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THE PARTIAL PURIFICATION OF SLOW REACTING SUBSTANCE OF ANAPHYLAXIS FROM RAT PERITONEAL ANAPHYLACTIC FLUID AND ITS SEPARATION FROM AN ARACHIDONIC ACID RELEASING SUBSTANCE

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Abstract—A method is described for the separation of Slow Reacting Substance of Anaphylaxis (SRS-A) from the other mediators of anaphylaxis. This method has the advantage of a high overall yield of biologically pure SRS-A, although chemical homogeneity is not obtained. Silicic acid chromatography of the material extracted by ethyl acetate revealed the presence of an arachidonic acid releasing substance (ARS). ARS is less polar than SRS-A and releases arachidonic acid and thromboxanes from perfused guinea-pig lungs. ARS can be distinguished from the peptide RCS-RF by its solubility in apolar solvents.

Slow Reacting Substance of Anaphylaxis (SRS-A) is a substance of unknown structure, released during anaphylaxis [1]. The established procedures for the isolation of SRS-A aim at producing a chemically homogenous preparation of SRS-A which can be used for structural studies. These procedures have disadvantages in that the overall recovery of SRS-A is low or variable [2–4] and often little evidence is presented showing that a homogenous preparation has been obtained. Although SRS-A is a potent compound, the preparation of relatively large quantities of active material is necessary for the study of its biological actions. Since none of the existing methods give consistently high yields, there is a need for a simple, high yielding method for the isolation of SRS-A. Such a preparation does not have to be chemically homogenous but it is necessary to establish that the preparation is free from contamination with other biologically active substances, particularly RCS-RF and substances which have similar actions to SRS-A.

SRS-A has been shown to release thromboxanes from perfused guinea-pig lung [5, 6], a property which is also shown by Rabbit Aorta Contracting Substance-Releasing Factor (RCS-RF) [5], a peptide released from guinea-pig lungs by anaphylaxis [7]. Although RCS-RF has been partially purified [7], the only property by which it can be distinguished from SRS-A is its lability on boiling [7], SRS-A being unaffected by this procedure [1].

This paper describes a simple method for the production of large quantities of SRS-A which are not contaminated by prostaglandins or by RCS-RF. A novel substance is also described which releases thromboxanes from guinea-pig perfused lungs.

MATERIALS

Oyster glycogen (Type II), ovalbumin (Grade III), arylsulphatase (Type V), atropine sulphate and arachidonic acid were all purchased from Sigma. Prostaglandin E_2 was obtained from the ONO Chemical Co. Ltd., amberlite XAD-2 was purchased from BDH Ltd, silicic acid (Bio sil A 100–200 mesh) from Bio Rad Ltd. and Whatman KC_{18} thin layer chromatography plates from Uniscience Limited. Indomethacin, mepyramine maleate and compound FPL 55712 were donated by Merck, Sharpe & Dohme, May & Baker Ltd. and Fisons Ltd., respectively. All other solvents and reagents used were of analar grade.

METHODS

The production of anaphylactic fluid containing SRS-A

Rat peritoneal anaphylactic fluid was prepared as described by Orange *et al.* [8] using male Wistar rats (200–300 g) pretreated with glycogen (20 ml 0.1% w/v i.p.) to increase peritoneal neutrophil levels. Rats were sensitized with 2 ml of a ¼ dilution of an IgGa antiovalbumin prepared as described by Orange *et al.* [8]. All animals were pretreated with indomethacin (1 mg kg^{-1} p.o.) 2 hr before challenge to inhibit prostaglandin synthesis [9] and to enhance SRS-A production [10, 12]. Two hours after sensitization the rats were challenged intraperitoneally with 5 ml ovalbumin (0.5 mg ml^{-1}) in heparinised Tyrodes' solution at 37°. Fifteen minutes later the animals were killed with CO_2 and the peritoneal fluid harvested.

