CONVERSION OF LEUKOTRIENES A₄ TO C₄ IN CELL-FREE SYSTEMS

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A procedure for assaying leukotriene C₄ synthase activity in cell-free extracts has been presented. Leukotriene A₄ methyl ester was as active a substrate as leukotriene A₄ (Na salt) for the synthesis. The methyl ester is the substrate of choice, because (1) it is more stable than the sodium salt, (2) it is not a substrate of epoxide hydrolase for leukotriene B₄ synthesis, and (3) it gives a lower blank than an equimolar concentration of leukotriene A₄. The enzyme activity in rat liver, guinea pig and human lungs, and human nasal polypl was chiefly membrane-bound, although the cytosol contained some activity.

Leukotriene A₄ occupies a pivotal position in the lipoxygenase pathway of arachidonic acid metabolism [1]. LTA₄ may be converted by an epoxide hydrolase (EC 3.3.2.3) to LTB₄, a potent chemotactic and chemokinetic agent for polymorphonuclear leukocytes or it may be converted by glutathione S-transferases (EC 2.5.1.18) to LTC₄, a component of the slow reacting substance of anaphylaxis. The extreme lability of LTA₄ in aqueous medium and at physiological pH has impeded biochemical studies with crude or purified enzyme preparations. Fitzpatrick et al. [2] reported that the compound can be stabilized to some extent with BSA. Subsequently, studies of conversion of LTA₄ to LTB₄ [3, 4] and to LTC₄ [5, 6] in cell-free extracts and purified enzyme preparations have appeared. This report represents an effort to study the conditions for the enzymatic conversion of LTA₄ to LTC₄ as well as this enzyme activity in some mammalian tissues as measured by RIA of LTC₄.

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Abbreviations used: BSA, bovine serum albumin; ENPP, 1,2-epoxy-3-(p-nitrophenoxy)propane; GSH, reduced glutathione; HPLC, high pressure liquid chromatography; LT, leukotriene; LTA₄Na and LTA₄Me, leukotriene A₄ (Na salt) and leukotriene A₄ methyl ester; LTC₄Me, leukotriene C₄ methyl ester; PBS, phosphate buffered saline; RIA, radioimmunoassay.
Materials and Methods

Reagents  ENPP was purchased from Aldrich Chemical Co., LTC₄Me from Calbiochem-Behring, [14,15-³H]LTC₄ from New England Nuclear Corp., and goat anti-rabbit immunoglobulin serum from Bio-Rad. The following reagents were received as gifts: 4-phenylchalcone oxide from Dr. B.D. Hammock, University of California, Davis; rabbit anti-LTC₄ serum from Dr. A.S. Rosenthal, Merck Institute for Therapeutic Research; and LTA₄Me from Dr. J. Rokach, Merck Frosst Canada Inc. All other reagents were of reagent grade.

Tissue extracts  Adult male rats of the Sprague-Dawley strain and guinea pigs of an out-bred strain were purchased from Charles River Laboratories. Human tissue specimens were obtained from the Surgical Services of the University Hospital. All the following preparations were done at 4°C. Subcellular fractions of a tissue were prepared from the homogenate in 0.25 M sucrose by differential centrifugation as described before [7]. In other experiments, tissue homogenates containing 300 mg tissue/ml in 0.15 M KC1 were centrifuged at 10,000 ×g for 1 h. The supernatant fluids were used for assaying the enzyme activity.

Glutathione S-transferase activity  When ENPP was used as substrate, the assay procedure of Habig et al. [8] was followed with the exception that assays were done at 37°C. Other conditions were the same as reported earlier [9]. Protein was measured by the procedure of Lowry et al. [10] with BSA as standard.

