BIOCHEMICAL INVESTIGATIONS OF SLOW REACTING SUBSTANCES FROM RAT BASOPHILIC LEUKEMIA-1 CELLS

A thesis submitted to the Graduate School of the University of Wisconsin-Madison in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

DAI-EUN SOK

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Date of Examination

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icosatetraenoic acid and 15-(S)-hydroxy-14(R)-S-cysteinylglycyl-5,8Z,10,12E-icosatetraenoic acid.

APPROVED: Charles J. Sih

Professor Charles J. Sih

DATE: December 1, 1982

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A thesis submitted in partial fulfillment of the requirements for the degree of

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To my family

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Abbreviations:

RBL-1, Rat basophilic leukemia-1; SRS, slow reacting substance; SRS-A, slow reacting substance of anaphylaxis; RP-HPLC, reverse phase high pressure liquid chromatography; SRS-Cys, 5-hydroxy-6-S-cysteinyl-7,9,11,14-icosatetraenoic acid (LTE); SRS-Cys-Gly, 5-hydroxy-6-S-cysteinylglycyl-7,9,11,14-icosatetraenoic acid (LTD or SRS-A); SRS-GSH, 5-hydroxy-6-γ-glutamylcysteinylglycyl -7,9,11,14-icosatetraenoic acid (LTC); 5,6-LTA (leukotriene A), trans-5(S),6(S)-oxido-7,9,-E-11,14-z-icosatetraenoic acid; SRS-NTFA-Cys-Gly, 5-hydroxy-6-S-N-trifluoroacetyl-cysteinylglycyl-7,9,11,14-icosatetraenoic acid; 5-HPETE, 5(S)-hydroperoxy-6E-8,11,14Z-icosatetraenoic acid; 5-HETE, 5(S)-hydroxy-6,8,11,14-icosatetraenoic acid; LTB, leukotriene B; 5,12-diHETE, 5,12-dihydroxy-6,8,10,14-icosatetraenoic acid; 5,15-diHETE, 5,15-dihydroxy-6,8,11,13-icosatetraenoic acid; 15-HETE, 15(S)-hydroxy-5,8,11Z,13E-icosatetraenoic acid; 15-PHETE, 15(S)-hydroperoxy-5,8,11Z,13E-icosatetraenoic acid; 14,15-LTA, (S)-trans-14,15-oxido-5,8Z-10,12E-icosatetraenoic acid; 5,6-DHA, 5,6-dehydroarachidonic acid; 15(S)-HEYA, 15(S)-hydroxy-8,11Z,13E-icosatrien-5-ynoic acid; 14,15-diHETE, 14,15-dihydroxy-5,8,10,12-icosatetraenoic acid; 8,15-diHETE, 8,15-dihydroxy-5,9,11,13-icosatetraenoic acid; ETYA,

5,8,11,14-icosatetraynoic acid; BW-755C, 3-amino-1-(3-trifluoromethyl phenyl)-2-pyrazoline hydrochloride; HTMP, 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical; p-NCS, p-nitrocatechol sulfate; p-NPS, p-nitrophenyl sulfate; HAC, acetic acid.

I. INTRODUCTION

Slow reacting substances (SRSs), which are known to be important mediators in bronchial asthma and other types of immediate hypersensitivities, are a family of acidic lipids that possess potent spasmogenic properties on several types of smooth muscles including human bronchi. SRS was first described by Feldberg and Kellaway (1938) as a smooth muscle contracting activity released from guinea pig lung after perfusion with cobra venom (1). Two years later, Kellaway and Trethesie (2) reported that slow reacting substances of anaphylaxis (SRS-A) was released from sensitized tissues when challenged by specific antigens. It was distinguished from histamine by the slow onset and its sustained nature of the response. This observation was confirmed by Brocklehurst with the demonstration (3) that antihistamines failed to antagonize the bioactivity of SRS. Since then, many reports on the chemistry and biology of SRSs have appeared in the literature (4-11). However, it was not until recently that the chemical structures of these substances were defined.

1. Isolation and characterization of SRS

Many of the early attempts to determine the structure

of these substances were carried out using immunologically generated SRS-A (6) or using SRSs released from unsensitized tissues or cells by calcium ionophore A23187 (7) or compound 48/80 (8).

Although the biological activities of the partially purified materials had been described, numerous attempts to characterize the chemical structures of SRSs had been unsuccessful, mainly because samples were impure and were available in only small quantities.

In 1962, Brocklehurst (4), developed a procedure for the partial purification of SRS-A by adsorption of SRS-A on charcoal and ether extraction (pH,3.0). Using this partially purified material, he showed that SRS-A was an acidic substance by its electrophoretic migration and that SRS-A was resistant to the action of proteolytic enzymes. Another SRS was shown to be released by compound 48/80 from cat paws by Anggard et al. (10). This non-immunologically generated SRS was found to be indistinguishable from SRS-A on the basis of the chemical and pharmacological properties.

In 1973, Orange et al. (11) attempted to purify the SRS-A from rats by chromatography on Amberlite XAD-2, silicic acid and sephadex G-50 or LH-20. The partially purified samples were shown to contain sulfur. Further, they noted that SRS-A activity was inactivated by limpet

arylsulfatase. Based on these results, Orange and his coworkers (12) suggested that SRS-A possessed a sulfate ester grouping.

Meanwhile, Orange and Chang (1975) noted that L-cysteine enhanced the antigen-induced formation and release of SRS-A (13). In 1976, Orange and Moore (14), further substantiated this finding using various in vitro and in vivo models. More recently, Bach and Brashler (15) reported the stimulation of SRS-A release from rat mononuclear cells by thiol containing compounds.

Engineer et al. (16) used various enzymes to characterize SRSs and they first noted the inactivation of SRS-A by soybean lipoxygenase. Jakschik et al. (17) showed that arachidonic acid was involved in the biosynthesis of SRS-A in RBL-1 cells. Morris et al. (18) were the first to publish an UV spectrum of SRS-A.

While these developments were in progress, Samuelsson et al. isolated 5,12-diHETEs and 5,6-diHETEs after the incubation of arachidonic acid with polymorphonuclear leukocytes. They recognized the similarities of the characteristic conjugated triene chromophores in these diHETEs and SRS-A, and suggested the existance of a biogenetic relationship between diHETEs and SRS-A.

In 1980, Murphy et al. (19) generated SRS from murine mastocytoma cells treated with calcium ionophore A23187

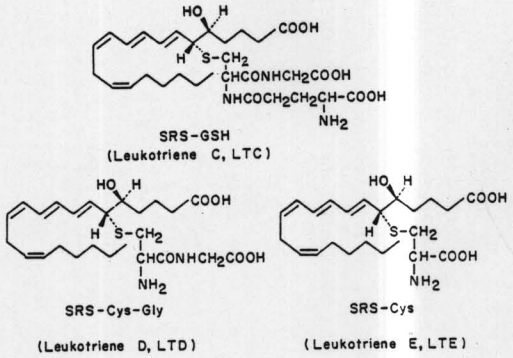
and L-cysteine. The compound was successively purified by chromatography on Amberlite XAD-7, silicic acid and by RP-HPLC. The UV spectrum of the purified sample and the antagonism of its bioactivity by FPL 55712 were the same as those reported for SRS-A.

Further labelling experiments demonstrated that arachidonic acid and cysteine were incorporated into the product. They reported that the structure of the major SRS from mastocytoma cells was 5-hydroxy-6-S-cysteinyl-7,9,11,14-icosatetraenoic acid, and termed it leukotriene C. However, this structural assignment was later found to be incorrect by comparison with a synthetic sample. In subsequent studies, leukotriene C was characterized by comparison with the properties of a synthetic specimen (HPLC, UV spectrum, reaction with soybean lipoxygenase and bioassay). The chemical structure of leukotriene C isomer was confirmed (20) by total synthesis to be 5(S)-hydroxy, 6(R)-S-glutathionyl-7,9-trans-11,14-cis-icosatetraenoic acid (LTC). A minor component was later identified as 11-trans-LTC.

Also in 1980, the British group (21) isolated a SRS from RBL-1 cells after incubation with L-cysteine, indomethacin, arachidonic acid and calcium ionophore A23187. Although the material showed the characteristic UV spectrum of SRS, its purity was questionable. From

mass spectroscopic data of the N-acetyl, trimethyl silyl ether, methyl ester derivative, they proposed the structure of this SRS material as 5-hydroxy-6-cysteinylglycyl-7,9,11,14-icosatetraenoic acid (LTD). Later, Orning et al. (22) isolated a SRS from RBL-1 cells. On the basis of its UV spectrum, enzymatic reaction with soybean lipoxygenase and amino acid composition, this SRS was reported to correspond to LTD, in agreement with the reports of Morris et al. (21). A small amount of 11-trans-LTD was also observed by this group.

Rat peritoneal cells were also reported to produce LTC and LTD after challenge with ionophore A23187 in the presence of arachidonic acid and L-cysteine (23).



In mid-1980, Houglum et al. (24) reported the chemical structures of two SRSs, generated from cat paws after challenge with compound 48/80. They were identified as SRS-Cys-Gly (LTD) and SRS-Cys (LTE) on the basis of chemical degradations, amino acid analyses, spectroscopic and enzymatic experiments, and by comparison with synthetic samples. It is noteworthy that SRS-Cys (LTE) was not obtained from the cells preincubated with L-cysteine by earlier workers.

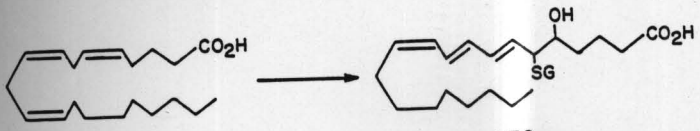
Recent reviews summarizing the identification of SRSs from other sources are now available (25-27).

This investigation describes the isolation and characterization of 6 SRSs from RBL-1 cells (SRS-GSH, SRS-Cys-Gly, SRS-Cys and their 11-trans isomers), and the effect of L-cysteine on the biosynthetic profiles of SRSs.

2. Biosythesis and Metabolism of SRS

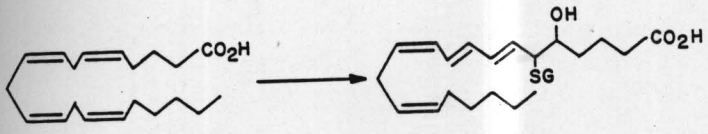
Recently, Jakschik and coworkers (28) showed that 5,8,11-icosatrienoic acid, and 5,8,11,14,17-icosapentaenoic acid were also converted enzymatically into active SRS-like compounds, which were identified as LTC_3 (29) and LTC_5 (30) in RBL-1 cells and mastocytoma cells, respectively. However, the native SRSs produced from the cells and tissues were shown to be LTC_4, LTD_4 and LTE_4, which are derived from endogenous arachidonic acid

Figure 1. Precursors of leukotriene Cs (SRS-GSHs). The unsaturated fatty acids 5,8,11-icosatrienoic acid, 5,8,11,14-icosatetraenoic acid (arachidonic acid), and 5,8,11,14,17-icosapentanoic acid are precursors of leukotrienes C_3, C_4, and C_5, respectively, where subscript is added to indicate the number of double bonds in the molecule. GSH, glutathione.



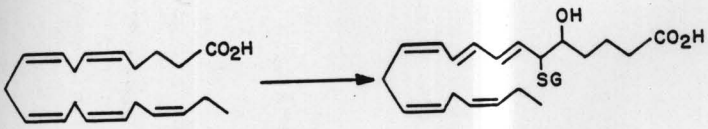
5,8,11- icosatrienoic acid

LTC_3



5,8,11,14- icosatetraenoic acid

LTC_4

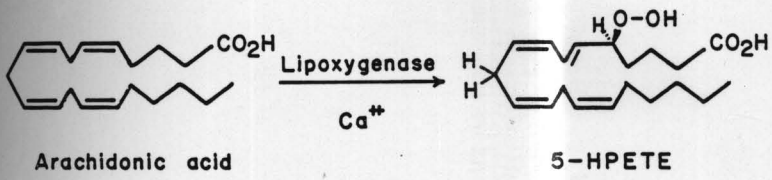


5,8,11,14,17- icosapentaenoic acid

LTC_5

A. 5-lipoxygenation of arachidonic acid and its inhibition

The biosynthesis of SRS from arachidonic acid involves an initial oxygenation of arachidonic acid by 5-lipoxygenase to form 5-HPETE.



Arachidonic acid 5-HPETE

The precursorial role of arachidonic acid in SRS formation was first demonstrated by Parker et al. (17), who reported that arachidonic acid was incorporated into SRS as evidenced by the comigration of radioactivity and bioactivity. This group also showed that ionophore A23187 markedly stimulated the formation of 5-HETE, the decomposition product of 5-HPETE. Subsequently, this ionophore was also reported to stimulate the production of 5-HETE and 5,12-diHETE in human PMNL (31), supporting the contention that calcium ions activate 5-lipoxygenase activity. Using a cell-free system, Jakschik et al. (32) showed that the 5-lipoxygenase activity of RBL-1 cells was

indeed stimulated by calcium ion in a concentration-dependent manner. Hence, the rates of formation of 5-HETE and 5,12-diHETE were markedly enhanced in the presence of calcium ions.

There were many reports (33-35) that ETYA inhibited the release of SRS-A in vitro and in vivo systems. It was proposed that this inhibitory effect of ETYA on SRS biosynthesis may be ascribed to the inhibition of 5-lipoxygenase. Especially in RBL-1 cells, the 5-lipoxygenation of arachidonic acid was found to be very sensitive to the action of ETYA (33). However, the effect of ETYA on the 5-lipoxygenase from other sources is variable from species to species (33-35). Furthermore, ETYA non-specifically inhibited 12-and 15-lipoxygenases, and prostaglandin synthetase (36). Consequently, many workers attempted to design more specific and more potent inhibitors of 5-lipoxygenase, that can block SRS biosynthesis (37).

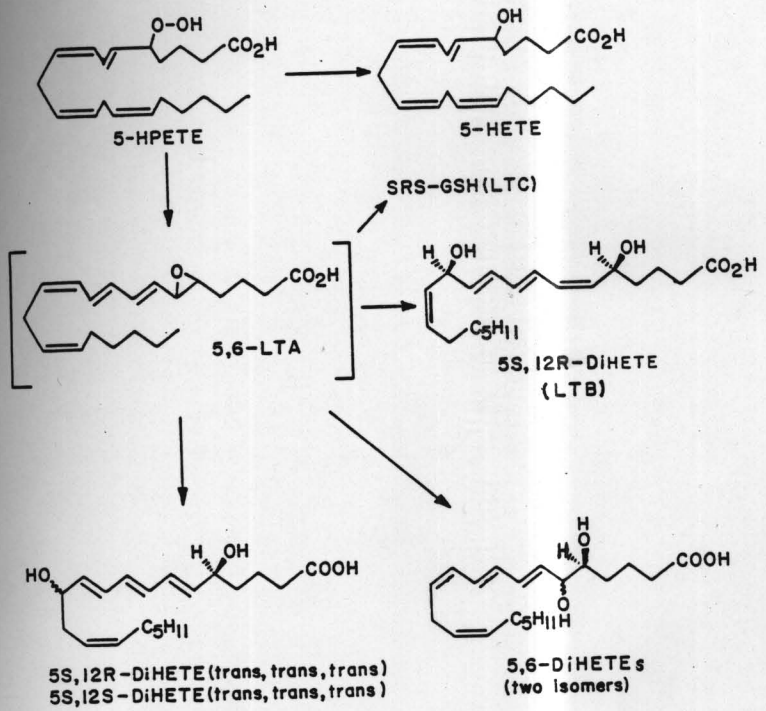
Hence, one phase of this study entails the use of acetylenic analogs of arachidonic acid as potential suicide substrates of 5-lipoxygenase to inhibit more specifically this first key enzyme of SRS biosynthesis.

B. Formation of 5,6-LTA and its conversion into SRS-GSH

Borgeat et al. (38) showed the presence of several 5-lipoxygenase products after exposure of arachidonic acid to human PMNLs. These were identified as: 5(S)-hydroxy-6-trans-8,11,14-cis-icosatetraenoic acid, 5(S),12(R)-dihydroxy-6,14-cis-8,10-trans-icosatetraenoic acid (LTB), 5(S), 12-dihydroxy-6,8,10-trans-14-cis-icosatetraenoic acids epimeric at C-12, and isomeric 5,6-dihydroxy-7,9,11,14-icosatetraenoic acids. The presence of the characteristic conjugated triene chromophore in these compounds combined with the isotopic oxygen incorporation data suggested the presence of an unstable intermediate in their formation. Trapping experiments with alcohols and other studies led to the identification of the allylic epoxide as 5,6-oxido-7,9-trans, 11,14-cis-icosatetraenoic acid (5,6-LTA) (39). This was later confirmed by comparison with synthetic specimens (40). Also, it was shown that LTB (39) was produced enzymatically from synthetic LTA, but other diHETEs were generated from LTA via non-enzymatic hydrolyses (Figure 2).

In 1980, Samuelsson et al. (40) showed the conversion of arachidonic acid into 5,6-LTA in human PMNLs by the isolation of (^{3}H)-5,6-LTA methyl ester admixed with synthetic 5,6-LTA methyl ester. They (41) also

Figure 2. Leukotrienes (LTs) biosynthesis in human polymorphonuclear cells.


O-OH
CO2H
5-HPETE
OH
CO2H
5-HETE
SRS-GSH(LTC)
O
CO2H
5,6-LTA
H OH
H OH
CO2H
C5H11
5S, 12R-DiHETE
(LTB)
HO
H OH
COOH
C5H11
5S,12R-DiHETE(trans, trans, trans)
5S,12S-DiHETE(trans, trans, trans)
H
H
O
COOH
O
C5H11H
5,6-DiHETEs
(two isomers)

demonstrated 5,6-LTA was transformed enzymatically by glutathione transferase in mastocytoma cells to form LTC, accompanied by variable amount of 11-trans-LTC. However, the mechanism of formation of 11-trans-LTC was not clearly understood.

Another segment of this investigation deals with the enzymatic formation of 5,6-LTA and its conversion into SRS-GSH in RBL-1 cells.

