The Biosynthetic Incorporation of the Intact Leucine Skeleton into Sterol by the Trypanosomatid Leishmania mexicana*

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Michael L. Ginger‡ §§, Michael L. Chance¶, Ian H. Sadler¶, and L. John Goad‡ **

From the § School of Biological Sciences, University of Liverpool, Life Sciences Building, Crown St., Liverpool L69 7ZB, United Kingdom, the ¶ School of Tropical Medicine, University of Liverpool, Pembroke Place, Liverpool L3 5QA, United Kingdom, and the ¶ Department of Chemistry, University of Edinburgh, King’s Buildings, West Mains Rd., Edinburgh EH9 3JJ, United Kingdom

The amino acid leucine is efficiently used by the trypanosomatid Leishmania mexicana for sterol biosynthesis. The incubation of [2-14C]leucine with L. mexicana promastigotes in the presence of ketoconazole gave 14α-methylgergosta-8,24(241)-3β-ol as the major sterol, which was shown by mass spectrometry to contain up to six atoms of 13C per molecule. 13C NMR analysis of the 14α-methylgergosta-8,24(241)-3β-ol revealed that it was labeled in only six positions: C-2, C-6, C-11, C-12, C-16, and C-23. This established that the leucine skeleton is incorporated intact into the isoprenoid pathway leading to sterol; it is not converted first to acetyl-CoA, as in animals and plants, with utilization of the acetyl-CoA to regenerate 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). An inhibitor of HMG-CoA synthase (L-659,699) blocked the incorporation of [1,14C]acetate into sterol but had no inhibitory effect on [U-14C]leucine incorporation. The HMG-CoA reductase inhibitor lovastatin inhibited promastigote growth and [U-14C]leucine incorporation into sterol. The addition of unlabeled mevalonic acid (MVA) overcame the lovastatin inhibition of growth and also diluted the incorporation of [1,14C]leucine into sterol. These results are compatible with two routes by which the leucine skeleton may enter intact into the isoprenoid pathway post-MVA.

Parasitic trypanosomatid protozoa of the genus Leishmania cause diseases in tropical and subtropical regions of the world. The treatment of leishmaniasis still relies upon the drugs introduced many years ago (1), which have toxic side effects, and there is now a great need for more effective new chemotherapeutic drugs (1, 2). This has prompted the search for new metabolic targets for drugs and has resulted in the recognition of sterol synthesis inhibitors as a potential candidate (2, 3). The importance of an active sterol biosynthetic pathway in trypanosomatids for growth and viability has been demonstrated using antifungal agents that are inhibitors of sterol biosynthesis. Thus, the imadazole- and triazole-based drugs (e.g. ketoconazole and itraconazole), which inhibit the 14α-methylsterol 14-demethylase, and the allylamines (e.g. terbinafine), which inhibit squalene epoxidase (2–11), have been shown to block sterol synthesis in a number of Leishmania and Trypanosoma species with retardation of growth and death of the parasite.

In our studies on sterol biosynthesis in Leishmania species, we have recently demonstrated (12, 13) that leucine is the major source of the carbon used for de novo sterol biosynthesis. By contrast, acetate or substrates from which acetyl-CoA is generated by metabolism (e.g. glucose, palmitic acid, alanine, serine, and isoleucine) are very poorly incorporated into sterol, although they are used efficiently for the synthesis of the fatty acid moieties of triacylglycerol and phospholipid. The utilization of leucine for sterol biosynthesis has been shown previously in animal tissues (14–18), plants (19–21), and fungi (22, 23). In animals and plants, a major route for leucine catabolism has been demonstrated to be located in the mitochondrion (24, 25). The pathway proceeds through the production of α-ketocaproate, isovaleryl-CoA, 3-methylcrotonyl-CoA, and 3-methylglutaconyl-CoA (Scheme 1) to give 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is then cleaved by a lyase to produce acetyl CoA and acetoacetate. The acetyl-CoA generated in this way can be either fed into the citric acid cycle or alternatively transported out of the mitochondrion into the cytosol, where the acetyl-CoA may be utilized for the biosynthesis of a range of compounds including fatty acids and isoprenoids such as sterols. The entry of the acetyl-CoA into the isoprenoid pathway requires the regeneration of HMG-CoA, which is then reduced to mevalonic acid (Scheme 1). Conclusive evidence that leucine enters isoprenoids in plants by this indirect route and involving production of acetyl-CoA has been provided by incubation of 13C-labeled leucine with a callus culture of Andrographis paniculata (19, 20). 13C NMR analysis of the 13C-enriched sesquiterpenoid and phytosterols (19, 20) produced by the callus showed unequivocally that the leucine was metabolized to acetyl-CoA and acetoacetate prior to incorporation into the isoprenoid pathway. We have demonstrated previously (12, 13) that [U-14C]leucine incubated with Leishmania mexicana and other trypanosomatid species was very efficiently incorporated into sterol and to some limited extent into fatty acids. However, by contrast, [1-14C]acetate was readily incorporated into fatty acids but poorly utilized for sterol production. These observa-

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‡ Recipient of a Biotechnology and Biological Sciences Research Council research studentship. Present address: Wellcome Unit of Molecular Parasitology, The Anderson College, University of Glasgow, Church St., Glasgow, G11 5SR, United Kingdom.

** To whom correspondence should be addressed: School of Biological Sciences, University of Liverpool, Life Sciences Bldg., Crown St., Liverpool L69 7ZB, United Kingdom. Tel.: 44 151 794 4343; Fax: 44 151 794 4349; E-mail: ljgo@liv.ac.uk.

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Sterol by the Trypanosomatid Leishmania mexicana
tions are incompatible with a route in *Leishmania* that requires leucine degradation to proceed to acetyl CoA before reutilization for isoprenoid production (19, 20). We therefore undertaken the studies described here to investigate the metabolic route whereby *L. mexicana* uses leucine for sterol biosynthesis, since this may produce evidence for a target for new antileishmanial drug development.

**MATERIALS AND METHODS**

**Chemicals**—[1,14C]Acetate, sodium salt (57 mCi/mmol), and [1,13C]leucine (299–314 mCi/mmol) were obtained from Amersham Pharmacia Biotech. [2,13C]Leucine (99% enrichment) was obtained from Promochem (Welwyn Garden City, UK). [2,13C]Acetate, [1-13C]glucose, ketoconazole, and mevalonic acid were obtained from Sigma-Aldrich. Compound L-659,699 was a gift from Merck.

