ABSTRACT:

[13-14C]Dihydroartemisinin was administered to male rats (35 µmol kg⁻¹, iv). Within 0–1 hr and 0–5 hr of dosing, 34.8 ± 5.2% (mean ± SD, N = 6) and 48.4 ± 5.9% of the radiolabel, respectively, was recovered in bile. Only 1.1 ± 1.2% was recovered in bladder urine after 5 hr. The principal metabolite (21.1 ± 9.3% of dose) was the biologically inactive dihydroartemisinin (DHA) glucuronide. The other metabolites were products of reductive cleavage and rearrangement of the endoperoxide bridge, a process known to generate reactive radical intermediates and abolish antimalarial activity. They were desoxy-DHA (3.3 ± 2.0%), its glucuronide (1.1 ± 1.0%), 3-hydroxydesoxy-DHA glucuronide (2.9 ± 1.8%), and the glucuronide of a ring-contracted tetrahydrofuran acetal isomer of DHA (6.3 ± 5.6%).

DHA, a sesquiterpene lactone endoperoxide that is the principal antimalarial constituent of the medicinal plant Artemisia annua (1). It has considerable activity in vitro and in vivo (2) and is more potent than artemisinin, but it is normally administered as either the methyl (artemether), ethyl (arteether), or 4-methylenebenzoate derivatives (2). Arteether’s major metabolites in rat plasma are 3α- and 9α-hydroxyarteether, 9α-hydroxy-DHA, DHA, and an isomeric THF acetate (see fig. 4) produced by A-B–ring contraction (3). Arteether is metabolized in the isolated perfused rat liver to DHA, desoxy-DHA, 9α-hydroxyarteether, and 9β-hydroxyarteether (4). Artemisinin compounds in chemical systems are highly susceptible to iron (II)-catalyzed reductive cleavage and rearrangement of the endoperoxide bridge (5, 6). These reactions yield, among other products, isomeric THF acetates and 3α-hydroxydesoxy derivatives (see fig. 4), exemplified by certain plasma metabolites of arteether in the rat (3). The isomerizations seem to proceed via oxy- and carbon-centered radicals that are the hypothetical mediators of antimalarial activity when formed within the parasite (1, 6) and of neurotoxicity when generated by the host (7). Reactive intermediates of antimalarial endoperoxides alkylate both parasite and host proteins (6, 7). Endoperoxide degradation in the malaria parasite is thought to be mediated by either free iron or heme (6), but the mechanism(s) of isomerization in mammalian cells remains unknown. Cleavage of the endoperoxide bridge is also effected by hepatic microsomes (8) and cytosolic NAD/NADH-dependent enzymes (9), and probably by simultaneous nonenzymic reactions (9).

DHA is metabolized by rat liver microsomes in the presence of NADPH to 3α-hydroxydesoxy-DHA, four unassigned monohydroxylated derivatives, and two unidentified products (10). Studies on the antimalarial activity of 9β-hydroxyarteether (3) suggest that at least some of these metabolites might retain substantial activity. We have characterized the biliary metabolites of DHA in rats with particular reference to products of isomerization and conjugation. Rapid conjugation of DHA is potentially of pharmacological importance, because it may limit the biological half-life of both DHA and any derivatives that can form DHA in vivo.

Materials and Methods

Chemicals. DHA was synthesized by a published method (11). It was obtained as an epimeric mixture resolvable by HPLC [methanol:acetic acid (1%), 4:1 v/v; Ultracarb 5 µm C₈ column; Rₙ: 8.5 and 11 min]. [14C]DHA (12.1 mCi mmol⁻¹) was also an epimeric mixture, but it was radiochromatographically homogeneous by TLC (hexane:ethyl acetate; 3:2 v/v; silica; Rₙ: 0.2). HPLC-grade solvents were products of Fisher Scientific Ltd. (Loughbourough, UK). The reagents for chemical synthesis were obtained from Aldrich Chemical Co. (Gillingham, UK).