C. Transformation of SRS-GSH into SRS-Cys-Gly and SRS-Cys

The metabolism of SRS appears to mimic the detoxification pathway of the glutathione conjugates of xenobiotic compounds. Glutathione conjugates are converted into cysteinylglycine conjugates, and then to cysteine conjugates (42).

Orning et al. (22,37) showed that SRS-GSH is converted into SRS-Cys-Gly after short time exposure to porcine γ-glutamyl transpeptidase. L-Serine-borate buffer, an inhibitor of γ-glutamyl transpetidase, inhibited the formation of LTD_4 and the enzymatic hydrolysis of γ-glutamyl p-nitroanilide in RBL-1 cells with I_{50} of 11 mM. These results suggested that the properties of γ-glutamyl transpeptidase in RBL-1 cells was similar to those of renal γ-glutamyl transpeptidase.

Further enzymatic conversion of LTD_4 into LTE_4 was first demonstrated by Sih's group in 1980 (24). It was demonstrated that the peptidase activity in limpet arylsulfatase preparation converted SRS-Cys-Gly (LTD_4) into SRS-Cys (LTE_4). In subsequent studies (43), they showed that a kidney peptidase cleaved SRS-Cys-Gly into SRS-Cys, and this cleavage was inhibited completely by 10^{-2}M of cysteine. However, the identity of these peptidases was not completely defined. Although the kidney was known to contain peptidases which were capable of hydrolyzing cysteinylglycine (44), it was not until recently that the peptidases that act on the S-derivatized cysteinylglycine were identified. In 1980, Curthoys et al. (45) showed that the purified aminopeptidase-M can cleave the peptide bond in S-benzyl cysteinylglycine. Another group (46) demonstrated that aminopeptidase-M is

responsible for the cleavage of S-carbamido methyl cysteinylglycine. Recently, another renal enzyme, dipeptidase, was shown to hydrolyze cystinylglycine (42). In this investigation, the enzymes involved in the sequential metabolism of SRS-GSH in RBL-1 cells are examined.

D. Inactivation of SRS-Cys-Gly (SRS-A)

A variety of arylsulfatases were known to inactivate the spasmogenic activity of SRS-A (47-51). It was suggested that arylsulfatases may be responsible for the inactivation of SRS-A *in vivo* following antigen-antibody reaction (47). Moreover, susceptibility to arylsulfatase inactivation was used until recently as an important criterion in the positive identification of SRS-A (12).

Austen and his group (47) reported that partially purified arylsulfatase B from human eosinophils inactivated SRS-A from human lung to the extent of 95%. The arylsulfatases from human lung and guinea pig lung also inactivated SRS-A (48,49). In the case of RBL tumor cells, both arylsulfatase A and B were reported to be involved in SRS-A inactivation (50). However, there was no clear correlation between arylsulfatase contents and the ability to destroy SRS-A. Furthermore, the inactivation of SRS-A by crude snail and limpet

arylsulfatase was relatively slow, incomplete and required high enzyme to substrate ratio (51). Interpretations of these results were further complicated by the use of impure enzymes and crude SRS preparations of varying compositions, generated by different methods.

In 1980, Sih's group defined the mechanism of SRS-A inactivation by limpet arylsulfatase (24). This enzyme preparation cleaved the peptide bond in the highly bioactive SRS-Cys-Gly to form the much less bioactive SRS-Cys. However, it was not known whether arylsulfatase *per se* or a contaminating peptidase was responsible for the hydrolysis of the peptide bond in SRS-A (SRS-Cys-Gly). After establishing the metabolism of SRS in RBL-1 cells, we examined the enzymatic inactivation of SRS-A (SRS-Cys-Gly) in RBL-1 cells, guinea pig lung, peritoneal eosinophils, and limpet arylsulfatase preparations (47-51).

II. EXPERIMENTAL

1. Materials

Type I soybean lipoxygenase (155,300 units/mg), sulfatase (Patella vulgata, 9.4 units/mg), γ-glutamyl transpeptidase (bovine, 4.0 units/mg), type IV-S leucine aminopeptidase (11 units/mg), prostaglandin B_2, p-nitrophenyl sulfate (p-NPS), p-nitrocatechol sulfate (p-NCS), adenosine triphosphate (ATP), histamine dihydrochloride, glutathione (GSH), L-cysteine, and arachidonic acid (99%) were products of Sigma. Eagle's minimal essential medium, fetal calf serum, newborn calf serum, penicillin, amphotericin B, and streptomycin were supplied by GIBCO. Sephadex G-150 and G-200, DEAE-Sephadex (A-25), SP-Sephadex (SP-500-120) and Percoll were purchased from Pharmacia. D-Penicillamine, 3-mercaptopropionic acid, and 4-Hydroxy-2,2,6,6-tetramethylpiperidinooxy (HTMP) free radical were supplied by Aldrich. L-[U-^{14}C]Cysteine hydrochloride (60.76 mCi/mmole), [^{3}H]-Arachidonic acid (131 Ci/mmol) and [1-^{14}C]-Arachidonic acid (53.9 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England. Amberlite XAD-7 and silicic acid (100 mesh) were from Mallinckrodt.

The following compounds were purchased from the manufacturers as indicated: N,O-Bis-(trimethylsilyl)

trifluoroacetamide (BSTFA, Pierce Chemical); trimethylchlorosilane (Tri-Sil); mepyramine maleate (ICN-K&K); FPL 55712 (Fisons, Ltd); 5,8,11,14- icosatetraynoic acid (ETYA, Hoffmann-LaRoche); ionophore A23187 (Eli Lilly); Crystalline bovine albumin (Calbiochem); DEAE-cellulose (DE-52, Whatmann). Purified rat renal dipeptidase and aminopeptidase-M were kindly supplied by Drs. T. M. McIntyre and N. P. Curthoys, Department of Biochemisty, University of Pittsburgh. Various SRSs and 5,6-DHA were supplied by Drs. V. Atrache and C.T. Hsu, respectively. 15-HPETE (52) and 15-HEY (52) were synthesized by published procedures.

A model M-6000 pump, equipped with an U6K injector and a model 440 UV absorbance detector (Water Associates) was used for high-pressure liquid chromatography (HPLC). 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 (25-37 μ, Whatman).

Radioactivity was detected with a Model 2002 scintillation spectrometer (Packard) and a Model 930 auto-scanner (Vangard).

Unless otherwise specified, evaporation to dryness refers to rotary evaporation <u>in vacuo</u> at 30°-35°. Room temperature was 22°C.

2. Bioassay

SRS bioactivity was assayed by a modification of the procedure reported by Chakravarty (54). A guinea pig (300-500g) of either sex was killed by a blow to the head. The peritoneal cavity was opened and the ileum tissue removed at approximately 10 cm from the terminus were immersed in a petri dish containing warm Tyrode's buffer with atropine only. A 2-4 cm section of ileum was washed with warm Tyrode's buffer, and then affixed to the assay apparatus where it was maintained at 37°C in 10 ml of Tyrode's buffer aerated with 5% of CO_2 in O_2. A frictionless horizontal lever, with approximately 12X magnification and 500 mg counterweight was utilized.

Histamine was utilized to standardize the ileum using the Tyrode's buffer with 10^{-6} M atropine only. The tissue was successively sensitized by histamine. After reproducible contraction response was obtained, a standard solution of SRS was added to the ileum suspended in Tyrode's buffer containing 10^{-6}M atropine and 10^{-6}M mepyramine. After determining the log-dose response curve for histamine, a similar curve of standard SRS was used for the quantitation of SRS sample.

One unit of SRS was defined as the amount of SRS that caused a contraction with a peak height equal to 5 ng of histamine base. For the higher accuracy, standard SRS

sample was assayed before and after the assay of unknown samples. The SRS activity was confirmed by the observation (55) that, at maximal contraction of the ileum, the addition of FPL 55712 (2-8 x 10^{-8}M) caused an immediate relaxation of the contracted ileum. The tissue was rinsed at least twice with fresh Tyrode's buffer after the test of each SRS sample, and the interval between samples was 4-10 minutes. For bioassay, samples were evaporated under N_2, and redissolved in Tyrode's buffer.

3. Growth of Rat Basophilic Leukemia-1 cells (RBL-1 cells)

RBL-1 cells were kindly provided by Dr. A. Kulczycki (Dept. of Microbiology, Washington University, St. Louis, MO). For maintenance, the cells were grown in Eagle's minimal essential medium supplemented with Earle's salts, 0.06% L-glutamine, and 10% fetal calf serum. For SRS production, RBL-1 cells were grown in spinner culture in Eagle's minimal essential medium supplemented with Earle's salts, L-glutamine (0.06%), fetal calf serum (3%), newborn calf serum (12%), penicillin G(100 units/ml), streptomycin (100 µg/ml) , and amphotericin B (0.25 µg/ml), in an atmosphere of 5% CO_2 in air.

4. Isolation and characterization of SRSs

A. Generation of SRS from RBL-1 cells

Cells were harvested by centrifugation (400xg, 10 min), washed once in buffer (150 mM NaCl/3.7 mM KCl/3.0 mM Na_2HPO_4/25 mM KH_2PO_4/0.9 mM $CaCl_2$/5.6 mM glucose, adjusted to pH 7.2 with 1 M NaOH), and suspended in this buffer to 1-1.5 x 10^7 cells per ml. After addition of L-cysteine (7.5 mM), the cell suspension was incubated for 2 min at 37°C with gentle shaking. Arachidonic acid dissolved in ethanol and ionophore A23187 dissolved in dimethyl sulfoxide were added to yield final concentrations of 3 μg/ml and 10 μg/ml , respectively. Separately, the reaction was carried out in the absence of L-cysteine. At the indicated time intervals, an aliquot was removed from the reaction mixture and frozen at -78°C. The aliquot was dissolved in Tyrode buffer and assayed on the guinea pig ileum for the determination of total activity. After 20 min of incubation at 37°C, the cells were sedimented (2,000xg, 5 min) and the supernatant was made 80% (vol/vol) in cold ethanol.

B. Purification of SRS

After 30 min at 0°C, the ethanol solution was centrifuged (10,000 xg, 20 min) and the supernatant was evaporated to dryness under reduced pressure. The residue was dissolved in 25 ml of distilled water, adjusted to pH

7.0, and applied onto an XAD-7 column (1.2 x 15 cm). The column was washed with 60 ml of distilled water and the SRS activity was eluted with 40 ml of 80% ethanol. After evaporaton of the solvent, the residue was dissolved in 6 ml of chloroform/methanol (9:1, vol/vol) and applied onto a silicic acid (0.5 g) column (1.3 x 1 cm) previously washed with 10 ml of chloroform. The column was successively eluted with 10 ml of chloroform, 10 ml of chloroform/methanol (95:5, vol/vol), 6 ml of methanol, and 4 ml of methanol/water (99:1, vol/vol). The SRS activity resided in the methanol and methanol/water fractions, which were combined and evaporated to dryness. The residue was dissolved in 0.1 ml of methanol/water (1:1, vol/vol) and applied onto a Radial-Pak C_{18} column. After the injection of samples onto a Radial-Pak C_{18} cartridge (0.8 x 10 cm) with precolumn, the column was eluted with a mobile phase of methanol/water (65:35, V/V), pH 5.4 (0.05% HAC + NH_4OH) at a flow rate of 2 ml/min. The effluent was collected and bioassayed. The peak 1 was rechromatographed over the same column, using a mobile phase consisting of methanol/water (55:45, V/V) containing 0.05% HAC, pH 5.4 at a flow rate of 2 ml/min. For chemical analyses and enzymatic studies, the active fractions were separately rechromatographed over the same column with a mobile phase, methanol/water (7:3, V/V),

pH 5.4 (0.05% HAC + NH_4OH).

C. Chemical analyses of purified SRSs

i) UV spectrum

UV spectra were recorded on a Cary 118 spectrophotometer with a wavelength calibration accuracy of ±0.1nm at 165-320 nm (24).

ii) Amino acid analyses

Ten nmoles of SRSs were suspended in 1 ml of 6 N HCl. Hydrolysis was carried out at 110°C for 24 hrs as described (56). After hydrochloric acid was removed using the Speed-Vac concentrator, the residue was dissolved in 20 µl of citrate buffer (pH 2.2) and the tube was washed again with 20 µl of the same buffer. The dissolved sample was then injected onto the Durrum D-500 automated analyzer.

iii) GC-Mass Spectra

RBL peak 1a of RP-HPLC was converted to its N-acetyl derivative by treatment with acetic anhydride/methanol (1:4, vol/vol) for 1.5 hr. It was then esterified with hydrochloric acid in methanol (0.4 M) and trimethyl silylated according to the procedure described by Morris et al. (21). Mass spectra were determined on a Finnigan model 4021-T instrument at 70 ev with an ionizing current of 0.35 mA.

For the GC-MS of desulfurized products, each peak of

SRSs (15-20 ug) was 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 desulfurized products were methylated with ethereal diazomethane, trimethyl silylated with Tri-Sil/BSA (Pierce), and subjected to GC-MS analysis as described above.

D. Bioactivities of SRSs and antagonism by FPL 55712

Dose response curves of SRS and histamine were obtained according to the method described before (24). For antagonism studies, SRS-Cys (100 p moles), SRS-Cys-Gly (2 p moles) or SRS-GSH (6 p moles) was added to the 10 ml Tyrode buffer, and the responses were recorded. FPL 55712 (10 ng/ml) was added at maximal contraction to antagonize the contractile activity of SRSs. Higher concentration of FPL 55712 (100 ng/ml) was used, where indicated.

E. Enzymatic Studies

The present SRS activation or inactivation was calculated compared with the boiled control incubated for the time intervals indicated.

i) Inactivation of SRSs with soybean lipoxygenase.

The reaction mixture contained 2.5 nmol (based on UV absorption at 280 or 278 nm, ε = 40,000 $M^{-1}cm^{-1}$) of SRS and 10 μg of soybean lipoxygenase in 1 ml of Tyrode buffer, pH 7.8 at 20°C. Spectra were recorded at various

time intervals with a Cary model 118 spectrophotometer.

ii) Incubation of SRSs with γ -glutamyl transpeptidase

The system contained; 0.8 units/ml of γ -glutamyl transpeptidase (bovine), 55 nmoles of SRSs (based on UV absorption at 280 mn), 10 mM cysteine and 10 mM $MgCl_2$ in 0.05 M Tris-HCl buffer, pH 8.5. Incubations were carried out at 37°C, and at the indicated time intervals an aliquot was removed, and used for the assay of residual activity.

iii) Inactivation of SRSs by bovine renal peptidase (Aminopeptidase-M preparation)

The system contained 0.06 units of enzyme and various quantities of SRSs in 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.2. Incubations were carried out at 37°C, and at the indicated time intervals an aliquot was removed from the reaction mixture and frozen at -78°C to terminate the enzyme reaction. This aliquot was dissolved in 1 ml of Tyrode buffer and the residual SRS activity was assayed. Thiol inhibition of the destruction of SRS-Cys-Gly by renal peptidase was carried out as described in Figure .

5. Biosynthesis and metabolism of SRS

A. Determination of 5-lipoxygenase activity and its inhibition

i) Cell-free extract

RBL-1 cells harvested as described previously (43) were washed with 50 mM phosphate buffer, pH 7.0, 1 mM EDTA, 0.1% gelatin and 14 μM indomethacin, resuspended to 5 x 10^7 cells/ml, homogenized with teflon homogenizer (5 strokes, at 4°C) and sonicated in Branson sonicator (70 watts, 45 secs). The homogenate was centrifuged at 10,000 x g for 10 min, and to the supernatant (1 ml) was added 0.6 mM Ca^{++} and [1-^{14}C]- arachidonic acid (50,000 cpm/ml, 40 μM), and the contents were then incubated at 37°C for 15 min. Separately, variable concentrations of calcium ion were included in the incubation mixture, as described in Table 3.

For the inhibition study, the 10,000 x g supernatant was preincubated with inhibitors, acetylenic acids at 15°C before the addition of arachidonic acid and calcium ion.

ii) Intact cell studies

RBL-1 cells were washed with the buffer described (43), and suspended to the cell density of 1.8-2.5 x 10^7/ml. The cells suspension was incubated with [1-^{14}C]-arachidonic acid (40 μM) and ionophore A23187 (10 μM) at 37°C for 15 min. For the inhibition study, the inhibitors

(acetylenic acids) were preincubated with cells 5 min before the addition of arachidonic acid at 37°C.

These enzymic reactions were terminated by the addition of one volume of acetone. The pH of the supernatant was adjusted to 3.2 with 10% formic acid and extraction performed twice with two volumes of chloroform. Thin layer chromatography was carried out on silica gel plate in solvent system A 9, the organic phase of ethyl acetate:2,2,4-trimethyl pentane: acetic acid: water (110:50:20:100). Radioactive zones on TLC plates were monitored by radioactive autoscanner (Model 930, Vangard Systems, Inc.), and were cut out and quantitatively assayed using liquid scintillation counting (32).

iii) Conversion of 15-HETE into 5,15-diHETE

The RBL-1 cell suspension (50 ml, 1.8-2.5 x 10^7/ml) in 6,5 mM phosphate buffer, pH 7.0 (43), was incubated with 15-HETE (20 µg/ml) at 37°C for 15 min. In a separate experiment, the incubation of RBL-1 cells with 15-HETE was carried out in the presence of Ionophore A 23187 (10 µg/ml). After 15 min, the reaction was terminated by the addition of 3 volumes of ethanol. After evaporation of ethanol, the aqueous layer was acidified (pH 3.0) and extracted with ethyl acetate. After evaporation, the residue was chromatographed over a C_{18}

column and the amount of 5,15-diHETE was quantitatively assayed (57).

B. Formation of [^{3}H]-5,6-LTA from [^{3}H]-arachidonic acid.

To the 100 ml of RBL-1 cell suspension (1.5-3.0 x 10^7 cells/ml) was added 15 µmol of [^{3}H]-arachidonic acid. After 30 sec incubation at 37°C, the reaction was quenched by adding 1.5 volumes of an alkaline 0.1 M borate buffer in methanol, cooled to 0°C. Another 30 sec later, the mixture was filtered to remove the precipitated proteins, and the filtrate was immediately poured into 2 volumes of a freshly prepared solution of diazomethane in diethyl ether which was kept at 0°C in an open beaker. After stirring the solution for 90 sec, ethyl ether (4 volumes) containing the free radical capture Tempol (10 mg/L) and 0.1 M sodium bicarbonate (4 volumes) and 0.02 M NaCl solution were added. The mixture was shaken and set to separate the ether phase, which was evaporated. Finally, the [^{3}H]-5,6-LTA methyl ester was coinjected with synthetic specimens into SiO_2 column (Figure 18).

