THE ROLE OF 'SRS-A'
(SLOW REACTING SUBSTANCE OF ANAPHYLAXIS)
AND OTHER ACTIVE SUBSTANCES
IN ALLERGIC AND INFLAMMATORY CONDITIONS

Thesis
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by

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Some of the results described in this thesis were presented as a preliminary communication before the British Pharmacological Society in July 1965 (Brooklehurst, W.E. and Marquis, V.C.).
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The aim of the work to be described has been to extend our knowledge of the pharmacology and possibly the chemical nature of SRS-A (Slow Reacting Substance of Anaphylaxis) which is present in the Tyrode solution flowing from the isolated perfused lung of the guinea-pig after anaphylactic shock. Before this aim could be achieved the methods of purification of SRS-A had to be improved.

A purification procedure has been developed with the following considerations in mind.

1) The use of organic solvents should be limited in order to minimize the possibility of chemical changes or contamination.

ii) Conditions which lead to quantitative or qualitative changes in the biological activity of the material should be avoided.

iii) The purified material should retain its biological activity.

iv) The method must be adaptable to handle the quantity of purified material needed for chemical characterisation.

The material has been used to compare SRS-A with the Slow reacting substance obtained from cat's paws perfused with histamine releaser substance 48/80 (Anggard et al., 1963) and also with that obtained
from peritoneal washings of the rat and guinea-pig following local sensitisation and challenge by intraperitoneal injection (Rapp, 1961). The purified material has permitted the application of large doses to a wide range of test preparations, and has been used for preliminary studies on the chemistry of SRS-A.
PART I

REVIEW
1. The discovery of SRS-A

In sensitised tissue the immediate type of antigen-antibody reaction leads to the release of histamine and of another smooth muscle stimulating substance described as SRS (Slow Reacting Substance). In 1936, Schild observed that the isolated uterus from a sensitised guinea-pig made refractory to histamine by adding high concentrations of the amine to the bath, still responded to the specific antigen. A few years earlier, Kellaway (1929) had reported a close similarity between the anaphylactic response and the effects of snake venom on guinea-pig uterus. Feldberg and Kellaway (1938) subsequently found that the effluent from perfused organs after injection of cobra venom contained both histamine and a substance causing slow contraction of the guinea-pig ileum. They called the substance SRS.

A substance having a similar effect on guinea-pig ileum is also formed by the action of snake venom on egg yolk (Feldberg, Holden and Kellaway, 1938): this has now been characterised as an unsaturated fatty acid (Vogt, 1957). In 1940 Kellaway and Trethewie showed that the anaphylactic reaction in perfused guinea-pig's lung leads to the appearance of a slow reacting substance. Substances causing a slow contraction of the guinea-pig ileum have also been found in the following:-
(a) Cat and dog plasma after intravenous administration of compound 48/80 (Paton, 1951).
(b) Wasp venom (Jacques and Schachter, 1954).
(c) Human urine (Gomes, 1955).

Brocklehurst (1952 at seq.) re-investigated the substance obtained from sensitised guinea-pig lung after anaphylactic shock. By 1952, potent and specific antihistamines had been developed and it was possible to abolish the response to histamine, allowing the contraction due to the SRS to be obtained separately. Brocklehurst found that the more specific antihistaminic drugs do not depress this contraction even at a concentration of $10^{-6} M$.

He established the separate identity and part of the pharmacological profile of this substance, and pending identification of its chemical nature, named it SRS-A (Slow Reacting Substance of Anaphylaxis) to distinguish it from other slow reacting substances, not necessarily identical to SRS-A.

Since 1958, many attempts have been made to isolate this substance or substances in a pure form. Only partial purification has been achieved and the various findings of the different workers will be presented later.

2. The source of SRS-A

It is well established that histamine is set free
from pre-existent stores which are rather slowly replenished. In contrast it has been shown that slow reacting substance or SRS-A is formed after the sensitised tissue is challenged (Brocklehurst, 1958; Chakravarty, 1960) and from this observation it has been suggested that it is present only as an inactive precursor.

Slow Reacting Substance is formed following anaphylaxis in many tissues and several species (Brocklehurst, 1958; Chakravarty, 1960), but lungs, aorta and great veins produce more of the substance than any other tissues tested, particularly in the guinea-pig. From this Brocklehurst (1960) advanced the idea that the substrate giving rise to the slow reacting substance must be particularly abundant in blood vessels. Uvnas and Thon (1959) have suggested that mast cells may be the source of slow reacting substance, as well as histamine since they found small amounts of SRS in rat basophils (blood mast cells) separated from other leucocytes by a flotation method. Earlier, Hogberg et al. (1956), Hogberg and Uvnas (1957; 1958), Uvnas (1958) presented the theory that compound 48/80, a histamine releaser, causes the degranulation of rat mast cells by activating a lytic enzyme attached to the mast cell membrane. This theory was based on observations concerning the influence of temperature, pH, ionic milieu and enzyme inhibitors on the action of compound 48/80 (Högberg and Uvnas, 1959) and anaphylaxis (Chakravarty, 1959, 1960;
Chakravarty and Uvnäs, 1960). The pattern of inhibition was consistent with the view that the enzyme was a phospholipase. If this theory is correct, histamine and the slow reacting substance should always be released in a constant ratio; this would strongly support the unitary concept that the mast cell is the site of histamine and SRS release during the anaphylactic reaction as well as by treatment with histamine releasing agents.

In the anaphylactic reaction, this is not easy to show because the system is complex and there is inevitably considerable biological variation. The intensity of the reaction as judged by the yield of active substances, will depend firstly on the level of sensitisation, secondly on the effectiveness of the enzyme steps leading from the union of antigen with antibody to subsequent release of active products, and thirdly on the levels of histamine and precursor of slow reacting substance in the tissue. These variables will apply even when the same organ is used and the experimental procedure is rigidly standardised. Additional factors come in when different tissues are used. These include the following:

(a) Rate at which the antigen can diffuse in and the products diffuse out
(b) Rate of inactivation of the enzymes involved in the reaction or of the pharmacological agents themselves
(c) The possible uptake of the active substances within the tissue (e.g. local adsorption which might well vary for acidic and basic substances).
Chakravarty (1960) showed that a correlation existed, but supported his argument with an experiment using litter-mate guinea-pigs, thus deliberately weighting the results in favour of correlation which may be quite unrelated to a unitary mechanism. Brocklehurst (Paris, 1958a; 1958) considers that the correlation is in general too poor to support any theory, and points to certain tissues such as the trachea where sometimes histamine alone is released during anaphylaxis. Riley and West (1955) and Mota and Vugman (1956) found a close correlation between the amount of histamine released by compound 48/80 and mast cell population of the tissue used. This indicated that the releasable histamine is located in the mast cell, but these workers concerned themselves only with histamine. Mota and Vugman in 1956 demonstrated that the mast cells of sensitised guinea-pigs disintegrated and disappeared following injection of antigen, and that the number of mast cells disappearing increases with rising concentration of antigen in vitro (Mota, 1958) and in vivo (Boreus, 1960). Boreus and Chakravarty (1960) have reported that the yields of both histamine and slow reacting substance in anaphylaxis, are related to the number of mast cells involved. A more recent study which cast doubts on the significance of mast cells in respect of SRS release was that of Humphrey and Rapp (1961). They showed that
little or no slow reacting substance is produced when histamine is freed from guinea-pig mesentery by anaphylaxis *in vivo*.

The events leading to histamine release and the formation of slow reacting substance must have much in common because the agents which reduce or enhance the yield of histamine, alter the yield of slow reacting substance in a roughly comparable degree (Austen and Brocklehurst, 1961; Chakravarty *et al.*, 1960). The inhibitory agents differ greatly in type, they include substances which destroy SH groups, esterase inhibitors such as phenol indole and DFP, fatty acids of C5 or longer, acidic polymers, and synthetic ester substrates of chymotrypsin. It seems most unlikely that all these agents act on the same enzyme step, and suggests that there are several stages each one essential to and quantitatively involved in the anaphylactic reaction and tissue damage. There is general agreement that the chain of events leading from antigen–antibody reaction to the release of histamine and slow reacting substance have an early part in common, but some workers contend that the final stages are different as shown by the different rates at which histamine and slow reacting substance leave the shocked tissues. Brocklehurst (1962b) visualises the mechanism of release thus

$\text{Ag} + \text{Ab}$

on cells  $\xrightarrow{1\rightarrow 2+\text{(}\ n \text{)}\text{}}$ enzyme steps $\xrightarrow{\text{brief}\ \text{H}\ \text{releaser}}$ Histamine $\xrightarrow{\text{producer}\ S\ \text{longer}}$ SRS-A
The above evidence indicates complexity, by involving several types of enzyme, but the intricate mechanism of the release of slow reacting substance is not known.

3. Pharmacological properties

Since the renewal of interest in slow reacting substance in 1952, many attempts have been made to purify the substance. The actions have therefore been investigated using (a) crude material obtained either from perfused or chopped tissues and (b) material partially purified by several different procedures. This has led to some confusion, since the manner of obtaining the crude material introduced a variable amount of lipid, and the various methods of purification remove different "interfering" substances, and may add some of their own.

(a) Slow reacting substance present in crude perfusate contracts only a limited number of isolated smooth muscle preparations (Table 1). It produces a very prolonged effect with slow relaxation after washing, and enhances the response to other agents applied shortly after it. There is no tachyphylaxis and there is at times sub-threshold stimulation which persists after washing. These features are accepted as characteristic of the substance.

(b) Partially purified extracts all contract the
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<tr>
<td>Goadby and Smith</td>
<td>Guinea-pig lung. Material obtained by perfusion after Ag/Ab reaction in vitro for 30 min.</td>
<td>Guinea-pig ileum</td>
<td>Contraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guinea-pig tracheal muscle</td>
<td></td>
</tr>
<tr>
<td>Anderson et al. (1965)</td>
<td>Guinea-pig lung. Material obtained by perfusion after Ag/Ab reaction in vitro for 30 min.</td>
<td>Guinea-pig ileum</td>
<td>Contraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Guinea-pig tracheal muscle</td>
<td></td>
</tr>
</tbody>
</table>
guinea-pig ileum, but show variable activity on other preparations (Table 2). The group led by Uvnäs in Stockholm, who used a sillicic acid purified SRS (both from cats' paws and guinea-pig lung) found that rabbit duodenum was contracted by some of the fractions, but tachyphylaxis developed (Anggard et al., 1963), it was therefore concluded that a fatty acid was present besides SRS.

In keeping with its plain muscle stimulant activity in vitro, the general effect of SRS-A on the respiratory system in vivo is that a broncho-constriction as shown by Berry and Collier (1964) in the guinea-pig and in human asthmatics as shown by Herxheimer and Streseman (1962). The response obtained in the guinea-pig occurred after pithing the spinal cord, and crushing the sympathetic nerves and vagi in the neck. These two groups of workers have used material from the same batch of purified SRS-A, but this sample contracted the rat uterus (Collier, personal communication), a property not found in the fresh crude perfusate (Brocklehurst, 1958; Chakravarty, 1960).

### Stability of Perfusate (SRS-A) and Different Extracts

Crude SRS-A loses potency slowly at room temperature. It retains 90% of its activity after boiling for 15 mins. at neutral pH (7-8) and is relatively stable in the crude form between pH 2 and 12 with a greater stability in alkaline solution (Brocklehurst, 1958; Chakravarty, 1959).
**TABLE 2**

Pharmacology of partially purified SRS-A from various workers.

<table>
<thead>
<tr>
<th>Worker</th>
<th>Source</th>
<th>Purification procedure</th>
<th>Isolated preparations</th>
<th>Pharmacological actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brocklehurst</td>
<td>Guinea-pig lung. Material obtained by perfusion during anaphylaxis in vitro.</td>
<td>Absorption of SRS-A, by partially activated charcoal, followed by elution with 8% butanol solution (or in some cases pyridine) and also wherever thought necessary addition of KCN.</td>
<td>Guinea-pig ileum</td>
<td>Contractions</td>
</tr>
<tr>
<td>(1956)</td>
<td></td>
<td></td>
<td>Human bronchioles</td>
<td></td>
</tr>
<tr>
<td>Chakravarty</td>
<td>Cat’s paw. Treated with compound 48/80 by perfusion (and in a few cases guinea-pig chopped lung, material obtained by diffusion)</td>
<td>Acid ether extract, followed by paper chromatography at 4°C on Whatman Nos 1, 3 and 4 using the following solvents: (a) n-propanol:ammonia:water (6:3:1)-ascending (b) methanol:chloroform-descenting (1:4) (c) ethyl acetate:acetic acid:water (3:1:1)-ascending (d)n-butanol:acetic acid:water (63:10:27)-descending in an atmosphere of N2.</td>
<td>Guinea-pig ileum</td>
<td>Contractions</td>
</tr>
<tr>
<td>(1960)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anderson et al.</td>
<td>Guinea-pig lung. Method of Brocklehurst.</td>
<td>Acid ether extracts after acidification of perfusate (ca pH 3) were used in columns. (a) Silicic acid column followed by elution with stepwise increase of ether in petroleum ether. (b) Aluminium oxide (chromatographic grade) followed by elution using increasing concentrations of methanol in diethyl ether.</td>
<td>Guinea-pig isolated ileum</td>
<td>Contractions</td>
</tr>
<tr>
<td>(1963)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Worker &amp; Source</td>
<td>Purification procedure</td>
<td>Isolated preparations</td>
<td>Pharmacological actions</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>------------------------</td>
<td>-----------------------</td>
<td>-------------------------</td>
<td></td>
</tr>
<tr>
<td>Berry and Collier (1964)</td>
<td>Absorption of histamine-free SRS-A by partially activated charcoal followed by elution with pyridine gave &quot;charcoal-purified&quot; SRS-A.</td>
<td>Guinea-pig ileum</td>
<td>Contractions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Removal of histamine from crude SRS-A by ion-exchange chromatography gave &quot;histamine-free SRS-A&quot;.</td>
<td>tracheal muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human bronchi-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>oles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
SRS-A is quite resistant to proteolytic enzymes, i.e. trypsin, chymotrypsin, pepsin and activated papain (Brocklehurst, 1958; Berry and Collier, 1964). Heating with oxidising (periodic acid) and reducing (reduced glutathione and sodium dithionite) agents also inactivate SRS-A (Chakravarty, 1959). The different methods of purification such as extraction with 80% ethanol or elution from charcoal, give samples which vary in their stability, but no precise information is available in the literature.

Differentiation from other active substances

SRS-A could be distinguished from histamine, acetylcholine, 5HT, bradykinin, substance P, ATP and certain lipid-like materials as shown on Table 3 (p. 16). Analysis shows that its effect is direct on the muscle; atropine and hexamethonium have no inhibitory effect on the contractions produced in the guinea-pig ileum, yet the response is severely reduced when the temperature falls below about 34°C. (Brocklehurst, 1958). Solubility in various solvent systems also distinguishes SRS-A from polypeptides like bradykinin (Rocha e Silva et al., 1949) or substance P (Buler and Gaddum, 1931; Pernow, 1953) which also have stimulant action on guinea-pig ileum (Gaddum, 1955).

Among the lipid-soluble acids, shown in Table 4
### TABLE 3

**Differentiation of SRS-A from other active substances**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Solubility</th>
<th>Thermo-stability</th>
<th>Biological effects on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90% EtOH</td>
<td>Acetone</td>
<td>Ether</td>
</tr>
<tr>
<td>Substance P</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Histamine</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Adenosine</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>Enteramine&lt;sub&gt;SH&lt;/sub&gt;</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Darmstoff</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SRS-A crude</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>SRS-A partially purified</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = soluble in dialysable thermstable in still active after contraction or pressure rise
- = insoluble in undialysable thermolabile in inactive after no response

(+) = as above to a lesser degree  ↓ = fall in pressure

0 = not available in references
**TABLE 4**

A comparison of the biological properties of lipid soluble acids

<table>
<thead>
<tr>
<th>Probable chemical structure</th>
<th>SRS-A</th>
<th>Prostaglandin</th>
<th>Irin</th>
<th>Dermatost</th>
<th>SRS from egg yolk</th>
<th>G-acid</th>
<th>Haemolytic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>a=Brocklehurst</td>
<td>Lipid soluble acid also water soluble small amounts phosphorous and nitrogen</td>
<td>Nitrogen free unsaturated hydroxy acid</td>
<td>Longchain unsaturated hydroxy-fatty acid</td>
<td>Acetalphosphatidic acid + acid phospholipids.</td>
<td>Unsaturated fatty acids</td>
<td>Longchain C₁₇:₃ octadecenoic acid</td>
<td>Cis-11-octadecanoic acid (cis-vaccenic acid)</td>
</tr>
<tr>
<td>b=Chakravarti</td>
<td>Appears in anaphylaxis (a) (b)</td>
<td>Seminal fluid and accessory genital glands most tissues</td>
<td>Iris</td>
<td>Intestine</td>
<td>Lecithin</td>
<td>Plasma</td>
<td>Plasma and other tissues</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>Effect on smooth muscles</th>
<th>Effect on blood pressure rabbit</th>
<th>Effect on RBC Haemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>G pig ileum</td>
<td>+ +</td>
<td>0 Fall</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit duodenum</td>
<td>+ +</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Rat small intestine</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Rat colon</td>
<td>0 +</td>
<td>+ (b)</td>
<td>0</td>
</tr>
<tr>
<td>Fowl rectal caecum</td>
<td>+ 0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Hamsters colon</td>
<td>0 0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit uterus</td>
<td>0 0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>G pig uterus</td>
<td>0 0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Rat uterus</td>
<td>0 0</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

+ = contraction  
0 = no effect
contraction of guinea-pig and rat uterus distinguishes prostaglandin and darmstoff from SRS-A. Prostaglandin, in addition, reduces rabbit's and cat's blood pressure, which is not affected by SRS-A. Contraction of hamster colon differentiates it from irin; in addition irin is thermolabile (Ambache, 1957) whereas as mentioned earlier SRS-A retains 90% of its activity after boiling for 15 minutes at neutral pH. The slow contracting substance produced by snake venom in egg yolk causes a fall in rabbit's blood pressure. Moreover, it is definitely known that both this substance and the smooth muscle stimulating principles isolated from plasma (G-acid and cis vaccenic acid) haemolyse red blood cells, whereas it is not yet confirmed that SRS-A has a haemolytic property, though Brocklehurst (1958) mentioned that some samples are weakly haemolytic.

