cells, phosphatidylcholine (PC**).

† Corresponding author: Dr. Julio A. Urbina, Laboratorio de Química Biológica, Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas, Apartado 21827, Caracas 1020A, Venezuela. Tel. 58-2-5011479; FAX 58-2-5011093.

** Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPM, sphingomyelin; SFA, saturated fatty acids; UFA, unsaturated fatty acids; LIT, liver infusion-tryptose medium; and AGE, aqueous garlic extract.

MATERIALS AND METHODS

The EP stock of *T. cruzi* was used throughout this study. The epimastigote form was cultivated in liver infusion-tryptose medium (LIT) supplemented with 5% newborn calf serum [12] at 28°, with strong (120 rpm) agitation. Cultures were initiated with 2×10^6 epimastigotes/mL, and the drug was added when the cell density reached 10^7 epimastigotes/mL. Cell densities were measured with an electronic

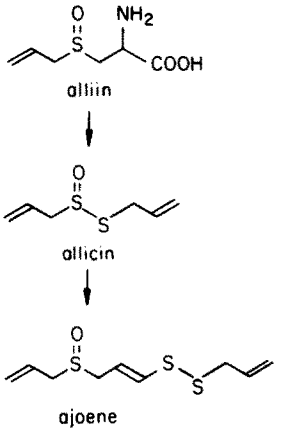

Fig. 1. Chemical structures of ajoene and its precursors. See Refs. 1, 3 and 5.

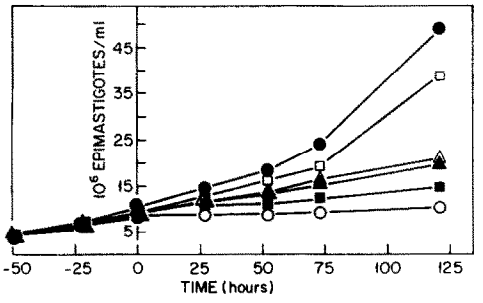

Fig. 2. Effects of ajoene on the proliferation of *T. cruzi* epimastigotes. The epimastigotes were grown in LIT at 28°, as described in Materials and Methods, in the absence (●) or presence of (□) 20 μM, (△) 40 μM, (▲) 60 μM, (■) 80 μM and (○) 100 μM ajoene.

particle counter (model ZBI, Coulter Electronics, Hialeah, FL) and by direct counting with a hemocytometer. Amastigotes were cultivated in VERO cells maintained in minimal essential medium supplemented with 2% fetal bovine serum in a humidified 95% air–5% CO_2 atmosphere at 37°, as previously described [13, 14]. The cells were infected with a 20:1 ratio of tissue culture-derived trypomastigotes to VERO cells for 2 hr and then washed three times with phosphate-buffered saline to remove non-adherent parasites; fresh medium with or without drug was added, and the cells were incubated for various periods of time. The medium was changed every 48 hr. Parasite proliferation was quantified by light microscopy as described before [13].

For the analysis of the effects of ajoene on the lipid composition of the epimastigotes, total lipids were extracted and separated into polar and neutral lipids by silicic acid chromatography as described [15]. The neutral lipids were fractionated by TLC, using Merck 5721 silica gel plates and heptane:isopropyl ether:acetic acid (60:40:4, by vol.) as eluent [14–16]. Additionally, the free sterols present in this neutral lipid fraction were separated and quantified by GLC using a 4 m by 2 mm (i.d.) column packed with 3% OV-1 on Chromosorb W (100–200 mesh) in a Varian 3700 gas chromatograph operating isothermally at 275°; the carrier gas was nitrogen at 25 mL/min. Flame ionization detection was employed using H_2 at 30 mL/min and air at 150 mL/min; detector temperature was 310°. The polar lipids were separated by one- and two-dimensional TLC; for one-dimensional separation high-performance TLC plates (Merck 5715) were used with chloroform:methanol:30% aqueous ammonia (17:7:1, by vol.) as eluent [17], while two-dimensional separations were carried out in Merck 5721 plates using in the first dimension chloroform:methanol:28% aqueous ammonia (17:7:1, by vol.) and in the second dimension chloroform:acetone:methanol:acetic acid (6:8:2:1, by vol.). Lipid phosphorous was determined both in total phospholipids and individual spots scraped from chromatograms, using the method of Ames and Dubin [18]. Free fatty acids esterified to total phospholipids or phospholipid fractions obtained from TLC separations were transformed to their methylesters by incubation in the presence of 2% H_2SO_4 in methanol at 60° for 1 hr and were analyzed quantitatively by GLC in a 2 m by 2 mm (i.d.) column packed with 10% SILAR GT on Chromosorb W (100–200 mesh) on the same equipment described above; the temperature program was: 150° for 10 min, followed by a linear temperature increment of 3°/min up to 205°, and then isothermally at this temperature for an additional 25 min. Nitrogen was used as the carrier gas at 8 mL/min, and flame-ionization detection was carried out as described above for the sterols.

