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Robert C. Murphy; Sven Hammarstrom; Bengt Samuelsson

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Leukotriene C: A slow-reacting substance from murine mastocytoma cells

(arachidonic acid/cysteine/calcium ionophore A23187/5,6-epoxy-7,9,11,14-icosatetraenoic acid)

ROBERT C. MURPHY*, SVEN HAMMARSTRÖM, AND BENGT SAMUELSSON

Department of Chemistry, Karolinska Institutet, S-104 01 Stockholm, Sweden

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ABSTRACT **Murine mastocytoma cells treated with calcium ionophore A23187 produced a slow-reacting substance (SRS) that caused guinea pig ileum to contract. The response was reversed by the SRS antagonist FPL 55712. On the basis of isotope incorporation experiments, spectroscopy, and chemical degradations, the SRS was identified as a cysteine-containing derivative of 5-hydroxy-7,9,11,14-icosatetraenoic acid. This amino acid was attached in thioether linkage at C-6. The SRS is structurally related to previously identified epoxy and dihydroxy metabolites of arachidonic acid in leukocytes. A common feature is the presence of a conjugated triene, and the name "leukotriene" has been introduced to designate these compounds. Leukotriene A (5,6-epoxy-7,9,11,14-icosatetraenoic acid) is an intermediate in the formation of leukotriene B (5,12-dihydroxy-6,8,10,14-icosatetraenoic acid) and is proposed to be a precursor also of leukotriene C, which is the SRS identified here.**

The term "slow-reacting substance" (SRS) was originally introduced for a smooth-muscle-contracting activity appearing in the effluent of perfused lungs after treatment with cobra venom (1). Kellaway and Trethewie (2) reported that SRS could also be released by immunological challenge of sensitized lungs. It has been suggested that SRS plays an important role as mediator in asthma and other types of immediate hypersensitivity reactions (reviewed in refs. 3 and 4).

Some structural features of SRS have been proposed (5–7). However, these studies have been severely hampered by the apparent impure state of the SRS preparations. Recently isolated cell suspensions such as rat peritoneal cavity cells (8–11) and rat basophilic leukemia cells (12) have been used for *in vitro* generation of SRS after stimulation with the calcium ionophore A23187. Also, advances in the purification of SRS by using high-pressure liquid chromatography (HPLC) have been reported (13–15). Reports on conversion of isotopically labeled arachidonic acid to SRS (8, 15–17) have not been convincing due to insufficient purification of the product. Moreover, the use of cyclooxygenase or lipoxygenase inhibitors in studies of SRS production has not provided unambiguous evidence for the role of arachidonic acid or these oxygenases in SRS biosynthesis (10, 16–18).

Increased SRS production in the presence of high concentrations of L-cysteine has recently been described (10). Furthermore, certain other mercaptans have been found to stimulate SRS production from leukocytes with A23187 (19). However, there have been conflicting reports as to whether ^{35}S-labeled cysteine can be incorporated into SRS (9, 20).

In this paper we report that the ionophore-induced SRS from mast cell tumor (MCT) leukocytes is a member of a group of compounds named leukotrienes (21). It is a cysteinyl derivative of arachidonic acid and is proposed to be formed from the recently described 5,6-epoxy-7,9,11,14-icosatetraenoic acid (leukotriene A) (22), an intermediate in the formation of dihydroxyicosatetraenoic acids (23–25).

MATERIALS AND METHODS

Materials. L-[^{35}S]Cysteine hydrochloride (116–137 Ci/mol), L-[3-^{3}H]cysteine (1.9 Ci/mmol), and [5,6,8,9,11,12,14,15-^{3}H$_8$]arachidonic acid (86 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England (1 Ci = 3.7×10^{10} becquerels). L-[*U*-^{14}C]Cysteine (290 Ci/mol) was obtained from New England Nuclear, Dreieichenhan, Germany.

The ionophore A23187 was a gift of R. Hamill of Lilly Research Laboratories, Indianapolis, IN, and the SRS antagonist FPL 55712 KA was supplied by Fisons Limited, Longborough, England. Lipoxygenase (EC 1.13.11.12), type I, was obtained from Sigma Chemical Co., St. Louis, MO.