Conversion of LTA₄ to LTC₄  The assay medium in a total volume of 100 μl contained 50 mM potassium phosphate buffer (pH 7.8), 5 mM GSH, 15 mM L-serine borate (pH 7.6) [11], 0.2% (w/v) BSA, 25 μM LTA₄Me (in 2 μl methanol), and tissue extract, the final pH being 7.6-7.8, adjusted if necessary. When LTA₄Na replaced LTA₄Me as substrate, it was generated from LTA₄Me with 1.25 M methanolic NaOH at 4°C for 3 h [3]. The reaction mixture was the same as that with LTA₄Me, except that 50 mM potassium phosphate buffer (pH 6.6) was used to keep the final pH within the desired range after addition of 2 μl of the alkaline LTA₄Na solution. In addition, 0.5 μM 4-phenylchalcone oxide, which served as a selective inhibitor of cytosolic epoxide hydrolase [12], was included.

The assay medium less LTA₄ (methyl ester or sodium salt) was equilibrated at 37°C in a water bath. After addition of LTA₄, the incubation was continued for 10 min. At the end of the incubation, 300 μl of ice-cold methanol was added. The mixture was stored at -20°C. Small aliquots (usually 10 μl or less) of the clear liquid were assayed by the RIA procedure described below. A control in which the tissue extract was omitted from the otherwise complete reaction mixture was included.

RIA of LTC₄  The procedure developed by Hayes et al. [13] was generally followed with a second antibody, goat anti-rabbit immunoglobulin serum, used to precipitate LTC₄-antibody complexes. All dilutions were made in PBS/BSA (0.14 M NaCl, 0.01 M potassium phosphate buffer (pH 7.3), 0.02% (w/v) sodium azide, 1% (w/v) BSA, and 0.1 mM phenylmethylsulfonyl fluoride, the last component being added just before use). The assay mixture contained: (1) 100 μl of rabbit anti-LTC₄ serum after suitable dilution, (2) 100 μl of standard LTC₄ or a solution to be assayed, and (3) 200 μl of PBS/BSA containing ³H-LTC₄ (5,000 cpm at 49% counting efficiency and specific activity of 36 Ci/mmoll) and 4 μl of normal rabbit serum as carrier. The mixture was incubated at 4°C for 2 h. At the end of the incubation, 500 μl of PBS/BSA containing 100 μl of the goat serum (titered for equivalence point) was added as the second antibody and the incubation continued at 4°C for 18 h. The mixture was then centrifuged at 1,000 ×g for 10 min. An aliquot of 800 μl of the supernatant fluid was mixed with 10 ml of Safety-Solve (Research Products International Corp.) and counted in a Packard 3320 liquid scintillation spectrometer. When the precipitates were used
for counting, they were washed twice with ice-cold PBS/BSA and dissolved in 100 µl of 0.1% (w/v) sodium dodecyl sulfate in 0.1 N NaOH. The alkaline solution was similarly counted.

**HPLC of LTC₄** The procedure of Mathews et al. [14] was used for analyzing samples after ethyl acetate extraction. The reverse phase column was packed with Nucleosil C₁₉ particles. The solvent system of methanol: water: acetic acid (65: 35: 0.02) was adjusted to pH 5.7 with NH₄OH.

**Results and Discussion**

More than 10% of the total radioactivity of the ⁳H-LTC₄ preparations estimated to be 98-99% pure by the manufacturer was not precipitable by the double antibody procedure, presumably due to deterioration of the labeled ligand on storage before shipment, and this percentage continued to increase gradually when stored at -20°C in our laboratory. Because of the continuing deterioration of the ³H-LTC₄, we used at least one calibration curve either of the supernatant fluid or of the precipitate after the second antibody precipitation with each assay in the RIA. Recovery of the total radioactivity in the supernatant fluid and pellet fractions for each amount of LTC₄ added ranged between 95-99%.

