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ON THE PREPARATION OF HIGHLY PURIFIED SLOW REACTING SUBSTANCE OF ANAPHYLAXIS (SRS-A) FROM BIOLOGICAL EXTRACTS

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1 Very highly purified (> 100,000 u/mg) slow reacting substance of anaphylaxis (SRS-A) has been prepared by reversed phase high pressure liquid chromatographic (HPLC) techniques.

2 High resolution liquid chromatography suggests that SRS-A may exist in at least three distinct forms which are possible tautomeric.

3 SRS-A collected by antigen challenge *in vivo* and by calcium ionophore-induced release *in vitro* are chromatographically indistinguishable.

4 Treatment of SRS-A with diazomethane but not sodium borohydride results in a loss of biological activity but treatment of the methyl ester with base results in a partial recovery of activity.

5 Highly purified SRS-A was examined by infrared and ultra-violet spectroscopy, and found to have a benzene-aromatic and probably an amino acid.

Introduction

The acronym 'SRS' (slow reacting substance) was originally coined in 1938 by Felberg & Kellaway, and by Feldberg, Holden & Kellaway to describe the substance(s) generated by the action of snake venom in perfused lungs or preparations of an egg yolk. The name was chosen because of the nature of the spasmogenic action of their unidentified principle which contracted gut muscle, but did so more slowly than histamine or acetylcholine.

Two years after the original discovery Kellaway & Trethewie (1940) reported that the generation of an SRS could be induced by antigen challenge. Brocklehurst (1953) concluded that the material generated during anaphylaxis was different from other SRSs and named this material 'SRS-A' (slow reacting substance of anaphylaxis).

In 1956, Vogt demonstrated that the original SRS was a fatty acid; however the identity of SRS-A was uncertain at the time, and has remained an enigma ever since. Because of its possible involvement in anaphylactic bronchoconstriction or immediate hypersensitivity-type reactions (cf. Austen & Orange, 1975; Adams & Lichtenstein, 1977) it would seem to be important to identify this mediator and to examine its pharmacology. The two major barriers to this seem to us to be firstly, that SRS-A is only produced in extremely small quantities and secondly that no really effective method exists for the extraction and purification of SRS-A.

We now describe an excellent method for the purification of SRS-A which should be of value both to pharmacologists wishing to study the biological activity of the pure material and to biochemists who wish to gain insight into the nature of this enigmatic mediator.

A partial account of this work was presented to The Society in March 1977 (Blackwell, Burka & Flower, 1977).

Methods

Generation of crude rat SRS-A

Crude rat SRS-A was generated either by antigen challenge *in vivo* (Orange, Valentine & Austen, 1968) or by the calcium ionophore technique *in vitro* (Bach & Brashler, 1974; Burka & Flower, 1979). When the material was not processed immediately, it was stored in buffer aqueous solutions at −20°C.

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Assay of SRS-A

Two methods were used: firstly, a slight modification of the method of Brocklehurst (1960), whereby two guinea-pig ileum preparations were suspended in series and superfused at 10 ml/min with Tyrode solution at 37°C. To improve selectivity of the tissues the superfusing fluid contained antagonists to histamine, 5-hydroxytryptamine, acetylcholine and catecholamines (Piper & Vane, 1969). The second method depends upon the fact that SRS-A releases a mixture of prostaglandin endoperoxides and thromboxane from guinea-pig isolated perfused lungs (Engineer, Piper & Sirois, 1977). For this assay, guinea-pig isolated lungs were prepared as described by Piper & Vane (1969). The preparations were perfused with Krebs solution (37°C) at 10 ml/min and the effluent from the lungs was used to superfuse in cascade strips of rabbit aorta and in some cases, rat stomach (Piper & Vane, 1969). Samples containing SRS-A were injected into the pulmonary artery and the resulting liberation of thromboxane A_2 was detected by the rabbit aortic strip.

Definition of a unit of SRS-A

SRS-A was quantitated by comparison with an 'in house' standard stored in aliquots at −20°C: when compared to a standard obtained from the laboratory of the late Dr R. P. Orange, 'one unit' was found to be approximately half the potency of his standard unit.

Methylation and de-methylation of SRS-A

We have discovered that SRS-A can be methylated (Me-SRS-A) and the biological activity recovered by removal of the methyl group at some subsequent time during the purification procedure. For methylation, the SRS-A was dissolved in methanol, 3 vol. of diethyl ether were then added, and a freshly prepared solution of ethereal diazomethane was added dropwise to the stirred mixture. Me-SRS-A is inactive on the guinea-pig ileum and also in the guinea-pig lung preparation, and since biological activity is the only way of measuring SRS-A it is necessary to remove the methyl group before assay. To do this we used base hydrolysis; SRS-A is stable in base, and in a typical 'reactivation' procedure we took a small aliquot of Me-SRS-A dissolved in methanol and took it to dryness in a small pyrex tube; 0.1 ml of 0.2 N NaOH was added, and the tube was tightly stoppered and incubated in a hot water bath at 75°C for 10 min. The basic solution was then neutralized and assayed. Time course studies (see Figure 1) indicated that 10 min was more than sufficient to recover biological activity.

Reduction of SRS-A with sodium borohydride

Samples of partially purified SRS-A were dissolved in methanol or water and an excess (1 to 5 mg) sodium borohydride added. After 15 min the sample was briefly acidified with 1 N HCl to destroy excess borohydride. The sample was then neutralized with base and assayed. In cases where the borohydride interfered with the assay procedure the material was desalted in a 0.5 g column of Amberlite XAD-2 as described later.

Arylsulphatase inactivation

Inactivation of partially purified SRS-A with arylsulphatase was carried out as described by Orange, Murphy & Austen (1974). Before bioassay, the sodium acetate buffer (pH 5.0) was neutralized with 1 N NaOH.

Thin layer chromatography of SRS-A and methyl SRS-A

This was conveniently performed on silica gel thin layer chromatography (t.l.c.) plates (ascending chromatography) or silica gel loaded paper descending chromatography) using the solvent system chloroform:methanol:2.5 N ammonium hydroxide (6:2:1, v/v: lower phase only). Biological activity was eluted from developed chromatograms with water and (after demethylation if necessary) detected by bioassay.

Purification procedure

Step 1, protein precipitation and desalting Crude SRS-A generated by either of the two methods referred to earlier was added to ice cold ethanol such that the final proportions were ethanol 80%, water 20% (v/v). After 10 min stirring of this solution on ice it was filtered through a glass sinter under vacuum to remove precipitated material (mainly protein in nature). In the case of small volumes (< 100 ml) centrifugation replaced filtration.

