A STUDY OF SLOW-REACTING SUBSTANCE FROM CAT PAWS

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

JOEL E. HOUGLUM

Degree to be awarded: December 19____ May 19 79 August 19____

Approved by Thesis Reading Committee:

Major Professor — Date of Examination: May 17, 1979

David Perlman

Carl K. Bachhuber

Dean, Graduate School

A STUDY OF SLOW REACTING SUBSTANCE FROM CAT PAWS

BY

JOEL E. HOUGLUM

(Under the supervision of Professor Charles J. Sih)

The optimum conditions for the release of SRS from cat paws induced by 48/80 and the calcium ionophore A23187 have been defined. The release of SRS was affected by agents known to alter cAMP and cGMP levels. Both epinephrine and aminophylline inhibited SRS release and a synergistic effect was observed when these agents were used together. Further, this synergism was reversed by propranolol and carbachol. These data suggest that cyclic nucleotides modulate SRS release. Arachidonic acid and indomethacin had no effect, but 5,8,11,14-eicosatetraynoic acid (TYA) inhibited SRS release.

A four step method of purifying SRS was developed which utilized XAD-7 resin, ethyl acetate extraction, disposable silica gel column, and preparative μC_{18} HPLC. These successive steps afforded 716,800 fold purification as determined by ^{14}C-methylation of the sample with a recovery of 14%. However, even at this stage of purity, a radiochromatogram scan of an 80,000 unit sample (methylated with ^{14}C-diazomethane) did not reveal a radioactive peak coinciding with the bioactivity. This purified material lost 97% of its bioactivity in two days at $-25^{0}C$.

It is well known that sulfate esters are readily solvolyzed by refluxing in anhydrous tetrahydrofuran or dioxane. However, SRS bioactivity was retained under these conditions, which raised the possibility that SRS is devoid of a sulfate ester function or that SRS sulfate is surrounded by neighboring hydroxyl groups. Although various crude arylsulfatase preparations inactivate SRS, commercial arylsulfatase preparations also contain phosphatase activity. Using alkaline disc gel electrophoresis, the sulfatase and phosphatase activities were partially separated and it was clearly shown that SRS inactivation was associated with the sulfatase activity.

An SRS inactivating enzyme was discovered in various crude phosphatase and phosphodiesterase preparations and this activity could not be attributed to the phosphatases or phosphodiesterases themselves. The SRS inactivating enzyme was characterized by a molecular weight of approximately 100,000, a pH optimum of 7, and inhibition by cysteine but not by K_2HPO_4, Na_2SO_4, $MgCl_2$ or EDTA. A comparison of the properties of fifteen other enzymes did not reveal the identity of this SRS inactivating enzyme.

APPROVED: _____

Professor Charles J. Sih

DATE: May 17, 1979

A STUDY OF SLOW REACTING SUBSTANCE FROM CAT PAWS

BY

JOEL EDGAR HOUGLUM

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

DOCTOR OF PHILOSOPHY

(Pharmacy)

at the

UNIVERSITY OF WISCONSIN-MADISON

1979

To my wife, Rita, whose continual encouragement, support, and love has helped me endure the frustrations and anxieties.

ACKNOWLEDGMENTS

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

TABLE OF CONTENTS

		Page
I.	INTRODUCTION	1
II.	EXPERIMENTAL	16
	A. Materials	16
	B. SRS Assay	18
	C. Cat Paw Perfusion	21
	D. Stability of SRS	23
	E. Purification of SRS	24
	F. Derivatization	28
	G. Hydrolysis of Methylated SRS	30
	H. Dioxane and THF Solvolysis	31
	I. Preparation of Sulfate Esters	33
	J. Enzymatic Studies	34
III.	RESULTS	40
	A. SRS Assay	40
	B. Cat Paw Perfusion	41
	C. Stability of SRS	58
	D. Purification of SRS	66
	E. Derivatization and Hydrolysis	80
	F. Dioxane and THF Solvolysis	90
	G. Enzymatic Studies	97
IV.	DISCUSSION	119
V.	REFERENCES	128

LIST OF TABLES

		Page
1.	SRS in the perfusate from cat paws.	42
2.	SRS^{cat} released by 48/80.	43
3.	Release of SRS^{cat} by ionophore A23187	44
4.	SRS^{cat} released at various perfusion rates.	50
5.	Effect of 2-deoxy-D-glucose on the release of SRS^{cat}	52
6.	Effect of epinephrine, aminophylline, and propranolol on the release of SRS^{cat}.	54
7.	Effect of phenylephrine and propranolol on the release of SRS^{cat}	55
8.	Effect of carbachol, epinephrine, and aminophylline on the release of SRS^{cat}.	56
9.	Effect of arachidonic acid on SRS^{cat} release. . . .	59
10.	Effect of indomethacin and TYA on the release of SRS^{cat}	60
11.	Stability of SRS^{cat} perfusate	61
12.	Purification procedures for SRS^{cat}.	69
13.	Purification of SRS^{cat}.	79
14.	Silylation of SRS^{cat}.	84
15.	Hydrolysis of silylated SRS^{cat}.	85
16.	Methylation of SRS^{cat}	87
17.	Stability of SRS^{cat} to KOH.	88

Page

18. Hydrolysis of methyl esters by pyridine 91

19. Thin layer chromatography of methylated SRS^{cat} treated with pyridine. 92

20. Solvolysis of sulfate esters by THF and dioxane . . 96

21. Solvolysis of SRS^{cat} by THF and dioxane 98

22. Activity of arylsulfatase and crude phosphatases on various substrates. 100

23. Effect of denatured enzymes on SRS^{cat} 105

24. Effect of enzymes on SRS^{cat} 117

LIST OF FIGURES

		Page
1.	SRS releasing factors and SRS antagonist.	3
2.	Modulation of SRS-A released from human lung. . . .	10
3.	Sequence of events for SRS-A release from human lung	13
4.	Constant perfusion of cat paws with 48/80 (1 μg/ml)	47
5.	Perfusion of cat paws with a single dose (25 μg) of 48/80.	49
6.	Stability of SRS at various pH's at 100^0 for 20 minutes	63
7.	Stability of SRS at various pH's at 22^0 for 2.5 hours	65
8.	Elution of SRS^{cat} from a μC_{18} column.	73
9.	Elution of SRS^{cat} from an overloaded μC_{18} column. .	75
10.	Elution of SRS^{cat} from a preparative μC_{18} column. .	78
11.	Radiochromatogram scan of SRS^{cat} after methylation with ^{14}C-diazomethane	82
12.	Solvolysis mechanism of sulfate esters by dioxane .	95
13.	Alkaline disc gel electrophoresis of arylsulfatase.	103
14.	Rate of SRS^{cat} inactivation by crude venom phosphodiesterase and crude acid phosphatase. . . .	107

Page

15. Inactivation of SRS^{cat} by various concentrations of crude alkaline phosphatase 109

16. Sephadex G-200 chromatography of SRS inactivating enzyme 111

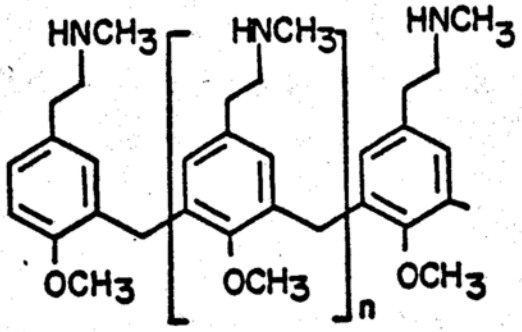
17. Effect of pH on the inactivation of SRS^{cat} by various enzyme preparations. 114

18. Inactivation of SRS^{cat} by crude enzyme preparations under various conditions 116

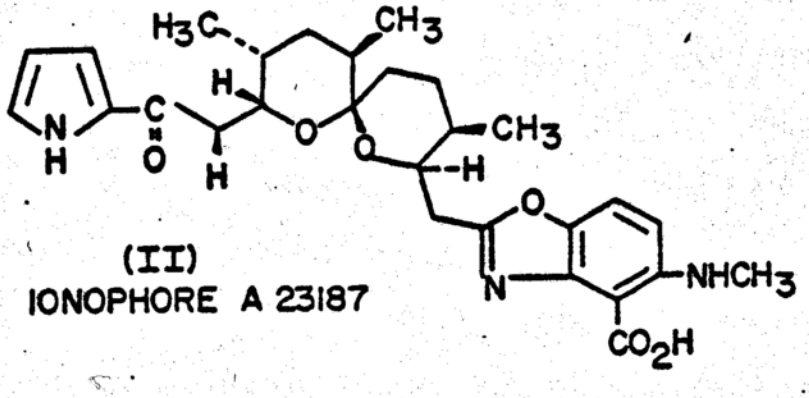
I. INTRODUCTION

In 1938 Feldberg and Kellaway (1) first described a substance that contracted the guinea pig ileum more slowly and with more sustained action than histamine, and named the compound slow reacting substance (SRS). Their source of this smooth muscle contracting substance was the effluent of guinea pig lungs treated with cobra venom. Kellaway and Trethewie (2) reported in 1940 that the effluent of sensitized guinea pig lungs also produced slow reacting substance when challenged with a specific antigen. SRS has since been released from many types of tissues and cell suspensions including guinea pig, rabbit, monkey, bovine, and human lungs (3-5), cat paws (6,7), isolated rat mast cells (8), human leukocytes (9), nasal polyps (10), human and rat leukemic basophils (11,12), and rat peritoneal fluid (13, 14).

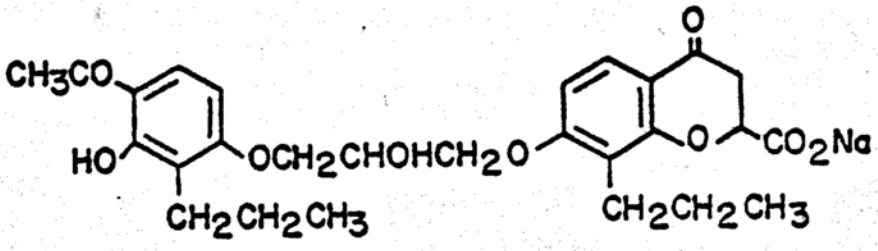
Unlike histamine, SRS has not been detected in tissues prior to challenge with antigens or releasing factors. This suggests that SRS exists in a precursorial state within the tissue (5,15). There are two standard methods used to release SRS: treatment with releasing factors (Figure 1) like compound 48/80 (6,16) or ionophore A23187 (11,12,17-19), and antigen-antibody interaction (2-4,9,13). To distinguish immunological from non-immunological release of SRS,



(I)
COMPOUND 48/80



(II)
IONOPHORE A 23187



(III)
FPL 55712

Brocklehurst (5) suggested that SRS released by antigen-antibody interactions be designated as SRS-A. Orange and Austen (20) introduced a suffix designation to denote the origin of the SRS so that SRS released immunologically from the rat peritoneal cavity, for example, is designated as $SRS\text{-}A^{rat}$.

The role of SRS in human physiology is not yet clear, but evidence indicates that SRS plays a significant role as a mediator during immediate hypersensitivity reactions such as asthma (21,22). When either whole segments or sliced tissues of an asthmatic human lung were brought in contact with sensitizing antigen, SRS-A was released. Prior to the addition of antigen, SRS-A could not be detected, even though other mediators such as histamine were detectable. When a tissue was challenged with antigen, SRS release was slower but more sustained than either histamine or eosinophil chemotactic factor of anaphylaxis (ECF-A) (5,10,21). In the sensitized guinea pig, lung tissue produced by far the greatest amount of SRS-A than any other of the nineteen tissues tested (5). Human bronchioles cut into rings and placed in an organ bath also produced a strong and sustained contraction when challenged with a single dose of SRS. When exposed to the appropriate antigen, bronchiole ring preparations from human asthmatics produced a similar prolonged contraction and released SRS-A into the suspending medium

(5). When asthmatic patients inhaled crude SRS-A^{gp} as an aerosol spray, a reduction of vital capacity resulted which was not apparent in the control subjects (23). Also, antihistamines are of very little value in the treatment of asthma which suggests the involvement of other potent bronchiole-constricting mediators.

During anaphylactic reactions, many other mediators are released, including histamine, prostaglandins, bradykinin, rabbit aorta contracting substance (RCS), ECF-A, and 5-hydroxytryptamine (5-HT). SRS was distinguished from these mediators through the use of inhibitors, enzymatic degradation, pH stability profiles, and differential bioassays. The contraction of guinea pig ileum by histamine and 5-HT, for example, was inhibited by antihistamines and atropine, respectively, although these inhibitors had no effect on the action of SRS. The ascending gerbil colon and estrous rat uterus were sensitive to bradykinin, but insensitive to SRS. Prostaglandins E_1 and E_2 relaxed human bronchial smooth muscle, whereas $PGF_{2\alpha}$ contracted the muscle and caused associated tachyphylaxis. SRS contracted bronchial smooth muscle without tachyphylaxis (20). SRS was distinguished from RCS (now identified as thromboxane A_2) by its ability to contract rabbit aorta and its greater instability (24). RCS-RF did not affect smooth muscle organs directly and, unlike SRS, was inactivated by carboxypeptidase (25). ECF-A was

identified as two tetrapeptides and was inactivated by digestion with pronase (22,26). SRS was resistant to trypsin, chymotrypsin, pepsin, activated papain (20,27), carboxypeptidase, leucine aminopeptidase, phospholipases A, B, C, D (20,28), pronase (20,29), and 15-hydroxyprostaglandin dehydrogenase (30).

SRS was soluble in water, methanol, and ethanol, slightly soluble in propanol (27), but was insoluble in chloroform or acetone. However, upon acidification of SRS^{cat} and $SRS\text{-}A^{gp}$ to pH 2-3, they could be extracted into diethyl ether or ethyl acetate and the activities could be partitioned back into aqueous alkali (28,30). $SRS\text{-}A^{gp}$ was unstable at room temperature and lost 50% of its activity within twenty-four hours at pH 7.5-9.5. Solutions of $SRS\text{-}A^{gp}$, $SRS\text{-}A^{rat}$, and $SRS\text{-}A^{hu}$ were reported to be less stable in acid than in base (20,31). Hydrogen peroxide rapidly destroyed the bioactivity. In the presence of 0.005% of H_2O_2, 50% of the bioactivity was lost in 30 minutes at 22^0 (14).

$SRS\text{-}A^{rat}$ was inactivated by two arylsulfatase preparations (32). Partially purified arylsulfatases from human lung (33) and human eosinophils (34) were shown to inactivate $SRS\text{-}A^{rat}$ and $SRS\text{-}A^{hu}$. This suggested a regulatory mechanism by which SRS-A was inactivated at the site of immediate hypersensitivity.

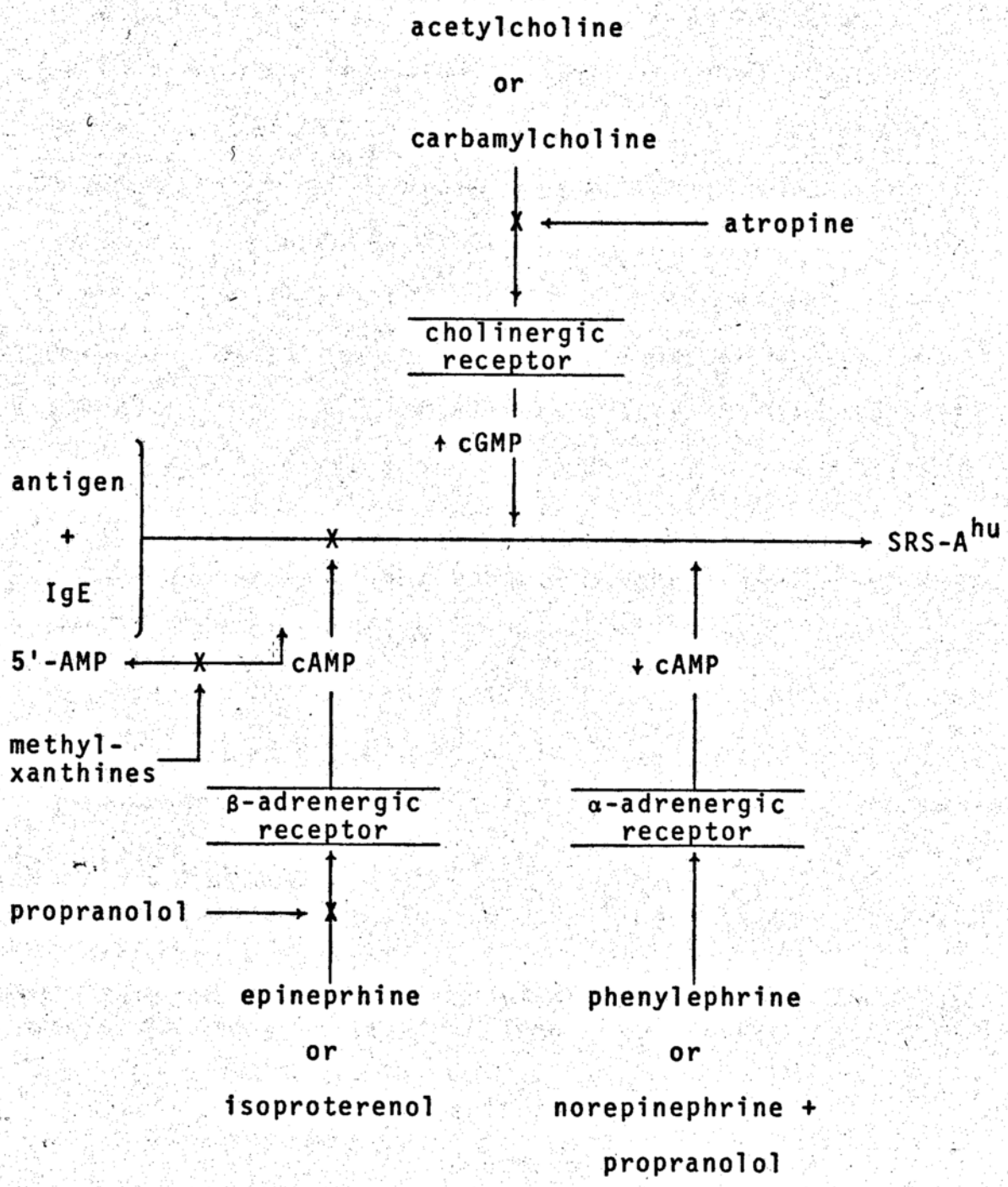
When administered intraperitoneally to sensitized rats before challenge with antigen, prostaglandin E_1 and E_2 inhibited the release of SRS-A (35). Other prostaglandins (A_1, A_2, B_1, $F_{1\alpha}$, $F_{2\alpha}$, and $F_{2\beta}$) were less effective. Since SRS-A was reported to stimulate the release of RCS and prostaglandins (24,36), a negative feedback by prostaglandins on SRS-A release was suggested. This contention is supported by the finding that in some tissues, a decrease in prostaglandin production and an increase in SRS release were noted upon the administration of indomethacin (36-38). This effect was suggested as a contributing factor in the aspirin sensitivity syndrome (characterized by bronchospasm) observed in some asthmatics after exposure to nonsteroidal antiinflammatory agents (38).

A potent SRS antagonist, FPL 55712 (Figure 1), inhibited the SRS response on the guinea pig ileum by 50% at 10^{-8} M (39). Other mediators, such as PGE_1 and $PGF_{2\alpha}$ were also inhibited by FPL 55712, but at a concentration 1000 times greater than that required to antagonize the effects of $SRS\text{-}A^{gp}$ and $SRS\text{-}A^{hu}$. In the intact animal, FPL 55712 had a very short half-life and was therefore unsuitable for *in vivo* studies. Inactivation by arylsulfatase, and antagonism by FPL 55712 are often used as characteristic properties of SRS.

Many *in vitro* systems were used to study the release

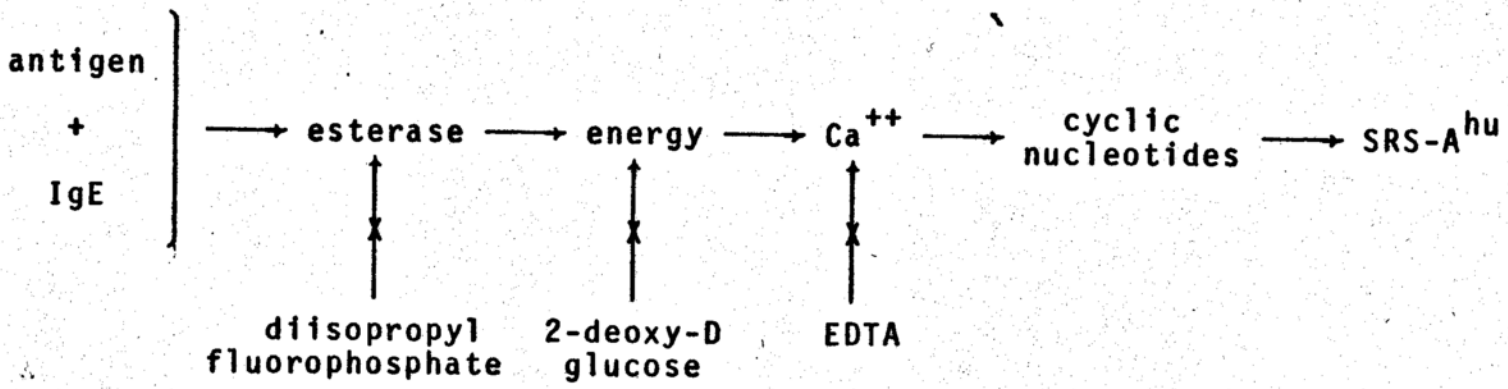
mechanism of the mediators of immediate hypersensitivity including SRS-A. Most of these studies were conducted using human lung (4,40), human basophils (11), human nasal polyp fragments (10), or rat peritoneal fluid (41). Evidence indicated that IgE was the major immunoglobulin responsible for SRS-A release in humans (4) and that mast cells and basophils were the primary cell types involved (9,13,42). The release appeared to be noncytolytic (43-45).