For the study of the *de novo* synthesis of lipids, the drug or solvent was added to the cultures and incubated for 24 hr; then 0.025 μCi of [^{14}C-U]acetate (New England Nuclear; 55 mCi/mmol) was added and incubation was continued for a further 48 hr. At this point, the lipids were extracted and analyzed as described above [14, 15]. The radioactive fractions obtained by TLC were detected by autoradiography with Kodak XRP-5 plates, scraped off, and counted by liquid scintillation spectrometry using an LKB Rack-Beta counter, operating at 80% efficiency for ^{14}C.

For electron microscopy studies, treated and untreated epimastigotes were processed as previously described [19, 20]. Briefly, the cells were washed by centrifugation at 1000 *g* for 10 min twice, collected and fixed in 0.05 M cacodylate buffer (pH 7.2) containing 3.5% saccharose, 1% glutaraldehyde, and 2% paraformaldehyde at 4° for 12 hr. Fixed cells were post-fixed in 1% OsO_4 in the dark. Fixed samples were embedded in Epon, and ultrathin sections were stained with uranyl acetate and lead citrate and observed using a JEOL JBM-100B electron microscope.

Synthetic ajoene was prepared and purified as described before [3]; it was added to cultures as dimethyl sulfoxide (DMSO) solutions. The final DMSO concentration in the culture medium never

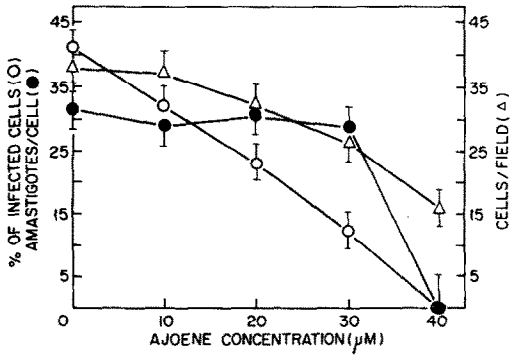

Fig. 3. Effects of ajoene on the proliferation of intracellular *T. cruzi* amastigotes, proliferating in Vero cells at 37°, as described in Materials and Methods. The per cent of infected cells (○), amastigotes per cell (●) or number of host cells per field (△), 96 hr after infection in the presence of the indicated concentrations of ajoene, are plotted. Each point represents the mean value of 15 microscopic fields examined (400×). Each bar represents one standard deviation.

Table 1. Effect of ajoene on the incorporation of [^{14}C-U]-acetate in different phospholipid fractions of *T. cruzi* epimastigotes*

Phospholipid fraction	Control	60 μM Ajoene
Phosphatidylserine	30.8	31.3
Phosphatidylinositol	38.2	41.7
Sphingomyelin	1.2	2.1
Phosphatidylcholine	25.4	16.5
Phosphatidylethanolamine	3.7	8.5

* Phospholipids were isolated from control and treated epimastigotes incubated in the presence of the indicated drug concentration for 24 hr and then with [^{14}C-U]acetate and the drug for a further 48 hr. The lipids were separated by TLC as described in Materials and Methods. The percentage of the total ^{14}C dpm incorporated in the phospholipid fraction found in each class is given.

exceeded 0.5% and had no effects on cell proliferation.