C. Formation of SRS-GSH from 5,6-LTA

5,6-LTA was prepared by hydrolysis of 5,6-LTA methyl ester (150 µg) in 250 µl of alkaline DME solution (DME:0.3 M LiOH = 1:1) at room temperature. The 5,6-LTA (70 µg) in the 125 µl alkaline DME solution was added to the RBL-1

cell suspension in the buffer, pH 7.0 including 5 mM serine and 20 mM borate buffer at 37°C. The contents were then incubated at 37°C for 20 min, and the reaction was terminated by the addition of ethanol (4 volumes). The purification of products was carried out as described before (Figure 19).

D. Metabolism of SRS in RBL-1 cells

RBL-1 cells (1-1.5 x 10^7 cells/ml) suspended in the buffer (43), were incubated with arachidonic acid (3 μg/ml) and ionophore A23187 (10 μg/ml) at 37°C with gentle shaking. At various time intervals (Figure 20), aliquots (50 ml) of the reaction mixture were removed, purified as described (43), and analyzed by HPLC [methanol/water (65:35, vol/vol) containing 0.05% acetic acid buffered to pH 5.4 with NH_4OH]. Separately, the aliquots were assayed for total bioactivity. The cells suspension was boiled after 40 min and was then incubated for another 6 hrs to give values of control samples (Table 4).

Where indicated, HTMP was added at 0.5, 5, or 50 μmol per 50 ml of cell suspensions after 40 min of incubation. Incubation was carried out for an additional 6 hrs as described (Table 4).

E. Transformation of SRS-Cys (λ_{max}280nm) into 11-trans-SRS-Cys (λ_{max}278nm) in the presence of L-[U-^{14}C] Cysteine.

To a RBL-1 cell suspension [3x10^8 cells per 20 ml of the buffer (43)] was added L-[U-^{14}C] cysteine (10μCi), L-cysteine (20μmole), and synthetic SRS-Cys(0.125μmol). The reaction mixture was incubated for 6 hr at 38°C. A control experiment with boiled RBL-1 cells was carried out in the same manner.

F. Transformation of SRS-GSH by γ-Glutamyl transpeptidase (Bovine)

The system contained 0.8 unit of γ-glutamyl transpeptidase, 55 nmol of synthetic SRS-GSH and 10 mM $MgCl_2$ in 1 ml of 0.05 M Tris-HCl buffer, pH 8.5. Incubation was carried out with or without 10 mM L-cysteine 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 Tyrode buffer and assayed quantitatively as described before. After 30 min, 4 ml of cold ethanol was added to the reaction mixture which was allowed to stand for 30 min at 5°C. After centrifugation (1,000xg, 5 min), the supernatant was evaporated to dryness. The residue was dissolved in 0.1 ml of methanol/water (1:1, vol/vol) and applied onto a C_{18} column for further purification (Figure 4).

G. Enzymatic inactivation of SRS-Cys-Gly

i) Arylsulfatase Assay

The rate of p-nitrocatechol formation was determined by measuring the absorbance at 515 nm (58) using a Gilford spectrophotometer (Model 240). Specific activities were expressed in unit (1 μmole product per hour) per mg of protein. Protein was determined by the method of Lowry (59) and the Biuret method (60) using bovine albumin as standard.

ii) SRS inactivation Assay

The system contained: 0.3 nmoles of SRS-Cys-Gly and enzyme in 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.2. The contents were incubated at 37°C and after 30 min incubation, an aliquot was removed and the residual SRS activity was bioassayed using the guinea pig ileum. One unit of SRS inactivating activity was defined as that amount of enzyme which destroys 0.1 nmoles of SRS-Cys-Gly in 30 min.

iii) Inactivation of SRSs by peptidases

The system contained 0.3 nmoles of SRSs and 0.15 units (77) of purified renal dipeptidase or aminopeptidase-M, or 5 units of SRS inactivating activity (RBL-peptidase or guinea pig lung peptidase) in 0.5 ml of 0.05M Tris-HCl buffer, pH 7.2. The contents were incubated at 37°C and at the indicated time intervals, an

aliquot was removed and frozen at -78°C to terminate the enzyme reaction. This aliquot was diluted to 1 ml of Tyrode buffer and the residual SRS activity was assayed. Values of control samples were obtained by incubation of boiled enzyme with SRS for the time intervals indicated.

iv) Purification of SRS-Cys-Gly inactivating peptidase from RBL-1 cells

RBL-1 cells (2×10^9) were harvested by centrifugation (400xg, 10 min), washed as previously described (43) and suspended in 0.2M acetate buffer, pH 5.7 to 5×10^7 cells per ml. The cell suspension was sonicated for 1 min at 4°C with a Branson sonifier and was then repeatedly frozen and thawed (4 times). After centrifugation of the mixture at 15,000xG for 15 min at 4°C, the supernatant (35 ml) was dialyzed against 0.01 M Tris-HCl buffer, pH 8.0 for 16 hours at 4°C. The dialyzate was applied onto a DEAE-cellulose column (1.6 x 26 cm), previously equilibrated with the same buffer. The column was washed with three void volumes of the same buffer and then eluted with a 400 ml of linear salt gradient to 0.4 M NaCl at 1 ml/min (Figure 26). Fractions (54-64) containing SRS inactivating activity were concentrated and applied onto a Sephadex G-200 column (1.6 x 70 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 7.0. The column was eluted with the same buffer at a flow

rate of 6 ml/hr (Figure 23). The eluate was concentrated, and then used for the SRS inactivation study.

v. Resolution of SRS-Cys-Gly inactivating activity from sulfatase activity

i) RBL-1 cells: Cells ($2x10^9$) were harvested and the cell extract was prepared (50) and dialyzed against 0.01 M Tris-HCl buffer, pH 8.0. The dialyzate was applied onto a DEAE-cellulose column as described in Figure 26.

ii) Guinea Pig lung: An extract of guinea pig lung (25 g) was prepared (49) and dialyzed against 0.01 M Tris-HCl buffer, pH 8.0. To the dialysate was added DEAE-cellulose (10 g), equilibrated with 0.01 M Tris-HCl buffer, pH 8.0. After 30 min, the mixture was filtered and the enzyme activity was eluted off the DEAE-cellulose with the same buffer containing 0.2 M NaCl. The filtrate (30 ml) after dialysis was chromatographed over a DEAE-Sephadex column (Figure 27).

iii) Guinea Pig Peritoneal Eosinophils: The cells, collected by peritoneal lavage of ether- anesthetized animals with 50 ml of saline, were centrifuged (400xg, 10 min). The cells were washed once with saline and suspended to 1-3 x 10^7/ml in a Percoll density gradient and centrifuged (61). The eosinophil-rich fraction (83%, density 1.090-1.100 g/ml) was washed once with saline and the pellet was stored at -20°C. The cells ($3.5x10^7$),

suspended in 2.5 ml of 0.1 M sodium acetate buffer, pH 5.7, were sonicated for 30 sec at 4°C. The cell extract was subjected to freezing and thawing (6 times) and was then centrifuged (600xg, 20 min). The supernatant was dialyzed against 0.01 M Tris·HCl buffer, pH 8.0 and the dialysate was applied onto a DEAE-cellulose column (Figure 28).

iv) Limpet arylsulfatase: The enzyme (5 mg), dissolved in 15 ml of 0.001 M sodium acetate buffer, pH 5.0 was applied onto a SP-Sephadex colum, and eluted as described (Figure 29).

III. Results

1. Isolation and Characterizations of SRSs.

When RBL-1 cells were incubated in the presence of L-cysteine (7.5 mM) and Ca^{++} ionophore A23187 the bioactivity of SRS increased rapidly and gradually leveled off with time reaching a plateau (Figure 3). After 20 min, the reaction mixture was centrifuged at 4°C and the supernatant was used for the isolation of SRS. Using a purification procedure (Table 1) consisting of XAD-7 column and silicic acid column chromatography, and RP-HPLC, three major SRSs were separated with an overall recovery of 19% by HPLC (Figure 4A) using a mobile phase consisting of methanol/water (65:35). Peak 1 had a retention time of 4.5-4.8 min, whereas peak 2a and 2b had elution times of 18-18.5 min and 20.5-21.5 min, respectively. When L-cysteine was omitted from the incubation, there was an initial increase in total contractile activity which slowly decreased with time and the types of SRS produced were altered. As shown in Figure 4B, peak 1 had the same retention time as peak 1 of Figure 4A, whereas peak 3a and shoulder 3b were eluted after 19.5-21 min and 23-24 min, respectively. Peak 1, which was observed in both cases, was further resolved into two peaks, 1a and 1b by additional RP-HPLC and

Figure 3. Time course of SRS formation in RBL-1 cells. The incubation and bioassay conditions were as described in Experimental. o-o; with 10^{-2}M L-cysteine, •-•; without L-cysteine. Average value of 3 experimental sets was taken.

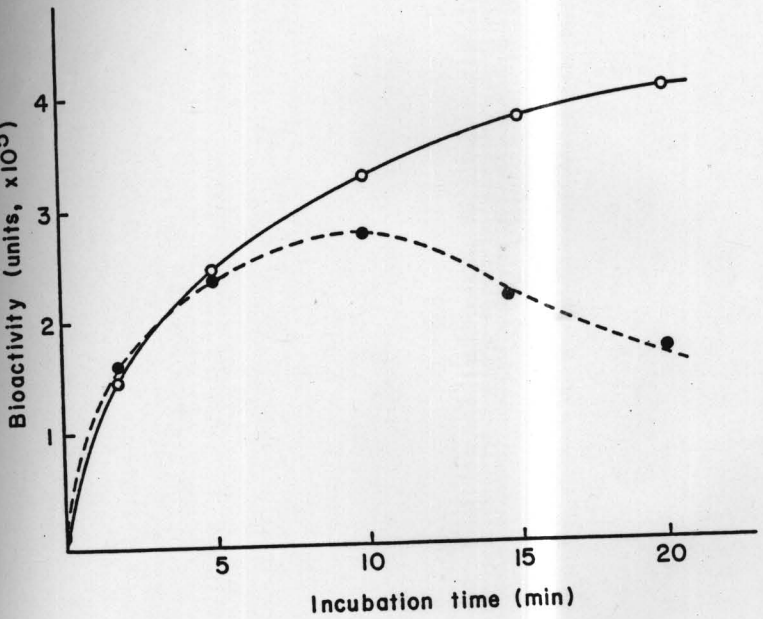

Bioactivity (units, x10⁵)
4
3
2
1
5
10
15
20
Incubation time (min)

Table 1. Purification of SRSs from RBL-1 cells.

Stage	Procedure	Total activity (units)		Recovery (%)	
		Control	+cysteine	Control	+Cysteine
1	Incubation mixture	160,000	400,000	100	100
2	Ethanol extract	120,000	350,000	75	88
3	XAD-7 column	95,000	250,000	59	63
4	Silicic acid column	70,000	180,000	44	45
5	RP-HPLC	21,700	77,000	14	19
	peak 1	6,000	8,000		
	2a	10,500	63,000		
	2b	-----	6,000		
	3a	4,700	-----		
	3b	500	-----		

Figure 4. Reverse-phase HPLC profiles of SRSs derived from RBL-1 cells. (A) Incubation with L-cysteine; (B) incubation without L-cysteine. Stationary phase: Radial-Pak C_{18}; mobile phase: methanol/water (65:35), pH 5.4 (0.05% acetic acid + NH_4OH). Flow rate: 2 ml/min. PGB_2, prostaglandin B_2.

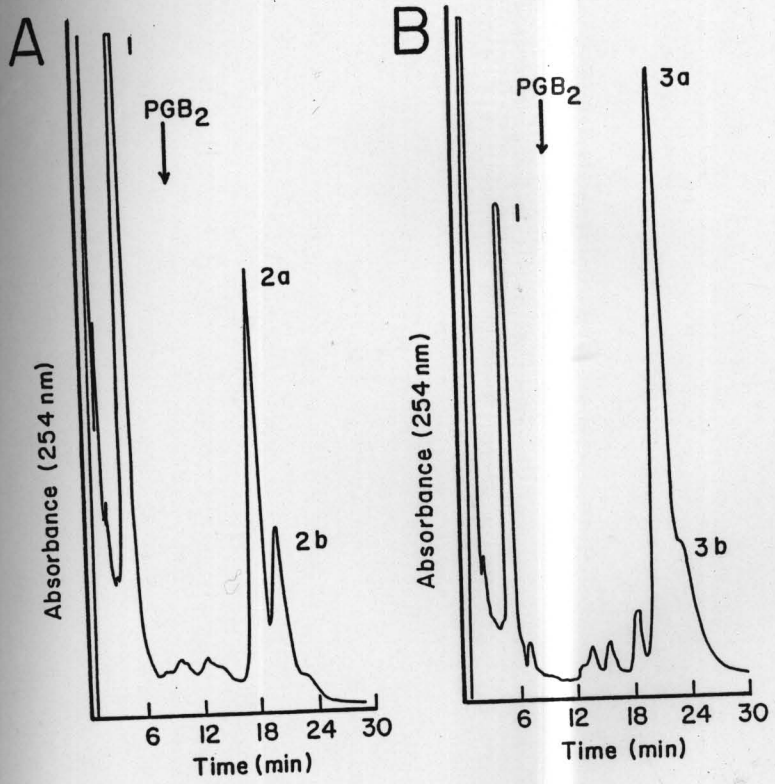
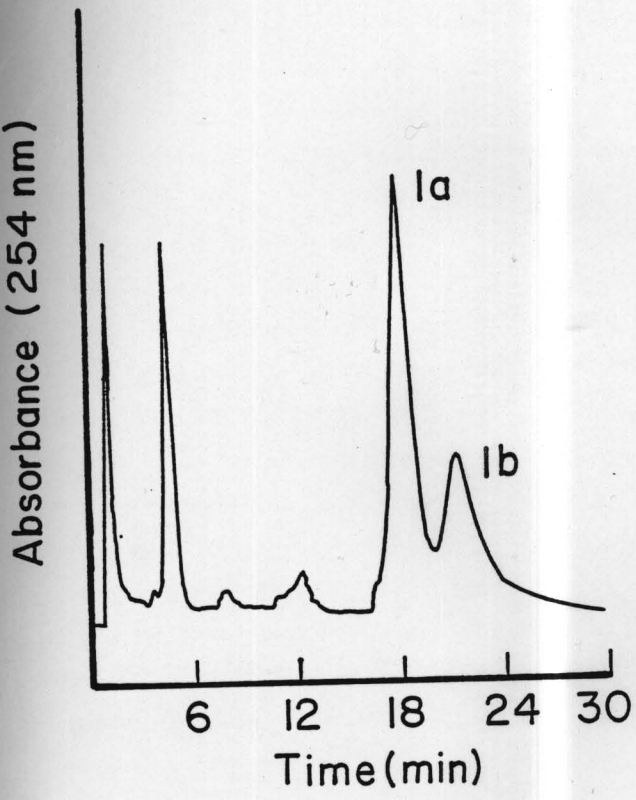

A
1
PGB2
2a
2b
Absorbance (254 nm)
6
12
18
24
30
Time (min)
B
PGB2
3a
1
3b
Absorbance (254 nm)
6
12
18
24
30
Time (min)

Figure 5. HPLC rechromatography of peak 1. Stationary phase: Radial-Pak C_{18}; mobile phase: methanol/water (55:45), pH 5.4 at a flow rate of 2 ml/min.


Absorbance (254 nm)
Ia
Ib
6
12
18
24
30
Time (min)

elution with methanol/water (55:45), pH 5.4 (Figure 5).

The UV absorption spectra of peaks 1a, 2a and 3a were found to be identical, exhibiting a maximum at 280 nm with shoulders at 270 nm and 292 nm (Figure 6). In contrast, peaks 1b, 2b and 3b all show an UV absorption maximum at 278 nm with shoulders at 268 nm and 290 nm. These spectral data strongly suggest the presence of conjugated triene chromophores in these SRSs, as was previously noted (19).

Amino acid analyses of peak 1a or 1b indicated that these molecules contained glutamic acid, cysteine and glycine. Approximately 1 mol of glutamic acid and 1 mol of glycine per mol of SRS were obtained, based on the UV absorption at 280 nm or 278 nm (ε_{280} or $_{278}$=40,000 M^{-1} cm^{-1}). The yield of half cystine was about 0.50 mol per mole of SRS. Peak 2a and 2b gave cysteine and glycine in a ratio of 0.5:1 whereas peak 3a and 3b afforded cysteine as the sole amino acid (Table 2).

The mass spectra of synthetic SRS-GSH and peak 1 after N-acetylation, methylation and trimethylsilylation showed identical fragment patterns (Figure 7). While the molecular ion (M^+) at 781 was not observed, a relatively high abundance peak at m/e 203 [$(CH_3)_3SiO^+=CH(CH_2)_3CO_2CH_3$] and prominent fragment ions at m/e 595 ($M^+-\cdot COCH_2CH_2CHNHAcCO_2CH_3$), 578 ($M^+-203$),

Figure 6. UV spectrum of SRSs from RBL-1 cells. The amount of SRSs was calculated from the absorbance at 280 nm or 278 nm (ε = 40,000$M^{-1}cm^{-1}$).