**Cell Culture**—The strain of *Leishmania* used in this study was *L. mexicana* (MNYC/62/BZ/M379). Promastigotes were cultured at 26 °C in HO-minimum essential medium (26), supplemented with 10% (v/v) heat-inactivated fetal calf serum.14C- and 13C-labeled substrates were added to the media to give the final concentrations indicated under "Results." Cell density of cultures was determined by counting using a Neubauer hemocytometer. Cultures were normally established with an "Results." Cell density of cultures was determined by counting using a Neubauer hemocytometer. Cultures were normally established with an

**Lipid Extraction and Analyses**—Parasite lipids were isolated after extraction with chloroform/methanol (2:1) as described previously (12, 27). Radioactive lipid extracts were analyzed by analytical TLC and radioscanning using silica gel TLC plates and chloroform/ethanol (98:2) as the developing solvent. Sterols were isolated and analyzed by GC or GC-MS as the TMS ether derivatives following previously described protocols (12, 27). Steryl acetates were prepared by treatment of the free sterol with pyridine/acetic anhydride (1:1) followed by usual work up of the steryl acetate. The steryl acetates were separated by preparative TLC on silica gel impregnated with 10% AgNO3 and developed with freshly distilled chloroform. Sterols were quantified by capillary GC analysis using 5a-cholestane as a standard.

**NMR Spectroscopy**—The NMR spectra were measured on deuteriochloroform solutions using a Varian INOVA 600 spectrometer operating at 599.9 MHz for protons and 150.9 MHz for 13C nuclei.1H NMR spectra were recorded using the following parameters: 7000-Hz spectrum width, 3-s preacquisition delay, 90° pulse, 0.936-s acquisition time, 70,000 transients, 64K data points No weighting function was used before Fourier transformation. Broad band proton-decoupled13C NMR spectra were obtained using the following parameters: 35,000-Hz spectrum width, 22° pulse, 0.936-s acquisition time, 10,000 transients, 64K data points, line broadening of 1 Hz before Fourier transformation.

**Isolation of 14a-Methylene[8,24(24)-dien-3β-ol—**Promastigotes were cultured for 72 h in the presence of 0.1 mg/ml ketoconazole (administered from a stock solution of 1 mg/ml in Me2SO) in HO-minimum essential medium. The sterols were isolated as previously described (12, 26), and the 14a-methylene[8,24(24)-dien-3β-ol was characterized by GC-MS and 1H NMR analyses. For the experiments studying [2-13C]leucine incorporation into the sterol, the medium contained [2-13C]leucine (78 mg/liter) in place of the unlabeled free leucine normally present. In total, 50 cultures (50 ml in each culture) were grown, and the cells were harvested in batches and extracted with 3 × 100 ml of chloroform/methanol (2:1) using procedures similar to those used for the smaller scale extraction (12, 27). The 13C-labeled

![Scheme 1](image)
N14a-methylergosta-8,24(24')-dien-3β-ol was then purified from the lipid by reversed-phase HPLC using an Econosphere C18 column (250 × 4.6 mm; inner diameter, 5 mm; supplied by Alltech) to separate it from cholesterol and other minor sterols. Compounds were eluted isocratically using acetonitrile/water (9:1), and sterols were detected by UV absorbance at 215 nm. The 14a-methylergosta-8,24(24')-dien-3β-ol was eluted at 26–30 min, and cholesterol was eluted at 32–36 min. The solvent volume of the eluate was carefully reduced by rotary evaporation, and the sterol was extracted into petroleum ether before taking to dryness and storage at −20 °C. The purity of the isolated 14C-labeled 14a-methylergosta-8,24(24')-dien-3β-ol (∼3 mg) was checked by GC-MS and then analyzed by 13C NMR spectroscopy.

RESULTS

Incorporation of [2-13C]Leucine into the Sterols of L. mexicana Promastigotes—We have utilized [2-13C]leucine to investigate if the leucine carbon skeleton is incorporated by L. mexicana directly into the sterols by reduction of HMG-CoA to MVA. The major sterols of L. mexicana are cholesterol obtained from the medium, and the biosynthesized ergosta-5,7,24(24')-trien-3β-ol (4, 12, 13) with smaller variable amounts of ergosta-5,7,22-trien-3β-ol, stigmasta-5,7,24(24')-trien-3β-ol, and precursors such as ergosta-7,24(24')-dien-3β-ol. A sterol mixture of this nature presents certain problems for the type of 13C NMR study envisaged. First, the complexity of the sterol mixture demands careful purification of one of the biosynthesized ergosta types of sterol so that signal assignments to 13C-enriched carbons can be made with accuracy and without ambiguity. Second, the major sterols of L. mexicana are Δ5,7-compounds that are notoriously unstable in small amounts due to oxidation, and this may cause difficulties in the purification, storage, and NMR analysis of these sterols. This problem has been considered in detail by Schepfer et al. (30), specifically in relation to the NMR analysis of Δ5,7-sterols. Finally, the success of the study depends upon the extent of enrichment of the biosynthesized sterol with 13C. A large pool of preexisting sterol from the inoculum will dilute the 13C-enriched sterol species and could make 13C-enriched carbons difficult to detect. Accordingly, we looked for a new approach to the problem and decided to use an inhibitor of sterol biosynthesis. An inhibitor was required that would cause the accumulation of a large amount of a relatively stable sterol intermediate that would normally occur in only trace amounts in the parasite, so dilution of the newly synthesized [13C]sterol by preexisting material would not be significant. Sterol biosynthesis inhibitors suited to this purpose are the imidazoles and triazoles types of antifungal drugs. These compounds block the action of the cytochrome P450-dependent 14a-methylsterol 14-demethylease with the result that the normal sterols are depleted and one or more 14a-methylsterols accumulate, often in large amounts (31). It has been demonstrated previously that antifungal imidazoles and triazoles also inhibit the 14α-demethylation step in sterol biosynthesis in several Leishmania species with the resulting appearance of 4α,14a-dimethylergosta-8,24(24')-dien-3β-ol and 14α-methylergosta-8,24(24')-dien-3β-ol in appreciable amounts (4, 6). These 14α-methylsterols are considerably more stable during isolation, storage, and NMR analysis than are Δ5,7-sterols. Consequently, we decided that using the 14α-demethylase inhibitor ketoconazole offered the best opportunity for the isolation of a pure 13C-enriched sterol undiluted by preexisting endogenous sterol, which was required for the 13C NMR analysis to determine the route of incorporation of leucine into the isoprenoid pathway.