The ethanol/water solution was next concentrated under vacuum in a rotary evaporator with the water bath temperature set at 50°C. The residue was then dissolved in approx. 1 l of distilled water and the pH adjusted to 10 with 1 N NaOH. After 1 h the pH was adjusted to neutrality with 1 N HCl: this 'base hydrolysis' step is included to destroy phospholipids, which would otherwise contaminate the final fraction. This procedure, as well as all subsequent operations, was performed in the cold room (ambient temperature 6°C). SRS-A containing solutions were desalted using columns of either Amberlite XAD-2, 6 or 8 or silicic acid with bonded octadecyltrichlorosilane. In the former case the resin was first washed with 10 to

20 vol. of double distilled water, acetone, and ethanol, equilibrated in water, and loosely packed into a glass column. The aqueous SRS-A solution was pumped through the column followed by 5 bed vol. of distilled water, and the SRS-A itself was then eluted with 10 bed vol. of 80% ethanol:20% water (v/v).

The procedure using the silicic acid with bonded octadecyltrichlorosilane was very similar: the packing was washed exhaustively in water and methanol and equilibrated in water. Application of SRS-A was again followed by 5 bed vol. of water to remove salts and the SRS-A recovered with 5 bed vol. of methanol.

In either case the solutions of desalted SRS-A were taken to dryness in a rotary evaporator as described before and redissolved in a minimal quantity of distilled water (adjusted to pH 7 to 8 with dilute ammonium hydroxide) for the next procedure.

Step 2: Sephadex chromatography The quality of the SRS-A extract was greatly improved if gel chromatography was included as one of the steps in the purification procedure. SRS-A has previously been reported to have a mol. wt. of 350 to 450 and we therefore selected Sephadex G-10 and G-25. The chosen gel was pre-swollen overnight in buffer. Ammonium bicarbonate or ammonium acetate (1% w/v) were the buffers of choice since they are volatile electrolytes and can be removed by lyophilization. In the case of ammonium acetate, the pH was adjusted to 7.5 with ammonium hydroxide or acetic acid. The sample was applied to the column in a minimal quantity of water, the eluting buffer pumped through the column and appropriate fractions collected. Each fraction was assayed for biological activity and u.v. absorbance (254 nm). Active fractions were pooled and lyophilized.

Step 3: Silicic acid chromatography Unisil graded silicic acid was slurried in *n*-hexane, packed into a column of suitable dimensions and washed with 5 column vol. of *n*-hexane. The lyophilisate from the previous step was dissolved in 5 ml methanol and 100 to 200 mg of Unisil was added and the slurry decanted into a small round-bottomed flask. The solvent was carefully removed on a rotary evaporator. This procedure results in the SRS-A being absorbed onto the Unisil, and when the methanol was completely removed the dry Unisil-SRS-A mixture was tipped out from the flask onto a piece of aluminium foil and applied to the top of the Unisil column. This procedure is necessary because SRS-A is not soluble in the equilibrating solvent (*n*-hexane).

Elution of the silicic acid column was begun with 5 column vol. of *n*-hexane, 5 column vol. of diethyl ether and 5 column vol. of ethyl acetate. This was followed by 2 column vol. of chloroform, 90% chloroform:10% methanol (v/v), 80% chloroform:20% methanol, and so on to 100% methanol. All fractions were collected, a small aliquot of each removed, the organic solvent was removed and the residues dissolved in buffer and assayed for biological activity. Active fractions were pooled. For those experiments in which the methyl ester was purified an identical procedure was used except that aliquots of the fractions were first de-methylated before assay.

Steps 4 and 5: Reverse phase high pressure liquid chromatography (HPLC) This technique is the cornerstone of the purification technique described in this paper. The instrument used here was the Waters model equipped with two model 6000A solvent pumps, a U6k injector, a model 440 dual channel u.v. absorbance detector and a model R-400 differential refractometer detector. For the running of gradients, the two solvent pumps were controlled by a model 660 solvent programmer.

The choice of column packing requires some consideration: although SRS-A can be chromatographed on Unisil silicic acid in an open column situation (as described above) it was soon established that the high efficiency silicic acid packings (μ-Porsasil) used with this machine were far too retentive, and the recovery of SRS-A from these columns was very small indeed (<5%), although the methyl ester may be chromatographed in this fashion by equilibrating the column with chloroform and eluting with increasing concentrations of methanol. For this reason reversed phase columns were used: two column packings were considered in particular, these were the μ-Bondapak-C-18 and μ-Bondapak-CN, which had bonded phases of octadecyltrichlorosilane and cyanopropylsilane respectively.

Two modes of chromatography were used with this system; gradient chromatography or isocratic chromatography. In the former case the columns were equilibrated with buffer or distilled water and eluted with increasing concentrations of methanol or ethanol, and in the second, the column was equilibrated with the eluting solvent (most usually, mixtures of methanol and water).

In either case serial fractions were collected after injection of the sample into the HPLC and tested for biological activity in the manner described. In the case of the methyl ester the samples were first demethylated as described.

Before injection into the HPLC all samples were filtered through a Waters sample clarification assembly, which removes particles >0.5 μm.

Analysis of final material

Quantities were insufficient for elemental analysis, but an attempt was made to characterize it by infra-red (i.r.) and ultra violet (u.v.) spectroscopy.

The material, dissolved in 0.5 ml methanol, was evaporated to dryness *in vacuo* in a platinum mortar and the residues made into a 5 × 1 mm rectangular KBr pellet for i.r. examination. The spectrometer was a PE 580, with computer facilities allowing storage and manipulation of data. The range used was 4000 to 350 cm^{-1} for a single overall scan taking 5 h, and the region 1850 to 650 cm^{-1} was scanned repetitively to confirm detail. The slow scan rate and repetition were required to reduce noise; scale expansion of 20× was finally used, which would correspond to a sample size of say 2 to 3 μg.

Subsequently, the KBr pellet was ground with methanol, and a u.v. spectrum obtained from the resulting solution evaporated to 0.2 ml, using a microcell. The instrument was a Beckman ACTA-CV.

The following chromatographic materials, drugs and chemicals were used: arylsulphatase, type V and sodium borohyride (Sigma); Unisil (Clarkson Chemical Co. Inc.); Sephadex G-10 (Pharmacia Fine Chemicals); Amberlite XAD-2, 6 and 8 (Rohm and Haas); histamine acid phosphate (BDH); silicic acid with bonded octadecyltrichlorosilane (Waters Ltd.); silica gel loaded paper, grade SG81 (Whatman) and 'Uniplate' t.l.c. plates precoated with silica gel G (250 μm) (Anachem). The calcium ionophore A23187 was a generous gift from Lilly.

All other reagents (buffer salts, chromatography solvents, etc.) were of 'Analar' grade or the highest purity obtainable.

