Br. J. Pharmac. (1979), **67**, 179–184

COMPARATIVE STUDIES ON IMMUNOLOGICALLY AND NON-IMMUNOLOGICALLY PRODUCED SLOW-REACTING SUBSTANCES FROM MAN, GUINEA-PIG AND RAT

H.R. MORRIS, PRISCILLA J. PIPER*, G.W. TAYLOR & J.R. TIPPINS*

Department of Biochemistry, Imperial College of Science & Technology, London SW7 2AZ and
*Department of Pharmacology, Royal College of Surgeons of England, London WC2A 3PN

1 Slow-reacting substance of anaphylaxis (SRS-A) was produced by antigen challenge of passively sensitized human lung and actively sensitized guinea-pig lung.

2 A slow-reacting substance (SRS) was prepared from the peritoneal fluid of rats treated with calcium ionophore A23187.

3 These substances were extensively purified by charcoal adsorption, Sephadex G-15 gel filtration, ether extraction and reverse phase high pressure liquid chromatography.

4 The three substances are pharmacologically, chemically and chromatographically indistinguishable.

5 Our data suggest that the same SRS entities are released from a variety of tissues and that these acidic lipids may have a wider physiological significance than just anaphylaxis.

Introduction

Slow reacting substance of anaphylaxis (SRS-A) is a primary mediator of immediate-type hypersensitivity reactions, biologically active in subnanogram quantities, which probably plays an important rôle in allergic bronchospasm in man. SRS-A was originally detected some 40 years ago in perfusate collected from guinea-pig lung during anaphylaxis (Kellaway & Trethewie, 1940). Subsequently, a number of slow reacting substances (SRSs) have been shown to be released from tissues by non-allergic stimuli, for example from perfused cat paw by the histamine releaser compound, 48/80 (Anggard, Bergqvist, Hogberg, Johansson, Thon & Uvnas 1963), from human lung by repeated freezing and thawing (Turnbull, Jones & Kay, 1976), and from human leucocytes and rat peritoneal cells by calcium ionophore A 23187 (Conroy, Orange & Lichtenstein, 1976).

To date, although some relatively crude comparisons have been made, severe difficulties with the purification and elucidation of structure of slow reacting substances have precluded a definitive answer to two important and related biological and pharmacological questions: Firstly, whether SRSs from various sources released by immunological and non-immunological stimuli are chemically related or indeed identical substances. Secondly, will structural studies on the more easily obtained guinea-pig SRS-A ($SRS\text{-}A^{g\text{-}p}$) or rat SRS ($SRS^{rat}$) be relevant to human SRS-A ($SRS\text{-}A^{man}$) and, by inference, to asthma? These important questions also have a bearing on the validity of using animal SRSs in the development and screening of drugs for therapeutic use in man.

Statements have been made previously suggesting the similarity or dissimilarity of certain SRSs (Takahashi, Webster & Newball, 1976), but in all cases these comparisons have been made on relatively impure preparations (our experience suggests they may have contained hundreds of compounds) using chromatographic methods which are not capable of the necessary resolution.

We have extended the purification technology for SRS-A, and in a recent paper (Morris, Taylor, Piper, Sirois & Tippins 1978) gave the first account of the use of reverse-phase high pressure liquid chromatography in this field. Inclusion of this procedure as a final step of purification removes many hundreds of contaminating compounds (as visualized by u.v. absorption of the column eluates) and yields, as far as can be determined, a single pure substance that fulfils the criteria for SRS-A activity.

In the present paper, we describe the comparative physicochemical properties of highly purified SRS-A produced by antigen challenge of actively sensitized guinea-pig lung, passively sensitized human lung, and SRS released from rat peritoneum by the calcium ionophore A 23187.

Part of this work has been communicated to the Seventh IUPHAR Congress, Paris 1978.