Removal of protein from peritoneal fluid

Intact cells were removed from the peritoneal fluid by centrifugation at 1400 *g* for 10 min. Four volumes of ethanol were added to the supernatant and the mixture left for 30 min at 5° to precipitate the protein. The precipitate was removed by centrifugation at 38,000 *g* for 30 min. The supernatant was collected and evaporated to dryness under reduced pressure at 60°.

Desalting and removal of histamine

(a) *Amberlite XAD-2.* A column of amberlite XAD-2 resin was prepared as described by Orange

et al. [2]. The residue obtained from the procedure described above was dissolved in a small volume of distilled water and added to the column. The column was eluted with 250 ml distilled water followed by 250 ml 80% ethanol in water. The ethanol was collected and evaporated to dryness at 60°.

(b) *Solvent extraction.* Dried deproteinised peritoneal anaphylactic fluid was dissolved in 10 ml distilled water. Sodium hydroxide was added to a concentration of 0.1M and the solution incubated at 37° for 30 min. The solution was then cooled, adjusted to pH 3.0 with 2 M HCl and partitioned six times with two volumes of ethyl acetate. The ethyl acetate phase was collected and taken to dryness. After extraction the aqueous phase was neutralised and taken to dryness.

Silicic acid chromatography

Silicic acid was activated by heating at 160° for 18 hr. 10 gram of silicic acid was slurried in *n*-hexane and poured into a column. Samples were dissolved in 1–2 ml 80% methanol and applied to the silicic acid column. The column was eluted sequentially with 80 ml of *n*-hexane ethyl acetate, acetone, *n*-propanol and finally with 100 ml ethanol: ammonium hydroxide (S.G. 0.88): water (60:30:10 by vol.). All fractions were collected and evaporated to dryness.

KC_{18} reversed phase thin layer chromatography

SRS-A was applied to KC_{18} thin layer plates in a small volume of 80% methanol. Plates were developed in 100% methanol. After development each plate was divided into 1 cm bands which were scraped off and eluted with methanol. The eluate was taken to dryness and assayed for SRS-A activity.

SRS-A was methylated by treatment with fresh ethereal diazomethane [11]. The resulting material was also subjected to KC_{18} thin layer chromatography as described above. After development the plate was divided into 1 cm bands which were scraped off, eluted with methanol and hydrolysed in 0.1M NaOH at 37° for 30 min to regenerate biologically active SRS-A before being assayed on guinea-pig ileum [11].

In some experiments the developed thin layer plates were sprayed with 20% sulphuric acid in methanol and heated in an oven at 160° until compounds on the plate appeared as black or brown spots.

Bioassay of material obtained

(a) *Guinea-pig isolated superfused ileum.* Material obtained from the above procedures were assayed against prostaglandin E_2 (PGE_2) using the guinea-pig isolated superfused ileum preparation. Preparations were superfused at 5 ml min^{-1} with a modified Krebs solution, gassed with 95% O_2/5% CO_2, containing atropine (10^{-6}M), mepyramine (3×10^{-6}M) and indomethacin (3×10^{-6}M). The Krebs solution was warmed to 37° before being superfused over the tissues. PGE_2 and samples for assay were infused into the superfusion fluid in a volume of 10 μl over a period of 15 sec. 100 units of SRS-A is defined as the amount of material required to produce a contraction of guinea-pig ileum equivalent to that produced by 100 ng prostaglandin E_2. This quantification of SRS-A is essentially similar to that used by other workers, except that prostaglandin E_2 has been used instead of histamine [2, 12].

(b) *Guinea-pig perfused lungs.* Guinea-pig lungs were perfused *in vitro* with modified Krebs solution at a rate of 5.0 ml min^{-1}. The perfusate was made to superfuse a rabbit aortic strip, to detect thromboxanes [6] and a guinea-pig ileum to detect SRS-A. To increase the specificity and sensitivity of these assay tissues the antagonists listed above were incorporated into the superfusate between the lungs and the assay tissues. Samples were infused into the Krebs solution in a volume of 100 μl over a period of 15 sec.