The release of SRS-A^{hu} was shown to be modulated by cyclic nucleotides (10,46) and various agents known to alter cyclic nucleotide levels (4,46). These experiments indicated opposing effects between cyclic 3',5'-AMP and cyclic 3',5'-GMP upon the release of SRS-A (Figure 2). Drugs which were known to increase cyclic AMP levels (β-adrenergic agents) decreased the amount of SRS-A released, whereas drugs known to decrease cyclic AMP levels (α-adrenergic agents) increased the amount of SRS-A released. Synergism was observed when methylxanthines (phosphodiesterase inhibitors) were used with β-adrenergic agents. Beta-adrenergic blockers also increased SRS-A levels. When cyclic GMP levels were increased either by the use of cholinomimetic agents (acetylcholine or carbamylcholine) or by the addition of 8-bromo-cyclic 3',5'-GMP, they caused an increase in SRS-A levels. The effect of the cholinomimetic agents could be blocked by the muscarinic blocking agent, atropine.


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

Other biochemical requirements for the release of SRS-A were also determined. Calcium ions, an intact glycolytic pathway, and serine esterase activation were all required for SRS-A^{hu} release. Kaliner and Austen (40) reported the biochemical sequence by which these factors modulate SRS-A release. They terminated the biochemical sequence, then started it again after certain inhibitors were added or removed. In general, the sensitized tissue was challenged with a specific antigen in the presence of an inhibitor, and then washed with a buffer containing a second inhibitor. The antigen and the first inhibitor were washed away at the same time. If the tissue subsequently released SRS-A, then the sequence had to have already progressed past the point of inhibition by the second inhibitor. The first inhibitor must therefore follow the second inhibitor in the biochemical sequence. If, after washing with the second inhibitor, the SRS-A release was blocked, the second inhibitor must follow the first in the sequence. The cascade of events leading to SRS-A release was determined by this technique to be as shown in Figure 3. This modulation of SRS-A release was reviewed recently (22,47).

The purification and structural elucidation of SRS were attempted (14,30,31), but the instability and limited quantities of SRS hampered the undertaking. The peritoneal fluid from 280 sensitized rats, challenged with antigen, was


antigen
+
IgE
esterase
energy
Ca++
cyclic nucleotides
SRS-Ahu
diisopropyl fluorophosphate
2-deoxy-D glucose
EDTA

reported to produce 308,000 units of SRS-A activity (one unit was defined as that amount which produced a contraction of the guinea pig ileum equal in amplitude to that produced by 5 ng of histamine). A partially purified sample of this material containing 100,000 units was estimated to weigh only 10-50 μg (14). Further, a decrease in SRS stability was observed with increased purification (14,27,31) even at low temperatures (14,31).

The different sources and ways of SRS generation have raised some question as to whether SRS is the same from all sources. Some physiochemical differences were reported, and the possible existence of more than one bioactive structure, even from the same source, was suggested. For example, acidified samples of SRS-A^{hu} did not extract into diethyl ether (31), whereas SRS^{cat}, SRS^{rat}, and SRS-A^{gp} did (28,30, 48). On the other hand, the same factors modulated the release of SRS-A^{rat} and SRS-A^{hu}, which suggested that both were derived from a common precursor. Takahashi (31) reported the separation of SRS-A^{hu} into four biologically active peaks. Although only one peak was inactivated by arylsulfatase, evidence was given for the existence of an arylsulfatase inhibitor in the other bioactive peaks. Using high pressure liquid chromatography, Morris (48) found that SRS-A^{gp} could be separated into two bioactive peaks, but suggested that these merely represented the protonated and

unprotonated forms of SRS. Rechromatographing of the original unprotonated sample under acidic conditions produced an elution pattern which corresponded to the protonated bioactive sample. The original protonated sample, however, was not rechromatographed under the acidic conditions.

Another stumbling block in the structural elucidation of SRS is researchers' inability to obtain a gas chromatogram or mass spectra with either chemical ionization or electron impact techniques (32). It was suspected that the existence of a sulfate ester on the molecule caused this problem.

This investigation was undertaken to examine the parameters affecting SRS release in the cat paw by compound 48/80; to compare the effects of various agents on the release of SRS in the cat paw and other systems; and to develop a purification scheme that would generate purified SRS to allow chemical structural studies.

II. EXPERIMENTAL

A. Materials and Methods

N,O-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce Chemical); trimethylchlorosilane (Tri-Sil), hexamethyl disilazane, pyridine (Pierce Chemical); N-methyl-N-nitroso-p-toluene-sulfonamide (MNTSA) (Aldrich); ^{14}C MNTSA (New England Nuclear); tris(2-hydroxypropyl)amine (THPA) or 1,1',1"-nitrilotri-2-propanol (Eastman Kodak); atropine sulfate (Mallinckrodt); mepyramine maleate (ICN-K&K); FPL 55712 (Fisons, Ltd.); 5,8,11,14-eicosatetraynoic acid (TYA) (Roche); ionophore A23187 (Eli Lilly); PGE_1 and PGE_2 (Miles Labs); L-epinephrine bitartrate (Winthrop); sotalol hydrochloride (Mead-Johnson); theophylline ethylenediamine (Merck); propranolol (Ayerst Laboratory); and indomethacin (Merck) were purchased from the manufacturers as indicated.

The following compounds were obtained from the Sigma Chemical Co.: butylated hydroxytoluene (BHT); hydroxystearic acid (HSA); p-nitrophenyl sulfate (p-NPS); p-nitrocatechol sulfate (p-NCS); p-nitrophenyl phosphate (p-NPP); glucose-6-sulfate (G-6-S); hexokinase; glucose-6-phosphate dehydrogenase (G-6-PDH); adenosine triphosphate (ATP); β-nicotinamide adenine dinucleotide phosphate ($NADP^+$); 2-deoxy-D-glucose (2-DG); L-phenylephrine hydrochloride; compound 48/80; arachidonic acid; histamine dihydrochloride;

glucose oxidase (type V, Aspergillus niger); o-dianisidine. All enzymes discussed under "Enzymatic Studies" were obtained from Sigma except horse radish peroxidase, grade B (Calbiochem) and purified venom phosphodiesterase (M. Laskowski, Sr., Roswell Park Memorial Institute).

A Model M-6000 pump, equipped with U6K injector (Waters Assoc.) was used for high pressure liquid chromatography (HPLC) equipped with a Model 77 double beam UV detector (Waters Assoc.). The analytical prepacked microparticulate (10 μ) HPLC columns (4 mm i.d. X 30 cm) that were used were μPorasil, μBondapak CN, and μBondapak C_{18} (Waters Assoc.). A microparticulate preparative column (9.4 mm i.d. X 50 cm), M9 ODS and ODS-2 (μC_{18}) was also used (Whatman). Other HPLC materials used were stainless steel columns (7 mm i.d. X 61 cm) containing Bondapak C_{18}/Corasil, 37-50 μ, and Bondapak C_{18}/Porasil B, 37-75 μ (Waters Assoc.) and precolumns (2 mm X 7 cm) containing Co:Pell ODS, 25-37 μ (Whatman).

Other chromatography materials used were: 7.5% polyacrylamide gels (Bio-Rad); silica gel (70-270 mesh) and cellulose powder (Brinkmann); neutral aluminum oxide (Waters Assoc.); Amberlite XAD-2 and XAD-7 (Mallinkrodt); LH-20 and Sephadex G-200 (Pharmacia); silica gel thin layer chromatography plates, 0.25 mm (Brinkmann); and C_{18} and silica gel Sep-Pak cartridges ("disposable columns") containing 40 mg and 70 mg of packing material, respectively (Waters Assoc.).

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

Enzymes were assayed by the use of a Model 14 recording spectrophotometer (Cary).

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

All solvents were redistilled from AR grade. Those used for HPLC were degassed either by filtration under vacuum or by warming and then cooled to room temperature.

All chromatographic solvents utilizing methylene chloride (or chloroform):methanol:water mixtures refer to the use of only the organic phase after equilibration at 4^0.

Unless otherwise specified, evaporation to dryness refers to rotary evaporation _in vacuo_ at 40^0-45^0. Room temperature was 22^0.

B. SRS Assay

SRS bioactivity was assayed by a modification of the procedure reported by Chakravarty (49). Guinea pigs (300-500 g) were killed by a blow to the head and ileum strips of approximately 2.5 cm were removed at approximately 10 cm from the terminus. The tissue was rinsed free of debris and the mesenteric tissue removed. The ileum sections were placed in water-jacketed 10 ml isolated tissue baths at 35^0.

The tissue was attached at the bottom to a hook on a glass rod, and at the top to a horizontal level counterbalanced with a 500 mg weight. The lever was on a fulcrum which allowed it to swing freely. Due to the position of the fulcrum, the tissues' contractions were magnified approximately twelve-fold and recorded isotonically on a smoked drum by a wire attached to the end of the horizontal lever. The tissue bath and Tyrode's solution were continuously gassed with 5% CO_2 in O_2.

Histamine was assayed in the presence of atropine (10^{-6} M) and an antihistamine (mepyramine, 10^{-6} M) was added during SRS assays. The tissue became more responsive to histamine after successive histamine doses, and eventually consistent contractions were obtained. After sensitization in this manner, the tissue more readily produced consistent contractions to SRS. One unit of SRS was defined as that amount of SRS which caused a contraction with a peak height equal to that of 5 ng of histamine base. After determining the log-dose response curve for histamine, a similar curve of a laboratory standard of SRS was determined from which other SRS samples were quantitated. To obtain a higher degree of accuracy, SRS samples under comparison were assayed on the same ileal strip and the closed bracket type assay (50) was used, in which the standard SRS sample was assayed before and after the assay of unknown samples.

Samples of SRS were periodically assayed in the presence of 2-4 X 10^{-8} M FPL 55712, a reversible SRS inhibitor (39), which was added 30 seconds before the addition of the SRS. This was done to give added assurance that the contractions were due to SRS.

After each SRS sample, the tissue was rinsed at least twice with fresh Tyrode's solution. The interval between samples was 6-10 minutes; Tyrode's solution was also added one minute before each sample. The ileums maintained their contractility for 2-8 hours, partly depending upon the regularity of use and quantity of SRS per dose.

Only aqueous samples were bioassayed. Samples that had been evaporated to dryness were redissolved in 0.1% NH_4OH because water did not always completely dissolve the sample. Large quantities (0.5 ml) of this NH_4OH concentration alone had no effect on the ileums' response.

When various chemical agents were perfused through the cat paws, the effects of the agents themselves on the assay system were determined. In the concentrations used, these chemical agents did not interfere with the accuracy of the bioassay except epinephrine which was assayed in the presence of 10^{-5} M sotalol. This β-adrenergic blocker inhibited the effect of epinephrine on the ileum. Compound 48/80 was previously reported to have no effect on the ileum at <2 μg/ml (49).

C. Cat Paw Perfusion

Cat paws were perfused by a modification of the procedure reported by Högberg (51). Cats of either sex (1.5-5 kg) were obtained through the University animal care system. All were examined by a veterinarian, treated prophylactically for infectious rhinotracheitis and distemper, and quarantined for ten days. The cats were anesthetized with approximately 50 mg/kg of sodium pentobarbital (intraperitoneally) and subsequently killed with an intravenous injection of 1 ml of saturated KCl. Immediately after the death of the animal, the main artery of each paw was cannulated with polyethylene tubing (0.023" i.d.). The paws were cut off above the ankle joint and hung from a hook in a water-jacketed jar which was maintained at 25°C by a water circulating pump. (This temperature was previously shown to promote optimum SRS release in the cat paw (7).) The paws were perfused with a salt solution containing 154 mM NaCl, 0.9 mM $CaCl_2$, 2.7 mM KCl, and 10% (v/v) 67 mM Sörensen phosphate buffer (pH 7). This "buffer-salt" solution was maintained at 25° and was simultaneously perfused through the set of 4 paws from each cat with a 4-channel peristaltic pump. The paws were perfused for 45 minutes to remove most of the blood. Any drugs to be perfused through the paws were added to the buffer for the last 30 minutes of this 45 minute period, unless otherwise stated. A stock solution (1 mg/ml) of the releasing factor,

compound 48/80, was diluted in buffer for administration to each paw. The 48/80 was administered either by constant perfusion (0.1-10 μg/ml) in the buffer or as a single, 1 ml dose (25-250 μg). The stock solution of releasing factor A23187 was made at 10 mg/ml in dimethylsulfoxide (DMSO). This stock solution was either added to the buffer (5 μg/ml) or given as a single dose (5-50 μg/ml). As reported by others (6,7), no significant SRS activity could be detected in any samples prior to the administration of a releasing factor. All samples were collected on ice as they dripped from the paw. The volumes from individual paws were monitored to ensure consistent flow rates. The samples of perfusate were either assayed on the same day or stored at -25° until assayed, usually the next day.

When the influence of various factors on the release of SRS were studied, either one paw or the average SRS (units/ml) of two paws from the same cat were used as the control. Similarly, another paw or the average SRS (units/ml) of two paws from the same cat were used as the experimental sample. The results were calculated as

$$\frac{\text{SRS units/ml perfusate in experimental}}{\text{SRS units/ml perfusate in control}} \times 100$$

to give the data as "% of control".

D. Stability of SRS

The stability of SRS was determined under various conditions. First, the stability of SRS extracted with 80% ethanol was determined. A portion of lyophilized perfusate was dissolved in water (20 ml) at a concentration of 1500 U/ml. Ethanol (95%) was added to make the final concentration of ethanol 80%. The solution was filtered through Whatman #2 filter paper as reported previously (30), and the filtrate was evaporated to dryness. Samples dried in this manner and reassayed repeatedly showed no loss of bioactivity. The residue was redissolved in water, and two equal 1500 unit quantities removed before the residue was again evaporated to dryness. Samples were stored at -25^0. Each sample was redissolved in water at various times and assayed to determine the loss of bioactivity.

The stability of stage 5E (see section II.E.) SRS at -25^0 and -196^0 was compared. The semi-purified samples were dissolved in methanol, divided equally, and then dried by a stream of nitrogen. Three such samples were assayed and the average (97 units) was used as the amount of activity existing on day one. After 68 days, the average SRS from three samples at each temperature was determined, and the percent of bioactivity remaining was calculated from these averages.

The stability of stage 1 SRS at -25^0, 4^0, and 22^0 was

compared. Perfusate (250 units/ml) from one cat was divided equally, and several samples were stored at each temperature.

The stability of stage 5B SRS stored under nitrogen was compared with SRS stored under air at -25°. SRS was placed in four tubes (800 units each) and evaporated to dryness. Two tubes were flushed with air and two with N_2. All four were immediately stoppered, wrapped with aluminum foil, and assayed after 62 days.

The stability of Stage 1 SRS at 100° for 20 minutes and at 22° for 2.5 hours at various pH's was determined. In three experiments, perfusate was divided equally and adjusted to various pH values using HCl or NaOH. Each was assayed to determine the SRS (units/ml) and then divided into two equal samples of 4 ml each. One sample at each pH was incubated at 100° 20 minutes, while the other was at 22° for 2.5 hours. The units/ml of each sample was again determined by bioassay and the percent of the original activity calculated.

E. Purification of SRS

XAD-7 was washed after each experiment with water, acetone, methanol, and again with water (1 volume X 3) to prepare it for reuse. Ice cold perfusate (4-4.5 liters) from 2 cats were passed over an XAD-7 column (2.2 X 17 cm). The

column was successively eluted with 100 ml of water, 20 ml methanol, and 125 ml methanol. Samples were evaporated to dryness and stored at -25°.

A column containing XAD-2 (1.5 X 28 cm) was washed with water. After the sample was applied, the column was successively eluted at 4° with 200 ml each of water, 80% ethanol, and butanol.

Diethyl ether was prepared immediately before use by washing three times with 1 M sulfuric acid (saturated with ferrous sulfate) and then distilling. The SRS was acidified to pH 2.5 with 0.1 M HCl and extracted 4 times with equal volumes of ether. The organic layer was extracted in a similar manner with 10% NH_4OH and evaporated to dryness. SRS was prepared in the same manner for extraction with ethyl acetate. The acidified SRS was extracted with ethyl acetate (1 volume X 4), the organic phase evaporated to dryness and then redissolved in 0.2% NH_4OH for bioassay.

Columns packed with Sephadex LH-20 (1.5 x 28 cm) were eluted at 4° with 250 ml of one of the following solvents: methanol, 80% methanol, 80% ethanol, 80% isopropanol, and n-butanol saturated with water.

Silica gel (25 g) was washed with solvent and poured into columns (1.5 X 30 cm). Samples were dissolved in the solvent before their application to the column. Elution was

done at 4^0 with either chloroform:methanol (1:1) or chloroform:methanol:water (65:35:10).

Cellulose columns (50 g) were prepared similarly, except that the solvents used were either ethanol:water (1:1) or chloroform:methanol:water (5:4:1, 7:2:1, 8:1:1, or 65:35:10).

All HPLC was conducted at room temperature and all columns were equilibrated with at least 20 column volumes of solvent before sample injection. Stainless steel columns were filled by the "tap-fill" method with either Bondapak C_{18}/Porasil B (fully porous) or Bondapak C_{18}/Corasil (pellicular). After the column was equilibrated, SRS was injected onto the column and eluted at 2 ml/min with either 50% methanol in water or a gradient of increasing methanol concentration (30% to 60%). UV absorbing impurities were monitored at 254 nm; and SRS was monitored by bioactivity. Elution from μ-silica gel was with methylene chloride: methanol:water (65:35:10 or 65:30:10). A gradient of increasing methanol concentration (45% to 60%) was used to elute the μC_{18} column. A stepwise gradient of 0.1% NaCl and water was used with the μCN column. The flow rate from these analytical columns was 1 ml/min. The μC_{18} preparative HPLC column was eluted with either a gradient of increasing methanol (40% to 95%) or with 50% methanol at a flow of 3 ml/min. The μC_{18} columns were protected from insoluble

materials by the use of a precolumn packed with C_{18} material. In addition, disposable silica gel or C_{18} columns were used to clean up crude samples for reverse phase HPLC. A stepwise gradient of water (1 ml), 50% methanol (5 ml), and methanol (5 ml) was used for the C_{18} column. A stepwise gradient of methylene chloride:methanol:water was used for the silica gel column as follows: 65:20:10 (3 ml), 65:30:10 (4 ml), and 65:40:10 (5 ml).

Unless otherwise specified, the SRS used for routine experiments was designated as follows:

Stage	Purification Sequence
1	Cat paw perfusate
2	XAD-7
3	Ethyl acetate extraction
4	Disposable column
A	C_{18} or
B	Silica gel
5	HPLC
A	µ-Silica gel or
B	µCN or
C	μC_{18} or
D	Preparative μC_{18} or
E	C_{18} (37-50 µ)

F. Derivatization

The SRS used in these experiments was partially purified through a stage 5 column.

The acetylation of SRS was carried out by the addition of pyridine and acetic anhydride (0.5 ml of each) to 1500 units of SRS. The mixture was incubated for 30 minutes at 22^0, and the excess reagent and solvent were then removed *in vacuo*.

To prepare the trimethylsilyl derivative of SRS, 200-300 µl of either TRI-SIL, BSTFA, or BSTFA plus pyridine (1:1) was added to a vessel containing SRS. The mixture was allowed to stand for 15 minutes at 22^0, and the solvent and unreacted silylating agent were then evaporated *in vacuo*. The existence of any unreacted SRS was determined by the addition of 0.1% NH_4OH to a portion of the silyl treated SRS, which was then assayed immediately.

Methylation of SRS was attempted by diazomethane (CH_2N_2) and by dimethyl sulfate (DMS). The method of generating CH_2N_2 was that reported by Schlenk for the methylation of fatty acids (52). Three vessels of 2 ml capacity were connected in sequence. Vessel A contained 2 ml of diethyl ether which was passed over a 1 X 9 cm column of neutral alumina immediately before use, vessel B contained 0.25 ml of 1 M KOH in 95% ethanol, and vessel C contained the SRS dissolved in 0.5 ml of 10% methanol in

ethyl acetate. Nitrogen was bubbled through vessel A which, after the addition of MNTSA to vessel B, carried the CH_2N_2 into the reaction vessel. After bubbling with nitrogen for 15 minutes, vessel C was removed, covered, and allowed to stand for 15 minutes at 22°. The excess CH_2N_2 and solvent were evaporated with a stream of nitrogen under hood. The SRS used for methylation was prepared either by acidification to pH 2.5 with 0.1 M HCL and evaporation to dryness or by acidification, extraction into ethyl acetate (1 volume X 3), and evaporation to dryness.

Methylation by DMS (distilled under vacuum) was that reported by Stodola for the methylation of carboxylic acids (53). The SRS was dissolved in 1 ml of methanol and, depending upon the amount of SRS being methylated, 1-4 µl of DMS and 19-76 µl of THPA (10 mg/ml) were added. The solution was placed on a steam bath for 15 minutes and then evaporated to dryness.

To methylate SRS (stage 5D) with ^{14}C-labelled CH_2N_2, the sample was dissolved in 1 ml of water, acidified with 0.1 M HCl to pH 2.5, extracted 3 times with 1 ml of ethyl acetate, evaporated to dryness, and redissolved in 50 µl of methanol and 450 µl of ethyl acetate. The methylation procedure was the same as that described above, except that vessel B contained 13 µCi (0.93 µmoles) of ^{14}C-labelled MNTSA. After methylation, the sample was evaporated with a

stream of nitrogen and redissolved in ethyl acetate. Two percent of the sample was added to 10 ml of Bray's solution to determine the amount of radioactivity. The remainder was spotted on a silica gel thin layer plate and developed in methylene chloride:methanol:water (65:15:10). The plate was scanned for radioactivity and then sections were scraped, extracted with methanol (2 X 4 ml), evaporated to dryness, and hydrolyzed with aqueous KOH as described below.

After methylation by DMS or CH_2N_2, the percent methylation was determined by adding 0.1% NH_4OH to a portion of the methylated sample and assaying for activity. Complete loss of bioactivity was considered to be 100% methylation.

G. Hydrolysis of Methylated SRS

After methylation, the solvent was evaporated and various concentrations of KOH were added. When aqueous base was used, the solution was neutralized with HCl, and then slightly alkalized in the NH_4OH. Neutralization of the KOH was necessary to avoid ileum contraction due to the base alone. When alcoholic KOH was used, the base was neutralized before evaporation. The percent regeneration of SRS bioactivity was calculated as the percent bioactivity after base hydrolysis, minus the percent bioactivity remaining after methylation. In this way, unreacted SRS was taken into account.

