The Structure Elucidation of Slow-Reacting Substance of Anaphylaxis

Graham W. Taylor

A dissertation submitted for the degree of Doctor of Philosophy of the University of London

Department of Biochemistry,
Imperial College of Science and Technology,
South Kensington,
London SW7.

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ABSTRACT

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Slow-reacting substance of anaphylaxis (SRS-A) is a primary mediator of immediate type hypersensitivity reactions probably playing a major role in allergic bronchospasm (asthma) in man. Although it was discovered forty years ago, its structure has remained unknown.

Slow-reacting substance of anaphylaxis from guinea-pig lung, together with non-immunologically generated slow-reacting substances (SRS's), have been purified to homogeneity for the first time by the introduction of reverse-phase high pressure liquid chromatography, with its attendant high resolution separative capability. It has been demonstrated that SRS-A possesses a characteristic ultraviolet spectrum arising from a triply conjugated triene chromophore, allowing for the first time a direct correlation between biological activity and a structural moiety in SRS-A.

Slow-reacting substance of anaphylaxis from guinea-pig lung has been compared extensively with material generated from human lung, rat peritoneum and rat basophil leukaemia cells and found to be pharmacologically, physico-chemically and chromatographically indistinguishable.

Microgramme quantities of SRS-A from guinea-pig lung and an SRS generated non-immunologically from rat basophil leukaemia (RBL-1) cells have been purified to homogeneity and characterised spectroscopically (mass spectrometry) and by analytical protein chemistry; the structure of these materials has been unequivocally defined as the novel peptidolipid:

5 - hydroxy - 6 - cysteinylglycinyl - 7,9,11,14 - eicosatetraenoic acid.

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for Sue

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I INTRODUCTION

Slow-reacting substance of anaphylaxis*A (SRS-A) is a biologically active material with smooth muscle contracting properties, immunologically released from lung during anaphylaxis; it is a primary mediator of immediate type hypersensitivity reactions, probably playing a major role in allergic bronchospasm in man. The involvement of SRS-A as a probable cause of the bronchoconstriction associated with asthma lends medical relevance to a study of the genesis, purification and structure elucidation of this material.

A slow-reacting substance (SRS)*B was first described in 1938 by Feldberg and Kelleway (1) who obtained smooth muscle contracting activity after treatment of various tissues (including guinea-pig lung) with cobra venom; on assaying this crude material on guinea-pig ileum, a slow and delayed contraction, distinct from that caused by acetylcholine or histamine, was observed.

A material with a similar tissue response was obtained two years later (2) from perfused sensitised guinea-pig lung, during anaphylaxis initiated by antigen challenge; this material was termed slow-reacting substance of anaphylaxis (SRS-A)*B to distinguish it from the non-immunologically produced material originally obtained.

*A Anaphylaxis is a condition of hypersensitivity to foreign proteins and arises from an antigen-antibody interaction

*B It is customary to refer to the "family" of materials with biological activity as originally described by Feldberg and Kelleway as slow-reacting substances (SRS's). Immunologically generated material is referred to as slow-reacting substance of anaphylaxis (SRS-A); when no distinction between SRS's and SRS-A is implied the term SRS(-A) may be used.

This distinction has been of historical importance because, until very recently, there was no evidence of chemical identity or otherwise of SRS or SRS-A from different sources. Only the immunologically generated material is an accepted model for asthma, which is usually triggered by antigen (e.g. pollen, house dust).

Although the immunologically and non-immunologically produced materials could not be pharmacologically distinguished, it was not possible in these early studies to show whether they were chemically identical. No evidence was adduced as to the structure of SRS(-A).

Following the work of Feldberg and Kelleway (1) and Kelleway and Trethewie (2), two distinct methods for generating SRS bioactivity have been developed, either as (a) a result of antigen-antibody reaction to produce SRS-A or (b) by action of non-immunological stimuli (e.g. mechanical, drugs etc.) producing an SRS :

(a) Immunologically generated slow reacting substance of anaphylaxis

SRS-A may be immunologically released from many tissues including human lung (3,5), but the most favoured tissue for large scale production is guinea-pig lung, (the guinea-pig is extremely prone to anaphylaxis).

In early studies, Brockelhurst (4) found that SRS-A was released concomitantly with histamine from actively sensitised guinea-pig lung on antigen challenge. It was distinguished pharmacologically from histamine, (and other mediators) by use of specific blocking agents (e.g. antihistamines) on the assay tissue. SRS-A is biosynthesised in an antigen-antibody reaction and is released immediately on formation (this is in contrast to histamine which is stored until release). Release of SRS-A in this system appeared to be independent of the nature of antigen used, and reached a peak after that of histamine.

Following this work, SRS-A was immunologically produced and pharmacologically characterised from a variety of tissues including human lung (5) human leukocytes (6) and rat peritoneum (7). Although indistinguishable in biological action from guinea-pig SRS-A, no evidence was adduced as to their chemical nature or co-identity.

Studies on the potentiation of release of SRS-A indicated a possible precursor role for thiols. Although nothing was known regarding the precursor of SRS-A, Orange and colleagues (5,8) found that exogenous L-cysteine or DL penicillamine led to an increased release of SRS-A from human leukocytes, and passively sensitised monkey and guinea-pig lung fragments. It was suggested that the effect arose at the antigen-antibody level but could not be attributed to either the redox potential of the sulphydryl compounds, or their involvement as precursors.

By the use of specific blocking agents Austen and Orange (9,10) elucidated the biochemical sequences leading to SRS-A release, and indicated the involvement of antigen binding, esterase activation together with an energy and calcium requirement; no further precursor data were available.

Turnbull et al (11), working with human lung suggested that SRS-A is present as a preformed mediator. These results, however, were based on the premise that such a 'preformed mediator' could be extracted by freezing and thawing and that this extraction would not lead to synthesis and release of an SRS; this, in the light of studies on the non-immunological release of SRS's, appears unlikely.

(b) Slow-reacting Substances

Slow-reacting substances have been obtained non-immunologically from various sources; many of the stimuli that release SRS are the same ones that release histamine. Chakravarty et al (12) used the histamine releasing compound 48/80 to obtain an SRS from cats-paw, this study being extended by Angaard et al (13), and Strandberg and Unvas (14). More recently (1974) Bach and Brashler (15) introduced the use of calcium ionophore A23187 (a material previously shown to cause histamine release); SRS was obtained on injection of the ionophore into rat peritoneum. This work has been extended by Conroy et al (16) for human leukocytes and Jakschick et al (17) for rat basophil-leukemia (RBL-1) cells. The use of ionophore A23187 has now become the standard method for non-immunological production of SRS's.

Purification of SRS(-A)

Although the slow-reacting substances appeared to be pharmacologically identical, statements regarding their co-identity could not be made on the impure material available; it was necessary to purify the SRS's from both a comparative and structural view point. Strandberg and Unvas (13) introduced a purification scheme for cats-paw SRS involving ethanol partition, ether extraction silicic acid and anion exchange chromatography. The partially purified material so obtained was subjected to chemical tests, to attempt to determine those functional groups essential for bioactivity (see later). In this study, the investigators noted that pre-incubation of the cats-paw with sulphydryl-blocking reagents prevented SRS release, further evidence linking thiols with SRS biosynthesis.

This purification scheme was extended by Orange et al (18) to include base hydrolysis, amberlite resin adsorption and sephadex LH20 gel filtration; the ether extraction step was discontinued following reported low yields. Material so obtained was dialysable, and had an apparent molecular weight of 350-450. Physico-chemical analysis was by mass spectrometry, and infra-red and ultraviolet absorption spectroscopy. The procedure introduced by Orange et al became, with minor modifications, the standard high yield method for preparing "purified" SRS(-A).

Structural Studies

The original observation that cobra-venom, (a source of phospholipases and phosphoesterases), caused SRS release, led to the suggestion that the material was lipid derived. Chemical activation studies on cats-paw SRS (13) indicated that unsaturation, hydroxyl and carboxyl groups were essential for 48/80 induced SRS bioactivity. Ether extraction of bioactivity from acidified (pH3) aqueous solutions (11) was consistent with the presence of a free carboxyl group(s) in SRS. The authors noted that the presence of amino groups could not be precluded by the chemical tests used, but appeared unlikely following the acidic nature of the material. Incubation with 15 hydroxy prostaglandin dehydrogenase did not reduce bioactivity. In this study the polarity of SRS was judged to be similar to that of a phospholipid; specific assays however indicated no significantly higher level of phosphorus in active

samples compared to controls.

In Orange's SRS-A purification scheme, biological activity was fully retained in the base hydrolysis step, thus ruling out the presence of some classes of compound (e.g. phospholipids). Electrophoretic data were consistent with the presence of carboxyl groups. Purified SRS-A obtained by Orange was analysed by mass spectrometry, i.r. and u.v. spectrophotometry, but no data of structural value were obtained.

SRS(-A) had been distinguished from other common mediators (5 HT, acetylcholine), various biologically active peptides (19), phospholipids (18), neuraminic acid (13) and known prostaglandins (11).

Incubation with arylsulphatase (arylsulphate sulphohydrolase E.C.3.1.6.1.) caused inactivation of SRS-A biological activity (20); SRS-A acted as a competitive inhibitor of aryl sulphatase destruction of known substrates of this enzyme (e.g. nitrocatechol sulphate), indicating that SRS-A was itself a substrate for arylsulphatase), and that inactivation was not a phenomenom of protein binding. It was suggested that arylsulphatase inactivation of SRS-A plays a significant role in its in vivo control. (21)

Arylsulphatases catalyse the cleavage of sulphate ester linkages from benzenoid rings; these data suggested that SRS-A and similarly inactivated SRS's (21) were lipid-sulphate derivatives.

No further structural data were available on SRS-A (or SRS's) at this time.

Although the variously produced slow-reacting substances (including SRS-A) appeared pharmacologically and physico-chemically identical, no direct high resolution chromatographic comparison had been undertaken. Some conflicting statements regarding the similarity (21) or dissimilarity(22) of SRS's had been made based on the physico-chemical data available. The existence of multiple forms of SRS(-A) biological activity had been reported (22) although these may have been due to artefacts of purification or as a result of adsorption to carrier molecules.

Purification, comparative and structural studies on SRS-A and SRS's were hampered by the low quantities of material available (active below the nanogram level (18)) and the necessity of using a biological assay for detection. The specificity of the bioassay was greatly improved by the introduction of the antagonist FPL 55712 (Fisons Pharmaceutical Laboratory (23)) which specifically and completely reversed the SRS-A induced contraction of guinea-pig ileum; similar antagonism was observed with non-immunologically produced SRS's. By inclusion of this antagonist in SRS(-A) bioassays, rapid differentiation of SRS(-A) on ileum from other spasmogenic materials was possible, FPL 55712 reversal of bioactivity becoming an important criterion for identification of SRS(-A).

FPL 55712 is unfortunately unsuitable for in vivo antagonism because of its lability.

Biological criteria for SRS-A

The major biological criteria for the identification of SRS-A are outlined below:-

SRS-A contracts guinea-pig ileum, guinea-pig trachea and human bronchus; it releases thromboxanes and prostaglandins from guinea-pig lung; the action of SRS-A is completely antagonised by FPL 55712.

Evidence for the role of SRS-A in asthma

The importance of studying the slow-reacting substances (in particular SRS-A) lies in their putative pathological role in asthma.

By development of antagonists (antihistamines) and enzyme inhibitors (asprin) it was shown that histamine and prostaglandins account for only a part of the bronchoconstriction associated with asthma. (24,25) The major cause of bronchoconstriction was believed to be associated with a mediator of unknown structure, obtained experimentally from lung on antigen challenge (SRS-A); its role has been indicated by many in vivo and in vitro studies:

It was observed by Berry and Collier that a partially purified (ex-charcoal) sample of SRS-A caused bronchoconstriction in the same species in vivo (19).

In an extension of this work, the contractile effect of SRS-A on the smooth muscle of the trachea and bronchioles in man was observed (26); human bronchiole smooth muscle appeared to be more sensitive to SRS-A than was tissue from other species (27). SRS-A prepared from guinea-pig lung and administered by aerosol to human asthmatics resulted in bronchoconstriction, suggesting SRS-A was 'species independent' (28).

It was observed that SRS-A appeared in the blood of asthmatic children after aerosol administration of antigen (29). It was argued that the plasma SRS-A must have derived from an antigen-antibody reaction on the lung surface, thus further implicating SRS-A in the asthma condition.

SRS-A also showed biological action not directly related to bronchoconstriction (e.g. lowering of blood pressure (30)).

The above tests were undertaken using crude SRS-A, and thus the biological effects observed may have derived from the undoubted impurities present. These data obtained implicating SRS-A in allergic bronchospasm in man could not be considered conclusive.

Summary

On commencing this study in late 1976 a role for SRS-A in the asthma condition had been suggested, although all data were obtained on crude samples; SRS-A had not been purified to homogeneity. Non-immunologically generated slow-reacting substances had been prepared and initial statements on their co-identity or dissimilarity with SRS-A had been made with the crude preparations available. No direct comparative study had been undertaken as no high resolution purification procedure was available.

The structure of SRS-A was unknown; however certain structural inferences had been made. SRS-A was probably not a known prostaglandin or phospholipid; chemical and enzymic inactivation data had indicated SRS-A was probably an acidic, unsaturated hydroxylipid, probably containing a sulphate ester moiety.

Further structural characterisation of SRS-A, essential for a full understanding of its putative role in asthma, awaited the preparation of pure material; this required the introduction of a more sophisticated purification procedure than hitherto available.

II RESULTS AND DISCUSSION

During this study (1976-80) a high resolution purification procedure for SRS(-A) was developed and SRS-A from guinea-pig lung, together with other slow-reacting substances (including human lung SRS-A) were purified to homogeneity; chromatographic, pharmacological and physico-chemical comparisons of the SRS's were undertaken.

Larger quantities of guinea-pig SRS-A and RBL-1 SRS were purified to homogeneity and analysed spectroscopically (including mass spectrometry) and with analytical protein chemical techniques. The complete covalent structures of these materials were deduced and are reported here.

1. Purification

The purification procedures available for SRS-A (1976) had not resulted in the generation of homogeneous material, and thus a new approach was required to deal with the small quantities (ng - μg range) of this reportedly labile material.

The following problems are encountered during the purification of very small quantities of biologically active molecules:

(a) losses by adsorption and absorption onto both vessel surfaces and impurities

(b) destruction by chemical or enzymic contaminants; the lability of many biologically active materials following release can result in unacceptable losses during purification

(c) samples may only be monitored by biological assay, with great reliance placed on the 'specificity' of such an assay.

The following purification scheme for guinea-pig SRS-A and related SRS's was designed to minimise losses through adsorption, destruction etc., whilst maximising physical data relevant to the structure of the material. Throughout this purification, salt-free buffers have been employed for compatibility with bioassay and mass spectrometric analysis.

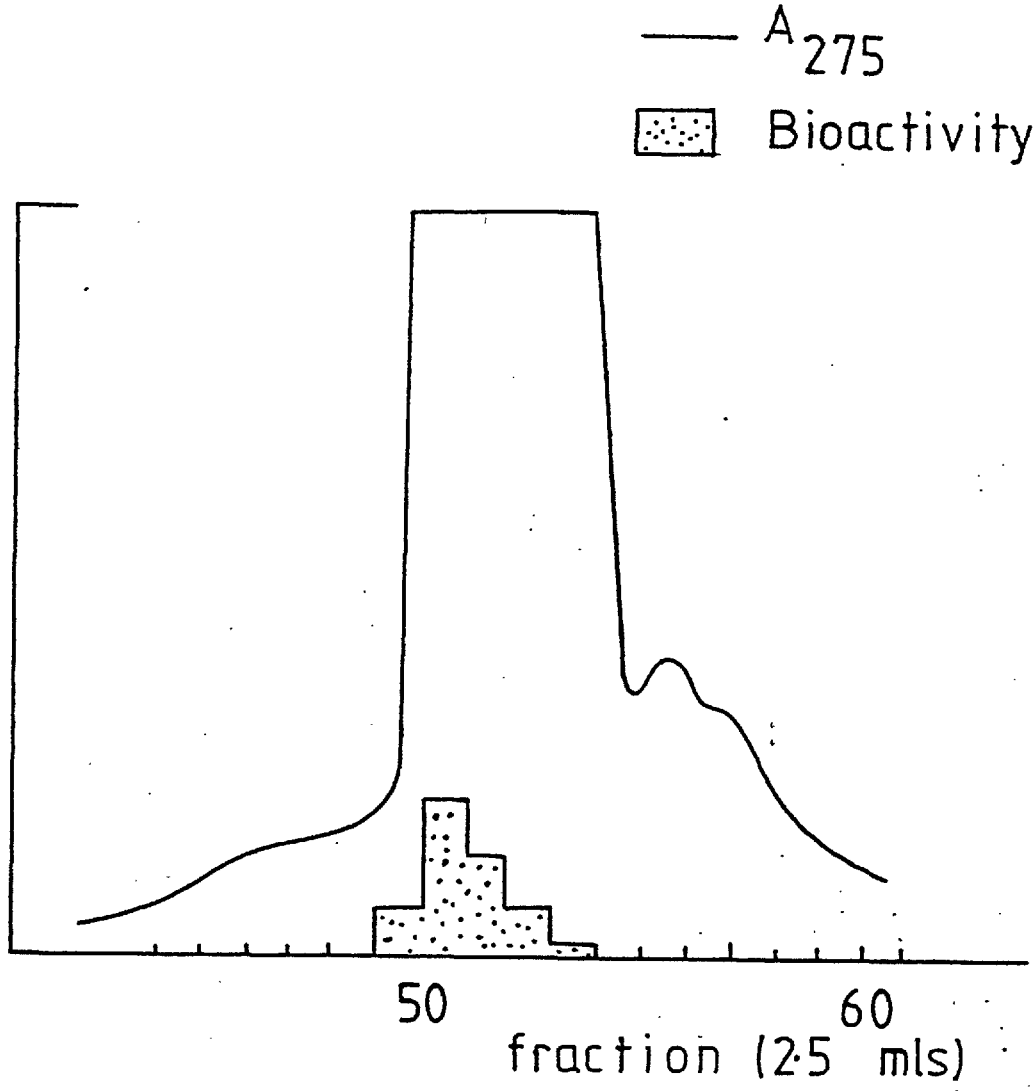

Figure 1

Sephadex G-15 gel filtration profile of guinea-pig SRS-A (methanol : ammonia : water, 2:2:1 v/v). Biological activity elutes as a single band (Vav/Vo = 2.3) together with tyrosine and other A275 absorbing material indicating the low molecular weight of this material.

Slow reacting substance of anaphylaxis and related SRS's were obtained from the Royal College of Surgeons as lyophylised powders (after an initial charcoal adsorption step), and stored in this laboratory, under nitrogen, at -20°C until required. This material was presumed to be virtually free of histamine and the major prostaglandins, as judged by biological and spectrofluorimetric assay.

The purification procedure developed in this laboratory for the purification of SRS-A is outlined below: (31).

(i) Sephadex G15 gel filtration
(ii) Ether extraction (PH3)
(iii) Reverse phase high pressure liquid chromatography

Samples were monitored for FPL 55712 - reversible biological activity (ileum assay) at each step.

(i) Sephadex G15 gel filtration: the reported molecular weight of SRS-A (200-500 (18)) is well within the fractionating range of Sephadex G15. Separation from salts and higher molecular weight impurities (>1000 mass units) would be possible in this system.

Biological activity elutes as a single peak on Sephadex G15 gel filtration together with tyrosine and phenylalanine (amino-acid analysis) which are responsible for the strong ultraviolet absorbance (A275) in the G15 profile [fig 17]. The elution position of SRS-A varied somewhat with the quantity of sample loaded (V/Vo = 2.2 - 2.6) with small batches of material eluting earlier than larger batches; an explanation for this behaviour is given in section 3:(iii).

The elution position of SRS-A (together with tyrosine) gives an indication of the low molecular weight of the material (200-400 daltons), however the possibility of retention of hydrophobic material, resulting in anomalous mol.wt. data, was noted.

The elution solvent (methanol: water: ammonia) led to enhanced stability of SRS-A during this purification step with yields substantially in excess of 90%.

(ii) Ether Extraction: The original observation that SRS-A could be extracted into ether at pH3 indicated that ether extraction might be a suitable step in the purification of this material. Although abandoned by other workers because of the low yields of SRS-A obtained, studies in this laboratory showed that rapid extraction at 0°C with peroxide-free ether resulted in a high yield extraction (>85%) with no biological activity remaining in the aqueous phase. Most of the solids present after G15 gel filtration (polar non-acidics and salts) were removed by this step. The poor yields obtained by others may have been due to peroxides present in the ether (resulting in oxidation of unsaturated linkages present (13)), acid inactivation of SRS-A or the state of purity of the sample (section 3 (iii)). Ether extraction at pH3 is consistent with the presence of free carboxyl groups (pk 3-4) in the molecule, but brings into question the existence of a sulphate ester group earlier postulated (20). A sulphate group would be expected to remain ionised at pH3 thus enhancing water solubility of SRS-A (See also sections 6,7, 9 ,12).