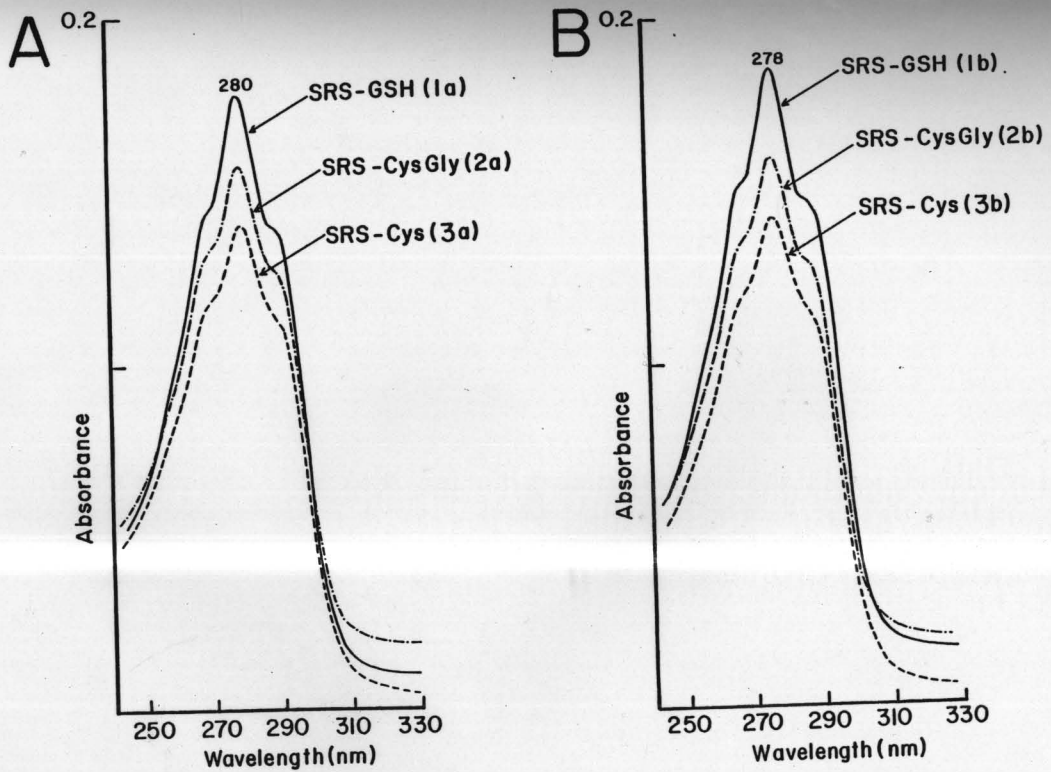

A
0.2
280
SRS-GSH (1a)
SRS-CysGly (2a)
SRS-Cys (3a)
Absorbance
250 270 290 310 330
Wavelength (nm)
B
0.2
278
SRS-GSH (1b)
SRS-CysGly (2b)
SRS-Cys (3b)
Absorbance
250 270 290 310 330
Wavelength (nm)

Table 2. Amino acid analysis of SRSs.

	Glutamic acid	Glycine	1/2 Cystine
Glutathione	5.37	5.63	5.06
1a	5.79	6.78	2.79
1b	5.96	6.77	2.65
2a	0.85	6.51	2.53
2b	0.72	6.21	2.35
3a	0.68	0.94	3.00
3b	0.43	0.80	3.08
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

* 10 nmoles of each compound was hydrolyzed with 6N HCl/0.5% phenol at 110°C in vacuo for 24 hrs. The results are expressed in nmoles based on the absorbance at 280 nm (ε=40,000).

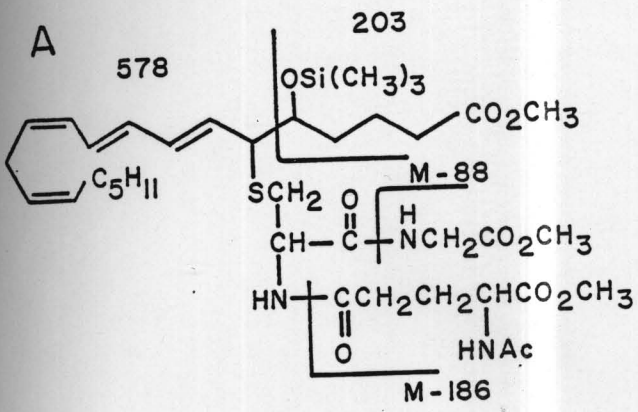
563 (m^+-203-15) and 507 (M^+-186-$\cdot NHCH_2CO_2CH_3$) were observed. The GC-MS analyses (Figure 7) of peaks 1a, 2a and 3a after hydrogenation over Raney nickel, followed by methylation and trimethyl silylation showed prominent peaks at m/e 399 (M^+-15); 383 (M^+-31); 313 [M^+-101, loss of $\cdot CH_2(CH_2)_2CO_2CH_3$] and a base peak at 203 [$(CH_3)_3SiO^+=CH(CH_2)_3CO_2CH_3$], consistent with published reports (19).

Soybean lipoxygenase is an enzyme that catalyzes lipoxygenation of unsaturated fatty acids containing two methylene interrupted cis double bonds at the ω 6 and ω 9 positions. Hence, it was used to locate the positions of the triene within these SRS molecules by observing the bathochromic shift during the triene to tetraene extension. Figure 8 shows that peaks 1a, 2a and 3a were oxidized by soybean lipoxygenase, as evidenced by the shift of UV absorption maximum from 280 nm to 308 nm. The oxidation of SRS-Cys and SRS-Cys-Gly was complete after 10 min and 20 min, respectively, resulting in a shift of absorption maximum from 280 nm to 308 nm. On the other hand, SRS-GSH was slowly oxidized by the lipoxygenase. Even after 20 min, the intensity of 308 nm peak did not exceed that of 280 nm peak. In contrast, 1b, 2b and 3b were found to be resistent to the action of this enzyme.

Figure 7. A)Mass spectrum analysis of N-acetylated, methylated and trimethylsilylated derivative of SRS-GSH (1a). (M^+,781)

B)Mass spectrum analysis of methylated and trimethylsilylated derivative of hydrogenated (over Raney nickel) SRSs. (M^+,414)

A


203
578
OSi(CH3)3
CO2CH3
C5H11
M-88
SCH2
O
H
CH—C—NCH2CO2CH3
HN—CCH2CH2CHCO2CH3
O
HNAc
M-186

B

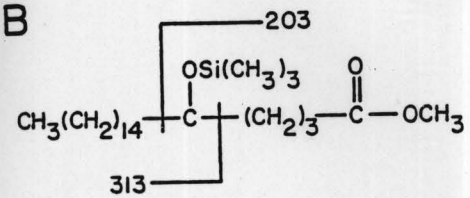
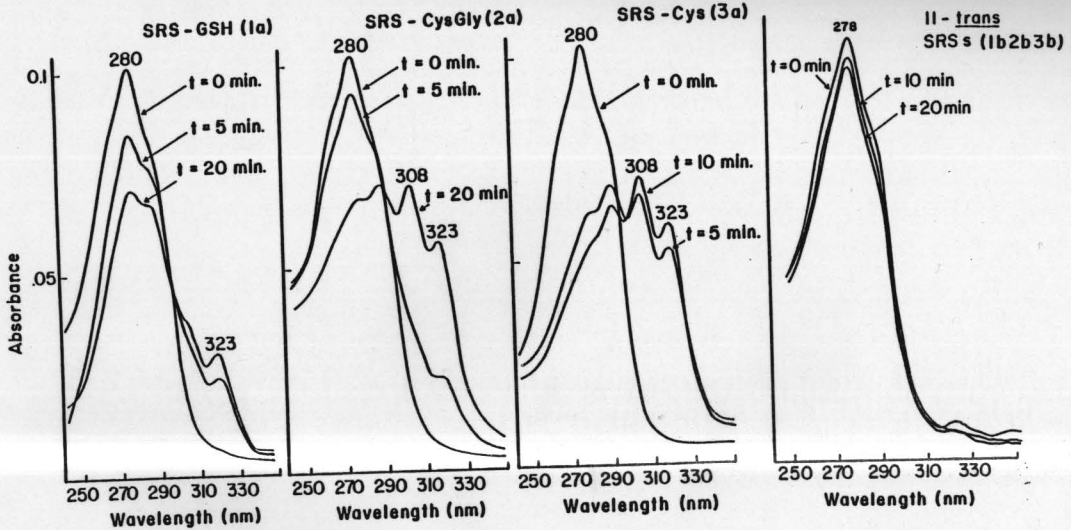

203
OSi(CH3)3
O
CH3(CH2)14—C—(CH2)3—C—OCH3
313

Figure 8. Ultraviolet spectra of RBL-1 SRSs before and after reaction with soybean lipoxygenase at indicated time intervals. Spectra were recorded in Tyrode buffer, pH 7.8.


SRS - GSH (1a)
SRS - CysGly (2a)
SRS - Cys (3a)
II - trans
SRS s (1b2b3b)
Absorbance
0.1
.05
280
308
323
276
t = 0 min.
t = 5 min.
t = 10 min.
t = 20 min.
t=0 min
250 270 290 310 330
Wavelength (nm)

On the basis of the foregoing data, we surmised that the structures of these SRSs are S-cysteinyl conjugates (glutathione, cysteinyglycine and cysteine) of icosatetraenoic acid. To confirm the chemical identities of these SRSs from RBL-1 cells, we compared the properties of natural SRSs with those of the synthetic samples. All three synthetic SRSs (SRS-GSH, SRS-Cys-Gly and SRS-Cys) had UV absorption maxima at 280 nm with characteristic shoulders at 270 nm and 292 nm. This observation confirms that the double bonds at C-7(8), 9(10), 11(12) in SRSs possess the trans, trans, cis configuration. The rates of spectral change upon treatment with soybean lipoxygenase and the retention times on HPLC of synthetic SRS-GSH, SRS-Cys-Gly and SRS-Cys were indistinguishable from the corresponding properties of peaks 1a, 2a and 3a, respectively. Also, the relative bioactivities of synthetic SRSs coincided with those of the corresponding natural SRSs, lending further support to our structural assignment (24).

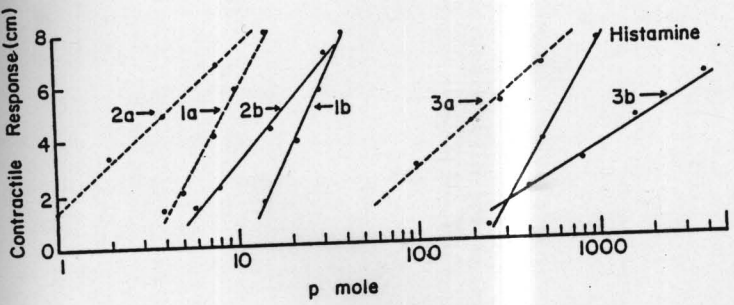
Peaks 1b, 2b and 3b have UV absorption maxima at 278 nm and were not oxidized by soybean lipoxygenase. These contrasting features suggested that the λ max 278 nm isomers differ from the λmax 280 nm SRSs in the geometry of the 11-double bond. Recently, another SRS component (λmax 278 nm) from mastocytoma cells was observed. Corey

and coworkers synthesized 11-*trans*-SRS-GSH (λ max 278 nm) and showed that the natural 11-*trans*-SRS-GSH is identical to the synthetic 11-*trans*-SRS-GSH, which has the double bonds of the conjugated triene system (C-7,9,11) in the *trans*, *trans* *trans* configuration. It was observed that 1b, 2b and 3b corresponded to 11-*trans*-SRS-GSH, 11-*trans*-SRS-Cys-Gly and 11-*trans*-SRS-Cys, on the basis of their UV absorption spectra, retention times on the HPLC, amino acid analyses and their resistance to the action of soybean lipoxygenase.

As shown in the dose-response curves of Figure 9, the relative contractile response (units/nmol) of guinea pig ileum to RBL-1 SRSs were as follows: SRS-GSH (1a), 1,100; SRS-Cys-Gly (2a), 4,700; SRS-Cys (3a), 75; 11-*trans*-SRS-GSH (1b), 350; 11-*trans*-SRS-Cys-Gly (2b), 800; 11-*trans*-SRS-Cys (3b), 9. It is noteworthy that the bioactivity ratio of each 11-*cis* isomer to 11-*trans* isomer was 3.1 for SRS-GSH, 5.9 for SRS-Cys-Gly and 8.3 for SRS-Cys. 11-*Trans*-SRS-Cys was found to be less potent than histamine as measured by its contractile activity on the guinea pig ileum.

SRS was known to contract guinea pig ileum more slowly and with longer duration than histamine, and FPL 55712 was used to antagonize the contractile activity of SRS. Maximal contraction for SRS-Cys-Gly (peak 2a) and

Figure 9. Dose-response curves for the action of RBL-1 SRSs on the isolated guinea pig ileum. The amount of SRSs was calculated from the absorbance at 280 nm or 278 nm, assuming $\varepsilon = 40{,}000 M^{-1} cm^{-1}$.


Contractile Response (cm)
8
6
4
2
0
2a
1a
2b
1b
3a
3b
Histamine
1
10
100
1000
p mole

SRS-Cys (3a) was reached considerably faster than SRS-GSH (1a) (Figure 10). FPL 55712 at a concentration of 10 ng/ml antagonized the contractile response of SRS-Cys-Gly and SRS-Cys by greater than 70%. However, higher concentration of FPL 55712 (100 ng/ml) was required to reverse the contractile activity of SRS-GSH.

Susceptibilities of SRSs to the action of γ-glutamyl transpeptidase and renal particulate peptidase may be used to distinguish various SRSs, because these enzymes are involved in the metabolism of GSH conjugates (42). As shown in Figure 11, exposure of SRS-GSH to the action of γ-glutamyl transpeptidase led to an increase of total bioactivity (3-4 times), whereas the enzyme had no effect on SRS-Cys-Gly or SRS-Cys. On the other hand, while the renal peptidase didn't affect the bioactivity of SRS-GSH or SRS-Cys, incubation of SRS-Cys-Gly with this enzyme resulted in a loss (90%) of bioactivity. L-Cysteine (10mM) or 6.5 mM phosphate buffer protected the inactivation of SRS-Cys-Gly. These results were similarly observed with 11-trans-SRSs (Figure 12). In addition, the inactivation of SRS-Cys-Gly was also protected effectively by D-penicillamine and 3-mercaptopropionic acid (Figure 13).

Figure 10. The contractile response of RBL-1 SRSs and its antagonism by FPL 55712 (10 ng/ml). The system contained: SRS-Cys (3a, 100 pmoles), SRS-Cys-Gly (2a, 2 pmoles) or SRS-GSH (1a, 6 pmoles) in 10 ml Tyrode buffer. FPL 55712 (10 ng/ml) was added at maximal contraction. Higher concentration (100 ng/ml) was used, where indicated.

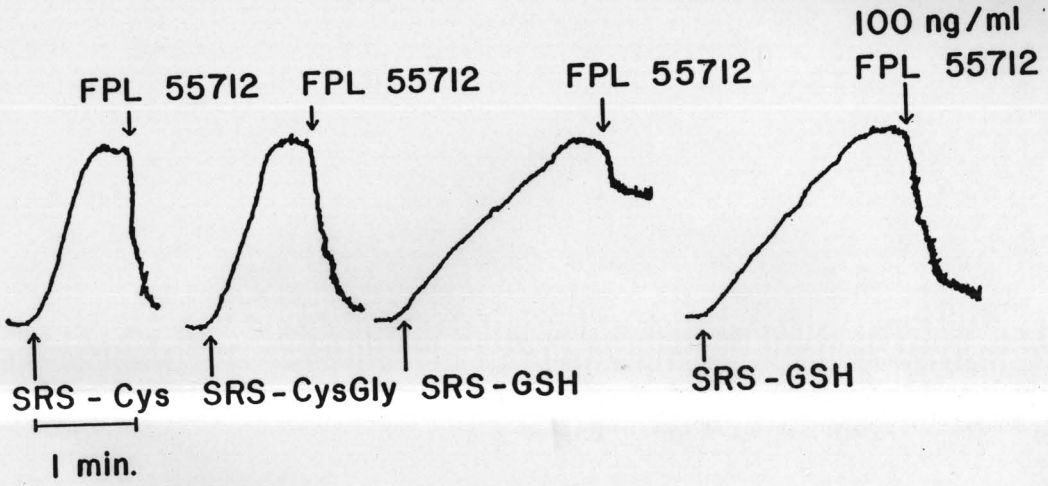

FPL 55712
FPL 55712
FPL 55712
100 ng/ml
FPL 55712
SRS - Cys
SRS - CysGly
SRS - GSH
SRS - GSH
1 min.

Figure 11. Activation of SRSs by γ-glutamyl transpeptidase (bovine kidney). The incubation was carried out in the presence of 10 mM L-cysteine.

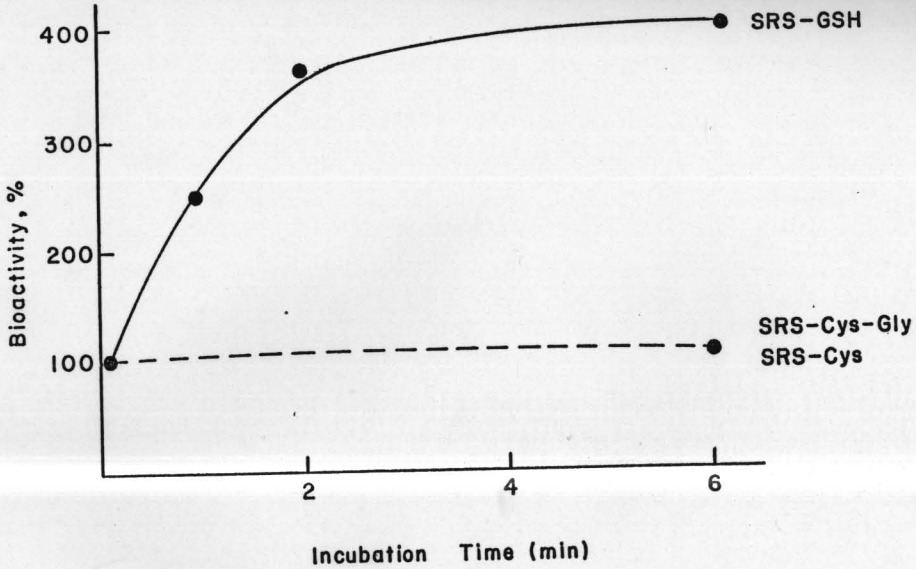

400
300
200
100
Bioactivity, %
SRS-GSH
SRS-Cys-Gly
SRS-Cys
2
4
6
Incubation Time (min)

Figure 12. Inactivation of SRS bioactivities by renal peptidase (type IV-S leucine aminopeptidase-M preparation). The system contained 10mM L-cysteine or 6.5 mM sodium phosphate buffer, pH 7.2, where indicated. The system contained 0.06 units of enzyme and various quantities of SRS (500 units of SRS-GSH and SRS-Cys-Gly, and 50 units of SRS-cys). Where indicated 10 mM L-cysteine or 6.5 mM sodium phosphate buffer, pH 7.2 was included.