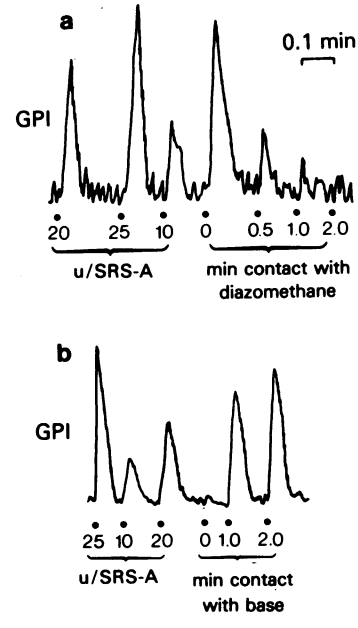

Figure 1 Destruction of biological (guinea-pig ileum contracting) activity of SRS-A by reaction with diazomethane, and reactivation by heating the ester with 0.2 N NaOH at 80°C. Each assay is preceded by three doses of standard SRS-A. The experiment illustrates the importance of the carboxyl group for the biological activity of SRS-A. GPI = guinea-pig ileum. See text for details.

Results

Comments upon the assay of SRS-A

Two assay systems were used for estimating SRS-A in this work and it is worthwhile briefly considering the relative merits of each. When superfused, the guinea-pig ileum had a somewhat faster response time than it would in an organ bath. When combined antagonists were included into the superfusion fluid the assay could also be rendered fairly specific, although prostaglandins still interfere with the estimations. Amongst the disadvantages of this tissue were the fact that it is sensitive to changes in salt and ion concentrations, and the fact that examining large numbers of column fractions is a fairly tedious procedure. An additional complication was that certain fractions contained a smooth muscle depressant factor which reduced the sensitivity of the ileum to SRS-A.

The guinea-pig isolated perfused lung, in contrast, was excellent for examining large numbers of column fractions very quickly. The preparation is very insensitive to the changes in salt and ion concentrations found in (say) the Amberlite column fractions and on the whole was more sensitive than the ileum. The lung does not respond to prostaglandins but does not distinguish between SRS-A and histamine since both of these mediators release thromboxane A_2. The great drawback of this assay is that it is qualitative only. The dose-response curve to SRS-A is very steep and is reminiscent of an 'all or none' response; this is in contrast to the guinea-pig ileum where reasonable dose-response curves were easily obtained and meaningful measurements of SRS-A could be made.

In summary, the latter preparation though useful should not be used in situations where a quantitative assessment is necessary, or where histamine is present in the extract.

Methylation of SRS-A

Figure 1 shows that SRS-A loses its spasmogenic activity after brief exposure to diazomethane. The methylation reaction proceeds very quickly being complete (in the presence of excess reagent) in 2 to 4 min at room temperature. The methyl ester was also 5 to 10 times less active than the parent acid as a

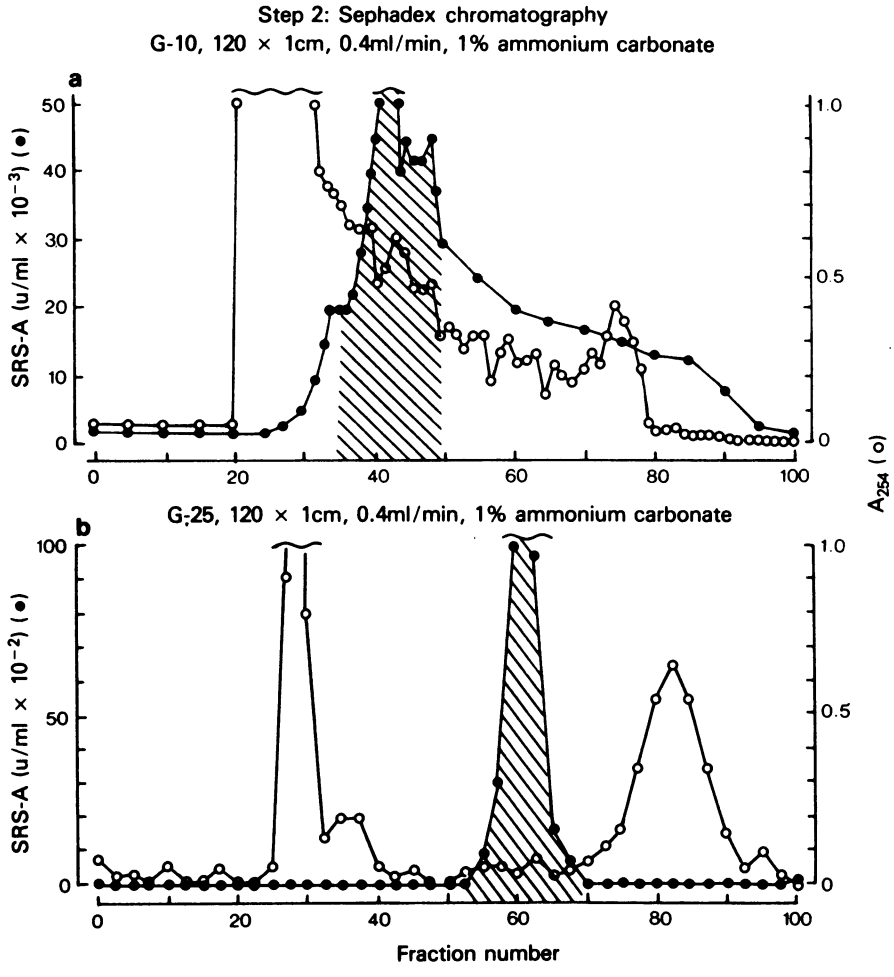

Figure 2 Chromatography of SRS-A on Sephadex is an early and important stage in the purification: (a) chromatography on G-25; (b) on G-10. The columns used were 120 × 1 cm, the flow rate was 0.4 to 0.8 ml/min and the buffer was 1% ammonium carbonate. Both columns separate SRS-A from a mass of high molecular weight material which appears in the void volume (indicated by the u.v. absorbance) but the G-25 gave a rather more discrete zone of biological activity. SRS-A chromatographs as a low mol. wt. compound of about 400 to 600 Daltons.

thromboxane releasing agent in the perfused lung assay.

The biological activity of SRS-A could be restored, although sometimes only partially, by exposing the methyl ester to 0.2 N NaOH and heating in a water bath. Usually biological activity was recovered within 5 min. The reason for the inconsistent recoveries was not clear and was not further investigated. One useful property of methyl SRS-A is that it can be easily extracted from aqueous solutions whether weakly acidic, neutral or basic. A solvent such as ethyl acetate is added to the aqueous solution and the two phases macerated by vortex mixing for 1 min; this is sufficient to extract 70 to 90% of the Me-SRS-A irrespective of the pH.