(iii) Electrophoresis: High voltage paper electrophoresis was carried out earlier in this laboratory; poor yields from the anodically mobile (therefore acidic) material at pH 6.5 led to the discontinuation of electrophoresis for the purification of SRS-A. The mobility of SRS-A at pH 6.5 is, however, consistent with a molecular weight of 500 (1 charge) or 1000 (2 charges) : M = - 0.27, Masp = -1.0 (31)

(iv) High pressure liquid chromatography: The physicochemical data on SRS-A available in 1976 indicated that SRS-A was of lipid origin (12). On considering the purification methods available for such hydrophobic compounds, it appeared that the method of choice would be reverse phase high pressure liquid chromatography. This relatively new technique has unquestionable advantages over classical

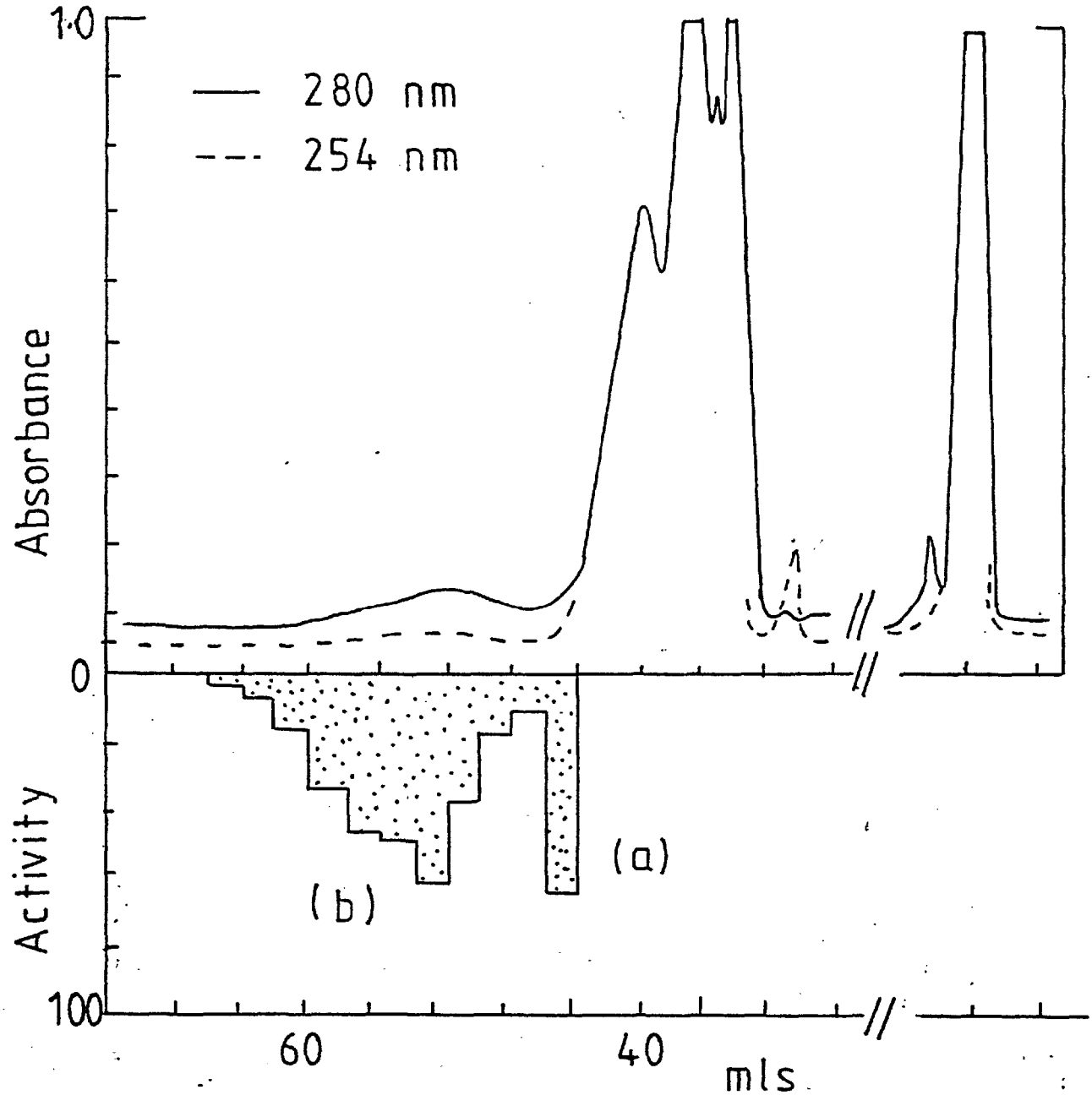

Figure 2

Reverse-phase HPLC-1 elution profile of guinea-pig SRS-A (μ Bondapak C_{18} , 30 x 0.4 cm); biological activity elutes as two distinct, but interconvertible peaks, and was always associated with A_{280} U.V. absorbance.

purification methods then used in this field, both in terms of resolution and speed (e.g. 32). A study of the known physico-chemical properties of SRS-A (e.g. solubility in less polar solvents on protonation) resulted in a suggested methanol: water HPLC elution system: SRS-A is fully soluble in methanol, but less soluble in water in its protonated form (ex ether). The elution system chosen was 50% aqueous methanol (10mls) followed by a linear gradient (20 mls) to methanol; SRS-A eluted in 80-100% methanol.well separated from other impurities. The design of a purification system based on this novel step led to the removal of many contaminating compounds (as judged by the u.v. absorption profile) yielding for the first time extremely pure material which fulfilled all the criteria for SRS-A activity.

In early studies in this system (methanol: water gradient) biological activity was observed to elute at the end of the gradient (100% methanol) in two well defined peaks [Fig 2]; the elution position being in accordance with the lipophilicity of the molecule (12). The two peaks of activity are interpreted as arising from protonated (b) and unprotonated (a) forms of SRS-A. On acidifying the unprotonated material (eluting earlier from the reverse phase column as would be expected for a more polar ionised carboxylic function), activity chromatographs in a position corresponding to protonated SRS-A.

The existence of these two forms of SRS-A is in agreement with the behaviour of this material during electrophoresis and ether extraction. It is also possible that some other reversible chemical change is occurring on acidification (e.g. lactonisation) leading to a change in the elution position. On increasing the volume of both ether and pH3 acetic acid, thus ensuring a good supply of protons in the ether phase, the unprotonated form of SRS-A was no longer observed.

During later purification studies, biological activity was observed to elute somewhat earlier from the column than before, presumably due to column aging (i.e. non-specific binding to the C18 resin with resultant loss of hydrophobicity); it was still possible, however, to obtain a similar degree of purity for SRS-A

produced (as judged by u.v. absorbance spectrum, section 2).

The average yield in the above purification is approximately 30-40% based on the assay of ex-charcoal material; the lowest yield step in the complete procedure is the initial charcoal adsorption step (Royal College of Surgeons, < 30% yield). Investigations into other methods suitable for use instead of charcoal were undertaken.

(v) Pre-purification studies: To overcome the poor yields associated with charcoal adsorption, studies into different adsorbents were undertaken. In studies by other groups, notably Orange et al (19), adsorption onto Amberlite resins (XAD2, XAD7) was used; this resulted in their hands, in good yields (>60%) of histamine-free SRS-A.

SRS-A was passed onto an XAD8 preparation in Tyrode, and the resin thoroughly washed with water to remove histamine, salts etc. On elution with ethanol however, little biological activity was obtained; most activity was associated with the aqueous phase. [Fig. 3] Inability to obtain the reported yields may have been due to the batch variability of XAD resins, or differences in handling the resin.

This experiment was repeated with glass beads, Biobeads S x 8 (common adsorbants), and with a Waters C18 sep pak (containing the same C18 hydrophobic phase found in reverse phase HPLC columns); In no case were reproducible yields obtained [Fig. 3] equivalent to those found for the charcoal adsorption step, and these studies were discontinued.

Ethanol precipitation is another early purification step widely used in the SRS-A purification field (17,18); this unfortunately requires such large volumes of solvent (2L per 20 guinea-pigs) that its use was considered inappropriate for our purpose.

Further studies on charcoal adsorption indicated that better yields (60%) could be obtained on re-using the charcoal. Presumably non-specific binding of SRS-A is occurring on the first use (thus blocking

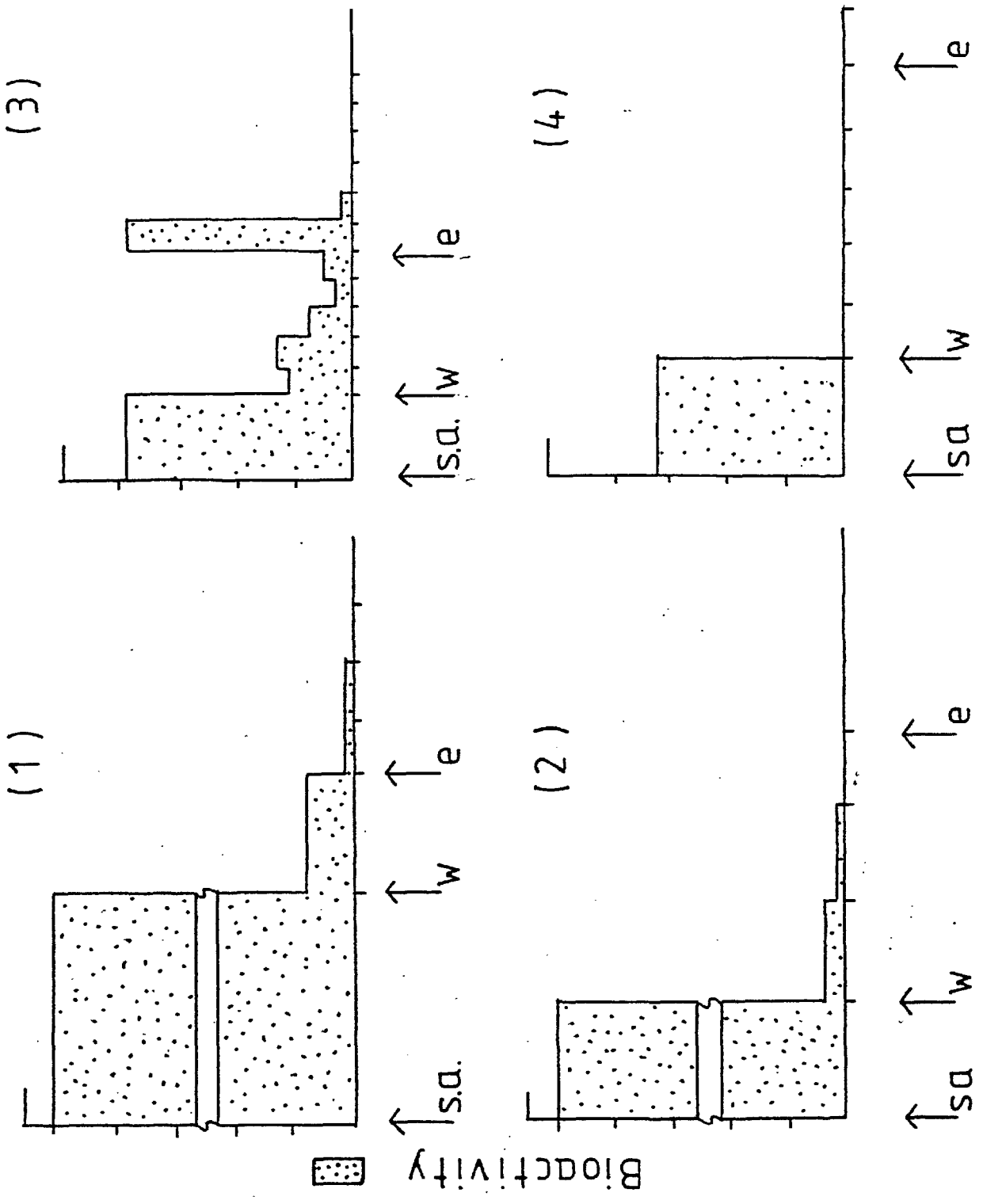

Figure 3

Pre G-15 purification studies on SRS-A with adsorbants other than charcoal:

1. Glass beads
2. Biobeads SX_8
3. Amberlite XAD_8
4. Waters Sep-pak

s.a.: sample applied
W : water wash
e : ethanol wash

In no case was SRS-A (substantially histamine free) obtained in yields greater than could be obtained with charcoal.

the active sites) leading to reduced binding on subsequent charcoal adsorptions. To denature proteolytic and other enzymes present on the re-used charcoal, it was boiled in water and stored under vacuum after each use. This method became the standard pre-purification step.

The full purification scheme reported here (the first involving reverse phase HPLC) is extremely rapid (48h total time); this is essential to avoid losses of the reportedly extremely labile SRS-A during storage etc.

2. Ultraviolet Spectrum

After high pressure liquid chromatography (section 1 (iv)) it was observed that SRS-A bioactivity always coincided with a weak u.v. absorbance at 280 and 254 nm (I 280 > I 254) at all elution positions. This indicated that SRS-A may have an ultraviolet absorption spectrum.

A large scale preparation of SRS-A from guinea-pig lung was undertaken resulting in $\sim$ 200 units of purified SRS-A. The full ultraviolet absorption spectrum of this material was taken in methanol (HPLC solvent), and a broad triplet was observed (λ max 280 $\pm$ 1nm with shoulders at $\sim$ 270 and 290 nm, Fig 4. (31)). The purity of SRS-A at this stage was evident from the symmetrical u.v. absorbance trace at both wavelengths ex HPLC, and the correlation with the observed u.v. spectrum. This is also apparent when this spectrum is compared with the only other published u.v. spectrum (which was obtained on only partially purified SRS-A) (Fig. 4 inset (18)). This earlier spectrum could not in fact provide evidence as to any structural moiety in SRS-A or whether SRS-A possessed a u.v. spectrum or not.

Initial speculation on structures suggested by our spectrum implied"aromatic or other highly conjugated chromophores" (31); further studies showed, by the subtraction of control-blanks, that the high end absorbance in the spectrum was probably due to impurity, and that SRS-A absorbed to give a broad triplet with λ max 280 $\pm$ 1nm. (33). The spectrum showed some resemblance with the

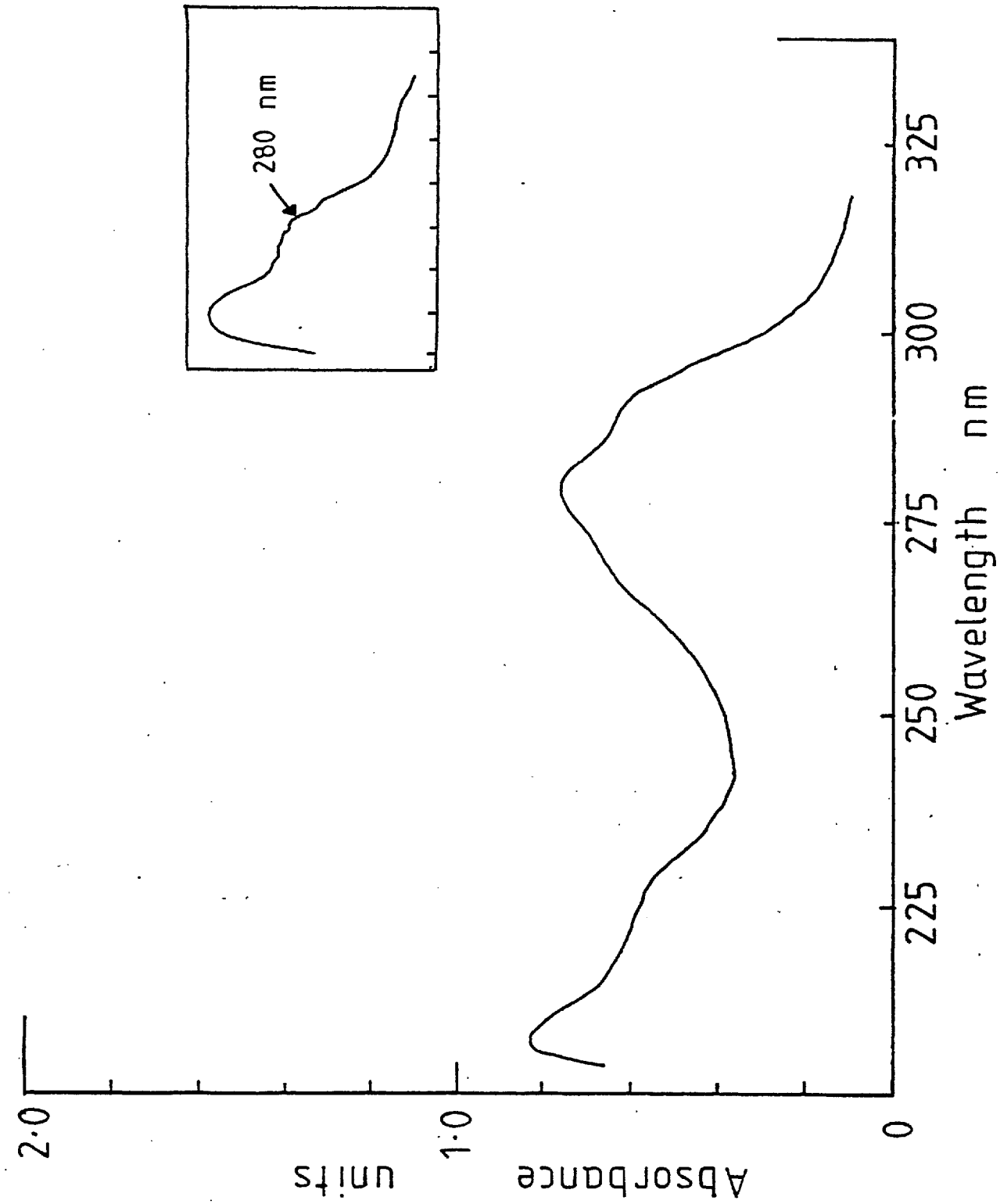

Figure 4

Full ultraviolet absorption spectrum of SRS-A in methanol (200 units, 10 mm path length) showing the characteristic triplet with λ max 280 ± 1 nm.

Inset: U.V. absorption spectrum of partially purified SRS-A taken from Orange et al (1973) (18).

spectra of certain hydroxylated conjugated trienes related to arachidonic acid (34) but with the λ max shifted bathochromically by 10 nm.

This spectrum is consistent with the presence of a conjugated triene modified by the attachment of a non-conjugated auxochrome. The fine structure observed in methanol in the spectrum discounts the presence of a conjugated ketone (PG B, for example) such structure collapsing to a smooth absorption peak in polar solvents (i.e. methanol).

3. Comparative Studies on SRS-A and SRS's

Slow-reacting substances (SRS's) produced by non-immunological stimuli have been obtained from many sources (e.g. 15, 16, 17) but the co-identity of these materials with SRS-A had not been shown. Statements have been made regarding the similarity of SRS's (21, 22) although this work was carried out on grossly impure samples, using chromatographic techniques incapable of the necessary resolution.

To determine whether these substances are chemically identical, and thus show the validity of animal studies to human SRS-A (and by inference to asthma), a comparative study between guinea-pig and human SRS-A with rat peritoneal SRS was undertaken. The SRS's were compared pharmacologically physio-chemically and chromatographically (HPLC). (33)

(i) Biological Properties: Tests carried out at the Dept. Pharmacology, Royal College of Surgeons indicated that the SRS's were pharmacologically indistinguishable.

(ii) Physico-Chemical Properties: Some basic physico- chemical properties of the SRS's were examined and the comparative data outlined below; the materials were indistinguishable by these tests:

i.	Stable to boiling	)
ii.	Stable to base	) ex charcoal
iii.	Destroyed by acid (pH 1)	)
iv.	Soluble in ether at pH 3	ex G15
v.	Water soluble at pH 7	

Lack of material, especially in the case of human SRS-A precluded further examination by activity destruction experiments.

(iii) Purification : Each SRS was purified to apparent homogeneity by the method reported (section 1). The amount of SRS's available in this study precluded observation in the ultraviolet at the HPLC stage, with all SRS elution positions reported here based on FPL 55712 - reversible guinea-pig ileum contractions.

The G15 gel filtration profiles, and comparative elution positions of the SRS's are shown in Fig. 5. Non-coincident activity profiles were observed suggesting greatly differing molecular weights of these species. Biological activity however always co-eluted with u.v. absorbing material at 275 nm, (tyrosine etc.) suggesting that the different elution positions are arising from a non-specific binding phenomenon, related to the purity of the samples used. On rechromatographing peak tubes of activity ex-G15 (the first step having greatly purified the SRS(-A)) a co-incident, and earlier, elution profile was obtained [Fig. 5a-inset].

It appears that very large or impure samples of SRS are retained more strongly by the column, (see also section 1 (i)), thus the more heavily contaminated human material (as judged by total solid present), elutes well after guinea-pig SRS-A or rat SRS.
This behaviour is interpreted as arising from non-specific binding of impurities to the gel and SRS(-A) resulting in differences in the elution position and apparent molecular weight of the SRS's.

Following gel filtration, the active samples were extracted into ice-cold peroxide-free ether. Good yields (>70%) were obtained in each case with no bioactivity remaining in the aqueous phase.

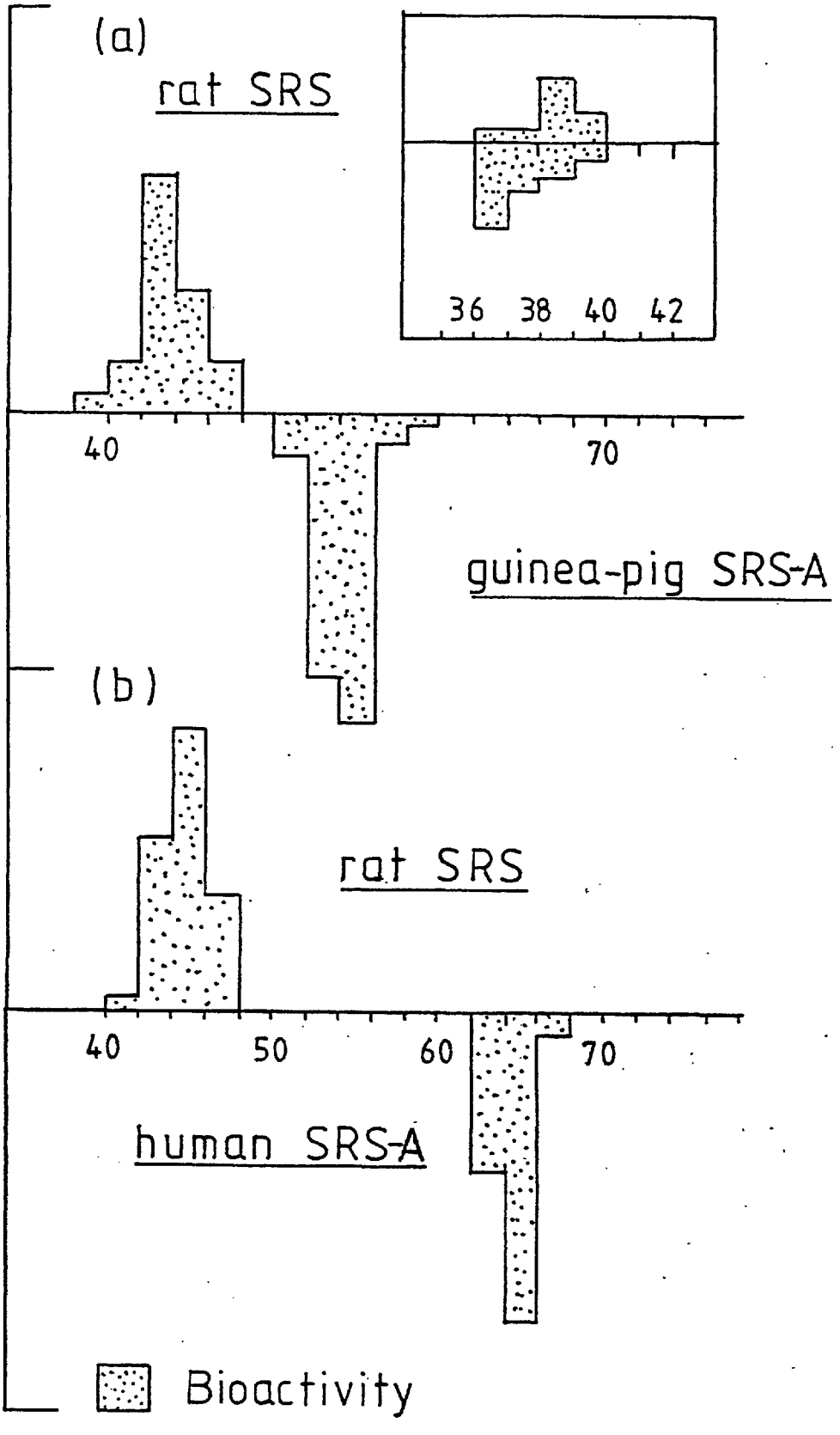

Figure 5

SRS(-A) comparative study: Sephadex G-15 bioactivity profiles of:

(a) guinea-pig SRS-A and rat peritoneal SRS. Inset: rechromatography on G15 of these materials showing co-elution.

(b) human SRS-A and rat peritoneal SRS.