RESULTS

Figures 2 and 3 show the effects of synthetic ajoene on the proliferation of epimastigotes and amastigotes of *T. cruzi*. In the epimastigotes (Fig. 2) proliferating in LIT at 28°, the compound slowed growth in a concentration-dependent manner: a 40-μM concentration of the compound was able to reduce growth by 50% in 48 hr, while 80 μM blocked growth immediately and 100 μM induced cell lysis in 24 hr. Figure 3 shows that amastigotes, proliferating inside VERO cells at 37°, were even more sensitive to this compound: 20 μM reduced the number of infected cells to 50% of those found in controls, while 40 μM eradicated the parasite from the host

Table 2. Effect of ajoene on the phospholipid composition of *T. cruzi* epimastigotes*

Phospholipid fraction	Control	60 μM Ajoene
Phosphatidylserine	21.9	22.7
Phosphatidylinositol	7.2	7.9
Sphingomyelin	14.1	8.4
Phosphatidylcholine	41.3	15.3
Phosphatidylethanolamine	15.5	45.6

* Phospholipids were isolated from control and treated epimastigotes incubated in the presence of the indicated drug concentration for 120 hr and analyzed quantitatively by TLC as described in Materials and Methods. Mol per cent values of the different phospholipid types are given.

cells. The number of VERO cells in the monolayer was also reduced markedly at the highest concentration of the compound, but the morphology of the cells was normal by both optical and electron microscopy (not shown); growth stasis probably mediated this effect.

Investigating the mechanism of the anti-proliferative effect of the drug, which is known to act at cell membranes [1, 4, 6, 21], we found that in the presence of 60 μM ajoene, which inhibited growth by 80% (Fig. 2), the specific activity of [^{14}C-U]acetate incorporated in the polar lipid (phospholipid) fraction during 48 hr was reduced to 24% of that found in control cells. The blockade in the phospholipid biosynthesis was highly specific. As can be seen in Table 1, the incorporation of the radioactive precursor was essentially unaffected in phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SPM), was reduced markedly in PC, and was *increased* concomitantly in phosphatidylethanolamine (PE), the direct precursor of PC. This led to a dramatic alteration of the phospholipid composition of the treated cells. Table 2 shows that although the relative amounts of PS, PI and SPM did not change when compared with controls after 120 hr in the presence of 60 μM ajoene, the proportion of PC dropped to a third of its value in non-treated cells, going from the most abundant species in the control cells to the least abundant phospholipid species in the cells treated with the drug. Table 2 also shows that, as expected from the data of Table 1, the increase in PE was proportional to the decrease in PC in treated cells. However, the total phospholipid content in control or treated cells did not differ significantly (1.75 vs 2.1% of the dry weight, respectively). The phospholipid headgroup composition of the treated cells suggested a highly unstable lipid bilayer due to the predominance of PE and negatively charged headgroups. This could lead to compensatory changes in the fatty acid composition of these lipids, and Table 3 shows that this was the case. In the presence of 60 μM ajoene, the proportion of saturated palmitic (16:0) and stearic (18:0) acids was increased markedly, whereas the relative amount of linoleic acid (18:2), the most abundant fatty acid in the control cells, dropped sharply 96 hr after the addition of the drug. Thus,

Table 3. Effect of ajoene on the fatty acid composition of the phospholipid fraction of *T. cruzi* epimastigotes*

Fatty acid	Control	60 μM Ajoene
16:0	11.0	20.2
16:1	1.5	3.1
18:0	13.1	39.3
18:1	34.1	15.6
18:2	38.5	19.2
20:0	1.8	2.6
SFA†	25.9	62.1
UFA‡	74.1	37.9
SFA/UFA	0.35	1.64

* Phospholipids were isolated from control and treated epimastigotes incubated in the presence of the indicated drug concentration for 96 hr; after conversion of the sterified fatty acids to their corresponding methyl ester, they were analyzed quantitatively by GLC as described in Materials and Methods. The percentage of each fatty acid in the total phospholipid fraction is given.

† Total saturated fatty acids.

‡ Total unsaturated fatty acids.

Table 4. Effect of ajoene on the free sterol composition of *T. cruzi* epimastigotes*

Sterol fraction†	Control	60 μM Ajoene
I	22.4	46.9
II	29.9	23.6
III	traces	traces
IV	26.0	12.1
V	21.7	14.1
u.e.	ND‡	3.3

* Free sterols were isolated from control and treated epimastigotes incubated in the presence of the indicated drug concentrations for 120 hr and analyzed quantitatively by GLC as described in Materials and Methods. Weight per cent values of the different sterol types are given.

† Fractions are identified as follows: I, cholesterol, incorporated passively from the culture medium, retention time 24.1 min; II, ergosterol, retention time 28.3 min; III, 24-methyl-5,7-cholest-dien-3-β-ol, retention time 30.4 min; IV, 24-ethyl-5,7,22-cholest-trien-β-ol, retention time 32.5 min; V, 24-ethyl-5,7-cholest-dien-3-β-ol, retention time 34.5 min; and u.e., unidentified sterol, retention time 38.7 min.