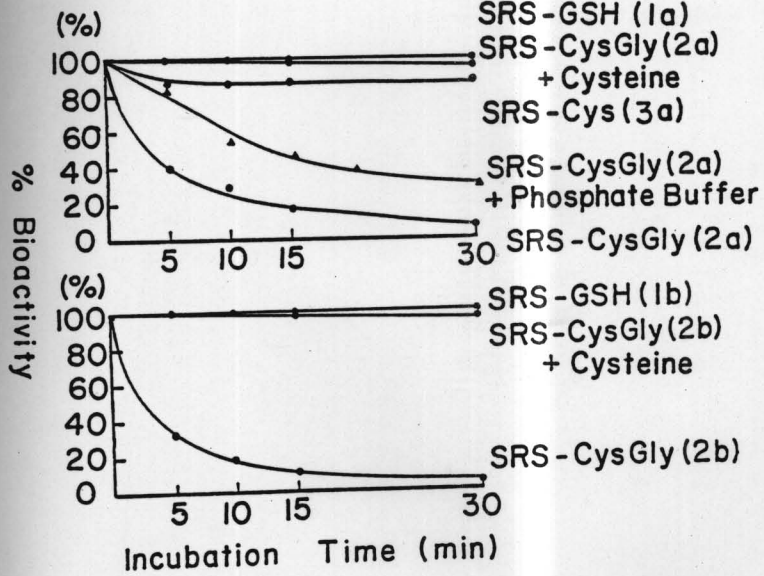
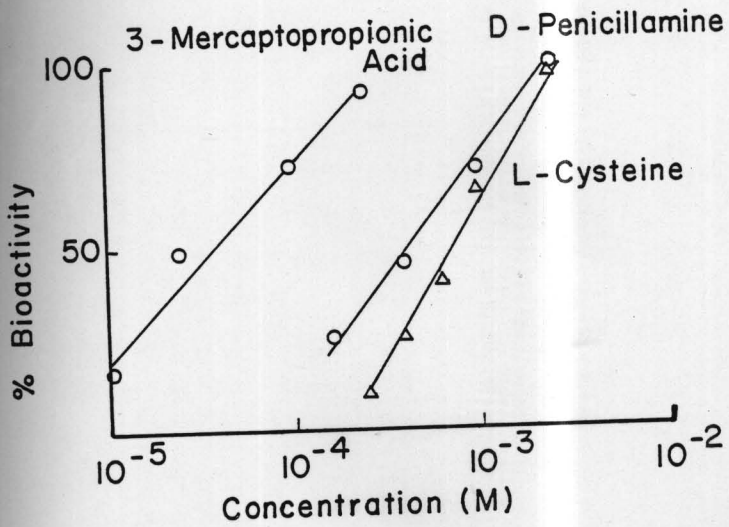

(%)
100
80
60
40
20
0
5
10
15
30
% Bioactivity
SRS-GSH (1a)
SRS-CysGly(2a)
+ Cysteine
SRS-Cys (3a)
SRS-CysGly(2a)
+ Phosphate Buffer
SRS-CysGly (2a)
(%)
SRS-GSH (1b)
SRS-CysGly(2b)
+ Cysteine
SRS-CysGly (2b)
Incubation Time (min)

Figure 13. Thiol inhibition of the destruction of SRS-Cys-Gly bioactivity by renal peptidase. The system contained 0.06 unit of leucine aminopeptidase (Type IV-S), 0.1 nmol of SRS-Cys-Gly, and the indicated concentrations of thiols in 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.2.


3 - Mercaptopropionic Acid
D - Penicillamine
L - Cysteine
100
50
% Bioactivity
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Concentration (M)

2. Biosynthesis and metabolism of SRS

A. 5-lipoxygenation of arachidonic acid and its inhibition

When [1-^{14}C]- arachidonic acid was incubated with cell-free extracts of RBL-1 cells, (32), the major product formed was found to be radioactive 5-HETE. The 5-lipoxygenase activity was enhanced by Ca^{++} in a concentration-dependent manner (Table 3). Intact RBL-1 cells also gave the same product after challenge with calcium ionophore A23187. Having developed an enzymatic assay of 5-lipoxygenase activity, we then turned our attention to examining the inhibition of 5-lipoxygenase during the initial stage of SRS biosynthesis.

Although BW-755C and some antioxidants (33) were found to inhibit 5-lipoxygenase activity, they were not selective in their mode of inhibition. ETYA, inhibits both lipoxygenases and cylooxygenase, non-specifically and irreversibly. Hence, we decided to examine the effectiveness of monoacetylenic acids as a selective mechanism-based inhibitor of 5-lipoxygenase.

When extracts of RBL-1 cells were preincubated with 5,6-DHA, the conversion of [^{14}C]-arachidonic acid into [^{14}C]-5-HPETE, as measured by the formation of [^{14}C]-5-HETE, decreased in a time dependent manner as shown in Figure 14. Under appropriate experimental conditions, the

Table 3. The effect of calcium ion on the 5-lipoxygenase.

Concentration of Ca^{++} (mM)	Relative Activity (%)
0	100
0.2	150
0.6	210
1.0	230
1.5	220

rate of inactivation was pseudo-first order over several half-lives. The half-life of the 5-lipoxygenase activity was in the range of 1.5-4.5 min, depending upon the concentration of 5,6-DHA. The kinetics of inactivaton was analyzed according to the procedure of Kitz and Wilson (69). By plotting the reciprocals of the pseudo-first order rate constants of inactivation ($1/k_{app}$) against the reciprocals of the 5,6-DHA concentration (1/5.6-DHA) for a series of values of 5,6-DHA, a linear relationship results, which allows evaluation of both K_i and k_3, since

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{K_i}{k_3[I]}$$

Under experimental conditions described in Figure 13, values of K_i = 15 μM and $k_3 = 8.3 \times 10^{-3}$ sec $^{-1}$ were obtained (Figure 14 insert).

Similar patterns of inhibition were also observed by following the rate of formation of 5,12-diHETEs. ETYA, a well established irreversible inhibitor of 5-lipoxygenase of RBL-1 cells , was approximately three times less potent than 5,6-DHA using a 5 min preincubation at 20°C (Figure 15).

Having established the inactivation of 5-lipoxygenase in cell extracts of RBL-1 cells, we next turned our attention to examining the inhibitory effects of 5,6-DHA

Figure 14. Pseudo-first order time course of inactivation of 5-lipoxygenase in RBL-1 cell extracts by 5,6-DHA. The cell extract was preincubated with 5,6-DHA for the indicated time intervals at 15°C. Insert: reciprocal plot of the observed pseudo-first order rate constant as a function of the concentration of 5,6-DHA.

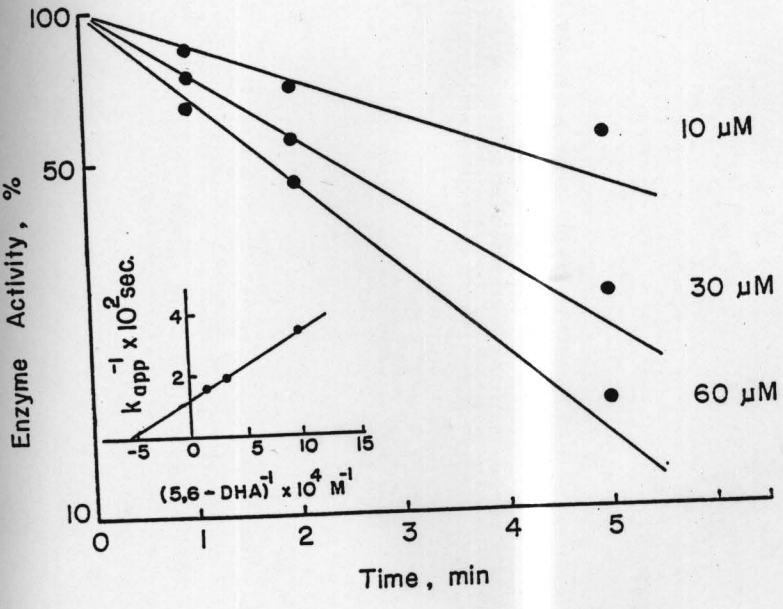
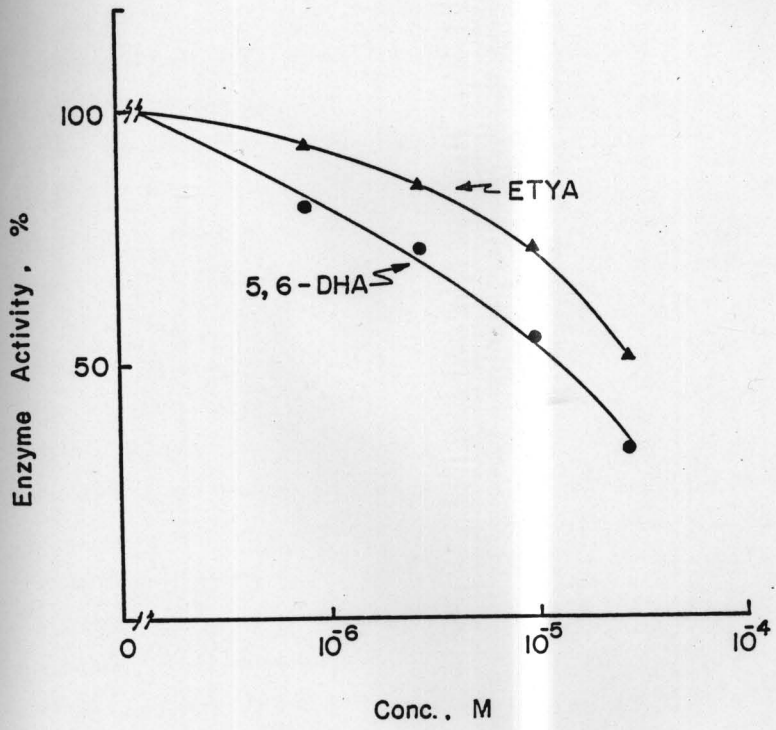

100
50
10
Enzyme Activity, %
10 µM
30 µM
60 µM
0
1
2
3
4
5
Time, min
$k_{app}^{-1} \times 10^2$ sec.
4
2
-5
0
5
10
15
$(5,6\text{-DHA})^{-1} \times 10^4\ M^{-1}$

Figure 15. Concentration-dependent inhibition of 5-lipoxygenase activity in RBL-1 cell extracts by 5,6-DHA and ETYA. The cell extracts were preincubated with the inhibitors for 5 min at 20°C.


100
50
Enzyme Activity, %
ETYA
5, 6 - DHA
0
10^{-6}
10^{-5}
10^{-4}
Conc., M

in intact RBL-1 cells. Unexpectedly, it was found that at 3 μM or below, 5,6-DHA enhanced the formation of 5-HETE but produced significant inhibition at 3×10^{-5} M. In contrast, both ETYA and 15(S)-HEYA inhibited the formation of 5-HETE very effectively ($[I]_{50}$ = 8 and 5 μM, respectively) and they did not stimulate the production of lipoxygenase products (Figure 16).

In an attempt to explain this anomaly, intact RBL-1 cells were incubated with 3 μM of [1-^{14}C]-5,6-DHA. It was found that more than 85% of the total radioactivity resided in the triglyceride and phospholipid fractions whereas [1-^{14}C]-15(S)-HEYA was not incorported to any significant amount into these fractions.
Moreover, the marked inhibition of 5-lipoxygenase by 15-HEYA as a suicide substrate was in agreement with the observation (Figure 17) that 15-HETE was converted into 5,15-diHETE by RBL-1 cells and that this conversion was enhanced 6 times in the presence of calcium ionophore A23187.

B. Formation of 5,6-LTA

Leukotriena A (LTA) is known to be enzymatically derived from arachidonic acid via the intermediate, 5(S)-hydroperoxy icosatetraenoic acid (5-HPETE). After [^{3}H]-arachidonic acid was exposed to RBL-1 cells in the presence of ionophore A23187 for 1 min, the products were

Figure 16. Inhibition of 5-lipoxygenase activity in intact RBL-1 cells by acetylenic analogs.

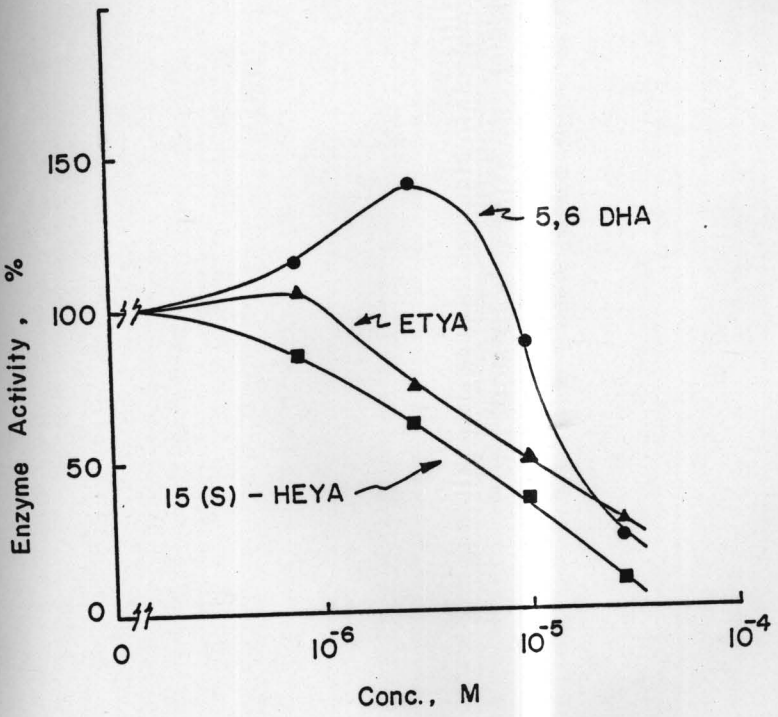

150
100
50
0
0
10^{-6}
10^{-5}
10^{-4}
Enzyme Activity , %
Conc., M
5,6 DHA
ETYA
15 (S) - HEYA

Figure 17. RP-HPLC profile of diHETE products from the incubation of 15-HETE with RBL-1 cells. In a separate experiment, Ionophore A 23187 was included. (·····); without ionophore, (—); with ionophore A23187 (10µg/ml). Radial-Pak 8C180 5µ column (0.8 x 10 cm) with C_{18} precolumn. Mobile phase: methanol-water (60:40), pH 5.4 (0.05% HAC + NH_4OH); flow rate, 2 ml/min.


Absorbance at 254 nm
10
20
30
Time (min)

methylated with diazomethane and analyzed by HPLC.

Figure 18 shows that the radioactivity resided mainly in the 5,6-LTA methyl ester, not in the other isomers, 9-Z-5,6-LTA methyl ester and 5(6)-cis-LTA methyl ester. These results demonstrate positively that the [^{3}H]-5,6-LTA formed from [^{3}H]-arachidonic acid in RBL-1 cells corresponds to 5(S)-trans-5,6-oxido-7,9-trans-11,14-cis icosatetraenoic acid, in agreement with the earlier results observed with 5,6-LTA from human PMNLs.

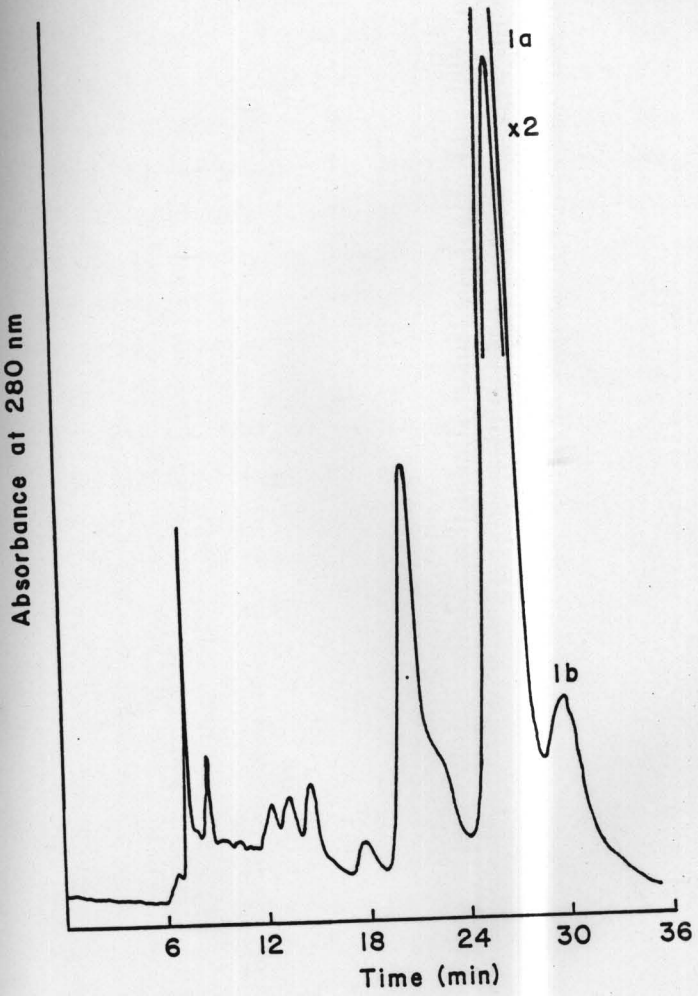
C. Transformation of 5,6-LTA into SRS-GSH

When synthetic 5,6-LTA was incubated with RBL-1 cells in the presence of serine-borate buffer, an inhibitor of γ-glutamyl transpeptidase, the major SRS (1a) was found to be indistinguishable from SRS-GSH on the basis of the retention time on HPLC, bioactivity, and UV absorption. However, the peak corresponding to the 11-trans SRS-GSH (1b) was very small (Figure 19). RBL-1 cells also converted 5 (S)-trans-5,6-oxido-7-trans-9,11,14-cis-icosatetraenoic acid and 5,6-cis-oxido-7,9-trans-11,14-cis-icosatetraenoic acid into SRS-GSH-like bioactive materials nonspecifically. In subsequent experiments (57), it was also observed that glutathione transferase in RBL-1 cells utilized 14,15-LTA as a substrate.