Aryl sulphatase inactivation of SRS-A

SRS-A was dissolved in acetate buffer (pH 5.0) and incubated with Aryl sulphatase (type V) at 37° for 2 hr. The ratio of SRS-A to enzyme was 7:1 (unit: unit) [15]. Following incubation, 4 vol. of ethanol were added and the mixture left for 30 min at 5°. The resulting precipitate was removed by filtration and the supernatant evaporated to dryness at 60°. The residue was reconstituted in a small volume of Krebs solution and assayed for biological activity.

RESULTS

(1) *Yields of SRS-A and removal of proteins*

In rats pretreated with glycogen and indomethacin and sensitized with an IgGa antiovalbumin prepared as described by Stechschulte *et al.* [14], yields of 1349 ± 518 (N = 12) units SRS-A (mean ± S.E.) per rat were obtained in the peritoneal anaphylactic fluid. These yields are comparable to those described by Stechschulte *et al.* [13] and Orange *et al.* [2]. Following removal of cellular material and protein from the crude peritoneal anaphylactic fluid by the addition of ethanol and centrifugation recoveries of SRS-A of 89 ± 19.5% (N = 12) were obtained.

(2) *Desalting and removal of histamine*

When SRS-A was desalted by passage through amberlite XAD-2 resin, the recovery of SRS-A was found to vary from 0 to 83% (Table 1). These data are contrary to the high yields obtained by Orange *et al.* [2], however, low or variable yields have been reported by other workers who have used this technique [14].

SRS-A behaves as an acid in that following acidification to pH 2–3, it can be extracted from aqueous solutions by apolar solvents such as diethyl ether [1, 17]. Therefore, SRS-A was extracted into ethyl acetate from an acidified aqueous phase. This solvent

Table 1. A comparison of the recoveries of SRS-A following desalting on Amberlite XAD-2 resin and by extraction with ethyl acetate at pH 3.0

	% recovery of SRS-A	
	Amberlite XAD_2	Ethyl acetate
Mean recovery (%)	24	55
S.E.	20	11
N	4	7

Table 2. The recovery of SRS-A from rat peritoneal fluid expressed as the mean recovery in units of SRS-A per animal and as the mean percentage of the amount of SRS-A in crude peritoneal anaphylactic fluid (mean ± S.E. of 6 experiments)

Stage of extraction	Mean recovery of SRS-A u/rat	± S.E.	N	%	± S.E.	N
Peritoneal fluid	1379	280	6	100	0	6
After removal of protein	930	50	6	77	11	6
After extraction into ethyl acetate	483	114	6	41	5	6
After silicic acid chromatography	734	278	6	50	13	6

extraction gives more consistent percentage recoveries of SRS-A than Amberlite XAD-2, the mean recovery being 54.6 ± 11.4% (N = 7) (Table 1). When the ethyl acetate and aqueous fractions were assayed for histamine it was found that all of the histamine was in the aqueous phase.

Silicic acid chromatography

Solvent extraction of SRS-A as described above would also extract other acid lipids and apolar substances. Of these prostaglandins, their precursor fatty acids and indomethacin (remaining from the peritoneal fluid) are pharmacologically active. Preliminary experiments revealed that indomethacin was eluted from silicic acid with *n*-hexane, prostaglandins and fatty acids with ethyl acetate or acetone and most phospholipids with *n*-propanol.

SRS-A was eluted from silicic acid with ethanol; ammonium hydroxide; water (60:30:10 by volume) as described by Orange *et al.* [2]. Yields of SRS-A of 80–100% were usually obtained following silicic acid chromatography. These yields are similar to those obtained by Orange *et al.* [2], but occasionally a yield greater than 100% was obtained (Table 2), which could be explained by the removal of factors which depress smooth muscle activity.

