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CONVERSION OF LEUKOTRIENES A_4 TO C_4 IN CELL-FREE SYSTEMS

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A procedure for assaying leukotriene C_4 synthase activity in cell-free extracts has been presented. Leukotriene A_4 methyl ester was as active a substrate as leukotriene A_4 (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 B_4 synthesis, and (3) it gives a lower blank than an equimolar concentration of leukotriene A_4. The enzyme activity in rat liver, guinea pig and human lungs, and human nasal polyp was chiefly membrane-bound, although the cytosol contained some activity.

Leukotriene A_4 occupies a pivotal position in the lipoxygenase pathway of arachidonic acid metabolism [1]. LTA_4 may be converted by an epoxide hydrolase (EC 3.3.2.3) to LTB_4, a potent chemotactic and chemokinetic agent for polymorphonuclear leukocytes or it may be converted by glutathione S-transferases (EC 2.5.1.18) to LTC_4, a component of the slow reacting substance of anaphylaxis. The extreme lability of LTA_4 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 LTA_4 to LTB_4 [3,4] and to LTC_4 [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 LTA_4 to LTC_4 as well as this enzyme activity in some mammalian tissues as measured by RIA of LTC_4.

Abbreviations used: BSA, bovine serum albumin; ENPP, 1,2-epoxy-3-(p-nitrophenoxy)propane; GSH, reduced glutathione; HPLC, high pressure liquid chromatography; LT, leukotriene; LTA_4Na and LTA_4Me, leukotriene A_4 (Na salt) and leukotriene A_4 methyl ester; LTC_4Me, leukotriene C_4 methyl ester; PBS, phos phate buffered saline; RIA, radioimmunoassay.

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Materials and Methods

Reagents ENPP was purchased from Aldrich Chemical Co., LTC_4Me from Calbiochem-Behring, [14,15-^{3}H]LTC_4 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-LTC_4 serum from Dr. A.S. Rosenthal, Merck Institute for Therapeutic Research; and LTA_4Me 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 KCl 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 LTA_4 to LTC_4 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 LTA_4Me (in 2 µl methanol), and tissue extract, the final pH being 7.6-7.8, adjusted if necessary. When LTA_4Na replaced LTA_4Me as substrate, it was generated from LTA_4Me with 1.25 M methanolic NaOH at 4°C for 3 h [3]. The reaction mixture was the same as that with LTA_4Me, 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 LTA_4Na 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 LTA_4 (methyl ester or sodium salt) was equilibrated at 37°C in a water bath. After addition of LTA_4, 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 LTC_4 The procedure developed by Hayes et al. [13] was generally followed with a second antibody, goat anti-rabbit immunoglobulin serum, used to precipitate LTC_4-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-LTC_4 serum after suitable dilution, (2) 100 µl of standard LTC_4 or a solution to be assayed, and (3) 200 µl of PBS/ BSA containing ^{3}H-LTC_4 (5,000 cpm at 49% counting efficiency and specific activity of 36 Ci/ mmol) 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 LTC_4 The procedure of Mathews et al. [14] was used for analyzing samples after ethyl acetate extraction. The reverse phase column was packed with Nucleosil C_{18} particles. The solvent system of methanol: water: acetic acid (65: 35: 0.02) was adjusted to pH 5.7 with NH_4OH.

Results and Discussion

More than 10% of the total radioactivity of the ^3H-LTC_4 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-LTC_4, 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 LTC_4 added ranged between 95-99%.