**Chemical properties of SRS-A**

The active material termed SRS-A has not yet been isolated, therefore the chemical identity remains unknown. Inability to isolate the material has been largely due to the small amounts available and changes in such properties as solubility, adsorption and passage through membranes as purification proceeds.

From the large number of chemical experiments which have been done with crude perfusates and also with
various extracts the following facts emerge.

(i) SRS-A is quite resistant to proteolytic enzymes like chymotrypsin, trypsin and papain (Brocklehure, 1958; Chakravarty, 1960; Berry and Collier, 1964); it is insoluble in butanol, it is therefore unlikely to be a polypeptide or protein.

(ii) It is acidic in nature (Brocklehure, 1962; Chakravarty, 1959) and has also been described as a lipid soluble acid (Uvnäs, 1962). The salt is very water soluble and the free acid (at pH 3) is less water soluble, it has been suggested that it probably exists as a sodium salt in the usual bath media. The actual partition ratio of the free acid between an aqueous and organic phase is dependent largely on the nature and amount of lipids present (Brocklehure, 1958; Chakravarty, 1960). This was shown clearly during attempts to find a suitable solvent pair for counter-current separation of SRS-A. When repeatedly extracted with amyl acetate, the aqueous phase at pH 3 retained a variable amount of the SRS-A, which could not be removed by the organic solvent, but when a little lecithin was added to the SRS-A solution and shaken, the activity could be extracted with amyl acetate.

(iii) The crude material is soluble in 80% ethanol but not in absolute ethanol. At acid pH (pH 3) it is extracted by peroxide-free ether, although the amount
in the ether phase is still less than that in the aqueous phase, unless there is lipid present to increase its solubility. When alcohol-extracted SRS-A is acidified (pH 3) it readily passes over to organic solvents like methyl ethyl ketone and ethyl acetate, and is slightly soluble in n-butanol, chloroform and petroleum ether (Chakravarty, 1959), from these facts SRS-A has been thought of as a lipid or lipid soluble acid. In 1960 Brocklehurst suggested that SRS-A might be a lipopolysaccharide.

In 1962, Smith suggested that it is much akin to methoxy-neuraminic acid, further proof was required before this suggestion could be accepted. In 1963, Anderson et al. put forward further evidence that this active substance may be glycosides of neuraminic acid. Their conclusion was based on the chromatographic behaviour of acid ether extracts of perfusate on silicic acid columns. The evidence for this was stated as follows:

(i) That fractions which gave glycoside positive reaction chemically also exhibited pharmacological action

(ii) That these fractions also gave positive tests for amino nitrogen.

In these tests glycoside reactions would be obtained when glycol radicals (-CH₂OH) or aminoglycols are present: from these findings the ratios of glycol to amino groups were calculated, this gave ratios of 3.2:1
and 7. The simplest possible structure for the active fractions having ratios of 1 to 3 might be:

Figure 1a)

![Diagram](image)

Inserted into

C-(CH₃)ₙ-COOH

Figure 1b)

![Diagram](image)

Neuraminic acid.
This structure, they argued would readily accommodate \( \text{CH}_2\text{OH}/\text{NH}_2 \) but the inclusion of up to two glycol and one amino glycol side chains, in a terminally substituted fatty acid is not known. Substitution at other points can obviously permit any ratio, and when the ratio of \( \text{CH}_2\text{OH}/\text{NH}_2 \) is 7, the structure of neuraminic acid or its derivative can be derived.

Gottschalk, in his book on sialic acids, stated that all tissues contain neuraminyl derivatives as a normal constituent, so that in the fractions tested by Anderson et al. it could be that they were investigating a side issue. Brocklehurst (1962) disagreed with these findings, arguing that there is as yet no known biologically active compound of neuraminic acid. Furthermore purified SRS-A gives neither colour reactions nor the infra red absorption spectrum characteristic of neuraminic acid and its derivatives.

Uvnäs (1962) stated that it is not a phosphatide and does not contain choline, but most of the material he studied was obtained from the cat's paws by the action of compound 48/80. Nevertheless these findings are in agreement with those from less refined experiments by others, using SRS-A from guinea-pig lung.

There is general agreement therefore by all workers in this field that this substance is not a protein or polypeptide, it is an acid which usually contains small
amounts of phosphorous and amino N, it is lipid soluble. Apart from these there is no agreement on any other features, and all the procedures for purification are empirical.

**Methods of purification**

*Empirical procedures.*—By 1958, Brocklehurst had already established that SRS-A was not destroyed during short periods of boiling at pH 7 to 8, and that it was relatively stable in the impure form (shock perfusate from lung) between pH 2 and 12 with somewhat greater stability in alkaline solution (see also Chakravarty, 1959). Attempts to establish the approximate size of the SRS-A molecule were quite unsuccessful because the substance has such a strong tendency to become adsorbed.

When active perfusate from lung was placed inside a dialysis sac very little SRS-A diffused out, it therefore seemed that purification could be achieved by incubation with trypsin followed by dialysis. The degradation products of protein and the salts were expected to pass through the sac, leaving the SRS-A inside. In fact, after destruction of protein SRS-A became distributed in equal concentration inside and outside the sac, with some overall loss attributable to adsorption on the cellophane (Brocklehurst, 1958). From this it was concluded that SRS-A was adsorbed
to protein and that the removal of the protein might facilitate the extraction and subsequent purification of SRS-A. Proteins can be removed by precipitation with 70 to 80 per cent ethanol or by extraction of the freeze-dried material with 80 per cent ethanol, the SRS-A remains in the alcohol provided that some water is present (Brocklehurst, 1958; Chakravarty, 1960). Precipitation of the proteins with acids (e.g. trichloracetic) removes all SRS-A from the solution, presumably by irreversible adsorption; activity lost in this way has never been recovered. When 20 volumes of acetone is used to precipitate the proteins together with any larger peptides and phospholipids, the SRS-A activity is all associated with the precipitate, whilst the neutral lipids and histamine remain in the acetone supernatant. The SRS-A is totally recovered when this precipitate is dissolved in physiological salt solution (e.g. Tyrode).

SRS-A free from both histamine and excessive salt is obtained by adsorption on partly activated charcoal, from which it is displaced by a saturated aqueous solution of n-butanol (Brocklehurst, 1958; Berry and Collier, 1964). Dilute ammonia or sodium hydroxide will also elute the SRS-A, as will 20 per cent solution of pyridine, but the bases also eluted some histamine.

The first successful electrophoretic purification on paper was achieved (Brocklehurst, 1958) by using
material partly purified by the charcoal method, and run in sodium phosphate buffer at pH 7.8 and ionic strength 0.3 molar. Migration towards the anode (+) showed that SRS-A was acidic. Losses were too high for this method to be used for large scale purification. Electrophoresis using a supporting medium was never successful; failure was attributed to the very strong tendency of SRS-A to become adsorbed. When the method of electrophoresis in a vertical density gradient (i.e. no solid stabilising medium) was available (Svensson et al., 1957; Charlwood and Gordon, 1958), SRS-A was found to travel towards the anode in a single band at pH 8.0. When the usual sucrose-water gradient and sodium phosphate buffer was replaced by a volatile system consisting of an ethanol-water gradient, and ammonium acetate buffer at pH 8.0, the electrophoretic migration of purified SRS-A was still satisfactory (Charlwood and Gordon, 1958). The volatile buffer vehicle was removed by evaporation of the alcohol at about 5°C, followed by dilution with water and freeze drying to volatilise the ammonium acetate. The biological activity so recovered was contained in a small amount of solid having a potency of at least 5 units/μg, but unfortunately the activity was rather unstable. This same technique was used by Anggard et al. (1963) in Stockholm on the SRS obtained both from cat's paws
and from sensitised chopped guinea-pig lungs.

Chakravarty in 1959, obtained good separation using ascending paper chromatography with n-propanol-ammonia-water (6:3:1) as the developing solvent in a nitrogen atmosphere. When the ammonia had been removed by drying, the SRS-A was localised as a discrete acid region by spraying with bromothymol blue.

Other methods tried in the purification of this substance included

(a) Ion exchange on synthetic resins, the weak cation exchange resin, Amberlite IRC50, which separated proteins of low molecular weight and high isoelectric point. The SRS-A was lost.

(b) Cellulose adsorbents, which provided hydrophilic supports with large surfaces for protein binding. Both the Diethylaminoethyl (DEAE) and Carboxymethyl (CM) varieties have been used with little success.

**Purification using techniques applicable to lipids.**—The preliminary observations of Brocklehurst (1958) and Chakravarty (1959) that their samples of SRS could be completely extracted with 80 per cent ethanol, and that they were soluble in methanol, n-propanol and water saturated n-butanol, suggested that SRS was of lipid character. Furthermore it was known that after acidification SRS-A passed over to diethyl ether and other organic solvents. The characteristic distribution
pattern obtained when aqueous solution of SRS-A at various pH's is shaken with ether is now well documented.

From these findings, many workers have assumed that SRS-A is a lipid and have employed classic methods of separation in organic solvents (Anderson et al., 1963; Anggard et al., 1963). Anderson et al. (1963) used chromatographic separation on silicic acid by Hirsch and Ahrens method (1960). Acid ether extracts were placed on the column, and eluted by stepwise changes in the concentration of ether in petroleum ether (B.P. 60-80°C.) followed by elution with ether and finally methanol. The collected fractions were examined for cholesterol, glycerides and phosphorus. They found that the glyceride test was positive in all fractions having biological activity. Losses incurred using this technique (up to 80 per cent) are too great to justify the use of this method. Anggard et al. (1963) also used acid ether extracts on a silicic acid column, but eluted with a sequence of solvents as follows: chloroform alone, chloroform + methanol (9:1), chloroform + methanol (1:1). Biologically active fractions were obtained with both the chloroform and chloroform + methanol (1:1), but the SRS-A like activity was obtained in the 1:1 eluates, whereas the activity in the chloroform fractions appeared to be due to a fatty acid. The percentage recovery was not stated.
PART II

EXPERIMENTAL
PREPARATION OF SRS-A FROM LUNG

The method used for obtaining the crude perfusate is almost identical to the method of Brocklehurst (1960), the details are given below for the convenience of the reader.

Experimental

sensitisation of guinea-pigs.—Guinea-pigs weighing 200 to 250 g, about three weeks old were injected with 100 mg crude egg albumen solution intraperitoneally and 100 mg subcutaneously. The animals were used 21 to 33 days later. At the beginning of this project, as a routine procedure after three weeks of sensitisation, the animals were subjected to anaphylactic shock by the aerosol technique developed by Herxheimer (1952). This procedure of shock "in vivo" was to check the sensitivity of the animals in the group to be used for the experiment.

If the animals were not sufficiently sensitive, booster doses of alum-adsorbed crystalline ovalbumin were given. 2 mg of this alum-adsorbed albumin in 3 ml normal saline was injected intraperitoneally as a booster once every 2 to 3 weeks. These animals were used 15 to 28 days after the last of such injections.

*The preparation of alum-adsorbed antigen is described in the Appendix.
Perfusion and shock of isolated lung.— The guinea-pig was killed by dislocation of the neck; the trachea was at once ligatured and the lungs with the heart attached were dissected out without handling them. An incision was made into both ventricles and through the right ventricle a flexible cannula was tied in the pulmonary artery so that the tip was proximal to the bifurcation of the pulmonary artery.

The preparation was suspended in a water jacketed chamber at 37°C, with the lungs inflated through a tracheal cannula to about two thirds of the "in vivo" volume. Tyrode solution, prewarmed to 37°C, entered through the pulmonary artery cannula the effluent being collected through the incision of the left auricle. Blood was flushed out by an initial flow of about 20 ml/min, and the rate was then reduced to about 2 ml/min. Collection started after 4 or 5 minutes when the perfusate appeared to be free from blood. Collected perfusate was kept on ice, and was tested on the same day, the assay being performed using guinea-pig ileum as described in the Appendix. After the assay on the samples had been performed, the samples were either pooled and freeze dried in 40 ml quantities or distributed in 3 ml quantities in ampoules and then freeze dried.
Preparation of "SRS-A" laboratory standards. - Two types of laboratory standards were used throughout this work.

(a) Crude perfusate standard

This was produced as described in the above paragraph. Sixty sensitised guinea-pig lungs were perfused and the perfusate leaving the lungs 2 minutes after shock was collected up to 10 minutes. The perfusates from individual lungs were pooled and divided into two aliquots. One aliquot was distributed in 3 ml quantities into ampoules, freeze dried and sealed under nitrogen and stored at -20°C.

(b) Alcoholic extract standard (AE standard)

The other half of the pooled perfusate from (a) was freeze dried in 40 ml quantities and extracted with 80 per cent alcohol. The alcohol was removed under reduced pressure in a rotary evaporator at 30°C, until a small volume was obtained. This concentrate was made up to the original volume with distilled water, distributed in 3 ml quantities into ampoules, freeze dried, sealed under nitrogen and kept at -20°C.
Fig. 2 (a) and (b) Typical assay on guinea-pig ileum of histamine and SRS-A content collected from sensitised guinea-pig lung. Atropine $10^{-7}$M present at all times and for SRS-A assay mepyramine $10^{-6}$M present.

$U'$, $U''$, $Y'$, $Y''$ are perfusates collected from sensitised guinea-pig lungs.

$\bullet$ = high dose of histamine 4.0 ng/ml;
$\cdot$ = low dose of histamine 2.0 ng/ml.
Fig. 2 (a)

Sample Dilution

Fig. 2 (b)
PURIFICATION OF SRS-A BY DEXTRAN GEL COLUMNS

DEAE and CM-derivatives of cross-linked dextran gels (G-25 and G-50 Sephadex) became available four years ago. There is already a large amount of information in the literature about them which could be useful in understanding the results of purification of SRS-A. DEAE-Sephadex grades A-50 and A-25 are reported to be penetrated by proteins comparable in size to pepsin (mol. wt. 35,000); the capacity for anion is 3-4 mE/g and the pK in 1M KCl about 8. CM-Sephadex C-50 and C-25 have a cation capacity of 4-5 mE/g and a pK about 4. Under appropriate conditions the gel from 1 g of dry powder will bind 1 to 2 g of protein. The Sephadex ion exchange derivatives are more homogeneous than the corresponding cellulose derivatives and have convenient handling qualities.

Factors influencing adsorption

Binding occurs at pH values between the isoelectric point of the protein and the pK of the ionising groups. The DEAE derivatives are effective below pH 8.0 and the CM derivatives above pH 4.0. The affinity for the ionised groups is determined by the number and distribution of the charges on the protein molecule. A large protein with a high charge density is most firmly
bound, high local concentration favours binding, as does low ionic strength (i.e. 0.2 molar or less).

When the ionic strength of the medium is moderate or high, the protein binding capacity of the adsorbent is reduced or abolished. The preparation of tissue extracts or exudates containing protein for adsorption commonly requires reduction of ionic strength and pH adjustment. This adjustment should be selective, and must be exact; this may be achieved by dilution, dialysis or gel filtration. Dialysis is inadequate unless very prolonged, dilution is only satisfactory when electrolyte composition is known. Dextran gel filtration retards the progress of small molecules in the column, and thus separates them from the proteins, which pass into the buffer solution with which the column was initially equilibrated. It will therefore give rapid and complete equilibration with buffer of chosen pH and ionic strength, and will usually remove unwanted small molecular constituents concomitantly.