In each case bioactivity coelutes with A_{275} absorbing material (tyrosine etc.).

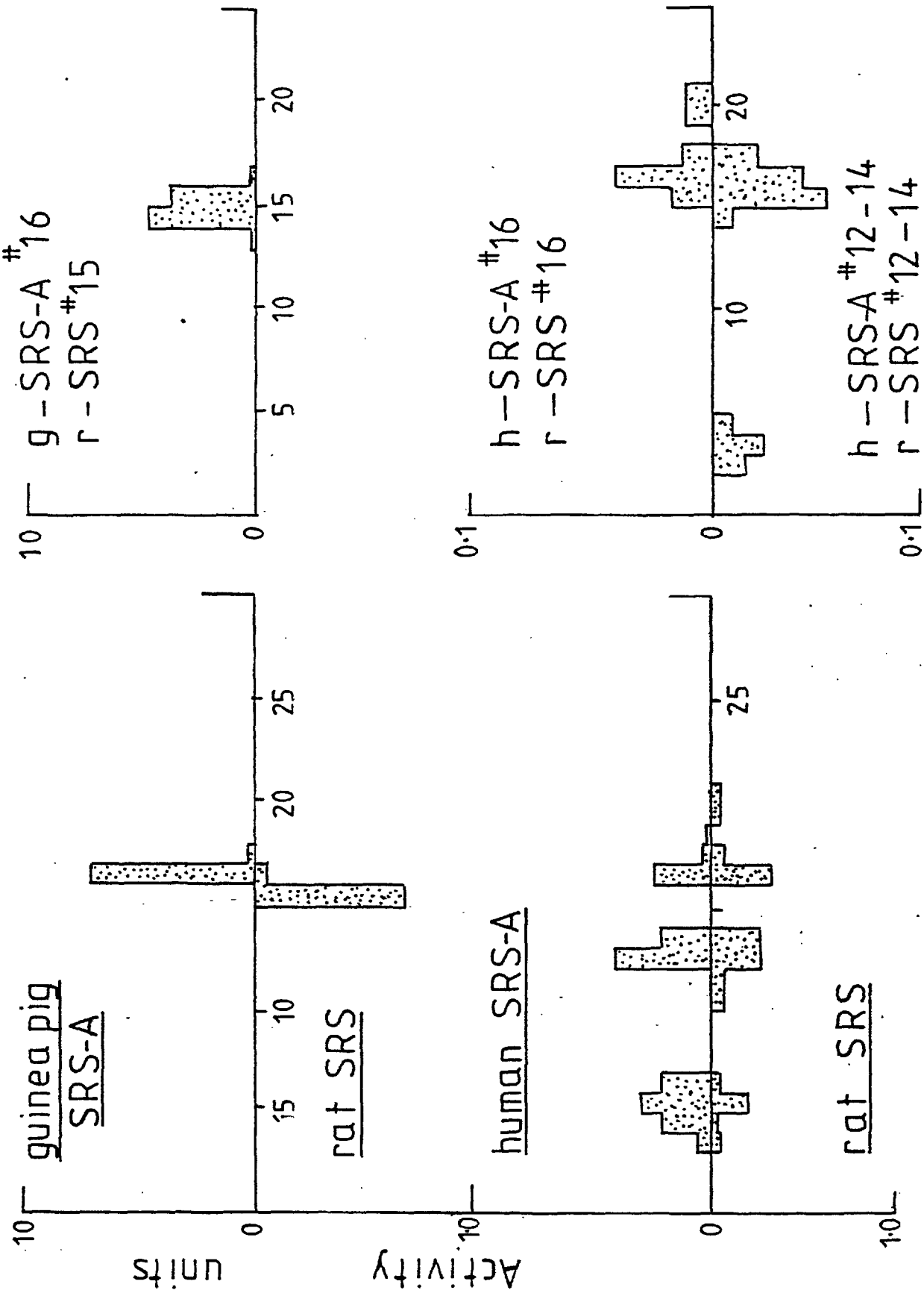

Figure 6

SRS(-A) comparative study: reverse-phase HPLC elution profiles of rat peritoneal SRS, human and guinea-pig SRS-A separately and mixed showing chromatographic co-identity of these SRS's (μ Bondapak C_{18} , 30 x 0.4 cm, 2 ml fractions).

The data on ether extraction of human SRS-A are in contradiction to the work of Takahashi et al (22) who found "human SRS-A, unlike that of cat and guinea-pig, could not be extracted into diethyl ether under acidic conditions". Extraction into ether does however depend on the state of purity of the sample; in these studies it was found that activity could not be extracted into ether unless the SRS(-A) was first partially purified (ex-G15). Attempts to extract neat lung effluent always failed, as a triphasic system was obtained, with most activity residing in the aqueous and middle gel-like phases. This behaviour is probably a result of surfactant impurities binding to SRS-A and enhancing its water solubility, and may explain the findings of Takahashi et al.

Active samples (ex-ether) were finally chromatographed on the reverse phase HPLC column (section 1 (iv)) in consecutive runs [Fig. 6]; to eliminate instrument fluctuations, equal quantities of SRS's from each peak tube were mixed and re-chromatographed.

Single bands of bioactivity, eluting in the same position, were observed in the rat/guinea-pig comparison. On mixing the materials, a single band of bioactivity was obtained, suggesting close chemical similarity of these SRS species.

On chromatographing the much smaller quantity of human SRS-A (with a deliberately low level of rat SRS as control) in the human SRS-A/rat SRS comparative study, multiple peaks of activity were observed (although bioactivity profiles were similar in both cases). On rechromatographing separate areas of bioactivity (i.e. ‡16 and ‡12-14), co-incidence was again noted. These multiple peaks of activity are interpreted as arising from artefacts produced during the purification of minute quantities of labile biological materials, and are not observed when chromatographing larger quantities (e.g. rat/guinea-pig comparison). Thus rat SRS and human SRS-A are also chromatographically similar in this system.

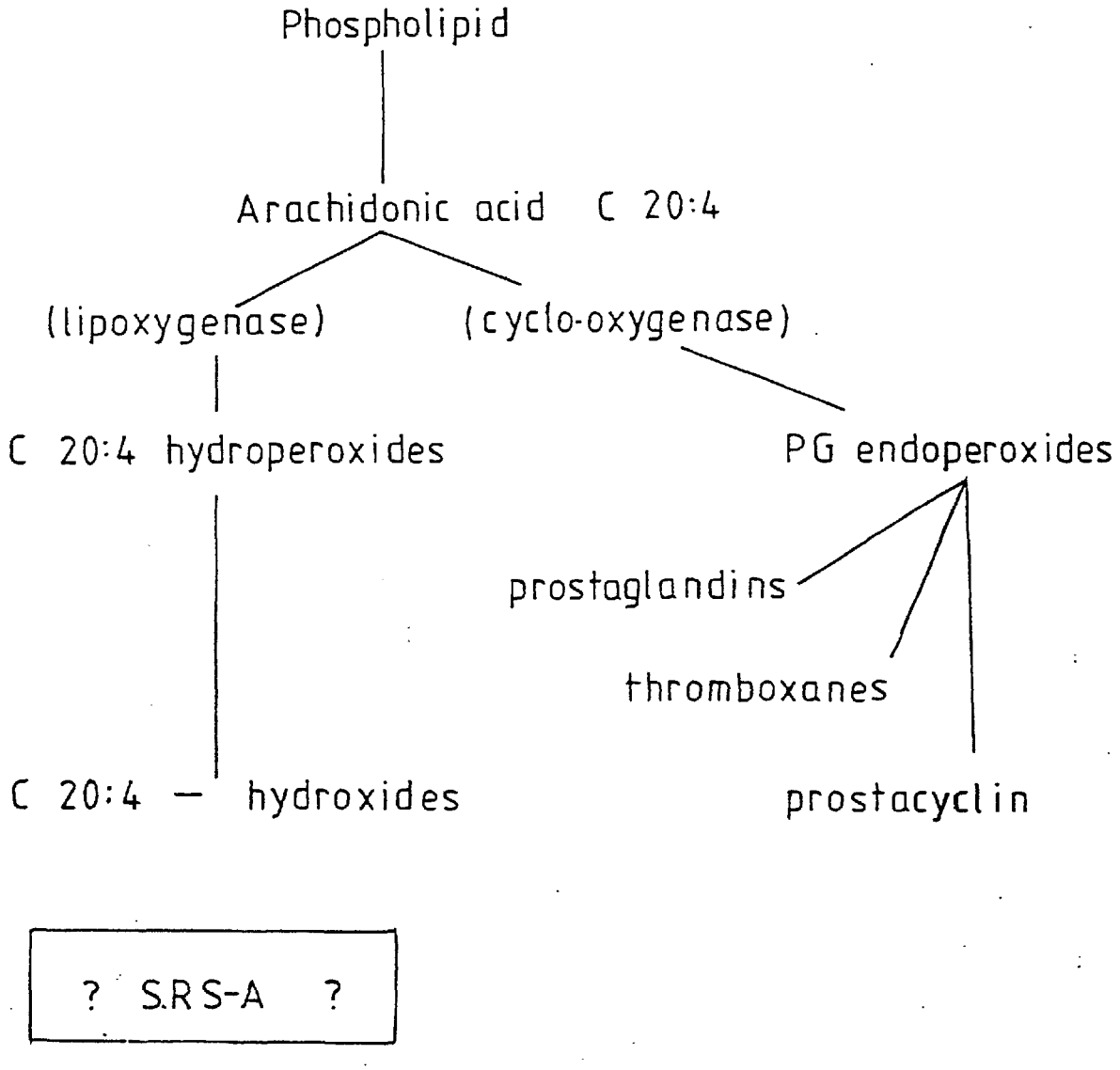

Figure 7

Metabolism of arachidonic acid in guinea-pig lung (1976). SRS-A release is potentiated by those drugs which block cyclo-oxygenase, but inhibited if the lipoxygenase pathway is blocked.

Guinea-pig SRS-A, human SRS-A and rat peritoneal SRS are pharmacologically, physico-chemically and chromatographically indistinguishable; this suggests these SRS entities are closely related (if not identical) and lends direct medical relevance to the use of animal SRS(-A) in studies related to the genesis of allergic bronchospasm in man.

4. Genesis of SRS-A

The putative precursor role of arachidonic acid was originally suggested independently by Bach and Brashler (35a) and Jakschick et al (35b) for non-immunologically generated SRS's; the latter group indicated a lipoxygenase-like enzyme in SRS biosynthesis.

Studies carried out together with colleagues at the Dept. Pharmacology, Royal College of Surgeons, extended these findings in a guinea-pig chopped lung system to indicate that immunologically generated SRS-A was also a lipoxygenase produced metabolite of arachidonic acid (36,37,38) [Fig. 7]. A possible precursor role for 5, 8, 11, 14, 17 eicosapentaenoic acid was also indicated.

At the time of this study, inconclusive data on incorporation of radiolabelled arachidonic acid into SRS's had been reported (34, 35, 39); in order to clarify the role of this acid, incorporation of 1 - ^{14}C - arachidonic acid into SRS-A in a guinea-pig chopped lung system was attempted.

5. Radioactivity Incorporation and Further Purification Step

(i) Radiolabelled arachidonic acid was incubated with guinea-pig chopped lung in the presence of indomethacin to prevent prostaglandin formation; (36) After an initial charcoal adsorption step (JRT, RCS) the "radiolabelled material" was mixed with guinea-pig SRS-A (from perfused lung) to act as a carrier during subsequent purification. The material was purified as detailed above (section 1). Radiolabel and bioactivity coincided in the G15 profile [Fig. 8] and under reverse-phase HPLC in three methanol water systems [Fig. 9]. However, on examination of the u.v. spectrum of the active material ex HPLC

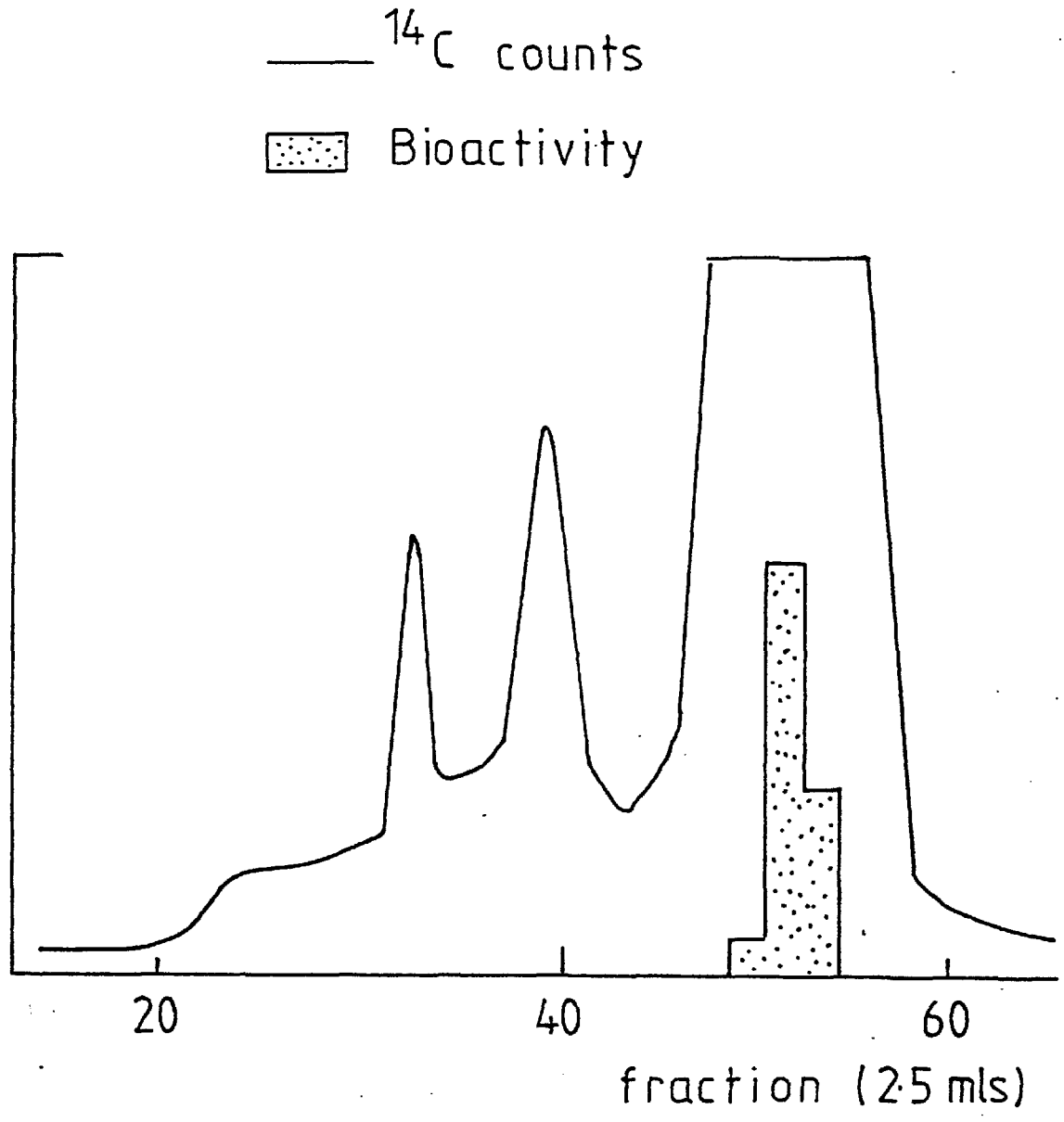

Figure 8

SRS-A radiolabel incorporation experiment (1-^{14}C-arachidonic acid): sephadex G-15 bioactivity/radiactivity profile.

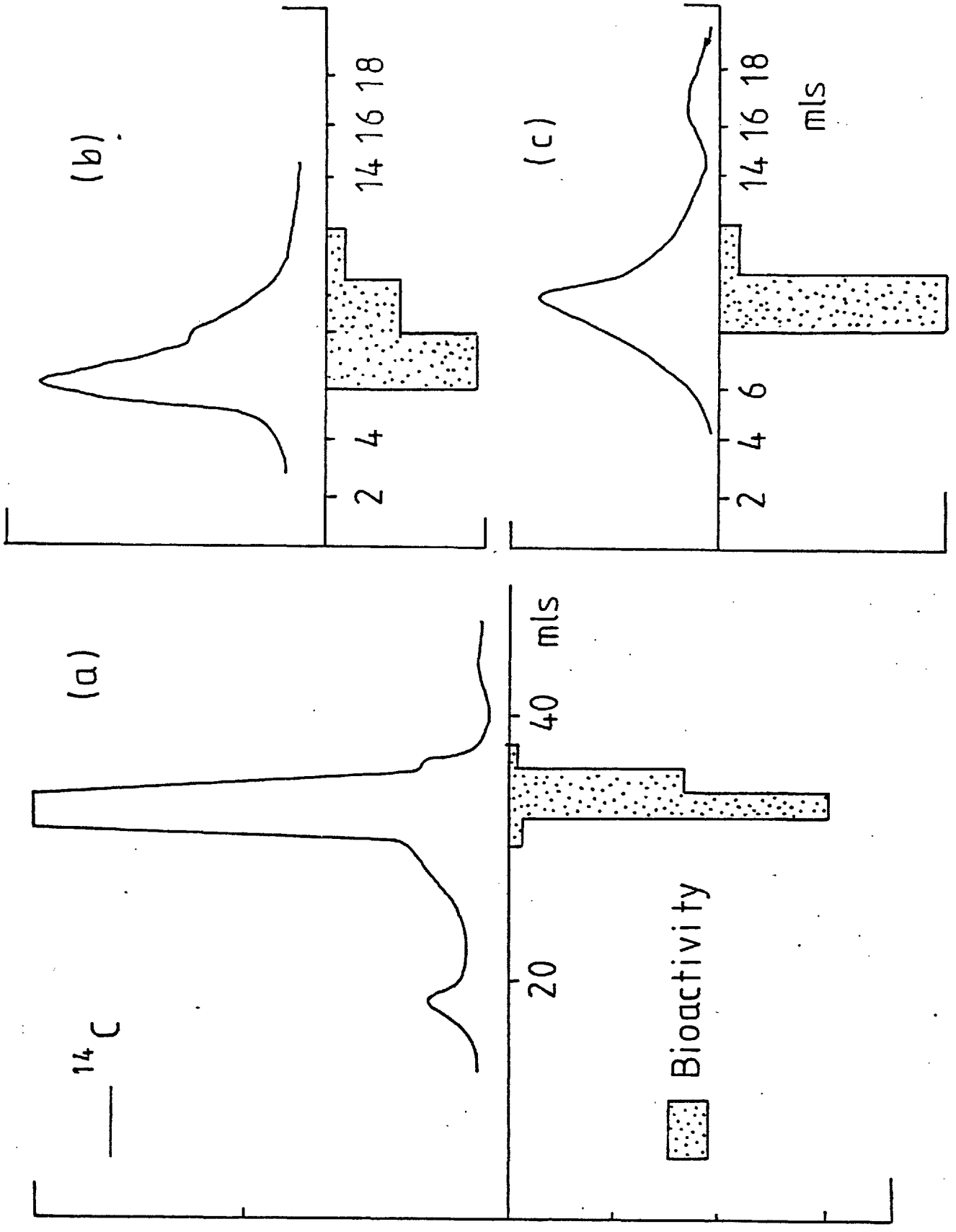

Figure 9

SRS-A radiolabel incorporation experiment (1-^{14}C-arachidonic acid): bioactivity/radioactivity elution profiles in reverse-phase HPLC (μ Bondapak C_{18} , 30 x 0.4 cm).

(a) 50% aqueous methanol (10 mls) followed by a linear gradient (20 mls) to methanol (HPLC-1 conditions).

(b) 85% methanol (aqueous) -isocratic elution.

(c) 75% methanol (aqueous) - isocratic elution.

In no case could bioactivity be separated from the ^{14}C radiolabel.

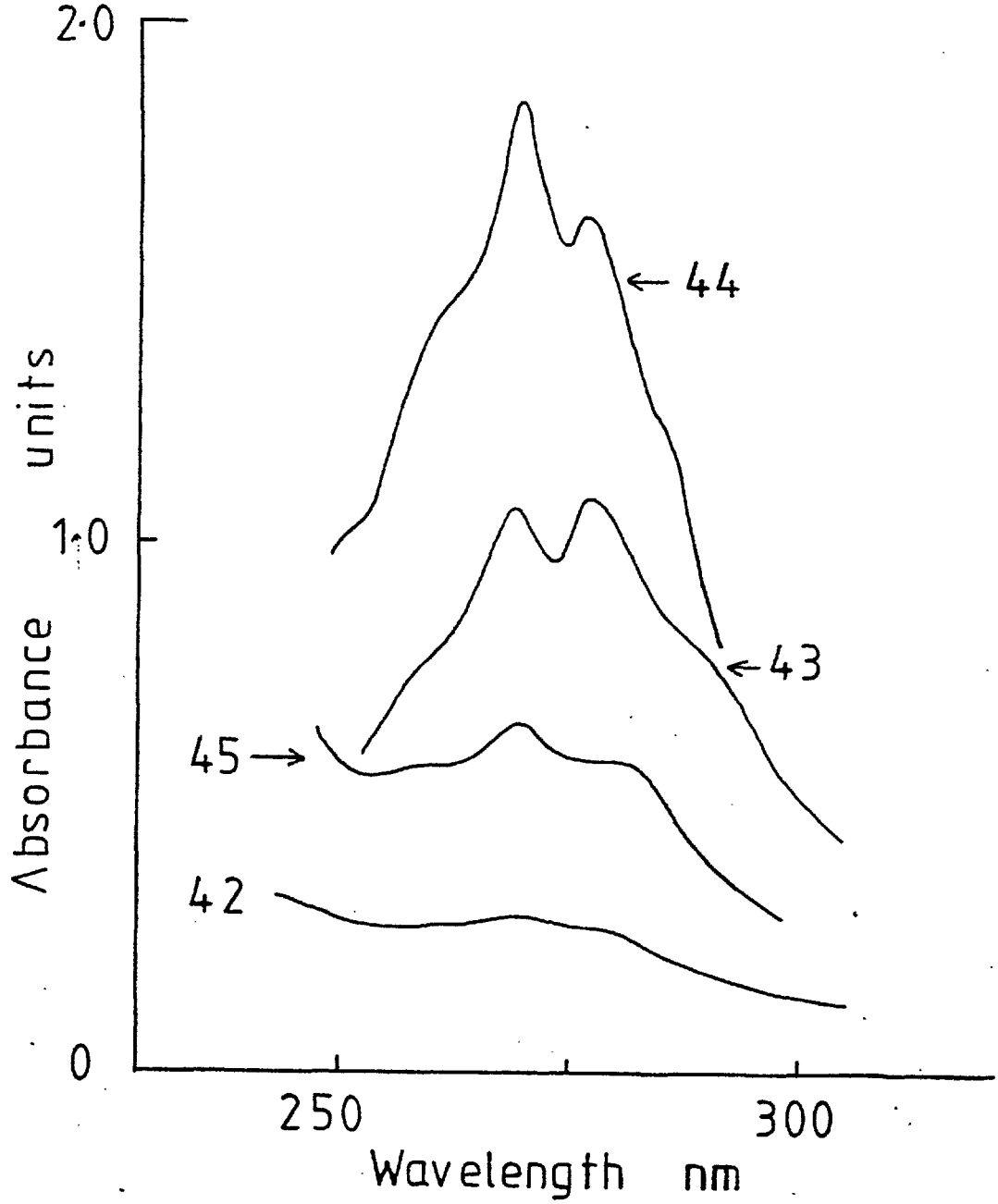

Figure 10

SRS-A radiolabel incorporation experiment (1 - ^{14}C - arachidonic acid): full U.V. spectra of biologically active material eluting from HPLC 1; the major peak of bioactivity is in tube 43.

it was apparent that SRS-A was contaminated with quantities of an impurity(ies) absorbing strongly at 270 ± 1 nm [Fig. 10]. The absorbance ratio I_{280}/I_{270} maximised co-incidentally with biological activity indicating an A280/bioactivity correlation as expected.