0007-1188/79/100179-06$01.00

Methods

Preparation of slow-reacting substances

SRS-A from human lung. Macroscopically normal, human lung was obtained from specimens resected for bronchial carcinoma. The tissue was washed in Tyrode solution and cut into small pieces (approx. 4 to 6 mm^3) and divided into 10 g portions. These were incubated overnight at room temperature in 15 ml serum from asthmatic patients sensitive to *Dermatophagoides pteronyssinus.* The serum was either used undiluted or diluted 2 to 4 times according to the IgE titre. The next day the lung pieces were washed three times with Tyrode solution, resuspended in 40 ml cysteine (10^{-3} M) in Tyrode solution and challenged for 15 min at 37°C with 1.5 mg freeze-dried extract of *D. pteronyssinus* (equivalent to 50,000 Noon units). The lung tissue was removed by straining through nylon mesh and the fluid extracted after centrifugation.

SRS-A from guinea-pig lung. SRS-$A^{g\text{-}p}$ was prepared as described by Engineer, Niederhauser, Piper & Sirois (1978a). Lungs of guinea-pigs (Dunkin-Hartley strain; either sex, 300 to 350 g) sensitized three weeks previously to ovalbumin (100 mg s.c. and i.p., Sigma Grade II) were perfused with Tyrode solution containing indomethacin (2.79×10^{-4} M) and cysteine (10^{-3} M). After challenge with ovalbumen (10 mg, Sigma, Grade III) the effluent was collected on ice and bubbled with nitrogen.

SRS-A from rat peritoneum. Calcium ionophore, A 23187, was initially dissolved in absolute ethanol and diluted with Tyrode solution to a final concentration of 5 μg/ml (1% ethanol). Rats (Sprague Dawley, male, 200 to 250 g) were lightly anaesthetized with ether and 1 ml cysteine 10^{-3} M injected intraperitoneally; after 2.5 min, 5 ml A 23187 (5 μg/ml) was injected intraperitoneally and left for 15 min. The rats were then killed by a blow on the head, exsanguinated and the peritoneal fluid harvested, centrifuged and extracted.

Quantitation of SRS-A and SRS

At all stages of preparation and purification SRS-$A^{g\text{-}p,man}$, and SRS^{rat} were assayed on stripped longitudinal smooth muscle of the guinea-pig ileum, superfused at 5 ml/min with oxygenated Tyrode solution at 37°C (Engineer *et al.*, 1978a) and blocked with mepyramine and hyoscine (final concentrations 10^{-6} M). Test samples of SRS-A and SRS were assayed against a partially purified laboratory standard preparation of SRS-$A^{g\text{-}p}$ and the results expressed in arbitrary units (Engineer *et al.*, 1978a), 0.068 ± 0.004 unit SRS-A are equivalent to 10 μg histamine (equivalent to 2 units SRS-A expressed by the method of Stechschulte, Austen & Bloch, 1967).

The SRS-A antagonist 4-oxy-8-propyl-4H-1-benzopyran-2-carboxylate (FPL 55712) (Augstein, Farmer, Lee, Sheard & Tattersall, 1973) 1 μg/ml was used to antagonise SRS-$A^{g\text{-}p,man}$ or SRS^{rat} at all stages of purification.

Purification

The purification of SRS (-A) was essentially as described by Morris *et al.* (1978).

a) *Charcoal adsorption.* The crude material was adsorbed onto animal charcoal (0.25 g/100 ml) and eluted in 80% ethanol after a water wash. After rotary evaporation the aqueous phase was freeze dried.

b) *Gel filtration.* A Sephadex G-15 column (180 × 1 cm) was run in methanol:water:ammonia (0.880), 2:2:1 v/v and the eluate monitored at 275 nm.

c) *Ether extraction.* The aqueous phase was adjusted to pH 3 and activity extracted into peroxide free diethyl-ether.

d) *Reverse-phase high pressure liquid chromatography (HPLC).* HPLC in the reverse phase was carried out on a μm Bondapak C_{18} column (Waters Ass.) under a linear gradient from 50% aqueous methanol to 100% methanol. The column eluate was monitored both at 254 and 280 nm. At all stages after the charcoal step, solutions were dried on a rotary pump. The position and concentration of SRS-A was determined by bioassay at all stages of the purification.