‡ ND, not detected.

the ratio of saturated to unsaturated fatty acids esterified to phospholipids increased by a factor of 4.7 after exposure to the drug. These changes were observed consistently in several independent experiments.

Studying the neutral lipid fraction, we found that the specific activity of [^{14}C-U]acetate incorporated in neutral lipids (measured as dpm/cell) was also reduced (by 89%) in the presence of 60 μM ajoene; furthermore, the percentage of the radioactivity incorporated in the neutral lipids found in the endogenous 4-desmethylsterols (separated by TLC as described in Materials and Methods) dropped from 16.3% in control cells to 6.1% in ajoene-treated cells. A quantitative analysis of the free sterols present in the two types of cell by GLC (Table 4) showed a drop in the ratio (w/w) of endogenous 4-desmethyl sterols to cholesterol from its normal value of 3/1 in control cells to 1/1 in treated cells; most of this drop was due to the reduction in content of 24-ethyl analogues of ergosterol, characteristic of *T. cruzi* (sterols IV and V of Table 4; see Refs. 14, 15 and 22).

When we correlated the biochemical changes described above in the epimastigotes with cellular alterations studied by electron microscopy, we found that the exposure to 40 μM ajoene for 96 hr led to a significant increase in the cell size when compared with control cells and swelling of the unique giant mitochondrion characteristic of kinetoplastid protozoa (see Fig. 4A–4C and Ref. 23); multivesicular bodies due to apparent fragmentation of the intracellular membranous system were also very prominent (Fig. 4D). Cells exposed to a 60 μM concentration of the drug displayed a grossly altered mitochondrion and endoplasmatic reticulum (Fig. 4E and 4F), while 100 μM led to a general breakdown of the intracellular membrane system and cell lysis (Fig. 4G and 4H).

DISCUSSION

Previous studies on the antifungal effects of aqueous garlic extracts (AGE) also found a marked effect of these crude preparations on the *de novo* lipid biosynthesis of *Candida albicans*, which correlated with its antiproliferative effects [8, 10]; moreover, Ghannoum [10] found that AGE induces in this fungus exactly the same changes in the phospholipid composition (decreases in PC and a concomitant increase in PE) and in the fatty acids esterified to total lipids (increase in 16:0, decrease in 18:2) that we found in the present study with *T. cruzi*. No quantitative estimate of the amount of ajoene in AGE is available, but if we take into account that the amounts of AGE required to elicit the above-mentioned effects are 2–3 orders of magnitude greater than those required to produce the same effects in *T. cruzi* by ajoene, this compound need only constitute 0.1 to 1.0% of AGE to explain its observed effects in fungi. Although ajoene inhibited the *de novo* biosynthesis of both neutral and polar lipids, the change in the sterol composition induced by the drug in treated cells does not seem sufficient to account for the observed effects on growth rate. This interpretation arises from previous studies of this group with specific ergosterol biosynthesis inhibitors, which have shown that no significant effects on growth rate and cell viability in *T. cruzi* are observed until the weight ratio of endogenous 4-desmethyl sterols to exogenous cholesterol drops from its normal value of *ca.* 3 to a critical value of *ca.* 0.25 [14, 15, 24]. As the drop in the relative content of endogenous 4-desmethyl sterols in the epimastigotes treated with 60 μM ajoene, which had less than 20% of the normal growth rate, was only to a 1:1 (w/w) ratio (Table 4), we concluded that the alteration of the sterol