The hydrolysis of sulfate methyl esters by pyridine was done at 22° for 30 minutes by the use of 0.4 ml of pyridine. The pyridine was removed under vacuum. The products were spotted, along with standards, on TLC and developed in methylene chloride:methanol:water (65:15:10). Visualization of the sulfate compounds was attained by the use of ceric sulfate spray, UV light, or by scanning the plate for radioactivity. The methylated SRS was treated with pyridine in the same manner and was spotted on TLC next to a control sample which had been incubated for 30 minutes with 0.4 ml of water instead of pyridine. The bioactivity from the TLC was determined by scraping the plate at 0.1 Rf intervals, extracting each fraction twice with 4 ml of methanol, and dividing each sample in half. The methanol extracts were evaporated, and one of each pair of fractions was dissolved in 0.1% NH_4OH. The other was hydrolyzed with 0.1 M aqueous KOH for 30 minutes at 22° and then neutralized before assay as described above.

H. Dioxane and THF Solvolysis

Samples that were refluxed with either tetrahydrofuran (THF) or dioxane were prepared either by evaporating to dryness and adding 1 ml of the cyclic ether or by adding 1 ml of the cyclic ether to a small volume (10-30 µl) of the aqueous sample. The samples were refluxed in THF or dioxane

under N_2 for 15 minutes with constant stirring. BHT (0.025%) was added to the dioxane where indicated. The THF already contained 0.025% BHT when purchased. The THF without BHT was prepared by refluxing with Na and benzophenone until a deep blue color persisted and then distilling it immediately before use. The THF and dioxane containing BHT were passed over a 1 X 9 cm column of neutral alumina immediately before use to remove peroxides and water. The BHT was not retarded by the column. The presence of peroxides was tested by adding an equal volume of 10% (w/v) KI to the solvent. A yellow color indicated the presence of peroxides.

Following reflux, the solvent was evaporated and the percent solvolysis determined. The extent of solvolysis of the sulfate esters of dodecyl alcohol, cholesterol, and 12-hydroxy stearate were estimated by TLC. The plates were developed along with standards in methylene chloride: methanol:water (65:25:10) and sprayed with 3 N ceric sulfate in concentrated sulfuric acid. The solvolysis of p-NPS and p-NPP was estimated by TLC (65:15:10) along with standards and visualized by UV. The solvolysis of SRS was followed biologically. The following assay for glucose was used to determine the solvolysis of G-6-S (54).

$$\text{glucose} \xrightarrow[\text{ATP}]{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{glucose-6-phosphate} \xrightarrow[\text{NADP}^+]{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+ \ (A_{340})$$

A standard curve of A_{340} (0.09-0.95) vs. µmoles glucose (0.05-0.5 µmoles) was used to determine the percent of G-6-S solvolyzed. Each 3 ml volume assay contained 30 µmoles $MgCl_2$, 1.6 µmoles ATP, 2 µmoles $NADP^+$, 0.6 µmoles citric acid, 3 units G-6-PDH, 3.2 units hexokinase, and 360 µmoles tris/HCl buffer (pH 7.5). The substrate was either glucose (0.05-0.5 µmoles) or G-6-S (0.5-5.0 µmoles). After reflux, the THF or dioxane was evaporated and the above mentioned assay components were added directly to the vessel containing the refluxed products. Refluxed G-6-S (1.6 µmoles) was also developed, along with standards, on cellulose TLC in 1-butanol:ethanol:water:NH_4OH (40:12:20:1), sprayed with acidic anisidine (3% in butanol), and heated to 115^0 for 10 minutes (55).

I. Preparation of Sulfate Esters

The sulfate esters of cholesterol and 12-hydroxy

stearic acid were prepared by using pyridine-sulfur trioxide according to the method of Sobel (56,57). Pyridine-sulfur trioxide (250 mg) was added to 1 g of cholesterol in 10 ml of methylene chloride and heated for 20 minutes at 67°. Forty ml of petroleum ether was added and the mixture cooled to 0° overnight. After filtration, 290 mg of white material was dissolved in 4 ml of cold (0°) chloroform and then filtered. Three volumes of petroleum ether was added to the filtrate and caused the pyridinium salt of cholesteryl sulfate to precipitate. After filtration, cholesterol could not be detected by TLC when developed in chloroform: methanol:water (65:25:10). Sodium and ammonium salts of cholesteryl sulfate were prepared from the pyridinium salt by dissolving in 10% aqueous NaCl or NH_4Cl, filtering, and washing with water (56). Cholesteryl [26-^{14}C] sulfate was prepared in a similar manner from [26-^{14}C] cholesterol. The final product yielded a specific activity of 0.23 μCi per μmole of cholesteryl [26-^{14}C] pyridinium sulfate.

J. Enzymatic Studies

Except where indicated, incubations of SRS with the enzymes were in 0.1 M buffer at the pH and temperature which defines the unit of enzyme activity. Units of enzyme activity are as defined by Sigma. The units of enzyme activity expressed for alkaline and acid phosphatases,

spleen and venom phosphodiesterases were determined experimentally (see below). The units for other enzymes were assumed to be those indicated on their respective labels. Assays for p-nitrophenol were made at 400 nm and for p-nitrocatechol at 515 nm (molar extinction of 1.9×10^4 and 1.24×10^4, respectively). Incubations were in 1 ml total volume and were terminated by adding 5 ml of 0.1 M NaOH.

Arylsulfatase, type V from Patella vulgata, was assayed at pH 5 (0.1 M acetate buffer) at 35^0 using 2.5 µmoles of p-NCS or p-NPS. To check for phosphatase activity in the type V arylsulfatase, 2 units of sulfatase was incubated with 2.5 µmoles of p-NPP for 30 minutes at 35^0.

Alkaline phosphatase, type I ("crude") and type VII ("purified"), was assayed at pH 10.4 (0.1 M glycine/NaOH) at 35^0 using 2.5 µmoles p-NPP. To check for sulfatase activity in the type I phosphatase sample, 10 units of this preparation was incubated for 30 minutes with 2.5 µmoles of p-NPS.

Acid phosphatase, type I from wheat germ ("crude") and type III from potato ("purified"), was assayed by incubation with 2.5 µmoles of p-NPP at pH 5 (0.1 M acetate buffer) at 35^0. Sulfatase activity was determined by incubating 1 unit of type I acid phosphatase with 2.5 µmoles of p-NPS for 30 minutes at 35^0.

Alkaline disc gel electrophoresis was performed at pH 8.9 (0.188 M Tris/glycine buffer) and acid disc gel

electrophoresis at pH 3.6 (0.188 M glycine/acetic acid buffer) in 7.5% polyacrylimide gels with two units of arylsulfatase (175 µg) per gel. Each gel was cut into 5 mm sections, crushed, and extracted with 1 ml of pH 5 acetate buffer (0.1 M) at 4°C for 14 hours. The extract from each section of one gel was divided in half and incubated for 3 hours at 35° with either p-NPP or p-NPS to determine the phosphatase and sulfatase activities, respectively. Incubations were terminated by adding 3 ml of 0.1 M NaOH. The extract from sections of another gel were incubated at 35° for 100 minutes with 185 units of stage 4B SRS.

Venom phosphodiesterase, type II from Crotalus adamanteus venom, was assayed by incubation with 2.5 µmoles of bis-p-nitrophenyl phosphate (bis-p-NPP) at pH 8.9 (Tris/HCl) at 35°. Contaminant sulfatase activity was determined by incubating 0.5 units of the enzyme for 120 minutes with p-NPS or p-nitrocatechol sulfate (p-NCS) at both pH 5 and pH 8.9.

Phosphodiesterase, type I from bovine spleen, was assayed by incubating with 0.5 µmoles of thymidine 3'-monophospho-p-nitrophenyl ester (TMP-3'-NPE) at pH 6.5 (Tris/maleate) at 35°. One unit was defined as the amount of enzyme that hydrolyzed 1 µmole of TMP-3'-NPE per minute at 35°. This corresponded to approximately 16 units using RNA-Core as substrate. Sulfatase activity was determined by

incubating 0.06 units of spleen phosphodiesterase for 130 minutes with p-NPS at both pH 6.5 and pH 5, or by incubating p-NCS at pH 6.5 at 35°.

Glucose oxidase activity was determined by measuring the oxidation of o-dianisidine at 500 nm (58). Fifty mg of glucose in 0.5 ml water was placed in a 1 ml sealed hypovial. In each of two sealed hypovials, 33 µg o-dianisidine, 2.5 units peroxidase, 0.01 units glucose oxidase, and 0.1 M acetate buffer (pH 5) were placed to make a total volume of 0.4 ml. One hypovial containing the enzymes and the one containing glucose were flushed with argon for 30 minutes. The other hypovial containing enzymes was flushed with air for 30 minutes. One hundred µl from the vial containing glucose was added to each of the other two vials. The hypovial containing the enzyme that had been bubbled with air turned dark brown almost immediately due to oxidation of the o-dianisidine. After 10 minutes and 30 minutes of incubation the amount of oxidation of o-dianisidine was determined in each case by adding a portion of the solution to a cuvette containing 10 mM KCN. The absorption was determined at 500 nm. It was previously determined that 10 mM KCN inhibited further oxidation of o-dianisidine.

The presence of oxidase activity in alkaline phosphatase was determined by using SRS substrate. The same general procedure was followed as described for determining

glucose oxidase activity, except that the substrate hypovial contained 1000 units of partially purified SRS. The two enzyme hypovials each contained 27 μg of crude alkaline phosphatase in pH 7 Tris/HCl buffer. One vial was flushed for 30 minutes with argon and the other with air. Assays for SRS activity were made directly on the ileum after the incubation period.

The effects of various factors on the SRS inactivating activity of arylsulfatase, venom phosphodiesterase, spleen phosphodiesterase, crude acid phosphatase, crude alkaline phosphatase, and 3',5'-cyclic phosphodiesterase were compared. Approximately 300 units of stage 4B SRS was incubated with each of the enzymes in a total volume of 0.5 ml. Unless otherwise specified, all incubations were at pH 7 (Tris/HCl) and 35^0. The final concentration of $MgCl_2$, EDTA, Na_2SO_4, and K_2HPO_4 was 10 mM. First, the rate of SRS inactivation by each enzyme was determined. The combination of enzyme and time that produced approximately 50% SRS inactivation was then used in all other incubations. Each incubation was run in duplicate. For convenience, one incubation from each experiment was assayed directly on the ileum, while the other incubation was stopped with 80% ethanol (final concentration), centrifuged, evaporated to dryness, and then bioassayed. The percent SRS inactivation was calculated comparing the SRS activity (units/ml) present

immediately after addition of the enzyme (zero time) and the SRS activity (units/ml) after incubation. This value (% inactivation) was compared with the control (pH 7, 35^0) to obtain the relative percent inactivation:

$$\frac{\text{\% inactivation of experimental}}{\text{\% inactivation of control}} \times 100$$

A Sephadex G-200 column (1.6 X 28 cm) was equilibrated with 0.05 M Tris/HCl, pH 7 buffer. The column was calibrated using blue dextran, crude alkaline phosphatase (3.6 mg), and ovalbumin (4 mg). Alkaline phosphatase, molecular weight of 115,000 (59,60), was assayed using p-NPP as described previously. Ovalbumin, molecular weight 45,000 (59, 61), was determined by its absorption at 280 nm. The elution of the SRS inactivating enzyme was determined by incubating each fraction off the G-200 column with 600 units of SRS for 30 minutes at 35^0.

III. RESULTS

A. SRS Assay

SRS^{cat} displayed the characteristic contraction on the guinea pig ileum as reported by others (6,49). After a latency period of 5-20 seconds, the contraction progressed gradually to reach a peak in 1-3 minutes. The tissue did not relax quickly after a single washing. This contraction was slow in comparison with, for example, histamine and prostaglandins E_1 and E_2, which had a latency period of about 5 seconds or less and reach peak contraction in less than 30 seconds. These compounds allow the tissue to relax quickly after only one washing. All samples of SRS assayed in the presence of 2-4 X 10^{-8} M FPL 55712 were inhibited 25-75%.

Cat paw perfusate was reported by Änggard to contain nonpolar compounds which are active on the guinea pig ileum (62). He identified the major component as PGE_2. An approximation from Anggard's results shows that only 0.2 picograms of PGE_2 were produced per SRS unit. Since on the guinea pig ileum, 5 ng of PGE_2 produces a contraction equal to 1 unit of SRS, the amount of PGE_2 produced by cat paws (<10 ng/paw) was too low to interfere with the bioassay of SRS in the crude perfusate.

B. Cat Paw Perfusion

Initial experiments were conducted to determine the optimum flow rate, perfusion time, and amount of chemical releasing factors for SRS production. The variability of SRS release among paws from the same cat was also examined.

Table 1 shows the results of experiments in which the perfusate from each paw was collected separately during a two hour period, after the introduction of compound 48/80. Flow rates were held constant in each experiment, but they varied between experiments from 1.3 ml/min to 3.3 ml/min. Similar results were reported by Strandberg (7) with 1 µg/ml of 48/80 for 80 minutes. On the average, paws from the same cat produced approximately the same amount of SRS, although occasionally, erratic SRS production by individual paws did occur.

Since the literature reported the use of both single dose (25 µg) and constant perfusion (1 µg/ml) of 48/80 to release SRS (4,5,30,32), both methods of 48/80 administration were compared. Table 2 showed that the same amount of SRS was produced. A ten-fold increase in 48/80 did not alter the amount of SRS released, but at 0.1 µg/ml the amount of SRS released was considerably decreased.

Others have successfully used the ionophore A23187 to increase SRS release in other tissues (11,17). As Table 3 shows, however, no increase was obtained at three concentrations

TABLE 1. SRS in the perfusate from cat paws.

	units/ml			
Cat	Right fore	Right hind	Left fore	Left hind
1	100	115	100	105
2	38	68	30	58
3	136	164	110	120
4	88	44	84	88
5	200	130	88	192
6	120	120	190	125
7	240	250	240	240

Each paw was perfused with 25 µg of 48/80 and then with buffer-salt solution for 2 hours.

TABLE 2. SRS^{cat} released by 48/80.

No. of expts.	48/80 in control paw	48/80 in experimental paw	Mean % of control (SEM)
3	25 µg	250 µg	101 (8)
4	25 µg	1 µg/ml	103 (18)
4	1 µg/ml	0.1 µg/ml	65 (12)
2	1 µg/ml	10 µg/ml	100 (3)

Samples were collected for 2 hours. Results were calculated in terms of:

$$\frac{\text{units/ml perfusate in experimental paw}}{\text{units/ml perfusate in control paw}} \times 100$$

SEM = standard error of the mean.

TABLE 3. Release of SRS^{cat} by ionophore A23187.

No. of expts.	A23187	Mean % of control (SEM)
3	25 µg	47 (7)
4	250 µg	95 (7)
2	500 µg	92 (15)
2	5 µg/ml	30 (8)

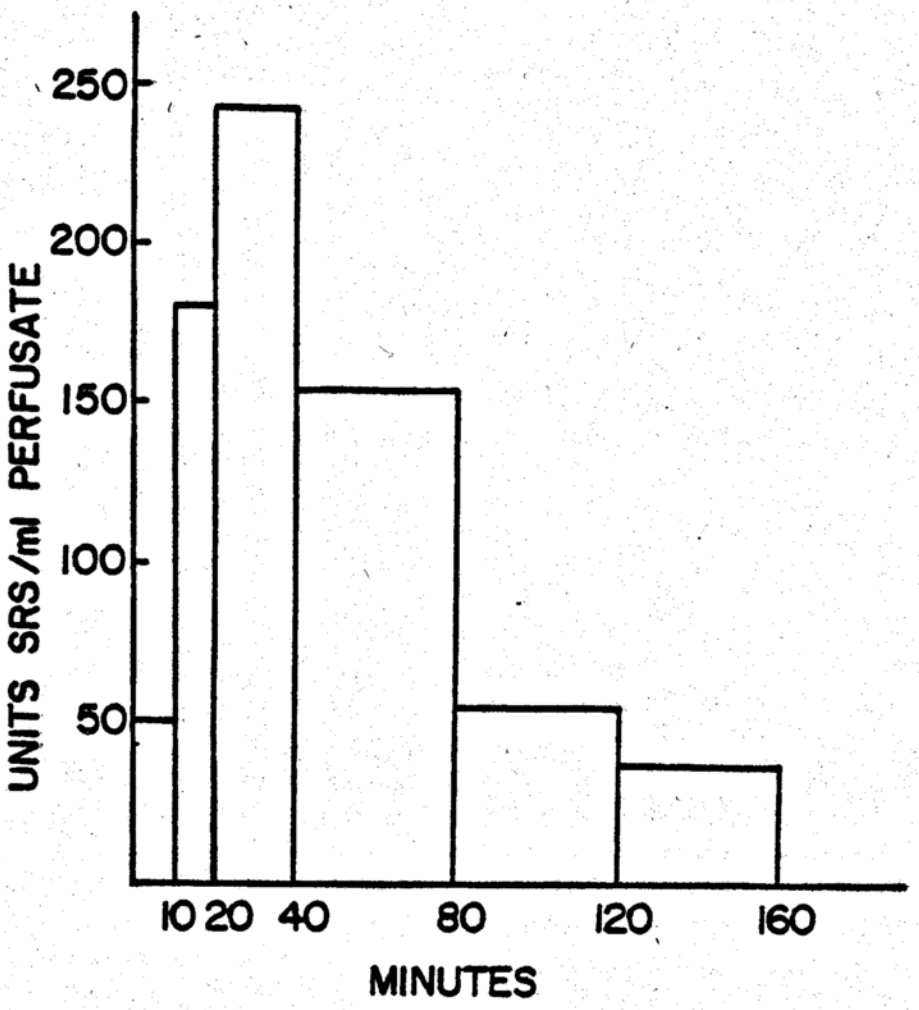
Control paws were perfused with a single dose (25 µg) of 48/80. Samples were collected for 2 hours.

of A23187 when compared with the SRS released by a 25 µg single dose of 48/80. Using concentrations of A23187 greater than 10 µg/ml, Jakschik (12) has reported a decrease in SRS release in rat basophilic leukemia cells. He suggested that this decrease may have resulted from increased cytotoxicity.

Figures 4 and 5 show the concentration of SRS in the perfusate at various time intervals with the constant 48/80 perfusion of 1 µg/ml or a single dose of 25 µg. The patterns of SRS released were virtually identical. Perfusion of a second dose of 48/80 (25 µg) at 80 minutes did not result in an increase in the amount of SRS. These observations are in agreement with those reported by Strandberg (7).

The total amount of SRS released at various perfusion rates is shown in Table 4. Within a given experiment, greater quantities of SRS were always obtained at the faster perfusion rate.

To obtain the maximum amount of SRS over a reasonable time period, cats were perfused for 3 hours at 2.5-3.5 ml/min. 48/80 was routinely used as a single dose (25 µg/paw) rather than a constant perfusion to avoid the possibility of concentrating large amounts of 48/80 during purification causing subsequent interference in the bioassay. Under these conditions, the average units of SRS obtained per cat from 40 experiments was 123,900 (range 22,400-510,980).


250
200
150
100
50
UNITS SRS/ml PERFUSATE
10 20 40 80 120 160
MINUTES

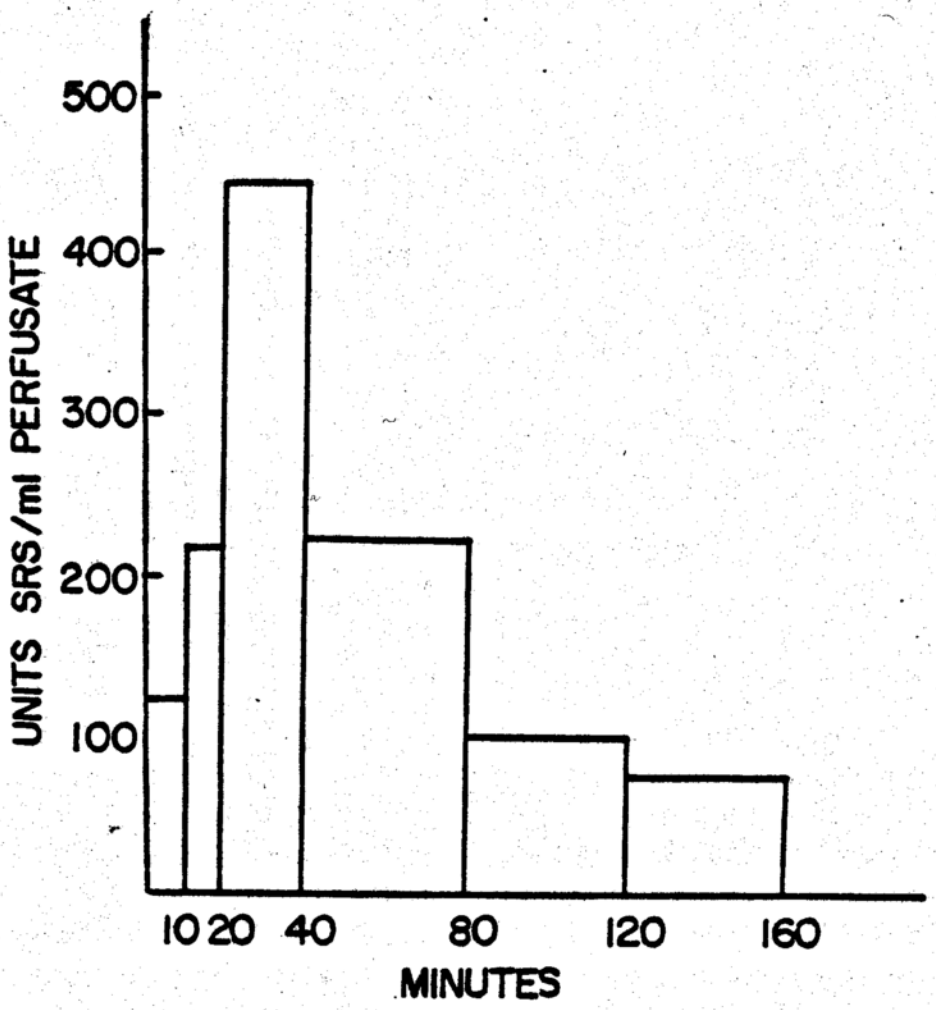

500
400
300
200
100
UNITS SRS/ml PERFUSATE
10 20 40 80 120 160
MINUTES

TABLE 4. SRS^{cat} released at various perfusion rates.

