Elucidation of carbon sources used for the biosynthesis of fatty acids and sterols in the trypanosomatid *Leishmania mexicana*

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Sterols are necessary for the growth of trypanosomatid protozoans; sterol biosynthesis is a potential target for the use and development of drugs to treat the diseases caused by these organisms. This study has used ¹⁴C-labelled substrates to investigate the carbon sources utilized by promastigotes and amastigotes of *Leishmania mexicana* for the production of sterol [mainly ergosta-5,7,24(24¹)-trien-3 β -ol] and the fatty acid moieties of the triacylglycerol (TAG) and phospholipid (PL) of the organism. The isoprenoid precursor mevalonic acid (MVA) was incorporated into the sterols, and the sterol precursor squalene, by the promastigotes of *L. mexicana*. However, acetate (the precursor to MVA in most organisms) was a very poor substrate for sterol production but was readily incorporated into the fatty acids of TAG and PL. Other substrates (glucose, palmitic acid, alanine, serine and isoleucine), which are

metabolized to acetyl-CoA, were also very poor precursors to sterol but were incorporated into TAG and PL and gave labelling patterns of the lipids similar to those of acetate. In contrast, the amino acid leucine was the only substrate to be incorporated efficiently into the squalene and sterol of *L. mexicana* promastigotes. Quantitative measurements revealed that at least 70–80% of the sterol synthesized by the promastigotes of *L. mexicana* is produced from carbon provided by leucine metabolism. Studies with the amastigote form of *L. mexicana* showed that in this case leucine was again the major sterol precursor, whereas acetate was utilized for fatty acid production.

Key words: leucine metabolism, lipid biosynthesis, parasitic Protozoa.

INTRODUCTION

The leishmaniases are endemic throughout many tropical and subtropical regions. The causative agents are pathogenic protozoans of the genus *Leishmania* (family Trypanosomatidae; order Kinetoplastida). The *Leishmania* parasite replicates as a flagellated promastigote in the extracellular environment of the digestive tract of infected female sandflies but divides as a non-motile intracellular amastigote within the macrophages of a mammalian host. Chemotherapy at present relies on drugs introduced many years ago [1] and there is a requirement for new, more effective drugs that are less toxic to the patient. The need to identify new potential chemotherapeutic targets for the treatment of leishmaniasis means that a greater understanding of intermediary metabolism in *Leishmania* species is desirable.

Previous investigations on the intermediary metabolism of trypanosomatid protozoans established that many of the glycolytic enzymes are uniquely localized within the matrix of a peroxisome-like microbody called the glycosome [2,3]. However, in trypanosomatids glucose is not always metabolized completely to yield CO₂: products of glucose metabolism such as acetate, alanine, glycerol, L-lactate and succinate are produced as end products and excreted by the cells [4–6]. A 10-fold increase in the β -oxidation of fatty acids and a low rate of glycolysis in axenic *L. mexicana* amastigotes, in comparison with cultured promastigotes, suggests that fatty acid metabolism provides an important source of energy generation for amastigotes [7]. The parasites can acquire a supply of amino acids by protease hydrolysis [8] of the proteins obtained from the host and it is apparent that amino acids have a major role in the intermediary metabolism and energy production of these protozoans. For example alanine, which has a role as an osmolyte [5], is metabolized to yield acetate

[9]; further oxidation to CO₂ indicates it to be an important energy source [5,10]. Other amino acids metabolized by trypanosomatids include proline [11] and leucine [11,12].

Fatty acids are required in the making of phospholipids for membrane assembly; detailed studies of the fatty acyl compositions of *Leishmania* lipids have been reported [13]. Sterols are also structural components in cell membranes, in which they interact with the fatty acyl moieties of phospholipids to regulate membrane fluidity. However, it has been suggested that other important metabolic roles exist for sterols in some organisms [13]. *Leishmania* species biosynthesize a variety of C₂₈ ergostatrienols and ergostadienols, plus smaller amounts of sterols with the C₂₉ stigmasterane skeleton [13]. The promastigotes of *Leishmania* sp. and their lesion amastigotes also contain a variable amount of cholesterol that is not a product of biosynthesis *de novo*. Lesion amastigotes obtain cholesterol directly from the mammalian host, whereas cultured cells can obtain cholesterol from lipoproteins present in the foetal calf serum that is added as a supplement to the culture medium. In the related organisms *Trypanosoma brucei* and *T. cruzi* the accumulation of low-density-lipoprotein-associated cholesterol has been shown to occur after receptor-mediated endocytosis of the low-density lipoprotein particles [14,15]. However, the uptake and accumulation of cholesterol alone cannot satisfy promastigote and amastigote requirements for sterol: the synthesis *de novo* of C-24-alkylated $\Delta^{5,7}$ -sterols is required.

Exposure of *Leishmania* cultures to antifungal inhibitors of sterol biosynthesis (e.g. the allylamine terbinafine and azoles such as ketoconazole) results in the perturbation of sterol biosynthesis and cell death [16–19]. In clinical trials, cytochrome P450-dependent C-14 demethylase inhibitors, ketoconazole and itraconazole, have produced favourable results in patients suffering

Abbreviations used: FAMES, fatty-acid methyl esters; HIFCS, heat-inactivated foetal calf serum; HMG-CoA, hydroxymethylglutaryl-CoA; MVA, mevalonic acid; PL, phospholipid; TAG, triacylglycerol.