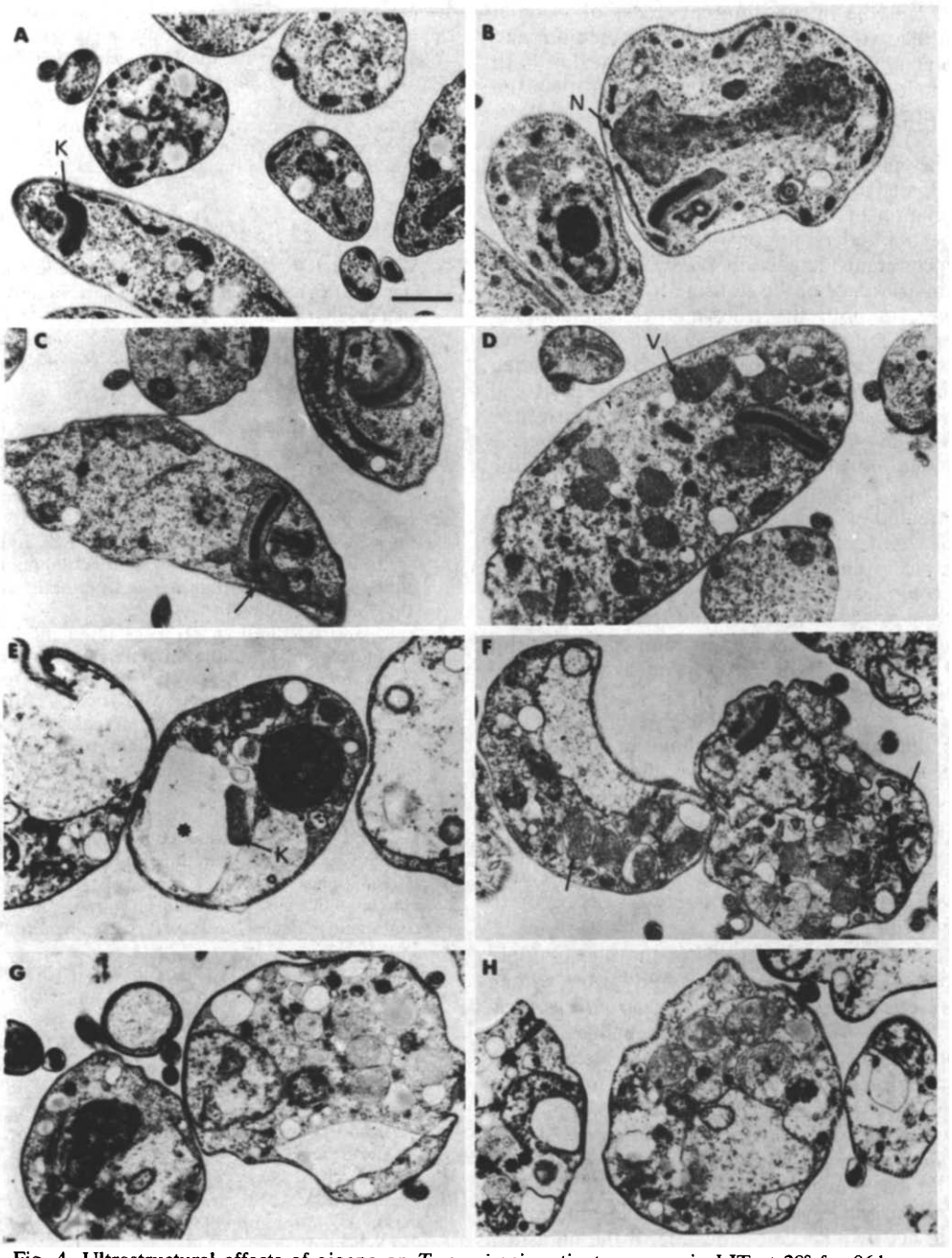

Fig. 4. Ultrastructural effects of ajoene on *T. cruzi* epimastigotes grown in LIT at 28° for 96 hr, as described in Materials and Methods. A and B: control (untreated) cells showing the characteristic kinetoplast–mitochondrion complex (K) and intranuclear mitosis (N) of kinetoplastid cells. C and D: cells exposed to 40 μM ajoene; note the increase of the cell size when compared with controls, the initial stages of the mitochondrial swelling (arrow) and intracellular bodies containing aggregated and/or fused membrane vesicles (V). E and F: cells treated with 60 μM ajoene; full swelling of the mitochondrion (asterisk) which appears as a vacuole containing the remanent of the kinetoplast (K) and large numbers of vesicle aggregates (arrows). G and H: cells treated with 100 μM ajoene; intense vacuolization and cell lysis. Bar: 1.5 μm.

content of the cells alone could not explain the observed effect on the growth rate.