**Selective elution**

Protein is eluted when the association with the binding sites is weakened by increased ionic strength or pH change. Both of these variables are used to obtain selective elution without recourse to extreme conditions. The conditions of high resolution are well
established (Peterson and Sober, 1962). The protein load should be small by comparison with the total capacity of the column. The sample should be applied in small volume or under conditions which allow tight adsorption to a long column of uniformly packed fine particles. The ionic strength and/or pH should be altered in a continuous gradient and not in discrete steps. This takes full advantage of adsorption equilibrium, the state in which partially adsorbed protein moves slowly down the column.

Stepwise elution with a graded series of buffer does not give high resolution but offers advantages in other ways. Large loads may be fractionated on short, wide columns with coarse particles and high flow rates. Rapid separation of components with markedly different adsorption characteristics occurs, e.g. an enzyme may be separated from its substrate.

**Experimental**

**Crude perfusate**

(a) *Ion exchange method.* - The preliminary treatment of the ion-exchanger before use is described in the Appendix, Section I.

5 ml quantities of crude perfusate at 4°C were stirred into a suspension of 2 g DEAE-A25 or A50 previously equilibrated with sodium phosphate buffer of
0.01 M concentration, pH 7.5 plus 0.01 M NaCl. The supernatant was decanted and tested on the guinea-pig ileum. The anion-exchange sediment was packed into a column (1.0 x 5.0 cm) and eluted with 200 ml buffer of the same ionic strength and pH, followed by 500 ml of buffer in which the sodium chloride concentration was continuously increased to 0.18 M.

The SRS-A activity (about 25 per cent) was eluted between NaCl concentration limits of 0.09 and 0.12. In each of the columns (six replicates for each type of anion exchanger) between 70 and 80 per cent of the SRS-A activity was lost. This low yield and the effects of the increment of sodium chloride on the test preparation makes this method unsatisfactory.

(b) Gel filtration method. In preliminary experiments dextran gel columns separated the salt from the protein fractions in the perfusates with little loss of SRS-A activity. This procedure also separated the histamine content of the perfusates from the mepyramine resistant activity (SRS-A), as shown in Figures 4 and 5.

10 ml of crude perfusate was layered beneath buffer on a column of medium grade G-50 Sephadex (2.5 x 18.0 cm) equilibrated with 0.01 M phosphate buffer (pH 7.5) at 4°C. The column was developed with the same buffer at a pressure head of 20 to 30 cm giving a flow rate of 60 ml/hour. The eluate was collected in
10 ml fractions using an automatic fraction collector. Optical density (275 mμ or 280 mμ) was taken as a measure of protein concentration and specific conductivity (m.mho/cm) as a measure of electrolyte concentration. The elution pattern is shown in Figure 4. For convenience the electrolyte concentration is shown as a continuous line. The biologically active fractions (SRS-A) are separated from the salt peak and coincide with the greater of the protein peaks in the first 80 ml. (To avoid repetition the method described above will be referred to as Stage I).

The reproducibility of the method was established by a series of experiments with various samples of crude perfusates collected from different batches of sensitised guinea-pigs. In these experiments three measurements were made on each fraction:

1) Specific conductivity m.mhos/cm.

2) The protein concentration using a solution of Ovalbumin (5 x crystalline) as standard.

3) The mepyramine-resistant activity (SRS-A) using the guinea-pig ileum as test preparation.

The loss in biological activity during "Stage I" was determined in separate experiments as occasion demanded using the laboratory standards to check on the SRS-A activity before and after column treatment. Of the 1500 units applied to a particular column 1320 units were recovered in the eluate ( > 80 per cent). Similar
percentage yields were obtained in most of the experiments. The yields were reduced when the column length was doubled or trebled.

Grades G-25 and G-75 Dextran gels were used and at a later stage in this work when the G-10 and 15 were available they were also used but were not superior to G-50. It was found that the G-50 Sephadex satisfied the more important criteria set out in the synopsis.

**Note** Twenty G-50 Sephadex and ten DEAE-Sephadex columns were run during the developmental stages of the method. The results for a few of these are tabulated in the Appendix. Although the same qualitative pattern occurs in each experiment the results have not been combined. Quantitative differences were due to the use of different batches of sensitised guinea-pigs.

**Alcoholic extract by gel filtration**

The recovery of the SRS-A activity obtained from fractions collected when the crude perfusate was passed through a column of G-50 Sephadex was good, but the fractions obtained from the chromatographic separation of the activity on both DEAE and CM- Sephadex were disappointing. It was therefore thought desirable
to attempt gel filtration of SRS-A which had been partly purified by alcohol extraction. Owing to its simplicity it was hoped there would be little or no contamination. Methods of purification using organic solvents are usually lengthy and may introduce peroxides and other undesirable impurities. This may have been the reason for the partial destruction of SRS-A and may have contributed to the varying stability of the product obtained by these methods.

The alcoholic extract of freeze dried perfusate gave a yield of at least 80 per cent and was substantially free from protein. Along with SRS-A, there was a small amount of lipids and salts, and traces of histamine. The alcohol was always removed under reduced pressure in a stream of nitrogen at about 30°C using a rotary evaporator. The remaining aqueous concentrate was then run through a column of G-50 Sephadex which had previously been equilibrated with the buffer (phosphate buffer of 0.01 M concentration).

(a) Gel filtration at pH 7.4.— Generally in these experiments the aqueous concentrate was made up to 10 ml with buffer, and the specific conductivity of the solution was measured. 8.0 ml of this solution was layered beneath buffer on a column of G-50 Sephadex (2.4 x 18.0 cm) equilibrated with 0.01 M phosphate buffer (pH 7.4) at 4°C. The column was developed with
the same buffer at a pressure head of 20 to 30 cm giving a flow rate of 60 ml/hour. The eluate was collected in 10 ml fractions. The specific conductivity of each fraction was taken as a measure of electrolyte concentration, and the amounts of K⁺ and Na⁺ were checked using a flame photometer. The biological activity was determined as described earlier. Twelve such columns were run.

In this set of experiments, as shown in Figure 6(a) the fractions containing biological activity were obtained in the middle part of the salt peak and the potency (activity per unit volume) was greatly increased as shown in Table 5. It was stable when stored at -15°C. As the purified material was later to be used in pharmacological tests, it was desirable to obtain the biological activity without appreciable amounts of salt in the samples. The amount of salt present in these fractions is far less than that in the starting material and a further advantage is that the composition is known. Since pH was known to influence the firmness with which SRS-A is adsorbed to starch, it was thought prudent to run the Sephadex (dextran gel) columns in other buffers.

(b) Gel filtration at pH 8.4.- The method is the same as described above except in the change of pH. Elution pattern is shown in Figure 6(b). On the six columns, the biological activity was found in the first
part of the salt peak and this activity was reasonably stable, but the potency of the combined eluates was not as high as those obtained for the previous columns using buffer at pH 7.4. The stability of the fractions was further investigated on freeze dried samples four weeks later. The apparent loss of activity was about 10 per cent but this is within experimental error.

(c) Gel filtration at pH 6.3.— In a series of eight experiments, the SRS-A activity was obtained in a sharp peak emerging later than the salt peak. The activity was relatively unstable, but when tested as collected from the columns it seemed that the potency had greatly increased, e.g. the total activity as assayed was often three times greater than that originally applied to the column, as shown in Figure 6 (c).
TABLE 5

Gel filtration of crude perfusate on G-50 Sephadex (2.5 x 18 cm.) phosphate buffer pH 7.4

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Activity in units</th>
<th>Specific Conductivity m./mhos/cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>1.24</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>1.24</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>1.24</td>
</tr>
<tr>
<td>4</td>
<td>210.0</td>
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<tr>
<td>5</td>
<td>165.0</td>
<td>1.24</td>
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<tr>
<td>6</td>
<td>41.5</td>
<td>1.24</td>
</tr>
<tr>
<td>7</td>
<td>28.0</td>
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</tr>
<tr>
<td>8</td>
<td>8.5</td>
<td>1.24</td>
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<tr>
<td>9</td>
<td>-</td>
<td>1.59</td>
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<tr>
<td>10</td>
<td>-</td>
<td>4.14</td>
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<td>11</td>
<td>-</td>
<td>6.77</td>
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<tr>
<td>12</td>
<td>-</td>
<td>10.66</td>
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<tr>
<td>13</td>
<td>-</td>
<td>8.76</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>6.04</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>3.04</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>1.63</td>
</tr>
<tr>
<td>17-30</td>
<td>-</td>
<td>1.26</td>
</tr>
</tbody>
</table>

Total 453.0 units) Increase 30%
% Yield 130

Ve=elution volume=10mls
Vo=void volume=12.5mls
Sample 10 ml 35 units/mls
Vol of each fraction 5.0mls
Fig. 3(a)  Part of an assay of fractions obtained after elution from DEAE-50 Sephadex column.

S' = 1.0 units SRS-A;  S'' = 1.5 units SRS-A

Fig. 3(b)  Anion exchange chromatography

Elution pattern of Stage I purified SRS-A applied onto DEAE-50 Sephadex column using a continuous elution system. Continuous line represents specific conductivity of salt (mmho cm"⁻¹) and histograms represent fractions containing biological activity.
**Fig. 3(a)**

Eluion Phosphate Buffer pH 7.4, 0.01M with molarity Gradient of NaCl 0.01M - 1.01M
Column DEAE Sephadex A-50, (15 x 20 cm)

**Fig. 3(b)**

Biological activity

Specific activity

Volume in ml
Fig. 4 (a) and (b) Elution pattern from G-50 Sephadex columns. Columns loaded with 20 ml crude perfusate. Continuous line represents specific conductivity (mho/cm).
Fig. 4 (c) G-50 Sephadex column: elution pattern of 10 ml volume of sensitised guinea-pig lung perfusate with phosphate buffer (0.01M, pH 7.4) containing NaCl 0.12 M.
Fig. 5 Assay on guinea-pig ileum bathed in Tyrode solution containing atropine $10^{-7}$M and mepyramine $10^{-8}$M at 37°C. Fractions collected after perfusate load on a column of G-50 Sephadex eluted with phosphate buffer (pH 7.4). Unlabelled contractions are those of laboratory standards containing 3.0 units/ml and 1.5 units/ml of SRS-A.
Fig. 6 (a), (b) and (c)

Elution pattern of alcoholic extract of freeze-dried perfusate on G-50 Sephadex column eluting with phosphate buffer of varying pH. Fractions containing biological activity shown as histograms.

(a) Phosphate buffer pH 7.4
(b) Phosphate buffer pH 6.4
(c) Phosphate buffer pH 8.5
Alteration of pH of buffer (Phosphate) on G-50 Sephadex
Sample Alcoholic Extract
Column (2.5x18cm) Buffer 0.01M Phosphate
10ml fractions

Alteration of pH of buffer (Phosphate) on G-50 Sephadex
Sample Alcoholic Extract
Column (2.5x18cm) Buffer 0.01M Phosphate
10ml fractions

Alteration of pH of buffer (Phosphate) on G-50 Sephadex
Sample Alcoholic Extract
Column (2.5x18cm) Buffer 0.01M Phosphate
10ml fractions
Fig. 7 Combination of Figure 6 (a), (b) and (c) showing shift in biological activity relative to salt peak.
ATTEMPTS TO STABILISE SRS-A PURIFIED BY GEL FILTRATION

The material obtained at pH 6.4 was highly active and therefore of considerable interest, but insufficiently stable for detailed pharmacological and chemical examination to be carried out on the purified SRS-A. It was thought therefore that ways of stabilising the material should be sought.

1. Alteration of the pH of the eluate containing SRS-A to alkaline conditions (pH 8.5 or 9.0)

The first procedure tried was changing the pH of the eluate collected, since it had been reported that SRS-A was fairly stable in alkaline conditions.

The columns were set up as previously described, with the eluting buffer at pH 6.4, and ionic concentration of 0.01 M. Immediately after collecting and testing the samples to ascertain where the active fractions were, the fractions were pooled and solution of Na₂HPO₄ (di-sodium hydrogen phosphate) of ionic concentration 0.01 M was added from a microburette to give a pH 8.5 or 9.0 using the pH meter to read the final pH. The sample was then tested, and freeze dried and stored.

The activity was still present after seven days, with an apparent loss of about 40 per cent. Some of this loss could have arisen from the biological test, but
it is believed that there had been a genuine loss of activity from the sample. Dilution of the sample before testing reinstated the pH to about 7.5 which is the pH of Tyrode solution.

(ii) The use of either silicone-treated test tubes or polythene test tubes

Although the above method of immediately altering the pH of the collected eluates met with reasonable success, it was thought worthwhile to try other methods. It is widely held that SRS-A is adsorbed onto wettable surfaces although no objective study appears in the literature. With this in mind it was thought that the use of repellent surfaces might be useful.

Glass test tubes were treated with Silicone "Repelcote" and dried at 110°C for one hour before use. The fractions were then collected in the usual way. In a parallel experiment untreated polythene test tubes replaced the usual glass test tubes.

The loss of activity in these two methods as assayed four and twenty-four hours after collection of the samples was about 40 per cent. No more loss of activity was detected after freeze drying in silicone-coated ampoules or flasks and testing later.
(iii) The use of antioxidants

(a) Glucose 20 mg/ml in final concentration
(b) Cysteine 50 µg/ml in final concentration
(c) Ascorbic acid 0.2 mg/ml in final concentration

As usual the final assay was performed on the guinea-pig ileum.

The solutions containing glucose were diluted with glucose-free Tyrode solution before bioassay, which was performed immediately after collection and also after storage at -20°C for 24 hours. An apparent loss of 50 per cent activity was recorded on average.

On testing samples into which cysteine had been added it was found that every sample elicited a contraction of the guinea-pig ileum. This prompted the direct investigation of cysteine on the test tissue. Cysteine hydrochloride adjusted to pH 7.5 with NaOH was found to contract the tissue at concentrations as low as 2 ng/ml.

Ascorbic acid was found not to have any effect on the activity.
Purification of SRS-A by Adsorption

Failure to achieve a reasonable recovery of SRS-A from Sephadex ion-exchanger columns led to an attempt to purify this substance using alumina. As mentioned in the main experimental introduction, Brocklehurst (1958) found that alumina Peter Spence Type H removed SRS-A activity from the ethanol solution and that much of what was adsorbed could be eluted with distilled water. He also found that material prepared in this way remained in solution when 20 volumes of acetone was added to an aqueous solution, also that ammonium sulphate did not cause precipitation. It was also insoluble in ether at neutral pH. These observations led Brocklehurst to conclude that since 20 volumes of acetone precipitated proteins, salts and some lipids from crude perfusate the concurrent removal of SRS-A was simply co-precipitation or adsorption, also that solubility in ammonium sulphate (60 per cent) supported his previous belief that SRS-A was not a peptide. Its insolubility in ether after alumina treatment led to the tentative conclusion that it was unlikely to be a free fatty acid.

In 1962, Anderson et al. used a chromatographic grade of alumina (B.D.H.) in an attempt to purify SRS-A. The whole guinea-pig lung perfusate at pH 7.5 was shaken with ether, because at this pH it has been reported that ether
did not extract the SRS-A activity (Brocklehurst, 1958; Chakravarty, 1960) but did remove lecithin and such lipids. After this preliminary treatment the aqueous layer was acidified to pH 3 and extracted with peroxide-free ether, at this stage the SRS-A passed into the ether phase. This extract was then layered onto an alumina column, after allowing the extract to percolate the column was eluted with methanol. From the pharmacological studies it was reported that the first ether effluent contained no SRS-A activity, but the methanol eluates contained the majority of the activity. Many substances, e.g. substance P, angiotensin, bradykinin, catechol amines, have been purified using alumina.

Preparation of chromatographically neutral alumina

For some applications, a neutral chromatographic adsorbent is required and experience with Sephadex indicated that it was desirable in this case. The neutral alumina was prepared from the manufactured standard Type "H" alumina (which is slightly alkaline) as follows:– The Type "H" alumina was slurried with tap water and heated to between 70 and 80°C. Dilute HCl was then added until the mixture was slightly acid (6.5). Heating was continued with constant stirring. After one hour, the stirring was stopped and the solids were allowed to settle. The supernatant liquor was
was then decanted. The alumina was again slurried with 2 per cent ammonium hydroxide, heated to between 70 and 80°C, and stirred continuously for 30 minutes. The liquor was again decanted. The alumina was repeatedly washed by decantation until chloride free, when tested with AgNO₃. As soon as no Cl⁻ ions were detected in the washing, a final wash was carried out using dilute acetic acid (pH about 6.5). Water was removed on a Buchner funnel and dried in an oven at 120°C, and then calcined for three hours at 600°C. After this the alumina was then ready for use. This type of alumina has been graded as Brockmann II.

1) Application to alcoholic extracts

5 g of the neutral alumina was slurried in 80 per cent ethanol and packed into a column of dimensions 1.0 x 10.0 cm and supported on a grade three sintered glass disc. After the column had packed down properly a disc of filter paper was set on top of the column. 80 per cent ethanol was allowed to percolate through the column, to ensure complete equilibration.

