CHARACTERIZATION OF SLOW REACTING SUBSTANCES FROM CAT PAWS

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by

JIN-KEON PAI

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Approved by Thesis Reading Committee:

______________________________ ______________________________
Major Professor Date of Examination

Carl K. Becker

______________________________ ______________________________
Dean, Graduate School

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By

JIN-KEON PAI

(Under the supervision of Professor Charles J. Sih)

Two slow reacting substances (SRSs) were released after perfusion of cat paws with Compound 48/80. Using a purification procedure consisting of XAD-7 column chromatography, ethyl acetate extraction, disposable silica gel column, and reverse-phase HPLC, SRS I and SRS II, were purified. On the basis of spectroscopic and enzymic experiments, chemical degradations, amino acid analyses, and comparison with synthetic samples, SRS I was characterized as 5-hydroxy-6-S-cysteinylglycyl-7,9,11,14-icosatetraenoic acid (SRS-CysGly), and SRS II as 5-hydroxy-6-S-cysteinyl-7,9,11,14-icosatetraenoic acid (SRS-Cys).

The smooth muscle-contractile activities of synthetic 5-hydroxy-6-S-glutamylcysteinylglycyl-7,9,11,14-icosateraenoic acid (SRS-GSH), synthetic SRS-Cys and SRS II were not inactivated by limpet arylsulfatase. However, this arylsulfatase preparation reduced activities elicited by both synthetic SRS-CysGly and SRS I at the same rate. This mode of inactivation is attributed to a contaminating

enzymatic isomerization of the C-11(12) double bond (11-cis → 11-trans), catalyzed by thiyl radical (RS•).

APPROVED: ______________________
Professor Charles J. Sih

DATE: 8/20/8_

To my parents
my wife
my sons

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Professor Charles J. Sih for his guidance and support during the course of this work.

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Abbreviations:

SRS, slow-reacting substance; SRS-A, SRS of anaphylaxis; HPLC, high-pressure liquid chromatography; 5-HPETE, 5-hydroperoxy-6-trans-8,11,14-cis-icosatetraenoic acid; SRS-GSH, 5-hydroxy-6-γ-glutamylcysteinylglycyl-7,9,11,14-icosatetraenoic acid (leukotriene C, LTC); SRS-CysGly, 5-hydroxy-6-S-cysteinylglycyl-7,9,11,14-icosatetraenoic acid (leukotriene D, LTD); SRS-Cys, 5-hydroxy-6-S-cysteinyl-7,9,11,14-icosatetraenoic acid (leukotriene E, LTE); leukotriene A, 5-trans-5,6-oxido-7,9,11,14-icosatetraenoic acid (LTA); HTMP, 4-hydroxy-2,2,6,6-tetramethyl-piperidinooxy free radical; GSH, glutathione.

I. INTRODUCTION

Bronchial asthma, which affects an estimated 6 to 8 million Americans, is a clinical state of heightened tracheobronchial tree reactivity to numerous stimuli (56). The many stimuli, which provoke asthmatic episodes, may do so by eliciting immune sequences, by acting directly as toxins or irritants, or by a combination of mechanisms. Of the potentially vast number of substances in the environment capable of inciting an asthmatic reaction, those which are antigenic are more significant because they evoke the immune response, and therefore elicit more severe symptoms.

Allergic bronchial asthma, and other allergic diseases such as hay fever and anaphylaxis, are examples of immediate hypersensitivity (Type I, Table 1). Such allergic reactions take place in specific target organs, such as the lungs, gastrointestinal tract, or skin. The immune processes that generate a hypersensitivity reaction represent the disease state referred to clinically as allergy. Immediate hypersensitivity reactions can result in a simple acute inflammatory response (urticaria), or they can cause complex reactions that may be systemic (anaphylaxis) or predominantly bronchial smooth muscular (asthma). The immune sequence consists of a

TABLE 1: Immune reactions (primarily pulmonary) (56).

IMMUNE REACTIONS
(Primarily Pulmonary)

Type of Immune Reaction	Time of Reaction	Examples of Antigen	Antibody or Cell	Clinical Examples
I Immediate hypersensitivity	15 to 30 minutes	Pollens, danders, foods	IgE	Extrinsic asthma, anaphylaxis, hay fever
II Cytotoxic response	—	Cell membrane, basement membrane	IgG, IgM (± complement)	Hemolytic anemia, Goodpasture's syndrome
III Antigen-antibody complexes (Arthus phenomenon)	6 to 8 hours	Moldy hay (thermophilic actinomycetes), other organic dusts	IgG (+ complement)	Allergic alveolitis (farmer's lung)
IV Delayed hypersensitivity (cell-mediated hypersensitivity)	48 hours	Tuberculoprotein, poison ivy extract	Sensitized lymphocytes, monocytes	Tuberculosis, contact dermatitis

sensitization phase and a subsequent challenge reaction, which produces the clinical syndrome of allergy.

In the sensitization phase, a genetically atopic patient is exposed to antigen (e.g., ragweed pollen, which is responsible for seasonal ragweed hay fever). Lysozymes from the respiratory mucosa digest the outer lipid-polysaccharide coating of the pollen, releasing water soluble proteins. As these proteins are absorbed, plasma cells within the lymphoid tissues of the upper or lower respiratory mucosa respond by forming a specific cytotropic antibody of the IgE class. These IgE molecules attach to the surfaces of the mast cells or of other cells, such as basophils.

After a latent interval of variable duration, a patient's reexposure to the specific antigen may result in an allergic reaction, which is characterized by the secretory release of pharmacologic mediators either from mast cells located in the respiratory mucosa or from circulating basophils. In the presence of certain cations, such as calcium, the specific antigen reacts with IgE on the membrane of sensitized mast cells or basophils, which respond by releasing pharmacologic mediators as slow-reacting substance of anaphylaxis (SRS-A), histamine, eosinophil chemotactic factor of anaphylaxis (ECF-A), serotonin, various kinins and prostaglandins (10)

(Fig. 1). Bronchial smooth muscle, blood vessels and mucous glands respond to these substances with muscular contractions, vasoconstriction, and hypersecretion of mucus along with an inflammatory response consisting of increased capillary permeability and cellular infiltration.

The release of mediators from mast cells is influenced or controlled by the intracellular concentration of cyclic AMP (10,55), which has been found to inhibit the immunologically induced release of histamine and other mediators. Thus, adequate concentration of cyclic AMP (as would result from β-adrenergic stimulation in conjunction with sufficient amounts of adenyl cyclase) will inhibit SRS-A release; inadequate concentrations of cyclic AMP, as with blockade or adenyl cyclase deficiency, will enhance SRS-A release and produce the clinical symptoms of asthma (Fig. 2).

The most thoroughly studied mediator is histamine, a vasoactive amine widely distributed in body tissues, particularly those of the lung. Histamine is concentrated as granules within tissue mast cells. The release of histamine causes increased capillary permeability and vasodilation, with resulting edema and infiltration by inflammatory cells. In vitro, histamine release can be

FIGURE 1. Schematic representation of mediator release in immediate hypersensitivity (Type 1) reactions.

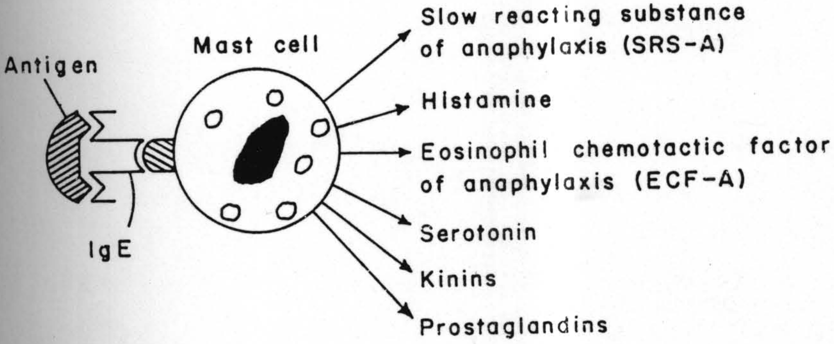
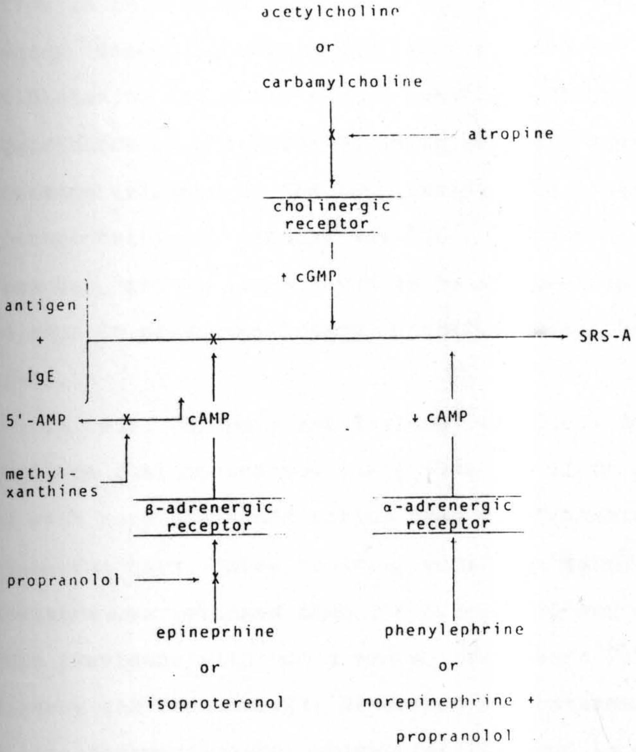

Antigen
Mast cell
IgE
Slow reacting substance of anaphylaxis (SRS-A)
Histamine
Eosinophil chemotactic factor of anaphylaxis (ECF-A)
Serotonin
Kinins
Prostaglandins

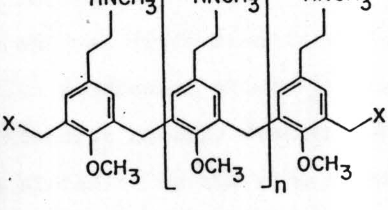
FIGURE 2. Opposing effects between cyclic 3',5'-AMP and cyclic 3',5'-GMP upon the release of SRS-A.


acetylcholine
or
carbamylcholine
atropine
cholinergic receptor
↑ cGMP
antigen
+
IgE
SRS-A
5'-AMP
cAMP
↓ cAMP
methyl-xanthines
β-adrenergic receptor
α-adrenergic receptor
propranolol
epineprhine
or
isoproterenol
phenylephrine
or
norepinephrine +
propranolol

demonstrated as occurring after leukocytes or lung tissues of allergic individuals are exposed to appropriate antigens. This experimental evidence implicates histamine action in the immune response. In vivo observations, however, detecting only minor clinical response to antihistamine drugs has raised questions about the significance of histamine in human asthma. Another substance released by the lung having more long-lasting bronchoconstrictor effects was later discovered. This spasmogen, SRS-A, is believed to be the most important mediator in producing bronchoconstriction of the asthmatic crisis.

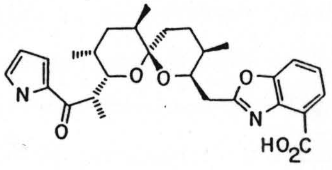
In 1938, Feldberg and Kellaway (1) first described a substance that contracted the guinea pig ileum more slowly and with more sustained action than did histamine; they coined the term, "slow reacting substance (SRS)." The substance was released from the guinea pig and cat lungs after treatment with cobra venom. Two years later, Kellaway and Trethewie(2) described a substance with similar pharmacological properties released in outflowing perfusate of sensitized guinea pig lungs during anaphylaxis induced by specific antigen challenge. On the basis of the slow and prolonged contraction produced on the guinea pig ileum, these authors suggested that this substance was different from histamine. Since then, SRS has been observed to be released from many types of tissues and cell suspensions, including guinea pig, rabbit, monkey, bovine and human lungs (3-5), cat paws (6,7), isolated mast cells (8), human leukocytes (9), nasal polyps (10), human and rat leukemic basophils (11,12), rat peritoneal fluid (13,14), mouse mastocytoma cells (15), and mouse macrophages (16).

Initially, SRS-A was produced from tissues sensitized by active immunization with a specific antigen, and subsequently from tissues passively sensitized by incubation with the appropriate antibody. The reaction of p-methoxy-N-methyl-phenethylamine with formaldehyde and hydrochloric acid produces a polymeric mixture known as Compound 48/80, which is thought to possess structures related to 1 (17,18). Unsensitized tissues perfused with Compound 48/80 release SRS-A as well as histamine through a mechanism which is not completely understood (19).



1 Compound 48/80

The calcium ionophore A23187 (2), produced by Streptomyces chartreusensis, has also been used to produce SRS-A in unsensitized tissue (11,20). Because no prior sensitization is required, the use of Compound 48/80 or A23187 simplifies the process of obtaining SRS-A for experimental work. Using the most discriminating procedures and pharmacological tests available, Brocklehurst (22) observed no difference between the SRS release by Compound 48/80 from cat paws (6,21) and SRS-A released via the antigen-antibody reaction of the guinea pig lung. He concluded that SRS could also be formed as a result of mild tissue damage other than via immunological reaction.