To remove the impurity, and to resolve the question of ^{14}C label incorporation, a further reverse phase HPLC step was developed. This step, in common with earlier purification stages, was designed to have the following important properties:

(a) salt-free buffers for compatibility with biological assay and mass spectrometric analysis

(b) reproducibility

(c) good yields and resolution.

A high resolution reverse-phase HPLC procedure using a volatile n-propanol: acetic acid elution system had been developed in this laboratory for the separation and characterisation of biologically important peptides (40); high yields of peptides of varying length were obtained in this system (> 90%). Studies were undertaken to determine the solubility and stability of SRS-A in propanol: acetic acid solvents.

(a) Solubility: SRS-A (as judged by bioassay) was completely soluble in solutions containing up to 50% n-propanol in acetic acid; little SRS-A was lost by adsorption to glass vessel surfaces in these solvents.

(b) Stability: Time course experiments showed that SRS-A was not appreciably inactivated by the acidic solvents used.

Initial studies into n-propanol:acetic acid elution systems showed that biological activity eluted in good yield (60%), as a single peak, at approximately 30% n-propanol. [Fig. 11]

In propanol acetic acid systems, SRS-A is fully protonated, and artefacts arising from protonation/deprotonation (section 1:(iv)) are not observed, thus the sample may be loaded in Tyrode (pH7, SRS initially unprotonated) without affecting the elution position.

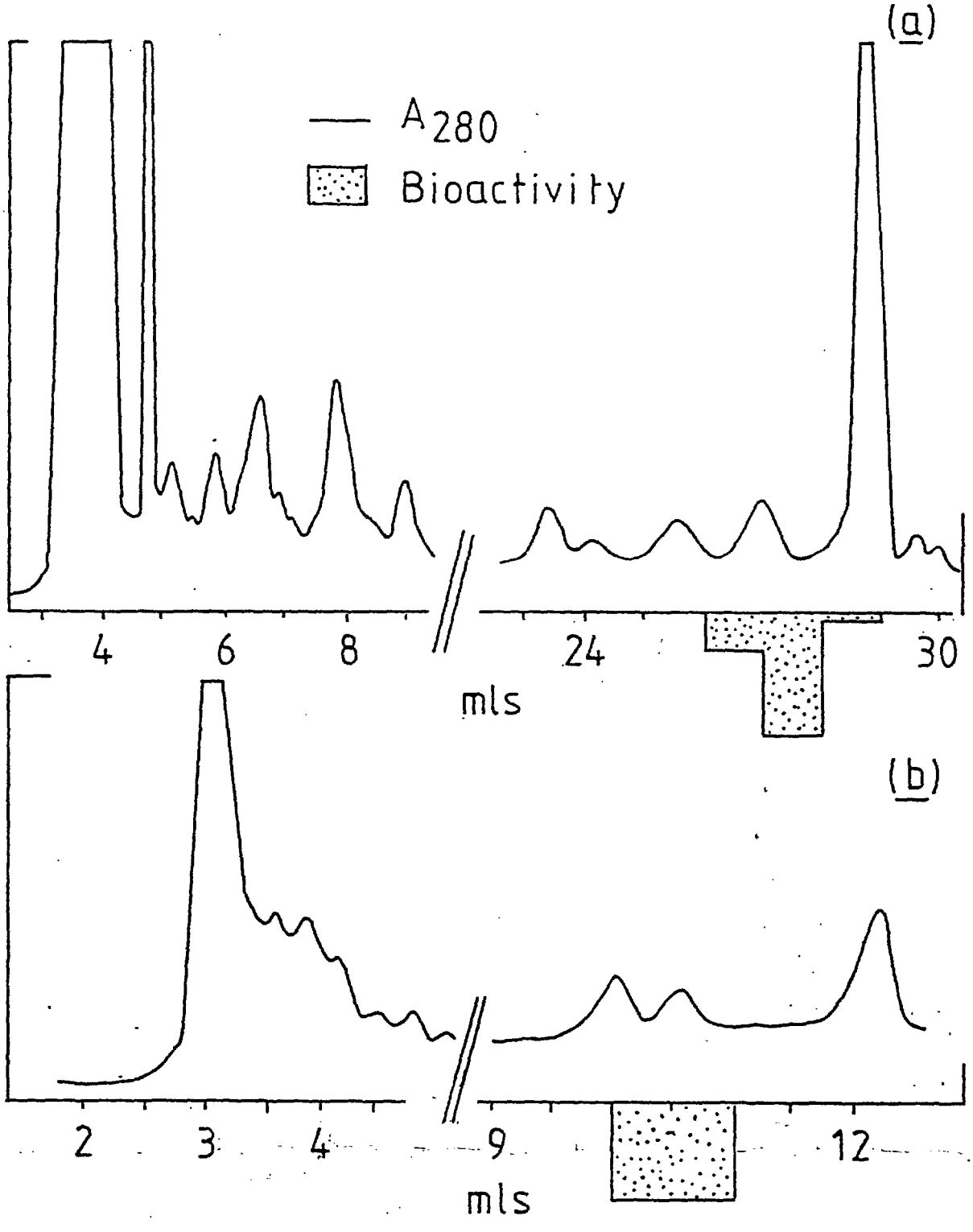

Figure 11

SRS-A radiolabel incorporation experiment (1 - ^{14}C - arachidonic acid): bioactivity/U.V. (A_{280}) profiles on reverse phase HPLC (μ Bondapak C_{18}, 30 x 0.4 cm) in n-propanol: acetic acid (5%) elution systems:

(a) n-propanol, 10% - 40% in acetic acid, over a linear gradient of 20 mls.

(b) n-propanol (30%), isocratically (10 mls) followed by a linear gradient (20 mls) to 40% n-propanol.

ex-ether SRS-A was used in each case.

From these studies, an elution system involving a linear gradient between 30% - 40% n-propanol in acetic acid (5%) was considered suitable for a final step purification of SRS(-A) (HPLC-2), and was used in all future SRS(-A) purifications.

Active material from HPLC-1 carrying a radiolabel (section 5, Fig. 9a) was chromatographed in the HPLC-2 system (propanol:acetic acid). Biological activity eluted as two distinct peaks (section 5:ii) co-inciding with A_{280} absorbance but was well separated from the peak of radioactivity which eluted much later [Fig. 12]; no incorporation of 1-^{14}C arachidonic acid was observed.

Inability to incorporate radioactive label appears at first sight to be in conflict with data on the arachidonic acid mediated potentiation of SRS-A release from lung. It has been shown however (41) that although exogenous arachidonic acid is incorporated into lung phospholipids it is not mobilised (and thus metabolised) on antigen challenge. It appears that radiolabel incorporation into SRS-A may not therefore occur on challenge of guinea-pig lung, and that the labelled impurities observed in the purification arise from non-specific metabolism or oxidation of added arachidonic acid. Potentiation of SRS-A release caused by exogenous arachidonic acid may then be a result of displacement of endogenous fatty acid at the site of action (which may then be metabolised to SRS-A).

Based on a minimum level of detection of $\sim$ 500 pg in the bioassay (u.v. absorbance data) any incorporation of label should have been observed; if the potency of this material is much higher than expected, the radiolabel incorporation might have been below the level of detection.

(ii) All future large scale purifications of SRS(-A) were extended to include the HPLC-2 step. In this system, four closely related A_{280} absorbing compounds were observed [Fig. 12, 13 Compounds I-IV] (38,42), biological activity on the guinea-pig ileum was associated with compounds I and II; the full u.v. spectra in methanol of I-IV are shown in Fig. 14.

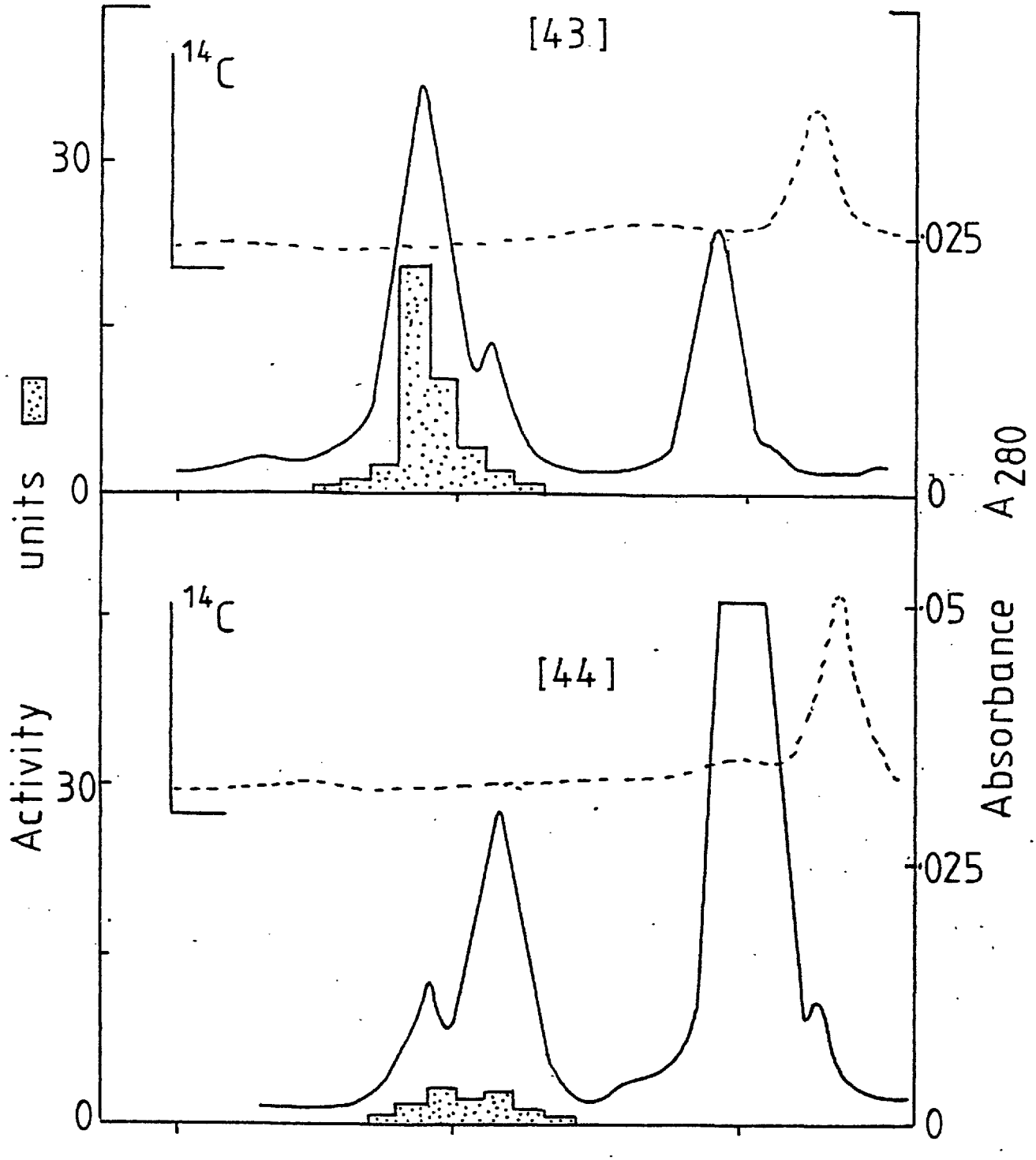

Figure 12

SRS-A radiolabel incorporation experiment (1- ^{14}C - arachidonic acid): HPLC-2 conditions - 30% n-propanol in acetic acid (5%), isocratically 20 mls) followed by a linear gradient (40 mls) to 40% n-propanol (μ Bondapak C_{18}, (30 x 0.78 cm). Tubes 43 and 44 ex-HPLC 1 were chromatographed separately; bioactivity was well separated from radioactivity.

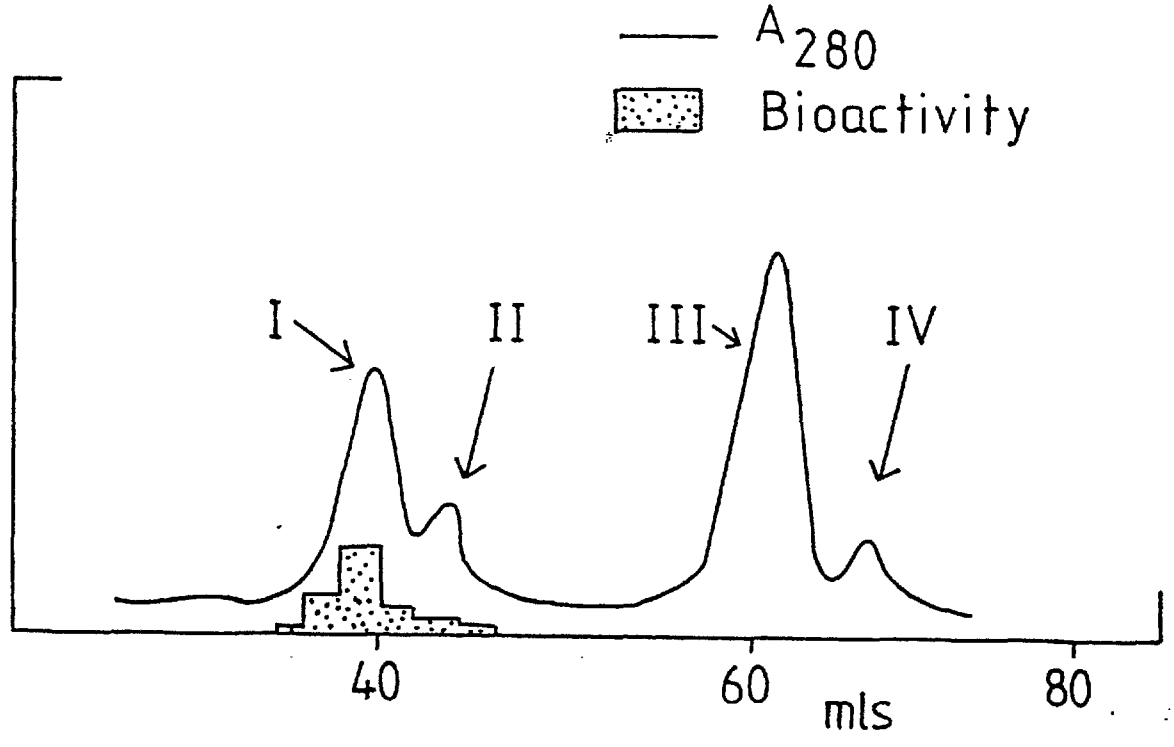

Figure 13

Guinea-pig SRS-A: purification by reverse-phase HPLC-2 showing four U.V. absorbing (A_{280}) compounds; bioactivity resides in compounds I and II (μ Bondapak C_{18}, 30 x 0.78 cm).

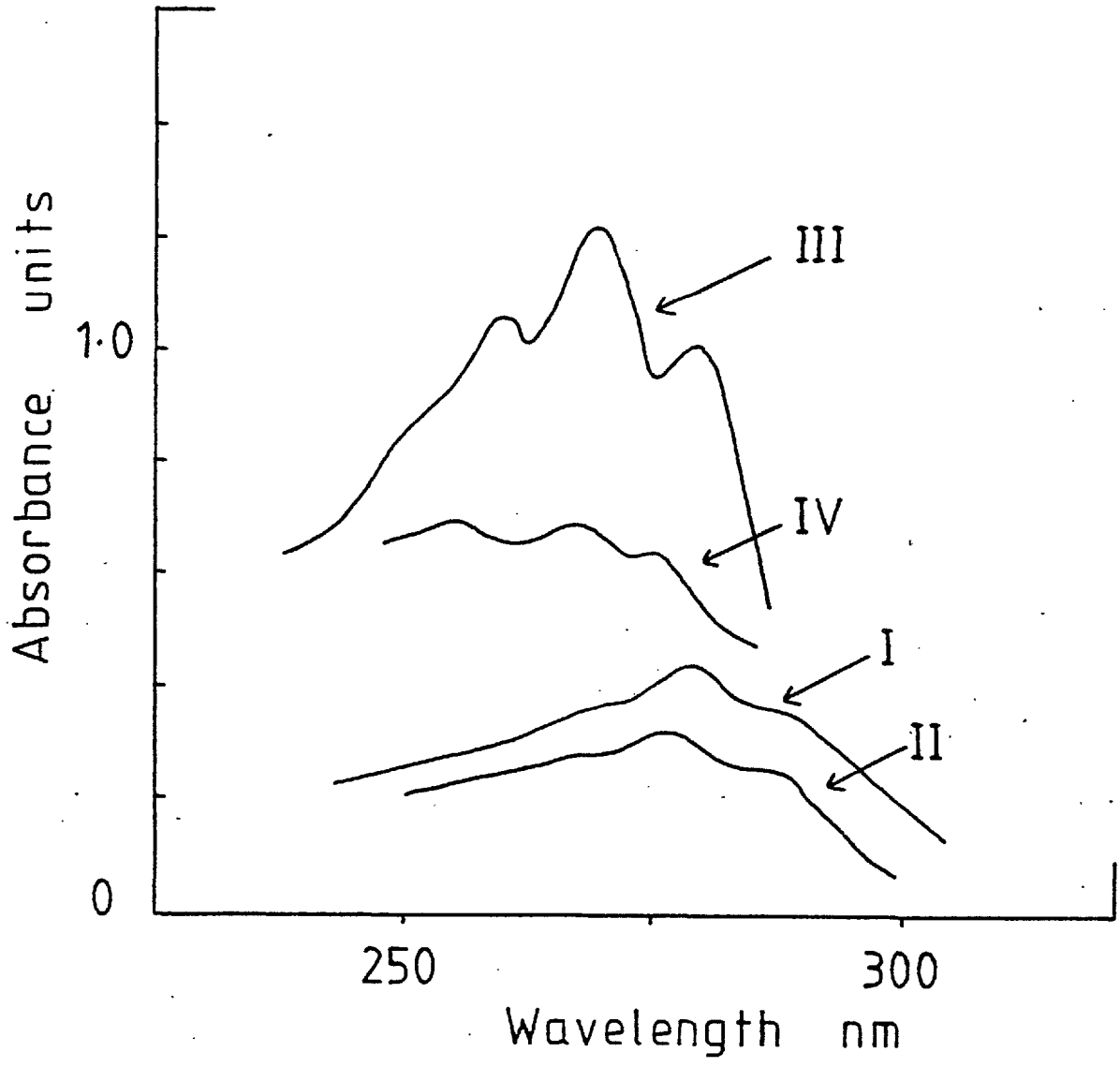

Figure 14

Full U.V. spectra of compounds I-IV from guinea-pig lung (ex HPLC-2) in methanol:

Compound I	:	λ	max	280 ± 1	nm
Compound II	:	λ	max	278 ± 1	nm
Compound III	:	λ	max	270 ± 1	nm
Compound IV	:	λ	max	268 ± 1	nm

SRS-A Large scale purification

	Activity units	Yield/step %	Yield %
Charcoal adsorption (380 animals)	8000	-	-
Sephadex G 15 gel filtration	8400	100%	100%
Ether extraction	5600	67%	70%
HPLC 1	3080	55%	39%
HPLC 2 peak I	1430	46%	18%
peak II	210	7%	3%
peak (I+II)	1630	53%	20%

Figure 15

Yields obtained during the purification of SRS-A in a recent large scale preparation; 10g of solids from guinea-pig lung yield approximately 10 μg of pure SRS-A.

Compound I (major peak of biological activity) shows a broad triplet in the u.v. (λmax 280nm), identical to the spectrum originally published for SRS-A (section 2 (31)): Compound II (λ max 278 nm) elutes immediately after SRS-A and has reduced biological activity; it has a u.v. spectrum resembling that of SRS-A, but shifted hypsochromically by 2-3 nm. This would be consistent with a cis-trans (43), or other closely related isomerisation, and could lead to the observed reduction in biological activity. Two compounds (III, IV), inactive on the guinea-pig ileum, elute from the column after SRS-A; the u.v. spectra (III : λ max 270 nm, IV: λ max 267 nm) are consistent with cis-trans-trans and all trans trienes respectively. The structure of III has been shown by mass spectrometry to be 5, 12 - dihydroxy - 6,8,10,14 - eicostetraenoic acid (section 8 (42))

From these data, the impurity absorbing at 270 nm in the radioactivity incorporation experiment (section 5:i, Fig. 10) is compound III and may arise from endogenous and exogenous arachidonic acid; larger quantities of this material appear to be present when exogenous arachidonic acid is added, suggesting perhaps, a greater release of enzymes producing III in chopped lung compared to perfused challenged lung.

This extended purification method is now standard procedure for purifying SRS(-A) in this laboratory; the yields in each step of the purification (from a recent large scale preparation) are given in Fig. 15.

6. Physico-Chemical Properties of SRS-A

To obtain further data relevant to the structure of SRS-A, the effect of various reagents on the biological activity of SRS-A was investigated. Such studies have the advantage of requiring minimal quantities of material of low purity but the data so obtained may not be considered conclusive; for example, inactivation of SRS-A by a specific chemical or enzyme may arise out of an unconsidered side-reaction leading to erroneous results (see sections 6g,7 regarding arylsulphatase inactivation of SRS-A).

Physico-chemical properties of SRS-A

Biological activity is stable to :

1. boiling
2. base treatment
3. borohydride reduction

Biological activity is destroyed by :

4. acid (pH 1)
5. acetylation
   - i. short acetylation in methanol : acetic anhydride
   - ii. acetylation with pyridine : acetic anhydride
6. Fluram
7. esterification with :
   - i. diazomethane
   - ii. methanol : hydrogen chloride
   - iii. methanol : boron trifluoride
8. catalytic hydrogenation (Ni_2B)
9. cyanogen bromide
10. arylsulphatase
11. lipoxygenase (plant)

Water soluble at pH 7

Ether soluble at pH 3

Migrates anodically on HVPE at pH 6.5

Figure 16

Physico-chemical properties of SRS-A based on chemical inactivation of biological activity (ileum assay).

Physico-chemical properties of SRS-A, based on activity destruction experiments are outlined in Fig. 16; from these properties certain structural features of SRS-A may be determined:-

(a) Stability to borohydride reduction indicates the absence of simple ketones, hydroperoxides and other readily reduced groups in SRS-A.

(b) Stability to base shows SRS-A is not a phospholipid or similar compound.