The phospholipid composition and metabolic activity of the different phospholipid species found in this work for the EP stock of *T. cruzi* (Tables 1 and 2) differ very significantly from the original report by Oliveira *et al.* [25] using the Y stock of the same organism. The origin of these different

findings must lie in the different stocks of *T. cruzi* used in the two studies, as the culture medium and growth conditions were essentially identical in both cases. In our study, the results suggest that the Greenberg's pathway (methylation pathway, Refs. 26 and 27) is very active in the synthesis of PE and PC in the epimastigotes of the EP stock and that the PS to PE step is rate-limiting, leading to a significant accumulation of PS. In this context it is probable that the marked reduction of the PC content and the concomitant increase in PE by ajoene are due to the interference of the transmethylation reaction. However, as both the trans-methylation and the Kennedy (CDP-choline pathway, Ref. 28) pathways have been shown to be active in the related Trypanosomatidae *Crithidia fasciculata* [29] and *Leishmania donovani* [30], work is currently underway to verify our hypothesis using specific radioactive precursors. The dramatic change in the headgroup composition of the phospholipids of *T. cruzi* treated with ajoene should lead to a very unstable lipid bilayer due to the predominance of PE and acidic phospholipids; thus, the predominance of PE could lead, particularly with polyunsaturated acyl chains, to the formation of non-bilayer hexagonal H_{II} phases [31, 32]. One way to compensate for this would be to reduce the proportion of unsaturated fatty acids present in these lipids [32] and this is exactly what was found (Table 3), supporting the notion that an important mechanism of action of ajoene against *T. cruzi* is the alteration of the phospholipid composition of the cells which leads to the destabilization of its cellular membranes, clearly verified by the ultrastructural results (Fig. 4). The strong mitochondrial swelling is also probably related to the depletion of endogenous sterols, as reported previously [9, 20].

The effects of modifications of the phospholipid composition on growth and viability vary among "lower" eucaryotes: although *Saccharomyces cerevisiae* seems to tolerate gross changes in its phospholipid headgroup composition, including an almost complete replacement of PC by mono- and dimethyl-PE [33], *Saccharomyces pombe* [33], *Neurospora crassa* [33], *Aspergillus nidulans* [34, 35], *Pyricularia oryzae* [36, 37] and *L. donovani* [30] seem to be much less tolerant. Many effective antifungal agents, such as iprobenfos and edifenphos, seem to act by a specific blockade of the parasite's phospholipid biosynthesis [34–38], which indicates that although these pathways are shared with the host, the particular enzymatic steps have enough differences to allow selective toxicity. In this sense, although ajoene does exhibit cytotoxic effects against cultured vertebrate cell lines, as can be seen from this work (Fig. 2) and that of Scharfenberg *et al.* [39], the doses required to elicit these effects are significantly higher than those that lead to complete eradication of the intracellular parasite. On the other hand, the drug seems to be well tolerated by vertebrates, both topically [39] and systemically in dogs [2]. Thus, ajoene appears to be a good model compound to explore the selective inhibition of phospholipid biosynthesis in *T. cruzi* and its potential chemotherapeutic value.

Acknowledgements—This investigation received financial support from the UNPD/World Bank/World Health Organization Programme for Research and Training in Tropical Diseases, Grant 900359. We thank Reneé Lira for skillful technical assistance, Mirtha Romano for her collaboration in the electron microscopy work, Jorge Rivas for photographic work, and Dr. Mahendra K. Jain for valuable suggestions on the manuscript.