Cell Suspensions. Human polymorphonuclear leukocytes (10^7 cells per ml) were prepared as described (24).

Mouse mast cell tumor (CXBGABMCT-1), a gift from Hans Bennich, Uppsala, Sweden, was carried as an ascites tumor in CXBG mice. Mice were injected intraperitoneally with 3–6 × 10^7 cells. After *ca* 2 weeks, MCT cells were harvested from the peritoneal cavity by a careful washing with 10 ml of incubation buffer (150 mM NaCl/3.7 mM KCl/3.0 mM Na_2HPO_4/3.5 mM KH_2PO_4/0.9 mM $CaCl_2$/5.6 mM dextrose, adjusted to pH 7.0 with 30% NaOH), placed on ice, and centrifuged in plastic tubes at 250 × *g* for 15 min at 2–5°C. The pellets were suspended in 10 ml of a buffered ammonium chloride solution (0.75%) for lysis of erythrocytes (24). After 5 min at 37°C the cell suspensions were centrifuged and the pellets were suspended and combined in incubation buffer at a final concentration of 10^7 cells per ml.

Incubation Conditions. The procedure for the production of SRS from MCT cells involved the addition of L-cysteine (0.01 M) to a cell suspension (10^7 cells per ml) warmed to 37°C. After 2 min, A23187 (2 mg/ml in ethanol) was added dropwise to make the final concentration of ionophore 10 μg/ml. After 20 min of gentle shaking, the cells were centrifuged and the supernatant was made 80% (vol/vol) in ethanol. This ethanol solution was stored for 12 hr at 4°C, filtered, and evaporated to dryness on a rotory evaporator at 40°C.

Conditions with radiolabeled precursors were slightly modified. [^{3}H]Arachidonic acid was added to the cell suspension 60 min prior to the addition of L-cysteine and ionophore. [^{35}S]-, [3-^{3}H]-, and [*U*-^{14}C]cysteine were added to the cell suspension and incubated for 15 min, followed by addition of the calcium ionophore and incubation for 20 min (addition of unlabeled cysteine omitted).

Bioassay. Assay of smooth-muscle-contracting activity was performed with the isolated guinea pig ileum in a 7-ml cuvette

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Abbreviations: HPLC, high-pressure liquid chromatography; LTC, leukotriene C; MCT, mast cell tumor; SRS, slow-reacting substance.

* Permanent address: Department of Pharmacology, University of Colorado Medical School, Denver, CO 80262.

with Tyrode's solution containing atropine (1 μM) and pyrilamine maleate (1 μM). One unit of SRS activity was arbitrarily defined as the contractile response when 100 μl of an in-house SRS standard solution was added to the cuvette. The SRS standard consisted of aliquots of the supernatant medium after ionophore challenge of human leukocytes. This supernatant had been freeze-dried in multiple vials and stored at −20°C. The lyophilized residue reconstituted with 1 ml of distilled water was the SRS standard solution. In over 50 different ileum preparations, this SRS unit corresponded in response to that of 5 ng of histamine hydrochloride. The procedures and precautions described by Orange and Austen (4) were followed in this bioassay.

Unknown samples (1–200 μl) were added to the cuvette and the responses were recorded. One criterion of SRS activity was that, at maximal contraction of the ileum, the addition of 0.1 μg of FPL 55712 caused an immediate relaxation of the ileum to greater than 50%.

Isolation and Purification. By following published procedures (6, 7), the residue soluble in 80% ethanol was hydrolyzed with 0.3 M NaOH for 30 min at 37°C. After neutralization the solution was passed over an XAD-8 anion-exchange column (Rohm and Haas, 5 × 8 cm); washed with 100 ml of H_2O, and eluted with 80% ethanol. The ethanol eluate was applied to a silicic acid column (4 g, Silicar CC-7, Mallinkrodt, St. Louis, MO) and sequentially eluted with 50-ml portions of ether/hexane 3:7 (vol/vol), ethyl acetate, 5% (vol/vol) methanol, 10% methanol, and 50% methanol in ethyl acetate, and methanol.