	Flow rate (ml/min)		Total units	
48/80	Slow	Fast	Slow	Fast
1 µg/ml	0.5	1.0	28,620	37,780
1 µg/ml	1.6	2.7	49,220	61,840
25 µg	0.8	2.4	10,020	16,600
25 µg	1.1	2.7	10,530	23,780
25 µg	2.1	3.4	74,840	84,730
25 µg	2.4	3.1	58,920	86,580

Samples were collected for 160 minutes.

As the cost of cats was considerable, and since it was possible to obtain rabbits and dogs used by other researchers, the paws from these other species were tested for SRS activity. Two dogs were used: four paws from one dog and two paws (fore and hind) from the second dog. After perfusion for 150 minutes and 90 minutes, respectively, with 48/80 (1 µg/ml), no SRS activity was detected in the perfusate from either dog. The hind paws of a rabbit were treated in the same manner, but SRS was not detected.

Modulation of the anaphylactic release of SRS-A^{hu} and SRS-A^{rat} by various agents was reported (4,10-12,40,41), but the modulation of the 48/80 release of SRS^{cat} has not been reported. In addition to the academic interest in determining biochemical parameters affecting SRS release, the ability to increase SRS release by chemical means would be of obvious importance from a preparative standpoint.

2-Deoxy-D-glucose (2-DG), a competitive inhibitor of phosphoglucoisomerase (63), was used to determine whether the SRS release process required energy. A dose-response relationship between the inhibition of SRS and the concentration of 2-DG, using both perfusion methods was obtained (Table 5). As with SRS-A^{hu}, these results indicate the necessity of an intact glycolytic pathway for maximum release of SRS^{cat}.

Since catecholamines and methylxanthines were known to

TABLE 5. Effect of 2-deoxy-D-glucose on the release of SRS^{cat}.

No. of expts.	48/80	2-DG (mM)	Mean % of control (SEM)
2	1 µg/ml	0.1	99 (4)
2	1 µg/ml	1	87 (0)
3	1 µg/ml	10	57 (13)
3	25 µg	10	65 (9)

Paws were perfused for 15 minutes with 2-DG before 48/80 administration. Paws were continually perfused with 2-DG during the collection period. Control paws were perfused with buffer-salt solution. Samples were collected for 2 hours.

affect cAMP levels (64-66), they were used to examine the modulation of SRS-A release in other tissues (4,10,40-42,46, 67). Although the cat paws were not responsive to 10^{-7} M epinephrine or to 10^{-4} M aminophylline (Table 6), a ten-fold increase in concentration, however, did result in a marked decrease in SRS release. In addition the combination of these two drugs at the threshold concentrations produced a synergistic inhibition of SRS release, which was reversed by the addition of a β-adrenergic blocker (propranolol). These results suggested that the SRS release by 48/80 was affected by the cAMP system of the cat paw. Phenylephrine, a potent alpha-agonist, inhibited SRS release (Table 7) but this inhibition was reversed by the addition of propranolol (10^{-5} M). Although phenylephrine is generally considered to be a selective alpha-agonist in most tissues, these results tend to suggest that the inhibition of SRS release by phenylephrine was the result of β-receptor stimulation, a result similar to that observed with SRS-A release from nasal polyps (10).

Carbachol, a cholinergic agent resistant to acetyl cholinesterase, has been shown to increase SRS-A release dramatically (10,46), presumably by increasing cGMP levels. Various concentrations of carbachol were used in the cat paws in another attempt to increase SRS release. Table 8 indicates, however, that at all concentrations of carbachol

TABLE 6. Effect of epinephrine, aminophylline, and propranolol on the release of SRS^{cat}.

No. of expts.	Epineph. (M)	Aminoph. (M)	Propr. (M)	Mean % of control (SEM) 30 min	120 min
2	10^{-7}			88 (8)	101 (15)
2	10^{-6}			30 (8)	60 (2)
2		10^{-4}			118 (6)
4		10^{-3}			66 (16)
6	10^{-7}	10^{-4}		68 (9)	68 (6)
7	10^{-6}	10^{-4}		30 (5)	42 (7)
2			10^{-5}	86 (10)	101 (1)
2	10^{-7}	10^{-4}	10^{-5}	94 (1)	96 (4)

Paws were perfused for 30 minutes with the drug(s) before the administration of 48/80 (25 µg). Samples were collected at 30 minutes and 120 minutes during which time the drugs were continually perfused. Control paws were perfused with buffer-salt solution and 48/80 (25 µg).

TABLE 7. Effect of phenylephrine and propranolol on the release of SRS^{cat}.

No. of expts.	Phenyleph. (M)	Propr. (M)	Mean % of control (SEM) 30 min	120 min
3	10^{-5}		44 (10)	51 (6)
4	10^{-6}		53 (14)	81 (17)
3	10^{-7}		91 (33)	75 (14)
2		10^{-5}	86 (10)	101 (1)
3	10^{-5}	10^{-5}	82 (8)	101 (9)

Paws were perfused for 30 minutes with the drug(s) before the administration of 48/80 (25 µg). Samples were collected at 30 minutes and 120 minutes during which time the drugs were continually perfused. Control paws were perfused with buffer-salt solution and 48/80 (25 µg).

TABLE 8. Effect of carbachol, epinephrine, and aminophylline on the release of SRS^{cat}.

No. of expts.	Carbachol (M)	Epineph. (M)	Aminoph. (M)	Mean % of control (SEM) 30 min	120 min
2	10^{-10}			100 (8)	89 (12)
1	10^{-8}			109	104
2	10^{-6}			100 (4)	136 (24)
1	10^{-5}			85	86
6		10^{-7}	10^{-4}	68 (9)	68 (6)
3	10^{-5}	10^{-7}	10^{-4}	92 (9)	94 (14)

Paws were perfused for 30 minutes with the drug(s) before the administration of 48/80 (25 µg). Samples were collected at 30 minutes and 120 minutes during which time the paws were continually perfused with the drugs.

tested, the amount of SRS released was not significantly different from the control paws. Carbachol did, however, reverse the inhibition of SRS release caused by aminophylline and epinephrine (Table 8), an indication of cAMP-cGMP involvement. It was conceivable that 25 μg of 48/80 released the maximum amount of SRS possible, whereas challenge of cells with antigen might have stimulated the cells sub-maximally and allowed modulation of the response with cholinergic and α-adrenergic agents. This assumption was strengthened by the data discussed earlier which showed neither larger doses of 48/80 nor A23187 were able to generate additional SRS. The relative levels of cAMP and cGMP appear to be important in the modulation of SRS^{cat} release induced by 48/80. SRS release was inhibited by both epinephrine and aminophylline; when a combination of these two compounds were used, a synergistic effect was noted; propranolol and carbachol reversed this synergism.

Jakschik reported a significant enhancement of SRS release from rat basophilic leukemic cells by the use of 10^{-7} M arachidonic acid (68). Eicosa-5,8,11,14-tetraynoic acid (TYA), a competitive and irreversible inhibitor of arachidonic acid oxidation by cyclooxygenase and lipoxygenase (69,70), inhibited SRS production (68,71), but conflicting results were obtained by others (72). Aspirin and indomethacin are also inhibitors of cyclooxygenase, but not

of lipoxygenase (69,70). These antiinflammatory agents were reported to have no effect in some systems on SRS release (68,73), whereas other systems showed either a decrease (71) or increase (3,36,74).

When cat paws were perfused for 30 minutes before 48/80 introduction and for 60 minutes after 48/80 with arachidonic acid (10^{-5}-10^{-7} M), no significant increase of SRS level was noted (Table 9). Similar results were obtained with A23187. Indomethacin at 5 X 10^{-6} and 5 X 10^{-5} M did not appear to affect SRS production (Table 10). TYA, however, consistently inhibited SRS release at 10^{-5} M.

C. Stability of SRS

The stability of SRS^{cat} was examined under various conditions. In duplicate experiments solutions of stage 1 SRS were stable at -25° for more than a week; samples at 5° and 22°, however, lost activity much faster (Table 11). The relative stability of stage 1 SRS at five different pH's at 100° for 20 minutes and at 22° for 2.5 hours is shown in Figures 6 and 7, respectively. Greater stability at alkaline pH was observed under both conditions. There was a slight decrease in stability at pH 7 at 100°, and a decrease in stability at pH greater than 9 at 22°. This differs with the results reported for $SRS\text{-}A^{gp}$ (20), where the activity was more stable at pH lower than 7.5 at room temperature.

TABLE 9. Effect of arachidonic acid on SRS^{cat} release.

No. of expts.	Releasing factor		Arach. acid (M)	Mean % of control (SEM)
	48/80	A23187		
3	50 µg		10^{-5}	111 (13)
4	50 µg		10^{-6}	106 (6)
2	50 µg		10^{-7}	101 (12)
2		250 µg	10^{-6}	118 (31)
2		5 µg/ml	10^{-6}	80 (7)

Paws were perfused with arachidonic acid for 30 minutes before and for 60 minutes after the administration of the releasing factor.

TABLE 10. Effect of indomethacin and TYA on the release of SRS^{cat}.

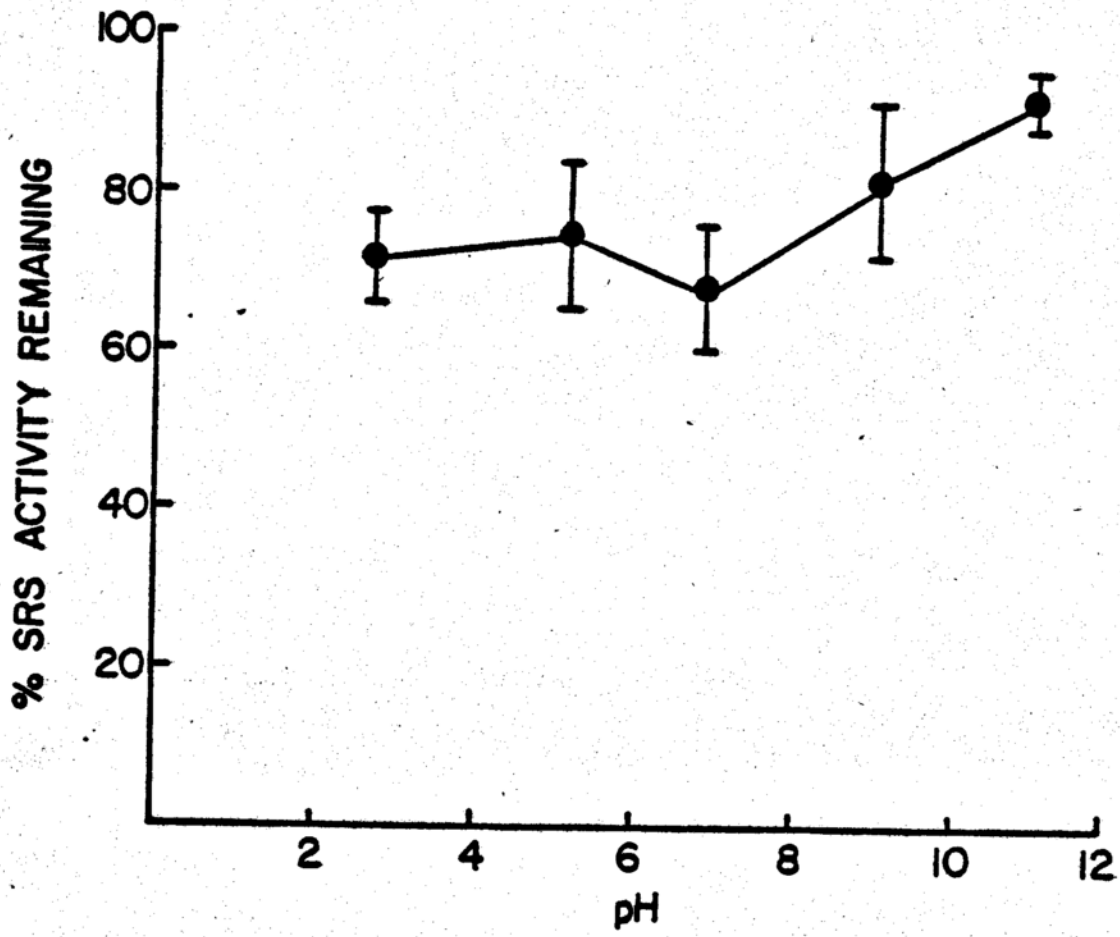
No. of expts.	Indomethacin (M)	TYA (M)	Mean % of control (SEM)
1	5×10^{-6}		108
1	5×10^{-5}		106
2		10^{-6}	114 (22)
4		10^{-5}	43 (8)

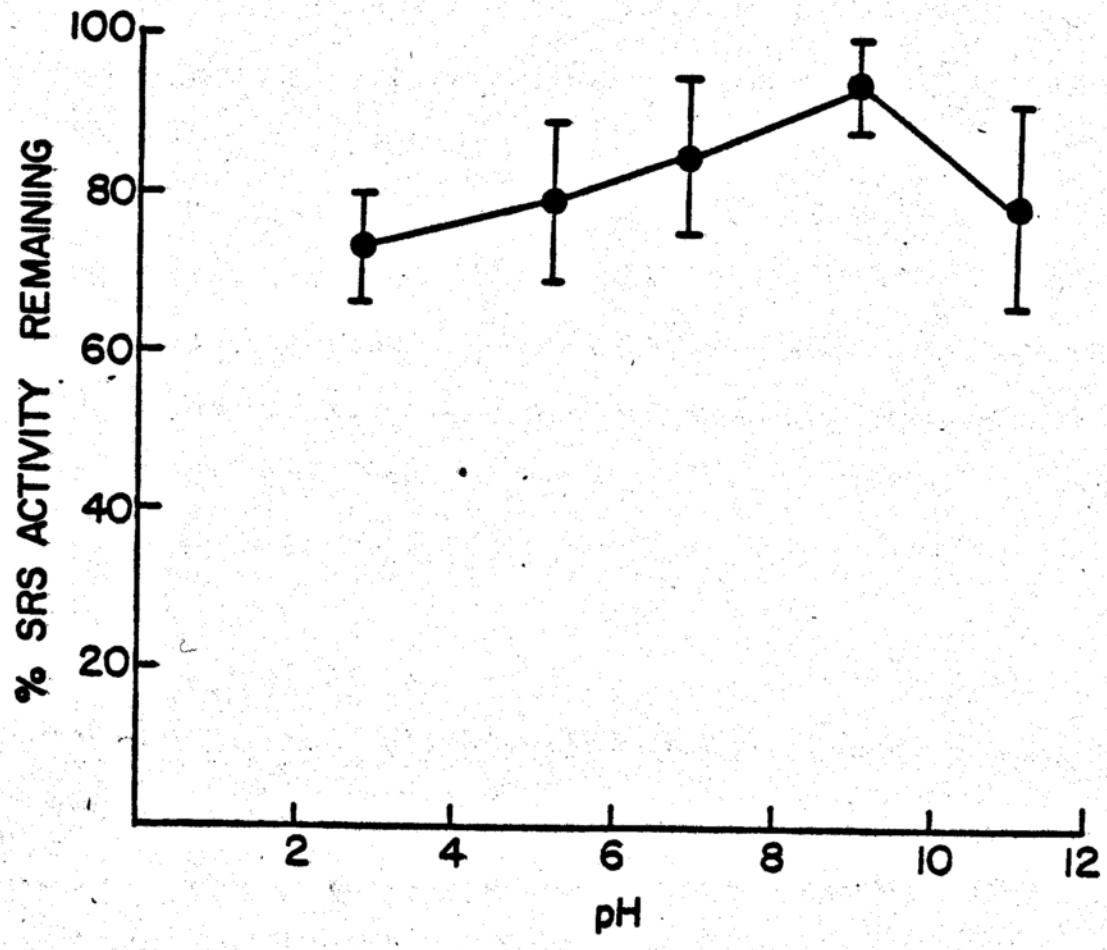
Paws were perfused with the drug for 30 minutes before and for 60 minutes after the administration of 48/80. Control paws were perfused with buffer-salt solution and 48/80 (50 µg).

TABLE 11. Stability of SRS^{cat} perfusate.

	% Activity remaining		
Days	22^0	5^0	-25^0
1	100	100	100
2	5		
10		49	91
21		30	67

All samples were stored as the perfusate solution (250 units/ml).


100
80
60
40
20
% SRS ACTIVITY REMAINING
2
4
6
8
10
12
pH


100
80
60
40
20
% SRS ACTIVITY REMAINING
2
4
6
8
10
12
pH

However, both SRS-A^{gp} and SRS-A^{rat} were more stable in base than in acid at 100^0 (20).

Lyophilized SRS^{cat} perfusate, purified by extraction with 80% ethanol, lost 50% of its activity after 55 days at -25^0. At this temperature stage 5E SRS also lost 47% of its activity (mean of 3 samples) after 68 days but when stored at -196^0 lost only 13% of its activity. Even when stored under nitrogen and protected from light, duplicate samples of SRS (stage 5B) still lost 77% of their activity (68 days at -25^0) as compared to a 75% loss in samples stored under air. A similar experiment also showed no significant difference (<2%) in retention of activity in samples stored under nitrogen after 30 days at -25^0.

D. Purification of SRS

There has been only limited progress towards the purification of SRS on a preparative scale (14,31,48,68) and only one report of SRS from cat paws (30). The following steps were employed for the purification of SRS from cat paws: (1) concentrating the SRS from its large volume of perfusate (>2 liters per cat); (2) desalting to remove the large amount of buffer salts (>10 g/l) and to remove easily precipitable proteins; (3) removing most of the dark colored material which comprises most of the sample weight; and (4) purifying the remainder to the highest possible extent,

ideally to an unweighable quantity.

Concentrating the SRS was previously accomplished by rotary evaporation or by lyophilization. These procedures are cumbersome and time consuming when large volumes are used, and they afford no increase in purity. As an alternative to this step, XAD-2, a polymeric polystyrene, was used as a means of concentrating and desalting SRS-A^{hu} and SRS-A^{rat} (14,31).

Although SRS^{cat} activity was efficiently adsorbed onto XAD-2 columns, the recovery of SRS^{cat} activity from the columns by elution with a variety of solvents was poor. On the other hand, the less hydrophobic resin, XAD-7 (polymeric acrylic ester) was found to be more suitable for our purpose. No SRS^{cat} activity was detected in the effluent after passing 4 liters of perfusate through the XAD-7 column. The SRS^{cat} activity was eluted from the resin with 125 ml of methanol with a 75% recovery in twelve experiments. This procedure provided a convenient method for concentrating and desalting the SRS^{cat} preparation. Histamine was weakly adsorbed onto the XAD-7 column and was eluted from the resin by aqueous washing. Based on bioassay, four cat paws afforded approximately 275 µg of histamine. Recently, XAD-7 was used to purify SRS from rat basophilic leukemia cells (12,68). In contrast to SRS-A^{rat} (14,75), SRS^{cat} could not be eluted from Sephadex LH-20 with a variety of solvents.

It has been reported (30) that SRS^{cat} activity could be extracted into diethyl ether and back into NH_4OH but the percent recovery of bioactivity was low (46%). The use of peroxide-free diethyl ether for extraction resulted in a slight improvement in recovery of SRS^{cat} activity (59%, mean of 10 experiments). However, it was found that ethyl acetate more efficiently extracted SRS^{cat} activity (79%) from the aqueous phase (4%) (Table 12).

Folch's method (76) for lipid extraction consisting of chloroform:methanol:water (8:4:3) was investigated for SRS purification. When 7500 units of SRS (purified by XAD-7) was partitioned between 4 ml of the aqueous phase and 6 ml of organic phase, 3600 units were recovered in the aqueous phase and 1200 units in the organic phase. When $CaCl_2$ (2 mg) was added, all the activity remained in the aqueous phase. At pH 2.7, SRS activity distributed between the organic and aqueous phases in a 1.7:1 ratio, respectively. This extraction method, therefore was inadequate as a purification procedure.

Silicic acid with a chloroform-methanol gradient was used to purify SRS^{cat} (7). A silica gel (25 g) column developed with chloroform:methanol:water (65:35:10) separated SRS from the dark colored contaminants much better than chloroform:methanol (1:1). The mean recovery of activity in 12 experiments with this chloroform:methanol:

TABLE 12. Purification procedures for SRS^{cat}.

Stage	Procedure	Weight/cat mg (n)	Mean % recovery (no. of expts.)
1	Perfusate[a]	1500 (6)	
2	XAD-7	40 (53)	75 (12)
3	Ethyl acetate extraction	5.4 (53)	79 (10)
4	Disposable column		
	A. C_{18}		70 (3)
	B. Silica gel	1.0 (30)	88 (3)
5	HPLC		
	A. µ-Silica gel	not weighable	53 (17)
	B. µCN	not weighable	77 (8)
	C μC_{18}	not weighable	79 (9)
	D. Preparative μC_{18}	not weighable	61 (6)

[a]Does not include the weight of buffer salts.

n = total number of cats from which the average was calculated.

water system was 60%. Cellulose was inferior to silica gel as support in separating the dark colored material from the bioactivity using the latter solvent system.

High pressure liquid chromatography is a rapid, efficient separation method and has been used to separate biologically-active acidic lipids, including prostaglandins (77). Until recently (48), no one had reported using HPLC for SRS purification. Before samples could be introduced onto HPLC columns, they were concentrated, desalted, and extracted with organic solvent as described under Experimental. Even after these treatments, the sample still contained material insoluble in small volumes of water, which were removed by filtration through small disposable columns of C_{18} or silica gel.