Enzymes

Arylsulphatase and lipoxygenase (Sigma) experiments were carried out as described by Engineer, Morris, Piper & Sirois (1978b).

Physico-chemical experiments

These were all carried out in glass tubes in a nitrogen atmosphere, at room temperature and in the dark unless otherwise stated.

Results

Biological activities

The first indication of a close relationship between SRS-A^{man}, SRS-$A^{g\text{-}p}$ and SRS^{rat} derives from a comparative study of the biological activities, summarized in Table 1.

SRS-A^{man}, SRS-A$^{g\text{-}p}$ and SRS^{rat} were found to behave identically with respect to contraction of guinea-pig ileum and release of both thromboxanes and prostaglandins (Engineer *et al.*, 1978a) at all stages of purification. All three preparations were found to contract guinea-pig trachea and human bronchus at the ex-charcoal state of purity. The ability of each preparation to contract guinea-pig ileum was examined after treatment of the ex-Sephadex G15 material with arylsulphatase, and in all three cases activity was equally destroyed. At all stages of purification the SRS-A^{man}, SRS-A$^{g\text{-}p}$ and SRS^{rat} ileum activities were fully antagonized by the specific SRS-A antagonist, FPL 55712. The ileum contracting activity of ex-G15, SRS-A$^{g\text{-}p}$, SRS-A^{man} and SRS^{rat} can be reduced (not completely destroyed under normal conditions) by treatment with commercial soya bean lipoxidase, (lipoxygenase).

Physico-chemical properties

The basic physico-chemical properties of the SRSs were examined under a variety of conditions, and the comparative data are shown in Table 2. There were no anomalies in the SRS-A^{man}, SRS-A$^{g\text{-}p}$ or SRS^{rat} preparations studied; all were stable to boiling in water, stable to mild base treatment (see footnote to Table 2) and were destroyed by mild acid treatment.

We found (Table 2) that in the three preparations, activity could be extracted in good yield (>80%) into peroxide-free diethyl ether from a pH 3.0 aqueous phase, with no activity remaining in the aqueous phase in either the SRS-A^{man}, SRS-A$^{g\text{-}p}$ or SRS^{rat} material studied.

Purification of SRS and SRS-A

After charcoal adsorption, SRS-A$^{man,g\text{-}p}$ or SRS^{rat} was subjected to Sephadex G-15 gel filtration, ether extraction and reverse phase HPLC. The HPLC is a particularly important step since, alone, it gave an estimated purification of over 1000 fold of the ether extract. In large scale purification runs with >1000 units of SRS-A$^{g\text{-}p}$ the bioactivity eluted as a single peak corresponding to an ultra-violet absorption, well separated from other u.v. absorbing material. The

Table 1 Comparison of the biological properties of human, guinea-pig and rat slow reacting substances

	Guinea-pig	*Rat*	*Man*	
Contracts guinea-pig ileum	(3)	(3)	(3)	
Contracts human bronchus and guinea-pig trachea	(1)	(1)	(1)	
Releases prostaglandins and thromboxanes	(3)	(1)	(1)	
Destruction by aryl-sulphatase	(1, 2)	(1)	(1)*	almost complete destruction after incubation with 1 mg/ml for 1 h.
Antagonized by FPL 55712 1 μg/ml	(3)	(3)	(3)*	
Destruction by lipoxygenase	(1)†	(1)	(1)*	100% destruction after incubation with 50 μg/ml for 1 h.

(1) ex-charcoal material used; (2) ex Sephadex G15 material used; (3) material from all stages of the purification used.
* from Seale & Piper (1978); † from Engineer *et al.* (1978b).