Reversed phase HPLC of the partially purified SRS was performed on a column (4.6 × 250 mm) of Nucleosil C_{18} and a column (10 × 500 mm) of Polygosil 60-D_5 C_{18} (both 5 μm particles, Macherey–Nagel Co., Düran, Germany). A variable wavelength ultraviolet spectrophotometer (Spectromonitor II from Laboratory Data Control, Riviera Beach, FL) and radioactivity monitor (scintillation detector, Berthold BF 5025, Weldbad, Germany) were used as detectors in series. The solvent used for the Nucleosil C_{18} column was methanol/water 65:35 (vol/vol) plus 0.01% acetic acid; the solvent for the Polygosil 60-D_5 was methanol/water 7:3 (vol/vol) plus 0.02% acetic acid, adjusted to pH 5.7 with ammonium hydroxide. Samples, from the silicic acid column were dissolved in 2 ml of methanol/water 1:1 (vol/vol) and injected onto the Polygosil column with a flow rate of 4.5 ml/min. The effluent was collected in fractions and tested for biological activity. Active fractions were dissolved in 200 μl of methanol/water and rechromatographed on the Nucleosil C_{18} column at a flow rate of 1.0 ml/min.

RESULTS

Purification and Isotope Incorporation. The purification procedure used for SRS produced by ionophore stimulation of MCT cells was an extension of published procedures (6). Recovery of biological activity in the methanol fraction from silicic acid chromotagraphy was typically 50% of the activity of the starting crude SRS. Two consecutive reversed phase HPLC purifications of the active material of the silicic acid separation stage provided essentially pure SRS. Fig. 1 shows the UV spectrum of the biologically active fraction collected during the final HPLC of MCT SRS. The spectrum had an absorbance maximum at 280 nm with shoulders at 270 and 292 nm. This HPLC fraction was highly active in the bioassay and the SRS response was inhibited by FPL 55712 (*inset A* of Fig. 1). Ten fractions were taken during elution of MCT SRS in another, final, HPLC purification and the absorbance at 280 nm and biological activity were determined. *Inset B* of Fig. 1 presents the linear semilogarithmic correlation between the absorbance and the SRS response. Three different bioassay determinations

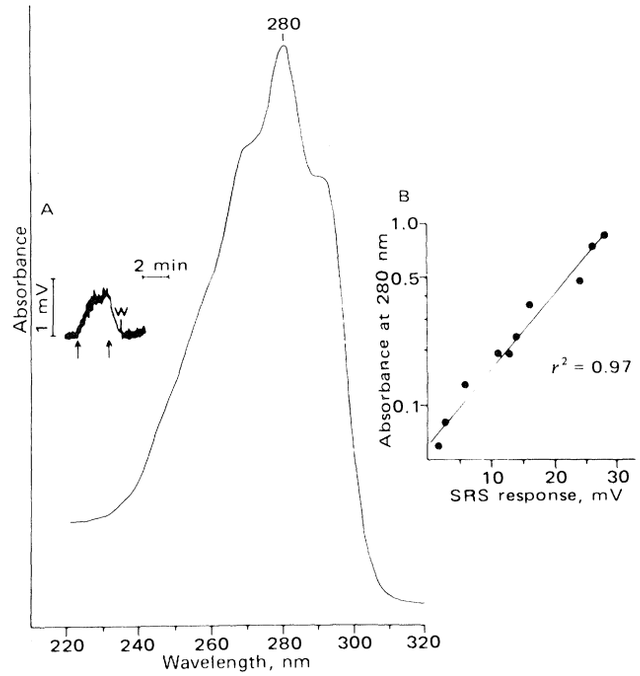

FIG. 1. Ultraviolet spectrum of the SRS (LTC-1) purified by reversed-phase HPLC from MCT. (*Inset A*) Recording of the contraction of the guinea pig ileum after addition to the organ of an aliquot of the UV-absorbing material from the MCT seen in this figure. First arrow, addition of LTC-1; second arrow, addition of FPL 55712; W, wash. (*Inset B*) Correlation between the absorbance at 280 nm of HPLC fractions collected during the elution of the MCT SRS and the plateau response from an isolated guinea pig ileum bioassay after addition of 1 μl of the collected fraction. The least-square linear regression line was calculated.

were made of these samples and the correlation coefficient for this relationship ranged from $r^2 = 0.87$ to 0.97.