Thin layer chromatography of SRS-A and methyl SRS-A

Both SRS-A and its methyl ester can be separated by t.l.c. or paper chromatography. Generally speaking t.l.c. was a more useful technique because of the shorter running times ($\simeq 2$ h compared with 6 to 8 h for descending paper chromatography). In our hands, SRS-A chromatographed on t.l.c. with an R_F approximately 0.65 whilst the methyl ester had an R_F of 0.90.

Significant band spreading was observed with SRS-A but not with the methyl ester, which always ran as a very sharp band. Recoveries from the plates were never greater than 60%.

Purification procedure

Step 1: Protein precipitation and desalting In this paper we have used two methods for desalting and partially purifying crude SRS-A extracts, Amberlite and octadecyltrichlorosilane chromatography. Each method has peculiar advantages and disadvantages. Amberlite XAD-2 resin is relatively cheap, fast flowing and will accept quite high loads. The chief disadvantage is the large variations in chromatographic properties encountered between batches, which led to inconsistent or poor recoveries of SRS-A. It was in an attempt to find a superior grade of resin that we tested XAD-6 and XAD-8. These resins appeared to suffer from the same problems as XAD-2 and in addition were difficult to clean: even after extensive prewashing a certain amount of material leaked out from the Amberlite into the SRS-A.

To try to improve this initial step we used a large column packed with silicic acid with octadecyltrichlorosilane. This had the advantage of very good and reproducible yields of SRS-A (80 to 90%) but suffered from the disadvantages of being expensive, rather fragile packing, and from the fact that it has a very slow flow rate. In this study we used Amberlite for large batches of crude SRS-A and silicic acid with bonded octadecyltrichlorosilane for small batches.

Amberlite resins are non-ionic and remove water-soluble organics particularly those with a structural hydrophobic component: the chief advantages of the Amberlite chromatography step from the purification viewpoint is that histamine as well as inorganic salts are not retained by the column and are thus removed from the extract at an early stage in the purification.

Step 2: Sephadex chromatography Two types of gel were found to be satisfactory; Sephadex G-10 and G-25. Figure 2 shows chromatograms of SRS-A obtained with these two gels. Both gels give adequate resolution of SRS-A from a large mass of high molecular weight material. The G-25 gives a more discrete resolution of the SRS-A and was therefore generally preferred.

Although these columns were not calibrated with marker compounds of known molecular weight it can be seen from the two figures that SRS-A chromatographed as a molecule of approx. mol. wt. 500 to 600.

Step 3: Silicic acid chromatography is an important step in the purification procedure described here, because it allows SRS-A to be separated from neutral lipids and fatty acids as well as the most biologically active (stable) prostaglandins; those of the E and F type.

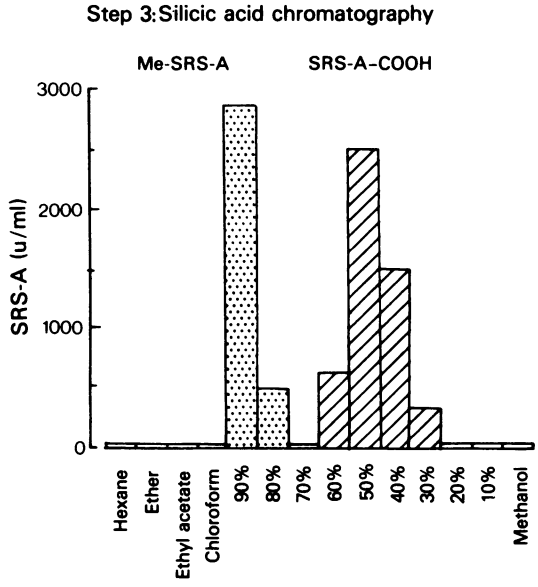

Figure 3 Silicic acid chromatography of SRS-A and its methyl ester. The Unisil column was prepared as described in the text and eluted with 2 to 5 column volumes of the solvents shown under the X-axis which mainly comprised mixtures of chloroform in methanol. SRS-A was chiefly recovered in the 50% chloroform in methanol fraction but the methyl ester (Me-SRS-A) was much less polar, and was usually found in the 90% (or even 100%) chloroform fraction.

Figure 3 shows that SRS-A (free acid form) elutes mainly in the 50% methanol:50% chloroform fraction. Control experiments with other radioactive materials such as fatty acids (arachidonic, oleic), phospholipids (phosphatidylcholine) and prostaglandins E_2 and $F_{2\alpha}$ showed that >98% fatty acids are eluted in the ether fraction (the remaining 2% in the ethyl acetate fraction) whilst prostaglandins E and F eluted in the 90% and 100% chloroform fraction. Occasionally there was a trace of E and F remaining in the 80% chloroform fraction but never in the 70% chloroform fraction. Phospholipids as represented by phosphatidylcholine (a phosphatide of intermediate polarity) were more of a problem. Large amounts were found (as much as 40% of the total applied) in the 'SRS-A fraction' (50% chloroform). Although separation from the most phospholipid occurs in the HPLC stages it is highly desirable to reduce the contamination at the earliest possible stage in chromatography. The fact that the silicic acid column does not completely resolve SRS-A from the phospholipids is a powerful argument for the base hydrolysis step

included at the beginning: of the products of phospholipid hydrolysis, the fatty acids would be well resolved by this column and the remaining fragments being very polar in nature would be expected to be retained by the column. Even so traces of organic phosphorous were occasionally detected in the final samples by i.r.

The chromatographic behaviour of SRS-A methyl ester is also seen in Figure 3. The ester is clearly separated from the free acid, and a silicic acid column is an excellent method for resolution of the two species.

Step 4: Reverse phase HPLC with gradient elution Both types of reverse phase chromatography packings that were tested here were found to be satisfactory and to be suitable for the separation of SRS-A. Table 1 briefly sums up the differences between the two packing materials. The chief difference between them was the greater retentive capacity of the C-18 packing and this material was the most frequently used in our purification. Whilst this manuscript was in preparation Morris, Taylor, Piper, Sirois & Tippens (1978) have also reported that this technique gives good results with SRS-A purification.

After some pilot experiments we settled upon the following conditions: the column was equilibrated in distilled water the pH of which had been adjusted to 7.5 with ammonium hydroxide. The filtered sample was dissolved in not more than 100 µl methanol and applied to the column. The column was eluted with water at 1 ml/min for 5 min and then with increasing concentrations of methanol. This gradient was controlled by a gradient programmer using Waters Curve no. 6; the time taken to run from 0% to 100% methanol was 30 min after which the column was eluted with 100% methanol for a further 20 min. SRS-A characteristically eluted between 40 to 60% methanol. The u.v. detector on the chromatograph clearly revealed that this step resolved SRS-A from a great many other compounds. Often 50 to 70 individual peaks were observed.