Table 2 The physico-chemical properties of human, guinea-pig and rat slow reacting substances

1. Stable to boiling	Ex-charcoal material used
2. Stable to base (0.1 M NaOH (aq), room temp., 0.52 h)	Ex-charcoal material used
3. Destroyed in 0.1 M HCl (aq) (room temp., 0.5 h)	Ex-charcoal material used
4. Ether soluble at pH 3.0	Ex Sephadex G15 material only

Experiments 1 and 2 occasionally gave conflicting results with different preparations of the same type of SRS.

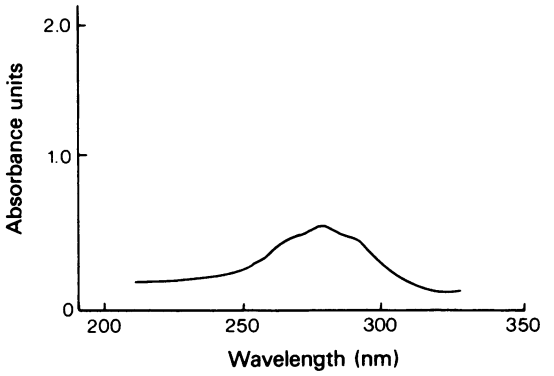

Figure 1 The full ultra-violet absorbance spectrum of guinea-pig SRS-A. Run in methanol, 10 mm path length, λ_{max} 280 ± 1 nm.

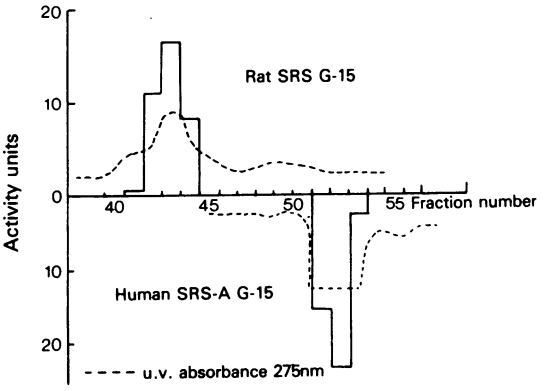

Figure 2 Comparison of the elution profiles of rat and human SRS-A on Sephadex G-15.

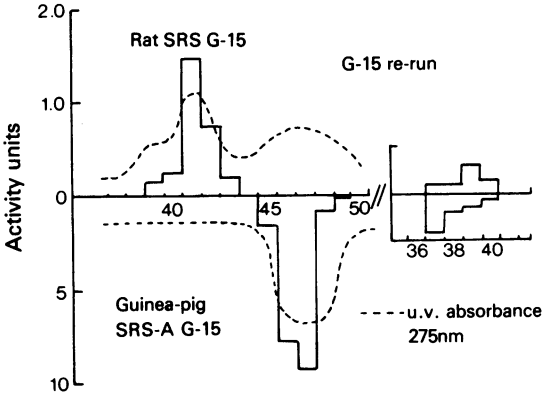

Figure 3 Comparison of the elution profiles of rat and guinea-pig SRS-A on Sephadex G-15, and the re-chromatography of these materials.

purity of the material at this stage can be judged by the u.v. spectrum shown in Figure 1 when compared with a spectrum of the previously most highly purified batch of SRS-A published by Orange and co-workers (Orange, Murphy, Karnovsky & Austen, 1973). The exact correspondence of bioactivity with u.v. activity eluted from the HPLC column in different positions under slightly different conditions indicates that the spectrum shown in Figure 1 is the u.v. spectrum of SRS-A. In any event the resolution and dramatically enhanced purification on HPLC provided a very sensitive probe for structural differences in SRSs studied below.

Comparative chromatographic properties of human SRS-A, guinea-pig SRS-A and rat SRS.

The quantities of SRS and SRS-As available for study precluded observation in the ultra-violet (at the HPLC stage) and all data reported here are based on FPL 55712-reversible guinea-pig ileum contracting activity.