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from cutaneous leishmaniasis caused by *L. major*, *L. mexicana* and *L. panamensis* strains [17]. These results suggest that the inhibition of sterol biosynthesis is a suitable target for the treatment of leishmaniasis. The use of sterol biosynthesis inhibitors [17–19] has contributed to knowledge of the section of the biosynthetic pathway after lanosterol but less is known about the earlier reactions of sterol biosynthesis in *Leishmania*. The 'classic' biosynthetic route for the production of sterols is through the isoprenoid pathway with the intermediates hydroxymethylglutaryl-CoA (HMG-CoA) and mevalonic acid (MVA). Acetyl-CoA is generally regarded as the prime source of carbon supplying this 'classic' isoprenoid pathway in mammalian cells. However, an alternative route to isoprenoids, which does not involve HMG-CoA and MVA but instead uses glyceraldehyde phosphate and pyruvic acid to yield isopentenyl diphosphate, has recently been described in some bacteria and the chloroplasts of plants [20]. Investigations with *Leishmania* species have resulted in low incorporations of [^{14}C]acetate and [^{14}C]MVA into sterols [13,21–25] but the nature of the primary carbon source for sterol biosynthesis in these organisms has not been clarified. We present here the results of detailed radiolabelling studies to identify the preferred carbon sources for fatty acid and sterol biosynthesis in the promastigotes and amastigotes of the human pathogen *L. mexicana*.

MATERIALS AND METHODS

Materials

L. mexicana (strain MNYC/62/BZ/M379) was used in this study. [$1\text{-}^{14}\text{C}$]Acetate (57 mCi/mmol), [$\text{U-}^{14}\text{C}$]leucine (299–314 mCi/mmol), [$\text{U-}^{14}\text{C}$]alanine (159.1 mCi/mmol), [$\text{U-}^{14}\text{C}$]serine (168 mCi/mmol), [$\text{U-}^{14}\text{C}$]proline (205.5 mCi/mmol), [$\text{U-}^{14}\text{C}$]glucose (400 mCi/mmol) (all obtained from Amersham, Little Chalfont, Bucks., U.K.), [$\text{U-}^{14}\text{C}$]isoleucine (342 mCi/mmol) and [$\text{U-}^{14}\text{C}$]threonine (241 mCi/mmol) (both obtained from NEN, Boston, MA, U.S.A.) were all supplied in 2% (v/v) ethanol. (3*R*)-[2- ^{14}C]MVA (54 mCi/mmol; Amersham), and [$\text{U-}^{14}\text{C}$]palmitate (824 mCi/mmol; Amersham), were supplied in toluene, which is toxic to *Leishmania* cultures. The radiochemical solutions were therefore evaporated to dryness under a gentle stream of N_2 at room temperature to prevent loss of the volatile solute, then an equal volume of ethanol was added to redissolve the radiochemical before administration to cultures. Freeze-dried (3*R,S*)-[2- ^{14}C]MVA (50 μCi , 72 mCi/mmol; Amersham), was dissolved in 500 μl of ethanol and stored at -20°C for use as required.

Cell culture

Promastigote cultures (10 ml) were grown at 27°C from an initial cell density of 10^6 cells/ml in 'HOMEM' medium [26] supplemented to 10% (v/v) with heat-inactivated foetal calf serum (HIFCS). Axenic amastigotes were grown at 32°C in Schneider's *Drosophila* insect medium (Sigma-Aldrich, Poole, Dorset, U.K.), adjusted to pH 5.5 and supplemented to 20% (v/v) with HIFCS [27]. Amastigote stocks were maintained by the sub-passage of existing cultures at an initial cell density of 5×10^5 cells/ml every 7 days. After incubations in the presence of radiolabelled substrates, cultures were harvested by centrifugation at 1400 *g* for 10 min. The cell pellet was washed three times with PBS solution before storage at -20°C until required. Cell densities were determined with a Neubauer haemocytometer and by counting under the microscope.

Lipid extraction

Cell pellets were resuspended in 1 ml of PBS solution, transferred to a vial containing 10 ml of chloroform/methanol (2:1, v/v) and stirred in the dark for 3 h. The organic and aqueous layers were left to separate overnight at 4°C . The lower organic phase was removed, dried over anhydrous Na_2SO_4 , transferred to a fresh vial and evaporated to dryness at 60°C under a steady stream of N_2 . The extract was stored at -20°C until required.

TLC analysis of lipid extracts

The incorporation of radioactivity into different lipid classes was determined by analytical TLC with Keiselgel 60G TLC plates (Merck) and radio-TLC scanning (Rapid Intelligent Radio TLC Analyser; Raytest). Portions of the total lipid extracts (15000–30000 d.p.m.) were applied to plates as a 1 cm band in chloroform/methanol (2:1, v/v). The lipids were separated with (1) chloroform, (2) chloroform until the solvent front ran halfway up the TLC plate followed by redevelopment with cyclohexane/diethyl ether (96:4, v/v) or (3) chloroform/ethanol (95:5, v/v). Radiolabelled phospholipid (PL) fractions were separated with chloroform/methanol/acetic acid/water (137:20:20:6, by vol.). Radiolabelled PL, sterol and squalene fractions were isolated by preparative TLC on silica gel (Keiselgel 60G) 0.5 mm thick. Bands were located by spraying with 0.0005% (w/v) berberine in ethanol and viewing under UV (254 nm). Phospholipids were eluted from the plate with chloroform/methanol (2:1, v/v); sterols and squalene were eluted with diethyl ether. Radioassay of the recovered materials from TLC showed that recoveries were 80–90% of the radioactive material applied to the TLC plate.