One most interesting observation was that SRS-A tends to split up into at least three active zones (see also Morris *et al.*, 1978). This is clearly illustrated in Figure 4 which shows a reverse phase chromatogram of a sample of SRS-A which had been stored in the deep freeze in methanol for 7 days. Originally, this sample chromatographed as a single discrete peak of biological activity with a retention time identical to that now occupied by the centre zone of biological activity. This phenomenon was reproducible and did not seem to depend upon the pH of the solution in which the SRS-A was stored.

The methyl ester of SRS-A can also be purified on this column. Characteristically it has a longer retention time (as one would expect from a less polar compound on a reverse phase column).

Step 5: Reverse phase HPLC using isocratic elution The final step of the purification technique again employs the HPLC, this time without using a gradient technique. Once again either the C18 or the CN packing material was suitable although the C18 was generally preferred because of its greater retentive capacity. One advantage of using isocratic elution is that an additional type of detector, the differential refractometer, can be used. It is not very sensitive, 10 µg quantities are mandatory, but does allow detection of gross impurities in the extract. Figure 5 shows a chromatogram of the major zone of biological activity from the previous step (see Figure 4). About 2 min after injection of the SRS-A there is a large deflection on the differential refractometer and a smaller one on the u.v. detector. This deflection is caused by the solvents in which the SRS-A was dissolved, eluting from the column. Thereafter the differential refractometer trace assumed a more or less steady baseline. Several

Table 1 Columns used for high pressure liquid chromatography of SRS-A

Brand name	*Packing*	*Mode*	*Polarity*	*Suitability*		*Solvents for*	
				SRS-A	*methyl ester*	*equilibration*	*elution*
µ-Porasil	Microparticulate silicic acid	N.P.	Very polar	Not suitable	Suitable	Chloroform	Methanol
µ-Bondapak-CN	As above with bonded cyanopropylsilane	R.P.	Intermediate polarity	Suitable	Suitable	Water	Methanol
µ-Bondapak-C18	As above with bonded octadecyltrichlorosilane	R.P.	Very non polar	Suitable	Suitable	Water	Methanol or ethanol

* N.P. = normal phase; R.P. = reversed phase.

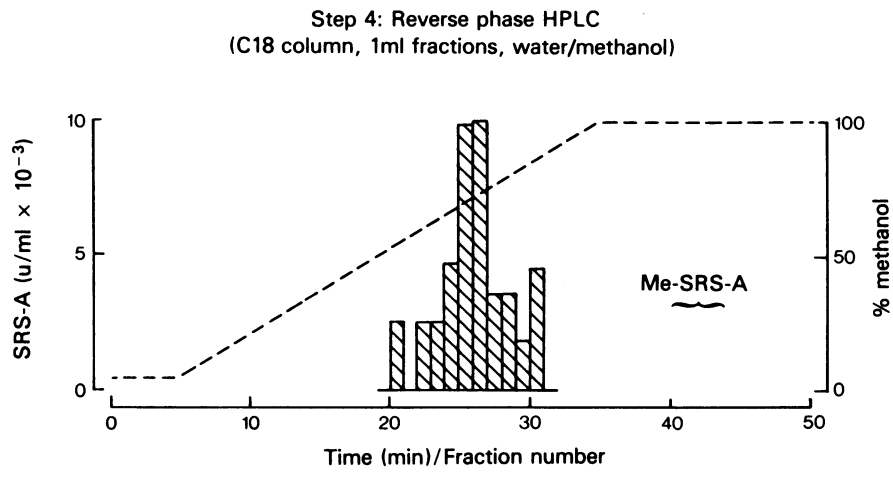

Figure 4 Reverse phase chromatography of SRS-A using a Waters high pressure liquid chromatograph. The column used was a μ-Bondapak C18 and was equilibrated with distilled water and then eluted with increasing amounts of methanol (broken line) at a flow rate of 1 ml/min using a Waters gradient controller running on programme no. 6. SRS-A bioactivity (indicated by hatched bars) often separated into 2 to 4 discrete fractions (3 are seen here) even if it had been previously purified by this technique. The figure also shows the position in the chromatogram normally occupied by the methyl ester (Me-SRS-A).

u.v. absorbing peaks are seen on the lower trace indicating the presence of at least 2 to 4 compounds even in this purified sample. SRS-A eluted with a retention time of about 11 min and always corresponded with a small deflection on the u.v. detector. However, there was no change in the refractive index detector indicating that SRS-A has some intrinsic u.v. absorbance at 254 nm but that the mass in this sample is too small (i.e. sub μg) for detection by the refractometer.

Purification of the methyl ester by straight phase HPLC

Although not suitable for purification of SRS-A itself because of its great retentive capacity, the μ-Porasil column can be used to purify the methyl ester of SRS-A. The μ-Porasil column is a straight phase column and was equilibrated with chloroform, the sample was applied dissolved in 100 μl methanol, and after 5 min elution with chloroform at 2 ml/min increasing concentrations of methanol in chloroform were pumped through the column running a linear gradient to 100% methanol in 30 min. Fractions were collected every half minute and aliquots of these were demethylated and tested for biological activity. Characteristically, two zones of biological activity were found, one very discrete zone (containing most of the biological activity) with a retention time of 29 min, ($\simeq$15% chloroform, 85% methanol) which corresponded to a deflection on the u.v. detector, and a fairly broad zone with a retention time of 14 to 20 min (80 to 50% chloroform:20 to 50% methanol) which contained only a small amount of activity. The latter was discarded, and the former used for further experiments.

Treatment of SRS-A with acid, base, arylsulphatase, hydrogen peroxide and sodium borohydride

Heating SRS-A to 80°C with 1 N HCl for 15 min completely destroyed its biological activity but when the same experiment was repeated with 1 N NaOH, greater than 70% of the biological activity remained.

SRS-A was inactivated by the enzyme arylsulphatase or by exposure to hydrogen peroxide but not by exposure to sodium borohydride. The chromatographic mobility of SRS-A was not changed by sodium borohydride strongly suggesting that there are no ketone groups on the molecule.

Differences between antigen and calcium ionophore generated material

No differences were detected between the qualitative biological activities in the ileum or lung assay of the two types of SRS-A: both were inactivated by acid (but not base) and by arylsulphatase. Both compounds also appeared to have the same chromatographic mobility.