The optimal pH for LTC_4 synthesis from LTA_4Me with the enzyme from a human lung extract was 7.8 (Fig. 1). The initial rate of LTC_4 synthesis lasted for about 20 min under the assay conditions used. Fig. 2 shows the relation between the

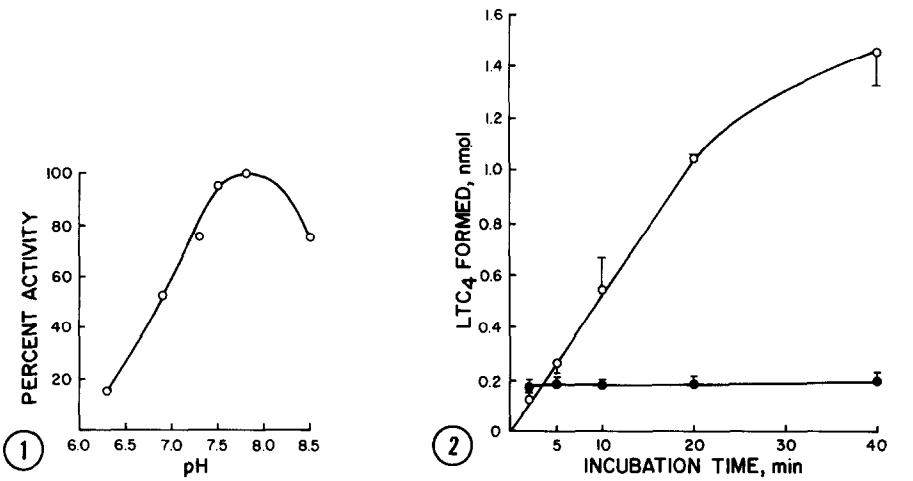

Fig. 1. Optimal pH for LTC_4 synthesis. The reaction mixture containing a human lung 10,000 g supernatant fluid was as described in Methods, except that L-serine-borate and phosphate buffer of different pH values were used. The controls contained no tissue extract and were not significantly affected by the pH changes.

Fig. 2. Relation between the length of incubation time and the amount of LTC_4 synthesized. The reaction mixture contained in 100 µl 4.3 nmol LTA_4Me and 34 µg protein from the 10,000 g supernatant fluid of a human lung. For other details see Methods. The values for LTC_4 (O) have been corrected by their respective controls (●).

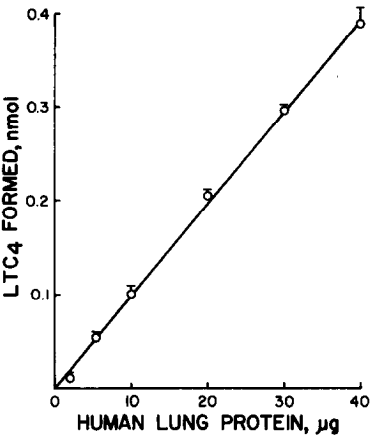

Fig. 3. Relation between the amount of tissue extract used and the amount of $\overline{LTC_4}$ formed. The reaction mixture contained in 100 µl 2.5 nmol LTA_4Me and variable amounts of a human lung extract and was incubated for 10 min. All values have been corrected for the blank, which was equivalent to 0.025 nmol LTC_4.

time period of incubation and the amount of LTC_4 synthesized in 100 µl of the reaction mixture. It is noteworthy that the various controls, in which boiled tissue extract or no tissue extract was used, gave a constant LTC_4-like activity independent of the length of the incubation time after the first 2 min. Hence, it would be advantageous to use the longest incubation time compatible with the initial rate in order to minimize the effect of the blank. For instance, the control values as a percentage of the overall synthesis at 10 and 20 min of incubation were, respectively, 25 and 15%. On the other hand, the control value was dependent on the concentration of LTA_4 in the reaction mixture. Increasing LTA_4 concentration from 5 to 50 µM increased appreciably the control value, although it increased the net synthesis of LTC_4 more (data not shown). The optimal concentration of LTA_4Me was found to be about 25 µM. Fig. 3 shows a linear correlation between the amount of tissue extract used and the formation of LTC_4. 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 LTC_4 equivalent.