Figure 2 shows the comparative elution positions of SRS^{rat} and $SRS\text{-}A^{man}$ on Sephadex G15. The 'peak tubes' were in fractions 43 and 52 respectively. Comparison of SRS^{rat} with $SRS\text{-}A^{g\text{-}p}$ gave a similar pattern of non-coincident elution profiles (Figure 3), which at first sight suggested that the SRSs have different molecular weights and therefore different structures. However, rerunning the peak tubes on G15 under the same conditions led to a coincident elution profile (Figure 3), which also corresponded with contaminating u.v. absorbing material (probably tyrosine as judged by amino acid analysis) which was eliminated upon further purification.

Following ether extraction of the active G15 fractions, samples were compared in consecutive runs on the HPLC column. Figure 4 shows the results of the $SRS^{rat}/SRS\text{-}A^{g\text{-}p}$ comparison. Small samples of the first runs were taken for bioassay; then to eliminate any instrument fluctuation error which may have occurred between runs, equal quantities of sample from each peak tube were mixed and re-chromatographed (Figure 4).

Although only single bands of bioactivity were observed in the $SRS^{rat}/SRS^{g\text{-}p}$ runs just described, we sometimes observed multiple bands of activity, particularly when very small quantities of SRS or SRS-A were purified. For example, chromatography of a much smaller sample of SRS^{rat} led to the multiple bands of activity (including the original at tube 16) seen in Figure 5a. However, the chromatography of a similarly small sample of $SRS\text{-}A^{man}$ gave an almost identical elution profile. Figure 5a thus illustrates the importance of trying to minimize all variables in this

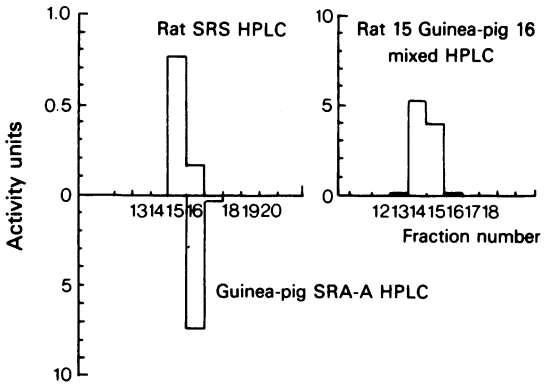

Figure 4 High pressure liquid chromatography profiles of rat SRS, guinea-pig SRS-A and mixed materials.

type of comparative work, even to the extent of processing similar amounts of biologically active samples.

Figure 5b shows how 'artefacts' may be produced particularly at this low level, but again importantly there is a consistency in the patterns observed.

Discussion

The biological properties of SRSs seen in Table 1 show a good correspondence, and we find no anomalies between SRS-A^{man}, SRS-A$^{g\text{-}p}$ and SRS^{rat} at this level. Interestingly, the guinea-pig ileum-contracting activity of all three preparations is destroyed by arylsulphatase; the ability of arylsulphatase to destroy SRS-A has led to a suggestion that SRS-A contains a sulphate moiety. Our data on the ether extraction of SRS, in which we observed complete extraction of SRS-A^{human}, SRS-A$^{g\text{-}p}$ and SRS^{rat} into the organic phase at pH 3.0, cast some doubt on the sulphate hypotheses. This organic solubility must be due to protonation of an acidic moiety(ies) thus removing charged lipophobic character; we would not however expect a sulphate group to be protonated at pH 3.0, suggesting that if the SRSs contain this group it must be shielded or the protonated form unusually stabilized by interaction with another part of the molecule. Ether solubility would then be more reasonably explained by protonation of a carboxyl group at this pH.