Reversed phase thin layer chromatography

SRS-A, prepared by solvent extraction and silicic acid chromatography, chromatographed as a single

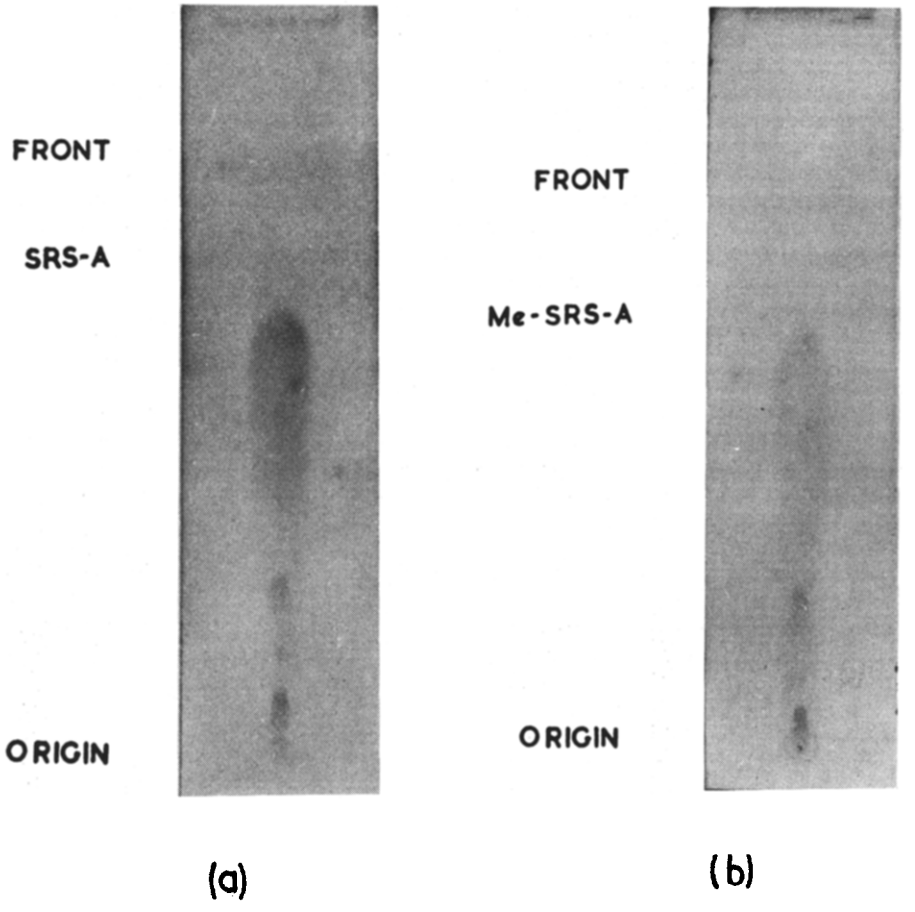

Fig. 1. KC_{18} thin layer chromatography of (a) SRS-A and (b) methyl SRS-A. Plates were developed in methanol and sprayed with 20% sulphuric acid in methanol. Compounds were revealed by heating at 160°.

Table 3. The effect of treatment with aryl sulphatase, compound FPL 55712 and boiling on the ability of SRS-A to contract guinea-pig ileum and to release thromboxane A_2 from perfused guinea-pig lung

Treatment	Activity (%) Ileum	Perfused lung
Control	100	100
Aryl sulphatase pH 5.0 at 37° for 2 hr	20	35
FPL 55712 10^{-6}M	36	11
Boiling at pH 8.0 for 10 min	96	100
Boiling at pH 5.0 for 10 min	27	40

biologically active substance on Whatman KC_{18} reversed phase thin layer plates. When plates were developed in methanol SRS-A had an R_f of 0.83 ($N = 6$), 70–100% of the biological activity being contained in this area of the plate. When chromatograms were sprayed with H_2SO_4: methanol (20: 80 v/v) and heated, no spot was obtained which corresponded to SRS-A (Fig. 1). However, substances with R_f's of 0.14, 0.18, 0.3 and 0.67 were detected.