Isolation and radio-HPLC analysis of free fatty acids

L. mexicana promastigotes were incubated with 15 μCi of [$1\text{-}^{14}\text{C}$]acetate (starting density of 10^6 cells/ml) for 72 h. In Expt. 1, 13.5% of the [^{14}C]acetate that was initially added was recovered in the lipids of the harvested promastigotes. After the addition of glycerol tripalmitate (250 μg) and myristic acid (250 μg) as carriers to a portion (1.12×10^6 d.p.m.) of the radioactive lipid it was heated at 70°C for 2 h with 5 ml of KOH (8%, w/v) in aqueous ethanol (80%, v/v). The solution was cooled and the neutral lipids (94700 d.p.m.) were extracted into light petroleum (boiling range $40\text{--}60^\circ\text{C}$). After acidification of the saponification mixture with 20% (v/v) H_2SO_4 , the free fatty acids (924000 d.p.m.) were partitioned into hexane. In Expt. 2, 10% of the radioactivity added was recovered in the promastigote lipid. A portion of this material (1110000 d.p.m.) was saponified to yield 96500 d.p.m. in the neutral lipid fraction and 915000 d.p.m. in the fatty acids and other saponifiable materials. The free fatty acids from each experiment (approx. 200000 d.p.m.) were converted into phenacyl ester derivatives [28] and analysed by reverse-phase HPLC with a Waters NovaPak C_{18} column (100 mm long $\times$ 8 mm internal diam., 4 μm particle size, 60 $\text{\AA}$ pore diameter). The elution programme employed a flow rate of 2 ml/min of acetonitrile/water (80:20, v/v) for 25 min, rising to acetonitrile/water (85:15, v/v) over the next 15 min, followed after 60 min by elution with 100% acetonitrile for a further 60 min. Fatty acid phenacyl esters were detected by UV absorbance at 254 nm; the radioactivity detector channel was set to detect ^{14}C .

GLC-MS analysis of sterols and fatty-acid methyl esters (FAMES)

Sterols were isolated, converted to trimethylsilyl ethers and analysed by GLC-MS [19]. Lipid extracts were transmethyated

to yield the FAMES by using 2.5% (v/v) H₂SO₄ in methanol (2.0 ml). Samples were heated for 2 h at 70 °C, then cooled to room temperature. The transmethylation mixture was neutralized by the addition of 5% (w/v) NaCl solution saturated with NaHCO₃; the FAMES were partitioned into hexane and then analysed with an HP-20m Carbowax capillary column (Hewlett Packard) (25 m × 0.32 mm × 0.3 µm film thickness). The temperature programme employed an initial temperature of 50 °C for 1 min, then rose at 50 °C/min to 120 °C. This temperature was held for 1 min before the column temperature was raised at a rate of 12 °C/min to 210 °C, which was maintained for 25 min. For all GLC-MS (Hewlett-Packard, model 5970/5890) analyses, helium was the carrier gas (12 lb/in²); the scan range was *m/z* 50–550, with a scan time of 2 s.

Characterization of [¹⁴C]squalene and [¹⁴C]sterol

Squalene (Sigma) carrier was added to the radioactive hydrocarbon fraction obtained by preparative TLC, to give an initial specific radioactivity of 129 d.p.m./µmol. The purification of squalene by thiourea adduct formation, followed by the recovery of squalene and the conversion of squalene to its hexachloride derivative, was then performed as described previously [29]. The [¹⁴C]sterol obtained by preparative TLC was mixed with authentic ergosta-5,7,22-trien-3β-ol (ergosterol; Sigma) to give an initial specific radioactivity of approx. 400 d.p.m./µmol; this mixture was acetylated at 70 °C for 2 h in 200 µl of acetic anhydride/pyridine (1:1, v/v). The sterol acetates were recovered, dissolved in 25 ml of ethyl acetate (HPLC grade), PtO₂ catalyst (7 mg of catalyst/50 mg of ergosterol acetate) was added and the mixture was stirred overnight at room temperature under H₂ gas. The platinum catalyst was removed by filtration and the hydrogenated sterol acetate was purified by column chromatography with alumina (grade III) eluted with light petroleum (boiling range 40–60 °C) followed by light petroleum/diethyl ether (97:3, v/v). The purity of the reaction product, 5α-ergost-8(14)-en-3β-yl acetate, was checked by GLC-MS and ¹H NMR spectroscopy before recrystallization to a constant specific radioactivity from methanol. MS *m/z* (relative intensities): 442 (100), 427 (28), 367 (15), 315 (15), 255 (20), 213 (25). ¹H NMR (440 MHz, chloroform-*d*): δ_H 0.83 (3H, *s*, H₃-18), 0.69 (3H, *s*, H₃-19), 0.93 (3H, *d*, H₃-21), 0.85 (3H, *d*, H₃-26), 0.78 (3H, *d*, H₃-27), 0.78 (3H, *d*, H₃-24¹), 2.00 (3H, *s*, H₃-acetyl).

Liquid-scintillation counting

Radioactivity was measured with a LKB Rackbeta scintillation counter linked to an Olivetti IBM24 computer; 50 µl aliquots of

[¹⁴C]lipids in diethyl ether were added to 5 ml of liquid-scintillation cocktail (0.05% 2,5-diphenyloxazole in toluene).

RESULTS

Sterols of *L. mexicana* cells

L. mexicana promastigotes grown in HOMEM supplemented with HIFCS were harvested at 72 h, when the cells were just entering the stationary phase of growth at a cell density of 2.4 × 10⁷ cells/ml. The sterols were identified by GLC-MS analysis; the results are shown in Table 1. As reported previously [21,30], the major sterol was ergosta-5,7,24(24¹)-trien-3β-ol, with smaller amounts of ergosta-7,24(24¹)-dien-3β-ol and stigmasta-5,7,24(24¹)-trien-3β-ol. These sterols were produced *de novo* by the parasite, whereas the cholesterol present in the cells is reported to be of exogenous origin [19] and to arise by uptake from the medium where it is supplied as a constituent in the foetal calf serum.

[1-¹⁴C]Acetate and [2-¹⁴C]mevalonate incorporation into lipids

The incorporations of acetate and mevalonate into the sterols of *L. mexicana* promastigotes were re-examined in view of the low incorporations noted previously [21–25]. The incorporation of [2-¹⁴C]mevalonate (0.14%) into total lipid was comparatively

Table 2 Incorporation of (3*R*)-[2-¹⁴C]mevalonic acid and [1-¹⁴C]acetate into the lipids of *L. mexicana* promastigotes

(3*R*)-[2-¹⁴C]Mevalonic acid or [1-¹⁴C]acetate were added to exponential-phase cultures of *L. mexicana* promastigotes grown in HOMEM [10% (v/v) HIFCS]. The cell density of each 10 ml culture when the radioisotopes were added was 12 × 10⁶ cells/ml. Cultures were harvested 24 h later, at which time the cell density was 17.1 × 10⁶ cells/ml for the [2-¹⁴C]mevalonic acid culture and 17.4 × 10⁶ cells/ml for the [1-¹⁴C]acetate culture. The lipids were extracted and separated by analytical TLC as described in the Materials and methods section. The percentage distribution of ¹⁴C in the lipids was determined by radio-TLC scanning.