Our data on the ether extraction of SRS-A^{man} in particular appears to conflict with a published account that 'Human SRS-A, unlike those of the cat and guinea-pig, could not be extracted into ether under acidic conditions' (Takahashi *et al.*, 1976). We found complete extraction, with no residual activity in the aqueous phase for both the human and guinea-pig SRS-A and the SRS^{rat}. However, we note that

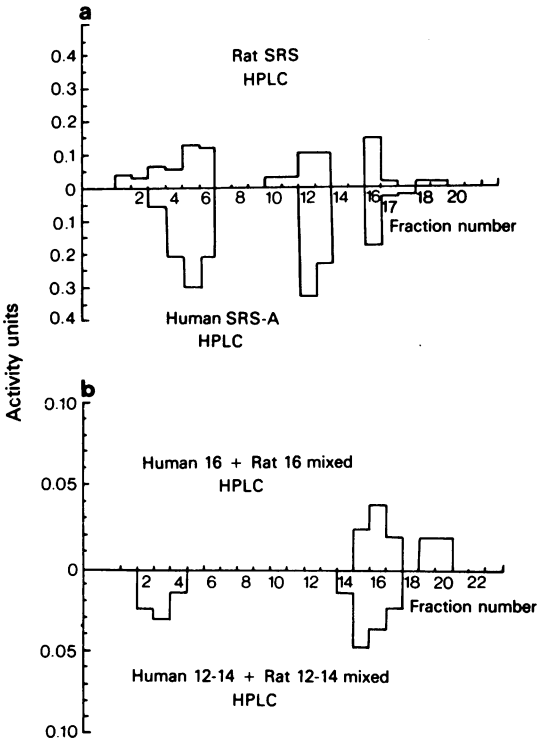

Figure 5 High pressure liquid chromatography profiles of rat SRS and human SRS-A. (a) Profile of separate materials; (b) profile of the mixed materials.

extraction does depend upon the state of purity of the preparation, and for example activity cannot be extracted from the excharcoal lung perfusate.

The partial destruction of biological activity by lipoxidase is an interesting observation, since the normal substrate specificity for the enzyme used is a class of unsaturated fatty acids containing a 1–4 *cis* diene unit. However, the possibility of impurities or side specificities in the commercial enzyme used cannot preclude the possibility of destruction of other functional groups in the molecule.

The u.v. spectrum of highly purified SRS-A$^{g\text{-}p}$ (Figure 1) would be quite compatible with a non-aromatic triply conjugated chromophore and it is interesting that there is a close resemblance to the spectra of certain hydroxylated unsaturated lipids (Bild, Ramadoss & Axelrod, 1977).

The comparative gel filtration data presented in Figures 2 and 3 appeared initially to provide strong evidence for molecular weight, and therefore structural differences between SRS-A^{man}, SRS-A$^{g\text{-}p}$ and SRS^{rat}. However, re-chromatography of the peak tubes gave coincidence of elution, and incidentally a

different elution position (Figure 3). Ultimately therefore we found good evidence that the three preparations have the same molecular weight, and that the initial anomalies are caused by varying amounts of impurities in the respective preparations, which appear able to solvate the SRS to a greater or lesser extent and affect the elution position of the SRS in the crude extract.

The validity and importance of the conclusions made in this paper on the comparative structures of SRS-A^{man}, SRS-$A^{s\text{-}p}$ and SRS^{rat} rests heavily on the high-resolution high pressure liquid chromatographic data presented in Figures 4 and 5. This methodology, clearly capable of resolving even small structural differences (such as methyl group position) allows the first valid comparison of SRSs since all previous ones have been made on what we now know to be grossly impure preparations, using chromatographic techniques incapable of the resolving power needed to distinguish between closely related structures.

The data in Figures 4, 5a and 5b give convincing evidence that SRS-A^{man}, SRS-$A^{s\text{-}p}$ and SRS^{rat} are indeed the same structures. We believe that the multiple activities observed in the necessarily smaller preparations of human and rat material (Figures 5a and 5b) are chemical artefacts (protonation, oxidation, complexes, lactone formation, isomerisation?) which might be expected to form when handling very small quantities of labile materials. This view is supported by the data in Figure 4 showing the absence of multiple activities in a much larger run of the rat SRS. The co-elution of the multiple activities of SRS^{rat} and SRS-A^{man} seen in Figure 5b gives further convincing evidence of identity of structure.