The ethanol extract of SRS-A (usually 20 ml) was run through the column and 10 ml fractions collected. When the height of the solution on top of the column was about 1 mm from the filter disc more 80 per cent ethanol (150 ml) was passed through the column before
eluting with whatever vehicle was desired.

(a) **Elution with distilled water.**—Double distilled water was used to elute the first five columns prepared. The fractions were collected in 10 ml quantities using an automatic fraction collector. Immediately the fractions were collected they were concentrated to a small volume using a rotary evaporator and low temperature (30°C). This procedure removes any remaining ethanol which may affect the final bioassay.

The results obtained from these experiments are on Table 6. It was found that using distilled water to elute following a previous wash by 60 per cent ethanol, the total SRS-A activity was obtained in the first three fractions after the change over as shown in Figure 8. This suggested that it was possible to elute the SRS-A activity with a lower concentration of ethanol.

(b) **Stepwise elution using decreasing concentrations of ethanol.**—In this series of experiments the adsorbed SRS-A was eluted by decreasing concentrations of ethanol. The elution sequence was started with 70 per cent ethanol, followed by 60 per cent, 50 per cent, 40 per cent and so on. The elution pattern (which is a combination of results obtained from six experiments) is shown in Figure 9. The fractions eluted with 20 per cent ethanol contained all the SRS-A activity.
This method has many advantages. The SRS-A activity is separated completely from histamine which passed through the column with the 80 per cent ethanol wash. Most commonly occurring phospholipids are reported to be eluted from alumina columns by ethanol concentrations between 45 per cent and 60 per cent (Lederer and Lederer, 1961; Lands, 1964; Hanahan et al., 1951). The amount of solid present after freeze drying the pooled active samples was very small. The samples on reconstituting in Tyrode solution later are very stable with minute salt concentrations.

(ii) Combined gel filtration and alumina adsorption

As described earlier the crude perfusate which was passed through a column of G-50 Sephadex and eluted with phosphate buffer at pH 7.4 yielded SRS-A active fractions before the salt peak. This material was now used in order to exclude the possible modification of the alumina column by salt. The fractions were pooled, freeze dried and extracted with 80 per cent ethanol. The biological activity was tested after each operation, to ascertain that there was no serious loss of activity. The alcoholic extracts then passed through columns of alumina and the SRS-A eluted with 20 per cent ethanol. The percentage yields were calculated for five such experiments and results are shown on Table 8.
(iii) Estimation of the capacity of alumina.— 5 g and 10 g of neutral alumina was loaded onto columns as previously described. 25, 50, 100 mls of samples containing 4.0 units/ml, 5.2 units/ml, and 10.0 units/ml of SRS-A were passed through the columns, and the eluted samples assayed. The results obtained are shown on Table 9.

From these experiments it was found that 1.0 g of this batch of alumina adsorbed between 90 and 100 units of SRS-A. This shows that the previous experiments, in all of which the SRS-A potency had not been as high as this had been completely adsorbed.
<table>
<thead>
<tr>
<th>Solvent</th>
<th>Vol. mls</th>
<th>Activity units/ml.</th>
<th>Total activity</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td>Fraction</td>
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<tr>
<td>1</td>
<td>10</td>
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<td>35</td>
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<tr>
<td>2</td>
<td>10</td>
<td>10.5</td>
<td>13.5</td>
<td>105</td>
<td>135</td>
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<td>152</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>76%</td>
<td>72%</td>
</tr>
</tbody>
</table>

TABLE 6

Distribution of SRS-A activity on alumina columns obtained after distilled water elution

Load 50 mls alcoholic extract (80% ethanol)
### TABLE 7

Distribution of SRS-A activity on alumina columns obtained from three experiments.

<table>
<thead>
<tr>
<th>Solvent % EtOH</th>
<th>Vol. mls.</th>
<th>Activity units/ml.</th>
<th>Total activity</th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Expt. 1</td>
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<td>Expt. 3</td>
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<tr>
<td>80</td>
<td>150</td>
<td>-</td>
<td>350 units</td>
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<tr>
<td>70</td>
<td>150</td>
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<td>150</td>
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</tr>
<tr>
<td>30</td>
<td>150</td>
<td>0.4 unit</td>
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</tr>
<tr>
<td>20</td>
<td>150</td>
<td>2.5 units</td>
<td>280 units</td>
<td>320 units</td>
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</tr>
<tr>
<td>10</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Sum</strong></td>
<td>300 units</td>
<td>330 units</td>
<td>190 units</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td><strong>Yield</strong></td>
<td>80%</td>
<td>79%</td>
<td>76%</td>
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</table>
**TABLE 8**

Combined gel filtration and alumina adsorption

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<th>Original crude samples</th>
<th>300 units</th>
<th>245 units</th>
<th>245 units</th>
<th>200 units</th>
<th>260 units</th>
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<tr>
<td>Total biological activity obtained from 0-50 Sephadex columns</td>
<td>Expt. 1 units 420</td>
<td>Expt. 2 units 350</td>
<td>Expt. 3 units 350</td>
<td>Expt. 4 units 280</td>
<td>Expt. 5 units 375</td>
</tr>
<tr>
<td>Extractable amount into 80% ethanol</td>
<td>370</td>
<td>320</td>
<td>315</td>
<td>250</td>
<td>330</td>
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<tr>
<td>Average loss 10%</td>
<td></td>
<td></td>
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<tr>
<td>Alumina column load</td>
<td>370</td>
<td>320</td>
<td>315</td>
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<td>330</td>
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<tr>
<td>Elution 20% ethanol</td>
<td>300</td>
<td>260</td>
<td>250</td>
<td>200</td>
<td>260</td>
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<tr>
<td>Recovery from original crude perfusate (%)</td>
<td>100</td>
<td>106</td>
<td>102</td>
<td>100</td>
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</table>
### TABLE 9

**Capacity of alumina. Absorbency of SRS-A on alumina**

<table>
<thead>
<tr>
<th>Number of experiments</th>
<th>Volume of alcoholic extract and SRS-A potency</th>
<th>Weight of alumina in grams</th>
<th>Total amount of SRS-A activity in effluent</th>
<th>% age SRS-A in effluent</th>
<th>units/g of alumina</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 mls</td>
<td>5</td>
<td>20 units</td>
<td>4.7%</td>
<td>100 units/g</td>
</tr>
<tr>
<td></td>
<td>5.2u/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>520 units</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4u/ml</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25 mls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 units</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10 units/ml</td>
<td>10</td>
<td>100 units</td>
<td>10%</td>
<td>90 units/g</td>
</tr>
<tr>
<td></td>
<td>100 mls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000 units</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 8 Elution pattern from alumina column (Peter Spence) Type H of alcoholic extract of crude perfusate in 80% ethanol. Dotted line represents distribution of biological activity.
Fig. 9  Elution pattern from alumina column (Peter Spence) Type H of alcoholic extract of crude perfusate in 80% ethanol, using decreasing concentrations of alcohol in water. Dotted line represents distribution of biological activity, obtained from two experiments.

Fig. 10  Elution pattern from alumina column (Peter Spence) Type H of alcoholic extract of crude perfusate in 80% ethanol, using 20% concentration of alcohol immediately after 80% ethanol wash. Results obtained from two experiments.
Fig. 9

Fig. 10
SRS-LIKE MATERIAL OBTAINED FROM CAT'S PAW

Paton (1951) showed that compound 48/80 released a little slow reacting substance as well as histamine from the skin of cats. In 1956 Hogberg et al. used this same releaser to release large quantities of histamine from the isolated perfused cat paws. In 1958 Chakravarty and Uvnäs found that along with histamine considerable amounts of a slow reacting substance were released from these perfused paws, using a very small amount of releaser. Work on this SRS-A was reported by Anggard et al. (1963). They concluded that the SRS from cat paws was similar or identical to that obtained during anaphylactic shock of the guinea-pig lung (SRS-A). They reported purifying this SRS using silicic acid chromatographic techniques developed by Wren (1960) and by Marinetti (1962).

If these claims were correct the cat paws would serve as a good starting material. It was therefore decided to investigate and use the methods already developed to compare this SRS with SRS-A.

Experimental

(1) Collection of samples

Isolated cat paws were perfused according to the technique described by Hogberg, Tufverson and Uvnäs (1956). A paw was mounted on a perspex plate and
perfused via the tibial artery with Tyrode solution at a temperature of about 36°C. All injections were made into the perfusion fluid just above its entry into the artery. 25 to 50 ng of compound 48/80 added to the perfusion fluid yields considerable amounts of histamine and "SRS". The responses of the paws however vary considerably from cat to cat, but paws from the same animal show a rather good quantitative correlation between the yields of both histamine and "SRS". The perfusion fluid was collected in tubes placed in ice and then kept frozen until required for testing.

Guinea-pig ileum was used as the test preparation. A time course of release of both substances was established and the results are shown in the Figure 12 with the sample of the tracing (Fig. 11).

(ii) Purification

These perfusates were treated in essentially the same way as those obtained from sensitised guinea-pig lungs. Columns were used to fractionate the crude perfusate, and also alcoholic extracts. Acetone precipitation, followed by reconstitution in Tyrode and alcoholic extraction was performed only to show that this method could be used.

(a) Treatment by gel filtration

Crude perfusate was passed through a column of
G-50 Sephadex of similar dimensions to those used for the guinea-pig lung perfusates. The buffer solution was phosphate, of pH 7.35 and ionic strength 0.01 M. The biological activity in the eluates was estimated on the guinea-pig ileum, electrolyte concentration was measured from specific conductivity readings and protein concentration was taken from spectrophotometric readings.

The alcoholic extracts were also treated in a similar manner to those used for SRS-A from lungs using pH changes of 7.45, 8.3 and 6.4 (phosphate buffer) to elute the columns.

(b) Treatment by alumina

A similar type of treatment was carried out for the alcoholic extract on a column of alumina.

The results which are illustrated by Figures 13, 14 and 15 and Tables 10, 11 show that the slow reacting substance obtained from the cat's paws behaves in a manner expected of SRS-A. More pharmacological tests were performed later (Section 3) to confirm that the substances were essentially similar.
TABLE 10

Time-course of SRS-like material obtained after administration of compound 48/80 (50 μg) into perfused cat's paws.

<table>
<thead>
<tr>
<th>Time in minutes</th>
<th>Units/ml</th>
<th>Total SRS-A activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 minutes</td>
<td>0.3 units/ml</td>
<td>= 30 units</td>
</tr>
<tr>
<td>10 minutes</td>
<td>2.5 units/ml</td>
<td>= 250 units</td>
</tr>
<tr>
<td>20 minutes</td>
<td>4.8 units/ml</td>
<td>= 480 units</td>
</tr>
<tr>
<td>30 minutes</td>
<td>9.0 units/ml</td>
<td>= 900 units</td>
</tr>
<tr>
<td>40 minutes</td>
<td>7.2 units/ml</td>
<td>= 720 units</td>
</tr>
<tr>
<td>50 minutes</td>
<td>2.2 units/ml</td>
<td>= 220 units</td>
</tr>
<tr>
<td>60 minutes</td>
<td>0.9 units/ml</td>
<td>= 90 units</td>
</tr>
</tbody>
</table>
TABLE 11

Distribution of SRS activity obtained from cat paw on alumina columns—obtained from five experiments

<table>
<thead>
<tr>
<th>Solvent % EtOH</th>
<th>Vol. in mls</th>
<th>Total activity</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
<td>Expt. 3</td>
<td>Expt. 4</td>
<td>Expt. 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>450 units</td>
<td>800 units</td>
<td>400 units</td>
<td>1000 units</td>
<td>650 units</td>
</tr>
<tr>
<td>80</td>
<td>200</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>100</td>
<td>30 units</td>
<td>40 units</td>
<td>10 units</td>
<td>50 units</td>
<td>33 units</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>330 units</td>
<td>580 units</td>
<td>330 units</td>
<td>850 units</td>
<td>500 units</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sum</td>
<td>360 units</td>
<td>620 units</td>
<td>340 units</td>
<td>900 units</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yield</td>
<td>80%</td>
<td>78%</td>
<td>85%</td>
<td>90%</td>
</tr>
</tbody>
</table>
Fig. 11 (a) and (b) Estimation of 10 minute samples of cat paw perfusate for SRS on guinea-pig ileum bathed in Tyrode solution containing atropine $10^{-7}$M and mepyramine $10^{-6}$M.

$S'$, $S''$ are laboratory standards containing 1.5 and 3.0 units/ml SRS-A.

1 = 10 minute sample
2 = 20 minute sample
3 = 30 minute sample
4 = 40 minute sample
5 = 50 minute sample
Fig. 11 (c) Estimation of 10 minute samples of cat paw perfusate for SRS on guinea-pig ileum bathed in Tyrode solution containing Atropine $10^{-7}$M and Mepyramine $10^{-8}$M. 
S', S" are laboratory standards containing 1.5 and 3.0 units/ml SRS-A.

1 = 10 minute sample
2 = 20 minute sample
3 = 30 minute sample
4 = 40 minute sample
5 = 50 minute sample

Fig. 12 Graphical representation of time-course of Histamine and SRS release from perfused cat paws after injection of 50 µg of compound 48/80.
Sample Dilution

Time Course of Histamine and SRS Release from Cats Paws after Compound 48/80

Fig. 12
Fig. 13 Elution diagram of cat paw perfusate on G-50 Sephadex. Fractions with biological activity shown as histogram. 10-20 ml perfusate load.

Fig. 14 Elution diagram of alcoholic extract of cat paw SRS on G-50 Sephadex. Fractions with biological activity shown as histogram. 10-20 ml perfusate load.
Fig. 15  Assay on guinea-pig ileum bathed in Tyrode solution containing atropine $10^{-7}$M and mepyramine $10^{-8}$M at 37°C. Fractions of cat paw perfusate run through a column of G-50 Sephadex eluted with phosphate buffer (pH 7.4).

$S' = \text{Laboratory standard SRS-A containing 2.0 units/ml}$

$S'' = \text{Laboratory standard SRS-A containing 3.5 units/ml}$
Fig. 15
Fig. 16 Comparative estimation of SRS-like material present in freeze-dried cat paw perfusate and alcoholic extract with laboratory standard.

$S' = 3.0$ units/ml

$S'' = 5.0$ units/ml

$B = \text{crude perfusate; } C = \text{alcoholic extract of } B$. 
SRS-LIKE MATERIAL OBTAINED FROM PERITONEAL WASHINGS OF RATS

Another source of SRS (?SRS-A) was reported by Rapp (1961). This was the peritoneal washings of rats following local sensitisation and challenge by intra-peritoneal injection. When this slow reacting substance was tested along-side that from the anaphylactic guinea-pig lung (supplied by Brocklehurst) it produced comparable contractions of the guinea-pig ileum, and Rapp concluded that both substances were the same. Histamine release and damage to mast cells were absent in these experiments.

All the SRS preparations that have hitherto been used were obtained 'in vitro', so this report of 'in vivo' release of an SRS was of special interest and prompted a series of experiments to determine whether this material was indeed SRS-A. Some preliminary findings had already been made by Lahiri (1962), who re-investigated Rapp's work to ascertain whether bradykinin was detectable under the conditions of this experiment and if not whether there was a significant increase in bradykinin-forming activity in rat peritoneal fluid after anaphylaxis.

Lahiri found that saline washes of the peritoneal cavity as performed by Rapp released a great deal of active material irrespective of antigen-antibody reactions, and was not suitable as bathing fluid in the
experiment. Tyrode solution and de Jalon solution were both found to be satisfactory, giving modest control values of biological activity. There was suggestive evidence of bradykinin release.

Experimental (1) Passive sensitisation and the collection of peritoneal washings

Male and female rats (100 to 200 g body weight) were injected intraperitoneally with 0.6 µg of purified γ-globulin containing antibody (see Appendix). Rabbit antibovine serum albumin or guinea-pig anti-ovalbumin was given intraperitoneally in 0.2 ml of Tyrode or de Jalon solution. Four hours later 2.0 mg antigen in 5-7 ml of the same solution was injected intraperitoneally. The rats were killed 5 minutes later, the abdomen was opened and the edges retracted and everted to avoid contamination with blood, and the peritoneal fluid removed by means of a polythene pipette. On some occasions a small midline slit was made in the abdominal wall, and the fluid collected in a polythene funnel without exposing the viscera. For control purposes, some animals were injected with antigen only, but no antibody, some others were injected with antibody only, and no antigen, and some with the Tyrode solution only.
(ii) **Assay and identification of active substances**

The recovered peritoneal fluids were kept on ice until estimated for histamine-like activity, SRS-like activity, and bradykinin-like activity, all of which were assayed on guinea-pig ileum. Assays for bradykinin were also performed on the rat uterus in oestrus, and rat blood pressure or rat duodenum. All the estimations were either carried out on the same day or within 24 hours of collection. In all but the very earliest experiments the fluid collected was spun at 4°C to remove any cells and debris.

(a) **Histamine assay**

A 2 plus 1 assay (Gaddum, 1953) was performed, using the terminal piece of guinea-pig ileum bathed in Tyrode solution containing atropine $10^{-7}$ g/L at a temperature of 37°C.