2 Calcium Ionophore
A23187

Although SRS was discovered in 1938, its characterization progressed slowly in the early 1950's. In 1953, Brocklehurst clearly distinguished the SRS activity from histamine by the slow onset and sustained nature of the response as well as by the failure of antihistamines to inhibit this activity (23). Thus, SRS-A is detected by biological assay on guinea pig ileum in the presence of an antihistamine (19). A review covering the pharmacology and chemistry of SRS was published in 1962 by Brocklehurst (24). Brocklehurst developed a method of extraction based on the ability of SRS-A to adsorb on partially activated charcoal and its subsequent displacement with n-butanol. He found that SRS may also be partially purified by reextraction of alcoholic extracts with ether at pH 3.0. The solubility of SRS in 80% methanol and ethanol as well as its resistance to proteolytic enzymes suggested that SRS was not a protein. Electrophoretic migration toward anode implied its acidic nature. However, subsequent progress was impeded by the increased lability with increased purity and the high loss of activity due to adsorption on supporting media (Table 2).

Despite considerable studies on SRS-A pharmacology and its mechanism of release, intensive chemical identification studies were not made until the mid 1970's.

Austen et. al. obtained SRS-A from the peritoneal cavity of passively sensitized rats and from human lung fragments. Using their purification techniques, they obtained a 40,000-fold purification, but no reliable information was derived from mass spectrometric analyses. After submitting the SRS-A preparations to

Table 2. Physico-chemical properties of SRS

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1. Stable to boiling at pH 7-8.
2. Better stability in alkaline solution.
3. Resistant to proteolytic enzyme.
4. Solubility in 80% methanol and ethanol.
5. Insoluble in acetone and chloroform.
6. Inactivated by peroxides of butanol or ether.
7. Electrophoretic migration towards anode (+) (sodium phosphate, pH 7,8).

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*According to Brocklehurst (18).

spark source mass spectrometry, they noted an abundance of sulphur (25,26). Also, they observed the enzymatic inactivation of SRS-A activity by limpet arylsulfatase. On the basis of the apparent specificity of arylsulfatase inactivation of SRS-A bioactivity and of their mass spectrometric experiments, they suggested that a sulphate ester group was present in the molecule. The presence of a sulphate moiety in the SRS-A molecule and resultant lowered vapor pressure appeared to explain their inability to obtain mass spectra with either chemical ionization or electron impact techniques (26).

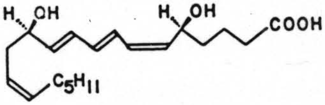
The spasmogenic activity of SRS-A was further shown to be inhibited by a variety of arylsulfatases from human lung (27), rat peritoneal cells (28) and human eosinophils (29). It was suggested that arylsulfatase could be involved in the process of SRS-A inactivation <u>in vivo</u> after an antigen-antibody reaction. Study of the kinetics of the reaction as well as of the effects of purified enzyme supported the specificity of arylsulfatase for SRS-A degredation. Moreover, susceptibility to arylsulfatase inactivation was used until recently as an important criterion in the positive identification of SRS-A.

The presence of sulphur in the SRS-A molecule was further indicated by observations that various sulphur-containing compounds such as cysteine, sodium sulfide and thioglycolate could enhance antigen-induced formation and release of SRS-A (30,31). More recently, SRS production from rat mononuclear cells was markedly enhanced by the addition of mercaptans to the incubations (32). Structure-activity studies revealed that mercapto-carboxylic acid is essential for the enhancement of SRS production, and that optimal activity is found in a molecule having a 2-carbon space between the carboxyl and mercapto group. But the critical question regarding the enhancing activity of the mercaptans in SRS-A production was whether any part of the mercaptans, and especially the sulfur atom, was incorporated into the SRS-A molecule. This question remained unanswered. An attempt to demonstrate such an incorporation, using ^{35}S-labeled cysteine, failed in one study by Orange and Chang (30). Another study by Dawson et. al. (33) using ^{35}S methionine or sodium sulphate, showed that a radiolabel traveled like SRS-A. Because the incorporation of radioactive sulphur was relatively low and the purification on Amberlite XAD-2 and silicic acid was not complete, their data provided at best only partial support to the hypothesis that SRS-A is a sulphur-containing compound.

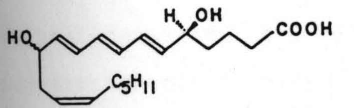
The possible involvement of arachidonic acid in SRS synthesis was studied by means of producing SRS in the presence of radiolabeled and unlabeled arachidonic acid and determining whether the yield of SRS was altered and whether radioactivity comigrated with SRS after purification. Jakschik et. al. (34) suggested that SRS-A could be synthesized from arachidonic acid. SRS appeared more polar than any of the known metabolites of arachidonic acid; it was suggested that SRS was a previously undescribed product of arachidonic acid metabolism, probably formed through a lipoxygenase pathway (35). Similar results were obtained by Bach et. al., using rat peritoneal mononuclear leukocytes (36). Although attempts to obtain radiochemically pure SRS from RBL-1 cells were successful, the success is a quantitatively minor one when viewed in the context of overall arachidonic acid metabolism in these cells. Only 0.1% to 0.5% of the arachidonic acid radioactivity originally added to the cells copurified with SRS (35). Even allowing for the moderate losses of SRS activity usually experienced during purification, the level of incorporation was considerably lower than expected. Also, the results are questionable because of insufficient purification of the product.

Using various enzymes to characterize SRS-A, Engineer et. al. (37) first noted the apparent loss of spasmogenic activity of guinea pig SRS-A after incubation with soybean lipoxygenase. Sirois _et. al._ showed that this enzyme was also capable of inactivating various preparations of SRS-A obtained from rat and mouse peritoneal cells as well as from passively sensitized chopped human lung (38). The inactivation of SRS-A by plant lipoxygenase was dependent upon concentration, time and temperature (39). These results strongly favored an enzyme-substrate reaction between SRS-A and lipoxygenase; on the basis of the specificity of this enzyme, the presence of a _cis_-_cis_-1,4-diene unit in the SRS-A molecule was postulated.

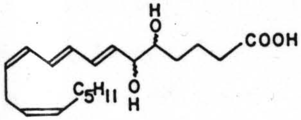
The most important step towards the structure-elucidation of SRS-A was the discovery of a new family of metabolites of arachidonic acid : the leukotrienes (LTs). Using polymorphonuclear leukocytes (PMNL) obtained from the peritoneal cavity of rabbits, it was found that 5(S)-hydroxy-6,8,11,14-icosatetraenoic acid (5-HETE) was the major arachidonic acid-derived product (40). This result is particularly interesting because it shows the existence of a new lipoxygenase pathway in leukocytes with specificity at the C-5 position. Additional study showed that more polar products were also formed. These were identified as: (a) 5(S),12(R)-dihydroxy-6,8,10,14-icosatetraenoic acid (LTB_4; Major product); (b) two additional 5(S),12-dihydroxy-6,8,10-_trans_-14-_cis_-icosatetraenoic acids epimeric at C-12; and (c) two isomeric 5,6-dihydroxy-7,9,11,14-icosatetraenoic acids (41,42).



5S,12R-DHETE
LTB$_4$



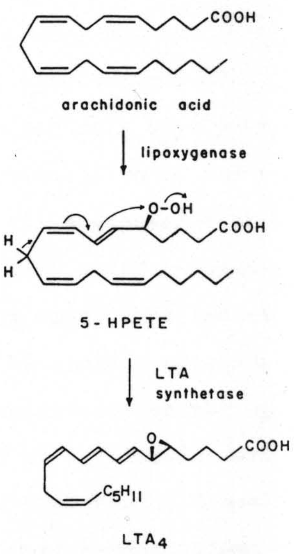
5S,12R-DHETE (trans,trans,cis)
5S,12S-DHETE (trans,trans,cis)



5,6-DHETE
(two isomers)

The structural similarities between LTB, the 5-hydroxy acid and the four dihydroxy metabolites (all carrying an hydroxyl group at C-5 with "S" configuration) strongly suggested a common pathway of biosynthesis. Using isotopic oxygen, it could be demonstrated that the oxygen of the hydroxyl group at C-5 originated from molecular oxygen, whereas the oxygen of the hydroxyl group at C-12 was derived from water (43). These observations suggested that an unstable intermediate was generated from arachidonic acid by the leukocytes. This intermediate could undergo nucleophilic attack by water, alcohols, and other nucleophiles. Trapping experiments with alcohols confirmed the existence of such an intermediate; indeed, after trapping with methanol or ethanol, 5-hydroxy-12-Oalkyl derivatives of arachidonic acid were isolated and identified. The intermediate was shown to be acid-labile and somewhat stabilized at alkaline pH. On the basis of these results, 5(6)-oxido-7,9,11,14-icosatetraenoic acid was proposed as the probable intermediate and was named Leukotriene A (LTA). The formation of the epoxide from arachidonic acid can be visualized as involving the initial formation of 5-hydroperoxy-6,8,11,14-icosatetraenoic acid (5-HPETE), the precursor of the 5-hydroxy acid. The epoxide is formed from 5-HPETE by abstraction of a proton at c-10, and elimination of hydroxyl anion from the hydroperoxy group (Scheme 1).

The notion that the unstable epoxide plays a role in the transformations of arachidonic acid in leukocytes was a prerequisite for the work leading to elucidation of the structure and biogenesis of slow reacting substance of anaphylaxis (SRS-A). Several pieces of experimental evidence led to the hypothesis that there is a biogenetic link between the unstable allylic epoxide intermediate and SRS-A. This is of particular interest, since previous studies had shown that the ionophore A23187 not only stimulates the synthesis of 5-HETE and 5,12-DHETE (44) but also stimulates release of SRS from leukocytes (45). SRS-A, as well as LTs, appear to be metabolites of arachidonic

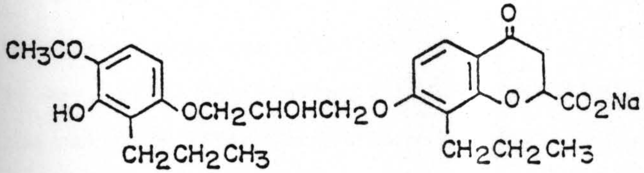


Scheme 1.Mechanism of formation of unstable intermediate, LTA_4.

acid. Indomethacin neither inhibit the leukocyte lipoxygenase responsible for the formation of 5-HETE (40) nor SRS-A biosynthesis (37). However, the hypothesis that a biogenetic link exists between SRS-A and the leukotrienes was only established after the publication of the ultraviolet spectrum of purified SRS-A by Morris *et. al.* (46). This spectrum revealed that SRS-A possessed the chromophore characteristic of every member of the LTs family: the conjugated triene.

The central position of LTA as the intermediate in the leukotriene pathway, and its susceptibility to chemical reactions with various nucleophilic agents, suggested its possible role as a precursor of SRS-A. Furthermore, the stimulation of SRS-A formation by thiols such as cysteine (30) suggested that SRS-A could result from the addition of a sulphur-containing compound to LTA to form a thioether bond. The addition of cysteine to LTA would be expected to yield a substance with chemical and chromatographic properties resembling those of SRS-A: a polar acidic lipid of a molecular weight around 500.

Based on this hypothesis, Samuelsson _et. al._ (47) prepared SRS from murine mastocytoma cells treated with ionophore A23187 and L-cysteine. The procedure used for isolation involved precipitation of protein with ethanol, alkaline hydrolysis, separation on Amberlite XAD-8 and silicic acid columns, and two reverse-phase HPLC steps. The material obtained by this procedure was essentially pure, showed an absorbance maximum at 280 nm, and produced a typical contraction of guinea pig ileum which was reversed by antagonist FPL 55712 (47). The UV spectrum resembled those of LTB, but its maximum was shifted 10 nm. It appeared identical to the spectrum published by Morris _et. al._ (46). Treatment of SRS with soybean lipoxygenase resulted in isomerization of the Δ^{14} double



FPL 55712

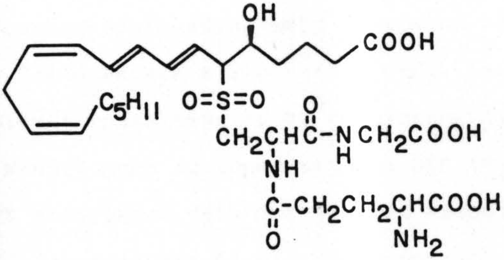
bond into conjugation with the conjugated triene, forming a tetraene, as evidenced by a bathochromic shift of 30 nm. This indicated the presence of a Δ^{11}-<u>cis</u> double bond and additional double bonds at Δ^{7} and Δ^{9} positions in SRS. Desulfurization of SRS by Raney nickel gave 5-hydroxyicosanoic acid, which indicated that the parent compound is an arachidonic acid derivative attached to cysteine via a thioether bond. The presence of the hydroxy group at C-5 in the fatty acid reinforced the hypothesis of a biogenetic relationship to LTA. Experiments with labeled precursors showed that arachidonic acid and cysteine were incorporated into the products. These findings represented a major step forward in the structure elucidation of SRS-A. At the Fourth International Prostaglandin Conference in May 1979 (48), Samuelsson reported the structure of an SRS from murine mastocytoma cells to be 5-hydroxy-6-S-cysteinyl-7,9,11,14-icosatetraenoic acid and termed it Leukotriene C (LTC). This structural assignment was later revised, and 5-hydroxy-6-γ-glutamylcysteinylglycyl-7,9,11,14-

icosatetraenoic acid was named LTC (49).