The possibility that arachidonic acid and cysteine were precursors of MCT SRS was investigated by using high specific activity radiolabeled species. Table 1 lists the incorporation of [^{3}H]arachidonic acid and [3-^{3}H]-, [*U*-^{14}C]-, and [^{35}S]cysteine into two biologically active HPLC components (named LTC-1 and LTC-2 in the table and below) with the characteristic UV spectra in Fig. 1. The HPLC elution of the components from experiment A (Table 1) absorbing at 280 nm and containing ^{35}S can be seen in the recordings of the UV monitor and radioactivity monitor in Fig. 2 *upper*. At a retention time near 32 min a component eluted that caused superposition of these two traces. Fig. 2 *lower* presents the bioassay results of fractions collected during this HPLC; the assays further superimpose at this retention time. The majority of the ^{3}H-containing component eluted one fraction prior to that where ^{35}S content, 280 nm absorbance, and biological activity maximized. A similar isotope effect was observed for [$^3H_5$]- and unlabeled prostaglandin B_2 and resulted in partial separation during HPLC.

Chemical Degradations. A sample of LTC-1 (10 μg) was subjected to oxidative ozonolysis (26). Analyses by gas–liquid chromatography/mass spectrometry revealed the presence of 1,5-pentane-dicarboxylic acid. To avoid loss of tritium at vinyl positions, reductive ozonolysis was performed on LTC-1 from experiment A in Table 1: A sample (8.4 × 10^4 dpm ^{3}H) was treated with ozone at −15°C for 2 min and with sodium borohydride (2 mg) at 0°C for 10 min and at 20°C for 40 min. 1-Hexanol (0.1 mg) was added and the acidified sample was extracted with diethyl ether. HPLC analysis of the extract (Fig.

Table 1. Incorporation of [$^3H_8$]arachidonic acid and [3-^{3}H]-, [*U*-^{14}C]-, and [^{35}S]cysteine into SRS from MCT cells after reversed phase HPLC purification[a]

Component	Retention time,[b] min	Exp. A[c] incorporation, dpm		Exp. B[d] incorporation, dpm		Exp. C[e] incorporation, dpm	
		[^{3}H]$C_{20:4}$	[^{35}S]Cys	[3-^{3}H]Cys	[^{35}S]Cys	[3-^{3}H]Cys	[*U*-^{14}C]Cys
LTC-1[f]	31.8	1,490,000 (0.027%)	410,000 (0.056%)	97,200 (0.014%)	181,000 (0.020%)	15,700 (0.0020%)	1910 (0.0022%)
LTC-2[g]	35.2	31,000 (0.006%)	13,200 (0.002%)	14,100 (0.002%)	34,400 (0.003%)	12,500 (0.0016%)	1030 (0.0012%)

Numbers in parentheses are calculated percent of added precursor incorporated.

[a] Mobile phase, 65:35 methanol/water. Prior HPLC used 70:30.

[b] Retention time calculated at the maximum of the UV absorbance.

[c] [^{3}H]Arachidonic acid ($C_{20:4}$) (250 μCi) in 2 ml of methanol was added dropwise to the cell suspension (9.6×10^9 cells) 60 min prior to A23187. [^{35}S]Cysteine (330 μCi) was added 15 min prior to A23187.

[d] [3-^{3}H]Cysteine (300 μCi) and [^{35}S]cysteine (400 μCi) were combined and added to the cell suspension (7.4×10^9 cells) 15 min prior to addition of A23187.

[e] [3-^{3}H]Cysteine (355 μCi) and [*U*-^{14}C]cysteine (39 μCi) were combined and added to the cell suspension (5×10^9 cells) 15 min prior to addition of A23187.

[f] UV spectrum with λ_{max} 280 nm, shoulders at 270 and 292 nm.

[g] UV spectrum with λ_{max} 278 nm, shoulders at 268 and 290 nm.

3*A*) showed a major radioactive component coeluting with carrier 1-hexanol. Gas–liquid chromatography before (not shown) and after trimethylsilylation (Fig. 3*B*) showed single radioactive components that cochromatographed with 1-hexanol and 1-hexanol trimethylsilyl ether, respectively.