Synthesis of Metabolite Standards. Desoxy-DHA was prepared from DHA (0.35 mmol in 15 ml freshly distilled methanol) by reduction in a bomb. Palladium on carbon (10 mg) was added and the suspension agitated with a magnetic stirrer. The bomb was flushed with hydrogen and maintained at 3.4 atm for 20 hr. The mixture was filtered over celite and evaporated to dryness under reduced pressure. The residue was purified by column chromatography [silica, 200–400 mesh; petroleum ether (40°–60°C)/ethyl acetate, 4:1 v/v] to give desoxy-DHA (0.18 mmol, 53%); m.p. 124°C. [1-14N]-NMR (300 MHz, CDCl₃), δ 3.15 (dq, J = 7.2 Hz, J₆ = 4.6 Hz, 1H), 1.95–1.65 (m, 5H), 1.64–1.45 (m, 1H), 1.45 (s, 3H), 1.2–1.15 (m, 3H), 1.13 (d, J = 8.0 Hz, 3H), 1.1–0.9 (m, 2H), 0.9 (d, J = 6.0 Hz, 3H); [6-13C]-NMR (75 MHz, CDCl₃), δ 108 (C₈), 98.5 (CH₃), 81.5 (C), 41.5 (CH), 34.5 (CH₃), 33.0 (CH₂), 32.5 (CH₂) 32.0 (CH), 23.0 (CH₂), 22.0 (CH₂), 21.0 (CH₂), 18.0 (CH₃), 11.5 (CH₃); IR (CDCl₃) 3695, 3601, 2957, 2929, 1793, 1468, 1385, 1247, 1095; LC/MS (Ultracarb 5 µm C₈; methanol:0.1 M ammonium acetate:80–65%, 20 min; Rₙ: 16.3 min): m/z 291 (M⁺+Na⁺)¹, 286 ([M⁺+H⁺]⁺), 13, 286 ([M⁺+H₃N⁺H₂O⁺]⁺, 14, 251 ([268-NH₃]⁺, 100).

3α-Hydroxydesoxy-DHA and its THF acetal isomer were obtained by...
FIG. 1. Antimalarial endoperoxides (1,2,4-trioxanes).

Artemisinin, =O; DHA, —OH; arteether, =OCH₂; arteether, =OCH₃; artesunate, =OCO(CH₂)₂CO₂H.

reduction of their artemisinan analogs prepared according to Jefford et al. (5). Artemisinin (1.7 mmol) and FeCl₃ • 4H₂O (1.7 mmol) in acetonitrile (15 ml) were stirred at room temperature for 15 min and filtered over celite. The combined filtrate and washings (dichloromethane and ethyl acetate) were evaporated. The residue was fractionated by column chromatography (silica, 200–400 mesh; petroleum ether (40°–60°C):ethyl acetate, 5:1 v/v] to give 3α-hydroxydesoxyartemisinin (0.27 mmol, 16%); m.p. 195–196; 1H-NMR (300 MHz, CDCl₃), δ 5.57 (s, 1H), 3.56 (br s, 1H), 3.14 (dq, J = 7.4 Hz, J = 4.7 Hz, 1H), 2.03–1.73 (m, 4H), 1.51 (s, 3H), 1.5–1.42 (m, 2H), 1.28–1.18 (m, 1H), 1.14 (d, J = 7.2 Hz, 3H), 1.1–0.9 (m, 2H), 0.87 (d, J = 6.4 Hz, 3H); IR (CHCl₃) 3581, 3024, 2953, 2930, 1744, 1449, 1380; LC/MS (methanol:0.1 M ammonium acetate:100 at 1 scan/5 sec. SIM (4 channels) was conducted with a dwell time of 0.2 sec and an interval delay of 20 msec. All data were processed via Masslynx II software (Micromass Ltd., Manchester, UK).