Preliminary experiments were first undertaken to determine the optimum conditions for incubation of the L. mexicana promastigotes with ketoconazole to accumulate a 14α-methylsterol in sufficient amount for isolation and 13C NMR analysis. The incubation of promastigotes of L. mexicana with ketoconazole (0.1 and 1.0 μg/ml) for 72 h followed by isolation and GC-MS examination of the sterols showed that, as anticipated, the ergosta-5,7,24(24')-trien-3β-ol found in the control was replaced by 14α-methylergosta-8,24(24')-dien-3β-ol in the ketoconazole-treated cultures (Table I). Cholesterol taken up from the medium was present in both the control and treated cells. The 14α-methylergosta-8,24(24')-dien-3β-ol was identified by the mass spectrum of the TMS ether and by the 1H NMR spectrum (6, 32). MS m/z (rel. intensity): 484 [M]+ (48), 469 [M-methyl]+ (100), 385 [M-methyl-part side chain]+ (15), 379 [M-MTMSOH]+ (79), 303 (36), 295 [M-TMSOH-methyl part side chain]+ (29), 281 (17), 227 [M-TMSOH-side chain and ring D]+ (28), 213 [M-methyl-TMSOH-side chain and ring D]+ (41). 1H NMR (chloroform-d): δ 0.70 s (H2-18), 0.94 s (H2-19), 0.92 d (H2-21), 0.88 s (H2-32), 1.01 d and 1.02 d (H2-26 and H2-27), 4.65 br s and 4.70 br s (H2-24).

Ketoconazole at 0.1 μg/ml retarded growth by only about 5% compared with the control, and the total sterol content of the treated cells was −70% of the control value. At the higher ketoconazole concentration (1.0 μg/ml), the growth was around 75% of the control, and the total sterol was about 50% of the control. In a further experiment, the L. mexicana promastigotes were incubated with ketoconazole (0.1 μg/ml) and [1-13C]leucine (2.6 μCi) for 72 h to ensure that the accumulating 14α-methylsterol was being biosynthesized from leucine derived from the medium rather than from some internal source of unlabeled precursor(s). The lipids were extracted and found to contain 4.2% of the radioactivity added to the culture medium, while analytical TLC with radioscanning showed that the 14α-methylsterol was the major labeled material. Recovery of the labeled sterol from the TLC plate, acetylation, and rechromatography by TLC on silver nitrate-impregnated silica.
gel showed that the radioactivity accompanied a material with the same Rf as 14α-methylergosta-8,24(24β)-dien-3β-ol acetate.

The above experiments showed that our approach to obtain a pure sterol for the 13C NMR analysis was feasible. Therefore, multiple cultures (50 × 50 ml) of L. mexicana promastigotes were grown in HO-minimum essential medium (plus 10% fetal calf serum) in which the free (i.e. nonprotein) leucine was replaced with [2-13C]leucine, and 0.1 µg/ml ketoconazole was added. The cells were cultured for 72 h and harvested, and the lipid was extracted. The total sterol was isolated from the lipid, and the 14α-methylergosta-8,24(24β)-dien-3β-ol was then separated from the cholesterol and other minor sterols by HPLC (see “Materials and Methods”). The cholesterol was shown by GC-MS analysis to contain only the natural abundance of 13C, and there was no detectable labeling from the [2-13C]leucine. This observation established unequivocally that the cholesterol in L. mexicana must be taken up from the medium and that it is not the product of de novo synthesis in the parasite. The purity of the isolated 14α-methylergosta-8,24(24β)-dien-3β-ol (~3 mg) was 95% as judged by GC analysis. The mass spectrum of the TMS ether showed clusters of ions for the molecular and fragment ions arising from several labeled species of the sterol containing from one to six 13C atoms (Fig. 1). Ions due to unlabeled sterol were very minor, showing that there was excellent incorporation of [2-13C]leucine into the sterol in accord with our previous studies with [U-13C]leucine incorporation, which had revealed that at least 80% of the sterol carbon originated from leucine (12, 13). The molecular ion region comprised a cluster of ions at m/z 484 (unlabeled sterol), 485, 486, 487, 488, 489, and 490, with the last two predominating (Table II). The fragment ion clusters at m/z 489–475 and 379–385, which arise by loss of a methyl and methyl and TMSOH from the [M]+ ion, respectively, showed a similar distribution of molecular species containing 1–6 13C-enriched positions (Fig. 1). The ions at m/z 304–307 showed fragments containing up to five 13C-enriched positions. The ions arising by the McLafferty loss of the terminal part of the side chain were at m/z 295–300 with the strong ion at m/z 300 showing the presence of five 13C-labeled positions in the fragment. The ion cluster at m/z 213–217 [M+ -side ring D-TMSOH] revealed up to four 13C-enriched carbons in the remaining fragment. The mass spectral results for the 13C-labeled sterol were compatible with the incorporation of up to six molecules of [2-13C]leucine into the sterol. Moreover, the fragmentation ions had labeling patterns revealing that one labeled position was in the side chain, one in the carbons of ring D, and the remaining four in the rings A, B, and C.

The 13C labeling patterns predicted for sterol derived from [2-13C]leucine by pathways involving either HMG-CoA breakdown to the acetyl-CoA level or directly by reduction of HMG-CoA to MVA are shown in Scheme 2. If the incorporation proceeds indirectly through the intermediacy of acetyl-CoA, the sterol will have 12 13C-enriched positions (C-2, C-4, C-6, C-8, C-10, C-11, C-12, C-14, C-16, C-20, C-23, and C-25). However, if incorporation results from direct conversion of HMG-CoA to MVA, then only six positions will be enriched (C-2, C-6, C-11, C-12, C-16, and C-23). Clearly, the mass spectral data revealing labeled sterol species containing up to six 13C atoms pointed to the latter labeling pattern. Accordingly, to determine the exact number of 13C atoms and their locations in the molecule, the labeled 14α-methylergosta-8,24(24β)-dien-3β-ol was purified by preparative HPLC and examined by 13C NMR spectroscopy (Fig. 2). The spectrum showed a very high enrichment of the compound with 13C and displayed six strong signals indicating the positions specifically labeled from the [13C]leucine. The assignments of these carbon signals were made by comparison with the reported 13C NMR spectra of other sterols (32).