REFERENCES

1. Apitz-Castro R, Cabrera S, Cruz MR, Ledezma E and Jain MK, Effect of garlic extract and of three pure components isolated from it on human platelet aggregation, arachidonate metabolism, release reaction and platelet ultrastructure. *Throm Res* **42**: 155–169, 1983.
2. Apitz-Castro R, Ledezma E, Escalante J, Jorquera A, Piñate FM, Moreno-Rea J, Carrillo G, Leal O and Jain MK, Reversible prevention of platelet activation by (E,Z)-4,5,9-trithiadodeca-1,6,11-triene-9-oxide (ajoene) in dogs under extracorporeal circulation. *Arzneimittelforschung* **38**: 901–904, 1988.
3. Block E, Ahmat S, Catafalmo JL, Jain MK and Apitz-Castro R, Antithrombic organosulfur compounds from garlic: Structural, mechanistic and synthetic studies. *J Am Chem Soc* **108**: 7045–7055, 1986.
4. Apitz-Castro R, Ledezma E, Escalante J and Jain MK, The molecular basis of the antiplatelet action of ajoene: Direct interaction with the fibrinogen receptor. *Biochem Biophys Res Commun* **141**: 145–150, 1986.
5. Jain MK and Apitz-Castro R, Garlic: Molecular basis of the putative 'vampire-repellant' action and other matters related to heart and blood. *Trends Biochem Sci* **12**: 252–254, 1987.
6. Apitz-Castro R, Jain MK, Bartoli F, Ledezma E, Ruiz MC and Salas R, Evidence for direct coupling of primary agonist–receptor interaction to the exposure of functional IIb–IIIa complexes in human blood platelets. Results from studies with the antiplatelet compound ajoene. *Biochim Biophys Acta* **1095**: 269–280, 1991.
7. Jain MK, Scanzello C and Apitz-Castro R, Wirkung des knoblauchs. Wahrheit und Dichtung. *Chem Unserer Zeit* **22**: 193–200, 1988.
8. Adetumbi M, Javor GT and Lau BHS, *Allium sativum* (garlic) inhibits lipid synthesis by *Candida albicans*. *Antimicrob Agents Chemother* **30**: 499–501, 1986.
9. Yoshida S, Kasuga S, Hayashi N, Ushiroguchi T, Matsuura H and Nakagawua N, Antifungal activity of ajoene derived from garlic. *Appl Environ Microbiol* **53**: 615–617, 1987.
10. Ghannoum MA, Studies on the anticandidal mode of action of *Allium sativum* (garlic). *J Gen Microbiol* **134**: 2917–2924, 1988.
11. San Blas G, San Blas F, Gil F, Mariño L and Apitz-Castro R, Inhibition of growth of the dimorphic fungus *Paracoccidioides brasiliensis* by ajoene. *Antimicrob Agents Chemother* **33**: 1641–1644, 1989.
12. De Maio A and Urbina JA, *Trypanosoma (Schizotrypanum) cruzi*: Terminal oxidases in two growth phases *in vitro*. *Acta Cient Venez* **35**: 136–141, 1984.
13. Urbina JA, Lazardi K, Aguirre T, Piras MM and Piras R, Antiproliferative synergism of the allylamine SF 86-327 and ketoconazole on epimastigotes and amastigotes of *Trypanosoma (Schizotrypanum) cruzi*. *Antimicrob Agents Chemother* **32**: 1237–1242, 1988.
14. Urbina JA, Lazardi K, Aguirre T, Piras MM and Piras R, Antiproliferative effects and mechanisms of action of ICI 195,739, a novel bis-triazole derivative on epimastigotes and amastigotes of *Trypanosoma (Schizotrypanum) cruzi*. *Antimicrob Agents Chemother* **35**: 730–735, 1991.