Me-SRS-A also chromatographed as a single biologically active substance on Whatman KC_{18} plates. This material had an R_f of 0.74 ($N = 4$), indicating that Me-SRS-A is less polar than SRS-A. When chromatograms were stained with H_2SO_4: methanol, no spot corresponded to Me-SRS-A but substances with R_f's of 0.06, 0.11, 0.23 and 0.7 became visible (Fig. 1).

This data indicates that although the ethanol–ammonium hydroxide–water fraction only contains one pharmacologically active substance—namely SRS-A—it contains at least four contaminants which probably constitute a greater proportion of the total amount of organic matter than does SRS-A. Furthermore, while SRS-A became less polar as a result of methylation, some of the contaminants also became less polar, indicating that these compounds may be methylated along with SRS-A.

Biological activity of material recovered

SRS-A was assayed against PGE_2 on the guinea-pig isolated superfused ileum preparation, in the presence of atropine, mepyramine and indomethacin, at each stage of its isolation. Table 2 depicts the mean recovery of SRS-A from 6 experiments. It is seen that an overall yield of 50% SRS-A is obtained, amounting to 734 U per rat.

The material isolated by the method described above has the properties of SRS-A in that its ability to contract the guinea-pig isolated ileum is destroyed following incubation with aryl sulphatase in pH 5.0 buffer for 2 hr at 37° [15] and it is antagonised by the specific SRS-A antagonist 8-propyl-7-[3-(4 acetyl-2 propyl-3-hydroxyphenoxy)-2-hydroxy-propoxy]-4-oxo-4H-benzopyran-2-carboxylic acid (compound FPL 55712) [16], as shown in Table 3. Purified material which had been characterized as SRS-A on guinea-pig isolated ileum also released arachidonic acid, measured as a release of the arachidonic acid metabolite thromboxane A_2 (RCS) on rabbit aortic strip preparations, from guinea-pig perfused lungs (Fig. 2). The ability of SRS-A to release thromboxane A_2 was abolished by incubation with aryl-sulphatase. The release was blocked by the specific SRS-A antagonist compound FPL 55712 (Table 3). Furthermore, boiling SRS-A at pH 8.0 for 10 min had no effect on the thromboxane releasing action of this material, demonstrating that it did not contain RCS-RF (Table 3) [7].

An Arachidonate Releasing Substance (ARS) was also detected in the material eluted from silicic acid columns with ethyl acetate. Like SRS-A, this substance released arachidonic acid metabolites from perfused guinea-pig lungs and contracted the guinea-pig isolated ileum. However, this substance appeared to be present in much smaller quantities than SRS-A, since relatively large amounts (usually the

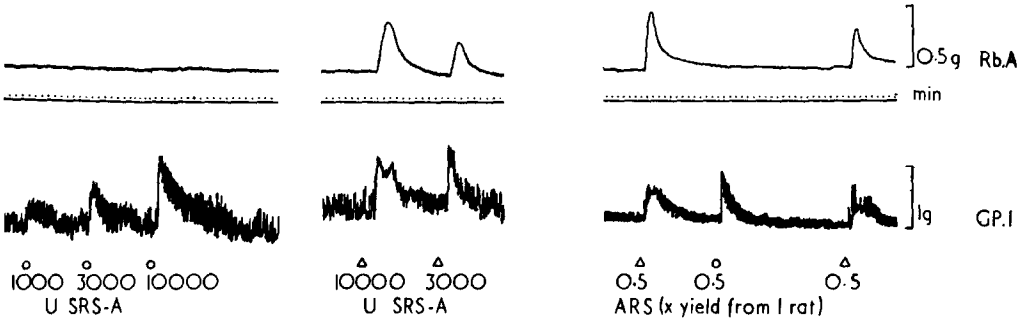

Fig. 2. Release of thromboxane A_2 (TxA_2) from guinea-pig perfused lung by SRS-A and ARS. The perfusate from the lungs was made to superfuse a rabbit aortic strip (Rb.A) and guinea-pig ileum (GP.I). SRS-A and ARS infused over the assay tissues (○) only contract the ileum, whereas when infused through the lung (△) both tissues contract, showing the release of TxA_2.

material obtained from one rat dissolved in 100 μl) were required to show an effect (Fig. 2).