The singlet signals at δ 25.2, 31.2, and 31.5 ppm were readily assigned to C-6, C-2, and C-23, respectively. The three signals centered at δ 21.7 (Fig. 2, inset A) comprised a singlet due to C-11 in molecules with no 13C enrichment at the adjacent positions (C-9 and C-12) and a doublet arising from coupling with C-12 in molecular species that were 13C-enriched at this position. Similarly, the signals centered at δ 30.9 were assigned to C-12 with a singlet in those molecular species lacking 13C-enrichment at C-11 or C-13 and a doublet due to coupling in molecules with 13C at position C-11. However, as shown (Fig. 2, inset B), each of the three signals arising from C-12 was further split by another 13C-13C long range coupling. The labeled position responsible for this coupling was assigned to C-16, the signal for which was at δ 28.1. Expansion of the signal at δ 28.1, which at first sight appeared to be a singlet, showed it was composed of a singlet plus a doublet. The splitting to give the doublet must have resulted from long range coupling in molecules labeled with 13C at C-12 as well as at C-16. The 13C labeling pattern determined in the 14α-methylergosta-8,24(24β)-dien-3β-ol was therefore entirely consistent with the leucine skeleton remaining intact during metabolism and incorporation into the isoprenoid pathway (Schemes 1 and 2). This is in striking contrast to leucine utilization in plants, where it is first degraded to acetyl-CoA before utilization in

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**FIG. 1.** The mass spectrum of the TMS ether of 14α-methylergosta-8,24(24β)-dien-3β-ol isolated from promastigotes of *L. mexicana* cultured for 72 h in the presence of ketoconazole (0.1 µg/ml) and [2-13C]leucine. The identities of the ions are described under "Results."
Sterol Biosynthesis from Leucine in L. mexicana

The sterols were analysed by GC-MS of their TMS ether derivatives as described in the Methods. The relative abundance of the ions has been corrected to take into account the natural abundance of $^{13}$C-carbon in the compounds. $M^+$, molecular ion; $M^++1$, molecular ion with one $^{13}$C atom; $M^++2$, molecular ion with two $^{13}$C atoms, etc.

| Compound | Base peak | $M^+$ | $M^++1$ | $M^++2$ | $M^++3$ | $M^++4$ | $M^++5$ | $M^++6$ |
|----------|-----------|-------|---------|---------|---------|---------|---------|---------|
| (a) 14α-Methylergosta-8,24(241)-dien-3β-ol from ketoconazole-treated promastigotes | m/z 474 | 484 | 485 | 486 | 487 | 488 | 489 | 490 |
| Relative abundance of ions | 100 | 0.6 | 1.4 | 4.6 | 11.2 | 27.3 | 43.2 | 42.6 |
| Proportion relative to $M^++5$ ion | 1.7 | 3.4 | 12.1 | 27.9 | 68.7 | 100 | 87.4 |
| (b) Ergosta-5,7,24(241)-trien-3β-ol from a promastigote culture | m/z 368 | 468 | 469 | 470 | 471 | 472 | 473 | 474 |
| Relative abundance of ions | 100 | 0.8 | 1.6 | 5.2 | 9.9 | 15.1 | 17.2 | 9.8 |
| Proportion relative to $M^++5$ ion | 4.7 | 9.3 | 30.2 | 68 | 87.8 | 100 | 57 |
| (c) Ergosta-5,7,24(241)-trien-3β-ol from an amastigote culture | m/z 368 | 468 | 469 | 470 | 471 | 472 | 473 | 474 |
| Relative abundance of ions | 100 | 16.9 | 1.7 | 4.6 | 7.4 | 8.6 | 13.9 | 6.9 |
| Proportion relative to $M^++5$ ion | 121.6 | 12.2 | 33 | 53.2 | 61.9 | 100 | 49.6 |

TABLE II

Mass spectra of the major sterols isolated after incorporation of [2-14C]leucine into the major sterol of (a) L. mexicana promastigotes incubated with ketoconazole (0.1 μg/ml), (b) L. mexicana control promastigotes, (c) L. mexicana amastigotes

The various fragment ion clusters were also consistent with the labeling pattern determined for 14α-methylergosta-8,24(241)-dien-3β-ol. The mass spectra of the ergosta-5,7,24(241)-trien-3β-ol TMS ether labeled from either [2-13C]acetate or [1-13C]glucose contained the molecular ion at m/z 468 for unlabeled compound, presumably produced from leucine, and a series of further molecular ions of diminishing abundance containing 1–10 $^{13}$C atoms (above this, the ions were too weak to determine whether molecular species with the theoretical maximum of 12 $^{13}$C atoms were present). The incorporation of acetate and glucose into the sterol was consistent with our previous investigations (12, 13), which indicated that acetate can provide up to 20% of the carbon needed for sterol production in L. mexicana but with the major portion (~80%) arising from leucine.

Incorporation of [2-13C]Leucine into Sterol by Amastigotes of L. mexicana—The above experiments were performed with L. mexicana promastigotes. We have previously reported that the amastigote form of this parasite can also use [U-14C]leucine for sterol biosynthesis (12). This has now been confirmed by incubation of L. mexicana amastigotes cultured in macrophages with [2-13C]leucine. The mass spectrum of the isolated ergosta-5,7,24(241)-trien-3β-ol, analyzed as the TMS ether, had a strong molecular ion at m/z 468 for unlabeled sterol, but this was accompanied by an ion of similar abundance at m/z 473 for sterol with five atoms of $^{13}$C, together with less abundant ions for molecules containing one, two, three, four, and six atoms of $^{13}$C (Table II) in proportions similar to that seen in promastigotes. The unlabeled ergosta-5,7,24(241)-trien-3β-ol must be

isoprenoid production (19, 20).