	[2- ¹⁴ C]Mevalonic acid	[1- ¹⁴ C]Acetate
Radioactivity added to culture (µCi)	2.6	10.0
Radioactivity incorporated into lipids (d.p.m.)	7.8 × 10 ³	1.31 × 10 ⁶
Incorporation into lipids (%)	0.14	5.9
Distribution of ¹⁴ C in lipids (%):		
Squalene	82.5	0
Steryl ester	0	3
TAG	0	52
Sterol	17.5	2
PL	0	43

Table 1 Sterol composition of *L. mexicana* promastigotes

Sterol identifications: 1, cholesterol; 2, unknown C₂₇ diene; 3, ergosterol (ergosta-5,7,22-trien-3β-ol); 4, ergosta-5,7,24(24¹)-trien-3β-ol; 5, ergosta-7,24(24¹)-dien-3β-ol; 6, [24(24¹)]-stigmasta-5,7,24(24¹)-trien-3β-ol; 7, [24(24¹)]-stigmasta-5,7,24(24¹)-trien-3β-ol. Values for the percentage composition and relative intensities of the ions in the mass spectra are typical of several experiments. Relative retention times (RRTs) are relative to cholesterol trimethylsilyl (TMS) ether on a 25 m HP1 GLC column (internal diam. 0.2 mm; film thickness 0.33 µm). Abbreviation: tr., trace.

Sterol	Composition (%)	GLC RRT of sterol TMS ether	Diagnostic ions in the mass spectrum: <i>m/z</i> (relative intensities)
1	8.6	1.00	458 (10%), 443 (4), 368 (24), 353 (11), 329 (26), 255 (5), 213 (3), 129 (17)
2	tr.	1.03	456 (100%), 441 (75), 253 (50), 213 (75)
3	tr.	1.08	468 (25%), 363 (30%)
4	78.0	1.13	468 (10%), 453 (1), 378 (10), 363 (40), 337 (27), 253 (4), 211 (8)
5	5.7	1.14	470 (6%), 455 (10), 386 (14), 343 (54), 255 (8), 213 (8)
6	tr.	1.20	482 (12%), 467 (1), 392 (7), 377 (44), 351 (24), 253 (3), 211 (9)
7	7.6	1.24	482 (11%), 467 (1), 392 (9), 377 (47), 351 (18), 253 (5), 211 (9)

Figure 1 Radio-TLC scans of the lipids isolated either from promastigote cultures of *L. mexicana* incubated for 72 h in the presence of [1-¹⁴C]acetate (a) [U-¹⁴C]glucose (b) or [U-¹⁴C]leucine (c) or from amastigote cultures incubated with [2-¹⁴C]MVA (d) or [1-¹⁴C]acetate (e)

In some incubations with [1-¹⁴C]acetate and [U-¹⁴C]glucose, such as those shown in (a) and (b), an unidentified labelled compound (U) was observed; however, in other incubations with these substrates this was not present. See the Materials and methods section for details of promastigote and amastigote culture, lipid extraction and radio-TLC procedures. PL were at the origin of the TLC plate. Abbreviations: ST, sterol; SE, steryl ester; SQ, squalene (at solvent front).

low (Table 2). TLC analysis established that the major labelled products were the sterols and squalene, which is the C₃₀ isoprenoid precursor to sterols. In contrast, although the [1-¹⁴C]acetate was considerably more efficiently incorporated (5–9 %) into lipids, only very low incorporation was observed into sterols and squalene (Table 2). The major labelled compounds chromatographed with triacylglycerol (TAG) and with polar material such as the phospholipids (Figure 1). The polar lipids were recovered by preparative TLC and subjected to further TLC examination with a solvent system that separated the PL types. Radioactivity was associated with phosphatidylserine (4.1 % of label), phosphatidylinositol (15.2 %), phosphatidylcholine (21.5 %), phosphatidylethanolamine (49.5 %) and

unidentified materials (9.7 %). The labelling observed in the TAG and PL fractions is consistent with the incorporation of the [1-¹⁴C]acetate into fatty acids and the subsequent esterification of the labelled fatty acids into TAG and PL.

Characterization of the ¹⁴C-labelled fatty acids

The identities of the TAG and PL fatty acid moieties were confirmed by GLC–MS analysis of the FAMES derived from the lipids of *L. mexicana*. The occurrence of C₁₈ compounds as the predominant fatty acids of the promastigotes of *L. mexicana* was in agreement with previous reports [13,25]. The labelling of the fatty acids of TAG and PL was established by saponification of

Table 3 Composition of the fatty acid moieties of TAG and PL of *L. mexicana* promastigotes and the incorporation of [1-¹⁴C]acetate into these fatty acids

The fatty acids were identified and quantified by GLC-MS of the FAMES derived from the *L. mexicana* lipids as described in the Materials and methods section. The distribution of radioactivity incorporated from [1-¹⁴C]acetate into the fatty acids of the lipids was determined by preparation of the phenacyl derivatives of the fatty acids and analysis by HPLC with radioactive monitoring (see the Materials and methods section for details). Fatty acids are identified in accordance with the nomenclature for chain length and degree of unsaturation. Abbreviation: n.d., not detected.