(b) **SRS-A like activity assay**

This was performed before and after chymotrypsin digestion of the aliquot. This was to eliminate any contribution which bradykinin-like material may make to the contractions elicited from the guinea-pig ileum during assay. The assay was usually performed on the same piece of ileum as used for the histamine assay. The Tyrode solution contained both atropine $10^{-7}$ g/L and mepyramine $10^{-8}$ g/L.
(c) Bradykinin-like activity

0.5 ml aliquots were poured into 1.5 ml of cold absolute alcohol to inactivate enzymes, stood for 30 minutes in ice, to give full precipitation of protein and then boiled for 5 minutes. After being centrifuged at 2,500 g, the supernatant was removed and evaporated under reduced pressure using a rotary evaporator and below 30°C temperature. Uteri from stilboestrol-treated rats of 200-300 g were used to assay the activity against synthetic bradykinin (Sandoz) as standard. Some of these samples were incubated with salt-free chymotrypsin (0.5 mg/ml of sample) for 30 minutes at 37°C to destroy bradykinin. The reaction mixture was then boiled for one minute and cooled ready for assay. Chymotrypsin alone, treated in the same way, and present in amounts in which it occurred in the reaction mixture did not have any oxytocic activity on the isolated guinea-pig ileum preparation.

As may be seen in Table 14 these five minute samples contained too little SRS to be sure that it was SRS-A, but there was bradykinin, which Rapp did not report and some histamine was also present. Tyrode solution gave only a very small spontaneous yield of histamine and it was therefore possible to show the modest release of histamine which occurred during the local anaphylaxis. It seemed possible that a larger yield of SRS-A would be
obtained by allowing the antigen-antibody reaction to continue for a period longer than 5 min.

(iii) **Time-course of release of active substances**

The details of this experiment are similar to those described above. The additional conditions are as follows:

Three rats were used for each period of time, and the time was arranged as follows: 0, 2.5, 5, 10, 15, 20, 30 and 60 minutes.

Chymotrypsin 0.5 mg/ml was used to hydrolyse both standard and control samples of bradykinin.

Three such experiments were performed and the results obtained are shown on Tables 12, 13, Figures 17, 18. It was found that the maximum yield of histamine was obtained at about 3 minutes, whereas the bradykinin maximum was at 15 minutes and the SRS maximum was 20 minutes. This SRS strongly resembled SRS-A pharmacologically. It was reasonable to suppose that when the guinea-pig ileum preparation was used any bradykinin present might lead to an over-estimate of SRS-A present, hence the use of chymotrypsin.

(iv) **Purification of SRS-like material**

The purification of the material collected 20 minutes after challenge with antigen was achieved with G-50 Sephadex. The experiments were essentially
similar to those described for the crude perfusate collected from lung (p. 29). The SRS-like activity from crude material travelled in advance of the salt peak, and when tested on the guinea-pig ileum it bore a strong resemblance to SRS-A.
TABLE 12

Histamine release in antigen-antibody reaction in the rat peritoneal cavity.

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Solution used intraperitoneally</th>
<th>Antibody</th>
<th>Antigen</th>
<th>Histamine-like activity obtained ng/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Saline</td>
<td>-</td>
<td>-</td>
<td>660.0 ± 36.0</td>
</tr>
<tr>
<td>2</td>
<td>Tyrode</td>
<td>-</td>
<td>-</td>
<td>42.0 ± 3.0</td>
</tr>
<tr>
<td>2</td>
<td>Tyrode</td>
<td>+</td>
<td>-</td>
<td>50.0 ± 4.9</td>
</tr>
<tr>
<td>2</td>
<td>Tyrode</td>
<td>-</td>
<td>+</td>
<td>62.0 ± 5.3</td>
</tr>
<tr>
<td>4</td>
<td>Tyrode</td>
<td>+</td>
<td>+</td>
<td>312.0 ± 4.8</td>
</tr>
</tbody>
</table>
TABLE 13

Assays of material similar to bradykinin, and expressed as the equivalent of synthetic bradykinin (Bk).

<table>
<thead>
<tr>
<th></th>
<th>Time in minutes (10)</th>
<th>ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control Group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyrode control</td>
<td></td>
<td>63.30</td>
</tr>
<tr>
<td>Antigen control</td>
<td></td>
<td>59.40</td>
</tr>
<tr>
<td>Antibody control</td>
<td></td>
<td>62.50</td>
</tr>
<tr>
<td><strong>Test Group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time (in minutes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td></td>
<td>64.87</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td>105.90</td>
</tr>
<tr>
<td>10.0</td>
<td></td>
<td>208.40</td>
</tr>
<tr>
<td>15.0</td>
<td></td>
<td>163.40</td>
</tr>
<tr>
<td>20.0</td>
<td></td>
<td>112.20</td>
</tr>
<tr>
<td>30.0</td>
<td></td>
<td>38.37</td>
</tr>
<tr>
<td>60.0</td>
<td></td>
<td>22.50</td>
</tr>
</tbody>
</table>

* In the test group, three rats were used for each time.
### TABLE 14

**SRS-like material obtained after 5 minutes challenge**

No. of rats in each sample = 6

3-12 units/ml.

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Average vol. collected from each rat</th>
<th>Mean total units of SRS</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>5 mls.</td>
<td>8.0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>6 mls.</td>
<td>13.0</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>5 mls.</td>
<td>10.0</td>
</tr>
</tbody>
</table>
Fig. 17  Diagram showing time-course of bradykinin release during antigen-antibody reaction in rat peritoneal cavity.

Fig. 18  Diagram showing time-course of release of bradykinin and other pharmacologically active substances during antigen-antibody reaction in rat peritoneal cavity.
PART III

PHARMACOLOGY
PHARMACOLOGY OF THE PURIFIED MATERIAL

Introduction

Brocklehurst (1958) used many isolated tissues nearly all composed of smooth muscle, to test the SRS-A in crude perfusates as well as the "purified" SRS-A obtained by the charcoal adsorption method. He found out that very few of these preparations gave any response to SRS-A, but he suggested that some of the preparations might respond to doses greater than he was able to use at that time. The results he obtained enabled him to distinguish SRS-A from many other substances; even so he obtained some anomalous results, like the bronchoconstriction in only one of six cats after intravenous injection of SRS-A. He concluded that this must be a special case since the cat probably had oedema of the lung following earlier doses of cocaine and adrenaline and suggested that the SRS-A activity was unmasked because the usual compensating mechanism was inoperative. He also thought that it was likely that SRS-A would be active on the uterus of both the rat and guinea-pig, and that this would provide a rational explanation of the experiments of Kellaway (1930) and Schild (1936) but he was unable to prove this due to the lack of highly potent material.
Now that comparatively pure and much more potent material had been obtained it seemed necessary to make a more comprehensive investigation of its pharmacological actions. Furthermore other actions of "purified" SRS-A have recently been reported (Berry and Collier, 1964; Goadby and Smith, 1964; Anderson, Goadby and Smith, 1963), and we wish to confirm these, since they might necessitate a re-appraisal of its role in allergic conditions.

The following isolated tissues and organs were selected for the pharmacological studies:

**Guinea-pig** ileum, uterus, veins, ureter, gall bladder bladder, heart, tracheal muscle

**Rat** uterus, duodenum, colon, stomach, bladder

**Rabbit** jejunum, heart, uterus

**Hamster** colon, ileum

**Fowl** rectal caecum, crop, oesophagus

**Cat** tracheal strip and chain

**Swine** splenic artery

**Human** bronchioles.

In addition changes in the cardiovascular and respiratory systems, and the production of reactions in the skin, were studied in intact guinea-pigs and rats. Table 20 - pages 167, 168 summarizes the more important pharmacological data.
(1) Guinea-pig ileum

The terminal ileum from guinea-pigs weighing between 200 and 500 g was suspended in Tyrode solution at 37°C with oxygen bubbled gently through the bath. The pull on the tissue was 0.5 g and the lever magnification about 6:1.

Results.— With mepyramine $10^{-6}$ M and atropine $10^{-7}$ M in the bath, active crude perfusates may be diluted with 40 to 100 times their volume of Tyrode solution (0.2 units/ml) and both the purified materials (G-50 Sephadex method on alcoholic extracts) at 0.1 unit/ml and (alumina column method on alcoholic extracts) at 0.3 units/ml, all producing a slow, well-maintained contraction of the tissue. As mentioned in the Section on purification, this preparation was used in the routine testing of eluates. When purified SRS-A is used the contraction produced is rather less slow and of shorter latency than that produced by the crude material.

Under the same conditions the ileum will contract to:

- Substance P 50 μg/ml
- Bradykinin 0.01 - 0.04 μg/ml
- 5-hydroxytryptamine 0.1 - 0.2 μg/ml

(2) Guinea-pig uterus

The uterine horn of a virgin guinea-pig of about 250 g was suspended in Tyrode solution containing
atropine $10^{-7}$M. Oxygen was bubbled through gently and the bath temperature maintained at $37^\circ$C. The lever gave a magnification of about 8:1 and exerted a pull of 0.5 g on the tissue.

**Results.** Although this preparation contracted to histamine 100 $\mu$g/ml and bradykinin 0.2 $\mu$g/ml it did not contract to purified SRS-A (0-50 eluate, 20 units/ml). Before mepyramine was added it contracted to a dose of histamine equivalent to that in the crude perfusate.

(3) Guinea-pig veins

As described by Sutter (1965).

**Preparation.** The animals were killed by a blow on the head. A segment of the external jugular vein, posterior vena cava (distal to the renal veins) or the anterior mesenteric vein (near its proximal end) was removed and immediately placed in oxygenated Krebs-Henseleit solution maintained at $37^\circ$C. The vein was cleaned of adherent tissue and in most experiments spiral or longitudinal strips were cut from the vein by hand. In some experiments rings were cut and three of these were tied together as a chain. The strips or rings were then suspended under 0.25 - 0.5 g tension in a 5 ml organ-bath containing Krebs-Henseleit solution maintained at $37^\circ$C and bubbled with 95 per cent oxygen and 5 per cent CO$_2$. When suspended the strips measured 2 - 3 cm by 2 - 3 mm; the rings had about the same gross dimensions.
Arrangements were such that two vein preparations could be mounted in the bath simultaneously, to provide a comparison of the two types of vein preparations exposed to identical drug concentrations. The veins were suspended in the bath for at least 30 minutes before exposing them to drugs, all doses of which were added in 0.2 ml saline. Results will be seen on Table 15, page 107.

(4) Guinea-pig ureter

Preparation.- Ureters were dissected from freshly killed guinea-pigs and placed in Tyrode solution. Connective tissues were removed by fine dissection. The ureters were then suspended in an isolated organ bath of capacity of 5 ml. The renal end was tied to the bottom of the hook in the bath, and the distal end was attached to the writing lever under a tension of 0.25 g. The bath was bubbled gently with O₂ and maintained at 37°C.

Results.- The preparation contracted to histamine 0.1 μg/ml, acetylcholine 0.1 μg/ml but not to 5-hydroxytryptamine 2 μg/ml, bradykinin 0.5 μg/ml and SRS-A (alumina) 6 units/ml. It gave inconsistent responses to histamine and ACh, sometimes failing to contract to a dose which at other times caused a strong contraction, as if obeying an all-or-none law sometimes giving maximal contraction.
(5) Guinea-pig gall bladder

The gall bladder was removed and placed in Tyrode solution. Attached liver tissues were removed by fine dissection. The common cystic duct was attached to the hook at the bottom of the bath and the apex of the preparation was attached to the writing lever. Tyrode solution was maintained at 37°C. The bath was bubbled gently with oxygen.

Results.— The tissue gave very small contractions to histamine 2 μg/ml, acetylcholine 50 μg/ml, bradykinin 0.2 - 0.4 μg/ml, but did not contract to 5HT up to 100 μg/ml. It contracted to purified SRS-A (G-50, 20 units: alumina, 30 units) contained in 0.5 μg of the freeze dried eluate.

(6) Guinea-pig bladder

Male and female guinea-pigs were killed by a blow on the head. The lower abdomen was opened along the midline to expose the bladder. A thread was tied around the urethra for attaching the bladder in the organ bath. Another thread was sewn through the vertex of the bladder for attaching the preparation to the writing lever which exerted a pull of 1.0 g and gave a magnification of 6:1. The bladder was set up in a 5 ml bath containing McEwan's solution (1956), the formula of which is given in the Appendix. The bath was aerated with 95 per cent O₂ and 5 per cent CO₂.
Results.— Acetylcholine 0.1 – 0.2 μg/ml caused a fairly rapid contraction (without latency) of the preparation. Bradykinin 0.5 μg/ml and 5 HT 4 μg/ml caused slow contractions, taking one minute to develop fully. The latency of these contractions was on average 10 secs. Histamine produced contractions at fairly high doses (10 – 20 μg/ml). Crude perfusates produced contractions only before the addition of mepyramine. SRS-A (G-50 eluate) 10 units did not produce any contractions. Higher doses (40 units) also did not affect the preparation.

(7) Guinea-pig heart

Preparation.— The animals were killed by a blow on the head, and the heart still beating was rapidly dissected out (with the lungs) and quickly placed in Locke's solution which had previously been gassed with a mixture of 95 per cent O₂ and CO₂ and chilled. The extraneous tissues were then quickly dissected off, leaving only the heart and the major vessels. The isolated heart was perfused by the Langendorf method with oxygenated Locke's solution at a constant pressure and a constant temperature below 37°C. The fluid passed into the aorta, through the coronary vessels and escaped from the inferior vena cava. Heart rate and coronary flow were continuously recorded.

Results.— The preparation responded to adrenaline 0.5 μg – increased heart beat; noradrenaline 0.5 μg, increased heart beat; isoprenaline 0.25 μg – coronary constriction; histamine 2, 4 and 10 μg; acetylcholine 5 μg – slowing of heart beat; SRS-A purified by alumina 20 and 40 units – variable response. After atropine and
mepyramine the responses to SRS-A were variable, nevertheless there was clearly constriction of the coronary vessels and an increase in amplitude of the beat. This was particularly marked for the SRS-A obtained from alumina.

(8) Guinea-pig tracheal chain preparations

A chain of 6 to 8 rings was cut from the trachea and linked together, as described by Castillo and de Beer (1947). Tissue from a guinea-pig of 400 - 600 g was used and suspended in Krebs-Henseleit solution at 37°C. Both the organ-bath and the reservoir were aerated with 95 per cent O₂ + 5 per cent CO₂. Bath fluids were replaced by displacement from below, using twice the volume necessary to fill the bath, and permitting the excess to overflow, since exposure to the air during draining of the bath appeared to disturb the degree of relaxation of the tissues. A very light frontal writing lever gave a magnification of about 15:1, and was vibrated to reduce friction. The pull on the tissue was 0.2 g.

Results. Very good contractions were produced by histamine, 1 - 2 μg/ml. In the presence of atropine 10⁻⁷ M and mepyramine 10⁻⁸ M, the chains did not contract to SRS-A (10 units/ml) purified by either method and obtained from both guinea-pig lung and cat's paws. The chain
preparation did contract to high concentrations of crude perfusates (1 in 5 dilution = 20 units/ml) and also to alcohol extracts 20 units/ml and very high doses of purified SRS-A 40 units/ml which may have contained undetermined salts.

(9) Rat Uterus

Preparation.- Uterine horns were taken from virgin rats in natural oestrus or pretreated with stilboestrol in arachis oil injected subcutaneously (10 μg/100 g body weight) 18 - 20 hours before use. A length of about 2 cm was suspended in a 2 ml organ bath containing oxygenated de Jalón's solution at 30° - 31°C. The de Jalón solution contained 10⁻³ g/ml atropine sulphate, and later 10⁻⁵ g/ml mepyramine was added. The lever gave a magnification of 6:1 and a pull on the tissue of 0.5 g. Active substances were in contact for 1 ½ to 2 minutes and the resting period of 5 minutes between doses included three changes of the bath fluid.

Results.- Quiescent tissue which had been contracting strongly to bradykinin 1 ng/ml or 5 HT 5 ng/ml was unresponsive to histamine 1 μg/ml, perfusate 1 in 10 dilution, or SRS-A 20 units/ml purified by both G-50 Sephadex method or alumina method.

(10) Rat Duodenum

Preparation.- The proximal 2 - 3 cm of duodenum from albino rats of 120 - 150 g body weight was suspended in de Jalón solution at 30 - 31°C in an organ bath.
The tissue had been stored at 4°C for 2 - 4 hours previously. The lever used exerted a tension of 0.5 g on the tissue and magnified the response about 15 times. In these experiments, the overflow technique of washing the tissue was used because this avoided unwanted stimuli leading to fluctuations in resting tonus.

**Results.**— The tissue contracted to acetylcholine 10 ng/ml, contracted to 5 HT 50 ng/ml, and relaxed to bradykinin 2 ng/ml, but gave no responses to histamine 10 µg/ml and SRS-A 10 units/ml.