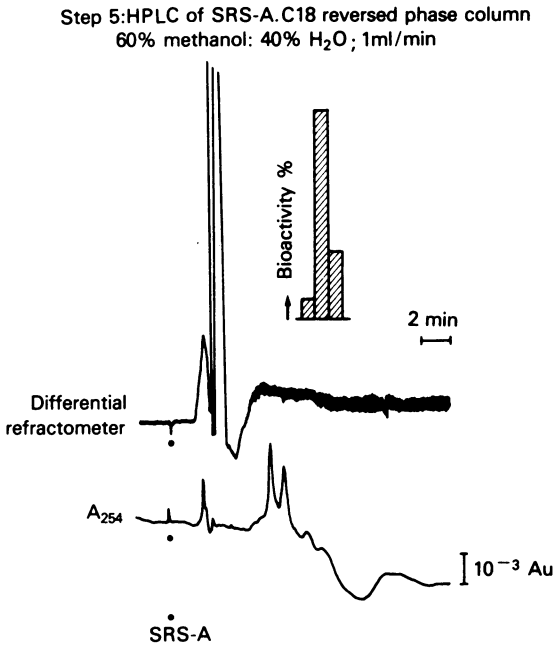

Figure 5 Reverse phase chromatography of SRS-A using the same columns as in Figure 4 but this time using a single solvent (60% methanol:40% water at 1 ml/min). The trace from the differential refractometer (attenuation × 4) and the u.v. detector (set at 254 nm) are shown together with the zone of biological activity (cross hatched columns) which characteristically eluted with a retention time of about 10 min, and always corresponded to a slight deflection of the u.v. detector in the experiment; about 1000 u has been injected. Au = absorbance units.

Examination of SRS-A by u.v. and i.r. spectroscopy

The most definite result was the presence of a benzene-aromatic component as deduced from the u.v. absorption which had a maximum at 275 mm. This was observed with both acid and methyl ester, while chromatography blanks gave no such absorption. The intensity corresponded to ~1.4 μg tyrosine in a 4000 u sample, though this amount could possibly be halved by background correction; (tyrosine is taken as a typical biological aromatic). If the aromatic is a component in a total molecular weight of 400, then there would be ≃3.1 μg of material in a 4000 u sample.

Infra-red spectroscopy was more of a problem because the blanks gave absorption, and this was variable. Chromatography using reverse phase columns gave much cleaner blanks than the straight phase μ-porasil column in so far as the former were essentially free from the silicic acid eluted from the latter, but there was also much interference from materials thought to be derived from the relatively large volumes of solvents used, even though these were of high grade. Study of the spectra of a number of blanks led to the conclusion that there was no carbohydrate or peptide present in SRS-A, and probably no lipid ester.

Possible diagnostic bonds which stood out were a pair at 1510, 1580 cm^{-1} which resembled the absorption of several amino acids, tyrosine in particular. Following computer-subtraction of water, a strong real maximum was evident at 3330 cm^{-1}, the absorption rising to this from 2500 cm^{-1} in the manner of COOH and/or N^+H. Less certain (because of the blanks) were 1250 cm^{-1} which in fact would suit phenolic C—OH of tyrosine, 1095 cm^{-1} and 1130 cm^{-1}. These latter could be from organic phosphate and inorganic sulphate respectively, though secondary or tertiary alcohol would be another possibility. Evidence for an aliphatic component was provided by CH absorption at 2860, 2920 cm^{-1} which was considerably stronger than in any of the blanks.

There was also a pair of bands at 1710, 1740 cm^{-1}, considerably weaker than the 1580 cm^{-1} band, suggesting carboxylic acid and ester. But 1740 cm^{-1} is the expected frequency of COOH in amino acid cation, $H_3N^+ \cdot CHR \cdot COOH$, and could represent a fraction of the bulk zwitterion. It is not certain that it is a true sample frequency, and in any case it is too weak to be the ester band of lipid giving the observed aliphatic CH: 1710 cm^{-1} could imply the presence of an ordinary carboxylic acid in addition to any amino acid present.

Regarding previous work, the bands at 1600, 1420 cm^{-1} reported by Orange, Murphy, Karnovsky & Austen (1973) can only be carboxylate if they derive from a hydroxy- or amino acid; simple carboxylates have their strong band near 1560 cm^{-1}. Allowing for different sample preparations, our 1580 cm^{-1} could well correspond to their 1600 cm^{-1}.

Discussion

Overall comments on the purification procedure

We have deliberately combined a selection of different chromatography techniques: non-ionic, size exclusion, as well as straight and reverse phase adsorption-partition chromatography in the hope that such a combination would give a better resolution than had been achieved hitherto.

Some idea of the efficiency of the various steps of the purification as well as the overall yields obtained may be gained from Table 2. It should be stressed that there was a considerable variation in the efficiency of the purification, and that the figures given in

the table are from one representative experiment. The variations which occur are chiefly due to the differences in the chromatographic efficiency of the Amberlite resin. Obviously a certain amount of error is inherent in the data because it relies upon the biological activity of SRS-A as a quantitative technique, and there are other agents in the extract which are SRS-A-like (for example, prostaglandins) as well as other factors which depress the response of the ileum to the contractile effect of SRS-A. The latter effect can be especially puzzling since it occasionally results in an apparent *increase* in the amount of SRS-A during some of the purification steps. In our experience this often occurs during HPLC and one may sometimes see an increase of as much as 20% of the total units of SRS-A after passing through the reverse phase column. Presumably this is because one is separating out an inhibitor. This phenomenon was not observed in the experiment shown in Table 2, but several other common features are seen: there is actually an apparent reduction in specific activity of the SRS-A following ethanol precipitation and Amberlite chromatography. One likely explanation for this is that the total activity measured in the crude fraction was not all SRS-A and thus when the bulk of this was removed during the first two stages, an apparent decrease in the specific activity resulted. The table illustrates the usefulness of Sephadex and silicic acid chromatography in eliminating a large amount of unwanted material, but really significant increases in specific activity of SRS-A are only seen after HPLC, where the specific activity is increased almost 20-fold in a single step. Unfortunately, the total mass of the material at this stage was very small and impossible to weigh on conventional balances. Thus the data on the final purification is probably an under-estimate. Losses of the pure material on HPLC columns are very small (see steps 6 and 7, Table 2) provided care is taken in recovery and sample manipulation to avoid losses during handling. Final specific activities of 100,000 u/mg or greater were regularly obtained with the techniques detailed here, but the final yield varied considerably from one batch to another. In the table a final yield of about 8% is reported; yields as high as 17% have been achieved but 8 to 9% is a more realistic estimate of the average figure.

Preparation of the methyl ester of SRS-A is a useful technique since it enables SRS-A to be purified in a slightly different manner from its free acid and then can provide a useful check on physico-chemical data: thus both free acid and methyl ester have u.v. absorption with maximum at 275 nm, and this independence of final purification procedure confirms existence of a u.v. chromophore in SRS-A. The methyl ester of SRS-A also has certain advantages such as its ability to partition readily into organic solvents from aqueous mixtures. While this manuscript was being prepared Jakschik, Falkenhein & Parker (1977) also reported that SRS-A could be methylated.