Fatty acid	Composition (%)	Distribution of radioactivity incorporated from [1- ¹⁴ C]acetate (%)	
		Experiment I	Experiment II
C _{14:0}	6.1	6.7	7.0
C _{16:0}	4.1	1.6	1.7
C _{16:1}	0.1	n.d.	n.d.
C _{18:0}	16.7	16.5	15.6
C _{18:1}	26.5	26.3	27.6
C _{18:2}	30.6	34.4	29.6
C _{18:3}	7.4	0.6	1.4
C ₂₀ acids	8.5	n.d.	n.d.
C _{22:6}	n.d.	2.4	6.5
Unidentified	—	11.5	11.6

a lipid extract obtained from *L. mexicana* after incubation for 72 h with [1-¹⁴C]acetate. Neutral lipids were first extracted from the saponification mixture by solvent partitioning (see the Materials and methods section) followed by acidification of the aqueous phase and re-extraction with solvent to obtain the free fatty acids. The recovered ¹⁴C-labelled fatty acids were converted to their phenacyl derivatives [28] and analysed by reverse-phase radio-HPLC to determine the distribution of ¹⁴C between the component fatty acids (Table 3). Radioactive peaks were observed that co-chromatographed with fatty acids in the chain length range C₁₄ to C₂₂; most of the radioactivity was associated with the C₁₆ and C₁₈ fatty acids (Table 3); linoleic acid (C_{18:2}) was the most heavily labelled. The labelling of the fatty acid moieties of TAG and PL with [1-¹⁴C]acetate was in accord with previous reports of fatty acid biosynthesis from labelled acetate by other *Leishmania* species [25,31]. It can therefore be concluded that fatty acids are produced in *L. mexicana* from acetyl-CoA, as in many other organisms.

Incorporation of ¹⁴C-labelled glucose, palmitic acid and amino acids into lipids

The very poor labelling of the sterols observed from [1-¹⁴C]acetate prompted us to investigate the nature of the principal carbon source utilized for isoprenoid and sterol production by *Leishmania* promastigotes. The cellular site(s) of the isoprenoid pathway in *Leishmania* or other trypanosomatids are not yet established. A key enzyme in the isoprenoid pathway, HMG-CoA reductase, was reported to be located in the endoplasmic reticulum of *Trypanosoma brucei* [32]. However, this enzyme was subsequently reported in *T. cruzi* to lack a membrane-spanning domain and to occur primarily in either the soluble fraction of *T. cruzi* [33] or the glycosomal fraction [34], which has analogy with its location in the peroxisomes of animal cells [35]. Recently the HMG-CoA reductase of *T. brucei* has been located as a predominantly mitochondrial matrix enzyme that is easily released into the soluble cell fraction when the mitochondria are ruptured during cell fractionation (N. Heise and F. R. Oppendoes, personal

communication). There are no reports on the distribution of this enzyme, or any other enzyme participating in the sterol pathway, in *Leishmania* species. The observations on the location of HMG-CoA reductase in trypanosomes suggest that the initial steps of the isoprenoid pathway might be compartmentalized in one or more cellular sites such as the glycosome or mitochondrion; this raises the possibility that exogenously supplied [1-¹⁴C]acetate might not reach the site of isoprenoid and sterol production. We wondered whether there might be various intracellular pools of acetyl-CoA and whether the acetyl-CoA required to supply the isoprenoid pathway might be generated *in situ* by substrate channelling from an appropriate precursor. Accordingly, *L. mexicana* promastigotes were incubated with several substrates that are potential suppliers of acetyl-CoA to determine whether any would act as an efficient carbon source for sterol and/or fatty acid production (Table 4).

[U-¹⁴C]Glucose is metabolized initially by the glycolytic pathway in the glycosome to yield 1,3-diphosphoglyceric acid, which can then be further metabolized in the cytosol and mitochondria to yield acetyl-CoA [2–6]. The fatty acid moieties of TAG and PL were efficiently labelled from [U-¹⁴C]glucose but, as with [1-¹⁴C]acetate, there was only a very low incorporation into sterols (less than 3%). This result also demonstrated that the recently discovered alternative bacterial and plant pathway to isoprenoids [20], which by-passes mevalonate, seems to be of little or no importance in the protozoan because this pathway utilizes glyceraldehyde phosphate and pyruvate, which are intermediates of the glycolytic pathway leading to acetyl-CoA.

[1-¹⁴C]Palmitic acid, which can be catabolized to produce [1-¹⁴C]acetyl-CoA by the β -oxidation route (a mitochondrial pathway in mammalian cells), gave high labelling of TAG and PL; however, no ¹⁴C label was incorporated into the sterols (Table 4). It was not determined to what extent the [1-¹⁴C]palmitic acid was incorporated intact into TAG or PL or after metabolism to [1-¹⁴C]acetyl-CoA for the resynthesis of new fatty acids. However, it was clear that palmitic acid did not supply a metabolite that could be utilized in the isoprenoid pathway and sterol synthesis.

Alanine, serine, threonine and isoleucine are ketogenic amino acids whose catabolism in mammalian liver generates acetyl-CoA, which can be utilized for the production of ketone bodies (acetoacetate or β -hydroxybutyrate) or the synthesis of fatty acids. In *L. mexicana* these ¹⁴C-labelled amino acids labelled the lipids in a similar pattern to that observed with [1-¹⁴C]acetate, [1-¹⁴C]palmitic acid and [U-¹⁴C]glucose, which was consistent with the catabolism of the amino acid skeletons to [1-¹⁴C]acetyl-CoA. However, there was again little incorporation into sterol. Two other amino acids, [U-¹⁴C]proline and [U-¹⁴C]valine, gave no incorporation into any of the lipids of *L. mexicana*. The former finding was unexpected because proline is reported to be a source of carbon and energy for some trypanosomes [6,11].