Enzymic Hydrolysis. Bile (100 μl) was diluted with 0.1 M sodium acetate (pH 5.0) (50 μl) and incubated with H-β-glucuronidase preparation (30 μl; 3 × 10⁸ units β-glucuronidase; Sigma Chemical Co., St. Louis, MO) at 37°C for 16 hr. Thereafter, the incubation was centrifuged to sediment precipitated material, and the supernatant was analyzed by LC/MS. Aliquots (50 μl) were eluted from an Ultrasound column with a gradient of methanol (60–85% over 20 min) in 0.1 M ammonium acetate. Concentrated solutions of aglycones were obtained by incubating two 250 μl aliquots of bile with β-glucuronidase (3 × 10⁸ units) and reconstituting the dry residue of the pooled extracts in methanol (100 μl) for SIM LC/MS. Recovery of radioactivity was 85–90%.

**Results**

Excetration and Distribution of [1⁴C]DHA. After intravenous administration of [1⁴C]DHA (35 μmol kg⁻¹) to rats, radioactivity was excreted into bile (fig. 2). Over 0–1 hr and 0–5 hr, respectively, 34.8 ± 5.2% (mean ± SD, N = 6) and 48.4 ± 5.9% of administered radiolabel were recovered in the bile (fig. 2). Only 1.1 ± 1.2% of the radiolabel was recovered in bladder urine after 5 hr. The kidneys, liver, and blood contained 5.2 ± 2.1%, 5.3 ± 2.4%, and 2.2 ± 0.4%,
The glucuronides of 3α-hydroxydesoxy-DHA (I), the THF acetate (II), and DHA (III) are represented by their ammonium adducts (b). Desoxy-DHA (V) and its glucuronide (IV) are represented by their common fragment ion ([desoxy-DHA + 1 - H2O]+) (c). Metabolites were eluted with a gradient of acetonitrile in acetic acid.

Each of the three isobaric glucuronides underwent distinctive fragmentation (table 1), which was interpreted with comparison with published spectra (3, 13, 14) and the spectra of the synthetic DHA derivatives.

Metabolite III yielded a fragment at m/z 267 representing loss of dehydroglucuronic acid and ammonia (14) from the ammonium adduct and dehydration of the resulting protonated aglycone. The subsequent losses of 46 amu and 58 amu have been attributed to excision of C-5 as formic acid and of C-3, C-4, and C-15 as acetone, respectively (13). No less than eight fragments in the spectrum (m/z 267, 249, 221, 207, 203, 189, 179, and 163) matched those obtained from DHA. The principal radiolabeled aglycones liberated by β-glucuronidase coeluted with the epimers of authentic DHA and gave matching ESP spectra: m/z 302 ([M+NH4]+), 284 ([M+NH4-H2O]+), 267 ([284-NH4]+), 221, 207, 203, 163 (base peak at 80 V).

Metabolite II underwent excision of acetic acid to give m/z 401, and subsequent loss of either H2O or CO, before loss of dehydroglucuronic acid (table 1). The preferential cleavage of acetic acid from an artemisinin derivative is diagnostic of a structure in which the A-B–ring system has rearranged to a 1,6-furo ring and an acetate group at C-5 (3, 8); it dominated the fragmentation of DHA’s synthetic THF acetate isomer. A minor aglycone liberated by β-glucuronidase and detected using SIM coeluted with the synthetic standard.

Metabolite I also yielded m/z 267, but no smaller fragments in either its full or daughter spectrum. This resistance to extensive fragmentation has been noted for a number of 3-hydroxylated desoxyartemisininoids (3, 8). The most polar deconjugated metabolite coeluted with synthetic 3α-hydroxydesoxy-DHA. Metabolite I is deduced to be either the C-3 or C-12 glucuronide of 3-hydroxydesoxy-DHA.

Metabolite V (fig. 3c) coeluted with synthetic desoxy-DHA and yielded a matching spectrum (table 1). The putative glucuronide of desoxy-DHA (metabolite IV, fig. 3c) was detected as an ammonium adduct at m/z 462 and as fragments at m/z 268 and m/z 251, but it was not seen as a fully resolved radiolabeled metabolite (fig. 3a). Trace quantities of DHA, detected by LC/MS ([M+NH4]+ at m/z 302), coeluted immediately after the desoxy-DHA.

The proposed pathways of [14C]DHA’s metabolism in the rat are schematized in fig. 4. Desoxy-DHA (metabolite V) and the aglycone of metabolite I are minor plasma metabolites of arteether in rats (3). The aglycone of metabolite II has not hitherto been reported as either a biliary or urinary metabolite of artemisininoids (3, 4).