(11) **Rat colon**

**Preparation.**— This was fundamentally as described by Toh (1952) and used by Brocklehurst (1955) and Chakravarty (1959) in their experiments. It consisted of the spirally striated section of the upper colon, suspended in de Jalon solution. The lever gave a magnification of 6:1 and exerted a pull of 1.0 g on the tissue. Oxygen was gently bubbled through the bath and the temperature was maintained at 32°C. The bath fluid was changed by displacement from below, since the tissue becomes unsteady if exposed to the air. Active substances were left in contact with the tissue for 30 - 40 seconds and there was a washing and rest period of 2 minutes between doses.

**Results.**— The tissue contracted to acetylcholine 20 ng/ml, 5 HT, 50 ng/ml, relaxed to bradykinin 4 ng/ml, Substance P, 2 units/ml, but gave no response to histamine 1 µg and SRS-A 20 units/ml purified by the Sephadex technique.

(12) **Rabbit ileum**

**Preparation.**— The rabbit was killed by a blow which
dislocated the neck. The sample of duodenum was taken about 12 inches from the stomach, washed, and set up in a 5 ml bath. The bathing fluid was Tyrode solution at 37°C, with oxygen slowly bubbled, and was replaced at 4 to 5 minute intervals. The lever exerted a pull of 1.0 g and gave a magnification of 6:1.

Results.—A positive response (consisting of an increase in the amplitude of rhythmic activity, and usually accompanied by a shortening of the muscle (when relaxed) was observed with each of the following substances:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetylcholine</td>
<td>0.2 – 0.4 µg/ml</td>
</tr>
<tr>
<td>substance P</td>
<td>10 – 50 µg/ml</td>
</tr>
<tr>
<td>5 HT</td>
<td>0.2 – 0.4 µg/ml</td>
</tr>
<tr>
<td>bradykinin</td>
<td>1.0 – 2.0 µg/ml</td>
</tr>
<tr>
<td>histamine</td>
<td>1.0 – 2.0 µg/ml</td>
</tr>
<tr>
<td>SRS-A (crude perfusate)</td>
<td>1 in 10 dilution, 2 units/ml</td>
</tr>
<tr>
<td>SRS-A (acetone precipitate)</td>
<td>1 in 20 dilution, 3 units/ml</td>
</tr>
<tr>
<td>SRS-A (alcoholic extract)</td>
<td>1 in 20 dilution, 2.5 units/ml</td>
</tr>
</tbody>
</table>

There was no response to:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRS-A (0-50 eluate)</td>
<td>0.5 – 0.8 µg/ml, 7.0 units/ml</td>
</tr>
<tr>
<td>SRS-A (alumina eluate)</td>
<td>0.3 – 0.7 µg/ml, 10 units/ml</td>
</tr>
</tbody>
</table>

The introduction of mepyramine 10⁻³ g/ml caused the crude perfusate to produce slower contractions, the alcoholic extracts behaved similarly, but the purified materials did not contract this tissue. At no time
did the tissue become tachyphylactic to the other spasmodgens as was observed by Uvnäs (1962) and Anggard et al. (1963).

(13) Rabbit heart

The organ was set up as described for the guinea-pig (7).

Results.— An increase in amplitude was observed but the rate and outflow remained unchanged. This was consistently seen in four preparations. Dose:
adrenaline 0.5 μg, noradrenaline 0.5 μg, isoprenaline 0.25 μg, carbachol 5 and 10 μg and SRS-A (alumina purified) 20 units.

(14) Rabbit uterus

Preparation.— About 2 cm of one uterine horn was removed from young non-pregnant rabbits. The tissue was set up in a 2 ml bath and bathed in either Tyrode or Krebs Henseleit solution at 37°C. The tissues bathed in Tyrode solution were more quiscent than those bathed in Krebs. The lever gave a magnification of 6:1, and exerted a pull of 0.5 g on the tissue.

Results.— There was no contraction produced on application of purified SRS-A 4 units/ml, although the preparation responded to acetylcholine 0.2 μg/ml, carbachol 0.1 μg/ml, 5-hydroxytryptamine 0.4 μg/ml.

(15) Hamster colon

This tissue was used by Ambache (1959, 1963) to differentiate irin (a lipid substance obtained from the iris of rabbits) from many other pharmacologically
active substances. From his findings he noted that the tissue was insensitive to histamine, and 5HT, whereas acetylcholine was very active on the tissue. Darmstoff and bradykinin responses were comparable with those of irin on the tissue. Brocklehurst (personal unpublished communication) used this tissue to differentiate SRS-A from SRS-S (prepared from swine lungs at the Merck Institute, and now identified as a prostaglandin) using various physiological solutions to bathe the tissue.

Preparation. Animals weighing between 120 and 160 g were killed by dislocation of the neck. The diagonally banded ascending colon was removed and washed, and 2 cm of this tissue was suspended in a 2 ml bath maintained at 34°C. The lever gave a magnification of 6:1 and exerted a pull of 0.5. The tissue was found to respond very well to drugs when bathed in either Tyrode or Page's (1955) solutions. Tyrode solution was used routinely, and the bath fluid was changed by the "overflow" method.

Result. Tissue does not contract to histamine, 2 µg/ml or Substance P, 5 units/ml. It is very sensitive to acetylcholine, 0.1 µg/ml, irin, 5 units/ml, darmstoff, 200 E.D.S, but weakly sensitive to crude SRS-A after atropine and mepyramine has been added, but not responsive to purified SRS-A (G-50 Sephadex) 20 units/ml.

(16) Hamster ileum

This preparation has not been used before.
The tissue was set up as described above, simply using the terminal ileum instead of the colon.

Results.— The tissue does not contract to 5 HT (10 μg/ml) and purified SRS-A (alumina 40 units/ml) but contracted to the acetylcholine 10 ng/ml, histamine 2 μg/ml, Substance P 5 units/ml, bradykinin 0.5 μg/ml, Irin 5.0 units/ml and concentrated 40% ethanol eluates obtained from alumina column.

This preparation has been described in detail by Goodall (1951) and a modification of this method has been employed. The organ was taken from young cockerels of 700 - 800 g and set up in Tyrode solution at 39°C with oxygen slowly bubbled through the bath. The lever gave a magnification of 10:1, and exerted a pull of 1.0 g. Oxygen was found to be preferable to O₂ - CO₂ mixture as used by Goodall (1951), since better relaxation of the tissue resulted.

Results.— In later experiments the tissue behaved very well. The tissue contracts well to Ach 10 ng/ml and substance P 10 μg/ml (8.36 units/mg). A very small response was obtained for histamine 1.0 μg/ml and a fairly large one for the crude perfusate 2.5 units/ml, before and after mepyramine, however the purified material did not contract the tissue at a dose level of 20 units/ml.

(18) Chick crop

This preparation is as described by Everett and Bowman (1964).

Preparation.— Chicks (silver link) 4 to 7 days old were starved overnight to empty the crop and then killed.
The crop was removed and placed in a petri dish containing Krebs Henseleit solution at room temperature. It was opened by a longitudinal incision in the oesophagus which continued into the crop and a strip of tissue about 3 mm wide was then cut from the middle of the opened crop at 90° to the first incision. A strip about 2 cm long was suspended in a 1 ml bath containing Krebs Henseleit solution at 32°C bubbled with O₂ - CO₂ mixture. An isotonic frontal writing lever gave a 12:1 magnification and exerted a tension of 4 g on the tissue. An interval of 30 - 60 minutes was allowed before beginning the pharmacological studies and during this time the muscle relaxed maximally. Drugs were applied at intervals of 6 - 9 min. and contact time was 2½ min. for SRS-A and 1 min. for the other drugs.

**Results.**—The tissue contracted well to 5HT 1.0 ng/ml, histamine 10.0 ng/ml, acetylcholine 10.0 ng/ml and crude perfusate 4 units/ml. The contraction to SRS-A was weak as compared with the response obtained on the guinea-pig ileum. When antagonists were used, mepyramine 10.0 ng/ml, atropine 10.0 ng/ml, and BOL 1 - 5 μg/ml, the responses to histamine, acetylcholine and 5HT were abolished. Doses up to 1.0 μg/ml 50 units of 0-50 SRS-A gave only a minimal contraction. The SRS-A from the cat's paw gave better contraction when 25 units/ml was used.
(19) **Chick oesophagus**

Chicks (Silver link) of 4-7 days old were starved overnight as was done for those of the crop preparation and then killed. The oesophagus was exposed together with the jugular veins. Gentle traction was applied in dissecting out the oesophagus, which was then placed in a petri dish containing Krebs Henseleit. A longitudinal incision was made from the upper part and a strip of tissue about 3 mm wide was cut from the middle of the opened oesophagus. A strip about 2 cm long was suspended in a 2 ml bath containing Krebs Henseleit solution at 32°C bubbled with $O_2 - CO_2$ mixture. An isotonic frontal writing lever gave a 12:1 magnification and exerted a tension of 4 g on the tissue. An interval of 60 minutes was allowed before beginning the pharmacological tests and during this time the muscle relaxed maximally.

Drugs were applied at intervals of 6 minutes and contact time was 2 minutes for SRS-A and crude perfusate and 1 minute for the other drugs. Spasmogens and antagonists used were the same as for crop.

**Results.**—The tissue contracted well to 5HT 5 ng/ml, acetylcholine 20 ng/ml and small contractions to histamine, 50.0 ng/ml, and crude perfusate, 10 units/ml. The contraction of crude perfusate was completely blocked by mepyramine, 10 ng/ml. Purified SRS-A /guinea-pig G-50 did not contract the tissue at 25 units/ml.
(20) Frog rectum

The frog was pithed and the whole rectum set up under a tension of 0.1 g and with a lever magnification of 12:1. A mixture of Tyrode solution two parts, with distilled water one part, was used as bath fluid with oxygen slowly bubbled and at 37°C. Under these conditions the tissue relaxed and no spontaneous activity was observed.

Results.-- Histamine 0.5 mg/ml was inactive but crude perfusate 2.5 units/ml and alumina SRS-A 40 units/ml produced very weak contractions which were maintained for a period of 60 - 90 seconds. The contraction was unaffected by the addition of mepyramine. No 0-50 purified SRS-A was available for testing.

(21) Swine splenic artery

Preparation.-- The method of Furchgott (1960) was used. The whole pluck of the swine was obtained from the slaughter house fresh, and the artery excised and placed in chilled Krebs Henseleit solution. Rings 0.4 cm wide were cut from the arteries and assembled as a chain, or alternatively, an artery of about 1.5 cm circumference was cut helically with sharp scissors to give a strip about 0.4 x 5 cm. The preparation was set up in oxygenated Krebs Henseleit solution at 37°C, the lever exerting 3 to 4 g tension and giving about 10:1 magnification. The response of this tissue was detected
by recording semi-isometrically so that the pre-loading of 4 g increased only very slightly for 2–5 per cent shortening. The tissue was washed every 3 minutes for 30 minutes before the addition of any drugs.

Noradrenaline 2 ng/ml and adrenaline 2 ng/ml caused contractions of the preparation, histamine 20 ng/ml relaxed it, SRS-A purified by both G-50 Sephadex 50 units/ml and alumina 30 units/ml relaxed the preparation. 5 HT 50 ng/ml and vasopressin 0.2 units/ml relaxed the preparation.

(22) Human bronchioles

Material. - The material was obtained as fresh as possible from the operating theatre and placed in previously aerated Krebs (1950) solution at 5°C as used by Brocklehurst (1955). It was sometimes stored in a large volume of fluid at 4°C overnight and the improvement in response which often followed this procedure was attributed to washing out of the anaesthetic and substances used in premedication. Tissue removed from young patients for bronchiectasis was sometimes used, but some atrophy was usually present.
Preparation.- Bronchioles of about 3 mm in diameter (4th branching) were found to be the most reactive, those of diameter 5 to 10 mm were less reactive, possibly due to the thickness of the muscle. Responses were never obtained from bronchioles of diameter less than 2 mm when relaxed. Chain of 4 to 8 rings were set up as described by Hawkins et al. (1951). A very light lever exerted a pull of 0.2 g and gave a magnification of 15:1: it was gently vibrated to reduce friction.

Results.- The preparation contracted strongly to histamine at concentrations of 0.1 µg/ml and acetylcholine at 0.5 µg/ml. Perfusate produced contractions which were sometimes greater than those produced on the ileum using an equivalent concentration in the bath. Mepyramine 10⁻⁶ M usually abolished contractions to histamine whereas those to perfusate were reduced less than 50 per cent. The purified materials (G-50 Sephadex 2 units/ml, alumina 3 units/ml) showed an overall increase of activity when compared with the crude perfusate. This increase was about three-fold, as had already been observed in tests using the guinea-pig ileum. (See page 85).
(23) **Cat tracheal muscle preparation**

(a) **Tracheal strip**

**Preparation**.- The trachea was cut into "rings" and the cartilage cut through on opposite sides. The cartilage was then used for the attachment of threads leading to the fixed support in the bath and the lever, leaving the circular smooth muscle strip extended between the two ends of the cartilage. Two such strips joined lengthwise, were suspended in Krebs-Henseleit solution in a 10 ml bath as described for the guinea-pig tracheal chain preparation. The lever was vibrated, and gave a magnification of about 6:1. It exerted a pull of 0.2 to 0.5 g on the tissue.

**Results.**- The tissue contracted vigorously to Ach (1 μg/ml), and to 5-HT (about 0.02 μg/ml). It gave a very small response to 40 μg/ml histamine which was abolished by mepyramine (1 μg/ml). After the addition of atropine (0.1 μg/ml) and mepyramine (1 μg/ml) both alcoholic extract of perfusate (20 units/ml) and alumina purified SRS-A 50 units contracted the preparation. The contraction was maintained for a period of 10 minutes.

(As illustrated in Figs. 34 (a) and (b))
Cardiovascular system

Results

The in vivo experiments were performed on rats of 200 to 300 g and guinea-pigs of 600 to 900 g. The animals were anaesthetised with urethane, 25 per cent aqueous solution was given 0.7 ml/100 g subcutaneously 45 min. before the experiment. Cannulae were inserted in the trachea, and the carotid artery; usually the jugular vein was preferred to the femoral. The animals were held with their necks supported from below to facilitate easy cannulation. Arterial and venous pressures were recorded by small displacement pressure transducers (Devices Type 4-326-L212). The intrathoracic pressure was recorded by making a small incision between the lower ribs well away from the sternum and introducing a 2 mm diameter polythene tube with the end open and 3 lateral holes near the end to avoid possible block (as seen in Fig. 37). Some saline was allowed to run through to prevent too much air entering the thorax and this was withdrawn when the manometer was coupled to the tube, so that the lung was re-expanded and the system was under slight negative pressure (about 2 mm of water). Changes in pressure were recorded by a Greer low pressure manometer. All the recording appliances were connected to the Devices recorder (Type M 4/62) giving a linear record.

The rectal temperature was maintained at approximately 37°C by means of heating lamps on the operating
Intravenous injections were made through a plastic cannula inserted into a jugular vein or the femoral vein. The results obtained are shown on Tables 16 and 17.

(25) Respiratory system

In the experiments where bronchial resistance was of particular interest, a modification of the Konsett and Rossler (1940) technique was used. The lungs were inflated by a Palmer small animal respiration pump driving a constant volume of air into the trachea for each stroke. The maximum ventilation pressure was regulated to 5 - 7 cm of water. The excess air, which did not enter the trachea, was directed to a closed cylinder, of about 500 ml capacity. Pressure rises in the cylinder during bronchoconstriction were small but could be recorded.

Results.- The results obtained are shown on Tables 16, 17. The intravenous injection of up to 4 µg of histamine into both rat and guinea-pig caused a fall in the arterial blood pressure. 0.3 µg of noradrenaline caused a rise in blood pressure, but no apparent effect on the bronchial resistance. Bradykinin 20 µg, 5-HT 2 µg caused falls in arterial blood pressure. In the rat SRS-A (GP)* 50 units and SRS-A (CP)* 20 units

*SRS-A (GP) means the slow reacting substance obtained from guinea-pig lungs; SRS-A (CP) means the slow reacting substance obtained from perfused cat paws.
(G-50 purified) and SRS-A (GP) 60 units (alumina purified) all caused prolonged falls in arterial blood pressure and a rise in the venous pressure. The effect of these doses on the bronchial resistance was very marked and it lasted for well over 4 min., compared with that exhibited by histamine it took three times as long to recover. In the guinea-pig 40 units SRS-A (GP), 25 units SRS-A (CP) (G-50 purified) and 60 units SRS-A (GP) (alumina purified) a fall in arterial blood pressure was observed, followed by a slight rise in venous pressure, but the effect on the bronchial resistance of the guinea-pig to these doses were not as pronounced as those seen in the rat.

These effects of both types of SRS-A (i.e. GP, CP) on the blood pressure and bronchiolar resistance were observed (even after mepyramine 2.0 mg/kg had been added). On autopsy of four of the rats, gross oedema of the visceral tissues was observed, especially in the small intestine and the spleen. The guinea-pigs did not show any of this damage seen in rats.