In May, 1979, we reinvestigated the purification of SRS from cat paws after perfusion with Compound 48/80. Several questions were posed:

a) Does the structure of an SRS depend on its cell origin or on the releasing stimulus? What is or are the structure(s) of SRS from cat paws? Is there a family of substances, all exhibiting SRS-like activity but different in chemical structure?

b) How is SRS-A inactivated by arylsulfatase? Would the sulfone-LTC (57) offer an explanation for



arylsulfatase inactivation of SRS-A?

c) A variety of the mercaptans were known to stimulate SRS production. What would be the mechanism of this stimulation? Are mercaptans incorporated into SRS as reported (47)?

II. EXPERIMENTAL

A. Materials and Methods

The following compounds or enzymes were obtained from the Sigma chemical company: Glutathione (GSH); L-cysteine; Arachidonic acid (99%); Type I soybean lipoxygenase (155,300 units/mg), Type V sulfatase (Patella vulgata); Histamine dihydrochloride and Compound 48/80. The following compounds were kindly supplied by the respective companies: Arachidonic ethyl ester (92%) (Hoffman La Roche); FPL 55712 (Fisons Limited); Ionophore A23187 (Eli Lilly). 4-Hydroxy-2,2,6,6,-tetramethyl-piperidinooxy (HTMP) free radicals was purchased from Aldrich. Chromatographic adsorbents consisted of Amberlite XAD-7 and Waters silica gel Sep-Pak cartridges, disposable columns containing 40 mg of packing material.

A model M-6000 pump equipped with an U6K injector and a model 440 UV absorbance detector (Waters Associates) were used for high-pressure liquid chromatography (HPLC). A microparticular reverse phase (C_{18}) preparative column (0.94 x 50 cm) of Partisil 10 M90DS (Whatman) was used for SRS purification. The radial compression separation system (RCSS) consisted of a Waters radial compression module (RCM-100) with a Radial-Pak C_{18} cartridge (0.8 x 10 cm), protected by a CO:Pell octadecylsilica precolumn (Whatman).

All solvents used for HPLC were degassed by warming and then sonicated with a Branson sonifier.

UV spectra were recorded on a Cary 118 spectrophotometer with a wavelength calibration accuracy of ± 0.1 nm at 165-300 nm.

Cat paws were perfused by the use of a Minipuls II peristaltic pump (Gilson Medical Electronics).

B. SRS Assay

SRS bioactivity was assayed on guinea pig ileum segments by a modification of the procedure reported by Chakravarty (50). Guinea pigs (300-500 g) were sacrificed by means of blows to the head and ileum strips of approximately 2.5 cm were removed at approximately 10 cm from the terminus. The tissue was rinsed free of debris with a 5 ml plastic syringe and the mesenteric tissue was removed. The ileum sections were placed in water-jacked 10 ml isolated tissue baths at 36°C. The tissue was attached at the bottom to a hook on a glass rod, and at the top to a horizontal level counterbalanced with a 500 mg weight. The lever was on a fulcrum which allowed it to swing freely. Due to the position of the fulcrum, the tissues' contractions were magnified approximately twelve-fold and recorded isotonically on a smoked drum by a wire attached to the end of a horizontal lever. The tissue bath and Tyrode's solution were continuously gassed with 5% CO_2 in O_2 (51).

Histamine was assayed in the presence of atropine (1 μM); SRS was assayed in the presence of atropine (1 μM) and mepyramine (1 μM). The tissue became more responsive to histamine after successive histamine doses, and eventually consistent contractions were obtained. After sensitization in this manner, the tissue more readily produced consistent contractions to SRS. One unit of SRS was defined as that amount of SRS which caused a contraction with a peak height equal to that induced by 5 ng of histamine base. After the log dose-response curve for histamine was determined, a similar curve of an in-house standard of SRS was derived and from this the other SRS samples were quantified. To obtain a higher degree of accuracy, SRS samples under comparison were assayed on the same ileal strip; a closed bracket type assay (52) was used, in which the standard SRS sample was assayed before and after assay of unknown samples. After each SRS sample, the tissue was rinsed at least twice with fresh Tyrode's solution. The ileum maintained contractile ability for 2-8 hours, depending upon the individual guinea pig ileum condition.

Only aqueous samples were bioassayed. Samples that had been evaporated to dryness were dissolved in 0.1% NH_4OH because water did not completely dissolve them. Large quantities (0.5 ml) of NH_4OH at this concentration had no effect on the ileum response. Samples of SRS were assayed in the presence of 20-40 nM FPL 55712, a reversible SRS inhibitor (53).

C. Cat Paw Perfusion

Cat paws were perfused by a modification of the procedure reported by Högberg et. al. (54). Cats (1.5-5 kg) of either sex were obtained through the University animal care system. All were examined by a veterinarian, treated prophylactically for infectious rhinotracheitis and distemper, and quarantined for ten days. The cats were anesthetized with sodium pentobarbital (50 mg/kg; intraperitoneally) and subsequently killed with an intravenous injection of 1 ml of saturated KCl. Immediately after the death of the animal, the main artery of each paw was cannulated with polyethylene tubing (0.6 mm inside diameter). The paws were cut off above the ankle joint and hung from a hook in a water-jacketed jar which was maintained at 25°C by a water circulating pump. The paws were perfused with 154 mM NaCl, 0.9 mM $CaCl_2$, 2.7 mM KCl containing 10% (vol/vol) Sőrensen phosphate buffer (Na_2HPO_4 + KH_2PO_4, 67 mM) at pH 7.0.

This buffered salt solution was maintained at 25°C and was simultaneously perfused through the set of four paws from each cat with a four-channel peristaltic pump. After the paws were perfused for 45 min to remove most of the blood, 50 μg of the releasing factor (Compound 48/80) was administered and the perfusion was continued for an additional 180 min. During this period, the effluents were collected by gravity drainage into flasks cooled to 4°C. As reported by others (6,7), no significant SRS activity could be detected in any samples prior to the administration of a releasing factor.

D. Purification of SRS

The ice-cold perfusate (4-4.5 liter) from two cats was passed over an XAD-7 column (2.2 x 17 cm). After the column was washed with 100 ml of distilled water followed by 20 ml of methanol, SRS was eluted with 125 ml of methanol. The elute was evaporated to dryness and stored at -25°C.

Crude SRS concentrate from 10 cats was pooled and dissolved in 50 ml of doubly distilled water, acidified to pH 2.5 with 0.1 M HCl, and extracted four times with equal volumes of ethyl acetate, which was evaporated to dryness under reduced pressure. The residue was dissolved in 0.7 ml of NH_4OH solution (pH 9.5), evaporated to dryness, and dissolved in 0.2 ml of $CH_2Cl_2/MeOH/H_2O$, 65:20:10 (vol/vol), before application onto a disposable silica gel column. The column was washed with 3 ml of $CH_2Cl_2/MeOH/H_2O$, 65:20:10 (vol/vol), and 2.5 ml of $CH_2Cl_2/MeOH/H_2O$, 65:30:10. SRS was then eluted with 4 ml of $CH_2Cl_2/MeOH/H_2O$, 65:40:10. After evaporation of the eluate to dryness, the residue was dissolved in 0.2 ml of $MeOH/H_2O$, 1:1, and chromatographed over a μC_{18} reverse phase preparative column of Partisil-10 (10 µm) M9ODS (Whatman), which was protected by CO:PELL ODS precolumn (Whatman). The solvent used for elution was $MeOH/H_2O$, 1:1, at a flow rate of 3 ml/min.

SRS I and SRS II from the preparative μC_{18} reverse-phase column was further purified by application onto a Radial-Pak C_{18} cartridge (0.8 x 10 cm). The cartridge was eluted with a mobile phase consisting of $MeOH/H_2O$ (70:30, vol/vol) plus 0.05% acetic acid, adjusted to pH 5.4 with NH_4OH, at a flow rate of 2 ml/min.

E. Inactivation of SRS by Soybean Lipoxygenase

SRS was dissolved in 1 ml of Tyrode's buffer at pH 7.8. This solution contained 5 nmol of SRS based on UV at 280 nm, $\varepsilon = 40,000$). The spectrum was plotted at 0 min in the absence of added enzyme. Soybean lipoxygenase (50 µg) was then added and spectra were recorded at various time intervals with a Cary model 118 spectrophotometer.

F. Inactivation of SRS by Arylsulfatase

The system contained 5 units of arylsulfatase (Type V) and varying amounts of SRS [500 units of SRS I or synthetic 5-hydroxy-6-S-cysteinylglycyl-7,9,11,14-icosatetraenoic acid (SRS-CysGly) or 5-hydroxy-6-γ-glutamylcysteinylglycyl-7,9,11,14-icosatetraenoic acid (SRS-GSH); 50 units of SRS II or synthetic 5-hydroxy-6-S-cysteinyl-7,9,11,14-icosatetraenoic acid (SRS-Cys)] in 1 ml of 0.1 M sodium acetate buffer at pH 5.7. Incubation was carried out at 37°C. At the indicated time intervals, an aliquot was removed from the reaction mixture and frozen at -78°C. This aliquot was dissolved in 1 ml of Tyrode buffer and the residual SRS activity was assayed. Control values were obtained by incubation of boiled enzyme with SRS for the time interval indicated.

To isolate the product after arylsulfatase inactivation, 20 μg of synthetic SRS-CysGly was incubated with 200 units of arylsulfatase (Type V) in 2 ml of 0.1 M sodium acetate buffer pH 5.7. After 30 min incubation at 37°C the reaction was stopped through the addition of 8 ml of ethanol. The mixture was cooled for 30 min at -25°C and then centrifuged. The supernatant was evaporated to dryness under reduced pressure. After adding small quantities of distilled water and acidifying to pH 3.0, it was extracted three times with ethyl acetate. HPLC separation was carried out on a Radial-Pak C_{18} cartridge (0.8 x 10 cm) using the radial compression modular system.

G. Amino Acid Analyses of SRS I and SRS II

Ten nmoles of SRS was suspended in 1 ml of 6N HCl. After the sample was frozen at -78°C, the tube was connected to a vacuum line and the system was evacuated using an oil pump (60 microns). When pressure of the system had reached 60 microns, the tube was withdrawn from the dry ice bath and the frozen solution was allowed to thaw slowly. As bubbles rise up in the tube, momentary immersion of the tube in the dry ice bath would break the bubbles and allow the liquid to fall back. The pressure was then increased to about 120 microns during this degassing process. When the pressure was back down to 60 microns, the tube was shaken to make certain that gas removal was complete, and then sealed off under vacuum with oxygen flame (78).

Hydrolysis was conducted at 110°C for 24 hrs. After the tube had cooled to room temperature, any liquid on the walls was spun down by gentle centrifugation. The tube was scored with a sharp file at a point belowed the tapered end and cracked by the use of a hot glass rod. HCl was removed using the Speed-Vac Concentrator (Savant Instrument). The concentrate was dissolved in 20 µl of citrate buffer (pH 2.2) and the tube was washed again with 20 µl same buffer. It was then injected onto the Durrum D-500 automated analyzer.

To determine NH_2-terminal residue, 6 nmoles of SRS I was dissolved in 10 µl of 0.2 M $NaHCO_3$ (pH 9.8). After the addition of DNS chloride (10 µl) (2.5 mg/ml in acetone) the tube was sealed with Parafilm. The tube was incubated for 1 hr at 37°C and then the mixture was evaporated with Speed-Vac Concentrator. To this mixture 50 µl of 6N HCl was added and the contents were heated at 105°C for 18 hr under vacuum. After concentrating to dryness with the Speed-Vac Concentrator, the sample was dissolved in 10 µl of 50% (vol/vol) pyridine and an aliquot was spotted on polyamide thin layer plates (7.5 x 7.5 cm). A marker plate was applied with DNS-Gly, DNS-Glu and DNS-NH_2 as standards. The plates were developed successively for 50 min in solvent 1 [1.5% (vol/vol) formic acid], dried and then developed for 1 hr in solvent 2 [benzene-acetic acid (9:1, vol/vol)]. After drying the plates were developed for another 1 hour in solvent 3 [ethyl acetate-methanol-acetic acid (20:1:1, vol/vol)] and dried. Solvents 2 and 3 were developed at

90 degrees to the direction of solvent 1 (79).

H. Desulfurization of SRS I and SRS II

Samples of purified SRS I and SRS II (15-20 μg, estimated by UV at 280 nm, ε = 40,000) were separately treated with 10 mg of Raney nickel (w-2) in refluxing ethanol for 30 min. The reaction mixtures were then filtered and concentrated under reduced pressure. The crude desulfurization products were first methylated with CH_2N_2, trimethylsilylated with Tri-Sil/BSA (Pierce), and subjected to gas chromatography-mass spectral analysis (3% OV-1, 6 ft column, 150-240°C at 8°/min) on a Finnigan model 4021-T instrument. Spectra were recorded at 70 eV with an ionizing current of 0.35 mA.