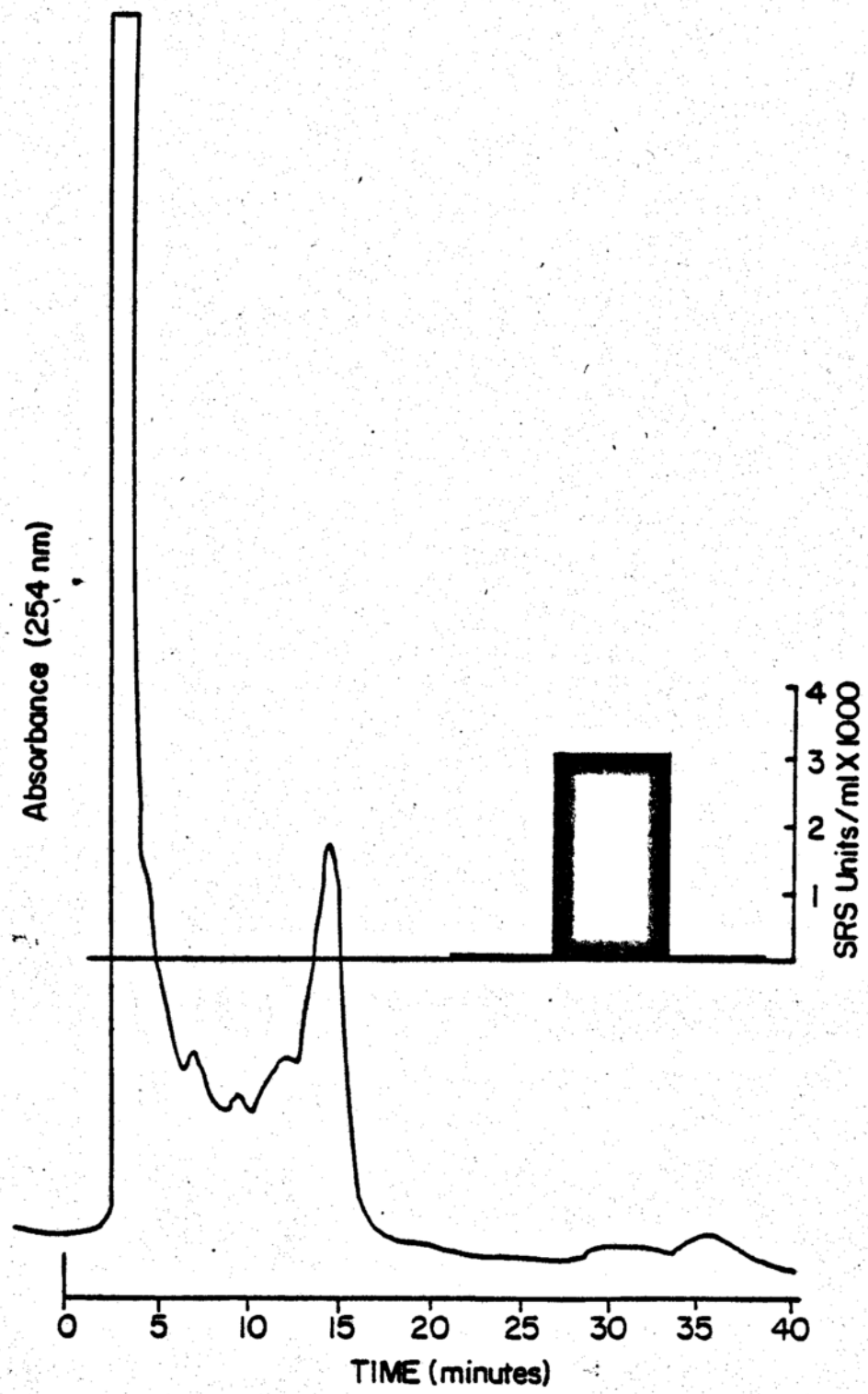
Microparticulate silica gel was initially used in an attempt to purify SRS by HPLC, but the percent recovery (53%, mean of 17 experiments) did not differ appreciably from that obtained by the use of conventional silica gel chromatography. The separation of SRS from the dark colored impurities was, however, considerably better. The UV absorbing impurities (254 nm) were eluted within 2 void volumes, whereas peak SRS activity was eluted between 4.5-5.5 void volumes of methylene chloride:methanol:water (65:35:10) solvent.

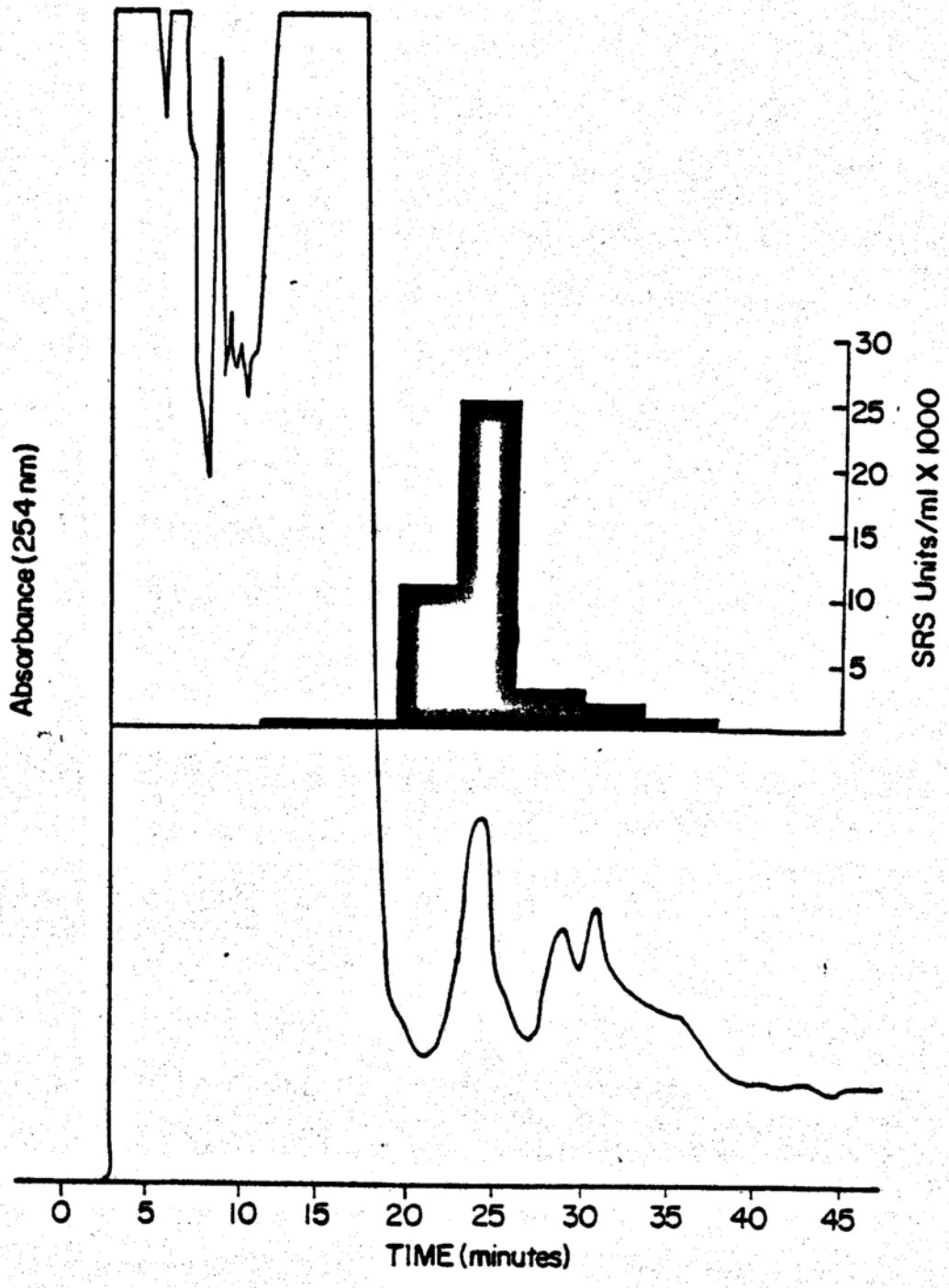
Reverse phase chromatography using μCN as support was

investigated as an additional purification technique. SRS activity was not retained by this column unless 0.1% NaCl was added to the aqueous solvent. Once retained, the SRS did not elute from the column until the NaCl was removed. Table 12 shows that the percent recovery with the μCN column (77%, mean of 8 experiments) was better than that obtained with μ-silica gel, but the resolution was poorer as the UV absorbing impurities overlapped with the SRS activity.

Improved separation of SRS activity from the UV absorbing impurities was attained (Figure 8) by the use of a microparticulate C_{18} column and a gradient elution system comprised of methanol in water (45% to 60%). The recovery from the μC_{18} column was 79% (Table 12). As with other analytical HPLC columns, however, the use of preparative amounts of SRS overloaded the column resulting in poor separation (Figure 9).

Bondapak C_{18}/Porasil (37-75 μ) was used because columns containing these larger particles of C_{18} have a greater capacity. This material, however, has a high percent (18%) C_{18} loading (the percent of available sites on the silica gel that are derivatized with octadecyl units) which more efficiently adsorbed the SRS. SRS was not eluted from the column, even with 100% methanol. Bondapak C_{18}/Corasil (37-50 μ) has a 2% loading of C_{18} from which SRS was eluted, but unfortunately the fewer theoretical plates, associated with


Absorbance (254 nm)
SRS Units/ml X 1000
1
2
3
4
0
5
10
15
20
25
30
35
40
TIME (minutes)


Absorbance (254 nm)
30
25
20
15
10
5
SRS Units/ml X 1000
0
5
10
15
20
25
30
35
40
45
TIME (minutes)

particles of this size resulted in poor separation.

SRS could not be eluted from M9 ODS-2, a preparative microparticulate C_{18} column, due to its high C_{18} loading (15%). However, a similar column (M9 ODS), which contained a 5% C_{18} loading, allowed the preparation of large quantities of highly purified SRS. Figure 10 shows the elution pattern of SRS and UV absorbing compounds from this preparative μC_{18} column in which the bioactivity separated from all but one UV peak. A water:methanol gradient (40% to 95%) was also unsuccessful in separating this UV peak from the bioactivity.

The purification of 560,000 units of SRS is shown in Table 13. After the preparative μC_{18} column, the sample weight was estimated by methylation with 11 µCi of ^{14}C-labelled diazomethane. The methylation procedure was found to be efficient by the methylation of 10 µg (33 picomoles) of 12-hydroxy stearic acid with 7.5 µCi of $^{14}CH_2N_2$ as a control. Prior to methylation, the SRS sample was extracted into ethyl acetate (80,000 units) and after methylation no bioactivity was detected. The methylated sample, after being dried and redissolved in ethyl acetate, contained 0.046 µCi of radioactivity. If one assumes that the sample at this stage of purity were monocarboxylic acids with an approximate average molecular weight of 400, this SRS sample would have contained 1.3 µg of material or 16 picograms per

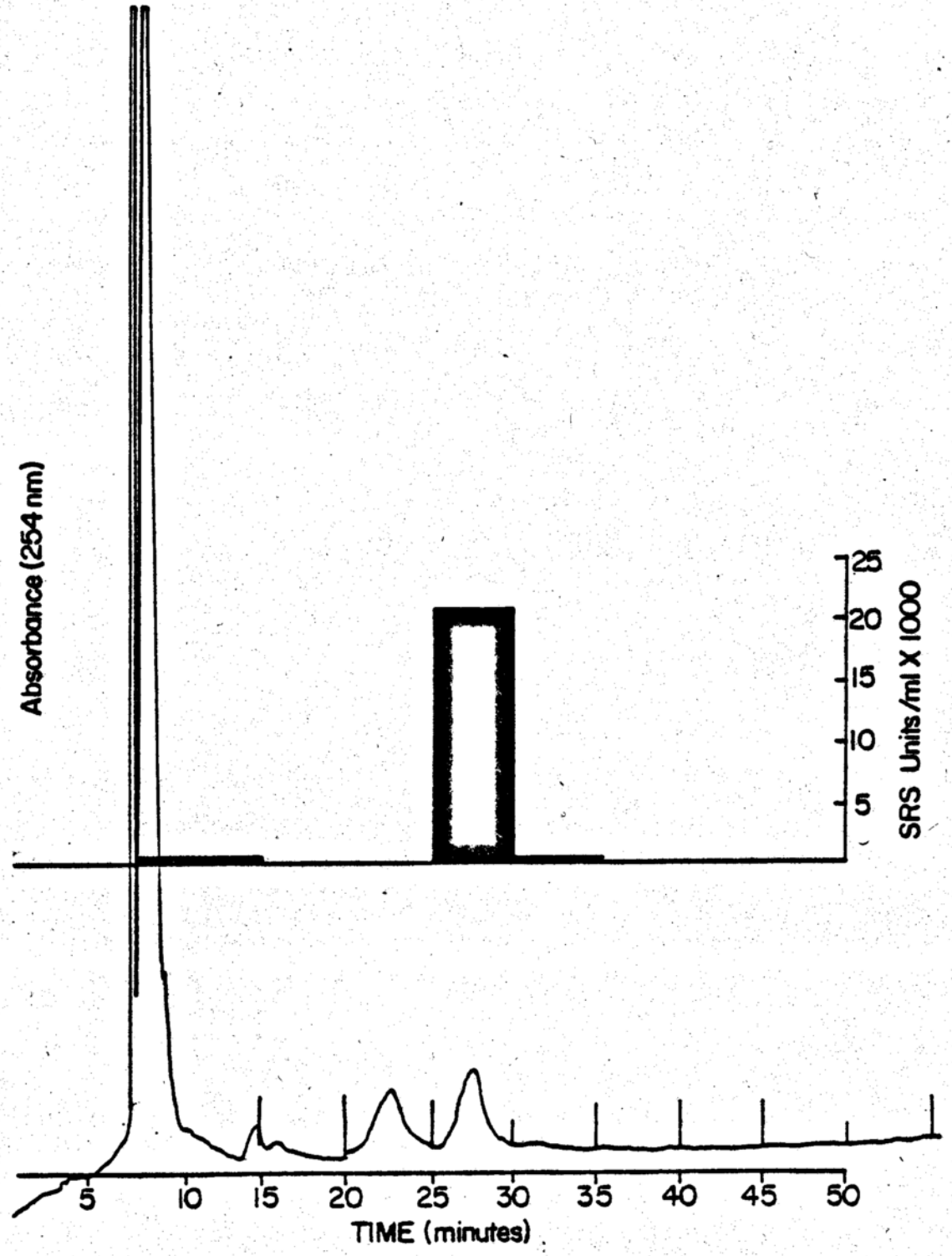

Absorbance (254 nm)
SRS Units/ml X 1000
25
20
15
10
5
5
10
15
20
25
30
35
40
45
50
TIME (minutes)

TABLE 13. Purification of SRS^{cat}.

Procedure	Units SRS	Percent recovery	Weight (mg)	Fold Purification
XAD-7	560,000		233	
Ethyl acetate extraction	345,000	62	30	4.8
Disposable silica gel column	250,000	45	6	17
Preparative μC_{18}	105,000	19		
	80,000		0.0013*	25,600

*Based upon methylation with $^{14}CH_2N_2$ and a molecular weight of 400.

unit of SRS bioactivity. A radiochromatogram scan of the ^{14}C-methylated SRS sample is shown in Figure 11 which showed the presence of only one major and three distinct minor radioactive peaks. Even at this stage of purity however, no radioactive peak coincided with the bioactivity obtained after base hydrolysis.

The purification of SRS by ethyl acetate extraction, disposable silica gel and μC_{18} HPLC column chromatography achieved a 25,600 fold purification (Table 13). XAD-7 produced, on the average, another 28 fold purification (Table 12), thus yielding a total purification of 716,800 fold and an overall recovery from the perfusate of 14%. This procedure, therefore, provides a convenient method for obtaining highly purified SRS. At this stage of purity, however, the SRS became very unstable. When stored at -25^0, either dry or in 0.1% NH_4OH, samples of SRS (stage 5D) lost 97% of their bioactivity in two days. This instability of purified SRS made the structural elucidation of the active molecule even more difficult. Other workers have also encountered decreased stability with increased purification (14,27,31), although no one has yet reported quantitative losses of this magnitude.

E. Derivatization and Hydrolysis

The preparation of SRS derivatives was investigated

Origin

with a view to utilizing the derivatized molecule advantageously during the purification process. The introduction of a radioactive group onto the SRS molecule would allow one to monitor SRS conveniently thus alleviating the tedious bioassay method when large numbers of samples are involved. Also, the preparation of more non-polar SRS derivatives may facilitate purification especially if one were able to regenerate SRS activity upon removal of the group introduced.

Acetylation of SRS^{cat} with acetic anhydride in pyridine resulted in total loss of bioactivity. This loss could not be attributed to pyridine since pyridine alone caused only 6% loss of bioactivity. This derivatization was not studied further, because only 7% of the activity was generated after treatment with 0.2 M NaOH for 30 minutes at 22^0.

Silylation of SRS (Table 14) by the use of BSTFA alone resulted in 66% inactivation of bioactivity, but BSTFA and pyridine or TRI-SIL (containing pyridine) resulted in almost complete inactivation (92%). The apparent silylated derivative was completely soluble in chloroform but not in hexane. Hydrolysis of silylated SRS was attempted by various means (Table 15). The best recovery of bioactivity (66%) was achieved by the use of aqueous 0.1 M HCl for 15 minutes at 22^0. Since the silylated derivative was still insufficiently volatile for gas chromatographic analysis, it was

TABLE 14. Silylation of SRS^{cat}.

	SRS units	Percent inactivation
BSTFA	3,800	66
BSTFA + pyridine	1,000	94
BSTFA + pyridine	12,000	90
TRI-SIL	2,000	92
TRI-SIL	3,800	96
Pyridine	12,000	6

200 µl of reagent and/or solvent were added to SRS for 15 minutes at 22°. Samples were dried and dissolved in NH_4OH immediately before assay to determine percent inactivation (i.e., silylation).

TABLE 15. Hydrolysis of silylated SRS^{cat}.

Solvent	Time (min)	Percent hydrolysis
Water	5	8
0.1 M HCl/water	5	58
	15	66
	30	66
Methanol	20	37
0.1 M HCl/methanol	20	58

Each sample contained 240 units of activity prior to silylation. HCl/water was neutralized with NaOH immediately before assay. HCL/methanol was evaporated to dryness and the sample dissolved in NH_4OH immediately before assay.

not pursued further.

The results of methylation by CH_2N_2 and DMS and regeneration of bioactivity by base are shown in Table 16. Both methods inactivated SRS equally well, but regeneration of activity was slightly greater after the use of CH_2N_2. A comparison of hydrolysis with 0.05, 0.1, 0.5, and 1.0 M aqueous KOH (30 minutes, 22^0) showed that the maximum regeneration of bioactivity was obtained with 0.1 M KOH for 30 minutes; hydrolysis for 15 minutes or 60 minutes produced less bioactivity. Heating to 50^0 regenerated only 3% more activity. SRS itself was found to be stable to 0.1 M aqueous KOH, but not to 0.5 and 1.0 M aqueous KOH or to 0.1 M alcoholic KOH after 30 minutes at 22^0 (Table 17). Hydrolysis of SRS methyl ester was therefore typically conducted at 22^0 for 30 minutes with 0.1 M aqueous KOH. CH_2N_2 was prefered rather than DMS, because excess reagents were easily removed by evaporation. The low recovery of bioactivity (39%) from the methylated SRS precluded its use as a preparative technique, but it was useful for some analytical experiments.

On silica gel TLC the Rf of methylated SRS was 0.35 whereas SRS remained at the origin when developed in methylene chloride:methanol:water (65:15:10) solvent. Bioactivity was detected at the origin before base hydrolysis and the amount of activity did not change upon base hydrolysis.

TABLE 16. Methylation of SRS^{cat}.

	No. of expts.	% Inactivation (range)	% Activity regenerated (range)
DMS	6	90 (70-100)	23 (11-35)
CH_2N_2	6	93 (87-100)	39 (16-76)

SRS ranged from 2,000-15,000 per sample for each methylation method. Hydrolysis was by 0.1 M KOH/water for 30 minutes at 22^0. KOH was neutralized with 0.1 M HCl before assay.

TABLE 17. Stability of SRS^{cat} to KOH.

Condition	Time (min)	SRS % remaining
0.1 M KOH/water	30	100
0.5 M KOH/water	30	75
1.0 M KOH/water	30	52
0.1 M KOH/ethanol	30	55
0.5 M KOH/ethanol	30	45

All incubations were at 22^0. KOH/water was neutralized before assay. Samples containing KOH/ethanol were neutralized, evaporated to dryness, and redissolved in NH_4OH.

Bioactivity was not detected at Rf 0.35 until after base hydrolysis and this activity was inhibited by FPL 55712. These results indicated that SRS was methylated by CH_2N_2 and that the SRS bioactivity was regenerated by base hydrolysis.

In an attempt to clarify whether a sulfate existed on SRS and to establish whether another methylatable group also existed (e.g., carboxyl), the CH_2N_2-treated SRS was hydrolyzed with pyridine to selectively remove any methyl group attached to a sulfate. The use of either 5% pyridine in diethyl ether or NaI in acetone (2 mg/ml) to hydrolyze steroid sulfate methyl esters to their respective steroid sulfates was reported (78). Regeneration of SRS bioactivity by pyridine would indicate the existence of a sulfate, but no carboxyl. A change in Rf after treatment of the methylated SRS with pyridine, with regeneration of bioactivity only after the addition of KOH would indicate the existence of both a sulfate and possibly a carboxyl group. No change in bioactivity or Rf would indicate either an inability to hydrolyze the sulfate ester or that no sulfate exists.

Model compounds were used to check the procedure. After methylation of 200 µg of cholesteryl sulfate with CH_2N_2, the product was treated with 5% pyridine in ether for 30 minutes at 22^0, but no hydrolysis was noted. 100% pyridine under the same conditions, however, did hydrolyze 95% of the cholesteryl sulfate methyl ester to cholesteryl

sulfate (Table 18), but when smaller quantities of the methyl ester were used, hydrolysis was found to be incomplete. For example, one μg of ^{14}C-labelled cholesteryl sulfate methyl ester under these conditions produced only 50% hydrolysis. Pyridine hydrolyzed p-nitrophenyl methyl sulfate, but not p-nitrophenyl methyl phosphate (Table 18). The use of NaI in acetone was ineffective in hydrolyzing small quantities of cholesteryl methyl sulfate.