Desulfurization of LTC-1 (from experiment A, Table 1) with Raney nickel proceeded smoothly in refluxing ethanol. A ^{3}H-containing product was extracted by diethyl ether, methylated by diazomethane, purified by silicic acid chromatography, and analyzed by gas/liquid chromatography after trimethylsilylation (column, 3% OV-17). The 30% ether in hexane eluate contained three radioactive components. A minor component was methyl arachidate. The major component corresponded to an equivalent chain length of 21.5 carbons. The mass spectrum is given in Fig. 4*A*. Ions were present at mass-to-charge ratio *m/e* 399 (parent ion M − 15), 367 (M − CH_3 − CH_3OH), 313 [M − 101, loss of $\cdot CH_2(CH_2)_2COOCH_3$], and a base peak at 203 [$Me_3SiO^+ {=}CH(CH_2)_3COOCH_3$]. There were also ions at *m/e* 311, 309, and 307, which indicated the presence of molecular species with one, two, and three double bonds from incomplete reduction. A mass spectrum of authentic 5-hydroxyarachidic acid (same derivative) was virtually identical to that of desulfurized LTC-1 (Fig. 4*B*).

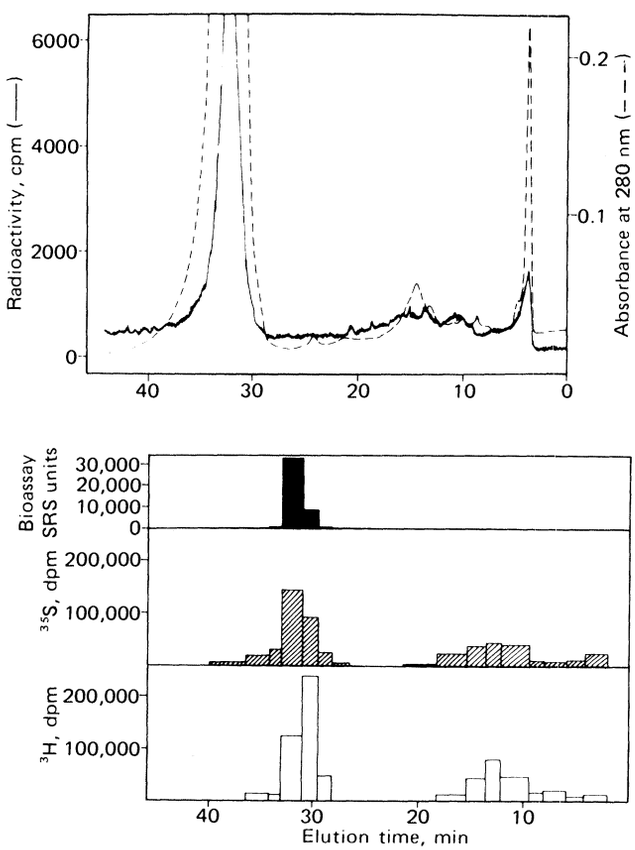

FIG. 2. Reversed phase HPLC [Nucleosil C_{18}, 5 μm; mobile phase 65:35 (vol/vol) methanol/water + 0.01% acetic acid] of MCT SRS (component LTC-1) previously purified by silicic acid chromatography and Polygosil C_{18} HPLC. The tumor cells were prelabeled with 250 μCi of [$^3H_8$]arachidonic acid and 330 μCi of [^{35}S]cysteine. (*Upper*) The radioactivity monitor and the ultraviolet monitor were connected in series at the effluent of the HPLC column. (*Lower*) Results of bioassay for SRS and calculated ^{3}H and ^{35}S contents from dual-channel scintillation counting of fractions collected during the chromatography.