15. Larralde G, Vivas J and Urbina JA, Concentration and time-dependence of the effects of ketoconazole on growth and sterol synthesis by *Trypanosoma (Schizotrypanum) cruzi* epimastigotes. *Acta Cient Venez* **39**: 140–146, 1988.
16. Van den Bossche H, Willemsens G, Cools W, Lauwers WFJ and Le Jeune L, Biochemical effects of miconazole in fungi. II. Inhibition of ergosterol biosynthesis in *Candida albicans*. *Chem Biol Interact* **21**: 59–78, 1988.
17. Cuzner ML and Davison AN, Quantitative thin layer chromatography of lipids. *J Chromatogr* **27**: 388–397, 1967.
18. Ames BN and Dubin DT, The role of polyamines in the neutralization of deoxyribonucleic acid. *J Biol Chem* **235**: 769–775, 1960.
19. Lazardi K, Urbina JA and de Souza W, Ultrastructural alterations induced by two ergosterol biosynthesis inhibitors, ketoconazole and terbinafine, on epimastigotes and amastigotes of *Trypanosoma (Schizotrypanum) cruzi*. *Antimicrob Agents Chemother* **34**: 2097–2105, 1990.
20. Lazardi K, Urbina JA and de Souza W, Ultrastructural alterations induced by ICI 195,739, a bis-triazole derivative with strong antiproliferative action against *Trypanosoma (Schizotrypanum) cruzi*. *Antimicrob Agents Chemother* **35**: 736–740, 1991.
21. Debouzy J-C, Neumann J-M, Hervé M, Daveloose D, Viret J and Apitz-Castro R, Interaction of antiaggregant molecule ajoene with membranes. An ESR and ^{1}H, ^{2}H, ^{31}P-NMR study. *Eur Biophys J* **17**: 211–216, 1989.
22. Beach DH, Goad LJ and Holtz GG Jr, Effects of ketoconazole on sterol biosynthesis by *Trypanosoma cruzi* epimastigotes. *Biochem Biophys Res Commun* **136**: 851–856, 1986.
23. De Souza W, Cell biology of *Trypanosoma cruzi*. *Int Rev Cytol* **86**: 197–283, 1984.
24. Urbina JA, Lazardi K, Marchan E, Visbal G, Aguirre T, Piras MM and Piras R, Mevinolin (lovastatin) potentiates the antiproliferative effects of ketoconazole and terbinafine against *Trypanosoma (Schizotrypanum) cruzi*: *In vitro* and *in vivo* studies. *Antimicrob Agents Chemother* **37**: 580–591, 1993.
25. Oliveira MM, Timm SL and Costa SCG, Lipid composition of *Trypanosoma cruzi*. *Comp Biochem Physiol* **55B**: 195–199, 1977.
26. Bremer J, Figard PH and Greenberg DM, The biosynthesis of choline and its relation to phospholipid metabolism. *Biochim Biophys Acta* **43**: 477–488, 1960.
27. Bremer J and Greenberg DM, Methyl transfering enzyme system of microsomes in the biosynthesis of lecithin (phosphatidylcholine). *Biochim Biophys Acta* **46**: 205–216, 1961.
28. Kennedy EP and Weiss SB, The function of cytidine coenzymes in the biosynthesis of phospholipids. *J Biol Chem* **222**: 193–214, 1965.
29. Palmer FBStC, Biosynthesis of choline and ethanolamine phospholipids in *Crithidia fasciculata*. *J Protozool* **21**: 160–163, 1974.
30. Herrmann H and Gercken G, Synthesis of phospholipids in *Leishmania donovani*. *Hoppe-Seyler's Z Physiol Chem* **361**: 1735–1742, 1980.
31. Gruner SM, Cullis PR, Hope MJ and Tilcock CPS, Lipid polymorphism: The molecular basis of nonbilayer phases. *Annu Rev Biophys Biophys Chem* **14**: 211–238, 1985.
32. Cullis PR, Hope MJ and Tilcock CPS, Lipid polymorphism and the roles of lipids in membranes. *Chem Phys Lipids* **40**: 127–144, 1986.
33. Hill JE, Chung C, McGraw P, Summers E and Henry SA, Biosynthesis and role of phospholipids in yeast membranes. In: *Biochemistry of Cell Walls and Membranes in Fungi* (Eds. Kuhn PJ, Trinci APJ, Jung MJ, Goosey MW and Copping LG), pp. 245–260. Springer, Berlin, 1990.
34. Kodama O, Yamada H and Akatsuka T, Kitazin P, inhibitor of phosphatidyl-choline biosynthesis in *Pyricularia oryzae*. *Agric Biol Chem* **43**: 1719–1725, 1979.
35. Kodama O, Yamada H and Akatsuka T, Edifenphos, inhibitor of phosphatidyl-choline biosynthesis in *Pyricularia oryzae*. *Agric Biol Chem* **44**: 1015–1021, 1980.
36. Craig GD and Peberdy JF, The effect of *S*-benzyl-*O*,*O*-di-isopropyl-phosphorothioate (IBP) and dicloran on the total lipid, sterol and phospholipids in *Aspergillus nidulans*. *FEMS Microbiol Lett* **18**: 11–14, 1983.
37. Craig GD and Peberdy JF, The mode of action of *S*-benzyl-*O*,*O*-di-isopropyl-phosphorothioate and of dicloran on the total lipid, sterol and phospholipids in *Aspergillus nidulans*. *Pestic Sci* **14**: 17–24, 1983.
38. Robson GD, Wiebe M, Kühn PJ and Trinci APJ, Inhibitors of phospholipid biosynthesis. In: *Biochemistry of Cell Walls and Membranes in Fungi* (Eds. Kuhn PJ, Trinci APJ, Jung MJ, Goosey MW and Copping LG), pp. 261–281. Springer, Berlin, 1990.
39. Scharfenberg K, Wagner R and Wagner KG, The cytotoxic effect of ajoene, a natural product from garlic, investigated with different cell lines. *Cancer Lett* **53**: 103–108, 1990.