Even though quantitative hydrolysis of the cholesteryl sulfate methyl ester was not achieved, 50% hydrolysis of methylated SRS would be easily detected. Incubation of methylated SRS in pyridine (400 μl) for 30 minutes at 22^0 did not generate bioactivity, and no significant change in the position of bioactivity on TLC plates was observed (Table 19). If hydrolysis of a sulfate methyl ester had occurred, bioactivity would reside at the origin because of the increase in polarity of the free sulfate ester. Therefore, either SRS does not contain a sulfate or hydrolysis of the sulfate methyl ester did not occur, even in 100% pyridine.

F. Dioxane and THF Solvolysis

Although arylsulfatases inactivated SRS, presumably by the hydrolysis of a sulfate ester (32-34), the removal of the sulfate by chemical methods would be easier and cleaner

TABLE 18. Hydrolysis of methyl esters by pyridine.

Methyl ester of	µg	Percent hydrolysis
Cholesteryl sulfate	200	95
	10*	50
	1*	50
p-Nitrophenyl sulfate	200	100
p-Nitrophenyl phosphate	200	0

Samples were incubated with 0.4 ml of pyridine for 30 minutes at 22^0. (*) radioactively labelled.

TABLE 19. Thin layer chromatography of methylated SRS^{cat} treated with pyridine.

R_f	SRS Units after hydrolysis: Pyridine treated	SRS Units after hydrolysis: Control
0.65-1.00	0	0
0.55-0.65	0	0
0.45-0.55	0	0
0.35-0.45	53	44
0.25-0.35	18	20
0.15-0.25	0	0
0.05-0.15	0	0
origin	0	0

Samples were incubated for 30 minutes at 22^0. The control was incubated with water and co-chromatographed with the pyridine treated sample. TLC was developed in methylene chloride:methanol: water (65:15:10). Hydrolysis was by 0.1 M KOH/water for 30 minutes at 22^0. No bioactivity was detected before hydrolysis.

for preparative purposes. Dioxane and THF were reported to solvolyze sulfates of aliphatic and aromatic sulfate esters (79-83), especially steroid sulfates. Refluxing sulfate ester in these solvents for 10 minutes quantitatively hydrolyzed the ester. Addition of water from 1% (82) to 10% (83) protected the solvolysis of sulfate esters. The mechanism of solvolysis of sulfate esters in dioxane is shown in Figure 12 (69). Apparently, the addition of water hydrates the sulfate and shields the molecule from dioxane.

The validity of this solvolytic procedure was examined using aromatic and aliphatic sulfate esters (Table 20). Addition of the sulfate esters as aqueous solutions to dioxane or THF helped the dispersion of the sulfate esters in the solvent. The sulfate esters of dodecyl alcohol, cholesterol, 12-hydroxystearic acid, and p-nitrophenol were all readily solvolyzed by refluxing with THF and dioxane. Addition of water to the extent of greater than 5% protected the solvolysis of sulfates as had been reported by other workers (82,83). Glucose-6-sulfate was virtually resistant to solvolysis by these cyclic ethers, presumably due to the presence of neighboring hydroxyl groups on the molecule. As expected, p-nitrophenyl phosphate was not solvolyzed.

When freshly distilled dioxane or THF was refluxed under nitrogen, significant quantities of peroxide was produced as revealed by the KI test. Since peroxides are known

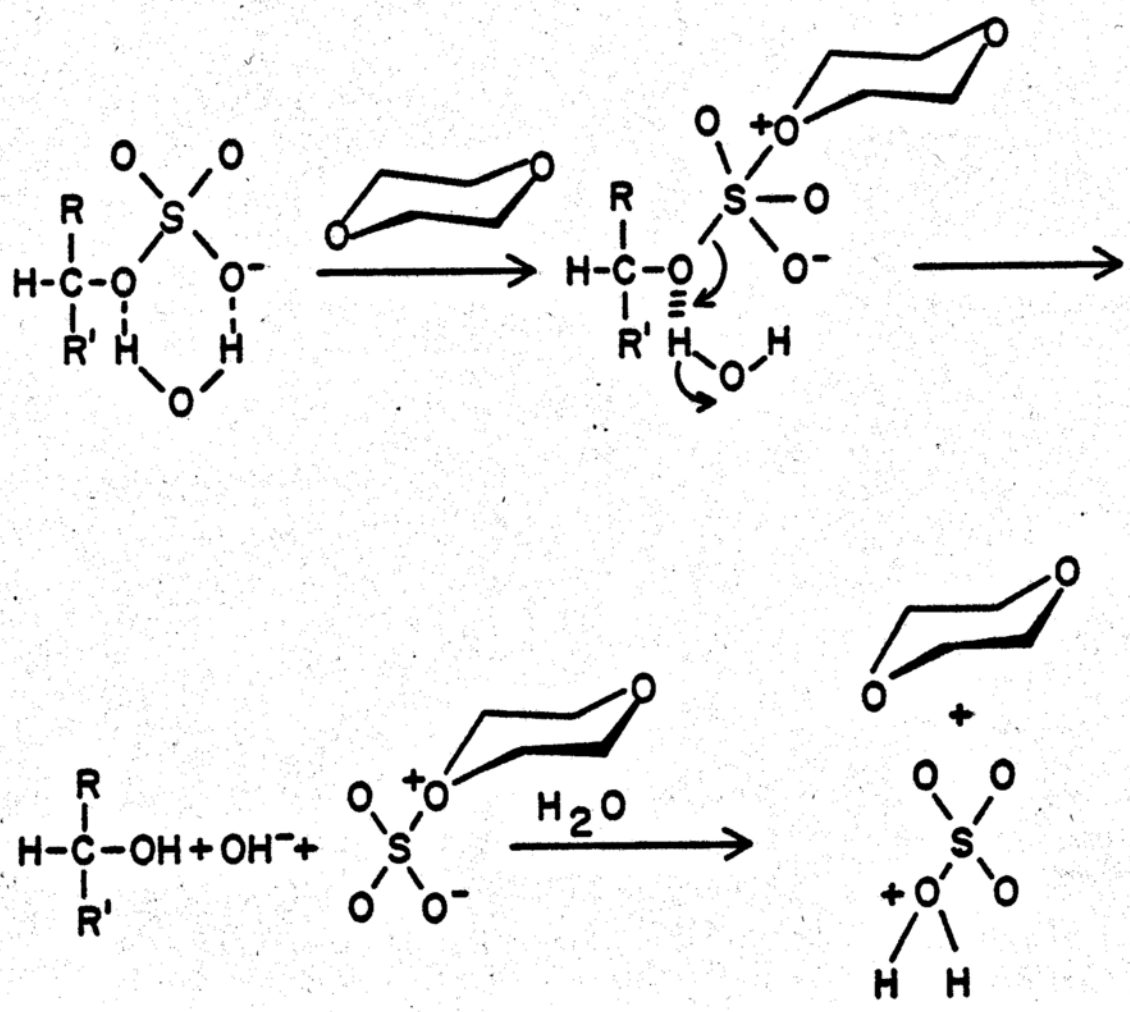


TABLE 20. Solvolysis of sulfate esters by THF and dioxane.

Sulfate esters of	Salt	% Water (v/v)	% Solvolysis THF	% Solvolysis Dioxane
Dodecyl alcohol	Na	0	100	90
	Na	3		100
	Na	5		100
	Na	25		5
Cholesterol	NH_4	0	100	
	NH_4	1	100	
	NH_4	5	40	
	C_5H_5NH	0	100	
12-Hydroxy stearate	Na	0	95	
	Na	5	100	
	Na	10	40	
	Na	20	5	
Glucose	K	0	2[a,b]	0[a,b]
	K	1	2[a]	3[a]
p-Nitrophenol	K	0	100	95
	K	1	100	100
	K	5	100	100
	K	10	0	0
p-Nitrophenyl phosphate	K	0	0	0
	K	1	0	10
	K	5	0	0

All samples were separated on TLC and visualized by either a spray reagent or by UV light except (a) was assayed for glucose by an enzyme spectrophotometric assay. All samples contained 50-150 µg of compound except (b) which contained 1.5 mg. Reflux was for 15 minutes under N_2 in 1 ml of solvent containing 0.025% BHT.

to destroy SRS activity (14,20), 0.025% of BHT was added to prevent peroxide formation. At this concentration BHT did not interfere with the bioassay of SRS. Table 21 shows that an average of 71% and 73% of SRS activity still remained after refluxing with THF and dioxane, respectively. In addition, similar results were obtained when SRS was refluxed in these ethers containing 25% water. Since all the standard sulfate esters, except glucose-6-sulfate, were completely solvolyzed under these conditions, this result raises the possibility that SRS is devoid of a sulfate ester function or that the SRS sulfate is surrounded by neighboring hydroxyl groups.

G. Enzymatic Studies

The relative substrate specificity of enzyme action provides a convenient method for the detection of functional group(s) present in SRS. For example, the inactivation of SRS by purified arylsulfatases from human lung (33) and eosinophils (34) strongly suggest the presence of a sulfate ester in SRS and recently the inactivation of SRS-A^{rat} by lipoxidase suggests the presence of a cis,cis-1,4-pentadiene structure (84). In an attempt to reveal the possible existence of other functional groups in SRS, the actions of a variety of enzymes were examined with SRS^{cat}.

SRS^{cat} was readily inactivated by a crude commercial

TABLE 21. Solvolysis of SRS^{cat} by THF and dioxane.

Expt.	% Water (v/v)	% SRS Remaining	
		THF	Dioxane
1	0	60	75
2	0	76	75
3	1	65	77
4	1	59	70
5	3	92	80
6	3	75	60
7	25	90	77
8	25	80	77

Each sample contained 200-500 units of SRS in 1 ml of solvent containing 0.025% BHT. Samples were refluxed for 15 minutes under N_2. Control samples were heated to the same temperature in water for 15 minutes.

arylsulfatase. This preparation, however, contained substantial amounts of phosphomonoesterase activity as it hydrolyzed p-NPP at a rate 22% that of p-NPS (Table 22). As crude acid or alkaline phosphatases, which did not hydrolyze p-NPS, inactivated SRS significantly at pH 5 and 10.5, respectively, it was suspected that SRS might possess a phosphate ester group. However, when SRS was exposed to 10 units each of purified acid and alkaline phosphatases, no significant inactivation of SRS occurred. Thus, it appeared that this inactivation was due to the presence of a contaminating enzyme in these crude phosphatase preparations.

Since the hydrolysis of p-NPP by arylsulfatase was not previously reported and that inactivation of SRS by arylsulfatase preparations was the major evidence to suggest that SRS contained a sulfate, it was important to determine whether the sulfatase and phosphatase activities in the arylsulfatase preparation were separable, and if so, which activity was responsible for the SRS inactivation. Acid disc gel electrophoresis did not separate the enzyme activities and the amount of phosphatase and sulfatase activity recovered was 70% and 54%, respectively. However, using alkaline disc gel electrophoresis, a partial separation of the phosphatase and sulfatase activities was achieved, for some gel sections were devoid of phosphatase activity but contained substantial sulfatase activity and the SRS

TABLE 22. Activity of arylsulfatase and crude phosphatases on various substrates.

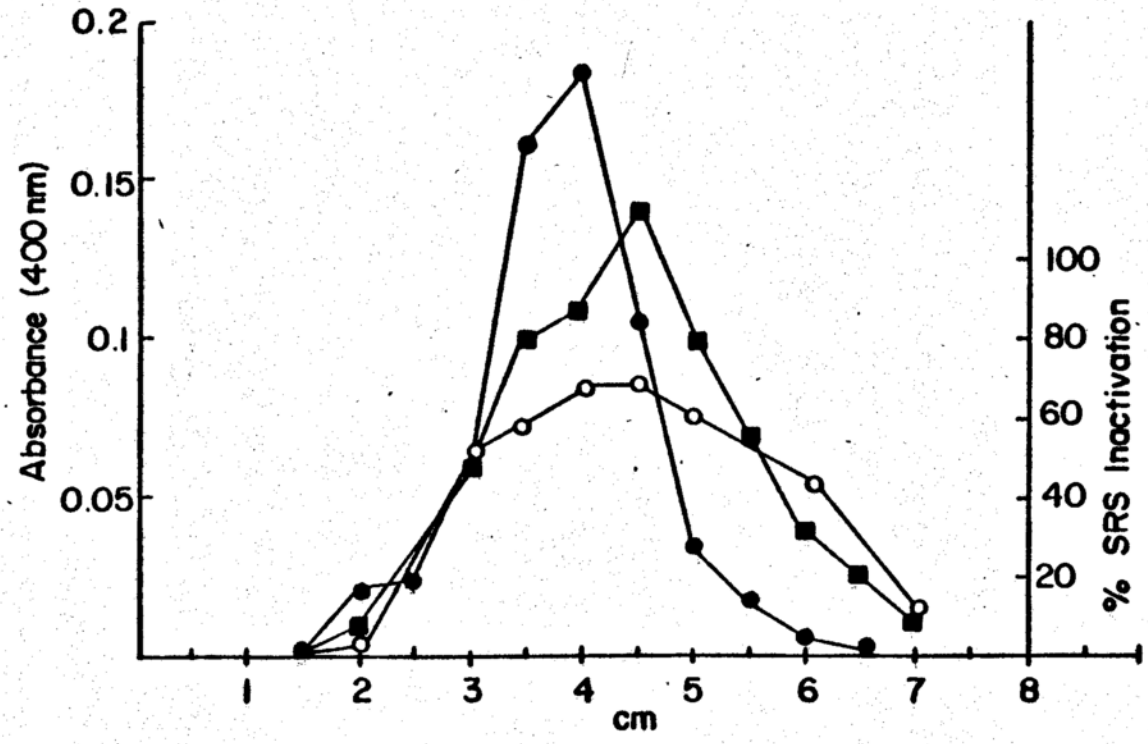
Enzyme	Units	Substrate	Incubation time (min)	pH	A_{400}	% SRS Inactivation
Arylsulfatase	2	p-NPS	30	5	0.46	
Arylsulfatase	2	p-NPP	30	5	0.10	
Arylsulfatase	1	SRS	30	5		35
Alkaline phosphatase	0.6	p-NPP	0.5	10.4	1.03	
Alkaline phosphatase	10	p-NPS	30	10.4	0	
Alkaline phosphatase	0.6	SRS	30	10.4		26
Acid phosphatase	1	p-NPP	1	5	3.10	
Acid phosphatase	1	p-NPS	30	5	0	
Acid phosphatase	10	SRS	15	5		26

All incubations were at 35^0 in 1 ml. Incubations with p-NPP and p-NPS were stopped by adding 5 ml of 0.1 M NaOH.

inactivation was associated with sulfatase activity (Figure 13). Under alkaline conditions, the sulfatase activity was rather unstable, as only 13% of the activity was recovered as compared to 61% for the phosphatase activity. This again suggests that these are two distinct enzymes responsible for the hydrolysis of sulfate and phosphate esters. Further, only sulfatase appeared to be involved in SRS inactivation.

Since crude acid and alkaline phosphatase preparations hydrolyzed bis-p-NPP and TMP-3'-NPE, one might suspect that the contaminant enzyme was a phosphodiesterase. While crude venom phosphodiesterase (0.2 units) destroyed SRS (900 units) bioactivity (45%), purified preparations of this enzyme did not inactivate SRS, even after hours of incubation. Crude spleen phosphodiesterase also inactivated SRS bioactivity but the rate of SRS inactivation was considerably slower than equivalent units of contaminant phosphodiesterase activity (measured with TMP-3'-NPE) in the crude alkaline phosphatase preparation. Crude 3',5'-cyclic phosphodiesterase preparation also inactivated SRS bioactivity. It is known that this cyclic phosphodiesterase is inhibited by theophylline with a K_i of 0.1 mM (85), but SRS^{cat} inactivation was not inhibited by 0.5-5 mM theophylline.

To determine whether the loss of SRS^{cat} bioactivity upon exposure to these crude enzyme preparations was enzymatic in nature, crude alkaline phosphatase and venom


0.2
0.15
0.1
0.05
Absorbance (400 nm)
100
80
60
40
20
% SRS Inactivation
1
2
3
4
5
6
7
8
cm

phosphodiesterase preparations were heated to 100°C for 10 minutes. The boiled enzyme preparations did not inactivate SRS bioactivity to any significant extent (Table 23). SRS was reported to adsorb onto protein and may be released by extraction with 80% ethanol (14,27,86). Therefore, after SRS inactivation by alkaline phosphatase and venom phosphodiesterase, the incubation mixture was extracted with 80% ethanol. Reassay of these extracts produced no additional SRS.

The rate of SRS inactivation by venom phosphodiesterase and acid phosphatase is shown in Figure 14. Inactivation of SRS (1,000 units/ml) occurred in a linear fashion.

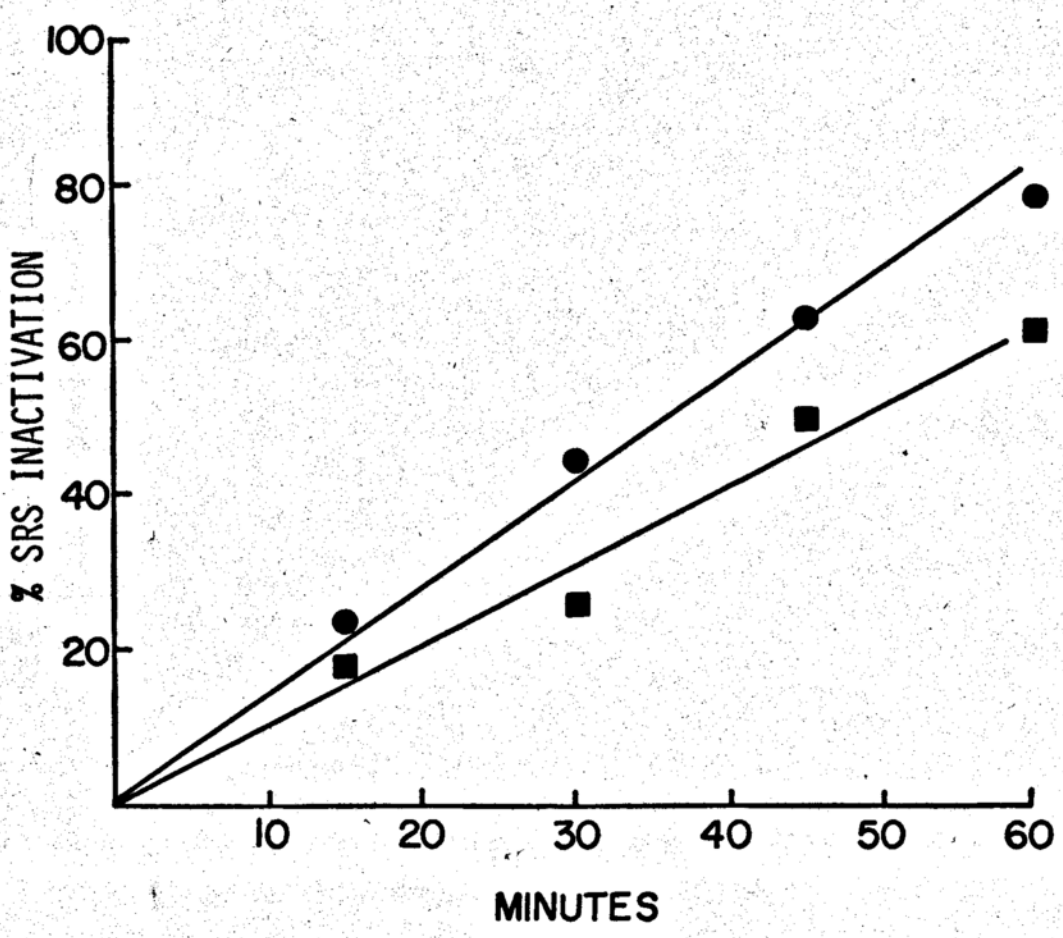
The percent inactivation of SRS at different enzyme/substrate ratios is shown in Figure 15. Each sample was incubated with increasing amounts of crude alkaline phosphatase for 30 minutes. The results showed a dose-response relationship between enzyme concentration and SRS inactivation.

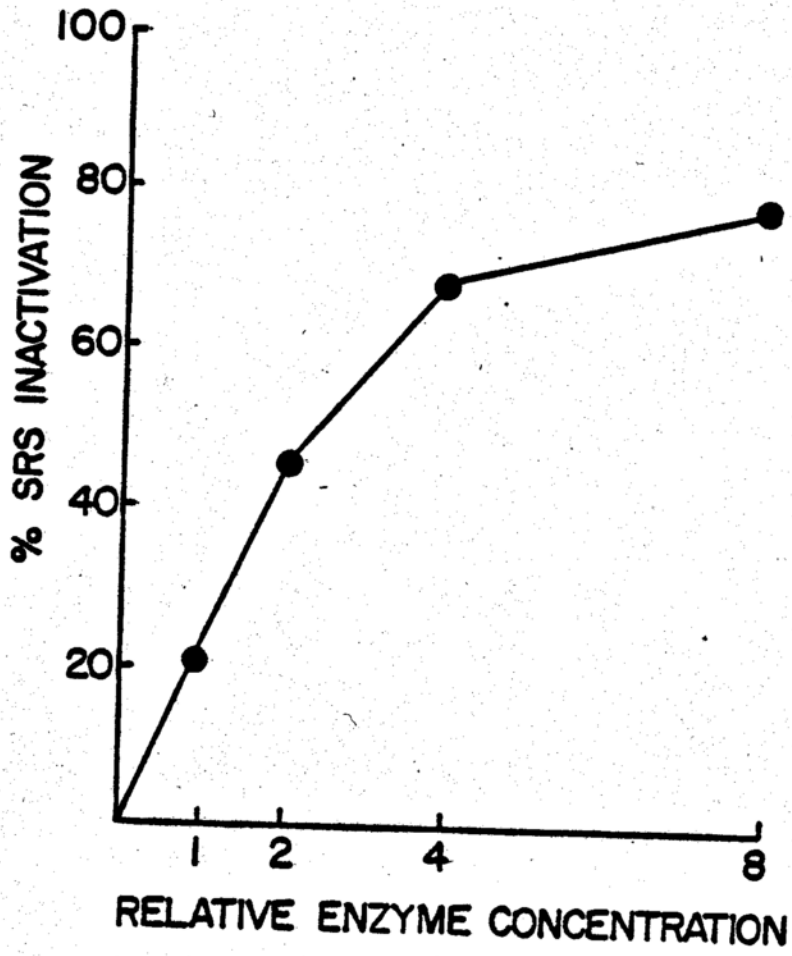
Sephadex G-200 gel filtration of crude alkaline phosphatase appears in Figure 16. The elution of the SRS inactivating enzyme indicated a molecular weight slightly less than that of alkaline phosphatase (115,000). These results, therefore, indicated that SRS was inactivated by enzyme(s) which apparently existed as a contaminant in the crude phosphatase and phosphodiesterase preparations.