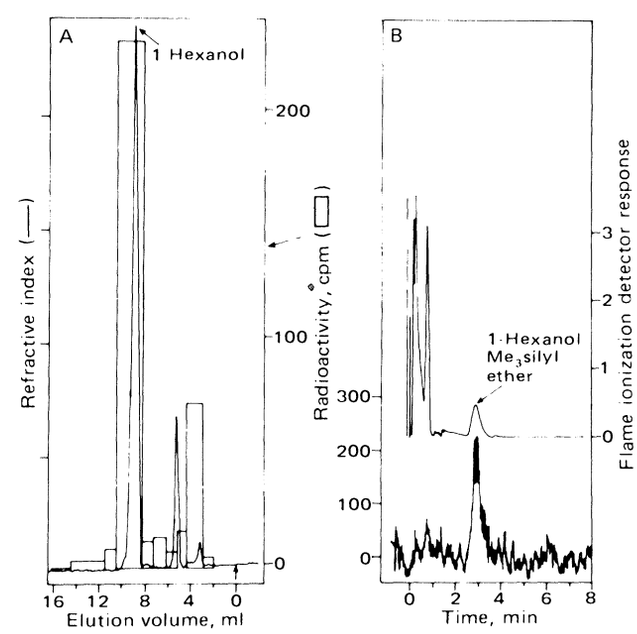

FIG. 3. Reductive ozonolysis of LTC-1 from [$^3H_8$]arachidonic acid. (*A*) HPLC of ether-extractable products. 1-Hexanol was added as internal reference. Stationary phase C_{18} μ-Bondapak; mobile phase 50% aqueous methanol + 0.01% acetic acid; flow rate 1 ml/min. Radioactivity was determined after addition of Instagel to HPLC fractions. (*B*) Gas–liquid chromatogram of ether-extractable products (trimethylsilyl derivative). 1-Hexanol was added prior to derivative formation. Stationary phase 1.6% OV-1 (Applied Science); column temperature 52°C.

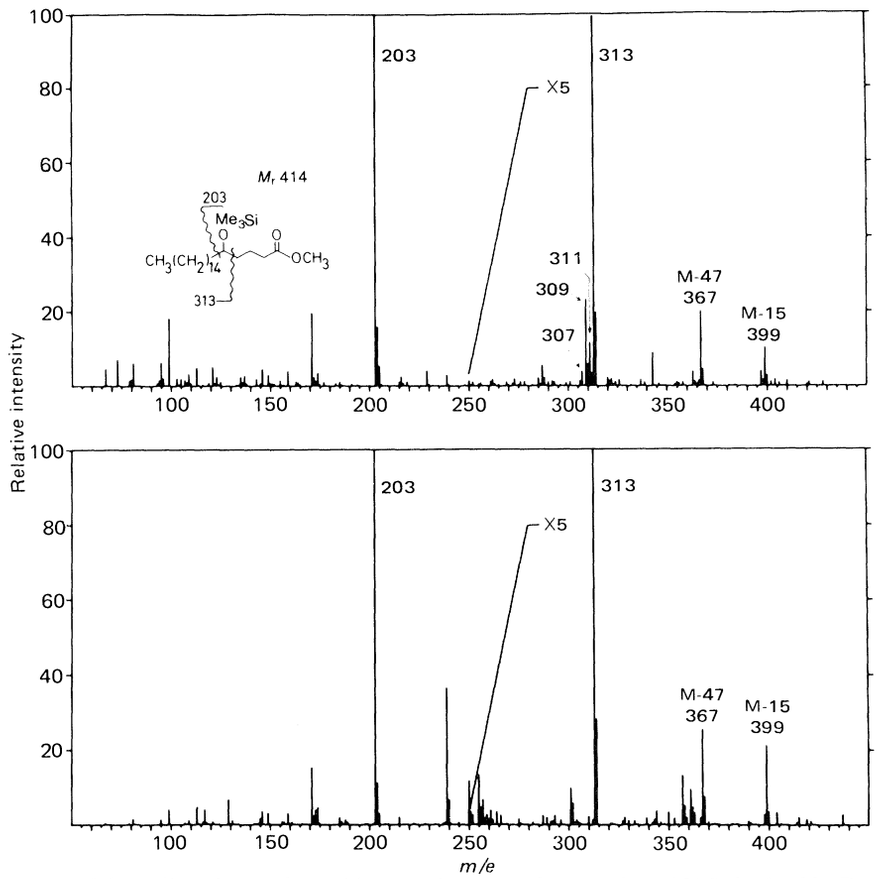

FIG. 4. (*A*) Mass spectrum of the radioactive product with an equivalent carbon number of 21.5 obtained by Raney nickel desulfurization of LTC-1 after methylation and trimethylsilylation. (*B*) Mass spectrum of the Me_3Si derivative of the methyl ester of 5-hydroxyarachidic acid.