Figure 18. HPLC separation of [^{3}H]-5,6-LTA methyl ester after the incubation of [^{3}H]-arachidonic acid with RBL-1 cells. Poracil column (10μ, 0.46 x 50 cm, Alltech). Mobile phase, hexane: ethyl acetate: triethylamine (100:0.7:0.7); flow rate, 1ml/min. After the first chromatography, the radioactivity fraction coeluting with synthetic 5,6-LTA methyl ester was reinjected onto the same column with synthetic LTA methyl ester isomers.

RADIOACTIVITY (cpm, ▨)
ABSORBANCE (280 nm, ——)
TIME (min)
0
200
400
60
50
40
30
20
10
0
A
B
C
A:
B:
C:
CO_2CH_3
C_5H_{11}
O

Figure 19. RP-HPLC profile of SRS-GSH derived from the incubation of 5,6-LTA with RBL-1 cells. 1a: SRS-GSH, 1b: 11-trans-SRS-GSH. Stationary phase (preparative μC_{18}), M9 ODS; (0.94 x 50 cm); mobile phase, methanol/water (65:35), pH 5.4 (0.05%HOAC + NH_4OH). Flow rate 3ml/min.


Absorbance at 280 nm
1a
x2
1b
6
12
18
24
30
36
Time (min)

D. Transformation of SRS-GSH into SRS-Cys-Gly and SRS-Cys

In the previous section, we showed that the serine-borate buffer blocked the conversion of SRS-GSH into SRS-Cys-Gly, and that L-cysteine inhibits the further metabolism of SRS-Cys-Gly into SRS-Cys. To establish the precursorial relationship of these SRSs, we examined the kinetics of SRS metabolism in RBL-1 cells. When RBL-1 cells were challenged with A23187 and arachidonic acid, there was an increase in total contractile activity, which slowly decreased with time (Figure 20). HPLC analyses revealed that SRS-GSH was the predominant SRS component at 2 min, and the major products after 10 min were SRS-GSH and SRS-Cys-Gly. After 20 min incubation, SRS-GSH and SRS-Cys-Gly decreased, and SRS-Cys was isolated as the major product. Further incubation (40 min) gave SRS-Cys as the main product with negligible amounts of SRS-GSH and SRS-Cys-Gly.

These results are consistent with the assumption that SRS-GSH is converted into the more bioactive SRS-Cys-Gly, which is in turn degraded into much less bioactive SRS-Cys by the peptidase. After establishing the precursorial relationship of SRS in RBL-1 cells, we turned to the GSH conjugates metabolizing system in kidney, which is known to contain γ-glutamyl transpetidase and peptidases. When synthetic SRS-GSH was exposed to γ-glutamyl

Figure 20. Time course of SRS metabolism in RBL-1 cells. For cis→trans isomerization study, the cells suspension was boiled after 40 min and then incubated further for 6 hrs to give control samples.

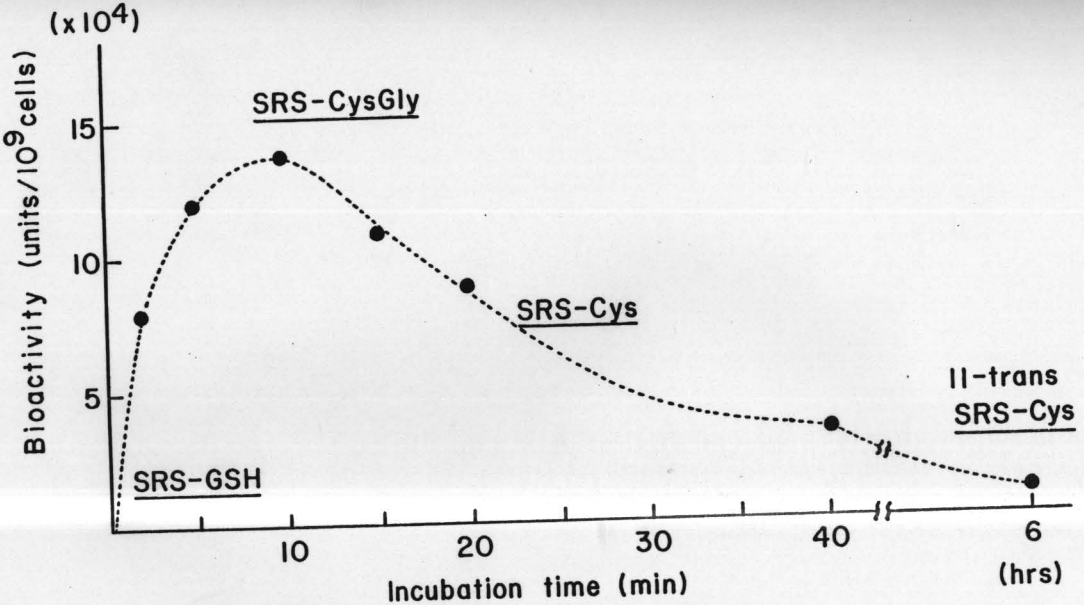
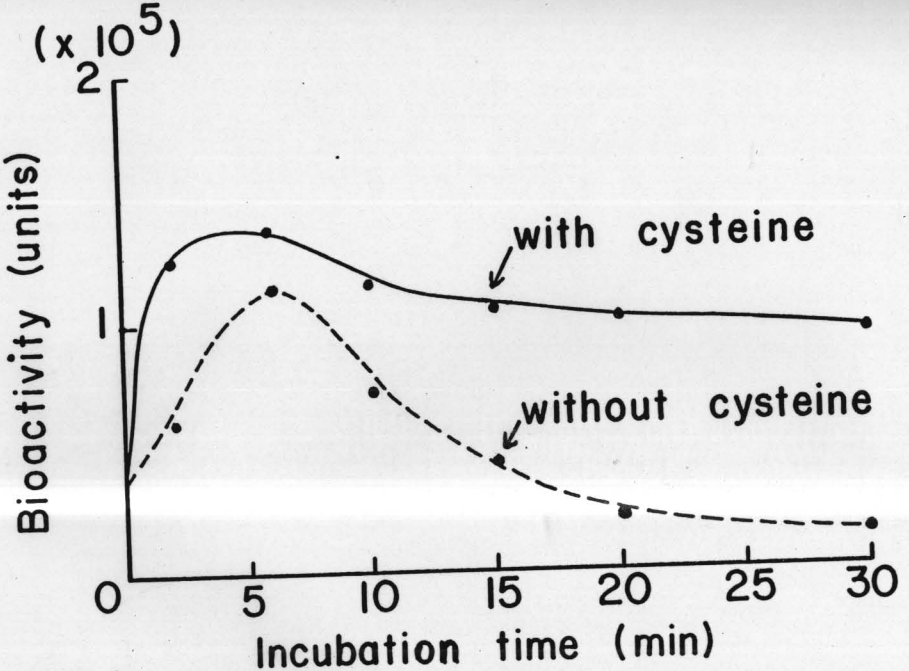

(x10^4)
Bioactivity (units/10^9cells)
15
10
5
SRS-CysGly
SRS-Cys
11-trans
SRS-Cys
SRS-GSH
10
20
30
40
6
(hrs)
Incubation time (min)

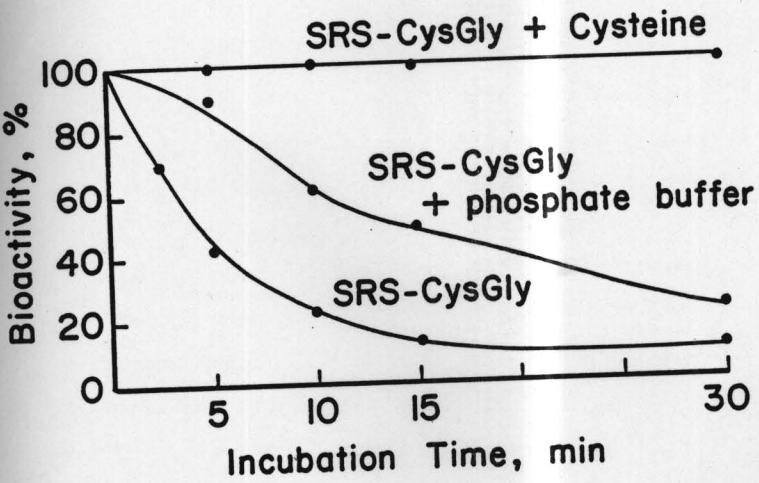
Figure 21. Transformation of SRS-GSH by γ-glutamyl transpeptidase (bovine kidney). Where indicated, L-cysteine (10mM) was included.


(x 10^5)
2
1
0
Bioactivity (units)
with cysteine
without cysteine
5
10
15
20
25
30
Incubation time (min)

transpeptidase (bovine kidney), the time course of SRS metabolism (Figure 21) was similar to that observed with RBL-1 cells. After 30 min, HPLC analyses of the reaction mixture revealed the formation of a product with retention time (19.5-21 min) and properties corresponding to SRS-Cys. This suggested that SRS-GSH was converted into SRS-Cys-Gly which was in turn transformed into SRS-Cys by a contaminating peptidase. The inclusion of L-cysteine blocked the conversion of SRS-Cys-Gly into SRS-Cys probably by inhibiting the peptidase. Consequently, SRS-Cys-Gly accumulated in the medium, accompanied by a small quantity of SRS-Cys.

The renal peptidase, which was responsible for the conversion of SRS-Cys-Gly into SRS-Cys, was also found to be cysteine-sensitive. However, it was not clear which type of peptidase was involved in the conversion of SRS-Cys-Gly into SRS-Cys, because two enzymes, renal aminopeptidase-M and dipeptidase were known to cleave cysteinylglycine conjugates. Figure 22 shows that SRS-Cys-Gly was rapidly inactivated by 0.15 units of purified rat renal dipeptidase; both L-cysteine (10 mM) and phosphate buffer (50 mM) inhibited this inactivation. On the other hand, purified rat renal aminopeptidase-M (0.15 units) did not inactivate SRS-Cys-Gly significantly. After having identified the renal dipeptidase as the

Figure 22. Inactivation of SRS-Cys-Gly bioactivity by purified rat renal dipeptidase (0.15 units). The system contained 10 mM L-cysteine or 50 mM sodium phosphate buffer, pH 7.2, where indicated.


SRS-CysGly + Cysteine
SRS-CysGly
+ phosphate buffer
SRS-CysGly
Bioactivity, %
100
80
60
40
20
0
5
10
15
30
Incubation Time, min

enzyme responsible for the inactivation of SRS-Cys-Gly, we decided to purify the peptidases in RBL-1 cells.

After chromatography of the RBL-1 cell extract on a DEAE cellulose column, two enzyme fractions possessing the SRS-Cys-Gly inactivating activities were noted (Figure 26). The major component (fractions 54-64) was further purified by rechromatography on a Sephadex G-200 column to remove the contaminating proteins (Figure 23). This partially purified enzyme (about 100 fold purification) readily converted SRS-Cys-Gly into SRS-Cys, but had no effect on SRS-GSH and SRS-NTFA-Cys-Gly (Figure 24). L-Cysteine (10 mM) markedly inhibited the inactivation of SRS-Cys-Gly, but 10^{-4} M bestatin, a well-known inhibitor of aminopeptidase, did not. L-Leucyl glycine was hydrolyzed by this enzyme at a much higher rate (90 fold) than L-leucyl-P-nitroanilide (45). These properties are reminescent of thiols-sensitive metallodipeptidases (62). It is conceivable that the major SRS-Cys-Gly inactivating peptidase in RBL-1 cells is a type of dipeptidase.

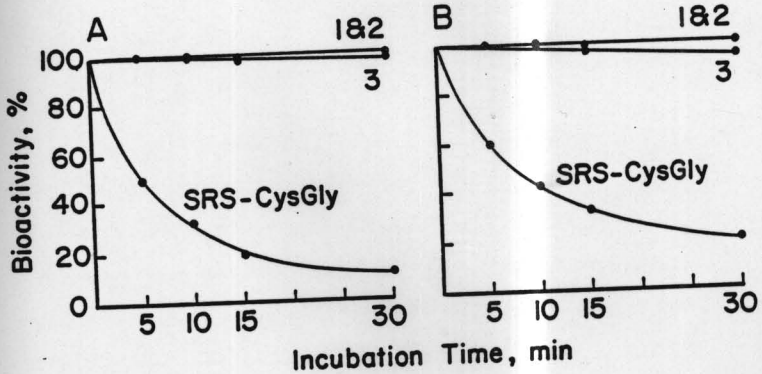
E. Formation of 11-trans-SRS-Cys

Further metabolism of SRS-Cys in RBL-1 cells was expected to result in the formation of N-acetyl SRS-Cys. However, even after prolonged incubation (400 min), no N-acetyl SRS-Cys was detected and, the major product was

Figure 23. Chromatography of SRS-Cys-Gly inactivating enzyme from RBL-1 cells on a Sephadex G-200 column (1.6 x 70 cm). The concentrate of fractions (54-64) from Figure 25, was applied on the column, which was eluted with 0.1M Tris-HCl buffer, pH 7.0.

Absorbance at 280nm (-----)
0.4
0.3
0.2
0.1
10
20
30
40
2.0
1.0
SRS inactivating activity, units/ml (●—●)

Figure 24. The rate of inactivation of SRSs by peptidases. The system contained 0.3 nmoles of SRSs and varying units of SRS inactivating activity [RBL-peptidase (5 units) or guinea pig lung peptidase (3 units)] in 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.2. A. RBL-peptidase; B. guinea pig lung peptidase. 1. SRS-GSH; 2. SRS-NTFA-Cys-Gly; 3. SRS-Cys-Gly + 10 mM L-cysteine.


A
1&2
3
SRS-CysGly
B
1&2
3
SRS-CysGly
Bioactivity, %
100
80
60
40
20
0
5
10
15
30
5
10
15
30
Incubation Time, min

11-*trans*-SRS-Cys, whose formation may account for the loss of bioactivity (Figure 20). Hence, we examined the mechanism of 11-*trans*-SRS-Cys formation. As can be seen in Figure 25, at 40 min, the major HPLC peak corresponds to SRS-Cys with retention time of 19.5-21 min. This peak gradually decreased with time, whereas the shoulder with the retention time (23-24 min) of 11-*trans*-SRS-Cys increased correspondingly with time (160 and 280 min). After 400 min, the ratio of 11-*trans*-SRS-Cys to SRS-Cys increased to 3:1.

Because a possibility exists that this isomerization may be the result of reversible equilibration of thiol between the C-6 and C-12 positions (Figure 31), we carried out the same incubation experiments using synthetic (±) SRS-Cys in the presence of [^{14}C]-L-cysteine and RBL-1 cells. The resulting 11-*trans*-SRS-Cys was isolated after 6 hrs by reverse-phase HPLC and was found to be devoid of any radioactivity. Thus, the formation of the 12-thio analog (position isomer) appears unlikely. Although at this juncture one would assume that this 11-*cis* to 11-*trans* isomerization at pH 7.2 may be enzyme catalyzed, a parallel experiment using boiled RBL-1 cells also revealed the formation of 11-*trans*-SRS-Cys from SRS-Cys. However, the amount of 11-*trans*-SRS-Cys produced by boiled cells was consistently lower than those of unheated cells. When

Figure 25. Isomerization of SRS-Cys into 11-trans-SRS-Cys in RBL-1 cells.

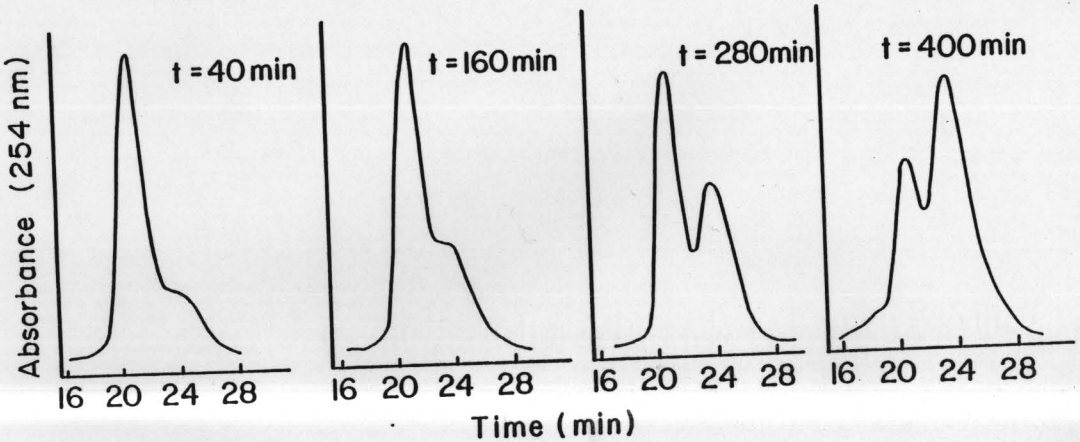

Absorbance (254 nm)
t = 40 min
t = 160 min
t = 280 min
t = 400 min
16 20 24 28
16 20 24 28
16 20 24 28
16 20 24 28
Time (min)

Table 4. HTMP inhibition of SRS-Cys isomerization in RBL-1 cells.

	Ratio of SRS-Cys isomers in the presence of HTMP				
	0 mM				
Isomers	Unboiled cells (12)	Boiled cells (4)	0.01 mM (1)	0.1 mM (2)	1 mM (3)
11-trans (278 nm)	65	35	30	15	8
11-cis (280 nm)	35	65	70	85	92

Numbers of experiments are given in parentheses. Unboiled cells were present in all experiments in which HTMP was added. Isomer ratios were calculated as described (63).

the antioxidant, HTMP was added to RBL-1 cells, the amount of 11-trans-SRS-Cys formed decreased as the HTMP concentration was raised. At a concentration of 10^{-3}M of HTMP, the ratio of 11-trans-SRS-Cys to SRS-Cys decreased to 8:92 as opposed to 65:35 in the absence of HTMP (Table 4). Based on these results, it appeared that 11-trans-SRS-Cys was most likely derived from SRS-Cys via a radical-catalyzed isomerization of 11,(12) double bond (63).