ARS was found to be soluble in ether and ethyl acetate, unlike RCS-RF [7] or SRS-A [1]. ARS can also be distinguished from SRS-A on KC_{18} reversed phase thin layer chromatography. When plates were developed with 100% methanol, ARS had an R_f of 0.6, whereas the more polar SRS-A had an R_f of 0.83. Furthermore, KC_{18} thin layer chromatography demonstrates that ARS is not arachidonic acid, which also elutes from silicic acid columns with ethyl acetate, since in the thin layer system described above, arachidonic acid has an R_f of 0.82.

DISCUSSION

All of the published procedures for the partial purification of SRS-A attempt to produce a chemically pure sample from which the structure of this mediator can be determined [2–4]. In most cases the procedures are lengthy, overall yield is low [3, 4] and evidence that chemical purity has in fact been obtained is often lacking [2–4].

The method described above is technically simple and gives a relatively high overall yield of SRS-A which is free from other known biologically active substances. Such a method has advantages for workers wishing to study the biological properties of SRS-A or SRS-A antagonists rather than determine its structure. The high overall yields obtained are due largely to the increased efficiency of desalting by solvent extraction. Although extraction from acidified aqueous solutions into ether have been reported before [17], yields are low when this procedure is attempted early in the extraction procedure [3]. Preliminary experiments with solvent extraction using ethyl acetate showed that the recovery of SRS-A became less variable and increased if the aqueous phase was subjected to alkaline hydrolysis before extraction.

SRS-A isolated by ethyl acetate extraction and silicic acid chromatography is free of histamine and prostaglandins, since histamine is not extracted into ethyl acetate and the prostaglandins are separated from SRS-A by silicic acid chromatography. Neutrophil chemotactic factor, (NCF-A) a protein, [18] and eosinophil chemotactic factor, (ECF-A) a tetrapeptide [19], are released from mast cells by anaphylaxis. Neither of these agents is likely to be a contaminant of SRS-A isolated by the above procedure, since NCF-A would be precipitated with other proteins by alcohol and ECF-A does not partition into ethyl acetate.

Rabbit aorta contracting substance-releasing factor (RCS-RF) is a polar substance, released by anaphylaxis, which releases prostaglandin precursors from perfused guinea-pig lungs [7], a property shared with SRS-A. RCS-RF is insoluble in ethyl acetate [7]; thus one would expect to find this substance in the aqueous phase following ethyl acetate extraction. No such activity was detected in the aqueous phase. The thromboxane releasing activity of SRS-A was not affected by boiling at alkaline pH, showing that it was not contaminated with RCS-RF since the latter mediator is destroyed by this procedure.

ARS appears to be similar to RCS-RF and SRS-A in that it releases thromboxanes from perfused guinea-pig lungs. However, it is not RCS-RF, since it is soluble in non-polar solvents, unlike RCS-RF [7]. Similarly, the less polar nature of ARS on silicic acid and KC_{18} reversed phase thin layer chromatography enables it to be distinguished from SRS-A.

Recently SRS-A released by a calcium ionophore has been separated into two fractions by fluorosil HPLC [20]. Both fractions are eluted from the columns by polar mixtures making it unlikely that either fraction is ARS. However, the possibility that one of these fractions may be the material termed RCS-RF [7] remains a possibility worthy of investigation.

In conclusion, the experiments described above show that slow reacting substance of anaphylaxis can be separated from other pharmacologically active substances released by anaphylaxis with high overall yields. Furthermore, a substance has been found in anaphylactic fluid, namely ARS, which releases arachidonic acid and its metabolites from perfused guinea-pig lung. This substance can be distinguished from anaphylactic mediators described by other workers, which have a similar profile of activity, on the basis of its physico-chemical properties. Therefore ARS appears to be a novel pharmacologically active substance released by anaphylaxis.

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