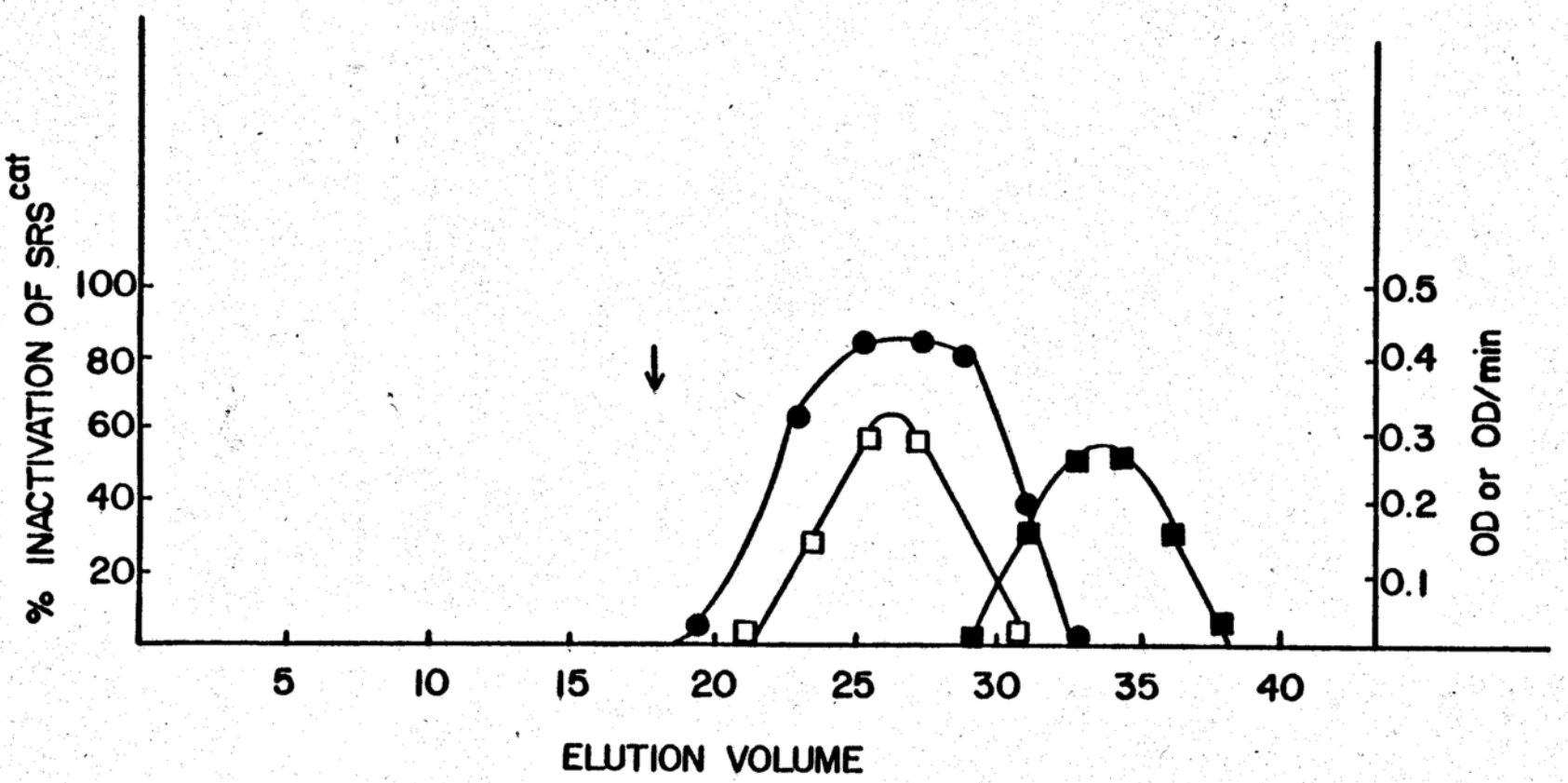
TABLE 23. Effect of denatured enzymes on SRS^{cat}.

Enzyme	Enzyme (units)	Incubation time (min)	SRS Units	
			Before incubation	After incubation
Venom phosphodiesterase	0.2	60	800	240
Boiled venom phosphodiesterase	0.2	60	800	800
Alkaline phosphatase	6.5	30	440	170
Boiled alkaline phosphatase	6.5	30	420	400

Enzymes were denatured by heating to 100^0 for 10 minutes. Incubations were at 35^0 in 1 ml at pH 8.9 and 10.4 for venom phosphodiesterase and alkaline phosphatase, respectively.


100
80
60
40
20
% SRS INACTIVATION
10
20
30
40
50
60
MINUTES

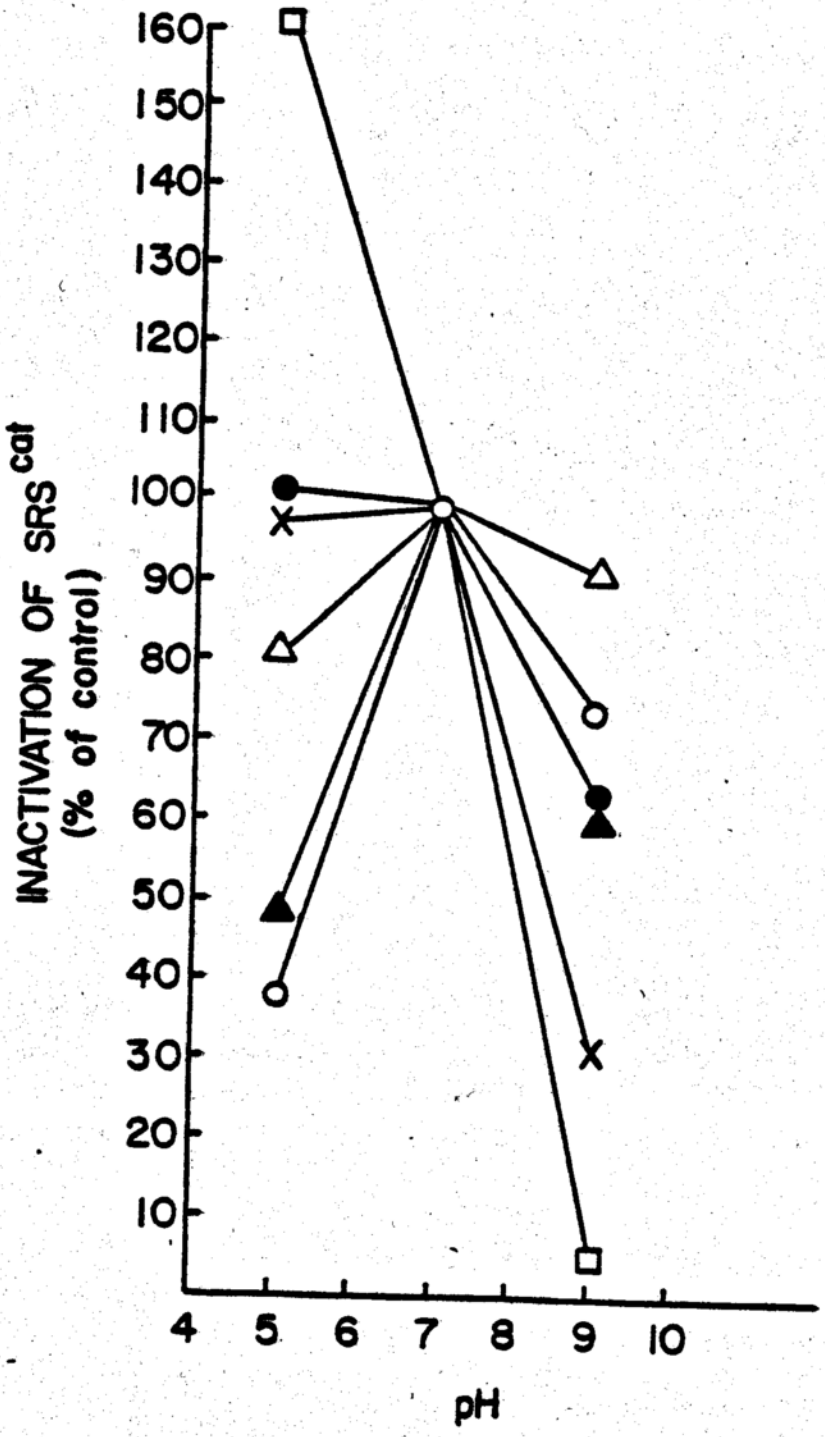

100
80
60
40
20
% SRS INACTIVATION
1
2
4
8
RELATIVE ENZYME CONCENTRATION


% INACTIVATION OF SRS cat
100
80
60
40
20
5
10
15
20
25
30
35
40
ELUTION VOLUME
0.5
0.4
0.3
0.2
0.1
OD or OD/min

It was unlikely that different enzymes were responsible for SRS inactivation in these five crude enzyme preparations. It was more probable that the same enzyme was contaminating all the preparations. Therefore, a comparison of SRS^{cat} inactivating ability of these five preparations and arylsulfatase under various conditions was undertaken. Due to the nature of the assay system, only differences of greater than 20% were considered to be significant. All values were compared with the percent inactivation of SRS at pH 7.0 and 35^0 for each enzyme preparation.

All the phosphate cleaving preparations exhibited a pH optimum for SRS^{cat} inactivation at pH 7.0, although acid phosphatase and 3',5'-cyclic phosphodiesterase preparations were equally active at pH 5.0 (Figure 17). Cysteine inhibited the enzymatic inactivation of SRS in all cases (Figure 18). Magnesium chloride, EDTA, Na_2SO_4, and K_2HPO_4 at 10 mM did not have a significant effect on SRS inactivation. As expected, arylsulfatase differed from the other enzymes in its pH optimum and inhibition by K_2HPO_4. Since there were no major differences among the five crude enzyme preparations, the existence of a common contaminating enzyme seemed likely.

In an attempt to further define the nature of the SRS inactivating enzyme, a number of other enzymes were evaluated for their abilities to inactivate SRS (Table 24).


INACTIVATION OF SRS cat
(% of control)
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
4
5
6
7
8
9
10
pH

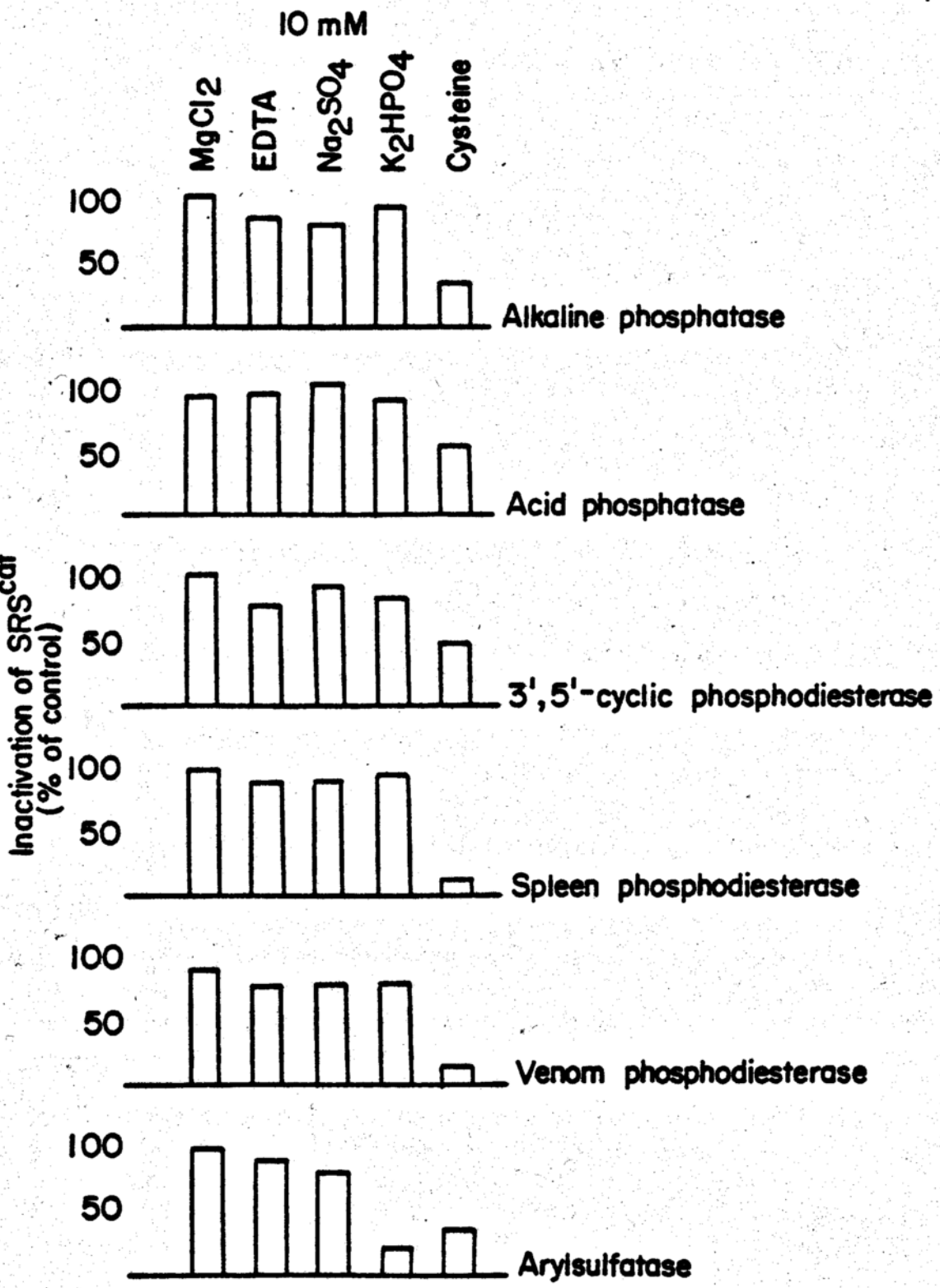

10 mM
MgCl2
EDTA
Na2SO4
K2HPO4
Cysteine
Inactivation of SRS^cat (% of control)
100
50
Alkaline phosphatase
Acid phosphatase
3',5'-cyclic phosphodiesterase
Spleen phosphodiesterase
Venom phosphodiesterase
Arylsulfatase

TABLE 24. Effect of enzymes on SRS^{cat}.

Enzyme	Units/ml	Temp.	pH	% SRS Inactivation[a]
β-Glucuronidase	100	35	6.8	negligible[b]
β-Glucosidase	10	35	5.0	negligible[b]
α-Glucosidase	10	35	6.8	negligible[b]
β-N-Acetyl glucosaminidase	5	22	5.0	negligible[b]
Lipase	362	35	7.7	negligible[b]
Phospholipase A_2	50	35	8.5	negligible[b]
Phospholipase C	12.5	35	7.5	negligible[b]
Phospholipase D	500	22	5.5	negligible[b]
Esterase	10	22	8.0	negligible[b]
5'-Nucleotidase	10	35	8.9	30
5'-Adenylic acid deaminase	1.75	22	6.5	negligible[b]
Adenosine deaminase	10	22	7.5	negligible[b]
Chondroitinase ABC	2.5	35	7.2	30
Chondro-6-sulfatase	1.0	35	7.5	10
Chondro-4-sulfatase	1.0	35	7.5	20

Stage 4A or 4B SRS (200-500 units) were incubated in 1 ml and bioassayed at 30 and 60 minutes. (a) Represent 60 minute incubations. (b) SRS inactivation of less than 5%/30 minutes.

Since the rate of inactivation by the crude alkaline phosphatase preparation was unaffected under anaerobic conditions, the possibility of an oxidase as the contaminating enzyme was thus eliminated. Although significant SRS inactivation was observed by some of the enzymes tested, none of these were likely to be the SRS inactivating enzyme in the five crude preparations. For example, 10 units of 5'-nucleotidase inactivated 30% of the SRS in 60 minutes, but there were only 0.006 and 0.002 units of 5'-nucleotidase in the venom and cyclic phosphodiesterase preparations respectively, used to obtain the same degree of SRS inactivation. Chondro-4- and -6-sulfatases and chondroitinase ABC inactivated SRS, but their activities were too low to be the contaminant SRS inactivating enzyme.

IV. DISCUSSION

The maximum quantity of SRS released from cat paws was obtained with 48/80 at 1 µg/ml or a single dose of 25 µg (Table 2). Although the release of SRS from other sources was enhanced considerably by the use of the calcium ionophore A23187 (11,17,18), this ionophore released SRS^{cat} at the same order of magnitude as 48/80 (Table 3). Faster flow rates and longer periods of perfusion significantly increased the amount of SRS^{cat} released (Table 4). The optimum conditions for the production of SRS from cat paws included a 45 minute perfusion to remove residual blood, followed by 25 µg of 48/80 and perfusing for three hours at 3 ml/min. This procedure generated an average of 120,000 units of SRS per cat. The large volume of perfusate was conveniently concentrated by XAD-7 chromatography which also removed histamine, 48/80, inorganic salts, and most of the proteins.

The antigen-stimulated release of SRS-A from sensitized tissue is modulated by the cAMP-cGMP system. Consequently the amount of SRS-A released from these tissues was greatly enhanced by the use of pharmacological agents which altered the levels of these cyclic nucleotides (9,10,43,54). If the cAMP-cGMP system was functional during the 48/80 induced release of SRS^{cat}, one would expect a change in the

production of SRS by the use of these pharmacological agents. Synergism between methylxanthines, which competitively inhibit cAMP catabolism to 5'-AMP, and β-adrenergic agonists would suggest the involvement of the cAMP system. Aminophylline and epinephrine exhibited a synergism in the cat paw system, which was reversed by propranolol, a β-adrenergic blocker (Table 6). Alpha-adrenergic agonists usually decrease cAMP levels and increase SRS-A release (43, 54), but in some tissues the α-agonist, phenylephrine, decreased SRS release, unless used in combination with propranolol which greatly increased the SRS-A above control levels (9,10). Similarly, phenylephrine decreased the amount of SRS^{cat} released but the addition of propranolol increased SRS^{cat} only to control levels (Table 7). Therefore, even though phenylephrine is usually considered a selective alpha-agonist, it seemed to possess some beta-agonist properties in this system. The levels of cGMP and immunologically released $SRS\text{-}A^{hu}$ have been increased by cholinergic agents (10,43), but the release of SRS^{cat} was unaffected by carbachol (Table 8). The inhibition produced by aminophylline and epinephrine, however, was reversed by carbachol, thus indicating a functional cAMP-cGMP modulating system similar to that of antigen-mediated $SRS\text{-}A^{hu}$. None of these pharmacological agents examined increased the levels of SRS^{cat}, an observation suggesting that the maximum amount of SRS available

in the paw was released by 48/80 alone. Also, as with immunologically released SRS-A^{hu}, the release of SRS^{cat} required an energy generating system as evidenced by the 2-deoxy-D-glucose inhibition of SRS release (Table 5). All of these results indicate that the biochemical sequence leading to the release of SRS-A^{hu} and the 48/80 initiated release of SRS^{cat}, are quite similar.

Arachidonic acid has been reported as a precursor of SRS (68,71). The major lines of evidence in support of this hypothesis are: an apparent incorporation of ^{14}C-labelled arachidonic acid into SRS; a significant increase in SRS bioactivity following the addition of arachidonic acid to SRS producing cells; and an inhibition of SRS release by TYA, an inhibitor of prostaglandin cyclooxygenase and lipoxygenase (69,70). In the cat paw, however, the amount of SRS released was not altered by arachidonic acid, but it is possible that in this system other precursors or cofactors may be required for de novo SRS synthesis. That TYA decreased the SRS release (Table 10) suggests a structural relationship between SRS and arachidonic acid. However, it is possible that TYA may be inhibiting an enzyme other than cyclooxygenase or lipoxygenase or affecting the levels of a modulating factor, rather than the synthesis of SRS directly. The only SRS modulating factors known to be influenced by

TYA, however, are the prostaglandins, some of which reportedly exert a negative feedback on SRS release (35,36, 38). The lack of inhibition by indomethacin appears to rule out the involvement of the cyclooxygenase system in SRS formation.

There are conflicting reports concerning the stability of SRS. For example, SRS-A^{hu} was reported to be unstable at -70° (31) whereas SRS-A^{rat} was reported to be stable for over a year at -80° (32). The storage of SRS at different stages of purity may cause conflicting results. Although qualitative data was not reported, increased instability was observed after partial purification of SRS (14,27,31). This same observation was made with SRS^{cat} as the half-life of ethanol extracted SRS was 55 days whereas SRS purified by preparative μC_{18} HPLC had almost total loss of activity after only two days at -25°. Temperature and pH also influenced stability (Table 11, Figures 6, 7). As reported for SRS-A^{gp}, SRS-A^{rat}, and SRS-A^{hu} (20,31), SRS^{cat} was more stable at alkaline than acid pH, and more bioactivity was retained in semi-purified samples kept at -196°. Protection from light and storage under nitrogen afforded no additional stability. Similarly, others have reported that highly purified SRS retained its bioactivity only for a few days, even when stored in the dark, under nitrogen, and at -70° (31).

Various purification schemes for SRS have been reported (14,30,31,48,68) but the small quantities of SRS available, its instability, conflicting and inconsistent reports of properties of SRS from different sources, and the occasional occurrence of multiple SRS bioactive peaks have hampered efforts towards its purification. Thus far, no suitable method is available to demonstrate sample purity.