**Discussion**

DHA underwent rapid glucuronolysis and biliary excretion; both epimers were conjugated but only β-DHA glucuronide retains even residual antimalarial activity (15). Isomeric conjugates of 3-hydroxydesoxy-DHA and a ring-contracted THF acetate were also formed, together with desoxy-DHA and its glucuronide, although in much smaller amounts.

The ring-contracted and 3α-hydroxydesoxy products of endoperoxide isomerization have previously been found in vivo only as minor plasma metabolites of arteether in rats (3); as 3α-hydroxydesoxyarteether, 3α-hydroxydesoxy-DHA, and the THF acetate isomer of arteether. Desoxy-DHA, but not desoxyarteether, is a metabolite of arteether in rats. Desoxyartemisinin is a urinary metabolite of artesinin in humans (4). Thus, the functional state of the C-12 oxygen exerts a qualitative influence on biotransformations of the endoperoxide moiety. Deoxyartemisinin, which lacks a C-12 oxygen, seems to be resistant to such biotransformations in vivo (16).

DHA is reported to be metabolized extensively to four hydroxylated derivatives by rat liver microsomes (10), but no hydroxylated metabolites were found in rat bile during the present study.
DIHYDROARTEMISININ METABOLITES

TABLE 1

ESP (LC/MS) mass spectra and daughter spectra of the biliary metabolites of [14C]DHA in male rats

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>R_f</th>
<th>Ions and Relative Abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α-Hydroxydesoxy-DHA glucuronide (I)</td>
<td>11.0</td>
<td>m/z 478 ([M+NH₄]⁺, 58), 267 ([M+NH₄-NH₂-176-H₂O]⁺, 100)</td>
</tr>
<tr>
<td>Daughter spectrum I</td>
<td></td>
<td>m/z 267 (100)</td>
</tr>
<tr>
<td>THF-acetate glucuronide (II)</td>
<td>16.5</td>
<td>m/z 478 ([M+NH₄]⁺, 56), 401 ([M+NH₄-NH₂-CH₃CO₂H]⁺, 23), 383 ([401-H₂O]⁺, 7), 373 ([401-CO]⁻, 100), 225 (401-176)⁻, 4, 207 ([225-H₂O]⁻, 6)</td>
</tr>
<tr>
<td>Daughter spectrum II</td>
<td></td>
<td>m/z 401 (5), 267 (59), 225 (100), 207 (53)</td>
</tr>
<tr>
<td>Daughter spectrum III</td>
<td></td>
<td>m/z 267 (100), 249 (2), 221 (10), 207 (2), 203 (4), 189 (2), 163 (18)</td>
</tr>
<tr>
<td>Desoxy-DHA glucuronide (IV)</td>
<td>21.5</td>
<td>m/z 462 ([M+NH₄]⁺, 63), 268 ([M+NH₂-176-H₂O]⁻, 6), 251 ([M+NH₄-NH₂-176-H₂O]⁺, 100)</td>
</tr>
<tr>
<td>Desoxy-DHA (V)</td>
<td>31.0</td>
<td>m/z 286 ([M+NH₄]⁺, 11), 268 ([M+NH₂-176-H₂O]⁻, 8), 251 ([M+NH₂-176-H₂O]⁺, 100)</td>
</tr>
</tbody>
</table>

Bile (0–1 hr collection) was eluted from a C₈ column with a gradient of acetonitrile in 1% aqueous acetic acid. Spectra were acquired at a cone voltage of 20 V. Daughter spectra of m/z 478 were generated by CID using argon. The fragment of mass 176 amu is dehydroglucuronic acid.

Although the biomimetic iron/heme-catalyzed isomerization of endoperoxides in chemical systems (5, 6) suggests the possibility of a similar nonenzymic process in vivo, the balance of probability favors enzymic catalysis as the dominant if not exclusive mechanism in nonparasitized animals: whenever the isomerization of organic hydroperoxides and endoperoxides in biological systems has been characterized, it has been shown that enzymatic catalysis predominates (17, 18). A third potential mechanism of isomerization in vivo is suggested by the apparently iron-independent transformation of DHA into a dialdehyde form in cell culture media. However, this isomer was not observed in the present study.