In summary, we believe that further clarification of the biochemistry of the SRSs must await the structure elucidation, now in progress. The data presented here suggest that SRS produced by non-immunological means is identical to that produced immunologically, and that structural studies on the more plentiful SRS-$A^{s\text{-}p}$ or SRS^{rat} will be relevant to human-SRS-A, and by inference to asthma. The comparative data presented here, for example non-immunological release of SRS^{rat}, also indicates a wider rôle for the same SRS than merely mediation of bronchoconstriction.

Since the immunologically and non-immunologically induced materials described above are indistinguishable it may no longer be necessary to describe the anaphylactic mediator as SRS-A, but rather to refer to all slow reacting substances as SRS.

We wish to thank the M.R.C. for financial support, Mr M.A. Palmer for excellent technical assistance, the Lilly Research Centre for the gift of A23187 and Fisons Ltd., for FPL 55712.

References

ANGGARD E., BERGQVIST U., HOGBERG B., JOHANSSON K., THON I.L. & UVNAS B. (1963). Biologically active principles occurring on histamine release from cat paw, guinea-pig lung and isolated rat mast cells. *Acta physiol. scand.*, **59**, 97–110.

AUGSTEIN J., FARMER J.B., LEE T.B., SHEARD P. & TATTERSALL M.L. (1973). Selective inhibitor of slow reacting substance of anaphylaxis. *Nature*, **245**, 215–217.

BILD G.S., RAMADOSS C.S. & AXELROD B. (1977). Multiple dioxygenation of lipids containing all cis 1,4,7-octatriene moieties. *Archs Biochem. Biophys.*, **184**, 36–41.

CONROY M.C., ORANGE R.P. & LICHTENSTEIN L.M. (1976). Release of slow reacting substance of anaphylaxis from human leucocytes by the calcium ionophore A23187. *J. Immunol.*, **116**, 1677–1681.

ENGINEER D.M., MORRIS H.R., PIPER P.J. & SIROIS P. (1978b). The release of prostaglandins and thromboxanes from guinea-pig lung by slow reacting substance of anaphylaxis, and its inhibition. *Br. J. Pharmac.*, **64**, 211–218.

ENGINEER D.M., NIEDERHAUSER U., PIPER P.J. & SIROIS P. (1978a). Release of mediators of anaphylaxis: inhibition of prostaglandin synthesis and the modification of the release of slow-reacting substance of anaphylaxis and histamine. *Br. J. Pharmac.*, **62**, 61–66.

KELLAWAY C.H. & TRETHEWIE E.R. (1940) The liberation of a slow reacting smooth muscle stimulating substance in anaphylaxis. *Q. J. exp. Physiol.*, **30**, 121–145.

MORRIS H.R., TAYLOR G.W., PIPER P.J., SIROIS P. & TIPPINS J.R. (1978). Slow reacting substance of anaphylaxis: purification and characterisation. *FEBS Letters*, **87**, 203–206.

ORANGE R.P., MURPHY R.C., KARNOVSKY M.L. & AUSTEN K.F. (1973). The physico-chemical characteristics and purification of slow reacting substance of anaphylaxis. *J. Immunol.*, **110**, 760–770.

SEALE P. & PIPER P.J. (1978). Release of prostaglandins and thromboxanes from guinea-pig lung by human SRS-A *Eur. J. Pharmac.*, **52**, 125–128.

STECHSCHULTE D.J., AUSTEN K.F. & BLOCH K.J. (1967). Antibodies involved in the antigen induced release of slow reacting substance of anaphylaxis in the guinea-pig and rat. *J. exp. Med.*, **125**, 127–147.

TAKAHASHI H., WEBSTER M.E. & NEWBALL H.H. (1976). Separation of slow reacting substance of anaphylaxis from human lung into four biologically active fractions. *J. Immunol.*, **117**, 1039–1044.

TURNBULL L.S., JONES D.G. & KAY A.B. (1976). Slow reacting substance as a preformed mediator from human lung. *Immunology*, **31**, 813–820.

(Received October 23, 1978. Revised February 12, 1979.)