Physiochemical data on SRS-A

It has previously been suggested that SRS-A and probably other SRSs as well, are low mol. wt. unsaturated fatty acid derivatives, containing a carboxylic acid, a sulphate ester, hydroxyl groups and may possibly contain an arachidonic acid transformation product (Bach, Brashler & Gorman, 1977; Jakshik *et al.*, 1977). Evidence for each of these ideas will now be

Table 2 A representative purification of SRS-A

Step no.	*Fraction*	*Total wt. (mg)*	*Total u*	*Sp. act. u/mg*	*% yield*	*Purification factor*
1	Crude fraction	1738.0	390,000	224.3	100	0
2	Ethanol precipitation and base hydrolysis	1450.0	254,000	175.2	65.1	0.7
3	Amberlite XAD-2	1007.0	186,000	184.7	47.6	0.8
4	Sephadex G-10	238.0	117,600	494.1	30.2	2.2
5	Silicic acid	9.9	56,000	5656.6	14.2	28.2
6	HPLC (1)	$\simeq$0.33	$\simeq$34,600	$\simeq$103,592.8	$\simeq$8.9	$\simeq$461.9
7	HPLC (2)	$\ll$0.3	31,000	$\gg$103,333.3	7.9	>460.7

The masses of SRS-A in Step 7 were certainly too small to weigh on a conventional balance but in one experiment 4000 units was subjected to i.r. spectroscopy and a mass of about 3 μg was estimated. This makes the final purification factor about 58,000× and the final specific activity about 1.3×10^6 u/mg.

discussed in relation to the data obtained in this paper.

Molecular weight The mol. wt. of SRS-A had previously been estimated by gel filtration as about 400 (Orange *et al.*, 1973), and the behaviour of SRS-A in our system would indeed suggest a compound of that approximate mol. wt.

Acidic-lipid nature Previous work strongly suggested that SRS-A was an acidic lipid. It was noted by Änggård, Bergqvist, Högberg, Johansson, Thon & Uvnas (1963) and Orange *et al.* (1973) that when protonated (i.e. by the addition of acid to an aqueous solution) SRS-A will pass into organic solvents such as diethyl ether. We have confirmed these findings but have not used them routinely as a purification technique because of the acid lability of SRS-A. The ability of SRS-A to pass into organic solvents when protonated is reminiscent of polar acidic lipids such as prostaglandins which might suggest that the acidic property is due to COOH. That SRS-A must be considerably more polar than these compounds, can be deduced from the relative mobilities on silicic acid chromatography and from relative solubilities: in the dry state SRS-A is not soluble in chloroform or any less polar solvent although it will dissolve in ethanol, acetone or (most readily) methanol. Corroborative evidence for its acidic nature comes from measurements of its electrophoretic mobility (Orange *et al.*, 1973) and from the observation that anion exchange chromatography may be used as a purification technique (Strandberg & Uvnas, 1971). Confirmation of carboxylic acid was given by observation of Strandberg & Uvnas (1971) that a reagent which reacts with carboxylic acids, N,N-carbo-di-*p*-tolylimide, at least partially reduced the biological activity of SRS-A: also diazomethane inactivates SRS-A and activity is restored by base hydrolysis (Jakschik *et al.*, 1977 have also reported a similar effect). Finally our spectroscopic evidence suggests an amino acid, and probably an additional carboxylic acid.

Aliphatic nature of SRS-A Confirmation of aliphatic character comes from the work of Strandberg & Uvnas (1971) who demonstrated that agents reacting with unsaturated double bonds (iodine monobromide, potassium permanganate) inactivated SRS-A. Deactivation by oxidation was also found by Orange and his associates (1973) and by ourselves. Both i.r. studies noted aliphatic C—H absorption. However, we have already remarked upon the difficulty of preparing an extract which is *completely* free of phospholipids. The i.r. band at 1095 cm^{-1} possibly assignable to organic phosphate, could be consistent with contamination by phospholipids, with consequent introduction of aliphatic material. Quite independent evidence that SRS-A is at least partially aliphatic comes from the experiments of Bach *et al.* (1977) and Jakschick *et al.* (1977) which show that arachidonic acid may be a precursor of SRS-A. Their conclusion is based largely upon two types of experiments, those in which known inhibitors of arachidonic acid metabolism are found to modify SRS-A generation, and those in which radioactive arachidonic acid has been shown to be incorporated into purified (or partially purified) SRS-A preparation.

With regard to the former line of evidence both groups give conflicting accounts of the effect of inhibitors on SRS-A generation. Thus Bach *et al.* (1977) report that ionophore-induced SRS-A production *in vitro* by mononuclear cells from rat peritoneal washing was blocked by inhibitors of the cyclo-oxygenase (which generates prostaglandins) such as indomethacin (Vane, 1971) as well as an inhibitor of endoperoxide metabolism, Azo analogue I (Gorman, Bundy, Peterson, Sun, Miller & Fitzpatrick, 1977), and an inhibitor of both cyclo-oxygenase and lipoxygenase pathways of endoperoxide metabolism, TYA (an analogue of arachidonic acid, cf. Hamberg & Samuelsson, 1974). By way of contrast Jakschik *et al.* (1977) using an *in vitro* ionophore-induced generation system with rat basophilic leukaemia cells reported that the cyclo-oxygenase inhibitor, indomethacin, did not block SRS-A production but that TYA did. Thus the conclusion of the first group, that SRS-A is a cyclo-oxygenase metabolite, differs from that of the second group who concluded that SRS-A is a metabolite of the lipoxygenase pathway. We have recently shown the situation to be even more complicated (Burka & Flower, 1979), in that SRS-A generated *in vivo* is enhanced by indomethacin but blocked by phenidone, an inhibitor of both cyclo-oxygenase and lipoxygenase pathways (Blackwell & Flower, 1978). When the same inhibitors were tested *in vitro* however, SRS-A release was inhibited by both indomethacin and phenidone. It is not possible at present to understand completely the effect of these inhibitors and it would be dangerous to draw any conclusions from the inhibitory action.

The other line of evidence supporting the idea that SRS-A is an arachidonate metabolite depends upon the finding that when cells preincubated with radioactive arachidonate are induced to release SRS-A, then a small amount of the radioactivity is associated with the SRS-A through various purification steps. Bach *et al.* (1977) showed that the radioactivity was associated with SRS-A through only a fairly crude purification (basically Amberlite and silicic acid), however, Jakschik *et al.* (1977) demonstrated in similar experiments that biological activity and radioactivity accompanied each other through two dimensional chromatography. In a series of unpublished experiments we have confirmed that radioactive arachidonic acid or a metabo-

lite does accompany SRS-A through purification steps 1 to 4 (we have not yet tested the final HPLC stage) but it is conceivable that this could be attributed to arachidonate esterified to phospholipids.