Because the inhibitor ketoconazole was used to facilitate the accumulation of the 14α-methylergosta-8,24(241)-dien-3β-ol used for the NMR study, there was perhaps a possibility that this drug could have perturbed the metabolic pathways through HMG-CoA. For example, it may have caused an over-expression of HMG-CoA reductase in response to the decline in the normal sterol (i.e. ergosta-5,7,24(241)-trien-3β-ol). This could have resulted in HMG-CoA being rapidly reduced before it could be cleaved by HMG-CoA lyase to acetyl-CoA and acetate. To check this point, promastigotes were cultured with [2-13C]leucine in the absence of ketoconazole. GC-MS analysis of the major sterols as their TMS ether derivatives showed that ergosta-5,7,24(241)-trien-3β-ol had a molecular ion cluster (m/z 469–475), indicating species of the sterol molecule containing from one up to a maximum of six $^{13}$C atoms (Table II) with a distribution of molecular species fairly similar to that found previously for 14α-methylergosta-8,24(241)-dien-3β-ol. The various fragment ion clusters were also consistent with the presence of labeled species containing $^{13}$C in the positions predicted from the labeling pattern determined for 14α-methylergosta-8,24(241)-dien-3β-ol. The mass spectra of the ergosta-5,7,24(241)-trien-3β-ol TMS ether labeled from either [2-13C]acetate or [1-13C]glucose contained the molecular ion at m/z 468.

SCHEME 2.

The $^{13}$C labeling patterns predicted in 14α-methylergosta-8,24(241)-dien-3β-ol produced by L. mexicana promastigotes from [2-13C]leucine if the biosynthetic pathway proceeds either indirectly from HMG-CoA to acetyl-CoA with subsequent regeneration of HMG-CoA from the acetyl-CoA and conversion to mevalonic acid (MVA) (a) or directly by reduction of HMG-CoA to MVA without the intermediary of acetyl-CoA (b).

[2-13C] Leucine

Indirect route (HMG CoA converted to MVA via acetyl CoA)
from the preexisting sterol pool in the amastigotes produced prior to exposure to [2-13C]leucine. In the 48-h incubation used for this experiment, the amastigotes will have undergone about two cell divisions; therefore, the amount of newly synthesized sterol labeled with 13C must be insufficient to dilute the unlabeled sterol to the extent seen with the promastigotes (Table II). Thus, it can be concluded that the promastigote and amastigote forms of the parasite both utilize leucine as a main carbon source for sterol biosynthesis that proceeds by the direct route.

Effects of an Inhibitor of HMG-CoA Synthase—An inhibitor of HMG-CoA synthase should block [1-14C]acetate incorporation into isoprenoids but have no effect on the direct incorporation of [U-14C]leucine into sterol (Scheme 1). Accordingly, [U-14C]leucine and [1-14C]acetate were incubated separately with L. mexicana promastigotes in the presence and absence of the compound L-659,699, a fungal metabolite, which is a competitive inhibitor of HMG-CoA synthase (33, 34). An analysis of the labeled lipid by TLC and radioscanning showed that after the incorporation of [1-14C]acetate, about 2–3% of the radioactivity was in sterol, with the remainder distributed between triacylglycerol (35%) and phospholipid (60%) as observed previously (12). However, incubation with [1-14C]acetate in the presence of L-659,699 (20 μg/ml) resulted in the triacylglycerol and phospholipid remaining labeled in about the same proportions as in the control, but there was a complete abolition of label from the sterol that was consistent with the inhibition of HMG-CoA synthase. The results obtained with the [U-14C]leucine incubations are presented in Table III and show that the presence of L-659,699 had no apparent inhibitory effect on the incorporation of radioactivity into total lipid and sterol or the distribution of radioactivity between the labeled products even at the highest concentration of L-659,699 (50 μg/ml). These results are consistent with the view that in L. mexicana the incorporation of leucine does not require breakdown to the acetyl-CoA level as an essential step.

When cells were cultured with [2-13C]leucine and increasing concentrations of the HMG-CoA synthase inhibitor L-659,699 followed by GC-MS analysis of the ergosta-5,7,24(24)-trien-3β-ol, the results provided evidence for the dual sources of carbon from either leucine metabolism or acetyl-CoA to fuel the isoprenoid pathway. In the absence of the inhibitor, the predominant molecular species of the sterol TMS ether (M+ at m/z 473) had five 13C atoms rather than six, indicating a contribution from unlabeled precursors probably via the acetyl-CoA

| L-659,699 concentration | 0 μg/ml | 10 μg/ml | 20 μg/ml | 50 μg/ml |
|-------------------------|---------|----------|----------|----------|
| dpm/10⁶ cells in total lipid | 2000 | 2180 | 1960 | 2300 |
| dpm/10⁶ cells in sterol | 1210 | 1300 | 1140 | 1400 |
| Distribution (%) of label in | | | | |
| Phospholipid | 15.8 | 19.0 | 20.3 | 16.3 |
| Sterol | 60.5 | 59.7 | 58.5 | 60.9 |
| Triacylglycerol | 5.4 | 5.7 | 4.2 | 5.8 |
| Steryl ester | 14.5 | 12.6 | 14.3 | 14.3 |
| Squalene | 3.8 | 2.9 | 2.7 | 2.6 |
| Cell number at end of incubation (×10⁶ cells/ml) | 14.1 | 12.5 | 14.1 | 12.7 |

TABLE III

**The effect of L-659,699 on the incorporation of [U-14C]leucine into the lipids of L. mexicana promastigotes**

Cultures (5 ml) of log phase cells (48-h growth, 8.4 × 10⁶ cells/ml) were incubated with [U-14C]leucine (1 μCi) in the presence or absence of L-659,699 at the concentrations shown. The cells were harvested after a further 24 h, and the total lipid was extracted and analyzed by TLC with radioscanning.
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**TABLE IV**
The effect of lovastatin on the incorporation of [U-14C]leucine into the sterols and other lipids of L. mexicana promastigotes and the sterol composition of the protozoa

In experiment (Expt.) 1, cultures (10 ml, 10.2 × 10^6 cells/ml) at midlog phase were incubated for 2 h with lovastatin at the concentrations shown. ([U-14C]leucine (2 μCi) was then added, and the cells were harvested after a further 22-h growth. The lipids were extracted, and the distribution of radioactivity in the component lipids was determined by separation using TLC as described under “Materials and Methods.” In experiment 2, cells were cultured as for experiment 1. The sterols were recovered from the lipid by preparative TLC and analyzed as their TMS ether derivatives by GC and GC-MS. tr indicates trace amount (<0.5%).