The above interpretation of this data is based on the requirement of these groups for biological activity.

(c) Short acetylation: on acetylating in the presence of methanol for 1-5 minutes, only those amino groups which are mainly in the unprotonated state are blocked (44). Thus amino groups α to carboxylic functions react rapidly whilst others (e.g. the ϵ amino group of lysine), as a result of their somewhat higher pK, are unaffected. The presence of methanol prevents O-acetylation. Thus the data on the inactivation of SRS-A by short acetylation suggest that an α amino group is present and essential for activity. Fluram destruction indicates that the amino group is primary (45).

Inactivation of SRS-A by N-acetylation appears to be in conflict with the work of Bach and Brashler (46,47) who showed that SRS-A release is potentiated by thiols including cysteine and derivatives but the presence of a free α amino group in the thiol was not essential for potentiation to occur. It is possible however that the thiols are not incorporated into SRS-A (8) but act by activation of suitable enzymes, or that the thiols are metabolised, and that the metabolites are so incorporated, resulting in an increased release of SRS-A.

Data on the possible presence of hydroxyl groups in SRS-A are not readily available from this type of experiment as the putative α amino group is more strongly nucleophilic (thus reacting preferentially and leading to inactivation of SRS-A).

(d) Treatment of SRS-A with reagents known to cause esterification (CH_3 OH/HCl., CH_2 N_2) results in loss of biological activity, indicating the presence of a free carboxyl group(s) in the molecule. This result is consistent with electrophoretic and ether extraction data.

(e) Cyanogen bromide is widely used in protein structure elucidation to generate chemical cleavage fragments at methionine residues; the reaction occurs via an initial nucleophilic attach of the thioether sulphur of methionine on CNBr. leading to the formation of a carbon-sulphur bond. Inactivation of SRS-A by this reagent suggests a thioether linkage may be present, (and essential for bioactivity), as reaction conditions were chosen to prevent reaction at other centres, e.g. the sample was in the protonated form to reduce reaction of the putative α amino group, and excess arachidonic acid added to quench any liberated bromine.

This is in agreement with studies by Parker et al, on a non-immunologically generated SRS (RBL-1SRS) who indicated, by chemical degradative and radioisotope studies, that sulphur was probably present in a thioether linkage. (48, 49)

(f) Soy bean lipoxygenase: The inactivation of SRS-A by soy bean lipoxygenase (first shown in this laboratory (50), 1977) indicated the presence of a cis-cis 1-4 pentadiene moiety in the molecule. The specificity of this enzyme (w6) together with the putative precursor role of arachidonic acid (section 4) would indicate that the $\triangle$ 14,15 double bonds of the precursor may be present in SRS-A. Recent data on the lipoxygenase inactivation of a non-immunologically produced SRS support this data (58).

(g) Arylsulphatase inactivation of SRS-A led many groups to postulate the presence of a sulphate ester group in the molecule (20). The ether solubility at pH3 (section 1 (ii)) appears to be in conflict with these data as a sulphate would remain ionised at pH3, conferring water solubility on the molecule. It was however noted that the sulphate may ion-pair with the postulated α amino group present (6c) and could lead to enhanced ether solubility.

It is also possible that inactivation is caused by a side-specificity of the enzyme (impurities are unlikely following the work of Wasserman (51)), or by recognition of part of the SRS-A molecule as a sulphate ester (e.g. sulphur-oxygen bond lengths and positions may 'mimic' a sulphate). To attempt to determine the cause of inactivation by this enzyme, experiments designed to re-sulphate inactivated SRS-A were undertaken.

7. Sulphations of arylsulphatase inactivated SRS-A

(i) Inactivated SRS-A was treated with two chemical sulphating reagents (pyridine: SO_3, NEt_3: SO_3) but no biological activity (ileum assay) was obtained. Standard compounds were treated under identical conditions and were converted to sulphate esters.

(a) Prednisolone:-on treatment with NEt_3:SO_3, the mobility of the material on t.l.c. changed; a new spot, with an Rf value in the same range as a sulphated steroid and bovine sulphatide, was observed. [Fig. 17] suggesting the standard had been sulphated.

(b) Tyramine:- Pyridine: SO_3 treatment, caused a shift in the u.v. spectrum compatible with the formation of tyramine O-Sulphate. HVPE at pH2.1 of "sulphated" tyramine showed a ninhydrin positive electrophoretically neutral spot, indicating the change in the u.v. spectrum was due to sulphation.

(ii) A triton X-100 solution of a rat-brain sulphating enzyme was prepared and used with arylsulphatase treated SRS-A. Traces of the detergent, present after ether extraction, badly affected the bioassay tissues and prevented observation of any active material if present. It was considered inappropriate to investigate

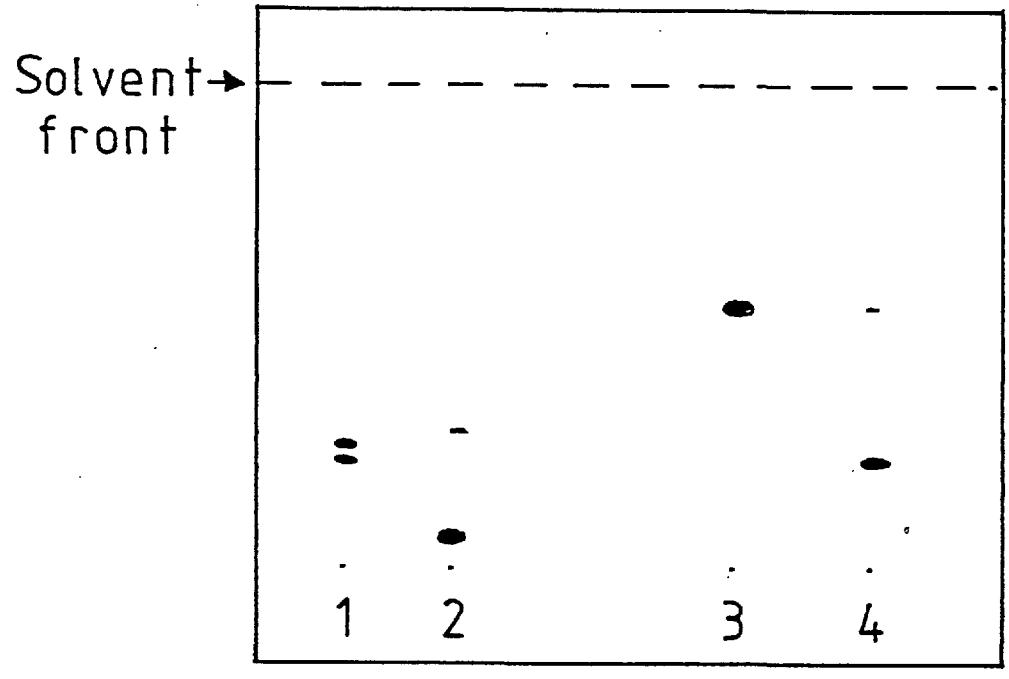

Figure 17

Resulphation experiments: thin layer chromatography on an HPTLC silica plate developed with chloroform: ethylacetate: methanol: ammonia (·880) - 10:5:5:2 v/v.

Samples: 1. commercial bovine sulphatide
2. 17-hydroxy-pregnelone-3-sulphate
3. Prednisolone
4. Prednisolone "sulphate"

Prednisolone is a medical synonym for 11β - 17α - 21 trihydroxypregna - 1,4 diene - 3,20-dione.
Spots were visualised with a Cu^{2+} /H_3PO_4 reagent.

further this enzymic system and the study was discontinued.

Inability to obtain biological activity in these experiments under conditions where sulphation of standards occurred, throws further doubt on the putative presence of a sulphate ester in SRS(-A). It was noted however, that sulphation may have been prevented by chemical or steric hindrances, or may even occur more rapidly at a second site. The presence of sulphate has now been resolved mass-spectrometrically (section 9,12)

8. Mass Spectrometric Identification of Compound III from guinea-pig lung (42)

Compound III is released together with SRS-A from perfused or chopped sensitised guinea-pig lung on antigen challenge; this suggested a common precursor for these materials (i.e. arachidonic acid, section 4). Compound III elutes after SRS-A on HPLC-2 (less polar) and has a u.v. spectrum consistent with a cis-trans-trans conjugated triene (43). Although compound III is inactive on the guinea-pig ileum, its relationship to SRS-A (u.v. spectrum, common precursor) made the elucidation of its structure an important preliminary to the identification of SRS-A.

The quantity of compound III available ($\sim 10\mu g$ based on u.v. data) suggested that a mass spectrum should be obtained from a suitable derivative. To determine the derivative of choice, the action of certain reagents on the chromophore of compound III was investigated. Neither acetylation (methanol: acetic anhydride) nor esterification (diazomethane) affected the u.v. spectrum; as these reagents inactivated SRS-A it appeared possible that they would also "inactivate" compound III without affecting the u.v. chromophore. Thus the "N-acetyl (1:1 d_3:h_3) - methyl ester" of compound III was prepared and this ester was trimethylsilylated to derivatise any free hydroxyl groups present. The mass spectrum of compound III was obtained by the mixture analysis method.

A mass spectrum was obtained [Fig. 18] at 180°C, absent in a control HPLC blank derivatised in the same way. No 1:1 isotope labelling was observed precluding the presence of a free α amino group in the molecule. An even mass molecular ion (m/e 494 no nitrogen) underwent the expected losses of $^{\bullet}CH_3$ (m/e 479) and $^{\bullet}OCH_3$ (m/e 463): ions at m/e 404 (M^{+} - TMSOH) and m/e 314 (404-TMSOH) shows the

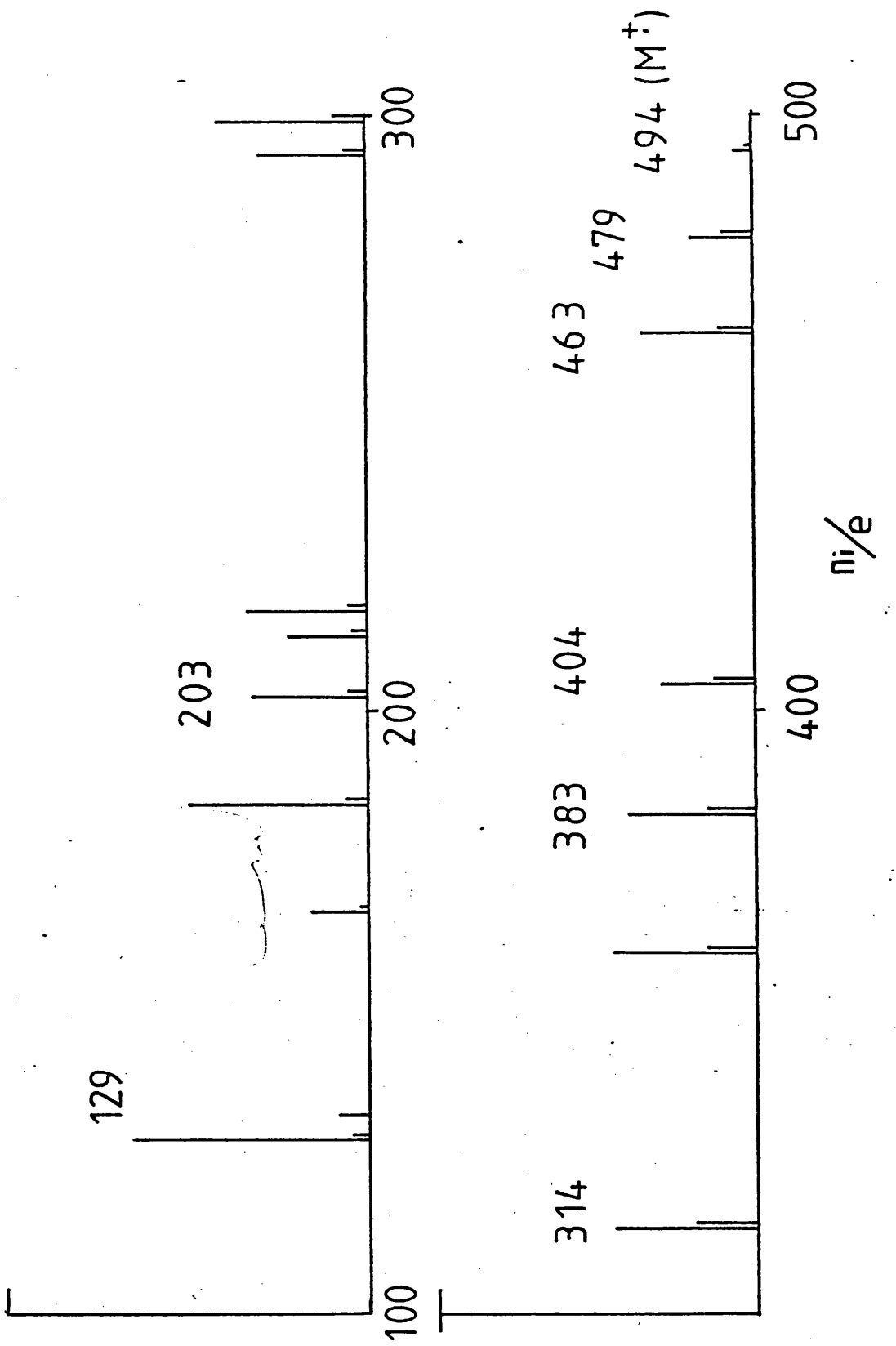

Figure 18

The electron impact mass spectrum of the trimethylsilyl ether of the N-acetyl (1:1 CH_3CO : CD_3CO) methyl ester of compound III at 180° C. No isotope labelling was observed.

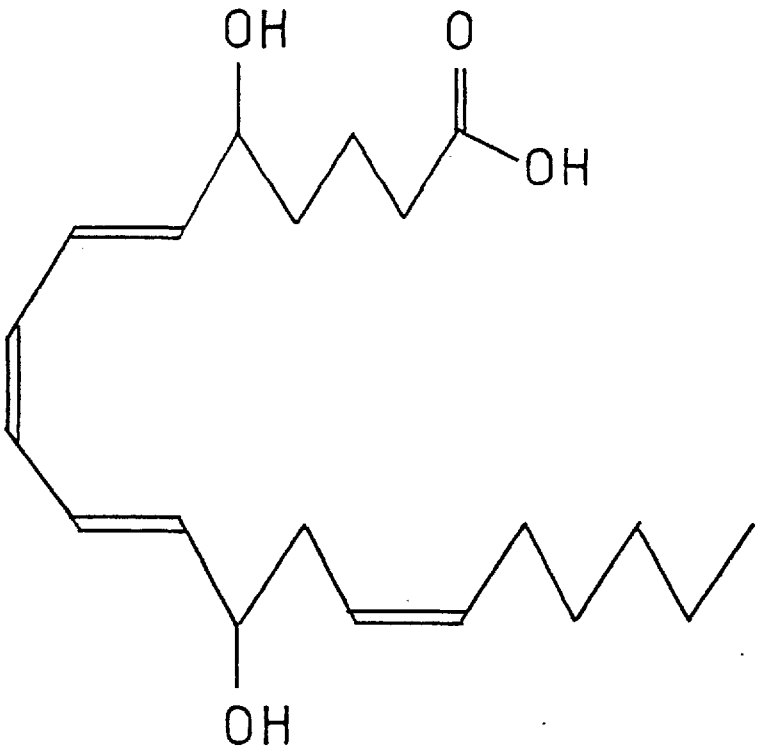

Figure 19

The covalent structure of compound III is 5,12-dihydroxy-6,8,10,14 eicosatetraenoic acid.

presence of two derivatised hydroxyl groups in the molecule; m/e 203 ($TMSO^{+}$ = CH $(CH_2)_3 CO_2 CH_3$) fixes one hydroxyl group at position C_5 (δ to carboxyl group); m/e 383 (M^{+} - $CH_3 (CH_2)_4$ CH = CH CH_2) shows compound III contains a C_8 unsaturated lipid chain (present also in arachidonic acid).

The molecular weight data together with the probable precursor role of arachidonic acid, indicate a $C_{20:4}$ lipid as the basic carbon skeleton of compound III. From the above data, the covalent structure of this material was deduced as 5, 12 - dihydroxy - 6, 8, 10, 14 - eicosetetraenoic acid [Fig. 19]. Cleavage α to the C_{12} TMSO-group would lead to the loss of a stabilised allylic radical (m/e 383); this ion is also able to lose TMSOH m/e 293). By fixing hydroxyl groups at C_5 and C_{12} , and one double bond at Δ 14,15, the conjugated triene moiety is fixed at Δ 6,7 Δ 8,9 Δ 10,11.

This was the first demonstration that this material was released from guinea-pig lung on antigen challenge and shows the presence of a C_5 specific lipoxygenase in the lung.

Independently of this work Borgeat and Samuelsson (52) reported the identification of this compound from human polymorphonuclear leukocytes on calcium ionophore stimulation; this material was subsequently termed Leukotriene B (LT-B) (53). They suggested LT-B arose through metabolising arachidonic acid to form a 5.6 oxido intermediate (Leukotriene A, LT-A)followed by hydroxylation of this epoxide. The mass spectra of the same derivative of compound III and LT-B were similar.

9. Purification and Structure Elucidation of an SRS from Rat Basophil Leukaemia (RBL-1) Cells

(i) Conclusions drawn from the data presented in previous sections are:-

1. Precursor role of arachidonic acid
2. Conjugated triene
3. Cis-cis 1-4 pentadiene
4. α Amino group(s)
5. Carboxyl group(s)
6. Thioether linkage
7. Sulphate group less likely

The quantity of SRS-A available had precluded extensive mass spectrometric studies required to elucidate the full covalent structure of this material, so investigations were directed towards more readily prepared SRS's.

(ii) Previous studies had indicated the pharmacological, physico-chemical and chromatographic (HPLC) similarity of guinea-pig and human SRS-A with rat peritoneal SRS (section 3 (33)) suggesting that studies on one SRS would be applicable to all SRS's. To augment our studies, and to act as a model for SRS-A, a slow-reacting substance was produced from rat basophil leukaemia cells (RBL-1 SRS) by the action of calcium ionophore A23187 (Mrs. M.N. Samhoun, R.C.S.). Studies on this SRS were undertaken for the following reasons:-

(1) the system was well understood (17,48,49)
(2) these cells readily proliferate
(3) large quantities of SRS may be generated (1 000 units/10^7 cells)
(4) the time course of SRS generation is greatly reduced compared to the production of guinea-pig SRS-A.
(5) handling problems are minimised (i.e. no animals to be housed/fed etc)
(6) purification is simpler as fewer by-products are generated.

These factors became apparent after initial studies on RBL-1 SRS and it was decided that this material would act as a suitable model for future studies on SRS-A.

(iii) RBL-1 SRS was produced at the Dept. Pharmacology Royal College of Surgeons (Mrs. M.N. Samhoun) and subjected to an initial charcoal adsorption purification step. It was received in this laboratory as a solid, (dried by rotary evaporation), and stored at -20°C until required. The lower level of impurities compared to those present in guinea-pig SRS-A was apparent even at this early stage of purification.

Biological properties of the SRS's

	Guinea-pig SRS-A	Human SRS-A	RBL-1 SRS	Rat SRS
Contracts guinea-pig ileum	+(2)	+(2)	+(2)	+(2)
Contracts human bronchus, guinea-pig trachea	+(2)	+(1)	+(2)	+(1)
Releases PGs, Tx	+(2)	+(1)	+(1)	+(1)
Antagonised by FPL 55712	+(2)	+(2)	+(2)	+(2)

(1) ex-charcoal material used

(2) material from all stages of purification used

Figure 20

Comparison of the pharmacological properties of guinea-pig SRS-A, rat basophil leukaemia (RBL-1) SRS and other slow-reacting substances.

(iv) Comparison of RBL-1 SRS with SRS-A:RBL-1 SRS was compared with SRS-A pharmacologically [Fig. 20] and physico-chemically by means of activity destruction experiments [Fig. 16] and shown to be indistinguishable. Samples of RBL-1 SRS and SRS-A were purified by the procedure outlined in sections 1 and 5 ; at each stage of the purification SRS and SRS-A co-eluted, suggesting close similarity (although not necessarily chemical co-identity) of these materials.

On Sephadex G15, RBL-1 SRS eluted as a single band of biological activity (V/Vo = 2.3) together with tyrosine and phenol red (arising from the cell culture medium). The active fractions from G15 gel filtration were chromatographed on the HPLC-2 system (section 5); the relative state of purity of the RBL-SRS preparation made the inclusion of the ether extraction and HPLC-1 steps unnecessary. On HPLC-2, biological activity eluted as two distinct peaks (I,II) corresponding with A280 absorbance, [Fig. 21 compounds I, II], eluting in the same position as SRS-A (I and II); a third compound (III) with a reduced A280/A254 ratio and inactive on the guinea-pig ileum, eluted after the biologically active peaks. The full u.v. spectra of compounds I-III in methanol is shown in Fig. 22.

Compound I (λmax 280 $\pm$ 1 nm) has a spectrum similar to that of SRS-A, and fulfils all the criteria for SRS-A bioactivity: Compound II (λmax 278 nm) has reduced bioactivity and appears to be an isomer (cis-trans) of I as judged by its related, but hypsochromically shifted (2-3 nm) u.v. spectrum: Compound III (λmax 268 nm) has a spectrum consistent with an all-trans conjugated triene. These spectra resemble those originally found in our SRS-A preparations (section 5,Fig. 14, I, II and IV). From the u.v. data it was apparent that RBL-1 SRS contained the same modified triene chromophore found in SRS-A. This chromophore was monitored in later studies to determine suitable derivatives for mass spectrometry.

(v) The specific activity of SRS-A was compared with that of RBL-1 SRS. SRS-A (660 units) was dissolved in methanol (300 μl) and the full u.v. spectrum taken; enough RBL-1 SRS was dissolved in the same volume of methanol to give the same strength u.v. absorption spectrum. Aliquots were removed from each solution and bioassayed.