Conversion by Lipoxygenase. LTC-1 (2 μg) was dissolved in 1 ml of Tyrode's buffer at 20°C and 10 μg of lipoxygenase was added. Ultraviolet spectra recorded before enzyme addition and after 5 and 60 min (Fig. 5) showed a clear bathochromic shift of the triene absorption by 30 nm. This indicates formation of a conjugated tetraene from LTC-1.

Amino Group Content. Part of the major fraction containing LTC-1 in Fig. 2 was quantitatively analyzed for the presence of amino groups by using fluorescamine (27). The sample estimated to be 4.5 nmol LTC-1 from the 280 nm absorbance (assuming ϵ = 40,000 M^{-1} cm^{-1}; cf. ref. 23) yielded fluorescence corresponding to 5.5 nmol of cysteine. Because adjacent HPLC fractions gave a background value corresponding to 1.1 nmol of cysteine, the amino group content of LTC-1 was 0.98 mol/mol.

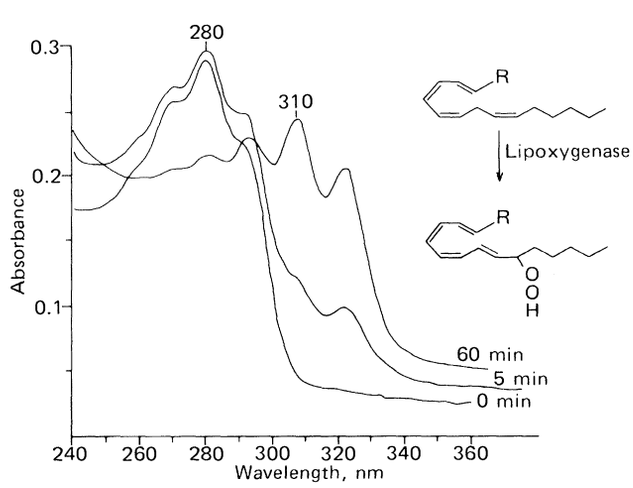

FIG. 5. Alteration of the UV spectrum of LTC-1 (2 μg) during incubation for the indicated times with 10 μg of lipoxygenase in Tyrode's buffer.

DISCUSSION

Experiments with isotopically labeled arachidonic acid and cysteine suggested that LTC-1 is derived from these compounds: Data obtained with [^{35}S]-, [3-^{3}H]-, and [*U*-^{14}C]cysteine (Table 1) indicated incorporation of sulfur, C-1, C-2, and C-3 and hydrogen at C-3. Furthermore, all carbon atoms of arachidonic acid and an amino group were present in LTC-1. The UV spectrum with λ_{max} at 280 nm and shoulders at 270 and 292 nm is similar to that of recently discovered dihydroxy metabolites of arachidonic acid with conjugated triene chromophores (25). The λ_{max} of LTC-1, however, is approximately 10 nm higher than the λ_{max} of the dihydroxy compounds, suggesting an auxochrome allylic to the triene in LTC-1.

The amino acid portion of the molecule was removed by Raney nickel, suggesting attachment by a C—S bond. The treatment also reduced the double bonds of LTC-1, because the UV absorbance was lost and the product obtained was mainly [^{3}H]-5-hydroxyarachidic acid. Incubation of LTC-1 with lipoxygenase converted it to a product with λ_{max} at 310 nm. The lipoxygenase requires a *cis,cis*-1,4-pentadiene structure with the methylene carbon atom at ω-8 (28). LTC-1 thus appears to have double bonds at ω-6 and ω-9. Further evidence for an ω-6 double bond came from reductive ozonolysis, which converted the [^{3}H]arachidonyl part of LTC-1 to [^{3}H]-1-hexanol in close to theoretical yield. The lipoxygenase experiment suggested that the conjugated triene of LTC-1 was extended to a conju-