F. Enzymatic inactivation of SRS-Cys-Gly (SRS-A)

The enzymatic inactivation of SRS-A by limpet arylsulfatase may be due to an inherent activity of arylsulfatase or that the preparation was contaminated with peptidase which hydrolyzed the peptide bond in SRS-Cys-Gly. It will be of interest to find out what types of enyzmes are responsible for the SRS-A inactivation in RBL-1 cells, guinea pig lung, peritoneal eosinophils, and limpet arylsulfatase preparation.

i) RBL-1 Cells. In the previous section, we demonstrated that L-cysteine inhibited that transformation of the highly bioactive SRS-Cys-Gly into the less bioactive SRS-Cys in RBL-1 cells. We surmised that a peptidase may be responsible for the inactivation of SRS-A. However, earlier workers had reported that arylsulfatases A and B from RBL-1 tumor cells (50)

Figure 26. Chromatography of RBL-1 cells enzymes on a DEAE-cellulose column (1.7 x 26 cm). The column was eluted with a 400 ml linear salt gradient to 0.4 M NaCl at 1ml/min. Fraction volumn: 4ml).

Absorbance at 280 nm (----)
Sulfatase activity, units/ml (o—o)
SRS inactivating activity, units/ml (•—•)
NaCl conc., M
Fraction
0
10
20
40
50
60
70
80
90
100
110
0.5
1.0
1.5
2.0
4.0
0.2

inactivated SRS-A. Consequently, a series of studies were undertaken to determine which type of enzyme(s) was responsible for SRS-A inactivation in cell systems. After chromatography of the RBL-1 cell extract on a DEAE-cellulose column, two enzyme fractions possessing SRS-Cys-Gly inactivating activity were noted (Figure 26). The major component (Fractions 54-64) was further purified by rechromatography on a Sephadex G-200 column to remove the trace of the minor component. The enzyme fraction had properties resembling those of dipeptidase. This enzyme readily converted SRS-Cys-Gly into SRS-Cys with a pH 7.0 optimum but had no effect on SRS-GSH and SRS-NTFA-Cys-Gly. L-cysteine (10^{-2}M) markedly inhibited the inactivation of SRS-Cys-Gly. The minor SRS-Cys-Gly inactivating peak (fractions 69-73) has the same SRS-Cys-Gly cleaving properties, except the L-cysteine (10^{-2}M) didn't protect the cleavage of SRS-Cys-Gly. The properties of arylsulfatase (fraction 66-71) resemble those of arylsulfatase A of RBL-1 tumor cells. The pH optimum of p-NCS hydrolysis was at 5.2; its molecular weight estimated by gel filtration was approximately 100,000; it was inhibited to the extent of 56% by 5mM ATP.

ii) Guinea Pig Lung. L-cysteine was used to enhance SRS-A levels in guinea pig lung tissue (18), which suggested the presence of a thiol-sensitive peptidase in

this system. However, the only SRS-A inactivating enzyme was ascribed to the action of arylsulfatase type II-B (49). When guniea pig lung extract was chromatographed over a DEAE-Sephadex column, the SRS-Cys-Gly inactivating activity did not coincide with the arylsulfatase activity (Figure 27). The peptidase peak (Fractions 48-60) as revealed by its ability to cleave SRS-Cys-Gly was further purified on a Sephadex G-200 column. Figure 24 shows the inactivation of SRSs by this partially purified enzyme. Like the RBL-1 peptidase, this enzyme didn't attack SRS-GSH and SRS-NTFA-Cys-Gly and was inhibited by 10^{-2}M L-cysteine. The arylsulfatase activity (Fractions 64-78) exhibited a 5.7 pH optimum for p-NCS hydrolysis, and was more active in cleaving p-NCS than p-NPS. These properties resemble those of arylsulfatase type II-B (15).

iii) Guinea Pig Peritoneal Eosinophils. It was proposed that eosinophils were attracted to the site of an immediate-type hypersensitivity reaction by ECF-A, and SRS-A was assumed to be inactivated by eosinophil arylsulfatase B. Since published data (47) implied that arylsulfatase type II-B was the only SRS-A inactivating enyzme in eosinophils, we decided to examine the validity of this supposition. Again, Figure 28 shows that the SRS-Cys-Gly inactivating peptidase activity (Fractions 41-48) as manifested by the hydrolysis of leucine p-nitroanilide

Figure 27. Chromatography of the enzymes from guinea pig lung on a DEAE-Sephadex column (1.7 x 30 cm). The extract of guinea pig lung was applied over the column, which was eluted with a 100 ml linear salt gradient to 0.2 M NaCl at a flow rate of 0.5ml/min. Fraction volume: 2 ml.

Absorbance at 280 nm (----)
Sulfatase activity, units/ml (o—o)
SRS inactivating activity, units/ml (•—•)
NaCl con., M
Fraction
0
10
20
40
50
60
70
80
90
0.2
2.0
4.0
6.0
1.0
2.0
4.0

Figure 28. Chromatography of the eosinophils enzymes on the DEAE-cellulose column (1.7 x 10 cm). The column was eluted with a 100 ml linear salt gradient to 0.2 M NaCl at a flow rate of 0.6 ml/min. Fraction volume: 2 ml.

Absorbance at 280 nm (-----)
Sulfatase activity, units/ml (o—o)
0.3
0.2
1.0
0
10
20
40
50
60
70
80
90
Fraction
NaCl conc., M
0.2
0.1
0.05
SRS inactivating activity, units/ml (•—•)

did not coincide with the arylsulfatase activity (Fractions 48-60) as assessed by the hydrolysis of p-NCS. Unfortunately, we were unable to conduct more in depth studies due to the small number of eosinophils.

iv) Limpet arylsulfatase. When limpet arylsulfatase was chromatograhped over an SP-Sephadex column (Figure 29), most of the SRS-Cys-Gly inactivating activity (fractions 34-37) was found not to coincide with arylsulfatase activity (fractions 38-41). The SRS-Cys-Gly inactivating peptidase had a pH 5,0 optimum and did not attack SRS-GSH but hydrolyzed SRS-NTFA-Cys-Cly slowly. This peptidase didn't hydrolyze leucyl-p-nitroanilide, and the sulfatase activity was inhibited by 70 μM ATP to the extent of 50%.

Figure 29. Chromatography of limpet arlysulfatase on a SP-Sephadex column (1.7 x 26 cm). The column was washed with a 100 ml pH gradient to pH 8.0 (0.01 M Tris-HCl buffer) at a flow rate of 12 ml/hr. Fraction volume: 2 ml. SRS-Cys-Gly inactivating activity was measured using 0.1 M sodium acetate buffer, pH 5.0.

Absorbance at 280 nm (-----)
0.8
0.4
Sulfatase activity, units/ml (o——o)
1.0
0.5
0
35
40
Fraction
pH (——)
7.0
5.0
4.0
2.0
SRS inactivating activity, units/ml (•——•)

IV. Discussion

1. Isolation and Characterization of SRSs

SRS-GSH, SRS-Cys-Gly and SRS-Cys and their corresponding structural isomers (λmax 278 nm) have now been isolated from RBL-1 cells. These SRSs and their respective isomers may be readily distinguished from each other by the properties shown in Table 5. These SRSs can be easily separated from each other by RP-HPLC on a Radial-Pak C_{18} cartridge. Generally, the 11-*trans* isomers of SRS migrated a little slower than their respective 11-*cis* isomers on RP-HPLC.

Using the guinea pig ileum bioassay, it was observed that SRS-Cys-Gly is 4 times more potent than SRS-GSH, which in turn is 14 times more active than SRS-Cys. However, the relative potency of SRSs is variable depending on the tissues used for bioassay. With the human, and guinea pig heart, LTD is about 10 times more potent than LTC, and LTC is in turn 200 times more potent than LTE (64). On the other hand, LTE is as potent as LTC on the guinea pig parenchymal strip or trachea (65). Generally, the 11-*trans* isomers of SRS are less potent than the 11-*cis* forms of SRS. These results are consistent with the recent observation that the geometrical arrangement of the hydrophobic C_{20} chain and

Table 5. Criteria to distinguish between SRSs.

	SRS-GSH		SRS-Cys-Gly		SRS-Cys	
	1a	1b	2a	2b	3a	3b
UV λmax (nm)	280	278	280	278	280	278
Specific Activity (units/nmole)	1100	350	4700	800	75	9
FPL 55712 antagonism (>75%, ng/ml)	100	100	10	10	10	10
Limpet Arylsulfatase	-	-	+	+	-	-
Renal peptidase	-	-	+	+	-	-
γ-Glutamyl transpeptidase	+	+	-	-	-	-
Lipoxygenase* (soybean)	+	-	++	-	+++	-

*(−); no enzymatic reaction, (+,++,+++); relative reaction rate

the polar peptide unit are important contributors to biological activity (66).

On the guinea pig ileum, the contractile responses elicited by SRS-Cys-Gly and SRS-Cys were reversed by greater than 75% by FPL 55712 at 10 ng/ml, whereas the contractile activity of SRS-GSH was antagonized by higher concentration of FPL 55712 (100 ng/ml). These results were observed similarly on other tissues, suggesting the possible existence of two types of SRS receptors (65).

There was a marked difference in the rates of oxidation of three SRSs catalyzed by soybean lipoxygenase. SRS-Cys was oxidized more rapidly than SRS-Cys-Gly by the enzyme. On the other hand, the oxidation of SRS-GSH was slower than that of SRS-Cys-Gly, manifesting the effect of the bulkiness of the peptide portion of the molecule on the rate of oxygenation.

The removal of the glutamyl group of SRS-GSH by γ-glutamyl transpeptidase results in the formation of the more bioactive SRS-Cys-Gly. On the other hand, the cleavage of SRS-Cys-Gly by renal peptidase results in the loss of bioactivity by greater than 90% (43).

It was generally believed that cysteine and other thiols enhanced the formation and the release of SRS (13-15), although the mechanism of this stumulation was not clearly understood. Our results showed that preincubation

of RBL-1 cells with L-cysteine led to an enhancement of total bioactivity by inhibiting the conversion of the highly bioactive SRS-Cys-Gly into the much less bioactive SRS-Cys, resulting in an accumulation of SRS-Cys-Gly. Later (24), a peptidase in the limpet arylsulfatase preparation was reported to be responsible for the inactivation of SRS-Cys-Gly. Our results showed that a microsome-bound peptidase (bovine kidney) is responsible for the inactivation of SRS-Cys-Gly. It is also interesting to note that L-cysteine and phosphate ions had a strong inhibitory effect on SRS-Cys-Gly inactivation by this peptidase. Therefore, it is likely that the combination of L-cysteine and phosphate buffer in the incubation results in accumulation of SRS-Cys-Gly. As shown in Figure 13, this inactivation was protected by not only L-cysteine, but also by 3-mercaptopropionic acid and D-penicillamine, thiols that had been also known to enhance SRS levels (15). Therefore, it is now clear the the primary action of L-cysteine in increasing SRS activity is to prevent the destruction of SRS-Cys-Gly by peptidase and not to enhance the production and release of SRS-A as had been assumed (13).

Very recently, one group reported that L-cysteine enhanced the conversion of SRS-GSH into SRS-Cys-Gly by activating the γ-glutamyl transpeptidase (29). However,

this observation is in disagreement with another report that the activation of this enzyme by L-cysteine was insignificant (67). Instead, we propose that any possible activation of γ-glutamyl transpeptidase by this amino acid is not due to L-cysteine itself, but rather due to L-cystine, its oxidation product, which was known to activate the enzyme because it is a good glutamyl group acceptor (68).

Considering the inhibitory role of L-cysteine on the cleavage of SRS-Cys-Gly by peptidases, it is understandable that SRS-Cys was not obtained from the cells treated with 10mM L-cysteine. However, it is unclear as to why 10 mM L-cysteine was included in the mastocytoma cells system, which generated only SRS-GSH (20). Furthermore, it was observed in RBL-1 cells that 10 mM L-cysteine decreased the formation of 5-lipoxidation products of arachidonic acid by one third. Accordingly, recent reports omit the use of L-cysteine for the generation of SRS in mastocytoma cells (70).

There are some arguments concerning the composition of SRSs. However, it seems likely that the composition of SRS depends on the type of cells (Table 6), the method of tissue preparation (71), or the incubation conditions. In contrast to the earlier reports that the SRS from RBL-1 cells corresponds to SRS-Cys-Gly, our results show clearly

Table 6. Identification of SRSs from different sources.

Source	Generation Method	SRS-GSH	SRS-Cys-Gly	SRS-Cys	Ref.
Cat Paw	compound 48/80		+	+	24
Horse eosinophils	Ionophore A23187	+	+		74
Human Lung	Antigen	small	+*		71
Human Peripheral neutrophils	A23187	+			41
Guinea pig lung	Antigen Cysteine		+		93
Mouse macrophage	Zymosan	+	small		75
Mouse Mastocytoma cells	A23187	+	small		19
Rat Basophilic leukemia cells	A23187	+	+	+	43
Rat peritoneal cells	Antigen	+	+	+	94
Rat peritoneal monocytes	A23187 Cysteine	+	+		23

*The specific activity was around 1/4 of that for SRS-Cys-Gly.

that the SRS from RBL-1 cells is a mixture of SRS-GSH, SRS-Cys-Gly and SRS-Cys, and their 11-*trans* isomers. Although this difference might be explained by the different cell growth conditions or SRS-generation conditions, our results were confirmed by recent reports that RBL cells generate variable amounts of SRS-GSH, SRS-Cys-Gly, and SRS-Cys (72). In our experiments, the replacement of fetal calf serum with newborn calf serum in growth medium or longer incubation increased the relative amount of SRS-Cys. Additionally, the level of γ -glutamyl transpeptidase or dipeptidase, and the possible native antibodies against these enzymes are expected to change the composition of SRS. Therefore, further studies are needed to define the factors that influence the relative amounts of these SRSs *in vivo*.

2. Biosynthesis and Metabolism of SRSs

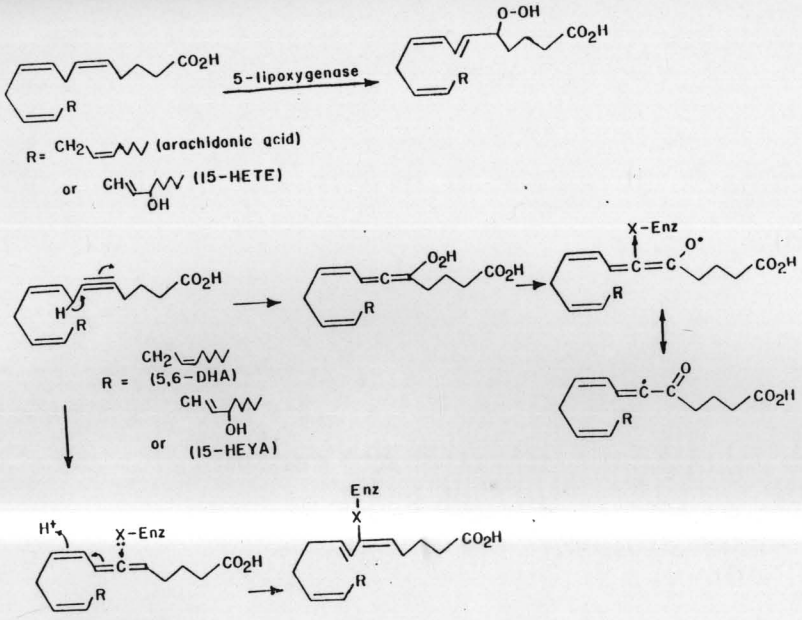
Lipoxygenation of arachidonic acid at C-5 leads to the formation of 5-HPETE, a precursor to the pivotal intermediate, 5,6-LTA. As reported by other workers (32), the incubation of arachidonic acid with RBl-l cell extracts led to the formation of 5-HETE, which was stimulated by calcium in a concentration-dependent manner. The calcium ion dependency of 5-lipoxygenase activity was confirmed by the observation that the conversion of 15-HETE into 5,15-diHETE was also enhanced (6 times) by ionophore A23187 (Figure 17).

ETYA had been reported to inhibit the formation of 5-HETE and SRS in RBL-1 cells. However, the inhibitory effects of ETYA lack specificity because of its inherent tetraacetylenic structure. Hence, we decided to investigate the effectiveness of monoacetylenic acids as a mechanism-based inhibitor of 5-lipoxygenase. In the cell-free system of RBL-1 cells, 5,6-DHA behaved as a typically specific irreversible inhibitor of 5-lipoxygenase, being approximately three times more potent than ETYA. However, in the intact RBL-1 cell system, 5,6-DHA instead stimulated the 5-lipoxygenase reaction at concentrations not more than 3 μM . A plausible explanation of this anomaly could be that 5,6-DHA is competing with

arachidonic acid for the synthesis of phospholipids, triglycerides and prostaglandins, so that more intracellular arachidonic acid became available for the biosynthesis of 5-HETE. This assumption is supported by the observation that [1-^{14}C]-5,6-DHA was readily incorporated into these constituents. This result poignantly reflects the complexity of designing mechanism-based inhibitors for intact cell systems. To overcome the metabolic diversity of 5,6-DHA, we prepared 15(S)-HEYA, which was not metabolized. Thus, we expected that 15(S)-HEYA might be more effective mechanism-based inhibitor of 5-lipoxygenase because 15-HETE was converted into 5,15-diHETE by this system. In RBL-1 cells, 15-HPETE was converted into 5,15-diHETE more effectively (6 times) than 15-HETE. However, the addition of 10 μM ionophore A23187 enhanced the transformation of 15-HETE into 5, 15-diHETE approximately 6-fold. The results of Figure 16 appear to be consistent with our expectation for 15(S)-HEYA, which effectively inhibited the 5-lipoxygenase even at relatively low concentrations.

Although the precise inhibitory mechanism of these acetylenic analogs was not defined, it is possible that 5-lipoxygenase reacts with 5,6-DHA to form a conjugated allene which then alkylates the nucleophile of the enzyme (Figure 30). Alternatively, 5,6-DHA is converted into an

Figure 30. Inhibitory mechanism of acetylenic acids.


5-lipoxygenase
R = (arachidonic acid)
or (15-HETE)
R = (5,6-DHA)
or (15-HEYA)
X-Enz
Enz

allenic hydroperoxide which decompose homolytically to generate alkoxy or carbon radicals which then covalently react with enzyme.