| Expt. 1 | Lovastatin concentration |
|---------|-------------------------|
|         | 0 (Control) | 5 μg/ml | 10 μg/ml | 50 μg/ml |
| Final cell density (cells × 10^6/ml) | 24.0 | 23.0 | 22.9 | 11.2 |
| dpm/10^6 cells in total lipid | 1210 | 910 | 690 | 150 |
| dpm/10^6 cells in sterol | 940 | 680 | 550 | 84 |
| Distribution (%) of radioactivity in | |
| Phospholipid | 7 | 13 | 14 | 33 |
| Sterol | 78 | 75 | 80 | 55 |
| Triacylglycerol | 6 | 8 | 6 | 7 |
| Steryl ester | 7 | 4 | tr | 0 |
| Squalene | 3 | 0 | 0 | 5 |

| Expt. 2 | Lovastatin concentration |
|---------|-------------------------|
|         | 0 (Control) | 5 μg/ml | 10 μg/ml | 25 μg/ml | 50 μg/ml |
| Final cell density | 14.0 | 16.2 | 16.4 | 13.3 | 7.3 |
| dpm/10^6 cells in sterol | 550 | 260 | 120 | 160 | 0 |
| Total sterol (ng/10^6 cells) | 190 | 140 | 120 | 90 | 30 |
| Sterol composition (%) | |
| Cholesterol | 12 | 15 | 20 | 24 | 59 |
| Ergosta-7,22-trien-3β-ol | 4 | 5 | 6 | 6 | tr |
| Ergosta-5,7,24(241)-trien-3β-ol | 72 | 64 | 57 | 48 | 41 |
| Ergosta-7,24(241)-dien-3β-ol | 3 | 5 | 4 | 3 | tr |
| Stigmastera-5,7,24(241)-trien-3β-ol | 10 | 10 | 13 | 19 | tr |

At an L-659,699 concentration of 1 μg/ml, the [M^+] ion at m/z 474 (containing six atoms of 13C) showed a small enhancement, while at 10 and 20 μg/ml concentration of inhibitor, the m/z 474 ion was the major one. This increase by 1 mass unit in response to inhibitor treatment was also seen in the main fragmentation ions, e.g. [M – TMSOH] at m/z 383 (no inhibitor) increased to 384 (plus inhibitor), [M – TMSOH-Me] at m/z 385 up to 386, [M + H] at m/z 41 to 42, and [M – side chain-ring D-TMSOH] at m/z 214 up to 215. These results can be explained by the inhibition of the HMG-CoA synthase, resulting in no contribution from the acetyl-CoA pool in the cell and all of the sterol then being derived from 13C-labeled leucine. Moreover, they are also consistent with unlabeled carbon introduced in the absence of HMG-CoA synthase inhibitor being largely derived from acetyl-CoA obtained from a carbohydrate, fatty acid, or ketogenic amino acid source rather than directly from unlabeled leucine of protein origin.

**Effects of Lovastatin, an Inhibitor of HMG-CoA Reductase**—
The effects of an HMG-CoA reductase inhibitor on the incorporation of leucine into sterol were tested using lovastatin, and the results are presented in Table IV. Lovastatin had little or no inhibitory effect on growth of the cultures at concentrations up to 5 and 10 μg/ml, but growth retardation became apparent as theLovastatin concentration was increased to 25 and 50 μg/ml. Lovastatin at 5 μg/ml caused an inhibition of incorporation of [U-14C]leucine into the total lipid and sterol. The extent of inhibition became progressively greater as the lovastatin concentration was increased to 50 μg/ml. This provided good evidence for either the intermediacy of HMG-CoA and the action of HMG-CoA reductase in the pathway for or the operation of a similar type of reductive reaction to that catalyzed by HMG-CoA reductase, which is sensitive to lovastatin inhibition.

Analysis of the sterols recovered from the incubations with lovastatin showed that there was a decline in the amount of sterol in the cells, which paralleled the decline in [U-14C]leucine incorporation. The decline in sterol concentration was accompanied by a change in the composition of the sterol mixture with a progressive increase in the proportion of cholesterol (cholesterol-5-en-3β-ol) and corresponding drop in the amount of ergosta-5,7,24(241)-trien-3β-ol. Cholesterol is not synthesized by the parasite but is derived from the fetal calf serum of the culture medium (12, 13), whereas the Δ^5^-sterols with a C-24 substituent in the side chain are produced by de novo biosynthesis in the protozoa (4). It was noticeable that in these experiments the proportion of stigmastera-5,7,24(241)-trien-3β-ol was observed to increase up to a concentration of 25 μg/ml lovastatin. This could be accounted for by the utilization of the 24-methylenesterol as substrate by the second C-24transmethylase; the 24-methylenesterols were then not being replaced because of the block in sterol production imposed by the lovastatin. Leishmania sp. may have a growth requirement for a sterol with the structural features of the endogenous sterols (i.e. a Δ^5^-ring system and a C-24 methylene, methyl, or ethylenide side chain) or alternatively perhaps only newly synthesized sterol can play some important role in sustaining cell growth (27). However, the inhibition of HMG-CoA reductase by lovastatin could also inhibit the production of other isoprenoid-derived compounds (e.g. dolichols, prenylated proteins, ubiquinone side chain). This could lead to growth inhibition by starvation of the cell of other vital compounds (e.g. dolichols, prenylated proteins) in addition to the sterols.

The addition of excess MVA to the L. mexicana culture was tested to determine the effect on the incorporation of [U-14C]leucine into sterol and on culture growth. The incorporation of MVA into sterols by Leishmania species has been demonstrated previously (4, 5, 11–13). Cultures (10 ml, 2 × 10^6 cells/ml) were incubated with [U-14C]leucine (2 μCi) in the presence or absence of MVA (1 mg/ml). The cultures were stopped after 72 h, the sterols were extracted, and the radioactivity was determined. The addition of MVA had a marked effect and significantly reduced (p = 0.005) the radioactivity incorporated from [U-14C]leucine into the sterol by about 30% (control, 370 (S.D. = 33.4) dpm/10^6 cells (n = 4); plus MVA, 220 (S.D. = 37.2) dpm/10^6 cells (n = 3)).
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from leucine metabolism. Moreover, the growth inhibition of the cells by lovastatin was reversed by the exogenous MVA (Fig. 3), showing that it could enter the isoprenoid pathway to provide the requirements of the cell for sterols and/or other essential isoprenoid-derived compounds.