I. Effect of Thiol Concentrations on the Chemical Isomerization of SRS

The reaction mixture consisted of 0.05 μmole of either synthetic SRS-Cys or SRS-GSH (59), 12.5 μmol of LiOH, and various amounts of GSH (0.5 and μmol) or cysteine (5 μmol) in 0.1 ml of dimethoxyethane/water (4:1, vol/vol). The samples were allowed to stand at 25°C for 4 hr, then were neutralized (1 M HCl) and evaporated to dryness under reduced pressure. The residue was analyzed by reverse-phase HPLC. The solvent system for SRS-Cys consisted of methanol/water (65:35, vol/vol) containing 0.05% acetic acid buffered to pH 5.4, and for SRS-GSH, methanol/water (55:45, vol/vol) containing 0.05% acetic acid buffered to pH 5.4.

J. Effect of HTMP on the Chemical Isomerization of SRS-GSH

The reaction mixture contained: 0.05 µmol of SRS-GSH, 12.5 µmol of LiOH, 5 µmol of GSH, and various amounts of HTMP (4-hydroxy-2,2,6,6,-tetramethylpiperidinooxy free radical; 0, 0.1, and 1 mM) in 0.1 ml of dimethoxyethane/water (4:1, vol/vol). After standing for 4 hr at 25°C, each sample was neutralized (1 M HCl) and evaporated to dryness, and the residue was analyzed by reverse-phase HPLC using a mobile phase of methanol/water (55:45, vol/vol) containing 0.05% acetic acid buffered to pH 5.4.

III.RESULTS

A. Purification of SRS from cat paws

Concentration of SRS preparations were previously accomplished by rotary evaporation or by lyophilization under reduced pressure. These procedures were cumbersome and time-consuming especially when large volumes were used. It was found that bioactivity of the perfusates was efficiently absorbed onto XAD-7 columns. Bioactivity was not detected in the effluent even after 5 liters of perfusate were passed through the XAD-7 column. The bioactivity was eluted from the resin with methanol, with a recovery of 75% (Table 3). This procedure provided a convenient method of concentrating and desalting the SRS activity from cat paw perfusions. Histamine was weakly adsorbed onto the XAD-7 column and was eluted from the resin by aqueous washing. It had been reported (30) that SRS bioactivity from cat paws could be extracted into diethyl ether and back into NH_4OH, but the percent recovery of bioactivity was low (46%). However, it was found that ethyl acetate more efficiently extracted bioactivity (80%) from the aqueous phase (Table 3).

Houglum used a silicic acid column eluted with a chloroform-methanol gradient to purify SRS from cat paws (7,51). A silica gel (25 g) column developed with cholorform/methanol/water, 65:30:10 (vol/vol) separated SRS from the dark colored contaminants much better than chloroform/methanol, 1:1 (vol/vol). The recovery of activity with the chloroform/methanol/water system was around 60%. A faster and convienient method using commercially packed disposable column was developed. After SRS was applied onto the disposable column, successive elutions of the column with methylene chloride/methanol/water, 65:20:10 (vol/vol) and methylene chloride/methanol/water, 65:30:10 (vol/vol) followed by methylene chloride/methanol/water, 65:40:10 (vol/vol) eluted SRS from the column with an overall recovery of 85% of the bioactivity.

HPLC was employed as the last step for SRS purification. SRS could not be eluted from M9 ODS-2, a preparative microparticulate C_{18} column, due to its high C_{18} loading (15%). However, a similar column (M9 ODS), which contained a 5% C_{18} loading, permitted the preparation of large quantities of highly purified SRS. Figure 3 shows the elution profile of SRS I and II from cat paws using preparative μC_{18} M9ODS column as the stationary phase and methanol/water, 1:1 (vol/vol) as the mobile phase. SRS I was eluted after 13 min and had a specific activity of 4,400 units/nmol (based on ε = 40,000 at 280 nm). SRS II was eluted from the column at 15.4 min

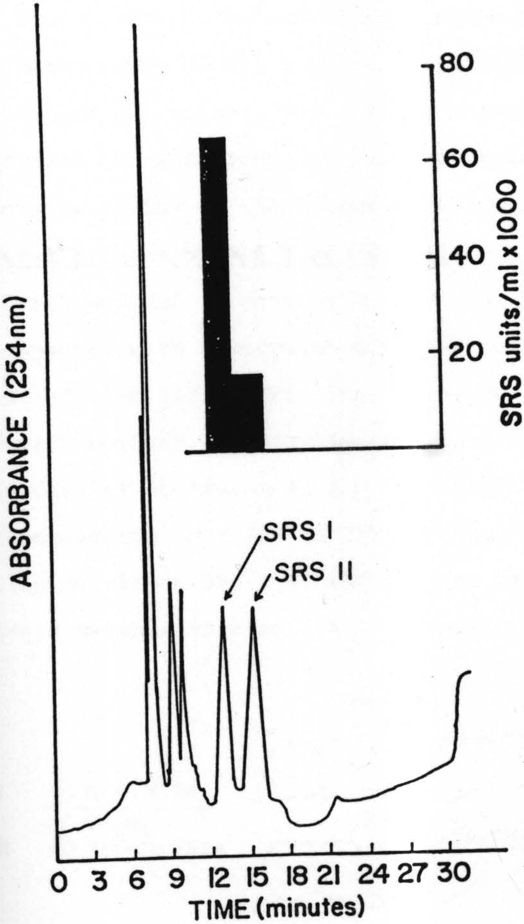
Table 3. Purification of SRS I and II

Procedure	Total Units	Weight (mg)	% Recovery	Specific Activity (units.mg)
Perfusate	533,000	12,000	--	0.044
XAD-7	400.000	302	75	1.325
Ethyl acetate extraction	320,000	24	80	13.333
Disposable column silica gel	272,000	1	85	272
Preparative μC_{18} HPLC			41	
SRS I	106,800	0.012*		8,900
SRS II	3,450	0.023*		150

---

*The amount of SRS I and SRS II were calculated from the absorbance at 280 nm, assuming $\varepsilon = 40,000$.

Figure 3. Reverse-phase HPLC of SRS I and SRS II. Stationary phase (preparative μC_{18}), M9 ODS; mobile phase, methanol/water, 1:1 (vol/vol); flow rate, 3 ml/min.

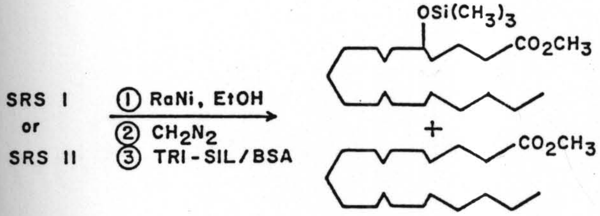

ABSORBANCE (254 nm)
SRS units/ml x 1000
80
60
40
20
SRS I
SRS II
0
3
6
9
12
15
18
21
24
27
30
TIME (minutes)

and had a specific activity of 65 units/nmol.

Using a purification procedure consisting of XAD-7 column, chromatography, ethyl acetate extraction, disposable silica gel column, and reverse-phase μC_{18} HPLC, we have purified two SRSs from cat paw perfusates with an overall recovery of 20% of the bioactivity (Table 3).

B. Structural Studies on SRS I and SRS II

Both SRS I and SRS II possessed identical UV absorption spectra with absorption maxima at 280 nm and shoulders at 270 and 292 nm (Fig. 4). Treatment of SRS I and SRS II with soybean lipoxygenase resulted in a rapid loss of bioactivity accompanied by a bathochromic shift of UV absorption maximum from 280 to 308 nm (Fig. 5). Hydrogenation of either SRS I or SRS II over Raney nickel afforded 5-hydroxyicosanoic acid and icosanoic acids.



5-Hydroxyicosanoic acid was identified by gas chromatograhpy/mass spectrometic analysis of the trimethylsilyl ether and methyl ether derivatives, which

Figure 4. Ultraviolet absorbance spectra of SRS I and SRS II in methanol.

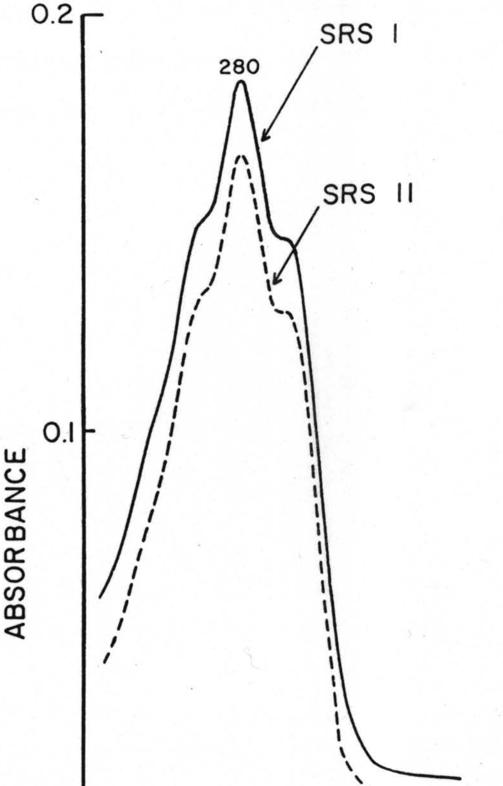
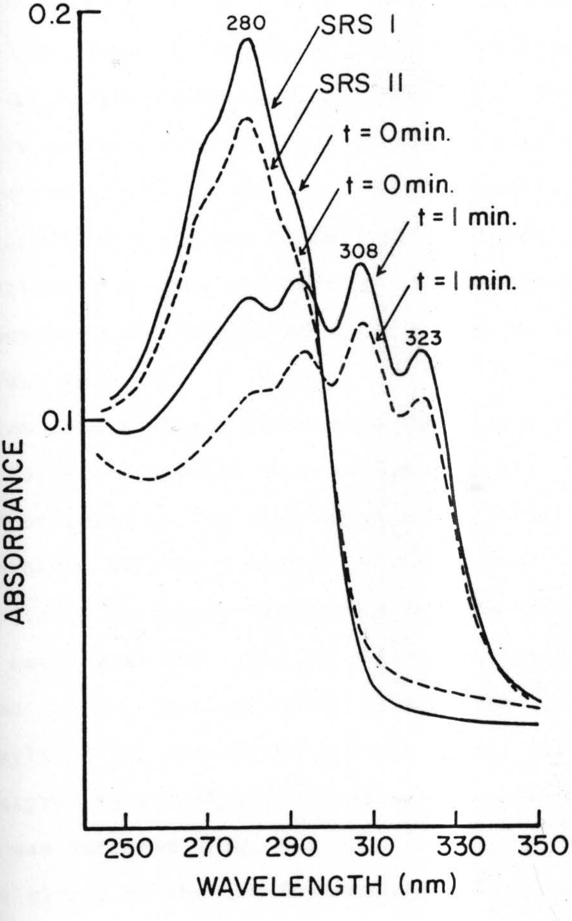

0.2
0.1
ABSORBANCE
280
SRS I
SRS II
250
270
290
310
330
WAVELENGTH (nm)

Figure 5. Ultraviolet spectra of SRS I and SRS II before (0 min) and after treatment with soybean lipoxygenase (5 min). Spectra were recorded in Tyrode's buffer.


0.2
0.1
ABSORBANCE
280
SRS I
SRS II
t = 0 min.
t = 0 min.
t = 1 min.
308
t = 1 min.
323
250
270
290
310
330
350
WAVELENGTH (nm)

showed prominent peaks at m/e 399 (M-15); 383 (M-31); 313 (M - 101, loss of $CH_2(CH_2)_2CO_2CH_3$) and a base peak at 203 $[(CH_3)_3Si\overset{+}{O}=CH(CH_2)_3CO_2CH_3]$ (Fig. 6). The same derivative of authentic 5-hydroxyicosanoic acid afforded a mass spectrum virtually identical to the spectra of desulfurized SRS I and SRS II derivatives. Icosanoic acid was identified as minor products by gas chromatography-mass spectrometric analysis of their methyl ester derivatives (Fig. 7).

Amino acid analyses after acid hydrolysis of SRS I gave cysteine and glycine in a ration of 0.5:1; SRS II afforded cysteine as the sole amino acid (Table 4).

Synthetic SRS-GSH contained glutamic acid, glycine and cysteine. Synthetic SRS-CysGly and SRS-Cys showed a similar ratio with SRS I and SRS II respectively. To determine the NH_2-terminal amino acid in SRS I, the sample was dansylated and hydrolyzed and the dansyl derivatives were analyzed by thin-layer chromatography; no dansyl-glycine was detected (Fig. 8).

The slopes of the log-dose-contractile response curves (Fig. 9) of synthetic SRS-Cys and SRS-CysGly coincided with those of SRS II and SRS I, respectively. The patterns of reversal of the contractile activity by FPL 55712 for SRS II and SRS I were found to be indistinguishable from those of corresponding synthetic

Figure 6. Mass spectrum of the Me_3Si derivative of the methyl ester of product after desulfurization of SRSs.