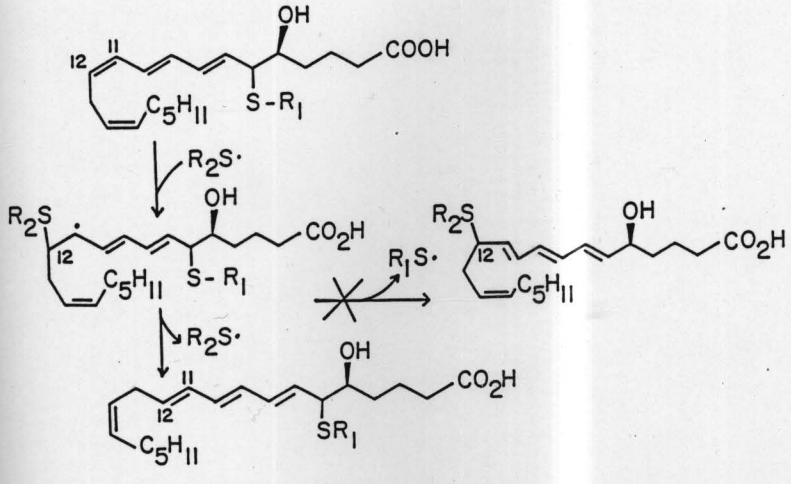
The enzymatic 1,7 elimination of 5-HPETE results in the formation of 5,6-LTA, which was first detected in human PMNLs (40). Our results show clearly the native 5,6-LTA formed from arachidonic acid via 5-HPETE in the RBL-1 cells corresponds to 5(S)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-icosatetraenoic acid, by comparison with synthetic specimens. Interestingly, this result differs from the biomimetic synthesis (73) of LTA methyl ester from 5-HPETE methyl ester which gives rise to two isomers, 5,6-LTA methyl ester and 9*Z*-5,6-LTA methyl ester.

In turn, the exposure of 5,6-LTA to RBL-1 cells in the presence of serine-borate buffer led largely to the formation of SRS-GSH with a trace amount of 11-*trans* SRS. These results were somewhat different from the earlier observation that SRS-GSH and 11-*trans*-SRS-GSH was found in variable ratios of 9:1 to 4:6 after incubation of 5,6-LTA with human leukocytes. This difference might be caused by the different purification procedure which included the alkaline treatment of sample (63). It was observed by Atrache *et al*. that the alkaline treatment of SRS samples containing thiols enhanced the 11-*cis*→*trans* isomerization of SRSs molecule (63).

Generally, SRS generating systems in which the L-cysteine and the alkaline treatment of sample were omitted, formation of 11-*trans*-SRS was diminished (70,74.75). Moreover, we showed the radical-catalyzed isomerization of SRS-Cys into 11-*trans*-SRS-Cys in RBL-1 cells after prolonged incubation.

On the basis of the foregoing results (Table 4), it appears that the 11-*trans*-SRSs are not formed by a separate independent pathway but rather they are most likely derived from their corresponding SRSs via nonenzymatic isomerization of the Δ^{11}-double bond. The higher amounts of 11-*trans*-SRS produced by RBL-1 cells than boiled cells is probably the result of an indirect effect. That is, living cells not only contain relatively high concentrations of GSH(10^{-3}M), but also enzyme systems that generate peroxides, known to enhance the formation of RS radicals from thiols. In turn, the RS radical adds reversibly at C-12 of SRSs to allow overall *cis*→*trans* isomerization of the Δ^{11}-double bond (Figure 31). Accordingly, these results support the proposition that 11-*trans*-SRS-GSH is derived directly from SRS-GSH, but not from 5,6-LTA. Although the properties of S-glutathione transferase in RBL-1 cells were not characterized, a recent report (67) showed that the S-glutathione transferase from RBL-1 cells is a particulate enzyme,

Figure 31. Proposed scheme for the formation of 11-*trans*-SRS catalyzed by thiyl radicals.


OH
COOH
12
11
C_5H_{11}
S-R_1
$R_2S\cdot$
R_2S
CO_2H
$R_1S\cdot$
SR$_1$

different from the soluble glutathione transferase from liver. Additionally, this enzyme utilized not only 5,6-LTA, but also 9-cis-5,6-LTA and 14,15-LTA, nonspecifically.

Both RBL-1 cells and kidney contained similar types of enzymes in the metabolism of SRSs. γ -Glutamyl transpeptidase converts SRS-GSH into SRS-Cys-Gly, which is in turn transformed into SRS-Cys by peptidases. No N-acetyl SRS-Cys was detected in the incubation mixture of RBL-1 cells indicating the absence of N-acetyl transferase in these cells. However, recent observations that incubation of SRSs with kidney, liver, or gall bladder extracts also did not form N-acetyl SRS-Cys, suggesting the SRS-Cys may be resistant to the action of N-acetyl transferase.

Based on the similarity of SRS metabolism in both systems, we conclude that the sequence of SRS metabolism in RBL-cells parallels the glutathione detoxification pathway, except that instead of N-acetyl SRS-Cys, 11-trans SRS-Cys was isolated as the final product after long periods of incubation. The properties of γ-glutamyl transpeptidase and dipeptidase of RBL-1 cells were similar to those of enzymes from kidney: γ-glutamyl transpeptidase was inhibited similarly by serine-borate buffer in both cases. The peptidases from both sources

were also inhibited to similar degrees by L-cysteine (78).

Although two peptidases, aminopeptidase-M and dipeptidase, were reported to cleave cysteinylglycine conjugates, it was not until recently that purified enzymes were available for the study of their substrate specificities. Our results show that while aminopeptidase-M did not cleave SRS-Cys-Gly significantly, purified dipeptidase was highly active in the cleavage of the peptide bond of SRS-Cys-Gly, indicating the important role of dipeptidase in regulating the level of SRS-Cys-Gly *in vivo*. Recently, our observations were confirmed by reports that renal dipeptidase is much more active in the cleavage of SRS-Cys-Gly than renal aminopeptidase-M. Moreover, this cleavage is inhibited by thiols-containing compounds, L-cysteine, D-penicillamine and cysteinyl-glycine (76-77). Therefore, it is concluded that the partially purified RBL-1 peptidase, active in the cleavage of the SRS-Cys-Gly peptide bond, is a type of dipeptidase based on substrate specificity, thiols-sensitivity and inhibition by phosphate ion.

After having established the conversion of SRS-Cys-Gly into SRS-Cys by dipeptidase, we then examined SRS-A inactivation in the RBL-1 cells, guinea pig lung and peritoneal eosinophils, and limpet arylsulfatase preparation. In all the systems studied, the SRS-Cys-Gly

inactivating peptidase activities were resolved from the arylsufatase activities, and these arylsufatases (fraction 75-80, Figure 26 and fractions 40-41, Figure 29) had no ability to inactivate SRS-Cys-Gly. These results suggest that both type A and type B arylsulfatases lack intrinsic peptidase activity to cleave R-Cys-Gly peptide bond. Although the precise nature of these SRS-Cys-Gly inactivating peptidases was not defined, their properties more closely resembled those of dipeptidases (substrate specificity and thiols-sensitivity). Also, the peptidase activity of limpet arylsulfatase preparation was observed to cleave SRS-Cys-Gly, but not SRS-GSH (78). Recently, this observation was confirmed by others that limpet arylsulfatase (5 units) inactivated SRS-Cys-Gly only (79). However, one group (80) claimed that SRS-GSH was also inactivated by a high concentration of limpet arylsulfatase (Type V, 20 units). This difference may be explained by our observation that crude limpet arylsulfatase (20 units) contained 0.15 milliunits of γ-glutamyl transpeptidase activity.

Recently, purified human placental arylsulfatase B was reported to inactivate Rat SRS-A to the extent of 50%. Based on the inactivation of Rat SRS-A by arylsulfatase B and the detection of sulfone group in the field desorption mass spectrum, they hypothesized that the

thioether at C-6 in SRS-A may be in the form of a sulfone (81). However, two groups (82,83) demonstrated that the sulfone form of SRS might be an artifact formed during purification of SRS. Therefore, SRS-A inactivation by human placental arylsulfatase-B was also due to the action of a contaminating dipeptidase, because the Rat SRS-A was reported to include SRS-Cys-Gly as a major component.

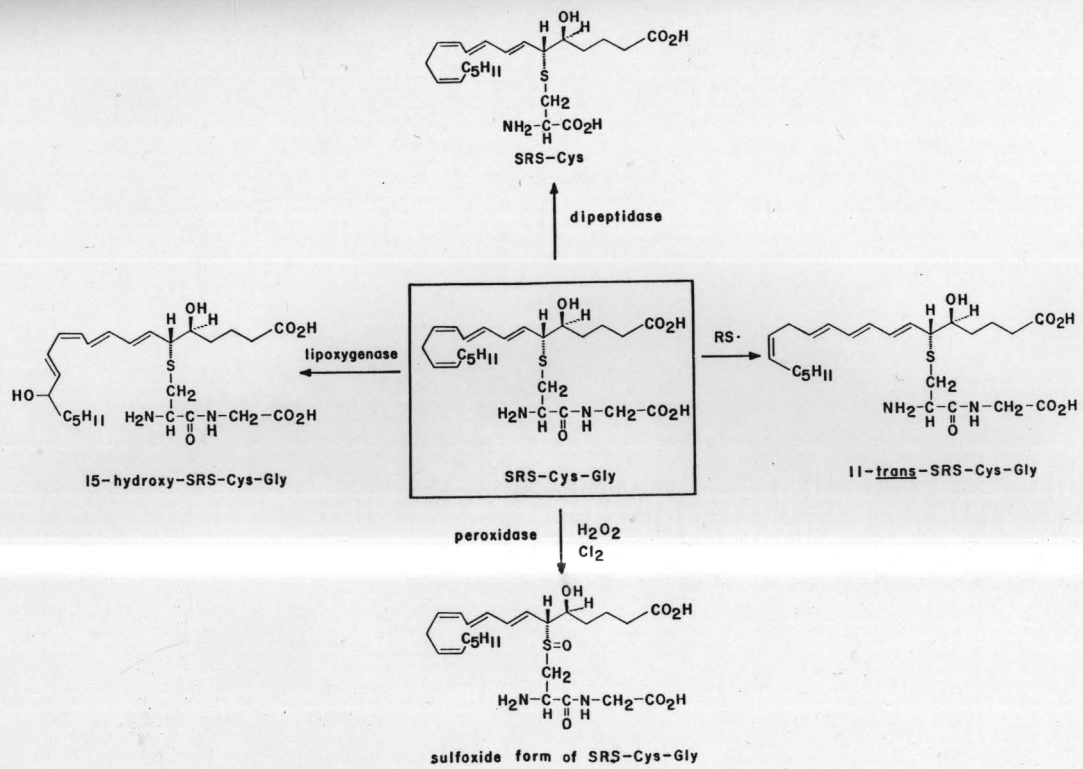
Subsequently, Kumagai et al. (79) reported that SRS-A from human lung was inactivated by placental arylsulfatase B, but neutrophil-derived SRS was not inactivated. This observation is also consistent with our contention, because neutrophil is known to produce SRS-GSH and human lung, SRS-Cys-Gly.

Other possible mechanisms of reducing the spasmogenic activities of SRSs could involve the isomerization of double bond (11-cis → 11-trans) of SRSs catalyzed by RS in cells. In addition, the lipoxidation at C-15 of SRSs by 15-lipoxygenase (16), and the peroxidase-catalyzed oxidation (84) of the sulfur atom in SRSs have been reported to inactivate the SRSs (Figure 32).

In summary, while the biosynthetic enzymes of SRS appear to reside in certain specialized cells, the enzymes responsible for the metabolism of SRS appear to be commonly shared by many cell types.

Recently, another series of leukotrienes was

Figure 32. Inactivation pathways of SRS-Cys-Gly.

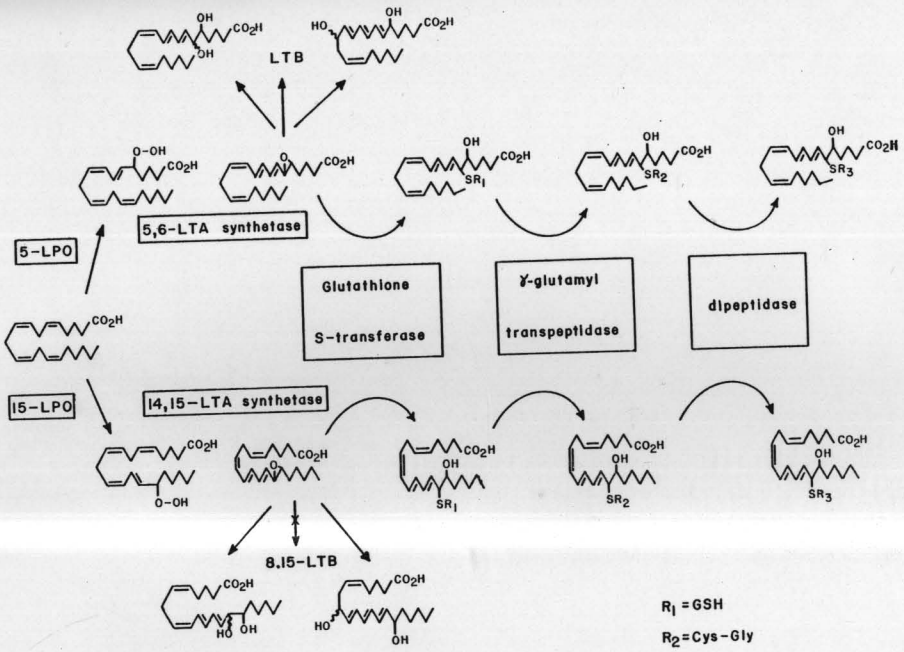

SRS-Cys
dipeptidase
15-hydroxy-SRS-Cys-Gly
lipoxygenase
SRS-Cys-Gly
RS·
11-trans-SRS-Cys-Gly
peroxidase
H_2O_2
Cl_2
sulfoxide form of SRS-Cys-Gly

uncovered after exposure of arachidonic acid (85) or (S)-15-HPETE to human leukocytes. Four dihydroxy acids were isolated and designated as 14,15-dihydroxy-5,8,10,12-icosatetraenoic acid (two isomers) and 8,15-dihydroxy-5,9,11,13-icosatetraenoic acid (two isomers). It was proposed that these compounds were derived from 14,15-LTA, which in turn was formed from 15-HPETE (57). This proposition was confirmed by recent observation that [^{3}H]-(S)-15-HPETE was converted enzymatically into 14,15-LTA and 14,15-LTA was tranformed into 14,15-diHETEs, 8,15-diHETE, and C(14)-sulfur-linked peptides (Figure 33) (57).

Although the physiological role of the 15-lipoxygenase pathway was not clarified completely, the products, 15HPETE and 15-HETE were reported to be inhibitors of 5-lipoxygenase (90). In addition 5,15-diHETE, the 5-lipoxidation product of 15-HPETE was proposed to be an inhibitor of Ca^{++} uptake in neutrophils (86). These observations may support the negative regulatory role of 15-lipoxygenation metabolites on the 5-lipoxygenation pathway.

The communication between cells and the interaction between lipoxygenases in cells may contribute to the formation of leukotrienes or diHETEs; 14,15-LTA was transformed into C(14)-sulfur-linked peptides by RBL-1 cells (57), and 15-HPETE was converted into 5,15-diHETE in

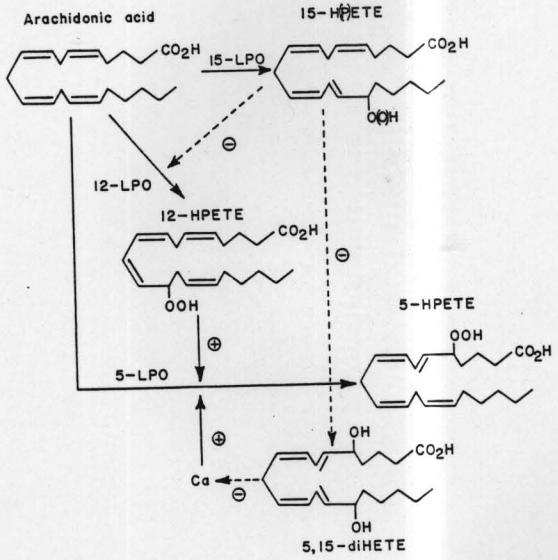
Figure 33. Two types of leukotrienes pathway.


LTB
5,6-LTA synthetase
5-LPO
Glutathione
S-transferase
γ-glutamyl
transpeptidase
dipeptidase
15-LPO
14,15-LTA synthetase
8,15-LTB
R1 = GSH
R2 = Cys-Gly
R3 = Cys

both RBL-1 cells and human leukocytes (87). It is interesting to note that 15-HPETE was more effectively converted into 5,15-diHETE than 15-HETE, suggesting that the peroxy group of 15-HPETE may activate the 5-lipoxygenase activity. The activating effect of peroxy group on the 5-lipoxygenase was also observed by others who reported the activation of 5-lipoxygenase activity by 12-HPETE. They also proposed an interaction between 5-lipoxygenase (human leukocytes) and 12-lipoxygenase from platelets (88). However, this proposition is somewhat questionable, because hemoglobin (89) in human blood is expected to decompose any extracellular 12-HPETE before entry into leukocytes. Instead, it is more probable that intracellular 12-HPETE may be involved in the activation of 5-lipoxygenase in the same cell, giving more emphasis to the interaction between intracellular lipoxygenases.

The interaction between lipoxygenases results in the positive or negative control of leukotriene formation via 5-HPETE. As shown in Figure 34, 12-HPETE activates the 5-lipoxygenase (88) whereas 15-H(P)ETE inhibits the 5-lipoxygenase and 12-lipoxygenase (90). As a result, the 15-H(P)ETE may decrease leukotriene formation directly by inhibiting 5-lipoxygenase and indirectly by inhibiting 12-lipoxygenase. In addition, other endogenous factors including platelet activating factor (PAF) (91) and

Figure 34. Regulation of leukotrienes formation by H(P)ETEs.


Arachidonic acid
15-HPETE
15-LPO
12-LPO
12-HPETE
5-HPETE
5-LPO
Ca
5,15-diHETE
LPO = lipoxygenase
⊕ = activation
⊖ = inhibition

phosphatidic acid (92) were proposed to affect leukotrienes biosynthesis via 5-HPETE. However, to determine which of these naturally occuring mediators (H(P)ETEs, diHETES, PAF and phosphatidic acid) are involved in the regulation of leukotrienes formation *in vivo*, further investigations are needed.

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Part II. Enzymatic Formation of 14,15-Leukotriene A and C(14)-Sulfur-Linked Peptides.