The optimal pH for LTC₄ synthesis from LTA₄Me with the enzyme from a human lung extract was 7.8 (Fig. 1). The initial rate of LTC₄ synthesis lasted for about 20 min under the assay conditions used. Fig. 2 shows the relation between the

![Graph 1: Optimal pH for LTC₄ synthesis](image1)

**Fig. 1.** Optimal pH for LTC₄ synthesis. The reaction mixture containing a human lung 10,000 g supernatant fluid was as described in Methods, except that L-serine-borate and phosphate buffer of different pH values were used. The controls contained no tissue extract and were not significantly affected by the pH changes.

![Graph 2: Relation between the length of incubation time and the amount of LTC₄ synthesized](image2)

**Fig. 2.** Relation between the length of incubation time and the amount of LTC₄ synthesized. The reaction mixture contained in 100 µl 4.3 nmol LTA₄Me and 34 µg protein from the 10,000 g supernatant fluid of a human lung. For other details see Methods. The values for LTC₄ (O) have been corrected by their respective controls (●).
Fig. 3. Relation between the amount of tissue extract used and the amount of LTC₄ formed. The reaction mixture contained in 100 µl 2.5 nmol LTA₄Me and variable amounts of a human lung extract and was incubated for 10 min. All values have been corrected for the blank, which was equivalent to 0.025 nmol LTC₄.

Figure 3 shows a linear correlation between the amount of tissue extract used and the formation of LTC₄. A simple comparison will show that lowering the concentration of the substrate from 43 (Fig. 2) to 25 µM (Fig. 3) reduced the blank from 0.18 to 0.025 nmol LTC₄ equivalent.

LTA₄Me has been shown to be enzymatically inactive as substrate for conversion to LTB₄ in rat basophilic leukemia cell cytosol [3] and in blood plasma from several mammalian species [4]. We have compared the efficacies of LTA₄Na and LTA₄Me as substrates for LTC₄ synthesis. Table 1 shows that the unnatural LTA₄Me was as effective a substrate as the natural LTA₄Na for the enzyme from human lung. Similar results have been obtained with human nasal polyp and guin-
Table 1. Comparison of LTA$_4$Na with LTA$_4$Me in LTC$_4$ synthesis

<table>
<thead>
<tr>
<th>Condition</th>
<th>LTA$_4$Na (nmol/min/mg protein)</th>
<th>LTA$_4$Me (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system (a)</td>
<td>3.71 ± 0.34 (6)</td>
<td>1.80 ± 0.10 (4)</td>
</tr>
<tr>
<td>Less tissue extract* (b)</td>
<td>2.58 ± 0.27 (4)</td>
<td>0.65 ± 0.07 (6)</td>
</tr>
<tr>
<td>Less GSH</td>
<td>0.16 ± 0.02 (4)</td>
<td>0.051 ± 0.010 (4)</td>
</tr>
<tr>
<td>Net synthesis (a-b)</td>
<td>1.13</td>
<td>1.15</td>
</tr>
</tbody>
</table>

*Not different from using boiled tissue extract.

The reaction mixture contained 100 μl 68 μg protein of a human lung extract and 6.8 nmol LTA$_4$Na or LTA$_4$Me. For other details see Methods. The values are expressed as nmol/min/mg protein and in terms of LTC$_4$ activity in RIA.

e.g. pig lung extracts (data not shown). The high concentration of LTA$_4$Na used in these experiments generated an extremely high blank. The blank was greatly reduced, however, with LTA$_4$Me as substrate, although it is still considered very high relative to the value obtained in the complete system. Fortunately, the problem can be resolved in a number of ways. First, since the concentration of LTA$_4$ alone determines the control value, lower concentrations of LTA$_4$Me should be used. Second, as pointed out earlier, increases in the time period of incubation and in the amount of tissue extract used will effectively lower the blank as a percentage of the overall synthesis. Reddy et al. [15] and Mannervik et al. [16] have reported that LTA$_4$Me was a substrate for LTC$_4$ synthesis by rat liver glutathione S-transferases.