The only labelled carbon source that was efficiently incorporated into sterol was [U-¹⁴C]leucine. Typically, approx. 50% of the ¹⁴C label incorporated into the lipids was found in the sterols; the remainder of the label was distributed between the TAG, PL, steryl ester and squalene fractions (Figure 1). The extent of incorporation of [U-¹⁴C]leucine into TAG and PL was somewhat variable between experiments but the sterols were always heavily labelled. Some of the ¹⁴C-labelled material in the polar lipid fraction co-chromatographed on TLC with PL standards (phosphatidylcholine, phosphatidylethanolamine) but there were other unidentified ¹⁴C-labelled products. The incorporation of label from [U-¹⁴C]leucine into TAG and PL is compatible with some of the amino acid's being metabolized to [1-¹⁴C]acetyl-CoA; indeed, the oxidation of leucine to CO₂ [12] shows that this metabolic route for the catabolism of some

Table 4 Incorporation of ^{14}C -labelled substrates into the lipids of *L. mexicana* promastigotes

Cultures (10 ml) had an initial cell density of 2×10^6 cells/ml in HOMEM [10% (v/v) HIFCS]. After addition of the labelled substrate, the cells were cultured for a further 96 h before harvest, except for the alanine and serine incubations, which were each cultured for 24 h during exponential growth. By analogy with other organisms it is assumed, for the calculation of percentage incorporation, that only the (3*R*,5*S*)-isomer of the (3*R*,5*S*)-[2- ^{14}C]mevalonate mixture will be utilized by the isoprenoid pathway enzymes. The lipids were isolated and separated by TLC as described in the Materials and methods section. Abbreviations: Sq, squalene; SE, steryl ester.

Substrate	Radioactivity added (μCi)	Cell density at harvest (10^6 cells/ml)	[^{14}C] in lipid ($10^{-3} \times \text{d.p.m.}$)	Incorporation (%)	Distribution of ^{14}C in lipids (%)			
					Sq/SE	TAG	Sterol	PL
[1- ^{14}C]Acetate	10	18.7	1500	6.8	2.1	46.8	1.4	49.8
(3 <i>R</i> ,5 <i>S</i>)-[2- ^{14}C]Mevalonate	4	22.0	21	0.5	15.7	12.5	42.9	28.9
[U- ^{14}C]Glucose	8	15.5	194	1.1	2.4	43.8	3.0	50.8
[U- ^{14}C]Palmitate	5	21.0	11400	100	2.0	43.3	0	54.7
[U- ^{14}C]Alanine	2	17.0	11	0.2	24.2	28.6	0	47.3
[U- ^{14}C]Serine	2	18.3	82	7.5	0	11.3	1.7	87.0
[U- ^{14}C]Threonine	2	16.1	50	1.1	1.8	42.3	2.2	53.7
[U- ^{14}C]Isoleucine	2	19.3	37	0.8	3.4	51.5	7.7	37.4
[U- ^{14}C]Leucine	2	24.2	246	5.5	8.1	8.0	51.1	32.7

Table 5 Characterization of the ^{14}C -labelled squalene and sterol products obtained from the incubation of *L. mexicana* promastigotes with [U- ^{14}C]leucine

The hydrogenated [^{14}C]steryl acetate purified by preparative TLC was identified by GLC-MS and ^1H NMR spectroscopy as 5 α -ergost-8(14)-en-3 β -yl acetate. Abbreviation: n.d., not determined.

Squalene characterization step	Specific radioactivity (d.p.m./ μmol)		
[^{14}C]Squalene purified by preparative TLC	129		
[^{14}C]Squalene purified from thiourea adduct	101		
[^{14}C]Squalene hexahydrochloride:			
First recrystallization	96		
Second recrystallization	84		
Third recrystallization	85		
Fourth recrystallization	78		
Fifth recrystallization	75		
Sterol characterization step	Specific radioactivity (d.p.m./ μmol)		
	Expt. 1	Expt. 2	Expt. 3
^{14}C -Sterols purified by preparative TLC	n.d.	123	167
Acetylated ^{14}C -sterols	174	142	n.d.
Hydrogenated [^{14}C]steryl acetate	137	142	124
Hydrogenated [^{14}C]steryl acetate purified by preparative TLC	142	n.d.	120
Hydrogenated [^{14}C]steryl acetate:			
First recrystallization	105	107	125
Second recrystallization	103	83	109
Third recrystallization	112	95	118
Fourth recrystallization	n.d.	87	n.d.

leucine must operate. Incorporation of some of the [^{14}C]acetyl-CoA into fatty acids would then result in the labelling of TAG and PL. The utilization of [U- ^{14}C]leucine for sterol synthesis was also examined with promastigote cultures of different ages and it was established that leucine was efficiently used as a sterol precursor during both the exponential growth and stationary phases of culture. At all four stages examined (cultures 0–24, 24–48, 48–72 and 72–96 h old), the distribution of ^{14}C label in the lipid fraction was similar (squalene and steryl ester, 7–21%; TAG, 5–12%; sterol, 48–69%; PL, 14–20%; other compounds, 1–4%).

Characterization of the ^{14}C -labelled squalene and sterol

It was essential to show that [^{14}C]leucine was incorporated into sterol and the precursor, squalene, and was not present in some

other unidentified co-chromatographing labelled compound(s). The ^{14}C -labelled squalene was characterized by the formation of the squalene thiourea adduct followed by the formation of squalene hexahydrochloride and crystallization to constant specific radioactivity (Table 5). The characterization of the ^{14}C -labelled sterol presented a problem because the material isolated from the *Leishmania* cells consisted of several components (Table 1) and certainly also contained trace amounts of other labelled precursor sterols. Moreover, the major sterols are $\Delta^{5,7}$ -sterols, which are notoriously unstable and in small amounts readily undergo oxidation during manipulation and storage. We therefore adopted a strategy of sterol characterization that would generate a single more stable derivative from as many as possible of the sterols in the labelled mixture. This was achieved by the addition of carrier ergosterol to the ^{14}C -labelled sterol mixture and purification of the sterols by preparative TLC, followed by

Table 6 Incorporation of ^{14}C -labelled substrates into the lipids of *L. mexicana* axenic amastigotes