(26) Changes of vascular permeability in the skin.

This was performed on both guinea-pigs and rats. The abdomens of the albino guinea-pigs and rats were clipped closely or shaved and the doses of neutral solutions of the test substances, diluted in Tyrode solution were injected intradermally. The volume of
all intradermal injections was 0.05 ml nominally and disposable sterile tuberculin syringes were found to be quite leak-proof and sufficiently accurate for this purpose (Colquhoun, 1964). Short bevel 26 gauge needles were used. The materials tested were histamine, 5-hydroxytryptamine, compound 48/80 and SRS-A purified on G-50 Sephadex the doses of which are shown on Tables 18, 19. The animals were pre-treated with an intravenous injection of 5 per cent pontamine sky blue solution 1.5 ml/kg, given approximately 10 min. before the intradermal injections. Guinea-pigs were injected into the dorsal vein of the penis, rats into the tail vein. Each test substance was given at two dose levels on the same animal, and the sites were randomised through the group of animals.

Results.— These are shown in Tables 18 and 19. Increased permeability was assessed by the blueing reaction.
TABLE 15
Summary of pharmacological effects of drugs on guinea-pig veins.

<table>
<thead>
<tr>
<th>Drug</th>
<th>External jugular vein</th>
<th>Posterior vena cava</th>
<th>Anterior mesenteric vein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine 0.1 μg/ml</td>
<td>0.1 μg/ml (+)</td>
<td>0.1 μg/ml (+)</td>
<td>0.1 μg/ml (+)</td>
</tr>
<tr>
<td>Adrenaline 10.0 pg/ml</td>
<td>10.0 pg/ml (+)</td>
<td>0.1 μg/ml (+)</td>
<td>0.1 μg/ml (+)</td>
</tr>
<tr>
<td>Noradrenaline 10.0 pg/ml</td>
<td>10.0 pg/ml (+)</td>
<td>0.1 μg/ml (+)</td>
<td>0.1 μg/ml (+)</td>
</tr>
<tr>
<td>Isoprenaline 10.0 pg/ml</td>
<td>10.0 pg/ml (-)</td>
<td>0.1 μg/ml (+)</td>
<td>1.0 μg/ml (+)</td>
</tr>
<tr>
<td>Acetylcholine 10.0 pg/ml</td>
<td>10.0 pg/ml (+)</td>
<td>0.1 μg/ml (+)</td>
<td>0.1 μg/ml (+)</td>
</tr>
<tr>
<td>Crude perfusate 10 units/ml</td>
<td>10 units/ml (+)</td>
<td>10 units/ml (+)</td>
<td>10 units/ml (-)</td>
</tr>
<tr>
<td>Purified SRS-A 0-50</td>
<td>Not tested</td>
<td>3 units/ml (+)</td>
<td>10 units/ml (-)</td>
</tr>
<tr>
<td>Guinea-pig lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alumina 5 units/ml</td>
<td>5 units/ml (+)</td>
<td>5 units/ml (+)</td>
<td></td>
</tr>
<tr>
<td>5HT 10.0 μg/ml</td>
<td>10.0 μg/ml (-)</td>
<td>0.1 μg/ml (+)</td>
<td></td>
</tr>
<tr>
<td>Bradykinin 1.0 μg/ml</td>
<td>0.1 μg/ml (+)</td>
<td>0.1 μg/ml (+)</td>
<td></td>
</tr>
</tbody>
</table>

+ = weak contraction
++ = strong contraction
- = no response
TABLE 16

Effect of purified SRS-A (alumina) and other drugs on the cardiovascular and respiratory systems.

GUINEA-PIG

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (37°C)</td>
<td>0.2 ml</td>
<td>Slight fall</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Histamine</td>
<td>2.0 µg</td>
<td>Fall*</td>
<td>Rise</td>
<td>Slight increase (+)</td>
</tr>
<tr>
<td>Histamine</td>
<td>4.0 µg</td>
<td>Increased fall*</td>
<td>Rise</td>
<td>Increase followed by a fall (++)</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>0.2 µg</td>
<td>Rise</td>
<td>Nil</td>
<td>Relaxation</td>
</tr>
<tr>
<td>Alumina eluates 80% ethanol concentrate</td>
<td>0.4 ml</td>
<td>Fall*</td>
<td>Nil</td>
<td>Increase (++)</td>
</tr>
<tr>
<td>70% - 30% ethanol, alumina eluates concentrates</td>
<td>0.4 ml</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>20% ethanol, alumina eluates concentrates</td>
<td>0.4 ml</td>
<td>Fall</td>
<td>Rise</td>
<td>Increase (+++</td>
</tr>
</tbody>
</table>

(+*) = Slight increase
(++) = Real increase
(+++) = Increase and tendency to convulse

*Blocked by mepyramine.
TABLE 17

Effect of purified SRS-A and other drugs on the cardiovascular and respiratory systems.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine</td>
<td>3 µg</td>
<td>Fall</td>
<td>Rise</td>
<td>Increase (++)</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>20 µg</td>
<td>Fall*</td>
<td>-</td>
<td>Slight increase (+)</td>
</tr>
<tr>
<td>SRS-A (GP) Sephadex G-50</td>
<td>50 units</td>
<td>Fall²</td>
<td>Rise</td>
<td>Increase (+++)</td>
</tr>
<tr>
<td>SRS-A (GP) (Alumina)</td>
<td>60 units</td>
<td>Fall²</td>
<td>Rise</td>
<td>Increase (+++)</td>
</tr>
</tbody>
</table>

(+) = Slight increase

(++) = Real increase

(+++) = Increase and tendency to convulse

*= Not blocked by mepyramine.
### TABLE 18

**Skin reactions (guinea-pigs)**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dose (given in 0.05 ml)</th>
<th>Area ((\text{cm}^2))</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Product of diameter)</td>
<td></td>
</tr>
<tr>
<td><strong>Histamine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5µg</td>
<td>0.35</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.70</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6.0µg</td>
<td>0.25</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trace</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trace</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><strong>5-HT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1600µg</td>
<td>0.31</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.70</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td>100ng</td>
<td>Trace</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trace</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><strong>U8/80</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>400ng</td>
<td>0.16</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trace</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td><strong>SRS-A</strong> (Purified sample on G-50 Sephadex)</td>
<td>3 units</td>
<td>1.08</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 units</td>
<td>0.5</td>
<td>+ + +</td>
</tr>
<tr>
<td><strong>Tyrode</strong></td>
<td>0.05 ml</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
### TABLE 19

**Skin reactions (rats)**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dose (given in 0.05 ml)</th>
<th>Area (a \cdot d) (product of diameter)</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histamine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25(\mu g)</td>
<td>Trace</td>
<td>.42</td>
<td>++</td>
</tr>
<tr>
<td>100(\mu g)</td>
<td>Trace</td>
<td>.14</td>
<td>+</td>
</tr>
<tr>
<td><strong>5 HT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6(\mu g)</td>
<td>0.5</td>
<td>0.35</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Trace</td>
<td>Trace</td>
<td>+</td>
</tr>
<tr>
<td>25(\mu g)</td>
<td>0.55</td>
<td>0.48</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Compound</strong></td>
<td>6(\mu g)</td>
<td>0.12</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Trace</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td><strong>48/80</strong></td>
<td>25(\mu g)</td>
<td>0.08</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Trace</td>
<td>Trace</td>
<td>+</td>
</tr>
<tr>
<td><strong>SRS-A</strong></td>
<td>3 units</td>
<td>Trace</td>
<td>+</td>
</tr>
<tr>
<td>(G-50 purified)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 units</td>
<td>0.45</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>0.60</td>
<td>0.20</td>
<td>++</td>
</tr>
<tr>
<td><strong>Tyrode control</strong></td>
<td>0.05 ml</td>
<td>Trace</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 19  Pharmacological effects of SRS-A, crude perfusate and other substances on isolated gall bladder of the guinea-pig bathed in oxygenated Tyrode solution at 37°C. Mepyramine 10^-6 M and atropine 5 x 10^-8 M added when required. P = Crude Perfusate.
Fig. 20 Pharmacological effects of SRS-A and other substances on isolated uterus of stilboestrol treated rat bathed in oxygenated de Jalon solution containing atropine $5 \times 10^{-5} \text{M}$ and mepyramine $10^{-6}$ at $31^\circ \text{C}$. Bath volume 2.5 ml.

Fig. 21 Pharmacological effects of SRS-A and other substances on isolated uterus of virgin guinea-pig bathed in oxygenated de Jalon solution containing atropine $5 \times 10^{-5} \text{M}$ and mepyramine $10^{-6}$ at $31^\circ \text{C}$. Bath volume 2.5 ml.
Fig. 20

Fig. 21
Fig. 22  Effects of purified SRS-A, crude perfusate obtained from sensitised guinea-pig lung on isolated human bronchioles bathed with Krebs solution (1950). Aerated with O₂/CO₂ mixture at 37°C.

Fig. 23  Effects of purified SRS-A, histamine and crude perfusate obtained from sensitised guinea-pig lungs on isolated guinea-pig tracheal chain preparation bathed in Krebs Henseleit solution. Aerated with O₂/CO₂ at 37°C.
MEPYRAMINE $10^{-6}$ M

Fig. 22

H = Histamine
P = Perfusate
AE = Alcoholic extract

Fig. 23
THE EFFECT OF PURIFIED SRS-A AND OTHER SUBSTS ON HUMAN BRONCHIOLES.

P = Perfusate
H = Histamine

0.2 ml P  20 μg H Mepyrr. 10^{-6} M.

40 μg H  0.2 ml P  0.1 ml SRS-A  0.2 ml SRS-A

0.4 ml P  20 μg Sub P 0.6 μg Bk 0.6 ml SRS-A (15 min)

Tissue from healthy lobe removed for carcinoma of the bronchus.
Eight rings cut from bronchioles 2.5 to 3.5 mm diameter
Suspended in 5 ml Krebs (1950) solution aerated with O_2 CO_2 at 37°C.
Atropine 10^{-9} g/ml present throughout.
Vertical lines denote change of bath fluid. Contact time five minutes.
0.1 ml SRS-A = 2 units biological activity
0.1 ml P = 8 units biological activity
Fig. 25(a) Effects of SRS-A and other drugs on the isolated perfused heart of guinea-pig. Perfusion fluid: oxygenated Locke solution at 37°C.

Fig. 25(b) Effects of SRS-A and other drugs on the isolated perfused heart of guinea-pig. Perfusion fluid: oxygenated Locke solution at 37°C.
Fig. 26 The effects of purified SRS-A, crude perfusate and other substances on isolated rat bladder bathed in McEwan's solution (vide Appendix), aerated with O₂/CO₂ mixture at 37°C. Atropine 10⁻⁶M, mepyramine 10⁻⁶ were added when required.

Fig. 27 The effects of purified SRS-A and other substances on isolated rat duodenum bathed in de Jalon solution at 31°C.
Figs. 28 and 29 Responses of rabbit jejunum to crude perfusate (CP), partially purified SRS-A (AE), purified SRS-A and acetone precipitated (AP). Perfusate bathed in Tyrode solution at 37°C bubbled with O₂.

CP 2 units/ml, AP 3 units/ml, AE 2.5 units/ml, SRS-A (G-50) 7.0 units/ml, SRS-A (alumina) 10 units/ml, histamine 0.5 μg/ml. Volume of bath = 5 mls.
Fig. 28

Fig. 29
Fig. 30 and 31 Responses of rabbit jejunum to crude perfusate (CP), partially purified SRS-A (AE), purified SRS-A, acetone precipitated (AP), bradykinin (Bk, 5 μg/ml), histamine (H, 0.5 μg/ml) and Substance P (10 μg/ml).

AP = 3 units/ml, AE = 2.5 units/ml.
Volume of bath = 5 mls.
Fig. 30

Fig. 31
Fig. 32 (a) and (b) Effects of injection of SRS-A, concentrated eluates obtained from alumina columns and other substances on the isolated perfused heart of the rabbit. Perfusion fluid is aerated Locke solution at 37°C.
Fig. 33  Comparison of pharmacological effects of SRS-A and other substances on isolated guinea-pig bladder bathed in McEwan's solution (vide Appendix) at 37°C and aerated with O₂/CO₂ mixture. Atropine 10⁻⁶M and mepyramine 10⁻⁶M were added as required.

Fig. 34  (a) and (b) Effects of SRS-A and other pharmacologically active substances on isolated cat tracheal strip preparation bathed in Krebs-Henseleit solution aerated with O₂/CO₂ mixture at 37°C.
Fig. 33

Fig. 34 (a)

Fig. 34 (b)
Fig. 35  Effect of purified SRS-A (G-50 Sephadex) on isolated splenic artery of swine bathed in oxygenated Krebs-Henseleit solution at 37°C.
Fig. 36 Effects of SRS-A, perfusate and other substances on isolated frog rectum bathed in a mixture of Tyrode solution with distilled water (2:1) at 37°C and aerated with O₂.
Fig. 37 Diagram showing point of incision of polythene cannula 2 mm in diameter with an open end and three lateral holes for recording intrathoracic pressure in an anaesthetised rat (and guinea-pig).
Interpretation of records made on Devices M 4/62 pen recorder and transducers (as in Figures 38-41)

A. Upper record shows spontaneous respiration recorded as the flow of air in the tracheal cannula, as shown in the sketch as

```
Animal

\[ \text{open air} \]

\[ \text{flow monitor} \]

\[ \text{to} \]

'Greer' differential manometer
```

The system is a simple version of the Frick flow tube, connected to the two sides of a sensitive differential manometer. Inspiration is represented by the downward sweep, and the excursion indicates the actual velocity of air inflow, which gives some idea of the inspired volume, but no indication of respiratory effort.

B. = time in seconds

C. = carotid arterial pressure measured with a Statham type small displacement high frequency pressure transducer. Pressure increases are represented by upward shift of the trace. Individual pulse excursions are seen (when the recorder is speeded up) superimposed on the slower and larger changes produced by thoracic venous pressures (and therefore cardiac filling) during the respiratory cycle.

D. Where there is a fourth record, this usually represents the thoracic (or jugular) venous pressure, recorded as in (C), but with the transducer slightly below the point of cannulation, and the recorder operating at maximum sensitivity. Rises of pressure are indicated by upward deflection.

Records of intrathoracic pressure have also been made in a similar manner, using a polythene tube (Fig. 37) as cannula. Summary of results shown on p. 168.
SRS-A (alumina) 26.1.65 and 28.1.65 were later estimated to contain 80 units/ml and 50 units/ml respectively. These values were based on assays carried out using guinea-pig ileum as described in the Appendix (p. 170).
Fig. 39(a)

Fig. 39(b)

20 units SRS-A(G-50)

0.2 ml Saline
0.2 ml Saline  40 ug Hist  40 units SRS-A  0.02 mg Bk  60 ug Hist

Fig. 40

40 units SRS-A  0.02 mg Bk

Fig. 41
PART IV

CHEMISTRY
CHEMICAL ANALYTICAL STUDIES ON PURIFIED SRS-A

SRS-A is usually described in the literature as a lipid soluble acid and recently it has been purified using lipid solvents and lipid techniques (Anderson et al., 1963; Anggard et al., 1963). In 1962, Smith reported that the pharmacological activity of SRS-A purified by acid ether extraction was due to a mixture of compounds, each of which contained polyhydroxy-, amino and carboxylic acid groups. He arrived at this conclusion from chromatographic separation carried out on silicic acid columns using an elution sequence of rising concentrations of methanol in chloroform as devised by Hirsch and Ahrens (1958). Smith also chromatographed the acid hydrolysates of perfusates on paper and using specific tests, reported the presence of neuraminyl derivatives. He concluded from these findings that SRS-A was a neuraminyl derivative.

The main weakness in the work of Smith (see Anderson et al.) was the small amount of biological activity used for fractionation. The estimates of the biological potency of the most active samples of SRS-A made by others, vary between 0.5 μg per "unit" and 0.001 μg as a "detectable amount", and "100 units" must therefore be less than 0.1 mg, probably very much less. Amounts of this order are too small to permit quantitative
chemical tests, and is therefore probable that contaminating substances gave the results reported by Smith.

All other workers have provided "negative evidence" concerning SRS-A, by showing that it fails to produce effects in a wide range of tissues or is not destroyed by various enzymes. The enzyme tests are the more acceptable, because there must be doubt concerning any chemical or colour reaction which fails, especially when the quantity of material under test is not known. Further chemical data are vital to this study, and those obtained with material purified by new methods (described on pages 35, 51) and possibly containing few or at least different contaminants seemed to be important. The SRS-A obtained from the guinea-pig lung and also the SRS obtained from cat paws by purification on dextran gel and alumina were examined for homogeneity using chromatographical methods. These tests mostly involved colour reactions and tested for specific radicals or groups along the lines of previous work, e.g. Chakravarty (1960) and Anderson et al. (1963).