LTA_4Me has been shown to be enzymatically inactive as substrate for conversion to LTB_4 in rat basophilic leukemia cell cytosol [3] and in blood plasma from several mammalian species [4]. We have compared the efficacies of LTA_4Na and LTA_4Me as substrates for LTC_4 synthesis. Table 1 shows that the unnatural LTA_4Me was as effective a substrate as the natural LTA_4Na for the enzyme from human lung. Similar results have been obtained with human nasal polyp and guin-

Table 1. Comparison of LTA_4Na with LTA_4Me in LTC_4 synthesis

Condition	Substrate	
	LTA_4Na	LTA_4Me
Complete system (a)	3.71 ± 0.34 (6)	1.80 ± 0.10 (4)
Less tissue extract* (b)	2.58 ± 0.27 (4)	0.65 ± 0.07 (6)
Less GSH	0.16 ± 0.02 (4)	0.051 ± 0.010 (4)
Net synthesis (a-b)	1.13	1.15

*Not different from using boiled tissue extract.

The reaction mixture contained in 100 µl 68 µg protein of a human lung extract and 6.8 nmol LTA_4Na or LTA_4Me. For other details see Methods. The values are expressed as nmol/ min/ mg protein and in terms of LTC_4 activity in RIA.

ea pig lung extracts (data not shown). The high concentration of LTA_4Na used in these experiments generated an extremely high blank. The blank was greatly reduced, however, with LTA_4Me 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_4Me 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_4Me 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_4Me was converted to LTC_4Me in human and guinea pig lungs, we used HPLC. Fig. 4 shows the retention times of LTC_4 and LTC_4Me to be, respectively, 9.8 and 18.3 min. HPLC of the reaction mixture containing initially LTA_4Me gave a peak cochromatographed with authentic LTC_4Me (tracings not shown). Hence, LTA_4Me was converted without hydrolysis of the ester bond in the lungs. The reaction mixture also yielded retention peaks other than LTC_4Me. Whether these compounds cross reacted with LTC_4 in the RIA has not been determined. γ-Glutamyltranspeptidase (EC 2.3.2.2) activity in the tissue

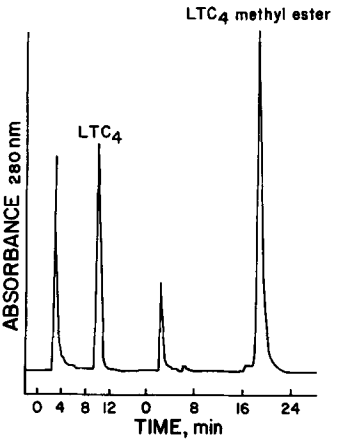

Fig. 4. Separation of LTC_4 from LTC_4Me in a reverse phase HPLC. The retention times of LTC_4 and LTC_4Me were, respectively, 9.8 and 18.3 min.

extracts assayed was not totally inhibited by L-serine-borate and partial conversion of LTC_4 to LTD_4 could not be prevented. However, because of the cross reactivity of LTD_4 in the RIA [13], the conversion did not entail a complete loss.

We next examined LTC_4 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 LTC_4 synthesis from LTA_4Na 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 LTC_4 synthase activity was membrane-bound. Jakschik et al.

Table 2. Glutathione S-transferase activity in subcellular fractions of some tissues

Tissue	Mitochondria		Microsomes		Cytosol	
	ENPP	LTA_4	ENPP	LTA_4	ENPP	LTA_4
Rat liver	43.3	1.5±0.2 (2)	79.3	2.3±0.2 (3)	81.3	1.1±0.1 (3)
Guinea pig lung	<2.0	5.3±0.7 (2)	25.8	11.1±0.7 (4)	31.7	0.52±0.04 (3)
Human lung	<2.0	9.5±2.1 (2)	77.4	13.0±0.7 (4)	3.4	0.90±0.06 (3)
Human nasal polyp	2.0	3.9±0.6 (2)	100	10.6±0.6 (2)	<2.0	0.14±0.03 (2)

The reaction mixture contained 50 μM LTA_4Na. For other assay conditions, see Methods. All values are expressed as nmol/ min/ mg protein.

[18] reported that LTC_4-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 LTC_4 [20,21], the activity with LTA_4 was higher in the lung than in the liver. We suggest that the latter activity be referred to as LTC_4 synthase (but not LTC_4 synthetase [22]) to distinguish it from the former activity, which is mainly concerned with detoxication.

Acknowledgments

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