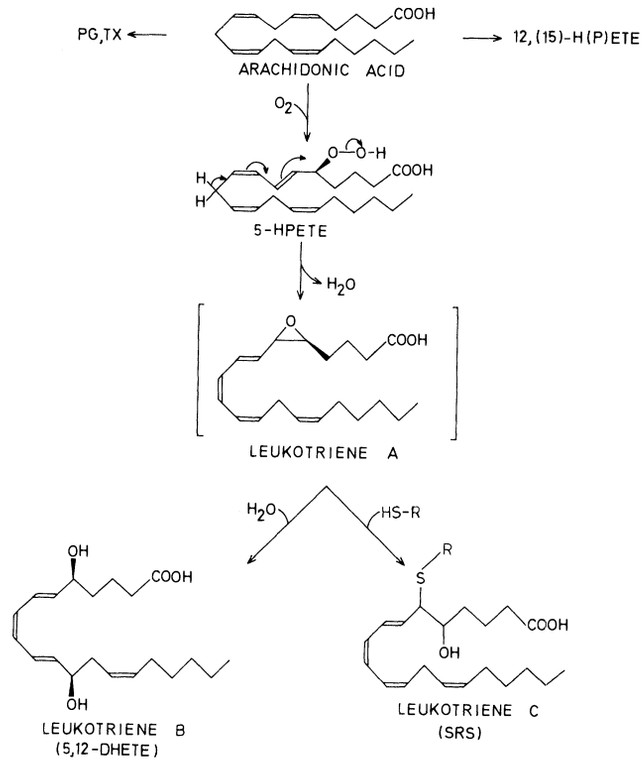

FIG. 6. Proposed scheme of transformations of arachidonic acid leading through 5-hydroperoxy-6,8,11,14-icosatetraenoic acid (5-HPETE) to the previously identified conjugated triene epoxide (22) (Leukotriene A) and 5,12-dihydroxyicosatetraenoic acid (23) (leukotriene B) in leukocytes and to SRS (leukotriene C) in murine mastocytoma cells. PG, prostaglandin; TX, thromboxane.

gated tetraene. Because the enzyme isomerizes ω-6 double bonds to ω-7 double bonds and introduces a molecule of oxygen at the ω-5 position, this requires two additional double bonds at ω-11 and ω-13. Therefore, the arachidonic acid moiety of LTC-1 is 5-hydroxy-7,9,11,14-icosatetraenoic acid.

Attachment of the amino acid to C-1 through C-5 and C-15 through C-20 of the 5-hydroxyicosatetraenoic acid is excluded from the products obtained after ozonolysis (1,5-pentanedioic acid and 1-hexanol). Substitution on the triene chromophore (C-7 through C-12) is unlikely because the λ_{max} of the ultraviolet spectrum should be at a higher wavelength (29). This leaves C-6, C-13, and C-14 as possible sites. A C—S— at C-13 or C-14 would presumably prevent conversion by soybean lipoxygenase. Moreover, sulfur substitution at either of these sites is not consistent with the UV spectrum of lipoxygenase-treated LTC-1 (29). Thioether bonds at allylic positions give bathochromic shifts of the same magnitude as that observed for LTC-1 compared to the dihydroxylated arachidonic acid metabolites in leukocytes (30). Therefore attachment at C-6 agrees with the UV spectrum and the other experimental data. Although the isotope experiments indicated incorporation of a whole cysteine molecule, the data do not rule out that the substituent at C-6 is a derivative of cysteine. Furthermore, the configurations assigned to the double bonds at C-7 and C-9 are tentative (Fig. 6).

The structural similarity of LTC-1 and recently described arachidonic acid metabolites in leukocytes (22–25) suggests that they have a common biosynthetic pathway, involving initially 5-hydroperoxy-6,8,11,14-icosatetraenoic acid (Fig. 6). The name "leukotriene" has been introduced for the conjugated trienes subsequently formed: leukotriene A is the unstable 5,6-epoxy-7,9,11,14-icosatetraenoic acid; leukotriene B is its enzymatic hydrolysis product, 5,12-dihydroxy-6,8,10,14-icosatetraenoic acid; and leukotriene C is the SRS described in this paper (21).

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