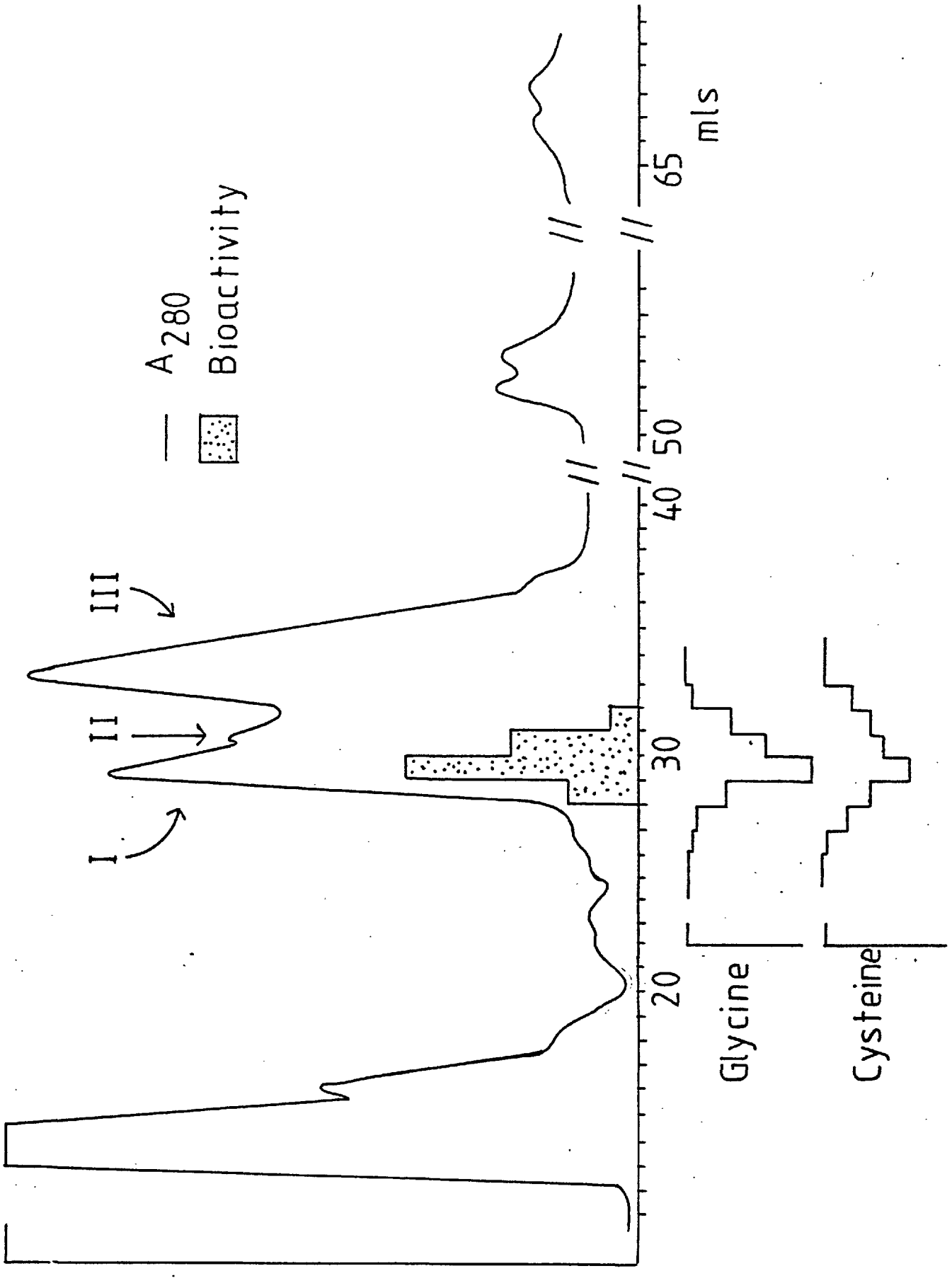

Figure 21

RBL-1 SRS: U.V. (A_{280})/bioactivity profile. Bioactivity is associated with compounds I and II; on strong acid hydrolysis the levels of glycine and cysteine rose and fell concomitantly with bioactivity and A_{280} absorbance.

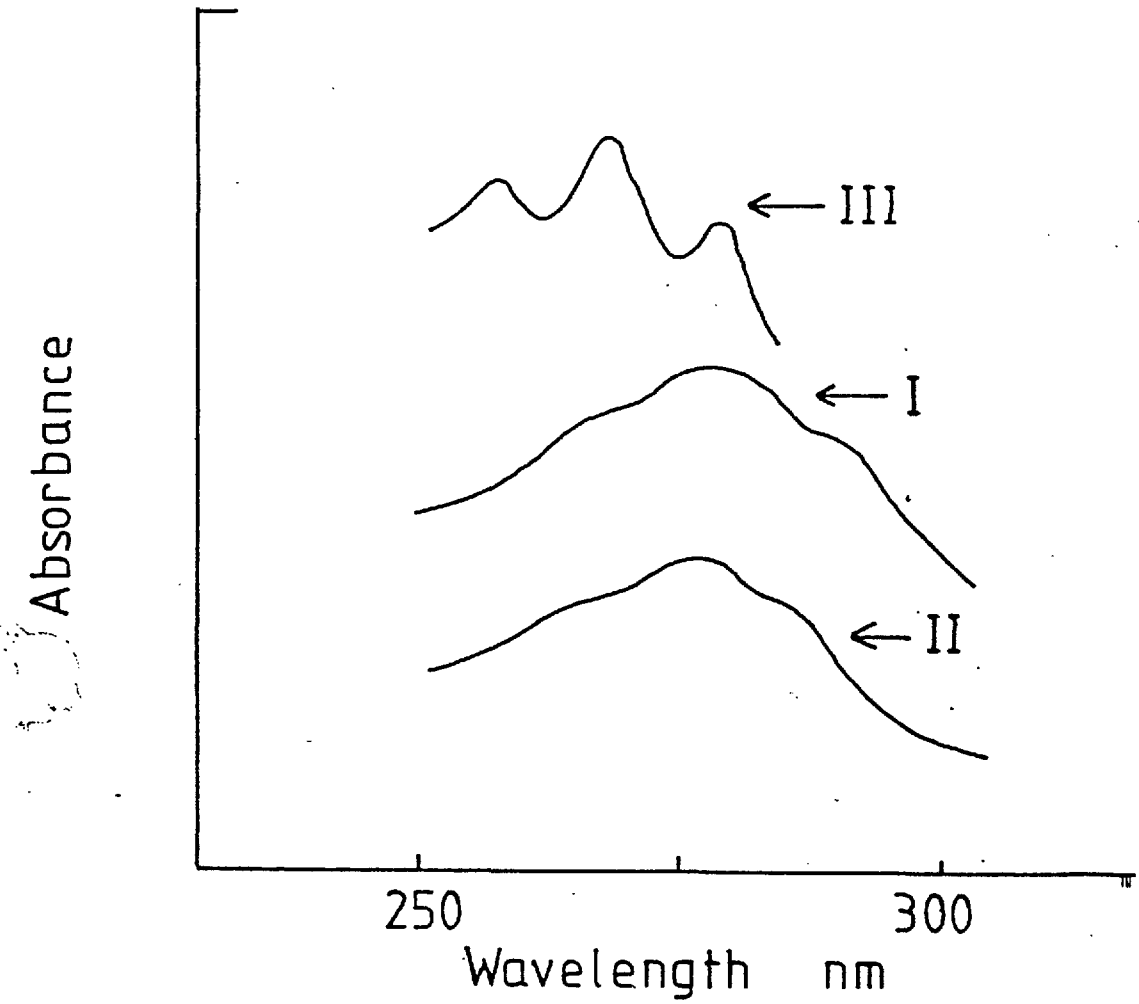

Figure 22

RBL-1 SRS: the full U.V. spectra of compounds I-III in methanol:

Compound I	max	$=\lambda$ 280 ± 1 nm
Compound II	max	$=\lambda$ 278 ± 1 nm
Compound III	max	$=\lambda$ 268 ± 1 nm

After correction for slight differences in volume, no significant difference in the biological activities (ileum assay) of SRS-A and RBL-1 SRS was observed.

A280 = 1.3

Volume = 300 μ l

ε est = 40,000 (for triene chromophore (41))

Cell path length = 1 cm

as A = ε c l

Concentration c = $\frac{1.3}{4 \times 10^4}$ M

$\sim$ 3×10^{-5} M.

= 10 nanomoles in cuvette (660 units)

Thus 1 unit SRS $\sim$ 15 picomoles
(See also amino-acid analysis data).

(vi) The short acetylation data (section 6) which suggested the presence of an α amino group in SRS(-A), together with reports of the possible incorporation of the amino acid cysteine into RBL-1 SRS (48) led to the suggestion that SRS(-A) may be of peptide origin; analytical protein chemical studies were undertaken on this material. RBL-1 SRS was purified as described and the three u.v. absorbing compounds obtained after HPLC-2 [Fig. 21]. Biological activity resided in compounds I and II. Aliquots were removed for amino-acid and N-terminal analysis.

Amino-acid analysis, after HCl hydrolysis of samples from individual tubes collected across peaks 1, II and III showed the presence of only two amino-acids, glycine and cysteine in relative ratios of approximately 1 : 0.75. Importantly the rise and fall of the amino-acid, U.V. and bioactivity profiles were co-incident for compounds I and II, with the amino-acid profile maximising in the peak-tube of compound I giving a value of approximately 1 nanomole of Gly and 0.75 nanomoles of Cys from the 200 units hyrolysed (5-10 picomoles per unit). Tubes corresponding to peak III were devoid of amino-acids above the 50 picomole level.

To determine the sequence of the two amino-acids, samples from individual tubes across peaks I, II and III (here using III as the control) were dansylated, hydrolysed and dansyl derivatives examined by t.l.c. (54). The results showed a fluorescent spot arising from a single N-terminus in both peaks I and II (control was blank), moving more slowly than dansyl glycine in all dimensions. Dansyl cysteine prepared as a standard in the presence of arachidonic acid gave a spot with identical mobility.

The co-incidence of amino-acids, U.V. absorbance and biological activity suggest that RBL-1 SRS is a peptidolipid. The amino-acid analysis and dansylation data are only compatible with the presence of the dipeptide cysteinylglycine in this molecule; these data preclude the sequence glycinylcysteine, or separate attachment of the amino-acids to the remainder of the molecule.

Inactivation of SRS by cyanogen bromide suggests that the peptide portion of SRS is bound through a thioether linkage; this is borne out by the mass spectrometric fragmentation pattern (section 9 vii).

(vii) Observations as to the precursor role of arachidonic acid in SRS-A (section 4) and RBL-1 SRS (35,48) suggested a tentative C20:4 carbon skeleton for SRS, thioether linked to the dipeptide cysteinylglycine. In order to define the full covalent structure of this material (with the low μg quantities available), mass spectrometric analysis of the intact molecule was required. Degradative and comparative studies (even with synthetic materials), are not capable of unequivocally defining structure; degradation may result in a substantial alteration of the covalent structure from which the original structure may not be deduced, and comparative work, however extensive, can never preclude coelution of dissimilar samples. Mass spectrometric analysis of the intact material overcomes these problems as each fragment ion defines part of the structure from which, by collating the information obtained from the fragments, the complete covalent structure may be obtained. The data is even more conclusive and rigorous if a molecular ion and thus a molecular weight can be obtained. The use of stable isotopes (e.g. $(CD_3\ CO)_2\ O$, $CD_3\ OD$) and accurate mass measurement

remove possible ambiguities in the interpretation. The mixture handling capability (56), specificity and sensitivity of mass spectrometry make it the method of choice for the analysis of complex unknown molecules. In comparative work, identical mass spectra from two compounds (i.e. identical ion abundance ratios at the same source temperature)are major criteria for co-identity.

To obtain a meaningful mass spectrum from small quantities (μg) of biological molecules such as SRS-A, handling techniques were developed to minimise losses through transfer, degradation etc; suitable derivatives, based on the chemical properties of the material were chosen to both enhance volatility and to aid interpretation of the resulting spectrum; such derivatisation must result in minimal by-product formation.

In a search for suitable derivatives the effect of various reagents (previously well studied in protein and lipid chemistry) on both the biological activity (ileum assay) and the triene chromophore was investigated. Under short acetylation conditions (acetic anhydride in methanol, 1 min., section 6) biological activity of compounds I and II was lost, but no change in the U.V. spectrum, either in λ max or absorbance, was observed. Treatment with methanolic HCl (0.4M) or diazomethane gave similar results. These results suggested that under the above conditions, the amino-acid and carboxyl functions were being blocked without modifying the conjugated triene present. Trimethylsilylation would be expected to block any putative hydroxyl group(s) (13) in the molecule.

Based on these results, the trimethylsilyl ether of the N-acetyl-methyl ester was considered to be a suitable derivative from which a mass spectrum might be obtained. Such a derivative would lead to increased volatility of the SRS (by removing the effect of hydrogen bonding) and direct fragmentation at and around the putative hydroxyl group.

To aid interpretation of the mass spectrum and differentiate SRS ions from background, the N-acetyl derivative was prepared using a 1:1 mixture of acetic anhydride and d_6 acetic anhydride. Ions containing the N-acetyl moiety would appear as 1:1 doublets, three mass units apart. (55) Two amino groups would yield a 1:2:1 pattern and so on.

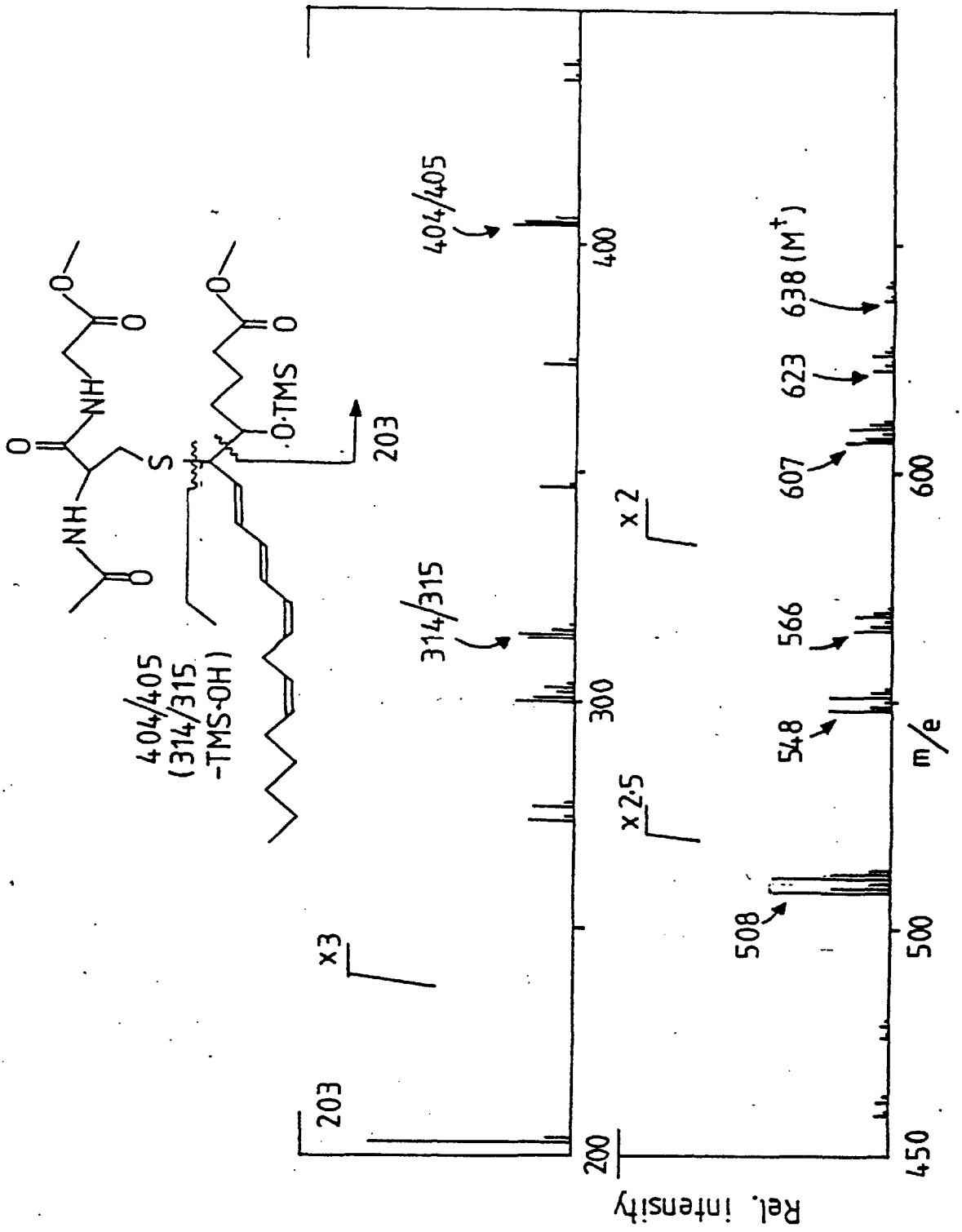

Figure 23

The electron-impact mass spectrum of the trimethylsilyl ether of the N-acetyl (1:1 CH_3CO : CD_3CO) methyl ester of RBL-1 SRS (compound I, 1000 units) at 240°C. The 1:1 isotope pattern is observed for those ions which contain a single derivatised amino group.

RBL-1 SRS (1000 units) was converted to the trimethylsilyl ether of the N-acetyl (1:1 d_3:h_3)- methyl ester and analysed mass spectrometrically (e.i. mode) by the mixture analysis method (56) over a temperature range of 120-350°C. A mass spectrum containing the 1:1 isotope label, (absent in control blanks) was observed at 200-240° C; this was the first mass spectrum of an intact SRS obtained and is shown in Fig. 23.

The mass spectrum of RBL-1 SRS clearly shows the 1:1 doublet molecular ion M^+ (m/e 638) (d) undergoing losses of $^{\cdot}CH_3$ (m/e 623) (d) and $^{\cdot}OCH_3$ (m/e 607) (d) consistent with an ester grouping. The ion (m/e 548) (d) arises via loss of trimethylsilanol (TMSOH) from the molecular ion showing the presence of a free hydroxyl group in the underivatised molecule. This hydroxyl group is positioned on the lipid portion of the molecule at position C-5 by the ion m/e 203 (TMS $\overset{+}{O}$ = CH $(CH_2)_3$ $COOCH_3$). This is a singlet ion i.e. no peptide portion.

From these data, by subtracting from the molecular ion the N-acetyl-methyl ester of cysteinyl glycine, (shown to be present), and the TMSO group, a mass corresponding to a C_{20} tetraunsaturated methyl ester is obtained as the lipid portion of the molecule (in agreement with other data on the precursor role of arachidonic acid). Our early data on lipoxygenase inactivation of SRS-A (50) and SRS (section 6) in common with recent data (58) indicated a cis 1-4 pentadiene, with one double bond at Δ 14,15 (ω 6) based on the ω 6 specificity of soy bean lipoxygenase. Since the mass spectrometric data specify a tetraene, one of the double bonds recognised by this enzyme must be in the conjugated triene chromophore which gives rise to the characteristic U.V. spectrum (section 2 (31)). This places the four double bonds at Δ 78, Δ 9,10 Δ 11,12 and Δ 14,15.

Consideration of the U.V. spectrum, and the bathochromic shift caused by allylic sulphur substitution (57) suggested the thioether linkage to the peptide is at C_6 ; this assignment is supported by the ions at m/e 508 (trimethylsilyl migration to C_6 with cleavage between C_5 and C_6) and m/e 566 (incomplete trimethylsilylation due probably to steric hindrance of the C_6 linked peptide moiety.

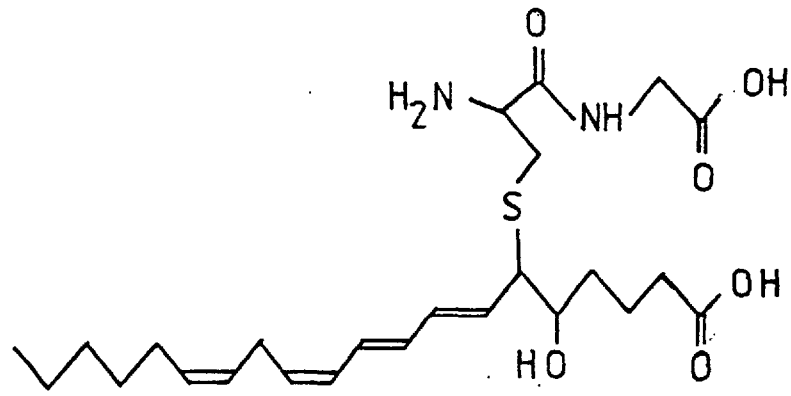

Figure 24

The covalent structure of RBL-1 SRS (compound I) deduced from spectroscopic and protein chemical analysis, is 5-hydroxy-6-cysteinylglycinyl-7,9,11,14-eicosatetraenoic acid.

Using the above data, the covalent structure of RBL-1 SRS was defined as the novel peptidolipid 5-hydroxy - 6 - cysteinyl glycinyl - 7,9,11,14 - eicosatetraenoic acid. [Fig. 24 (59)]. This structure differs from a recently reported synthetic material with SRS like activity (Leukotriene C (58) section 11). The stereochemistry of RBL-1 SRS cannot be defined mass spectrometrically on the vanishingly small quantities of material available; however, a few deductions may be drawn:-

(1) The specificity of lipoxygenase suggests the ω 6 and ω 9 double bonds are in the cis-cis conformation.

(2) Compounds I and II are related in the U.V. spectra by a 2-3 nm hypsochromic shift; this is consistent with a cis-trans isomerisation (section 5 (42)). The mass spectrum of the same derivative of compound II shows no major differences to that of compound I, as would be expected for a geometrical, rather than positional, isomer.

(3) No further isomers were observed in the HPLC-2 profile (as judged by full U.V. spectra) suggesting that II is the most stable all-trans isomer and that I is a trans-trans-cis or related isomer. [Fig. 24].

The complete stereochemistry will be determined by direct comparison with synthetic material.

(viii) The assignments of remaining ions in the mass spectrum were confirmed by isotope labelling experiments (d_9 TMS) and mass measurement. Comparison between the TMS and d_9-TMS derivatives of I are shown in Fig. 26 ; those ions which contain the TMS moiety are shifted by 9 mass units [Fig. 25, 26]. The ions at m/e 405/404 are singlets (thus no peptide portion) and shift upon d_9 TMS labelling (therefore contains the TMS group). These ions arise from cleavage at the thioether linkage (C_6-S) with or without proton transfer to the sulphur (404, 405 respectively).