In Expt. 1, amastigote cultures (10 ml , 5×10^5 cells/ml) in Schneider's *Drosophila* medium were maintained at 32°C for 144 h when they were in the exponential-growth phase. The ^{14}C -labelled substrates were then added and the cultures were maintained for a further 48 h before being harvested. The lipids were extracted and analysed by the same methods as used for the study of promastigotes. In Expt. 2, lesion amastigotes were isolated (10^6 cells/ml, total volume 10 ml) and cultured axenically for 67 h (32.4×10^6 cells/ml); then $[1\text{-}^{14}\text{C}]\text{acetate}$, $[2\text{-}^{14}\text{C}]\text{mevalonate}$ or $[U\text{-}^{14}\text{C}]\text{leucine}$ was added and the cells were harvested after a further 48 h of culture (65.2×10^6 cells/ml) before isolation of the lipid. The percentage distribution of ^{14}C in the lipids was determined by radio-TLC scanning as described in the Materials and methods section. Abbreviations: Sq, squalene; SE, steryl ester.

Expt.	Substrate	Radioactivity added (μCi)	Incorporation (%)	Distribution of ^{14}C in lipids (%)			
				Sq/SE	TAG	Sterol	PL
1	$[1\text{-}^{14}\text{C}]\text{Acetate}$	10	8.8	6.8	9.4	2.5	81.3
	$(3R,S)\text{-}[2\text{-}^{14}\text{C}]\text{Mevalonate}$	4	1.1	28.8	0	60.7	10.5
	$[U\text{-}^{14}\text{C}]\text{Glucose}$	8	0.2	6.0	13.1	2.1	78.8
	$[U\text{-}^{14}\text{C}]\text{Palmitate}$	5	50	3.0	11.2	0	85.8
	$[U\text{-}^{14}\text{C}]\text{Leucine}$	2	0.8	24.8	0	63.3	11.9
2	$[1\text{-}^{14}\text{C}]\text{Acetate}$	5	11.6	2.8	14.3	4.7	77.2
	$(3R,S)\text{-}[2\text{-}^{14}\text{C}]\text{Mevalonate}$	4	0.8	16.4	0	59.9	17.2
	$[U\text{-}^{14}\text{C}]\text{Leucine}$	2	0.5	13.0	0	54.8	32.2

acetylation and hydrogenation to yield the common product $5\alpha\text{-ergost-8(14)-en-3}\beta\text{-yl acetate}$, which was then crystallized to constant specific radioactivity (Table 5). Clearly the individual identities of the component sterols were lost by this procedure but this was secondary to the importance of establishing with confidence that the protozoan was synthesizing sterols with the ergostane skeleton. The observation that both the labelled squalene hexahydrochloride and the ergost-8(14)-en-3 β -yl acetate could be crystallized to constant specific radioactivity demonstrated unequivocally that leucine does indeed serve as a carbon source for sterol production *de novo* in *L. mexicana*.

Estimation of the contribution of leucine to sterol production

Having established that leucine is readily incorporated into sterol, we wished to estimate how much of the total sterol in a promastigote culture was produced from leucine-derived carbon in comparison with that made from carbon from other sources that provide acetyl-CoA as the starting carbon source for the 'classic' cytosolic and peroxisomal isoprenoid pathway. To determine the proportion of sterol produced from leucine-derived carbon it was necessary to determine (1) the total amount of sterol produced by the cells from all carbon sources during the culture period and (2) the amount of sterol produced only from the $[U\text{-}^{14}\text{C}]\text{leucine}$.

(1) The total amount of sterol produced by the cells during the culture period was determined by quantitative GLC analysis of the isolated sterol, with $5\alpha\text{-cholestane}$ as the standard. The amount of sterol present in the initial inoculum of *L. mexicana* cells makes a minor contribution (less than 3%) to the amount of sterol recovered from the culture after 72 h of growth. The mean value obtained from four experiments was $280 \pm 0\text{ pmol}/10^6\text{ cells}$.

(2) The amount of sterol synthesized by the cells from $[U\text{-}^{14}\text{C}]\text{leucine}$ -derived carbon was calculated by using the expression:

$$\frac{\text{d.p.m. in sterol synthesized by } 10^6 \text{ cells}}{\text{specific radioactivity of } [U\text{-}^{14}\text{C}] \text{ leucine in medium}} \times \frac{36}{27} \times \frac{1}{6}$$

The specific radioactivity of the $[U\text{-}^{14}\text{C}]\text{leucine}$ (typically approx. $373\,000\text{ d.p.m.}/\mu\text{mol}$) available to the parasite for sterol synthesis was estimated from the amount of free unlabelled leucine present in the HOMEM; no allowance was made for any further dilution by leucine that might have been released by proteolysis from the

proteins in the HIFCS supplement, because this could not be quantified. It was assumed that the ^{14}C incorporated would be distributed evenly between the six isopentenyl units used to produce one molecule of sterol. The factor 1/6 is required because 6 mol of leucine are needed to produce 1 mol of sterol; the factor 36/27 makes a correction for the six carbon atoms lost in the decarboxylation of six molecules $\alpha\text{-oxoisohexanoate}$, which is an intermediate produced in the steps from leucine to HMG-CoA, and the loss of three ^{14}C -labelled angular methyl groups in the conversion of the intermediate lanosterol into the ergostane skeleton. The mean value obtained from four experiments for the amount of sterol produced from $[U\text{-}^{14}\text{C}]\text{leucine}$ was $218 \pm 21\text{ pmol}/10^6\text{ cells}$.

These results showed that at least 70–80% of the sterol was produced from leucine-derived carbon. This might be an underestimate because, as mentioned above, the possible contribution of leucine released by the proteolysis of endogenous and exogenous proteins (obtained from the foetal calf serum added as a supplement to the medium) have not been taken into account when calculating the specific radioactivity of the leucine substrate used by the cells for sterol synthesis.