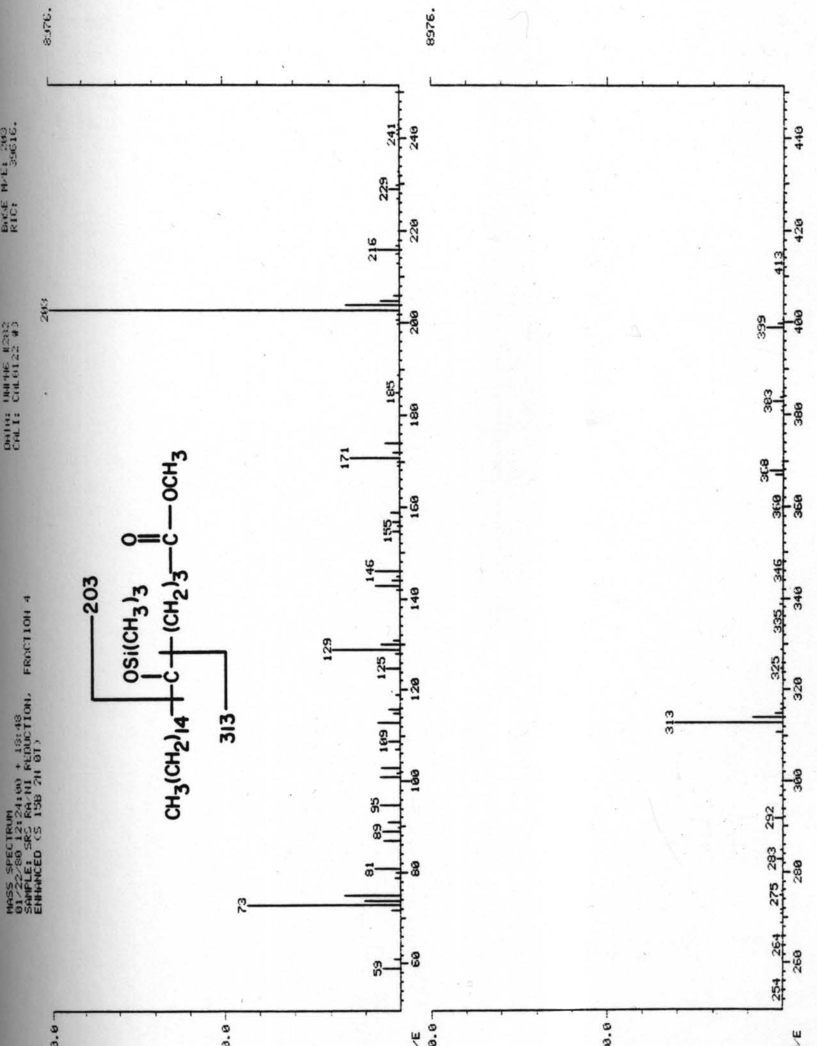

MASS SPECTRUM
01/22/80 12:24:00 + 18:48
SAMPLE: SRC RA/NI REDUCTION, FRACTION 4
ENHANCED (S 15B 2N 0T)
DATA: UNIMHE #282
CALI: CAL0122 #3
BASE M/E: 203
RIC: 39616.
8976.
100.0
50.0
M/E
59
73
81
89
95
109
125
129
146
155
171
185
203
216
229
241
60
80
100
120
140
160
180
200
220
240
254
264
275
283
292
313
325
335
346
360
368
383
399
413
260
280
300
320
340
360
380
400
420
440
203
313
OSi(CH3)3
CH3(CH2)14
C
(CH2)3
C
O
OCH3

Figure 7. Mass spectrum of the methyl ester derivative of icosanoic acid.

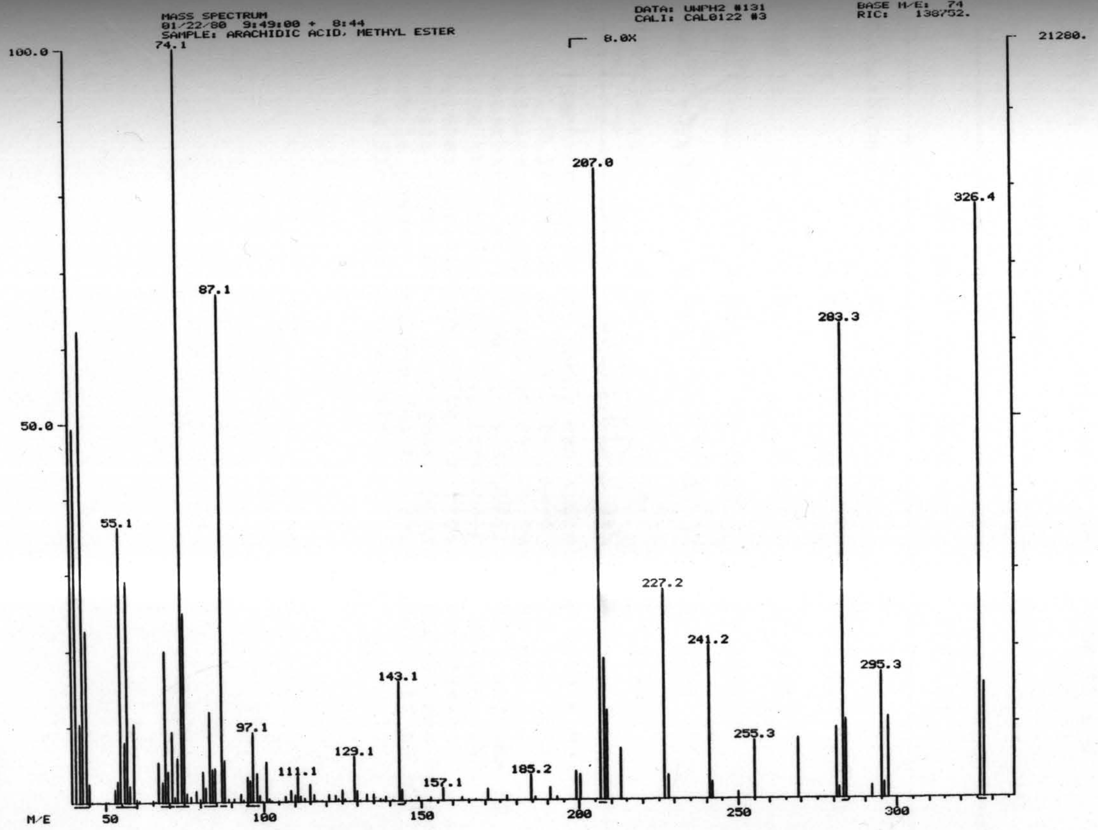

MASS SPECTRUM
01/22/80 9:49:00 + 8:44
SAMPLE: ARACHIDIC ACID, METHYL ESTER
DATA: UWPH2 #131
CALI: CAL0122 #3
BASE M/E: 74
RIC: 138752.
8.0X
21280.
100.0
50.0
M/E
50
100
150
200
250
300
74.1
55.1
87.1
97.1
111.1
129.1
143.1
157.1
185.2
207.0
227.2
241.2
255.3
283.3
295.3
326.4

Table 4. Amino acid compostion of SRS I, SRS II and synthetic SRSs.

	Glutamic acid	Glycine	1/2 Cystine
Glutathione	5.37	5.63	5.06
SRS I	0.26	4.50	2.24
SRS II	0.37	0.98	3.05
SRS^{syn}-GSH	6.00	6.38	3.30
SRS^{syn}-CysGly	0.35	5.73	2.98
SRS^{syn}-Cys	0.42	0.76	3.16

*Each compound (10 nmol) was hydrolyzed with 6 M HCl/0.5% phenol at 110°C in vacuo for 24 hr. The results are expressed in nmoles based on the absorbance at 280 nm ($\varepsilon = 40,000$). SRS I and SRS II from the preparative C_{18} reverse-phase column was further purified by using the radial compression module (RCM-100). Stationary phase: Radial-Pak C_{18}; mobile phase: methanol/water, 70:30 (vol/vol) pH 5.4 at a flow rate of 2 ml/min.

Figure 8. Chromatography of DNS-amino acids on polyamide thin layer plates. Solvent: 1, 1.5% (vol.vol) formic acid; 2, benzene-acetic acid (9:1, vol/vol); 3, ethyl acetate-methanol-acetic acid (20:1:1:, vol/vol).

A) Control plates with DNS-Gly, DNS-Glu and DNS-NH_2.

B) SRS I after reaction with DNS chloride and then acid hydrolyzed.

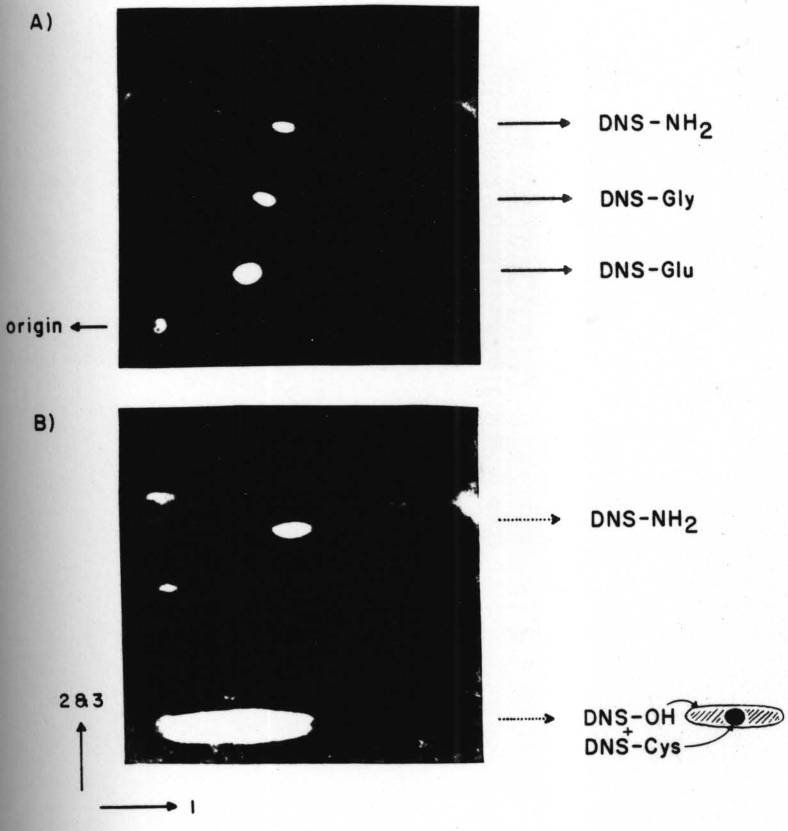
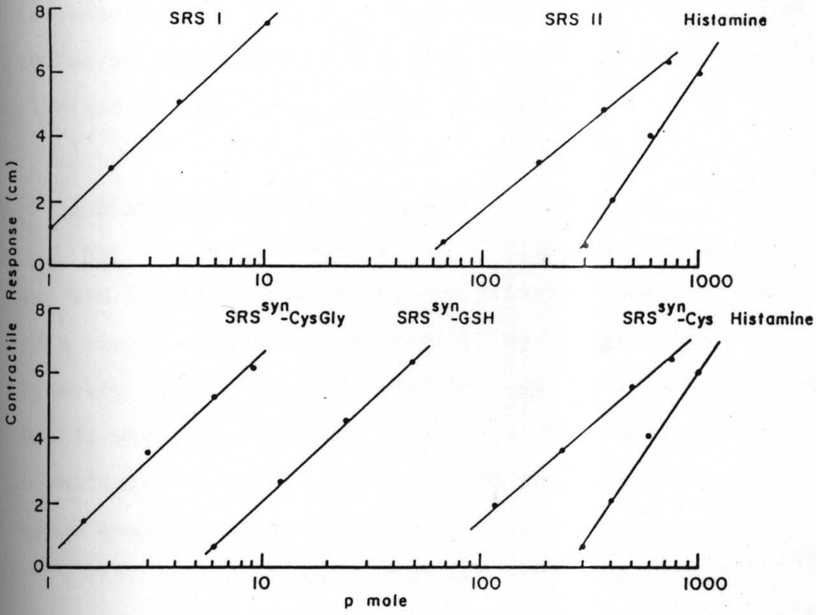

A)
DNS-NH$_2$
DNS-Gly
DNS-Glu
origin
B)
DNS-NH$_2$
DNS-OH
+
DNS-Cys
2&3
1

Figure 9. Dose-response curves for SRS I, SRS II, synthetic SRSs and histamine on isolated guinea pig ileum. The amount of SRS was calculated from the absorbance of 280 nm, assuming ε = 40,000.


SRS I
SRS II
Histamine
SRS^{syn}-CysGly
SRS^{syn}-GSH
SRS^{syn}-Cys
Histamine
Contractile Response (cm)
0
2
4
6
8
1
10
100
1000
p mole

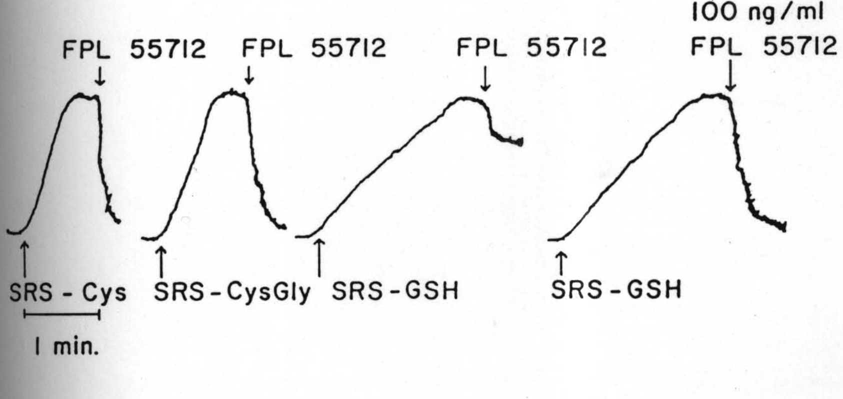
derivatives (Fig. 10). On the other hand, synthetic SRS-GSH had a specific activity of 650 units/nmol but its pattern of contractile responses was only partially reversed by FPL 55712 at a concentration of 10 ng/ml. At 100 ng/ml, FPL 55712 reversed the contractile activity elicited by SRS-GSH by greater than 70%.