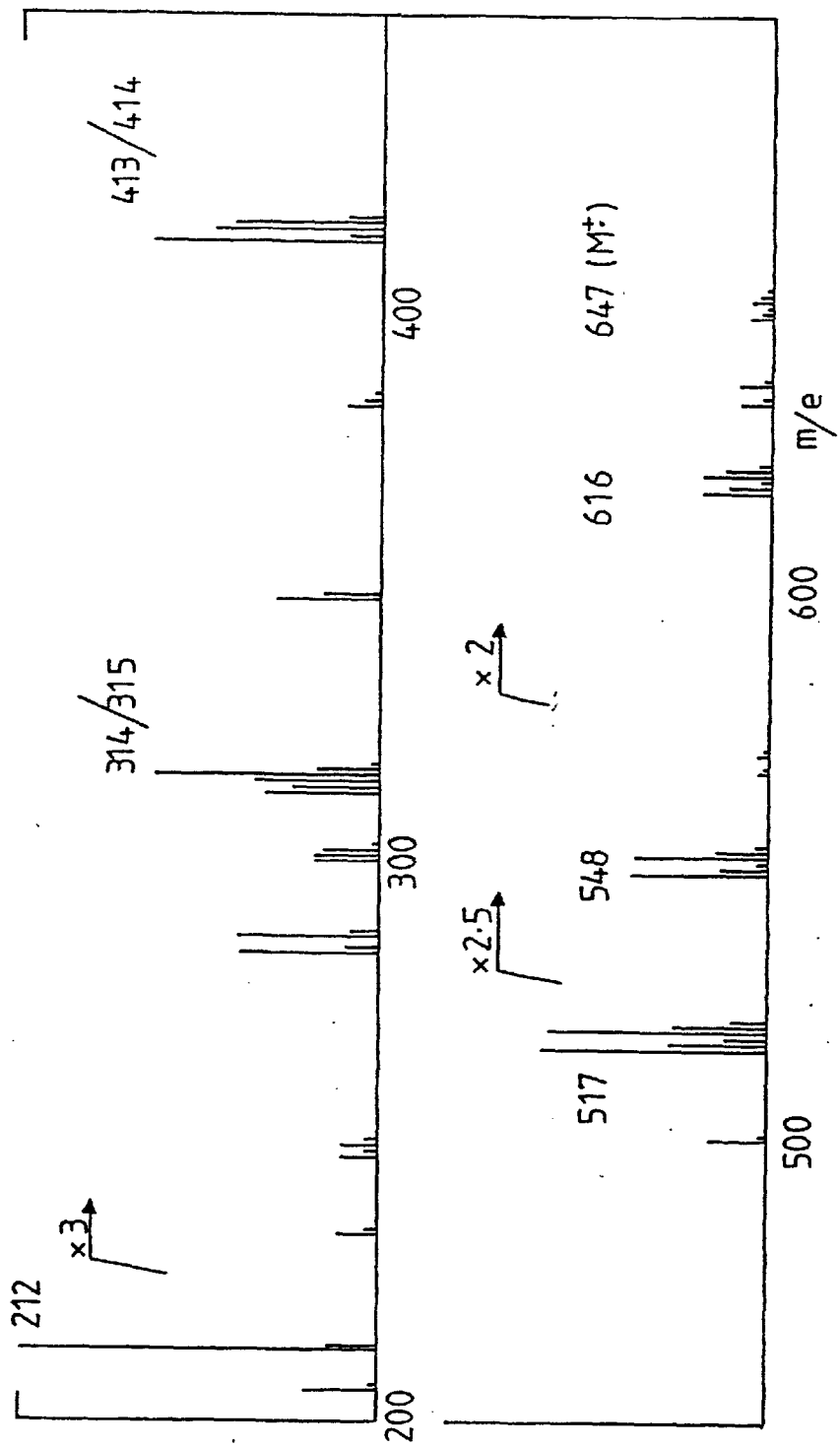

Figure 25

The electron-impact spectrum of the d_9-trimethylsilyl ether of the N-acetyl (1:1 CH_3CO : CD_3CO) methyl ester of RBL-1 SRS (compound I).

m/e (TMS)		m/e (d_9 - TMS)
638 (d)	$M^{\ddagger}$	647
623 (d)	$M^{\ddagger} - \cdot CH_3$	632
607 (d)	$M^{\ddagger} - \cdot OCH_3$	616
566 (d)	$(M : OH)^{\ddagger}$	566
548 (d)	$M^{\ddagger}$ - TMS OH	548
508 (d)	$M^{\ddagger} - HCO(CH_2)_3COOCH_3$	517
405 (s)	$M^{\ddagger} - \dot{S}CH_2CH(H)NHCO \cdot CH_3$	414
404 (s)	$M^{\ddagger} - \dot{S}CH_2CH(CONHCH_2COOCH_3)NHCO \cdot CH_3$	413
315 (s)	m/e 405 - TMS OH	315
314 (s)	m/e 404 - TMS OH	314
203 (s)	$TMS\overset{+}{O}=CH(CH_2)_3COOCH_3$	212

Figure 26

Comparison of the major ions observed in the TMS and d_9 TMS derivatives of RBL-1 SRS [see figures 23,25]; ions which contain the TMS moiety shift by nine mass units on d_9 labelling.

(d) : doublet
(s) : singlet

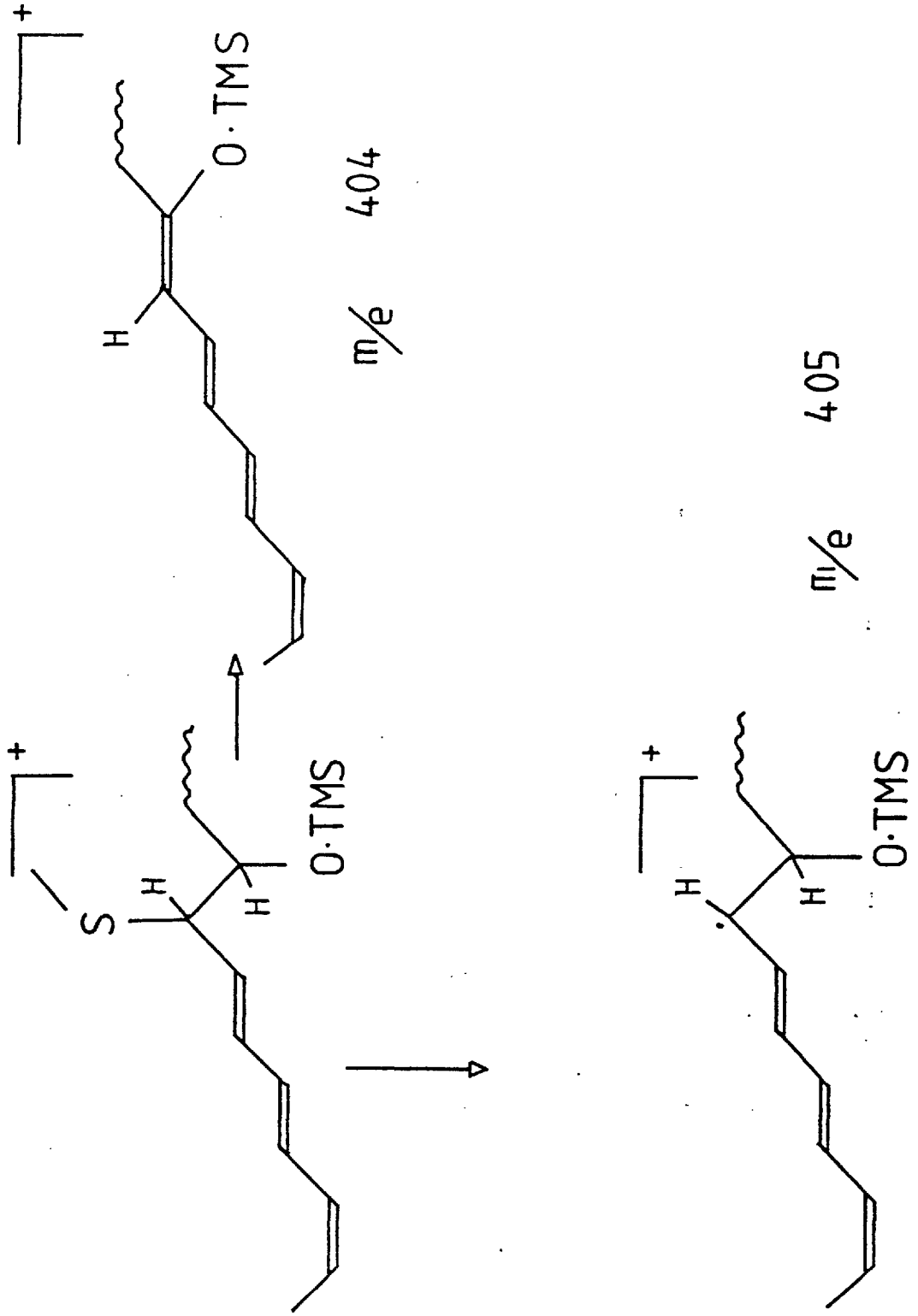

Figure 27

Proposed ion structures for m/e 405 and m/e 404 [fig. 23].

The ions m/e 315/314 are singlets but are not affected by d_9 TMS labelling and thus contain neither the peptide nor hydroxyl portions of the molecule. m/e 315/314 probably arise from loss of trimethyl silanol (TMS-OH) from m/e 405/404. The relative ratios of these ions ($I_{404} > I_{405}$, but $I_{315} > I_{314}$) may be rationalised by considering the proposed ion structures [Fig. 27]; m/e 405 is a radical ion and less readily formed than the conjugated tetraene ion m/e 404. Loss of TMSOH however would be easier from the radical ion than from the conjugated tetraene ion m/e 404, leading to the ratio $I_{315} > I_{314}$. d_9 TMS isotope labelling is consistent with the interpretation given to m/e 638, 623, 607, 566 and 548.

The ion at m/e 508 (d) contains both peptide and hydroxyl portions of the original molecule; it did not appear to arise from a simple cleavage either from the molecular ion or observed daughter ions. Two possible routes for the formation of m/e 508 were considered:-

(a) Concerted loss of $^{\bullet}C_5H_{11}$ (radical elimination at Δ 14,15) and $^{\bullet}COOCH_3$ (from the methyl ester).

(b) Cleavage at C_6-C_5 with migration of the TMS group to C_6 or sulphur: this migration does not occur normally for TMS-O ethers but could arise through interaction of the TMS moiety with the thioether linkage.

Quantities of SRS available precluded extensive studies required to unequivocally identify m/e 508; however, sufficient material was available for an accurate mass measurement.

Calculated masses of the two possible ions are:

(a) 508.2427

(b) 508.2791

Resolution of 40,000 was obtained on the mass spectrometer, sufficient to separate these two possibilities, and the ion at nominal mass m/e 508 peak matched against a triazine reference peak.

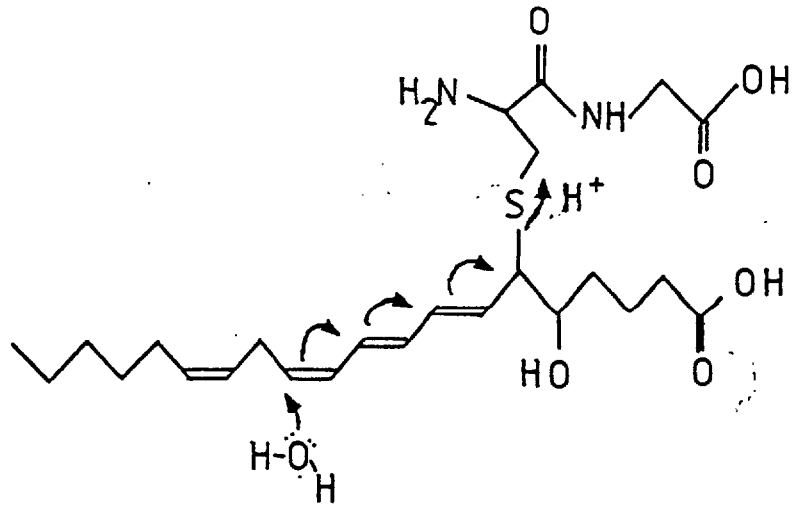

Figure 28

Proposed mechanism for strong acid catalysed cleavage of C_6-S thioether linkage in RBL-1 SRS (compounds I and II)

The measured mass of this peak (5 ppm error) was consistent with pathway "b" for formation of m/e 508. This is further evidence fixing the position of thioether linkage β to the hydroxyl group.

An important inconsistency with the data reported here and the proposed structure of RBL-1 SRS is the generation of cysteine, and dansylcysteine, following strong acid hydrolysis. Cleavage of thioether linkages does not normally occur in acid (e.g. the thioether linkage of methionine is preserved). The release of cysteine is attributed to acid catalysed nucleophilic attack at C_{12} C_{10} or C_8 (a possible mechanism is shown in Fig. 28): this should result in cleavage at C_6-S liberating the peptide or amino-acid. Such a mechanism could provide a chemical route for the generation of of 5, 12 or related dihydroxy eicosatetraenoic acids.

The absence of any other groups on the molecule (e.g. sulphoxide, glycine amide etc.) is clearly shown by the mass spectrum; it would be difficult if not impossible to discount such groups by degradative and comparative studies.

The mass spectrum also shows that a sulphate ester group is not present in RBL-1 SRS (sections 1,6,7); the inactivation of this material by arylsulphatase must arise from some previously unreported side specificity of the enzyme, possibly related mechanistically to the proposed pathway of thioether cleavage in strong acid.

10. Glutathione detoxification pathway

Consideration of the structure proposed for RBL-1 SRS suggests that it arises as part of the glutathione detoxification pathway (60) involving nucleophilic attack of glutathione or cysteinylglycine on a 5,6 oxido, or hydroperoxy, metabolite of arachidonic acid probably in the presence of a glutathione S-transferase [Fig. 30]. The major SRS produced in the RBL-1 cell system is the cysteinyl glycinyl metabolite, there was no evidence for the existence of a glutathionyl SRS, although occasionally small peaks of biological activity, eluting much earlier than SRS in HPLC-2, were observed; the elution volume (16 mls) would be consistent with a more polar SRS. The glutathionyl SRS, if released, is present in our cell supernatant in very low quantities (1%) (see also section 12).

Further steps on the glutathionyl detoxification pathway would be expected to lead to the formation of the cysteinyl and N-acetyl cysteinyl metabolites of SRS (60). Following HPLC-2 purification, substances, inactive on the guinea-pig ileum, with triene U.V. chromophores were observed; these were not further characterised.

11. Leukotriene C

Independently of this work Samuelsson and colleagues (Karolinska Institute, Stockholm, Sweden) isolated a non-immunologically produced slow-reacting substance from murine mastocytoma cells and termed it leukotriene C (LT-C). This material possessed the characteristic U.V. spectrum originally published for SRS-A (section 2). A structure for leukotriene C (LT-C) was proposed, from degradative and radiolabelling studies, as 5 hydroxy-6-cysteinyl-7,9,11,14-eicosatetra enoic acid (61); our independent mass spectrometric studies clearly indicated at the time that this structure was incompatible with our data and the structure was later withdrawn (53,58). A second structure was later published, (concomitant with the RBL-1 SRS work reported in section 9), based on chromatographic comparison with synthetic material. The revised structure was claimed to be the glutathionyl derivative - i.e. 5-hydroxy - 6-γ glutamyl cysteinyl glycinyl - 7,9,11,14 - eicosatetraenoic acid; this material is reported to be formed from a 5,6 oxido intermediate (leukotriene A), the immediate precursor of 5,12-dihydroxy - 6,8,10,14 eicosatetraenoic acid (LT-B, section 8) (62). The complete stereochemistry of this material has now been determined by synthesis (63,64).

Parker and colleagues, in similar degradative studies (RBL-1 SRS) also suggested a glutathionyl structure for an SRS; the presence of a hydroperoxy, or similar moiety was argued (65). In neither case was definitive chemical or spectroscopic evidence on the intact molecules obtained. The definitive covalent structure reported here differs from that of both of the above SRS's . The relevance of these findings will be discussed later (section 12).

12. The structure of Slow-Reacting Substance of Anaphylaxis from guinea-pig lung

(i) Following the structure elucidation of the major SRS from rat basophil leukaemia (RBL-1) cells as 5 hydroxy 6 cysteinyl glycinyl 7,9,11,14 eicosatetraenoic acid (section 9 (59)), attention was again focussed on the more medically relevant SRS-A, in the knowledge that $\sim$ 1000 units of SRS-A correctly derivatised was likely to yield a mass spectrum.

(ii) SRS-A from guinea-pig lungs was purified to homogeneity (sections 1,5). Biological activity was associated with two A280 absorbing peaks (HPLC-2 I and II Fig. 13). Compound I (SRS-A) was pharmacologically, physico-chemically and chromatographically identical to RBL-1 SRS I. (Section 9). Aliquots from the peak tubes of biological activity (together with inactive controls) were subjected to amino-acid analysis and N-terminal analysis (dansylation, Mrs. C.M. Jones - I.C.).

Amino-acid analysis of compounds I and II showed the presence of the amino-acids glycine and cysteine; compound III was devoid of amino-acids above the background level as expected (section 8). N-terminal analysis (dansylation) showed a fluorescent spot on t.l.c. corresponding to dansyl-cysteine (section 9). These data show that SRS-A and RBL-1 SRS contain the same peptide moiety - 'cysteinylglycine'.

(iii) Initial studies with SRS-A indicated that both N-acetylation and esterification destroyed biological activity without affecting the U.V. spectrum; it was decided to convert SRS-A (compound I, 600 units) to the TMS ether of the N-acetyl-methyl ester under the same conditions which resulted in a derivative of RBL-1 SRS suitable for mass spectrometry.

N-terminal labelling with acetic anhydride and d_6 - acetic anhydride (1:1) was again employed to enable differentiation of SRS-A ions from background ions, and to aid interpretation. A deliberately smaller quantity of RBL-1 SRS (500 units) was converted to the same derivative (but without the isotope label) to act as a control sample.

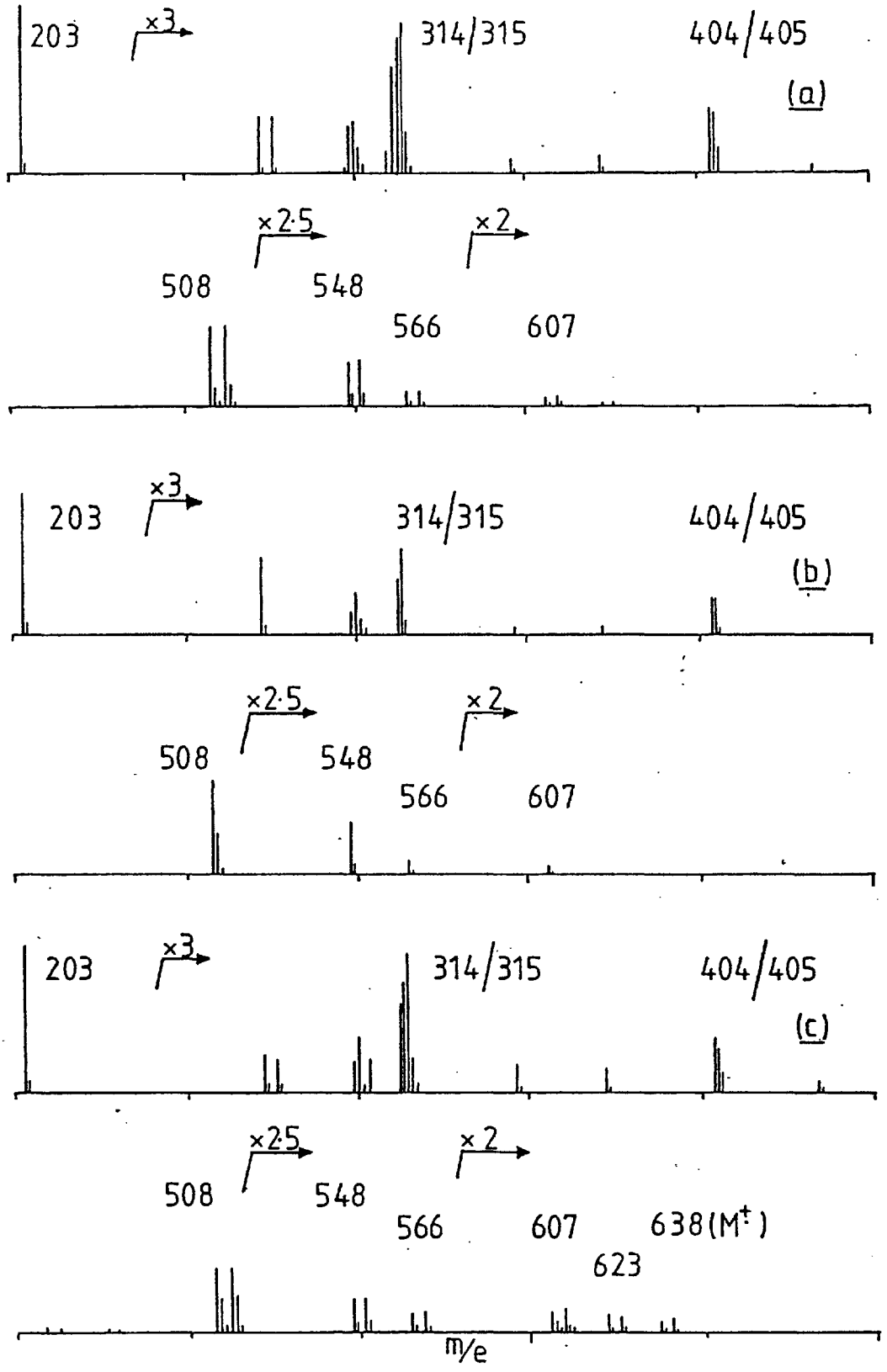

Figure 29

Comparison of the mass spectra obtained from guinea-pig SRS-A (compound I) and RBL-1 SRS (compound I) at 240° C.

(a) SRS-A (600 units) as the trimethylsilyl ether of the N-acetyl (1:1 CH_3CO : CD_3CO) methyl ester; no molecular ion was observed.
(b) RBL-1 SRS (550 units) as the same derivative, but without the 1:1 d_3:h_3 isotope labelling.
(c) The mass spectrum from which the structure of RBL-1 SRS (compound I) was deduced (1000 units, section 9).

The spectrum of SRS-A (followed by the control SRS sample) was recorded using the mixture analysis method, over a temperature range of 120 - 350° C. A spectrum, containing 1:1 doublets separated by 3 mass units, was obtained at ~ 240° C. This was the first mass spectrum of a derivative of SRS-A obtained. The mass spectrum produced is compared with the spectrum from RBL-1 control, and with the previously reported spectrum of RBL-1 SRS (59) (section 9) [Fig. 27]. The spectra unequivocally show that SRS-A from guinea-pig lung is identical to RBL-1 SRS, since the same fragment ions are observed in the same relative abundance ratios at identical ion source temperatures. The figure illustrates the considerable difficulty of determining structure on the small quantity of SRS-A available from the lung, since in contrast with the previous work on RBL-1 SRS, at this lower level the molecular ion was not observed. This in no way affects the interpretation of the spectrum as:-

(a) No molecular ion was observed in the RBL-1 SRS control

(b) The full structure may be built up by combining the fragment ions. For example, m/e 607 (d) corresponds to $M^{\dagger}$ - $^{\bullet}OCH_3$ (containing both lipid and peptide moieties), m/e 548 ($M^{\dagger}$ - TMSOH) shows the presence of a hydroxyl group), m/e 405/404 (s) and m/e 315/314 (s) (-TMSOH) define the lipid portion of the molecule and m/e 203 (s) positions the hydroxyl group at C_5.

Using the same arguments put forward in section 9 to define the structure of RBL-1 SRS (e.g. molecular weight data, presence of triene chromophore, inactivation by ω6 soy bean lipoxygenase etc), together with the pharmacological, physico-chemical , chromatographic and, most importantly, mass spectrometric co-identity of SRS-A with RBL-1 SRS, the complete covalent structure of this important immunologically generated material:
"Slow-reacting substance of anaphylaxis from guinea-pig lung"
is defined as:-

5 - hydroxy-6-cysteinylglycinyl - 7,9,11,14 - eicosatetraenoic acid (66).