**DISCUSSION**

The incorporation of leucine into cholesterol by animal tissues has been demonstrated (14–18), and it was reported that in rats this proceeded with the prior breakdown of the leucine to acetate (14). Likewise, the incorporation of leucine into a plant sterol was also shown to require the catabolism of the leucine to acetyl-CoA before being incorporated into the isoprenoid pathway (19). By contrast, we have now established unequivocally by MS and 13C NMR methods that the trypanosomatid L. mexicana can incorporate the leucine skeleton intact into the isoprenoid pathway for sterol production without breakdown first to acetyl-CoA. This could occur by a pathway leading to HMG-CoA, and the HMG-CoA could then be directly reduced to MVA by HMG-CoA reductase (Scheme 1). The fact that some label from [U-14C]leucine appears in the fatty acids of triacylglycerols and phospholipids (Tables III and IV) (12) is best explained by the formation of intermediary HMG-CoA, which can yield labeled acetyl-CoA by the action of HMG-CoA lyase. A mitochondrial HMG-CoA reductase has recently been characterized in *Leishmania* and *Trypanosoma* species (35, 36). The existence of this enzyme could provide the opportunity for a portion of any HMG-CoA produced from leucine metabolism to be reduced to MVA and thus channeled directly into the isoprenoid pathway for sterol biosynthesis. The operation of pathways from leucine and acetyl-CoA, which merge at HMG-CoA, followed by reduction to MVA, is consistent with the following observations. (i) Inhibition of HMG-CoA synthase does not lower leucine incorporation into sterol (Table I). (ii) Lovastatin inhibits the incorporation of leucine (Table IV) into sterol. (iii) Labeled MVA is incorporated into *Leishmania* sterol (4). (iv) Excess unlabeled MVA added to the culture lowers the incorporation of [2,14C]leucine into sterol. (v) Added MVA overcomes the inhibitory effect of lovastatin on *Leishmania* growth (Fig. 3). However, there is an alternative route for the direct incorporation of leucine into sterol that would also be compatible with some of the above criteria. This requires the reduction of dimethylcrotonyl-CoA to dimethylallyl alcohol, followed by phosphorylation to yield dimethylallyl diphosphate, which is a constituent of the isoprenoid pathway (Scheme 1) and is interconvertible with isopentenyl diphosphate by an isomerase-catalyzed reaction. This route would effectively be a reversal of the mevalonate shunt that has been demonstrated in some animal tissues (24, 37, 38). The reduction of dimethylcrotonyl-CoA to dimethylallyl alcohol would be mechanistically similar to the conversion of HMG-CoA to MVA and could perhaps be catalyzed by the HMG-CoA reductase or a very similar enzyme that may also be susceptible to lovastatin inhibition. If leucine carbon is being channeled along this route, the lowered incorporation of [2-14C]leucine by added unlabeled MVA could be explained in two ways. Either the conversion of MVA into an appreciable unlabeled pool of dimethylallyl diphosphate/isopentenyl diphosphate results in dilution of the leucine-derived radioactive dimethylallyl diphosphate/isopentenyl diphosphate or the MVA is converted into excess sterol that may inhibit leucine utilization by a feedback inhibition mechanism.

The utilization of the intact leucine skeleton for sterol production may make an important contribution to the metabolic economy of the *Leishmania* cell. The use of leucine could spare the need for acetyl-CoA produced from glucose or fatty acid catabolism, which would therefore remain available for energy production or other biosynthetic reactions. However, amino acids derived from the breakdown of exogenous proteins are recognized as important energy and carbon sources in trypanosomatids (39–43). Leucine and other amino acids (glutamate, proline) are reported to be taken up readily from the growth medium by *Leishmania* sp. and *T. cruzi* and catabolized to provide acetyl CoA or other metabolites that can be oxidized to provide energy (39–43). Our work has shown that several *Leishmania* species, *Trypanosoma cruzi*, and *Endotrypanum monterogrrei* can all utilize leucine as a carbon source not only for sterol production but also for fatty acid biosynthesis (12, 13). Part of the HMG-CoA produced from leucine could be channeled into breakdown by HMG-CoA lyase (Scheme 1) to produce the acetyl-CoA needed for the synthesis of fatty acids. Additionally, this route of leucine catabolism could provide some acetyl-CoA for oxidation in the tricarboxylic acid cycle (41, 43, 44) or to support the mitochondrial acetate-succinate CoA transferase cycle for the generation of ATP and acetate (45). We have demonstrated that the metabolism of [U-13C]leucine by *L. mexicana* promastigotes produces 13CO2.2 Part of this 13CO2 will arise from the decarboxylation of the labeled leucine (Scheme 1) and by C-4 demethylation of a labeled sterol intermediate (4, 32), but some could arise from the oxidation of acetyl-CoA generated from the leucine (43, 44). Clearly, there must be coordinated regulation of the metabolism of amino acids, glucose, and fatty acids to maintain the balance of acetyl-CoA needed for cell metabolism under conditions of varying availability of these substrates to the promastigote or amastigote forms of the *Leishmania* parasite.

The key position of HMG-CoA in the catabolism of leucine and in the production of isoprenoids poses questions regarding the cellular compartmentation and regulation of the pathways and enzymes involved in HMG-CoA metabolism. In animals and plants, leucine breakdown is a mitochondrial event (24, 25). It has been reported that leucine aminotransferase and α-ketoisocaprate dehydrogenase are present in cytosolic and mitochondrial preparations from *T. cruzi* (46). Similarly, we have found that leucine aminotransferase is located in the mitochondrion of *L. adleri*.2 These facts suggest that leucine

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2 M. L. Ginger, M. L. Chance, and L. J. Goad, unpublished observations.
3 B. Smythe, A. Jones, M. L. Chance, and L. J. Goad, unpublished results.
catabolism to produce HMG-CoA could be located in the mitochondrial or trypanosomatid. HMG-CoA lyase is a mitochondrial enzyme in the mammalian liver cell (47), and there is an HMG-CoA synthase also located in the mitochondrion of mammalian cells (47), but these enzymes have not yet been studied in trypanosomatids. The remaining enzyme of HMG-CoA metabolism, HMG-CoA reductase, is associated mainly with the endoplasmic reticulum in mammalian cells. The HMG-CoA reductase of trypanosomatids has been studied in Trypanosoma brucei (48), T. cruzi (49, 50), and Leishmania major (35, 51) and variously described as either a microsomal, a glycosomal, or a soluble enzyme. However, recent investigations have now revealed that the HMG-CoA reductase of T. cruzi and L. major (51) and T. brucei (36) are predominantly located in the mitochondrion. Thus, the mitochondrion may be perhaps a major cellular site for the first stages in the production of isoprenoids in trypanosomatids. Certainly, a mitochondrial location of HMG-CoA reductase could provide for an efficient integration of the isoprenoid pathway with a mitochondrial leucine degradation sequence of reactions and thus facilitate the efficient utilization of leucine carbon for sterol synthesis.