C. Inactivation by Arylsulfatase

The contractile activities of synthetic SRS-CysGly and SRS I were inactivied at approximately the same rate by a limpet arylsulfatase preparation, whereas the bioactivities of synthetic SRS-GSH, synthetic SRS-Cys, and SRS II were not diminshed (Fig. 11). The small loss of bioactivity by SRS II is due to the contamination of a trace quantity of SRS I in the SRS II sample.

When synthetic SRS-CysGly was incubated with arylsulfatase for 30 min, a less polar product was formed (Fig. 12). Its retention time on the radial compression Radial-Pak C_{18} column (11 min; $MeOH/H_2O$, 7:3, pH 5.4), coincided with that of SRS-Cys. Amino acid analysis of this product showed only cysteine, indicating that the glycine residue was cleaved off from the substrate SRS-CysGly.

Figure 10. The contractile response of SRSs and its antagonism by FPL 55712. The system contained: SRS-Cys (SRS I, 1,000 pmoles), SRS-CysGly (SRS II, 2 pmoles) or SRS-GSH (6 pmoles) in 10 ml Tyrode buffer containing atropine sulfate (1μM) and mepyramine maleate (1μM).



FPL 55712
FPL 55712
FPL 55712
100 ng/ml
FPL 55712
SRS - Cys
SRS-CysGly
SRS-GSH
SRS-GSH
1 min.

Figure 11. Inactivation of SRS bioactivity by arylsulfatase.

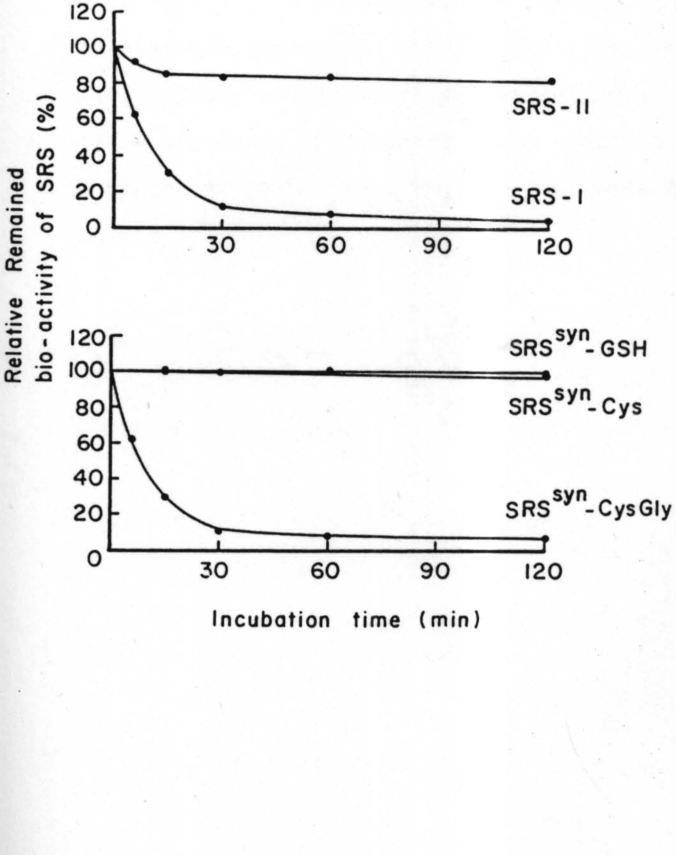
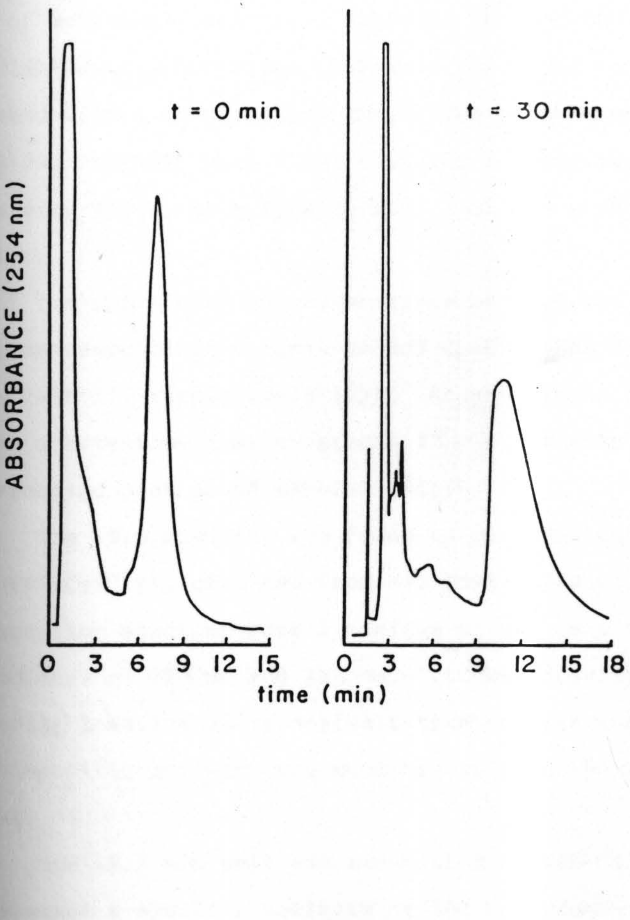

Relative Remained bio-activity of SRS (%)
120
100
80
60
40
20
0
30
60
90
120
SRS - II
SRS - I
SRS^{syn}- GSH
SRS^{syn}- Cys
SRS^{syn}- CysGly
Incubation time (min)

Figure 12. HPLC elution profile of SRS-CysGly after exposure to arylsulfatase. HPLC was carried out on a Radial-Pak C_{18} liquid chromatography cartridge (0.8 x 10 cm) using a radial compression separation system (RCM-100). The mobile phase consisted of methanol/water, 70:30 (vol/vol), containing 0.05% acetic acid buffered to pH 5.4 with NH_4OH; flow rate, 2.5 ml/min.


ABSORBANCE (254 nm)
t = 0 min
t = 30 min
0
3
6
9
12
15
0
3
6
9
12
15
18
time (min)

D. 11-trans SRS-Cys from cat paws

After the samples were purified through the disposable silica gel column, they were dissolved in 0.1 ml of methanol/water (1:1, vol/vol) and applied onto a Radial-Pak C_{18} cartridge (0.8 x 10 cm). The cartridge was eluted with a mobile phase consisting of methanol/water (65:35, vol/vol) plus 0.05% acetic acid, adjusted to pH 5.4 with NH_4OH, at a flow rate of 2 ml/min were obtained (61).

Two peaks with retention times of 18.5 min and 19.5 min corresponding to those of SRS-CysGly (SRS I) and SRS-Cys (SRS II) respectively (61). An additional shoulder with a retention time of around 22 min was also present, resembling peak 4b of RBL-SRS (61).

The 18.5 min peak was found to be identical to of SRS I (SRS-CysGly), obtained from cat paws. Its ultraviolet absorption spectrum gave a maximum at 280 nm with shoulders at 270 nm and 292 nm. Its bioactivity was readily inactivated by arylsulfatase preparations (59). Its specific activity was much higher than the 19.5 min peak.

The 19.5 min peak was identified as SRS-Cys, which possessed a specific activity of 100 units/nmol and a ultraviolet absorption maximum 280 nm with characteristic shoulders at 270 and 292 nm. It was virtually unattacked by limpet arylsulfatase (59).

The ultraviolet absorption spectrum of the SRS with retention time of 22 min showed a maximum of 278 nm with shoulders at 268 nm and 290 nm (Fig. 13). Like SRS-Cys, this material was not inactivated by arylsulfatase preparations, and amino acid analysis gave no glycine or glutamic acid.

SRS-Cys was readily oxidized by soybean lipoxygenase, resulting in a shift of the absorption maximum from 280 nm to 308 and 323 nm, characteristics of triene to tetraene extension (Fig. 14). In contrast, no significant spectral shift was observed when SRS-Cys (λ_{max}278 nm) was used as substrate. These contrasting features suggested that λ_{max} 278 nm structural isomer differs from the λ_{max} 280 nm SRS in the geometry of the 11,12 double bond.

E. Chemical Isomerization Studies of SRS

We repeatedly observed that when (±)-leukotriene A methyl ester was reacted with an excess of GSH and the resulting SRS-GSH methyl ester (ester in icosanoic moiety) was saponified (0.1 M K_2CO_3) with prior purification, a mixture of SRS-GSH and 11-*trans*-SRS-GSH was obtained. The amount of 11-*trans* isomer formed varied from experiment to experiment, but generally was in the range of 10-20%.

Figure 13. Ultraviolet spectrum of the 11-<u>trans</u> SRS-Cys (<u>trans</u> isomer of SRS II) obtained after reverse-phase HPLC with a Radial-Pak C_{18} cartridge.

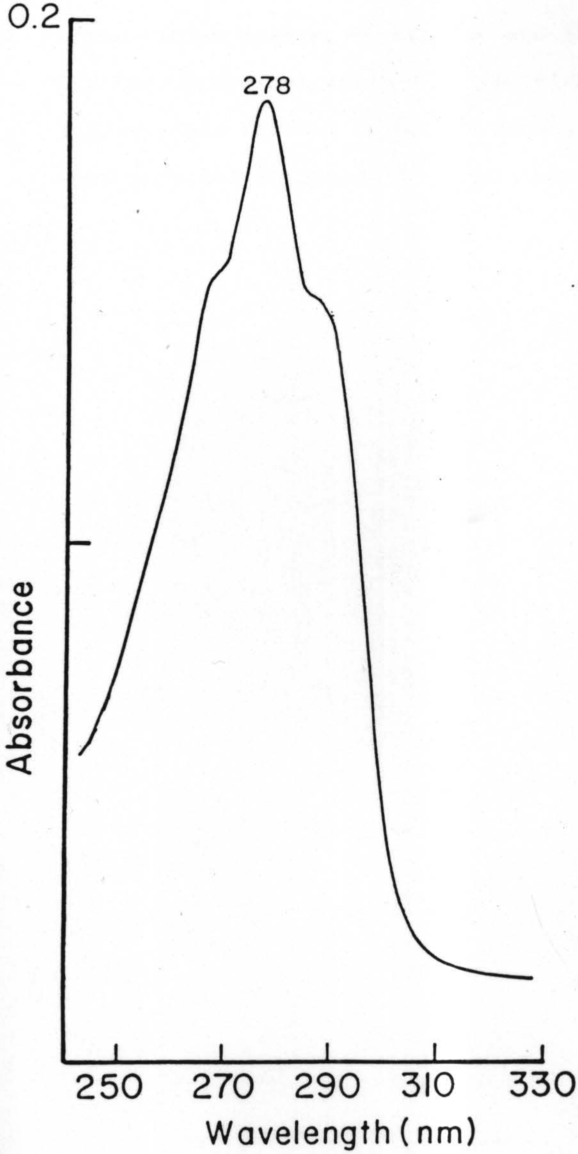
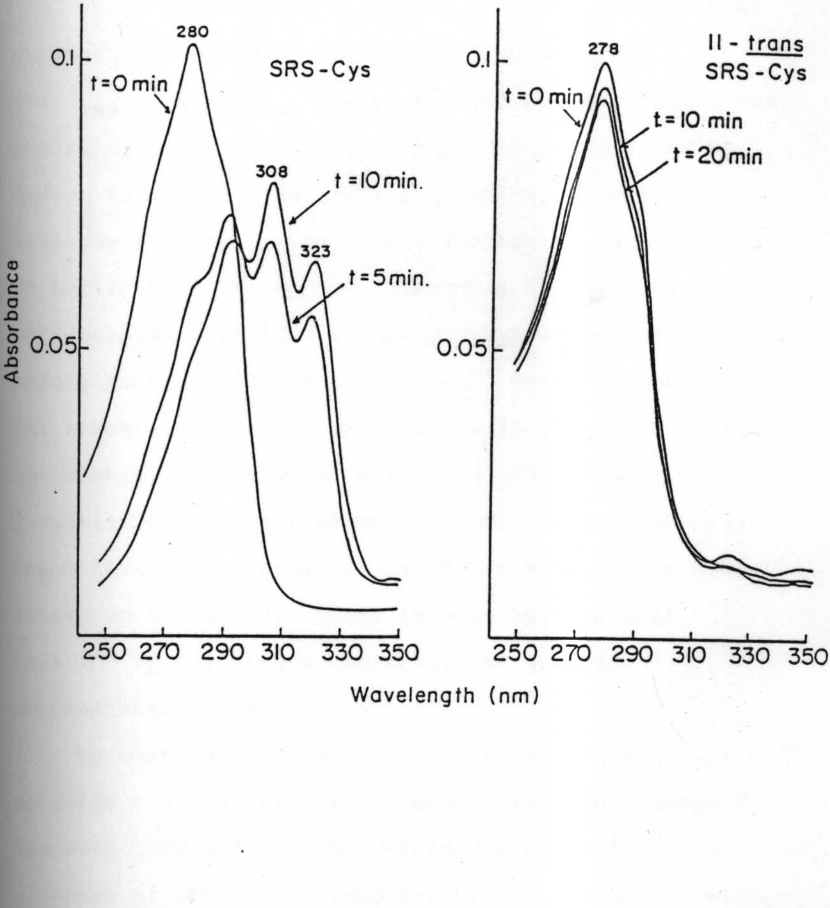

0.2
278
Absorbance
250
270
290
310
330
Wavelength (nm)

Figure 14. Ultra violet spectra of SRS-Cys and 11-*trans* SRS-Cys before and after reaction with soybean lipoxygenase for the indicated times. Spectra were recorded in Tyrode's buffer, pH 7.8.