The presence of sulphate (section 1, 6,7) is completely discounted, as in the possibility for example of glycineamide, (which is difficult to discount by chemical or enzymic methods (62,64)). The mobility of SRS-A on high voltage paper electrophoresis at pH 6.5

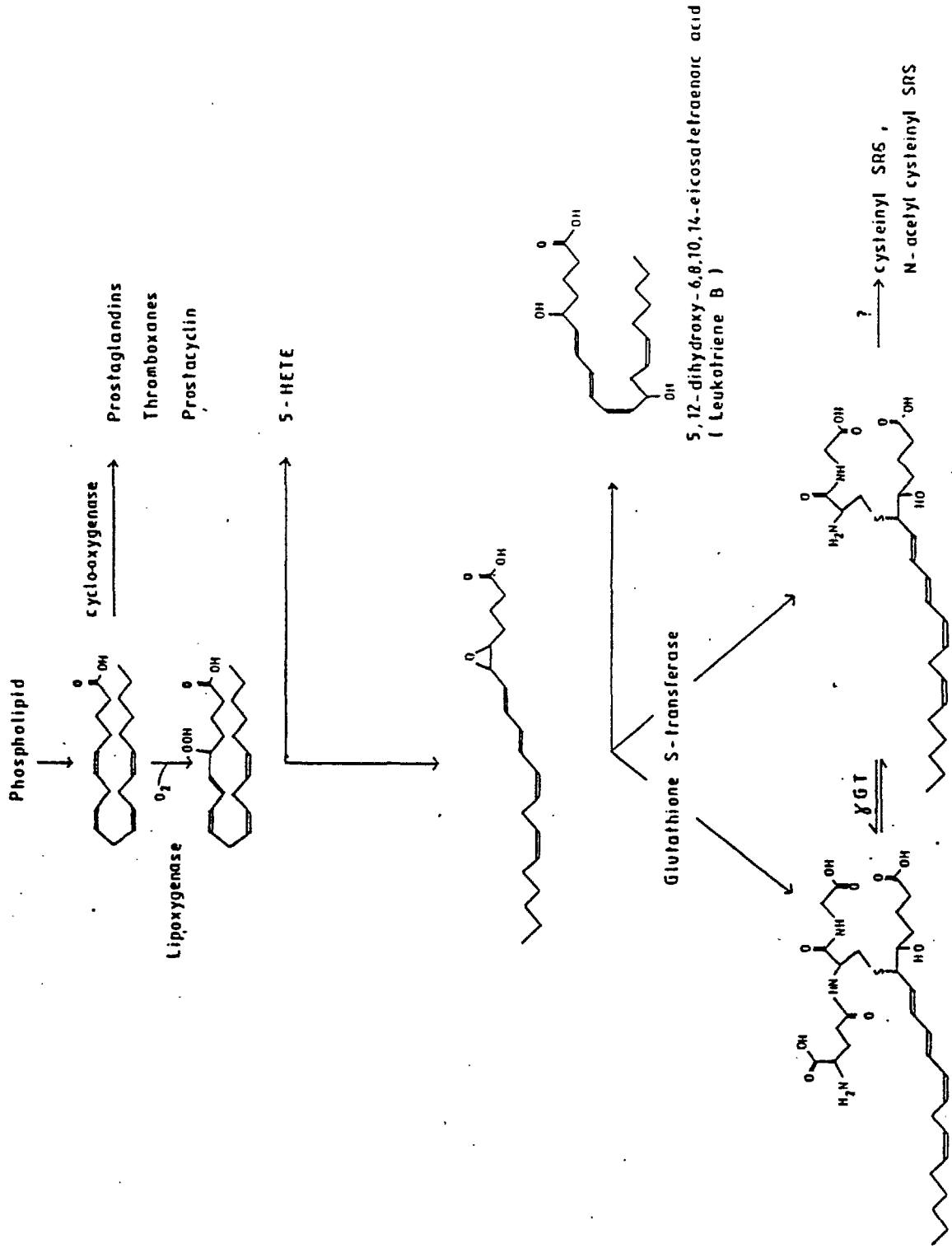

Figure 30

Proposed biosynthetic pathway for the biosynthesis of SRS(-A). The role of glutathione-s-transferase and γ-glutamyl transferase in the biosynthetic pathway has not yet been elucidated. Further steps on this pathway would be expected to lead to the cysteinyl and N-acetylcysteinyl derivatives of SRS(-A).

(M = - 0.27, Asp = - 1.0 section 1 (31)) is fully consistent with the proposed molecular weight of the material, based on the dipeptidyl structure. From these data, the existence of the natural methyl ester is precluded (e.g. the monomethyl ester would be neutral at pH 6.5).

(iv)The pharmacological, physico-chemical and chromatographic co-identity of guinea-pig and human SRS-A (section 3) indicate that the human material is also of the dipeptidyl structure.

(v) It is a point of considerable interest that the structure proposed here for SRS-A derived immunologically from lung is identical to that previously described in section 9 for RBL-1 SRS, released by non-immunological stimulation, from malignant basophils. It would appear that both the antigen-antibody interaction and calcium ionophore treatment lead to a similar activation of the lipoxygenase/glutathione detoxification system, resulting in SRS production [Fig. 30].

(vi)The proposed role of glutathione detoxification in SRS-A biosynthesis [Fig. 30], together with the recent chromatographic and synthetic studies on leukotriene C (62,63) would indicate a role for the glutathionyl derivative in the lung. There is no evidence for such a glutathionyl-SRS released from guinea-pig lung by antigen challenge.

The possibility of having lost a major biologically active component of any putative glutathionyl SRS(-A) during purification may be discounted because:-

(a) all fractions from various column eluates were monitored for bioactivity.

(b) where partitioning took place (i.e. at the charcoal adsorption and ether extraction steps), no biological activity was found in the aqueous solutions.

Thus the major slow-reacting substance released immunologically from lung on antigen challenge is the dipeptidyl material. It is not known whether this material is biosynthesised as such, or arises in vivo from the glutathionyl derivative by the action of

glutamyl transferase (γ GT) [Fig. 30]. It is known that γ GT is involved in glutathione regulation and translocation through the cell membrane; but its role, if any, in SRS-A synthesis and release is not known.

It is interesting that Samuelsson and colleagues in their studies on mouse mastocytoma cells have observed only the glutathionyl SRS (LT-C); they found no evidence for cysteinylglycinyl SRS (SRS-A). It is possible that species differences are responsible for this (e.g. lack of γ GT) however the data presented earlier (sections 3,9) indicate that this is unlikely as the same dipeptidyl SRS is found in human, guinea-pig and rat suggesting SRS(-A) is species independent.

Ionophore treatment does not lead to LT-C formation as the data above on RBL-1 SRS show (section 9), thus presumably the dipeptidyl (SRS-A)/LT-C differences must arise from conditions of cell handling (incubation times etc). Initial studies in this laboratory (67) have shown the presence of γ glutamyl transferase in both cells and lung perfusates; it is possible that the di- and tripeptide metabolites are readily interconvertible, with SRS-A being rapidly formed from LT-C in the presence of this enzyme. The reverse reaction (SRS-A→ LTC) is of course also possible when excess glutathione is present.

It is interesting to note that N-acetylation greatly reduces SRS-A activity. If formation of a peptide bond has such an effect, it is to be expected that the glutathionyl - SRS will be less active biologically than SRS-A. No data on the specific activity of LT-C are, as yet, available.

The role of enzymes in the genesis and metabolism of SRS-A (glutathione S-transferase, γ glutamyl transferase etc) is not yet understood. Investigations into the status of these enzymes is at present under way in this laboratory.

13. Biological properties of pure SRS-A

Purified SRS-A has been tested by colleagues at the Royal College of Surgeons and has the pharmacological properties associated with partially purified material (including contraction of the human bronchus). In vivo studies are now under way.

Based upon U.V. absorbance, and amino-acid analysis data the specific activity of SRS-A is approximately 5-10 ng/unit: 10-20 picomoles/unit . On the guinea-pig ileum assay, the minimum level of detection (0.02 units) is approximately 100 pg (200 femptomoles), this represents a 50-100 fold increase of potency over histamine.

14. Concluding Remarks

A rapid high resolution purification system was developed for the purification of slow-reacting substance of anaphylaxis and related SRS's (31,33,42). Homogeneous material was obtained, with which it was demonstrated that SRS(-A) possessed a characteristic U.V. spectrum (31,42); this discovery has proved invaluable in locating SRS's (eg 58) and providing a cross-correlation between biological activity and a chemical entity in the molecule.

Both guinea-pig SRS-A and RBL-1 SRS were purified in sufficient quantity (5-10μg) to allow spectroscopic and analytical protein chemical investigations into their structure. It has been demonstrated for the first time that a mass spectrum can be produced from undegraded SRS-A and RBL-1 SRS, showing their co-identity and unequivocally defining their full covalent structure as 5-hydroxy-6-cysteinylglycinyl - 7,9,11,14 - eicosatetraenoic acid (59,65). This structure differs from that of a recently reported synthetic compound with SRS-like properties (63) (leukotriene C).

Comparative studies between human and guinea-pig SRS-A indicate that the human material is also of the dipeptidyl structure.(33).

The important definition of the structure of SRS-A described here, opens the way towards a new and enlightened study into the genesis and role of SRS-A and thus into the pathology and therapy of asthma.

Footnotes

(1) During the final stages of preparation of this manuscript synthetic SRS-A has been synthesised by Dr. J. Rokach and colleagues (Merck-Frosst Laboratories, Montreal, Canada). We have undertaken extensive pharmacological, physico-chemical, chromatographic and mass spectrometric comparisons of SRS-A with this stereospecific synthetic material (74). The stereochemistry of SRS-A has now been defined as:

5-(S)-hydroxy-6-(R)-cysteinylglycinyl-7,9-(trans)-11,14-(cis)-eicosatetraenoic acid.

Further studies have shown that SRS-A is at least an order of magnitude more potent on the guinea-pig ileum than is leukotriene C.

(2) Recent work by Samuellson and colleagues (75) on the structure elucidation of the SRS from RBL-1 cells has confirmed the dipeptidyl structure reported earlier (section 9, (59)); conclusive mass spectrometric data was not however obtained in this study.

III EXPERIMENTAL

1. Extraction of slow-reacting substances

Extraction of SRS(-A) and an initial charcoal purification step were carried out by J.R. Tippins (Dept. Pharmacology, Royal College of Surgeons of England, London).

(i) Guinea-pig SRS-A: From perfused sensitised guinea-pig lungs on antigen challenge by the method of Engineer et al (1978) (36).

(ii) Human SRS-A: From sensitised chopped human lung, with or without added exogenous fatty acid, on antigen challenge (37).

(iii) Rat peritoneal SRS: From cells washed from rat peritoneum treated with a calcium ionophore A 23187 (15).

(iv) Slow-reacting substances from rat basophil leukaemia cells were prepared by Mrs. M.N. Samhoun (RCS) by a modification of the method of Jakschick et al (35).

Slow-reacting substances were obtained as a lyophilised powder and stored in our laboratory @ -20°C under nitrogen until required.

2. Preparation of "radiolabelled" SRS-A

Five guinea-pig lungs previously sensitised with ovalbumin (J.R.T. - Royal College of Surgeons) were chopped into 1 mm^3 pieces and washed free of blood in Tyrode solution.

The lung pieces were incubated at 37°C in Tyrode solution in a shaking water bath, and indomethacin (50 μg Mercke, Sharpe and Dohme) and 1-^{14}C arachidonic acid (50 μC; Amersham radiochemical centre U.K.) added after 5 mins and 15 mins respectively. After a further 30 mins incubation, the lung pieces were challenged with ovalbumin (Sigma grade III, 10 mg) for 15 mins; the supernatant was removed by filtration for charcoal adsorption purification (J.R.T.).

The drugs used were added in Tyrode solution; arachidonic acid was initially solubilised in ethanol (200μl) and added to Tyrode solution to obtain a finely divided suspension.

The 'radiolabelled' SRS-A was bulked with guinea-pig material (prepared from perfused lung), for purification.

3. Purification

(a) Gel Filtration

Biologically active material from the charcoal purification step was bulked in methanol: water: ammonia (0.880) [2:2:1 v/v, M:W:A] and rotary evaporated dry at 30-35° C. The dried material was taken up in 2 mls of the same solvent, centrifuged at 1000g for 5 mins to remove insoluble salts, excess charcoal etc, and loaded onto a Sephadex G15 column (180 x 1cm, Pharmacia) under gravity. The column was eluted with M:W:A at the rate of 12 mls/h; the eluate was monitored at 275 nm (Cecil spectrophotometer) and by bioassay. Active samples were bulked and dried on a rotary evaporator at 30-35° C.

(b) Ether extraction

Active material was dissolved in ice cold aqueous acetic acid pH_3 ; (the pH was re-adjusted if necessary) and extracted twice with an equal volume of ice cold, freshly distilled, peroxide free diethyl ether. The ethereal phase was dried under nitrogen and finally on a vacuum pump.

(c) High pressure liquid chromatography (HPLC)

HPLC was carried out on a Waters instrument fitted with a reverse-phase μ Bondapak C_{18} column (analytical or semi-analytical size).

Solvents were of A.R. quality; methanol was used directly from the bottle, n-propanol was distilled from ninhydrin and aqueous solvents (water, 5% acetic acid) purified by passage through the C_{18} column. Aqueous solvents were degassed on a water pump before use.

(i) HPLC-1 methanol : water gradient: The ex-ether material was dissolved in methanol (2-300μl) and mixed with an equal volume of water in the loading syringe. The sample was loaded onto the column [U6K injector, Waters Ass. Ltd.] and eluted isocratically in 50% aqueous methanol (5 mins) followed by a linear gradient (10 mins) to 100% methanol. Samples were dried on a vacuum pump.

(ii) HPLC - 2:n-propanol : acetic acid water gradient: Active material from the initial HPLC step was loaded in 30% n propanol in 5% acetic acid (aq) (2-500 μ l) and eluted isocratically in this solvent (10 mins) followed by a linear gradient (20 mins) to 40% propanol in acetic acid (aq). Samples were dried as rapidly as possible on a vacuum pump.

In both cases the columns were pumped at 2 mls/min with fractions of convenient size being collected. The column eluate was monitored at 254 and 280 nm and by bioassay.

The above purification steps were carried out at room temperature in subdued lighting.

The ether extraction and HPLC-1 steps were not used in the purification of RBL-1 SRS.

4. Pre-purification steps

(i) XAD8 adsorption

XAD8 (5g Rohm and Hass) was washed according to the method of Orange et al (18) for XAD2 and loaded into a column (2½ cm x 2½ cm) in water.

Rat peritoneal SRS (30 mls) was loaded onto the column and washed with 200 mls water followed by 100 mls of ethanol. Fractions (10 ml) were dried on the pump and bioassayed.

(ii) SX8 adsorption

SX8 beads 90g (Bio-rad labs) were swollen in dichloromethane and washed with n propanol, ethanol, 50% aqueous ethanol and water (100 mls); the beads were loaded into a column (10 x 2.5 cm) in water.

Guinea-pig SRS-A (400 mls = 20 animal preparation) was loaded onto the column in water and washed with water (100 mls) and finally ethanol 100 mls. Fractions (25 mls) were bioassayed.

(iii) Glass beads adsorption

Guinea-pig SRS-A (400 mls) was shaken with glass beads (4g Sigma) for 30 mins at room temperature; the beads were packed into a column 7 x 1 cm and washed with water and ethanol. Fractions (5 ml) were bioassayed.

(iv) Sep-pak adsorption

Rat peritoneal SRS (40 mls) was adjusted to pH7 with 5% acetic acid and forced through a C_{18} sep-pak (Waters Ass. Ltd.); the sep-pak was washed with water (60 mls) and ethanol(40 mls). Fractions were bioassayed.

(v) Ethanol precipitation/ether extraction

Guinea-pig SRS-A (400 mls) was cooled in ice and four volumes of ice-cold ethanol added with stirring; the precipitate formed was filtered off with mild suction (No. 3 sinter) and the supernatant dried at 30-35° C on a rotary evaporator. The material was dissolved in acetic acid (pH_3) and extracted twice with an equal volume of ether. The phases formed were dried on the pump and bioassayed.

5. U.V. Spectra

U.V. spectra were recorded in methanol (10 mm pathlength) (300-500 μ l) on a Unicam SP800 spectrophotometer; the spectra were calibrated with a holmium reference.

6. Scintillation counting

Scintillation counting was carried out on a Beckman LS 230 liquid scintillation counter. Samples were dissolved in methanol (500 l) and added to 5 mls of 2,5 di-(5 - t - butyl - 2 benzoxazolyl) - thiophene in toluene (0.6% w/v).

7. Chemical destruction experiments

Samples of active material (5-10 units) at various stages of purification were treated as follows:-

(a) Short acetylation: The sample was taken up in one drop of water, to which was added 200 μl of methanol : acetic anhydride (4:1 v/v). The reaction was terminated immediately by removal of reagents on a vacuum pump.

(b) Acetylation: The sample was treated with pyridine : acetic anhydride (1:10 v/v) for 30 mins.

(c) Fluram treatment: Fluram 1 mg/ml in acetone (200 μl) was added to a solution of SRS(-A) in methanol and allowed to stand at room temperature for 30 mins.

(d) Methylation: Ethereal diazomethane was freshly prepared in a millimole-size wheaton generator (Pierce and Warriner U.K. Limited) by the method of Fales et al (68) and allowed to react with methanolic solution of SRS(-A) for 30 mins. Excess reagents were removed under nitrogen.

(e) Methylation: Methanolic HCl was prepared either by addition of "aristar" HCl (BDH) to methanol to give a 0.4 M solution or by passing dry HCl gas from a cylinder into methanol until the solution became noticeably warm; after cooling the solution was added to SRS(-A) and allowed to stand for 30 mins.

(f) Methylation: A solution of BF_3 in methanol (BDH) was added to the sample and incubated at 37° C for 30 mins.

(g) Catalytic hydrogenation: Nickel boride (Ni_2B) catalyst was prepared by the method of Paz et al (1970) (69); a methanolic solution of SRS(-A) was treated with 5 mg of the catalyst at room temperature and 55°C for 30 mins. Excess catalyst was removed by centrifugation; it was washed in warm methanol and the washings and supernatant combined.

(h) Cyanogen Bromide treatment: CNBr (10 mg) was dissolved in 1 ml. water and washed twice with chloroform; the aqueous phase was treated with an ethanolic solution (50 μl) containing arachidonic acid (100 mg). An aliquot ($\sim \frac{1}{10}$) of CNBr solution was added to protonated SRS-A in water (containing 100 ng of arachidonic acid); the solution was allowed to stand for one hour before multiple lyophilisation to remove excess CNBr.

(i) Acid treatment: SRS(-A) was dissolved in 0.1M HCl (aq) and incubated at 37° C for 30 mins.

In each of the above reactions, carried out at room temperature (20° C) unless otherwise stated, the reaction was terminated by removal of reagents on a vacuum pump, and samples re-dissolved in Tyrode solution for bioassay.

(j) Base treatment: SRS(-A) was dissolved in 0.1M NaOH (aq) and incubated at room temperature, 37° C and 100° C for 30 mins. The solution was neutralised with acetic acid and bioassayed directly.

(k) Borohydride reduction: The sample in water was treated with alkaline solution of sodium borohydride (0.5 mg/ml pH10) and incubated at room temperature for 30 mins. The reaction was stopped and excess borohydride neutralised with acetic acid; the sample was bioassayed directly (at pH 7-8).

In each case the reactions were carried out in the dark under nitrogen; aqueous solvents were gassed with nitrogen prior to use. Both control and reagent blanks were run at the same time. The reactions were carried out at various stages of purification; in order to solubilise samples after the ether extraction stage (i.e. protonated), SRS(-A) was dissolved in a minimum quantity of methanol and mixed with excess water in the relevant reactions.

8. Enzymic Inactivation of SRS(-A)

(i) Arylsulphatase treatement *

SRS(-A) (5-50 units) was incubated with 50-100 milliunits of arylsulphatase (Limpet, Sigma type V, 11.7 units/mg) in pH5 acetate buffer (200 mM) at 37° C for one hour.

(ii) Lipoxygenase treatment *

SRS(-A) (5-50 units) was incubated with 10 μg of soy bean lipoxygenase (Sigma type IV) in 50 mM oxygenated Ambic buffer (pH 8.7) for 30 mins at 37° C.

In each of the enzyme digests, control samples, containing boiled inactive enzyme, and reagent blanks were run concurrently. The reactions were terminated by lyophilisation.

* 1. Arylsuphatase: 1 unit will hydrolyse 1 μ mole of nitro catechol sulphate per hour at pH5 at 37° C.

* 2. Lipoxygenase: 1 unit will cause an increase in A_{234} of 0.001 per minute at pH9 at 25° C with linolenic acid as substrate.

9. Resulphations

(i) Chemical: Sulphating reagents pyridine SO_3 and $NEt_3{:}SO_3$ were prepared as described in Fieser and Fieser (I) (70) and Tserng et al (71) respectively. Samples of arylsulphatase inactivated SRS(-A) together with standards (including Tyrosine, Tyramine, Prednisolone, 0.1 mg, from our stocks) were dissolved in acetonitrile or dry dimethylformamide and incubated with excess sulphating reagent at 37° C or 80° C for one hour (in the dark under nitrogen). The reaction was terminated by addition of water and excess reagents removed on a vacuum pump.

(ii) Enzymic: A Triton X-100 solution of a liver sulphating enzyme was prepared from 5 rats by the method of Rao et al (1977) (72) and used in the attempted resulphation of SRS-A in an imidazole buffer (pH 7). After incubation, the solution was acidified with acetic acid to pH 3.5 and extracted with 2 volumes of peroxide free diethyl ether.

10. Thin layer chromatography for Sulphation experiment

Resulphation experiments: The sample $\sim$ 0.5 μg was loaded in 5 μl of acetonitrile onto high performance (HPTLC) silica plates (Whatman); the plates were developed in chloroform; ethyl acetate: methanol: ammonia (0.880) 10:5:5:2 v/v. The plate was stained with Cu^{2+} /H_3PO_4 reagent (73).

11. Mass Spectrometry

Mass spectrometry was carried out on a Kratos MS50 mass spectrometer fitted with high field magnet. The electron impact (EI) mode was used. Samples, after derivatisation, were loaded in chloroform onto a hollow quartz tip; the accelerating voltage was 8 KV with 70 eV electron beam energy. The method of mixture analysis, over a temperature range of 120-350° C was used and spectra were recorded on U.V. sensitive chart paper.

12. Derivatives for Mass Spectrometry

Trimethylsilyl ether of N-acetylmethyl ester.

(a) N-acetylation: short acetylation procedure (methanol: acetic anhydride 4:1 v/v 1 min): section 7a: experimental.

(b) Esterification: ethereal diazomethane (15 mins, room temperature).or methanol-HCl (0.4M , 15 mins): section 7 d,e.

(c) Trimethylsilylation - sample was dissolved in chloroform (50μl) and a solution of pyridine: bis (trimethylsilyl) trifluoracetamide: trimethyl chlorosilane (1:6:1) (20μl) added; the reaction was carried out at room temperature under nitrogen for 30 mins and terminated by removal of reagents under vacuum.

Isotopically labelled reagents were used as required.

13. Biological Assay

Bioassays were performed by colleagues at Dept. Pharmacology, Royal College of Surgeons. Samples were assayed on guinea-pig ileum in the presence of mepyramine, hyoscine and indomethacin (36). Biological activity is quantitated in terms of arbitrary units defined in terms of an initial batch of partially purified guinea-pig SRS-A. The tissue response may be measured in terms of histamine:

0.034 units SRS(-A) = 5 ng histamine.

All active samples are tested with the specific SRS(-A) antagonist FPL 55712 (Fisons(23)).

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