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REFERENCES

1. Croft, S. L., Urbina, J. A., and Brun, R. (1997) in Trypanosomiasis and Leishmaniasis Biology and Control (Hide, G., Mottram, J. C., Coombs, G. H., and Holmes, P. H., eds) pp. 245–258, CAB International, Wallingford, UK
2. Van den Bossche, H. (1993) Microbiol. Eur. Nov./Dec. 20–28
3. Chance, M. L., and Goad, L. J. (1997) in Trypanosomiasis and Leishmaniasis Biology and Control (Hide, G., Mottram, J. C., Coombs, G. H., and Holmes, P. H., eds) pp. 163–176, CAB International, Wallingford, UK
4. Haughan, P. A., and Goad, L. J. (1991) in Biochemical Protozoology (Coombs, G. H., and North, M. J., eds) pp. 312–328, Taylor and Francis, London
5. Berman, J. D., Holz, G. G., Jr., and Beach, D. H. (1984) Mol. Biochem. Parasitol. 12, 1–13
6. Goad, L. J., Holz, G. G., Jr., and Beach, D. H. (1985) Mol. Biochem. Parasitol. 15, 257–279
7. Urbina, J. A., Payares, G., Molina, J., Sanoja, C., Liendo, A., Lazardi, K., Piras, M. M., Piras, R., Perez, N., and Blanco, A. (1988) in Sterol Biosynthesis Inhibitors: Pharmaceutical and Agrochemical Aspects (Berg, D. and Plemp, M., eds) pp. 79–119, Ellis Harwood Ltd., Chichester, UK
8. Rosenthal, J., Angel, A., and Farkas, J. (1974) J. Protozool. 21, 185–193
9. Domenech, C. E., Giordano, W., Avalos, J., and Cerda-Olmedo, E. (1996) Eur. J. Biochem. 239, 720–725
10. Urbina, J. A., Lazardi, K., Marchan, E., Visbal, G., Aguirre, T., Piras, M. M., and Ryley, J. F. (1996) J. Chem. Soc. Perkin Trans. 1, 249, 66–71
11. Blum, J. J. (1991) J. Protozool. 38, 527–531
12. Blum, J. J. (1994) J. Biol. Chem. 269, 2555–2566
13. Bloch, K. (1944) J. Biol. Chem. 155, 255–263
14. Wittcoff, R. H., and Agar, S. R. (1977) J. Biol. Chem. 252, 1012–1018
15. Hida, A., Uehijima, Y., and Seyama, Y. (1998) J. Biochem. (Tokyo) 124, 648–653
16. Haughan, P. A., Chance, M. L., and Goad, L. J. (1995) Biochem. J. 308, 31–38
17. Suga, T., Tange, K., Ichiko, K., and Hirata, T. (1988) Phytochemistry 19, 67–70
18. Chichester, C. O., Yokoyama, H., Nakayama, T. O. M., Lacton, A., and Mackinney, G. J. (1995) J. Biol. Chem. 270, 3922–3926
19. Frohlich, T. B., and Bei, R. (1987) J. Biol. Chem. 262, 2555–2566
20. Pena-Diaz, J., Montalvetti, A., Camacho, A., Gallego, C., Ruiz-Perez, L. M., and Gonzalez-Pacanowska, D. (1997) Biochem. J. 324, 397–405
21. Goad, L. J., and Akihisa, T. (1997) Mol. Biochem. Parasitol. 85, 219–226
22. Weinstock, S. B., Kopito, R. R., Endemann, G., Tomera, J. F., Marinier, E., Murray, D. M., and Brunengraber, H. (1994) J. Biol. Chem. 269, 8939–8944
23. Anderson, M. D., Che, P., Song, J., Nicolau, B. J., and Wurtele, E. V. (1998) Plant Physiol. 118, 1127–1138
24. Blum, J. J. (1994) J. Biol. Chem. 269, 2555–2566
25. Goad, L. J., Holz, G. G., Jr., and Beach, D. H. (1984) Mol. Biochem. Parasitol. 12, 1–13
26. Blum, J. J. (1999) Biochem. J. 338, 569–582
27. Pena-Diaz, J., Montalvetti, A., Camacho, A., Gallego, C., Ruiz-Perez, L. M., and Gonzalez-Pacanowska, D. (2000) Biochem. Parasitol. 107, 110–117
28. Montalvetti, A., Perez, N., Wincker, P., and Ryley, J. F. (1996) Eur. J. Biochem. 239, 441–443
29. Heath, M., and Opperdoes, F. R. (2000) J. Protozool. 47, 193–199
30. Blum, J. J. (1993) Parasitol. Today 9, 118–122
31. Blum, J. J. (1994) J. Bioenerg. Biomembr. 26, 147–155
32. Urbina, J. A. (1994) Parasitol. Today 10, 107–110
33. Blum, J. J. (1991) J. Protozool. 38, 527–531
34. Tielens, A. G. M., and Van Hellemont, J. J. (1998) Parasitol. Today 14, 265–271
35. Van Hellemont, J. J., Opperdoes, F. R., and Tielens, A. G. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2038–2041
36. Montamat, E. E., Arauzo, S. S., and Blanco, A. (1987) J. Mol. Biochem. Parasitol. 22, 165–183
37. Hegardt, F. G. (1999) Biochem. J. 338, 29–40
38. Pena-Diaz, J., Montalvetti, A., Camacho, A., Gallego, C., Ruiz-Perez, L. M. & Gonzalez-Pacanowska, D. (1997) J. Biol. Chem. 272, 619–626
39. González-Pacanowska, D., Pena-Diez, J., Montalvetti, A., Hurtado-Garcia, R., and Ruiz-Perez, L. M. [1999] Mem. Inst. Oswaldo Cruz 94, Suppl. II, 18