Enzymic endoperoxide reduction activity, as determined by the NADH/NADH-dependent conversion of arteether to desoxy-DHA, has been found in rat liver cytosol (9), but the rate of arteether turnover in hepatic microsomes is an order of magnitude greater (8, 10). Arteether is metabolized inter alia to 3α-hydroxydesoxyarteether and 3α-hydroxydesoxy-DHA by rat liver microsomes (8), and DHA, notwithstanding the reported predominance of its monohydroxylation in microsomes, is metabolized partly to 3α-hydroxydesoxy-DHA (10). Whereas the enzymes that catalyze the isomerizations have not been identified, the closely related mechanisms of prostacyclin and thromboxane synthases suggest the involvement of P450. These heme-thiolate enzymes, located in the vascular endothelium and thrombocytes, respectively, belong to superfamilies that are distinct from microsomal P450 (17). They catalyze the isomerization of prostaglandin 1,3-endoperoxides to prosta
cyclins and inter alia thromboxanes, respectively, although thromboxane synthase possesses latent monoxygenase activity. Conversely phenobarbital-induced rat hepatic P450 metabolizes the endoperoxide prostaglandin H₂ (19). The proposed mechanisms (17) consist of the same initial reactions as the iron-mediated isomerizations of prostaglandin (19) and artemisininoid (6) endoperoxides: a regiospecific iron (III)-oxygen interaction leading to homolytic scission of the dioxygen bond with formation of an oxygen-centred radical that rearranges to a carbon-centred radical. Oxidation of the latter by an iron (IV) species and further rearrangement yields the isomeric product. A schematic pathway for the

- **Table:** The table lists the biliary metabolites of [14C]DHA in male rats, including their retention times (R_f), mass spectra (m/z), and daughter spectra.

- **Fig. 4:** The figure illustrates the biliary metabolites of [14C]DHA in male rats.

- **Text:** The drug was given intravenously as a mixture of C-12 epimers (~2:1 from LC/MS). Dashed arrows indicate multistep pathways/rearrangements. Numerals in parentheses refer to chromatographic peaks in fig. 3. G. glucuronic acid moiety; metabolite I, either C-3 or C-12 glucuronide. Metabolite III was the major metabolite.

Within the malaria parasite in vitro, artemisinin endoperoxides undergo activation via iron-mediated reductive cleavage to radical intermediates generally considered to be the agents of parasiticidal activity (7); the iron, either in free form or as heme iron, is released during the intracellular digestion of hemoglobin by the parasite. Nevertheless, the stable isomerization products formed in uninfected humans and animals—which signify the intermediary of radical species (5)—have yet to be found in parasite cultures.

2 J. L. Maggs et al., unpublished data.
conversion of DHA to its THF acetate isomer that derives from the isomerization of prostaglandin endoperoxides (17, 19) is shown in fig. 5. The metabolites of arteether have short plasma half-lives in rats: 9–34 min and 14 min for DHA (3). This now seems to be, in part, a reflection of rapid glucuronylation of DHA and the other lactol metabolites. Such conjugation may be expected of DHA formed from any O-ether and esterified derivatives of DHA via glucuronolysis. (+)-Dideoxartemisinin (16) has been projected as the lead compound for artemisinoids having enhanced activity in vivo by virtue of greater chemical stability and consequently a longer half-life (21). The findings reported herein suggest that (+)-dideoxartemisinin’s superior activity might be equally derived from an incapacity to undergo clearance via glucuronolysis.

Acknowledgments. [14C]DHA was prepared by Dr. J. A. Kepler, Research Triangle Institute, NC, and was obtained through Dr. J. G. Edwards, Department of Pharmacology and Therapeutics, University of Liverpool. The LC/MS system was purchased with a grant from the Wellcome Trust. We thank Miss S. Newby for her assistance with the animal experiments and Miss S. Oliphant for preparing the manuscript.

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