Table 1 also shows that the high control value was not the result of non-enzymatic degradation of LTA$_4$, but rather of non-enzymatic reaction of LTA$_4$ with GSH, since in the absence of GSH very little LTC$_4$-like activity was measured. The concentration of GSH in the reaction mixture taken for RIA of LTC$_4$ was insufficient to cause cross reaction.

To determine whether LTA$_4$Me was converted to LTC$_4$Me in human and guinea pig lungs, we used HPLC. Fig. 4 shows the retention times of LTC$_4$ and LTC$_4$Me to be, respectively, 9.8 and 18.3 min. HPLC of the reaction mixture containing initially LTA$_4$Me gave a peak cochromatographed with authentic LTC$_4$Me (tracings not shown). Hence, LTA$_4$Me was converted without hydrolysis of the ester bond in the lungs. The reaction mixture also yielded retention peaks other than LTC$_4$Me. Whether these compounds cross reacted with LTC$_4$ in the RIA has not been determined. Y-Glutamyltranspeptidase (EC 2.3.2.2) activity in the tissue
LTC₄ methyl ester

![HPLC Separation of LTC₄ from LTC₄Me](image)

**Fig. 4.** Separation of LTC₄ from LTC₄Me in a reverse phase HPLC. The retention times of LTC₄ and LTC₄Me were, respectively, 9.8 and 18.3 min.

Extracts assayed was not totally inhibited by L-serine-borate and partial conversion of LTC₄ to LTD₄ could not be prevented. However, because of the cross reactivity of LTD₄ in the RIA [13], the conversion did not entail a complete loss.

We next examined LTC₄ synthase activity in subcellular fractions of rat liver, guinea pig lung, human lung and nasal polyp. Human nasal polyps were examined because of the presence of high concentrations of certain arachidonic acid metabolites in this tissue [17]. Table 2 shows the results in the mitochondria, microsomes, and cytosol. Included for comparison is the activity with ENPP as substrate. The highest specific activity for LTC₄ synthesis from LTA₄Na occurred in the microsomal fraction, followed by the mitochondrial fraction. The specific activity in the cytosol was the lowest in all 4 tissues. These results clearly show that most LTC₄ synthase activity was membrane-bound. Jakschik et al.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Mitochondria</th>
<th>Microsomes</th>
<th>Cytosol</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ENPP</td>
<td>LTA₄</td>
<td>ENPP</td>
</tr>
<tr>
<td>Rat liver</td>
<td>43.3</td>
<td>1.5±0.2 (2)</td>
<td>79.3</td>
</tr>
<tr>
<td>Guinea pig lung</td>
<td>&lt;2.0</td>
<td>5.3±0.7 (2)</td>
<td>25.8</td>
</tr>
<tr>
<td>Human lung</td>
<td>&lt;2.0</td>
<td>9.5±2.1 (2)</td>
<td>77.4</td>
</tr>
<tr>
<td>Human nasal polyp</td>
<td>2.0</td>
<td>3.9±0.6 (2)</td>
<td>100</td>
</tr>
</tbody>
</table>

The reaction mixture contained 50 μM LTA₄Na. For other assay conditions, see Methods. All values are expressed as nmol/min/mg protein.
reported that LTC₄-forming enzyme activity of rat basophilic leukemia cells was localized in the 10,000 g pellet and very little activity was found in the 10,000 g supernatant fluid. Their results would thus exclude the microsomes as a major locale of the enzyme. Whether the neoplastic transformation has brought about a modification in the subcellular localization of this enzyme activity is not known.

There seems a correlation between the predominance of certain glutathione S-transferase isoenzymes and their physiological functions in a tissue. Since liver is generally considered a major site of detoxication [19], the activity with ENPP was higher in the liver than in the lung. Conversely, since airway smooth muscles are a target tissue of LTC₄ [20,21], the activity with LTA₄ was higher in the lung than in the liver. We suggest that the latter activity be referred to as LTC₄ synthase (but not LTC₄ synthetase [22]) to distinguish it from the former activity, which is mainly concerned with detoxication.

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**References**