Absorbance
0.1
0.05
280
t=0 min
SRS-Cys
308
t =10 min.
323
t=5 min.
250 270 290 310 330 350
278
II - trans
SRS-Cys
t=0 min
t=10 min
t=20 min
250 270 290 310 330 350
Wavelength (nm)

However, pure SRS-GSH and SRS-Cys were not transformed into their correpsonding 11-*trans* isomers when treated with an excess of base such as LiOH in 4:1 DME-H_2O (Table 5).

Thus, we surmised that perhaps the residual thiol present in the crude mixture was required for the $\lambda_{280\ nm} \rightarrow \lambda_{278\ nm}$ isomer transformation. Under basic conditions, the thiol anion, RS^-, may be oxidized by oxygen to generate RS· radicals, which are known to catalyze the *cis* → *trans* isomerization of double bonds. To verify this assumption, synthetic SRS-Cys and SRS-GSH were treated with LiOH in 4:1 DME-H_2O in the presence of thiols such as GSH and L-cysteine. Table 5 shows that in the absence of thiols, no apparent 11-*trans*-SRS were detectable. Addition of 5×10^{-3}M GSH enhanced the formation of 11-*trans* isomers of SRS-GSH and SRS-Cys, which further increased as the GSH concentration was raised to 5×10^{-2}M. These results suggest that this Δ^{11} -*cis* to *trans* isomerization is thiol concentration dependent.

To confirm that this *cis*-*trans* isomerization at C-11 proceeds via free radical intermediates, we treated SRS-GSH with LiOH and dimethoxyethane/water (4:1) in the presence of GSH and various quantities of the antioxidant, 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical

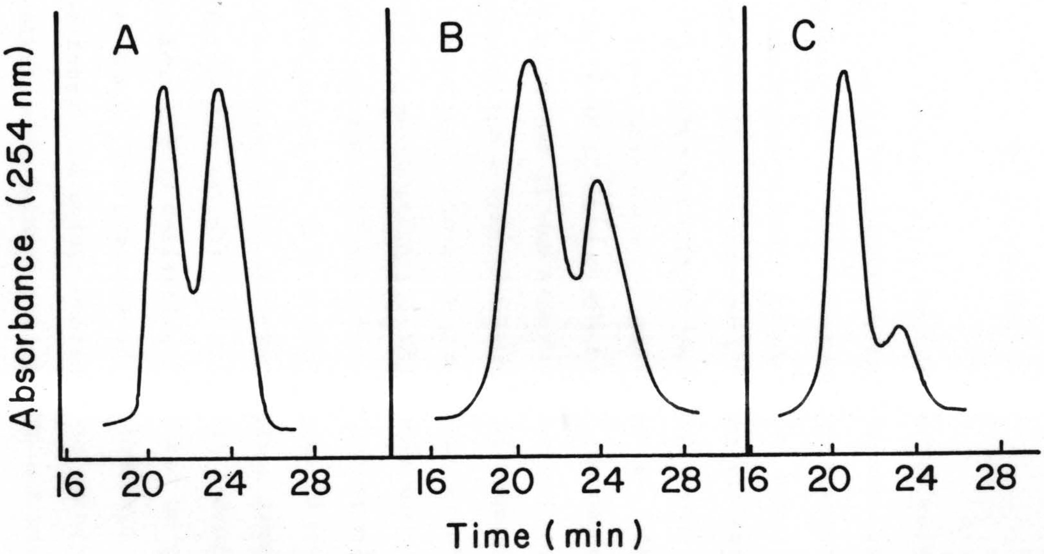
Table 5. Effect of thiol concentration on the chemical isomerization of SRS-Cys and SRS-GSH.

	GSH [M]				L-Cysteine [M]
	0	5×10^{-3}	5×10^{-2}		5×10^{-2}
				with HTMP	
Ratio of SRS-Cys					
$\frac{11\text{-trans (278 nm)}}{11\text{-}\textit{cis}\text{ (280 nm)}}$	$\frac{0}{100}$(3)*	$\frac{15}{85}$(2)	$\frac{35}{65}$(2)	$\frac{10}{90}$(2)	$\frac{30}{70}$(1)
Ratio of SRS-GSH					
$\frac{11\text{-trans (278 nm)}}{11\text{-}\textit{cis}\text{ (280 nm)}}$	$\frac{0}{100}$(2)	$\frac{40}{60}$(2)	$\frac{55}{45}$(2)	$\frac{15}{85}$(2)	

*No. of experiments. The ratio of SRS was calculated from the absorbance at 278 and 280 nm, assuming = 40,000.

(HTMP). Figure 15 clearly demonstrates that as the concentration of the antioxidant was increased, the amount of 11-*trans*-SRS-GSH decreased.

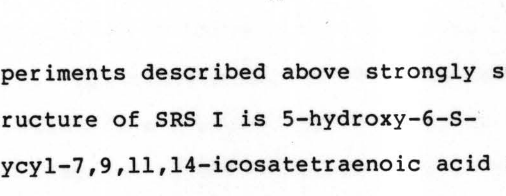
Figure 15. HPLC elution profile of SRS-GSH and 11-trans SRS-GSH, showing the effect of HTMP on the chemical isomerization. (A) No HTMP; (B) 0.1 mM HTMP; (C) 1 mM HTMP.


A
B
C
Absorbance (254 nm)
16
20
24
28
16
20
24
28
16
20
24
28
Time (min)

IV. DISCUSSION

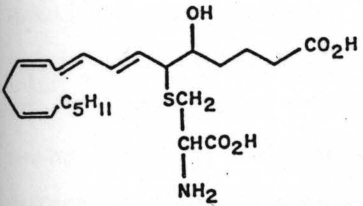
Perfusion of cat paws with Compound 48/80 produced SRS I and SRS II whose UV absorption spectra were similar to those of Leukotriene C (49) and characteristic of conjugated triene chromophores. Treatment of SRS I and SRS II with soybean lipoxygenase shifted the absorption maximum from 280 to 308 nm, suggesting the presence of a *cis, cis* -1,4-diene unit within these molecules. The bathochromic shift of 30 nm suggested that the conjugated triene of SRS I and SRS II was extended to a conjugated 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, two additional double bonds must be present at the ω-11 and ω-13 position. 5-Hydroxyicosanoic acid was identified as the main product obtained by hydrogenation of either SRS I and SRS II after Raney nickel desulfurization (47,62). This result indicated that the arachidonic acid derivative is linked to cysteine by a thioether bond. The presence of the hydroxyl group at C-5 in the fatty acid moiety confirms the putative biogenetic relationship between LTA, 5-HETE and SRSs. Therefore, these results suggested that SRS I and SRS II contain 5-hydroxy-7,9,11,14-icosatetraenoic acid as the fatty acid portion of the molecule but differ in the thioether substituent as C-6.

Amino acid and NH_2-terminal sequence analyses revealed that the amino acid portion of the molecule in SRS I is cysteinylglycine, and in SRS II, cysteine. Although the mechanism for the generation of cysteine after strong acid hydrolysis is not understood, it was apparent that the thioether linkages in these molecules were cleaved because synthetic SRS-GSH likewise afforded cysteine upon acidic hydrolysis (Table 4). Cleavage of thioether linkage does not generally occur in acid, but a possible explanation for the release of cysteine might be that the acid catalyzed nucleophilic attack at C-12, C-10, or C-8 resulting in the liberation of the peptide or amino acid (63).

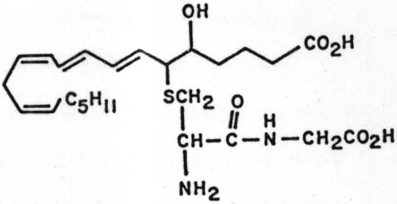


The experiments described above strongly suggested that the structure of SRS I is 5-hydroxy-6-S-cysteinylglycyl-7,9,11,14-icosatetraenoic acid and that of SRS II is 5-hydroxy-6-S-cysteinyl-7,9,11,14-

icosatetraenoic acid.



SRS II



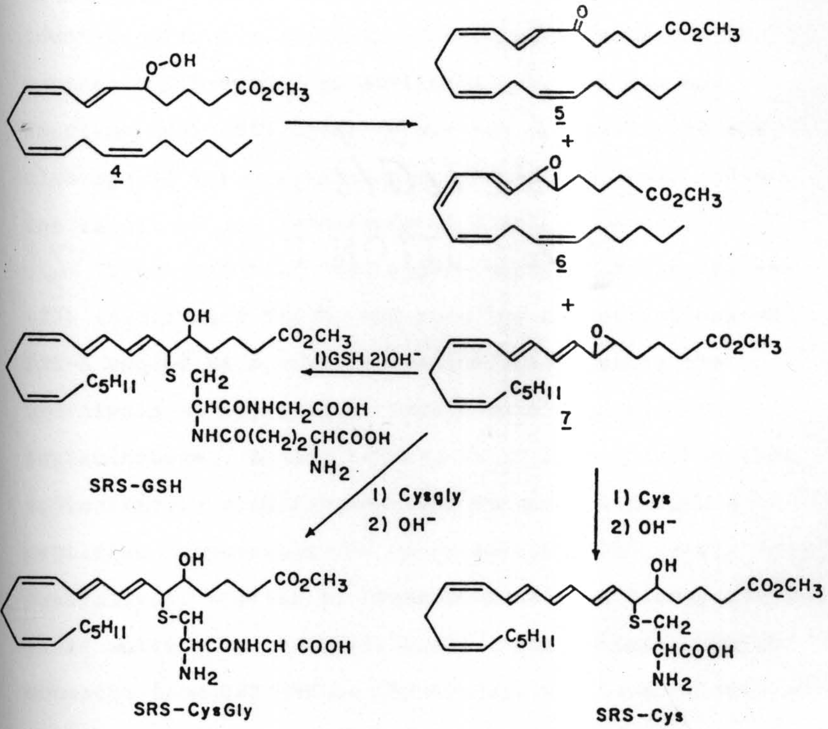
SRS I

The presence of a sulfate group in SRS-A had been proposed on the basis of arylsulfatase inactivation experiments (26,29), and the susceptibility to arylsulfatase inactivation had been an important criterion in the positive identification of SRS-A. Because SRS I also is readily inactivated by arylsulfatase (Fig. 11) and it was possible that some labile group in SRS I and SRS II was lost during chemical degradative studies, we decided to verify their identities by chemical synthesis.

To confirm the chemical identities of SRS I and SRS II from cat paws, we decided to devise a biomimetic synthesis of the key intermediate, leukotriene A, using readily available (±)-5-HPETE methyl ester as starting material (66). The transformation of (±)-5-HPETE methyl ester into leukotriene A methyl ester requires the selective abstraction of a C-10 proton and activation of the hydroperoxyl group at C-5. Because t-butyl arylpersulfonate (67) and trifluoroperacetate (68) had been prepared, we reacted (±)-5-HPETE methyl ester with methanesulfonyl chloride and a hindered base such as methylcyclohexylamine (to minimize 1,2-elimination) at -78°C to give in 15% yield the desired leukotriene A methyl ester <u>7</u>, whose spectral properties were in good agreement with those derived from independent chemical synthesis (64,65). A small amount of the dienone <u>5</u> was formed as a result of a 1,2-elimination side reaction and, surprisingly, a large amount of <u>6</u> was also formed (69) (Scheme 2).

SN_2 displacement of the epoxide <u>7</u> by the respective nucleophiles (cysteine, cysteinylglycine and glutathione) followed by base hydrolysis, gave the corresponding adducts as pairs of diastereomers (5S,6R;5R,6S)(70). Although we were unable to separate the two diastereomers of the synthetic derivatives at that time, it is noteworthy that the properties of the diastereomeric mixtures of synthetic SRS-Cys and SRS-CysGly agreed well with the corresponding properties of SRS II and SRS I, lending further support to our structural assignment.

The inactivation of SRS-A by arylsulfatase had been a long-standing enigma. The availability of synthetic substances of known chemical identities allowed us to define this mode of inactivation. Although the



Scheme 2. Biomimetic synthesis of SRSs.

bioactivities of synthetic SRS-GSH, SRS-Cys and SRS-II were not inactivated by the limpet arylsulfatase preparation, the contractile activities of synthetic SRS-CysGly and SRS I were destroyed at approximately the same rate by the arylsulfatase (Fig. 11). Because SRS-Cys was identified as the reaction product after exposure of synthetic SRS-CysGly to arylsulfatase, one can now conclude that this inactivation was the result of the cleavage of the cysteinyl-glycine peptide bond, and not the result of the hydrolysis of a sulfate ester.