Hydroxyl groups The presence of hydroxyl groups within SRS-A was suggested by Strandberg & Uvnas on the basis of experiments in which reagents which react with hydroxyl groups were observed to inactivate SRS-A (phenyl isocyanate and acetic anhydride in pyridine). Orange and his co-workers also observed a hydroxyl band in their i.r. spectra (3400 cm^{-1}) and in one study we too have observed absorption in a similar band (3300 cm^{-1}) which would tend to confirm the presence of one or more hydroxyl groups in SRS-A. There is no evidence that this is a primary alcohol, but it could be phenolic in nature.

Sulphate ester The possibility that SRS-A contains a sulphate ester was first suggested in 1974 by Orange, Murphy & Austen. The unsatisfactory behaviour of SRS-A during mass spectrometry led these workers to speculate that SRS-A could contain a highly polar group such as phosphate or sulphate. This idea was supported by two findings: firstly, that an arylsulphatase enzyme inactivated SRS-A and secondly, when subjected to spark source mass spectrometry SRS-A was found to contain a preponderance of ^{32}S and ^{40}Ca ions. The ability of arylsulphatases to degrade SRS-A has been confirmed by several studies including the current one, Bach *et al.* (1977), Jakschik *et al.* (1977), Wasserman & Austen (1976) and Takahashi, Webster & Newball (1976). Under most circumstances one would regard enzymatic inactivation data with a certain amount of circumspection: the enzyme used in the original (as well as some subsequent) studies was derived from limpets and this may be contaminated with certain other enzymes such as carboxypeptidases (Bach, Jones & Kay, 1975) which could be active when large masses (i.e. mg quantities) of arylsulphatases are added to relatively small masses of SRS-A. However, several groups of workers have also used purified, or partially purified preparations of enzymes from other sources all of which appear to be active (Wasserman, Goetzl & Austen, 1975; Wasserman & Austen, 1976; Orange & Moore, 1976). Furthermore Wasserman & Austen have demonstrated that the hydrolysis of the synthetic substrate *p*-nitrocatecholsulphate by human lung arylsulphatase was competitively inhibited by SRS-A.

In the i.r. spectroscopy data reported here was an absorption at 1125 cm^{-1} which might be attributed to inorganic sulphate. It is unlikely that inorganic sulphate *per se* could have accompanied SRS-A all the way through the purification procedure we have described (there was none in the solvent blanks). It is more likely that the inorganic sulphate arose through the decomposition of the SRS-A. It is well known that SRS-A is somewhat unstable when pure and that enzymatic removal of the sulphate ester (see above) will also inactivate SRS-A. It is not therefore unreasonable to suggest that during the preparation of SRS-A for i.r. spectroscopy (i.e. manufacture of a KBr disc and repeated scanning of the sample at somewhat above room temperature) the sample decomposed and that it does so by losing its sulphate group, thus giving rise to an inorganic rather than an organic sulphate signal in the i.r.

Possibility of aromaticity We report here that purified SRS-A has u.v. absorption with maximum at 275 nm and this taken together with i.r. bands at 1510 and 1585 cm^{-1} suggest the possibility of a tyrosine-like aromatic. This finding is interesting since it naturally leads one to think of the possibility of an aromatic sulphate being present in SRS-A. Tyrosine-*O*-sulphates are of course found in peptides such as gastrin, caerulein and cholecystokinin, and it is interesting that removal of the sulphate ester abolishes smooth muscle contractile activity (Bertaccini, 1969; Ondetti, Rubin, Engel, Pluscec & Sheehan, 1970). This is, of course, consistent with the notion of inactivation by arylsulphatase enzymes which (as the name suggests) display hydrolytic activity towards a wide range of phenolic sulphates. It may be dangerous to draw too many firm conclusions from this fact since some of these enzymes can also apparently hydrolyze cerebrosides and some other sugar sulphates (Jerfy & Roy, 1973; Helwig, Farooqui, Bollack & Mandel, 1977) and it seems likely that this is the endogenous substrate for the enzyme. There was no indication of carbohydrates in i.r. analysis and an aromatic sulphate appears to be more likely. Interestingly, aromatic sulphates are known to be unstable especially in mild acidic media but are stable in base (Bettelheim, 1954; Coburn, Mahuren, Schaltenbrand & Sallay, 1978): observations which also apply to SRS-A.

In summary the picture of SRS-A which emerges from the data presented here and from that of other workers is of a low mol. wt. compound; containing a phenolic hydroxyl group and a carboxylic acid function. The balance of evidence is in favour of there being a sulphate group possibly an aromatic sulphate ester as well as an aliphatic component. If the mol. wt. of the components for which we have reasonable evidence (allowing one carboxyl, one hydroxyl, one benzene aromatic and one sulphate ester) are calculated then a total of almost 250 Daltons is accounted for. The actual mol. wt. of SRS-A is probably approx. twice this figure, the remainder could be made up with aliphatic residues.

It is difficult to assess the significance of our observation that pure SRS-A can be rechromatographed as up to four active fractions. A similar phenomenon has

previously been observed with human SRS-A (Takahashi *et al.*, 1976): it could be different salts or zwitterions of SRS-A, or tautomeric forms or active metabolites. There could be a 'family' of SRS-As derived from the same precursor, analogous to the prostaglandins.

Conclusion

A method of preparing very highly purified SRS-A is described. Rat SRS-A has been purified almost 60,000 times to specific activities of about 1.3 u/ng. No difference was detected between the SRS-A generated by antigen challenge or calcium ionophore. SRS-A has also been purified as the biologically inactive methyl ester. The physicochemical evidence suggests that SRS-A is a low mol. wt. substance with a phenolic hydroxyl and carboxyl groups, an aliphatic component and possibly an aromatic sulphate ester.

Note added in proof

Since this paper was written evidence has been presented by Samuelsson and his colleagues at the International Prostaglandin Conference (Washington, May 1979) that the 'SRS' generated by cells in the presence of calcium ionophore and cysteine is in fact a cysteine adduct of arachidonic acid (5-hydroxy-6-sulphidocysteinyl-7,9,11,14-eicosatetraenoic acid). The parent compound (the 5'6 epoxide of arachidonic acid) is presumably formed by a lipoxygenase.

It is not clear whether this SRS is identical to the SRS-A described in this manuscript but such a structure could certainly accommodate much of the data described here, e.g. the presence of an amino acid, a hydroxyl, an additional carboxylic acid, aliphatic chain and lack of ketone. The u.v. absorbance which we attributed to an aromatic compound could have derived from the conjugated triene system of the proposed molecule. Presumably these conjugated double bonds could not be *cis* as this would have been immediately apparent from the i.r. It is not clear where the inorganic sulphate could originate from except perhaps breakdown or oxidation of the molecule.

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