Sterol and fatty acid production in amastigotes of *L. mexicana*

Following the recognition of leucine as a major precursor for sterol production in free-living promastigotes of *L. mexicana*, it was of interest to examine the roles of leucine and acetate in the synthesis of sterol and fatty acids in the amastigote form, which resides in the macrophage cell of the parasite's host. The study of amastigote lipid metabolism is difficult while the amastigote is associated with the host macrophage because the latter cell will obviously contribute to the metabolism of any substrates supplied. However, a method has been developed [27] that allows the axenic culture of amastigotes of *Leishmania*; we employed this type of amastigote culture in our investigations. We first demonstrated that the amastigotes have the capacity for sterol biosynthesis by incubation with $[2\text{-}^{14}\text{C}]\text{MVA}$, which was incorporated into the sterols (Figure 1). The results (Table 6) of incubation with $[1\text{-}^{14}\text{C}]\text{acetate}$ and $[U\text{-}^{14}\text{C}]\text{leucine}$ showed clearly that the cultured amastigotes also utilize leucine preferentially for sterol production, whereas acetate (as acetyl-CoA) provides the carbon for the synthesis of the fatty acid moieties of TAG and PL. However, it is notable that the proportion of the label from all substrates entering TAG compared with that in PL was

much lower than that found in the promastigote incubations (Table 4 and Figure 1). This implies that the rate of synthesis of TAG might be lower in the amastigotes than the promastigotes. The observation of numerous lipid particles in the promastigote forms, but not the amastigotes, of *L. mexicana* and *L. major* [36] suggests that TAG storage is a more important metabolic feature of the promastigote. In some experiments with both promastigotes (Table 4) and amastigotes (Table 6), a portion of the incorporated ^{14}C label derived from $[2-^{14}\text{C}]\text{MVA}$ co-chromatographed with the PL but this material has not been investigated further.

DISCUSSION

Several amino acids, including leucine, have been recognized as being important energy sources in trypanosomes; our results now establish that, for *L. mexicana*, leucine is also the major

source of carbon required for sterol biosynthesis, whereas some ketogenic amino acids, together with glucose, can provide the acetyl-CoA needed for fatty acid biosynthesis. We have extended these observations to show that leucine can be utilized to varying extents by other trypanosomatids (several *Leishmania* species, *Endotrypanum monterogeii*, *Crithidia fasciculata*, *Trypanosoma cruzi* and *T. brucei* (M. L. Ginger, M. L. Chance and L. J. Goad, unpublished work). Periodically over the past 50 years, leucine has been reported to act as a precursor to various products of the isoprenoid pathway in micro-organisms, plants and animals. Bloch [37] was the first to demonstrate that leucine was incorporated into cholesterol in rats; subsequently leucine was confirmed as a precursor of cholesterol in muscle and adipose tissue in man [38] and rats [39–41]. Leucine is incorporated also into various other products of the isoprenoid pathway such as β -carotene by the fungus *Phycomyces blakesleeana* [42], sterol by *Gibberella fujikuroi* [43], geraniol, squalene and β -amyrin by

Scheme 1 Metabolic pathway for the conversion of leucine into HMG-CoA and acetyl-CoA and an outline of the isoprenoid biosynthetic route for the synthesis of sterols

α -Ketoisocaproic acid, α -oxoisohexanoic acid.

plants [44], and paniculide and sterols by the plant *Andrographis paniculata* [45]. Leucine is metabolized by loss of the amine group to give α -oxoisohexanoic acid, which is then decarboxylated to produce isovaleryl-CoA, followed by dehydrogenation, carboxylation and hydration to yield HMG-CoA (Scheme 1). This HMG-CoA could theoretically enter directly into the isoprenoid pathway and be reduced by HMG-CoA reductase to MVA. However, the results of ^{13}C -labelling experiments established that in *A. paniculata* the HMG-CoA is first cleaved by a lyase (Scheme 1) to produce acetyl-CoA and acetoacetate (this reaction also occurs in the mitochondria of mammalian liver to produce the ketone bodies). The acetyl-CoA is then reutilized in *A. paniculata* to restore HMG-CoA, which proceeds through the isoprenoid pathway [45]. A possible reason for the need for this indirect incorporation of leucine by prior catabolism to acetyl-CoA could be the cellular compartmentalization of metabolic pathways that requires a shuttle of substrates between compartments. Leucine catabolism reactions in mammalian cells are described as mitochondrial [46] and in plants as peroxisomal [47] or mitochondrial [48], whereas the isoprenoid pathway operating for sterol synthesis with HMG-CoA and mevalonate as intermediates is cytosolic or peroxisomal [35,49] for the steps leading up to squalene. The sites of sterol biosynthesis and leucine metabolism in trypanosomatids are not yet established but there are some relevant published results. HMG-CoA reductase has been described as a predominantly soluble or glycosomal enzyme in *T. cruzi* [33,34], whereas in *T. brucei* it has been reported to be mainly either microsomal [32] or mitochondrial (N. Heise and F. R. Oppendoes, personal communication). Leucine aminotransferase and α -oxoisohexanoate dehydrogenase in *T. cruzi* have been assigned cytosolic and mitochondrial locations [50]. Therefore the possibility exists that leucine catabolism and the reduction of HMG-CoA leading into the isoprenoid pathway could occur together in the mitochondrion of trypanosomatids. However, more information is required to establish the roles of the glycosome and endoplasmic reticulum in isoprenoid and sterol production in the protozoan. There is also the possibility of a requirement for 'shuttles' to transfer HMG-CoA or other substrates utilized for isoprenoid biosynthesis from one intracellular site to another. The observation that leucine is incorporated into fatty acids and can be oxidized to CO_2 as an energy source [12] reveals that leucine must be broken down to some extent to acetyl-CoA (Scheme 1). The transfer of this acetyl-CoA (and also the acetyl-CoA generated from other ketogenic amino acids and glucose) to the site of fatty acid biosynthesis, which is not yet established, also needs to be clarified.

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