It was reported that highly purified arylsulfatase (27) inactivated SRS-A, and very low concentrations of SRS-A served as a competitive inhibitor during the hydrolysis of arylsulfate esters catalyzed by arylsulfatase. Alternatively, it is also possible that arylsulfatase preparations were contaminated with a peptidase responsible for the cleavage of S-Cys-Gly derivatives, similar to those found in the kidney (27). It is notewrothy that γ-glutamyl transpeptidase, which converts from SRS-GSH to SRS-GysGly, and leukotrienes were also reported present in kidney tissues (71,72).

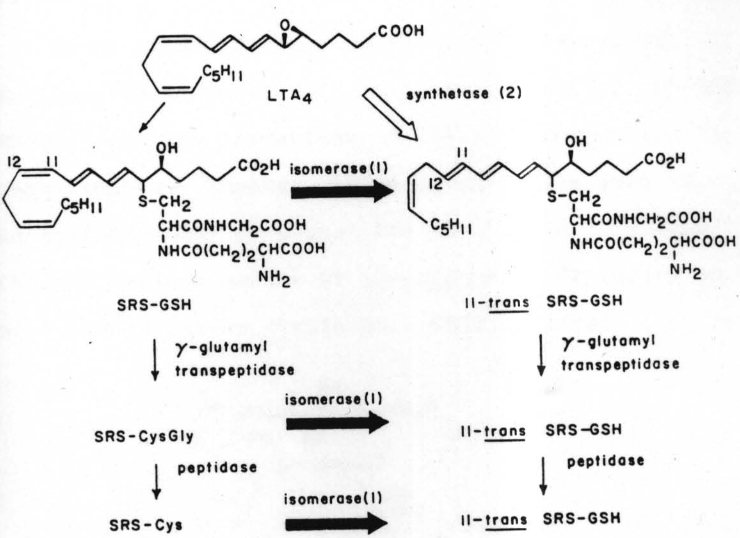
When limpet arylsulfatase was chromatographed over an SP-Sephadex column, the SRS-CysGly inactivating peptidase was found not to coincide with arylsulfatase activity (73). The peptidases in rat basophilic leukemia cells

(RBL-1), guinea-pig lung, and peritoneal eosinophils preparations were shown to inactivate SRS-CysGly but not SRS-GSH and SRS-Cys. The SRS-CysGly inactivating peptidases were resolved from arylsulfatases in these cell systems. Further, the arylsulfatase did not inactivate SRS-CysGly. The purified kidney brush border dipeptidase was highly active in SRS-CysGly inactivation (73). Consequently, one can conclude that limpet arylsulfatase was contaminated with peptidase.

Houglum's observation (51) is noteworthy. He observed that SRS was inactivated by crude preparations of phosphatase enzymes (alkaline and acid phosphatase, venom, spleen, and 3',5'-cyclic phosphodiesterases). The ability of these crude enzyme preparations to inactivate SRS could not be attributed to their respective phosphatase or phosphodiesterase activities and a common contaminating enzyme seemed more likely, because the influence of pH, EDTA, $MgCl_2$, K_2HPO_4, Na_2SO_4, and cysteine was similar for each enzyme preparation. Based upon gel-elution chromatography on Sephadex G-200, the SRS inactivating enzyme in crude alkaline phosphatase possessed a molecular weight of 100,000. This value is identical to the reported molecular weight of dipeptidase (Mr=105,000) (77).

Recently, it was reported that a SRS from murine mastocytoma cells, previously designated as leukotriene C-2 (LTC-2) with a UV absorption maximum at 278 nm, was identified as 5(S)-hydroxy-6(R)-S-glutathionyl-7,9,11-*trans*-14-*cis*-icosatetraenoic acid (11-*trans*-LTC)(60). Because we also reported the identification of the three structural maxima at 278 nm (61), we reinvestigated the cat paw system with a radial compression module HPLC system. Ultraviolet absorption maxima at 278 nm was isolated at the retention time of 21 min as a shoulder peak of SRS-Cys (61). The retention time and other characteristics were similar to those of 11-*trans* SRS-Cys (4b peak) from RBL-1 cell. Enzymatic studies indicated that this material was indeed 11-*trans* SRS-Cys (No inactivation by arylsulfatase, no spectral change with soybean lipoxygenase and no initial activation after γ-glutamyl transpeptidase).

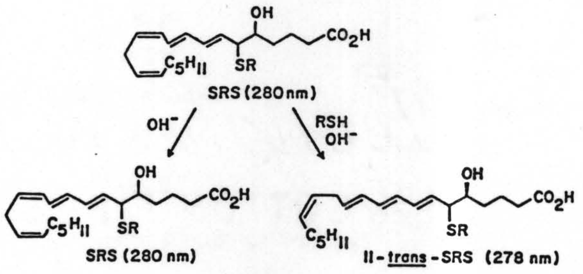
Because these 11-*trans* isomers of SRS have been isolated from several different natural sources, the question arose as to whether they are biosynthesized enzymatically or produced by chemical means. Several plausible mechanisms for their formation may be envisioned (Scheme 3) : 1) An enzyme converts SRS-GSH into 11-*trans* SRS-GSH, which is then converted into 11-*trans* SRS-CysGly by γ-glutamyl transpeptidase; 11-*trans* SRS-CysGly is hydrolyzed to 11-*trans* SRS-Cys by a peptidase. This



Scheme 3. Possible enzymes for the formation of 11-trans SRSs.

isomerase may also be nonspecific, capable of transforming all three SRSs into the corresponding 11-*trans* isomers; 2) 11-*trans* SRS-GSH is biosynthesized via a separate enzymatic pathway from leukotriene A; 3) a chemical isomerization involving the reversible addition of thiyl radical (RS•) to the triene system at C-12 results in the cis → trans isomerization of the Δ^{11} double bond. Many examples of such interconversions have been reported (74,75).

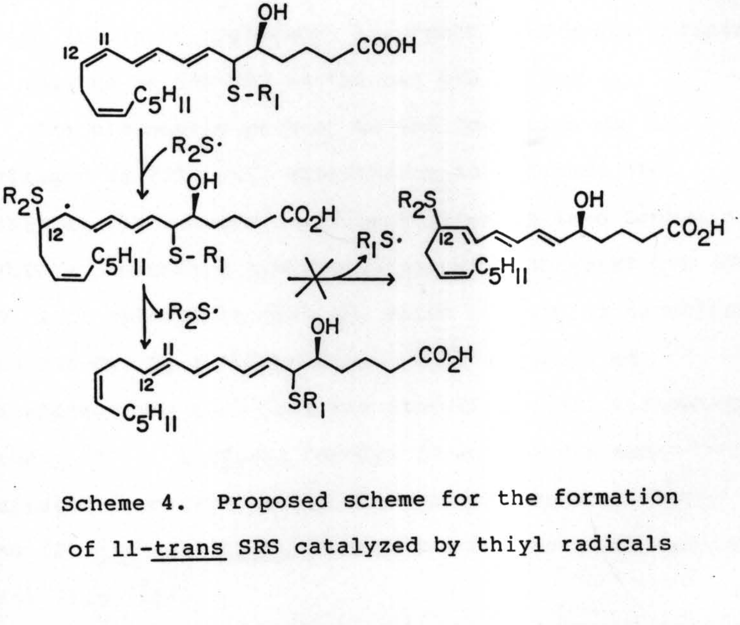
Under strongly basic conditions [excess LiOH, dimethoxyethane/water (4:1,vol/vol)], purified SRS-GSH and SRS-Cys were not isomerized to their corresponding 11-trans isomers. However, addition of thiols such as glutathione (GSH) or L-cysteine to this basic medium produced various amount of 11-trans SRS, depending on the thiol concentration (Table 5). This chemical



isomerization was inhibited by the radical scavenger HTMP (Fig 15). This inhibition suggests that the thiyl radical (RS•) is added reversibly to the triene system at C-12, resulting in the overall cis → trans isomerization of the 11,12 double bond.

Because a possibility exists that this isomerization may be the result of reversible equilibration of thiol between the C-6 and C-12 positions (Scheme 4), we carried out the incubation experiment using synthetic (±)-SRS-Cys in the presence of ^{14}C-L-cysteine and RBL-1 cells (76). The resulting 11-trans SRS-Cys was isolated after 6 hrs. by reverse-phase HPLC and was found to be void of any radioactivity. Thus, the formation of the 12-thio analog (position isomer) appears unlikely.

Although at this juncture one may suspect that this



Scheme 4. Proposed scheme for the formation of 11-trans SRS catalyzed by thiyl radicals

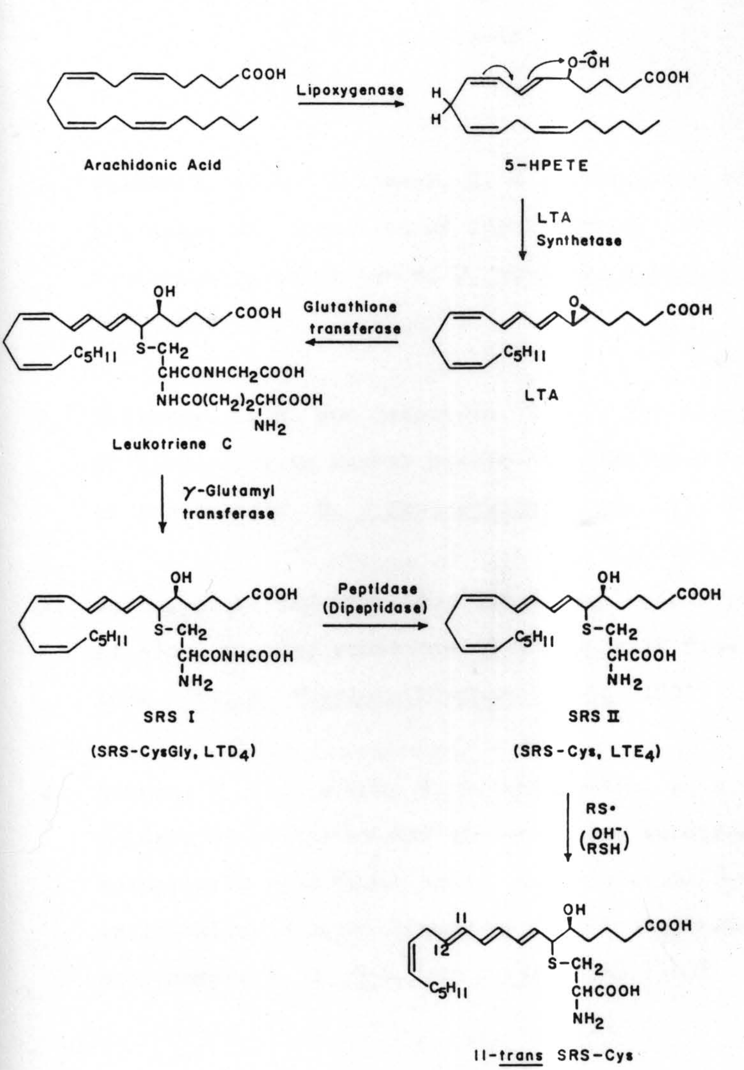
11-cis to 11-trans isomerization may be enzyme catalyzed, a parallel experiment using boiled RBL-1 cells also revealed the formation of 11-trans SRS-Cys from SRS-Cys. On the basis of the foregoing results, it appears that the

11-trans SRSs are not formed by a separate independent pathway (Scheme 4) but rather are most likely derived from their corresponding SRSs via nonenzymatic isomerization of the Δ^{11} double bond.

It is noteworthy that we have been unable to find SRS-GSH (LTC) and LTB in cat paws, even though we carefully searched for these compounds using known standards. The fact that different tissues may possess varied levels of γ-glutamyl transpeptidase might explain the absence of SRS-GSH in the cat paw system.

The biogenetic pathway of SRS formation may be envisaged as follows: Glutathione transferase (80) catalyzes the conversion of Leukotriene A into Leukotriene C (LTC); γ-Glutamyl transpeptidase (81) converts LTC (SRS-GSH) into SRS-CysGly (SRS I), which in turn is transformed into SRS-Cys by a dipeptidase. This sequence of transformations parallels the glutathione detoxification pathway (80). 11-Trans SRS-Cys is formed via nonenzymatic isomerization of the C-11(12) double bond (11-cis → 11-trans), catalyzed by the thiyl radical (RS$^\cdot$) (Fig. 16).

FIGURE 16. Biogenetic scheme of formation and degradation of SRS I and SRS II.


COOH
Lipoxygenase
Arachidonic Acid
O-OH
H
H
COOH
5-HPETE
LTA
Synthetase
OH
COOH
Glutathione
transferase
C5H11
S-CH2
CHCONHCH2COOH
NHCO(CH2)2CHCOOH
NH2
Leukotriene C
O
COOH
C5H11
LTA
γ-Glutamyl
transferase
OH
COOH
C5H11
S-CH2
CHCONHCOOH
NH2
SRS I
(SRS-CysGly, LTD4)
Peptidase
(Dipeptidase)
OH
COOH
C5H11
S-CH2
CHCOOH
NH2
SRS II
(SRS-Cys, LTE4)
RS•
(OH⁻ RSH)
11
12
OH
COOH
C5H11
S-CH2
CHCOOH
NH2
11-trans SRS-Cys

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