After investigating various solvent extraction methods and column chromatography packing materials, a four step method was developed which generated highly purified SRS^{cat} as determined by sample weight, UV_{254}, and ^{14}C-labelling. This purification scheme (Table 13) utilized XAD-7 resin, ethyl acetate extraction, silica gel disposable column, and preparative μC_{18} HPLC to generate an overall 716,800 fold purification. Even at this stage of purification, ^{14}C-methylation and TLC of this purified SRS failed to show a correlation of a radioactive peak with bioactivity.

SRS was eluted from C_{18} reverse phase HPLC with 50% methanol but could not be eluted from columns containing a high percent C_{18} loading. In contrast to $SRS\text{-}A^{rat}$ and $SRS\text{-}A^{hu}$ (14,31,75), SRS^{cat} could not be eluted from Sephadex LH-20 or XAD-2 resins. This difference could be due to either a chemical structural difference among the SRS's from these sources or a difference in the composition of sample contaminants since some variations in the physical behavior

of SRS at different purification stages were reported (14, 48). Although SRS is a very polar molecule, its efficient adsorption onto reverse phase column packing suggests that it possesses a significant number of hydrophobic groups.

Derivatization of SRS by various methods was attempted with a view to facilitating the purification process with less polar derivatives. Although SRS was methylated by both CH_2N_2 and DMS, quantitative recovery of bioactivity was not obtained after base hydrolysis. It is conceivable that multiple sites on the SRS molecule were methylated and quantitative hydrolysis at each site by 0.1 M KOH was not achieved. SRS was apparently acetylated with acetic anhydride but bioactivity could not be regenerated. Inactivation of SRS by acetic anhydride was reported previously (30), indicative of hydroxyl and/or amino group(s) on the molecule. Quantitative silylation of SRS was also obtained but only when pyridine was used as solvent (Table 14).

The suggestive evidence that SRS contains a sulfate ester rests on its nonvolatile, highly polar character, and the inactivation of its bioactivity by several different type IIB (87,88) arylsulfatase enzyme preparations (32-34). However, phosphate esters are also nonvolatile, highly polar molecules, and their hydrolysis by sulfatases is conceivable. Since sulfate diesters are more easily hydrolyzed than phosphate diesters, the use of pyridine to hydrolyze CH_2N_2-

treated SRS was used in an attempt to confirm the existence of a sulfate ester. However, 100% pyridine did not change the behavior of methylated SRS on TLC, indicating that no ester hydrolysis occurred. On the other hand, methylation of the sulfate probably occurred since the Rf of the methylated SRS was much greater than would be expected for a sulfate monoester. However, since the hydrolysis of the model sulfate diester was not quantitative, these results are only suggestive.

Another characteristic of sulfate ester is their solvolysis in cyclic ether solvents, but when SRS^{cat} was refluxed with either THF or dioxane, only partial loss of bioactivity resulted (27-29%), most of which was attributed to an effect other than solvolysis (possibly undetectable amounts of peroxides) since an average of 15-23% of the bioactivity was also lost when the refluxing cyclic ether contained water at a concentration (25%) known to inhibit solvolysis (Table 21). These results suggest that SRS contains either a phosphate, or a sulfate with neighboring hydroxyl groups, for only these types of model compounds were resistant to solvolysis (Table 20).

Although SRS is inactivated by arylsulfatase preparations from various sources, there is only one report of the inactivation of SRS by a purified arylsulfatase (33), which contained two protein bands on disc gel electrophoresis.

Other researchers typically used crude arylsulfatase preparations. Since our studies revealed substantial amounts of phosphomonoesterase activity in commercially available limpet arylsulfatase, and that our data could not confirm the existence of a sulfate (Tables 19, 21), it was necessary to determine whether the phosphatase activity could be separated from the sulfatase activity. Acid disc gel electrophoresis failed to separate the activities but alkaline disc gel electrophoresis did separate the phosphatase from the sulfatase activity and the SRS inactivation was associated with the sulfatase activity.

The investigation of phosphatase enzymes revealed that SRS was inactivated by enzymes in crude preparations of alkaline and acid phosphatases, and venom, spleen, and 3',5'-cyclic phosphodiesterases. These enzymes were distinct from arylsulfatase in their pH optimum, inability to hydrolyze p-NPS, and the lack of inhibition by K_2HPO_4. The ability of these crude enzyme preparations to inactivate SRS could not be attributed to their respective phosphatase or phosphodiesterase activities and a common contaminating enzyme seemed likely since the influence of pH, EDTA, $MgCl_2$, K_2HPO_4, Na_2SO_4, and cysteine was similar for each enzyme preparation. Based upon gel-elution chromatography on Sephadex G-200, the SRS inactivating enzyme in crude alkaline phosphatase possessed a molecular weight of

approximately 100,000. A study of fifteen other enzymes failed to identify the SRS inactivating enzyme in the crude phosphatase and phosphodiesterase preparations (Table 24).

The discovery of the SRS inactivating enzyme was significant, although its importance will be more evident when the nature of its activity is identified. Not only will its identity give another clue to the structure of SRS, but its usefulness in fragmenting the SRS molecule for structural studies could also be investigated. Like arylsulfatase, this SRS inactivating enzyme may be of value in differentiating SRS from other bioactive molecules.

V. REFERENCES

1. Feldberg, W. and C. H. Kellaway, Liberation of histamine and formation of lysolecithin-like substance by cobra venom, J. Physiol (London), 94, 187(1938).
2. Kellaway, C. H. and E. R. Trethewie, The liberation of slow reacting smooth muscle-stimulating substance in anaphylaxis, Q. J. Exp. Physiol., 30, 121(1940).
3. Burka, J. F. and P. Eyre, The immunological release of slow-reacting substance of anaphylaxis from bovine lung, Can. J. Physiol. Pharmacol., 52, 1201(1974).
4. Orange, R. P., W. G. Austen and K. F. Austen, Release of histamine and slow-reacting substance of anaphylaxis from human lung. I. Modulation by agents influencing cellular levels of cyclic 3',3'-adenosine monophosphate, J. Exp. Med., 134, 136s(1971).
5. Brocklehurst, W. E., The release of histamine and formation of a slow reacting substance (SRS-A) during anaphylactic shock, J. Physiol. (London), 151, 416 (1960).
6. Chakravarty, N., B. Högberg and B. Uvnäs, Mechanism of the release by compound 48/80 of histamine and of a lipid-soluble smooth muscle stimulating principle ("SRS"), Acta Physiol. Scand., 45, 255(1959).

7. Strandberg, K., Release of histamine and formation of slow reacting substance in the cat paw induced by compound 48/80, Acta Physiol. Scand., 82, 47(1971).

8. Uvnäs, B., Lipid spasmogens appearing in connection with histamine liberation, Biochem. Pharmacol., 12, 439 (1963).

9. Grant, J. A. and L. M. Lichtenstein, Release of slow reacting substance of anaphylaxis from human leukocytes, J. Immunol., 112, 897(1974).

10. Kaliner, M., S. I. Wasserman and K. F. Austen, The immunologic release of chemical mediators from human nasal polyps, N. Engl. J. Med., 289, 277(1973).

11. Lewis, R. A., E. G. Goetzl, S. I. Wasserman, F. M. Valone, R. H. Rubin and K. F. Austen, The release of four mediators of immediate hypersensitivity from human leukemic basophils, J. Immunol., 114, 87(1975).

12. Jakschik, B. A., A. Kulczycki, Jr., H. H. MacDonald and C. W. Parker, Release of slow reacting substance (SRS) from rat basophilic leukemia (RBL-1) cells, J. Immunol., 119, 618(1977).

13. Orange, R. P., D. J. Stechshulte and K. F. Austen, Immunological and biological properties of rat IgE. II. Capacity to mediate the immunologic release of histamine and slow-reacting substance of anaphylaxis (SRS-A), J. Immunol., 105, 1087(1970).

14. Orange, R. P., R. C. Murphy, M. L. Karnovsky and K. F. Austen, The physiochemical characteristics and purification of slow-reacting substance of anaphylaxis, J. Immunol., 110, 760(1973).

15. Chakravarty, N. and B. Uvnäs, Histamine and lipid soluble smooth-muscle stimulating principle ("SRS") in anaphylactic reaction, Acta Physiol. Scand., 48, 302 (1960).

16. Baltzly, R., J. S. Buck, E. J. DeBeer and F. J. Webb, A family of long-acting depressors, J. Am. Chem. Soc., 71, 1301(1949).

17. Bach, M. K. and J. R. Brashler, In vivo and in vitro production of slow reacting substance in the rat upon treatment with calcium ionophores, J. Immunol., 113, 2040(1974).

18. Conroy, M. C., R. P. Orange and L. M. Lichtenstein, Release of slow reacting substance of anaphylaxis (SRS-A) from human leukocytes by the calcium ionophore A23187, J. Immunol., 116, 1677(1976).

19. Chaney, M. O., P. V. Demarco, N. D. Jones and J. L. Occolowitz, The structure of A23187, a divalent cation ionophore, J. Am. Chem. Soc., 96, 1932(1974).

20. Orange, R. P. and K. F. Austen, Slow reacting substance of anaphylaxis, Adv. Immunol., 10, 105(1969).

21. Brocklehurst, W. E., The role of slow-reacting substance in asthma, Adv. Drug Res., 5, 109(1970).

22. Austen, K. F., Reaction mechanisms in the release of mediators of immediate hypersensitivity from human lung tissue, Fed. Proc. Fed. Am. Soc. Exp. Biol., 33, 2256 (1974).

23. Herxheimer, H. and E. Stresemann, The effect of slow reacting substance (SRS-A) in guinea-pigs and in asthmatic patients, J. Physiol. (London), 165, 78P (1963).

24. Piper, P. J. and J. R. Vane, Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs, Nature (London), 223, 29(1969).

25. Nijkamp, F. P., R. J. Flower, S. Moncada and J. R. Vane, Partial purification of rabbit aorta contracting substance-releasing factor and inhibition of its activity by antiinflammatory steroids, Nature (London), 263, 479 (1976).

26. Goetzl, E. J. and K. F. Austen, Purification and synthesis of eosinophilotactic tetrapeptides of human lung tissue: Identification as eosinophil chemotactic factor of anaphylaxis, Proc. Nat. Acad. Sci. USA, 72, 4123 (1975).

27. Brocklehurst, W. E., Slow reacting substance and related compounds, Prog. Allergy, 6, 539(1962).

28. Anggard, E., U. Berquist, B. Hogberg, K. Johansson, I. L. Thon and B. Uvnäs, Biologically active principles occurring on histamine release from cat paw, guinea pig lung and rat mast cells, Acta Physiol. Scand., 59, 97 (1963).

29. Orange, R. P. and K. F. Austen, Slow reacting substance of anaphylaxis in the rat, pages 196-206, in: Cellular and Humoral Mechanisms in Anaphylaxis and Allergy, edited by H. Z. Movat. Basel:Karger, N.Y., 1969.

30. Strandberg, K. and B. Uvnäs, Purification and properties of the slow reacting substance formed in the cat paw perfused with compound 48/80, Acta Physiol. Scand., 82, 358(1971).

31. Takahashi, H., M. E. Webster and H. H. Newball, Separation of slow reacting substance of anaphylaxis (SRS-A) from human lung into four biologically active fractions, J. Immunol., 117, 1039(1976).

32. Orange, R. P., C. R. Murphy and K. F. Austen, Inactivation of slow reacting substance of anaphylaxis (SRS-A) by arylsulfatases, J. Immunol., 113, 316(1974).

33. Wasserman, S. I. and K. F. Austen, Arylsulfatase B of human lung, J. Clin. Invest., 57, 738(1976).

34. Wasserman, S. I., E. J. Goetzl and K. F. Austen, Inactivation of slow reacting substance of anaphylaxis by human eosinophil arylsulfatase, J. Immunol., 114, 645 (1975).

35. Koopman, W. J., R. P. Orange and K. F. Austen, Prostaglandin inhibition of the immunologic release of slow reacting substance of anaphylaxis in the rat, Proc. Soc. Exp. Biol. Med., 137, 64(1971).

36. Engineer, D. M., P. J. Piper and P. Sirois, Interaction between the release of SRS-A and of prostaglandins, Br. J. Pharmacol., 57, 460P(1976).

37. Jakschik, B. A., L. H. Lee, G. Shuffer and C. W. Parker, Arachidonic acid metabolism in rat basophilic leukemia (RBL-1) cells, Prostaglandins, 16, 733(1978).

38. Walker, J. L., The regulatory function of prostaglandins in the release of histamine and SRS-A from passively sensitized human lung tissue, Adv. Biosci., 9, 235(1972).

39. Augstein, J., J. B. Farmer, T. B. Lee, P. Sheard and M. L. Tattersall, Selective inhibition of slow reacting substance of anaphylaxis, Nature (London), New Biol., 245, 215(1973).

40. Kaliner, M. and K. F. Austen, A sequence of biochemical events in the antigen-induced release of chemical mediators from sensitized human lung tissue, J. Exp. Med., 138, 1077(1973).

41. Koopman, W. J., R. P. Orange and K. F. Austen, Immunochemical and biologic properties of rat IgE. III. Modulation of the IgE-mediated release of slow-reacting substance of anaphylaxis by agents influencing the levels of cyclic 3',5'-adenosine monophosphate, J. Immunol., 105, 1096(1970).

42. Ishizaka, T., K. Ishizaka and H. Tomioka, Release of histamine and slow reacting substance of anaphylaxis (SRS-A) by IgE-anti-IgE reactions on monkey mast cells, J. Immunol., 108, 513(1972).

43. Lichtenstein, L. M. and A. G. Osler, Comparative studies of histamine release and potassium efflux from human leukocytes, Proc. Soc. Exp. Biol. Med., 121, 808 (1966).

44. Lichtenstein, L. M. and A. G. Osler, Studies of the mechanisms of hypersensitivity phenomena. IX. Histamine release from human leukocytes by ragweed pollen antigen, J. Exp. Med., 120, 507(1964).

45. Hastie, R., The antigen-induced degranulation of basophil leukocytes from atopic subjects, studies by phase contrast microscopy, Clin. Exp. Immunol., 8, 45(1971).

46. Kaliner, M., R. P. Orange and K. F. Austen, Immunological release of histamine and slow reacting substance of anaphylaxis from human lung. IV. Enhancement by cholinergic and alpha adrenergic stimulation, J. Exp. Med., 136, 556(1972).

47. Goadby, P., Current aspects of pharmacology: Asthma, anaphylaxis and allergy, Pharm. J., 216, 92(1976).

48. Morris, H. R., G. W. Taylor, P. J. Piper, P. Sirois and J. R. Tippins, Slow-reacting substance of anaphylaxis, purification and characteristics, FEBS Lett., 87, 203 (1978).

49. Chakravarty, N., A method for the assay of slow reacting substance, Acta Physiol. Scand., 46, 298(1959).

50. Dale, H. H. and P. P. Laidlaw, A method of standardising pituitary (infundibular) extract, J. Pharmacol. Exp. Ther., 4, 75(1912).

51. Högberg, B., G. Thufnesson and B. Uvnäs, Histamine liberation produced in the perfused paw of the cat by 48/80 and extracts from jellyfish (Cyanea capillata) and eelworm (Ascarishurum bricoides) from swine, Acta Physiol. Scand., 38, 135(1956).

52. Schlenk, H. and J. L. Gellerman, Esterification of fatty acids with diazomethane on a small scale, Anal. Chem., 32, 1412(1960).

53. Stodola, F. H., Base-catalyzed preparation of methyl and ethyl esters of carboxylic acids, J. Org. Chem., 29, 2490(1964).

54. Kaplan, N. O., Enzymatic determination of free sugars, in: Methods in Enzymology, ed. by S. P. Colowick and N. O. Kaplan, Vol. 3, pp. 109-110, Academic Press, N.Y., 1957.

55. Guiseby, K. B. and P. M. Ruoff, Monosaccharride sulfates. I. Glucose-6-sulfate. Preparation, characterization of the crystalline potassium salts, and kinetic studies, J. Org. Chem., 26, 1248(1961).

56. Sobel, A. E. and P. E. Spoerri, Steryl sulfates. I. Preparation and properties, J. Am. Chem. Soc., 63, 1259(1941).

57. Sobel, A. E., I. J. Drekter and S. Natelson, Estimation of small amounts of cholesterol as the pyridine cholesteryl sulfate, J. Biol. Chem., 115, 381(1936).

58. Huggett, A. St. G. and D. A. Nixon, Enzymatic determination of blood glucose, Biochem. J., 66, 12p(1957).

59. Andrews, P., Estimation of the molecular weights of proteins by Sephadex gel-filtration, Biochem. J., 91, 222(1964).

60. Engström, L., Studies on calf-intestinal alkaline phosphatase. I. Chromatographic purification, microheterogeneity and some other properties of the purified enzyme, Biochim. Biophys. Acta, 52, 36(1961).

61. Reiland, J., Gel filtration, p. 291, in: Methods in Enzymology, ed. by W. B. Jakoby, Vol. 22, Academic Press, N.Y., 1971.

62. Änggård, E. and K. Strandberg, Efflux of prostaglandin E_2 from cat paws perfused with compound 48/80, Acta Physiol. Scand., 82, 333(1971).

63. Wick, A. N., D. R. Drury, H. I. Nakada and J. B. Wolfe, Localization of the primary metabolic block produced by 2-deoxyglucose, J. Biol. Chem., 224, 963(1957).

64. Sutherland, E. W. and A. G. Robison, Metabolic effects of catecholamines. A. The role of cyclic-3',5'-AMP in responses to catecholamines and other hormones, Pharmacol. Rev., 18, 145(1966).

65. Butcher, R. W. and E. W. Sutherland, Adenosine 3',5'-phosphate in biological materials. I. Purification and properties of cyclic 3',5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3',5'-phosphate in human urine, J. Biol. Chem., 237, 1244 (1962).

66. Bourne, H. R., L. M. Lichtenstein, K. L. Melmon, C. S. Henney, Y. Weinstein and G. M. Shearer, Modulation of inflammation and immunity by cyclic AMP, Science, 184, 19(1974).

67. Orange, R. P. and K. F. Austen, Drug-induced modulation of the immunologic release of histamine and slow reacting substance of anaphylaxis, Int. Arch. Allergy and Appl. Immunol., 41, 79(1971).

68. Jakschik, B. A., S. Falkenhein and C. W. Parker, Precursor role of arachidonic acid in release of slow reacting substance from rat basophilic leukemia cells, Proc. Nat. Acad. Sci. USA, 74, 4577(1977).

69. Flower, R. J., Drugs which inhibit prostaglandin biosynthesis, Pharmacol. Rev., 26, 33(1974).

70. Hamberg, M. and B. Samuelsson, Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets, Proc. Nat. Acad. Sci. USA., 71, 3400 (1974).

71. Bach, M. K., J. R. Brashler and R. R. Gorman, On the structure of slow reacting substance of anaphylaxis: Evidence of biosynthesis from arachidonic acid, Prostaglandins, 14, 21(1977).

72. Dawson, W. and R. Tomlinson, Effect of cromoglycate and eicosatetraynoic acid on the release of prostaglandins and SRS-A from immunologically challenged guinea pig lungs, Br. J. Pharmacol., 52, 107P(1974).

73. Piper, P. J. and J. R. Vane, Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs, Nature (London), 223, 29(1969).

74. Liebig, R., W. Bernauer and B. A. Peskar, Prostaglandins slow-reacting substance and histamine release from anaphylactic guinea-pig hearts and its pharmacological modification, Naunyn-Schmiedeberg's Arch. Pharmacol., 289, 65(1975).

75. Orange, R. P. and K. F. Austen, The immunological release of chemical mediators of immediate type hypersensitivity from human lung, in: Progress in Immunology, edited by B. Amos, Academic Press, N.Y., 1971.

76. Folch, J., M. Lees and G. H. Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem., 226, 497(1957).

77. Cooper, M. J. and M. W. Anders, High pressure liquid chromatography of fatty acids and lipids, J. Chromatogr. Sci., 13, 407(1975).

78. McKenna, J. and J. K. Norymberski, Steroid sulphates. Part II. Cholestan-3β-yl methyl sulphate and cholesteryl methyl sulphate, J. Chem. Soc., 3893(1957).

79. McKenna, J. and J. K. Norymberski, Steroid sulphates. Part I. Some solvolytic reactions of the salts of steroid sulphates, J. Chem. Soc., 3889(1957).

80. Mayers, G. C., M. Pousada and T. H. Haines, Microbial sulfolipids. III. The disulfate of (+)-1,14-docosanediol in Ochromonas danica, Biochemistry, 8, 2981(1969).

81. Haines, T. H., The chemistry of the sulfolipids, pages 299-344, in: Progress in the Chemistry of Fats and Other Lipids, ed. R. T. Holman, Vol. XI, Part 3, Pergamon Press, N.Y., 1971.

82. Cohen, S. L. and I. B. Oneson, The conjugated steroids. IV. The hydrolysis of ketosteroid sulfates, J. Biol. Chem., 204, 245(1953).

83. Grant, G. A. and D. Beall, Studies on estrogen conjugates, Recent Prog. Horm. Res., 5, 307(1950).

84. Sirois, P., Inactivation of slow reacting substance of anaphylaxis (SRS-A) by lipoxidase, Prostaglandins, 17, 395(1979).

85. Butcher, R. W. and E. W. Sutherland, Adenosine 3',5'-phosphate in biological materials. I. Purification and properties of cyclic 3',5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3',5'-

phosphate in human urine, J. Biol. Chem., 237, 1244 (1962).

86. Middleton, E., Jr. and G. B. Phillips, Distribution and properties of anaphylactic and venom induced slow-reacting-substance and histamine in guinea pigs, J. Immunol., 93, 220(1964).

87. Roy, A. B., The synthesis and hydrolysis of sulfate esters, pages 205-235, in: Advances in Enzymology, Vol. 22, edited by F. F. Nord, Interscience Publishers, Inc., N.Y., 1960.

88. Roy, A. B., The hydrolysis of sulfate esters, pages 1-19, in: The Enzymes, Vol. 5, edited by P. B. Boyer, Academic Press, N.Y., 1971.