**Paper chromatography**

The first technique used was a modification of that used by Chakravarty (1960). Samples of (a) crude perfusate (5 mg dry wt), (b) material obtained from columns of G-50 Sephadex (1 mg dry wt) and (c) the concentrated eluates from columns of alumina (200 units),
were applied to a Whatman No. 1 paper, which had already been equilibrated with the solvent system consisting of n-propanol - ammonia - water (90:5:5). The chromatogram was run at 4°C for 24 hours using the ascending technique, after which the paper was dried, examined under ultra-violet light and later sprayed with a solution of bromothymol blue in Na₂CO₃ solution.

Some pale blue spots were observed at the solvent front when the chromatogram was examined under ultra-violet light, and on spraying with the indicator solution yellow spots on a blue background were observed at about Rf 0.6. On testing a parallel chromatogram the yellow acidic region contained the biological activity.

**Observations on the behaviour of purified 'SRS' on thin layer chromatography.**- 'Silica Gel G' and 'Silica Gel H' plates were prepared by methods described by Stahl (1956). Controls included normal guinea-pig lung, sensitised guinea-pig lung lipids and lipid fractions obtained from egg yolk as described in 1955 by Rhodes and Lea (see Appendix 2). Specific colorimetric tests were carried out for total phosphorus, cholesterol, glycerides and lipid phosphorus. The apparatus used was described by Vogel *et al.* (1962). A Desaga applicator spread silica gel G (E. Merck, Darmstadt, West Germany) at a known thickness (250 μ) on glass plates 20 cm x 20 cm. These were allowed to
dry, and then the silica gel was activated by heating for 1 - 2 hours, in an oven at 105°C. The known volumes of lipid were applied to the origin with a tuberculin syringe under a steady stream of oxygen-free nitrogen. Plates were chromatographed at 4°C for 4 hours in the appropriate solvent system. The following solvents systems were tried:

Solvents

(a) **Phospholipids**

(i) \( \text{CHCl}_3 : \text{MeOH} : \text{H}_2\text{O} \) (85 : 25 : 4)

(ii) di-isobutyl ketone : acetic acid : water
     
     (80 : 50 : 7)

(iii) chloroform : methanol : 7 N ammonium hydroxide
     
     (65 : 25 : 4)

(iv) n-propanol : ammonium hydroxide : water
     
     (90 : 5 : 5)

(v) chloroform : methanol : acetic acid : water
     
     (65 : 25 : 8 : 4).

(b) **Neutral Lipids**

(i) petroleum ether : diethyl ether : acetic acid
     
     (90 : 10 : 1 v/v)

(ii) n-propanol : ammonium hydroxide : water
     
     (90 : 5 : 5).

The best results were obtained with chloroform : methanol : water. After chromatography the lipids were detected by either placing the plates in a tank of iodine vapour,
when the lipids were stained brown after exposure for 1 min. (Randerath, 1963) or spraying with 50 per cent sulphuric acid and placing the plates in an oven at 220° for 20 min. to char all organic matter (as shown diagrammatically in Fig. 42). All the test samples, i.e. fractions of SRS-A from G-50 Sephadex and alumina columns gave the blue colouration, but not as intense as those of lung lipid extracts and egg phospholipids.

After several trials with various solvent systems, it was found that the addition of acetic acid (about 8% v/v) to the chloroform:methanol:water system gave far better resolution of the phosphatides and purified samples of SRS-A, than the chloroform: methanol:water mixture alone. This was the system adopted for use with silicic acid plates for the remainder of this work (Fig. 44). The plates were chromatographed as described earlier (p. 130) and sprayed with solution of phosphomolybdic acid, i.e. 10 per cent solution of ammonium molybdate in sulphuric acid. In order to investigate the correspondence of biological activity and chemically detectable material, duplicate plates were run; one serving for the chemical identification of the groupings present in purified SRS-A samples with separated egg phospholipids and one for biological tests. After spraying, the spots were allowed to develop by heating the plates at 80°C for 20 min. After ascertaining the sites of the spots,
pharmacological tests were carried out using scrapings from the corresponding band of the second plate. The activity was eluted with Tyrode solution and tested on isolated guinea-pig ileum.

SRS-A samples purified on alumina, and other alumina samples which had been subjected to a lipid solvent extraction procedure (Appendix, Section II) were investigated. Biological activity was obtained in Tyrode eluates from the origin of both the chloroform extracted and the treated alumina purified material. The active region gave little, if any, colour reaction for phosphatides, although with the chloroform extract spots giving positive reactions for phosphatides were present which had Rf values corresponding to sphingomyelin, phosphatidyl choline and a slight colour for phosphatidal ethanolamine. All these were found to be biologically inactive.

Summary

The present work leaves the chemical identity of SRS-A still unknown. Now that it is possible to move some of the contaminants from the biological activity and still retain the characteristic of the activity (i.e., slow contraction of the atropinised and mepyraminised guinea-pig ileum without tachyphylaxis) more work on its chemical identity is envisaged.
Fig. 42  Diagrammatic representation of chromoplate after treatment with iodine vapour or 50% v/v H₂SO₄.

Marker mixture:
- Lipophosphatidyl choline
- Sphingomyelin
- Phosphatidyl choline
- Phosphatidyl ethanolamine

$B =$ Chloroform extract of 80% ethanol wash

$B' =$ 80% ethanol wash

$C =$ Chloroform extract of 20% ethanol eluate (SRS-A fraction)

$C' =$ 20% ethanol eluate (SRS-A fraction)
AE = Alcoholic extract of freeze dried perfusate

Egg P = Egg phospholipid

Fig. 43 Chromatplate of SRS-A obtained from alumina column and alcoholic extract using n-Propanol: Ammonia: H₂O (90:5:5).
Origin - Biological activity in C and C'
1 = Polar phospholipids, 2 = Lysophosphatidyl choline, 
3 = Sphingomyelin, 4 = Phosphatidyl choline, 
5 = Phosphatidyl serine, 6 = Phosphatidyl ethanolamine. 
Solvent front - Neutral lipids if any.
Fig. 44 Chromatograph of different fractions obtained from an alumina column of 80% alcoholic extract of crude perfusate using CHCl₃:MeOH:Acetic acid:H₂O (65:25:8:4) as solvent.

EP = Crude egg phospholipid extract (CHCl₃/MeOH 1:1)
A = Chloroform extract of 80% ethanol effluent
A' = 80% ethanol effluent
B = Chloroform extract of 80% ethanol wash
B' = 80% ethanol wash
P = Methanol extract of purified egg phospholipids
C = Chloroform extract of 20% ethanol eluate (SRS-A fraction)
C' = 20% ethanol eluate (SRS-A fraction)
P' = Chloroform extract of purified egg phospholipid
PART V

DISCUSSION
DISCUSSION

This work was undertaken in the belief that SRS-A plays a wider role in allergic and inflammatory phenomena than had previously been shown. In order to demonstrate that SRS-A had a wider range of pharmacological activity it was essential to obtain a more purified product than had been used in earlier work. Recently developed methods of separation offered a reasonable chance of achieving greater purity, and it was hoped that the product might even be sufficiently pure to justify investigation of its chemistry. The criteria for a purification procedure (p. 1) give an indication of the difficulties previously encountered and the shortcomings of some earlier work.

The great instability of the SRS-A purified to a high degree by chromatography on cellulose columns could be attributed to peroxides in the organic solvents used or perhaps to free radicals from the ammonia or the cellulose. Alternatively the instability could have been the result of exposing the active substance to unsuitable pH or to agents which could react with it, as when acetic acid was used. It was also possible that the pure SRS-A was strongly adsorbed to glassware, or at worst that the activity was intrinsically unstable and that it was only stable in the crude form because it was protected by the presence of other substances.
The latter possibility could only be explored by using methods which as far as possible excluded the others. Some of the recently introduced methods of chromatography and especially the use of gel-filtration could be used without the use of organic solvents, and these were considered to be the first choice for trial. These methods also permit changes of the salt content of the product and it was hoped that gel filtration might give a product practically free from salt, which would be most suitable for chromatography on treated paper or thin layer silica gel. Furthermore if gel filtration was practicable, it would give an indication of the size of the SRS-A molecule which has so far defied evaluation except for its ability to pass through cellophane, which only limits the maximum to a molecular weight of about 20,000.

Gel-filtration of the crude perfusate on G-50 Sephadex using low ionic buffer solutions, left the SRS-A with the greater protein fraction as shown in Figure 4. The activity travels with this protein fraction in the exclusion volume of the column and has been separated from both the salt and the histamine content of the perfusate. As was shown in the separation pattern (p.444), more protein was eluted later on but this was not associated with any SRS-A activity. It might be instructive to know what types of protein SRS-A associates
with, in order to understand its distribution in vivo. However, this may be easier to follow when the chemistry of SRS-A is better understood, and was not pursued further. There is no published work on the association of SRS-A with protein and the results with gel filtration show clearly that this does occur. The material obtained at this stage showed a slight increase in total biological activity. A similar increase in potency was reported by Gaddum and Smith (1962) who separated nucleotides on dextran gels. The use of distilled water as the moving phase of a few of these columns gave separation patterns similar to those using buffer, but the product was not sufficiently stable. This could be due to lack of ions which are reported to be responsible for the stabilisation of proteins.

Materials obtained by this method of purification have been found to be very stable, this could be due to the presence of protective moities such as lipids associated with proteins. The separation of histamine and the alteration of the salt content by purification on dextran gels made it feasible to separate the SRS-A from the protein by anion and cation exchange techniques. Experiments were performed (1) using the carboxymethyl Sephadex columns with acidic buffer systems and increment of the NaCl concentrations, (2) using DEAE-Sephadex columns with neutral to alkaline buffer systems. These
attempts met with little success.

It is possible to remove the SRS-A activity from the associated protein by ethanol extraction of the active fractions obtained by gel filtration (Stage I) without any destruction of SRS-A. On testing this extracted material on the ileum and comparing it with the standards, showed the same characteristic contraction of SRS-A, except that it had a shorter latent period of contraction than the standards. This could be explained by the reduction in size of the protein SRS-A complex, which permit more rapid diffusion in the bathing fluid close to the tissue. It is unlikely to be due to the removal of inhibitory substances since a concomitant increase of total activity would be expected if this was the case. The yields obtained in these experiments have been very consistent.

As mentioned earlier attempts to fractionate material containing protein (from Stage I) on the Sephadex anion and cation exchangers were disappointing. The reasons for this low yield are not clear, but one suggestion that would be advanced is that adsorption onto the column leads to some denaturation of the protein with which SRS-A activity is associated. It is known that acid denaturation of protein with trichloracetic acid or metaphosphoric acid leads to total loss of SRS-A, presumably by "irreversible" adsorption. Less drastic procedures might similarly lead to loss of SRS-A. To
exclude the influence of protein, SRS-A which had been partly purified by alcoholic extraction was submitted to gel filtration. A two-stage extraction of freeze-dried crude perfusate by 80 per cent ethanol gave a yield of at least 80 per cent of SRS-A activity. The effect of pH when this extract was passed through G-50 Sephadex was surprising. When the columns were at pH 7.4, the activity was found in the last half of the salt peak and its potency had greatly increased. When the columns were run with buffer at pH 8.4, the activity was obtained in the first half of the salt peak, its potency was not as high as the active fractions collected at pH 7.4. The active fractions collected at both pH's 7.4 and 8.4 were moderately stable and retained about 50 per cent of their activities after three months. When the column was run at pH 6.4 the activity was obtained after the salt peak, and was even more potent than the materials from previous columns, but it was unstable. This instability could be due to many factors some of which could be readily assessed. One was the exposure of the active substance to unsuitable pH. As mentioned in the review, it was known that SRS-A in its crude state and partially purified form was more stable in alkaline than in acidic conditions. When the active material leaving the column at pH 6.5 was immediately buffered to pH 8.5 with sodium phosphate the stability
was improved, but still was not good enough for long-term studies. This improvement does nevertheless show that SRS-A should never be stored at acid pH, and that any process exposing it to acid should be of minimal duration. In any case the use of buffer introduces extra salt, which is generally undesirable. Losses by adsorption also seemed likely when very small amounts of pure material were handled. Attempts to prevent this by the use of siliconed glassware or polythene containers showed that adsorption was an important contributory factor but not the principal cause of loss. Since adsorption is a problem at this level of purification, it must clearly be guarded against in all attempts to produce material of high purity.

Brooklehurst (1956) reported that after hydrogenating SRS-A purified by adsorption on partially activated charcoal, activity was not lost until the sample was exposed to air. From this he concluded that the loss of activity might be due to autoxidation. Glucose was used as a mild antioxidant supposedly devoid of biological activity. The SRS-A treated with glucose had a lower biological activity, but this remained steady during storage for four weeks. The reason for this initial loss was not investigated, but it is possible that even 'analar' glucose has some adverse effect on the tissue. Cysteine, because of its ability to contract the test
tissue had to be ruled out. Cysteine was used by Berry and Collier (1964) in the perfusion fluid during the shock of guinea-pig lungs. It was intended to boost the yield of SRS-A, but may have contributed to the actions of their purified material on rat uterus and guinea-pig tracheal muscle preparations, which were not stimulated by the SRS-A used in the present study. The most acceptable method yet found is to collect the fractions in either silicone-coated test tubes or polythene tubes at low temperatures and to freeze-dry the active fractions immediately.

The increase in potency is unlikely to be due to a true increase of SRS-A although the purified material was more active on tissue and thus gave an enhanced value in biological assay. It is known that the purified material has been dissociated from a large molecule. This must make for increased rate of diffusion and may also permit more easy access to "receptors" by permitting free orientation of the molecule. Features of this sort are indicated by the reduced latency of the contraction. Contractions produced by the most highly purified material reach a maximum more quickly and then do not maintain it for very long in comparison with the crude samples. All these features are compatible with the suggestion that when equal concentrations of SRS-A are present in the bath, a pure sample will be more effective than a crude one.
Clearly this makes nonsense of biological assay, and it must be expected since the standard and test samples are no longer alike. Nevertheless in this study there is as yet no alternative. The most serious misconception will not arise from processes which lead to an apparent increase in material, but rather from steps which appear to give a good yield, but in fact give a low yield of more active material, which hides the loss.

The shift relative to the salt peak of the biologically active fractions on altering the pH of the moving phase on columns of Sephadex of the same dimensions prevented any evaluation of the molecular weight of SRS-A. At pH 6.4 it appeared that the molecule was smaller than those of the salts present because it travelled through the gel more slowly. Fortunately no such unlikely conclusion is necessary because it is clear that processes other than molecular sieving are at work. Changes in pH should have no effect on the relative position of small molecules undergoing gel filtration. The observed difference must therefore be attributed to retardation of SRS-A in the gel by a chromatographic process, resulting from the attraction between the dextran and SRS-A. Such attraction would vary greatly with pH.
Attempts to use ion-exchange materials had been so disappointing by comparison with the simple processes of adsorption and elution, that it was thought worthwhile to try the purification of SRS-A on active alumina and possibly silicic acid. Early work by Brocklehurst (1958) had suggested that alumina was useful and the process adopted was based on these early methods. Brocklehurst simply washed the alumina with water before use and found a large amount of unwanted salt in his eluted active material. The salt did not seem to be inherent in the use of alumina, attempts were therefore made to remove all soluble material before the experiments were performed. Thorough boiling with acid and alkali and also thorough washing with distilled water removes a lot of the free ions which are present in the commercial material. This is an improvement on the samples used by Brocklehurst (1958). The final calcination at 600°C for 3 hours removed any remaining traces of organic material and free radicals. These are capable of occupying the active spots on the column and have been reported to hinder the adsorption of most materials.

The final alumina beads are neutral which avoids fluctuation in pH which is liable to adversely affect good separation, and might cause degradation. Alumina which had been thoroughly washed with acid lost none of its ability to take up SRS-A from 80 per cent ethanol.
When water was used to elute the SRS-A the total biological activity was obtained in the first three tubes which must have contained a mixture of ethanol and water. We therefore investigated the elution with decreasing concentrations of ethanol in water and found that the elution occurred between 20 per cent and 25 per cent ethanol concentration. This method has many advantages. The SRS-A activity is separated completely from histamine which passed through the column with the 80 per cent ethanol. Most of the commonly occurring phospholipids are eluted from alumina columns by ethanol concentrations between 45 per cent and 60 per cent (Hanahan et al., 1951). The amount of solid present after evaporating the alcohol and freeze-drying the pooled active fractions was very small. The samples which have a potency of up to 30 units/mg are very stable when reconstituted with Tyrode solution.

The recovery of SRS-A obtained by the combination of the gel filtration and alumina adsorption in the five experiments performed was equal to the amount of SRS-A in the crude sample. The increase in total activity obtained after the Sephadex G-50 column alone thus disappeared during alumina adsorption, whereas direct purification of crude perfusate on alumina results in only small loss. The most likely possibility therefore seems to be that the SRS-A protein-complex obtained
after gel-filtration has a configuration which combines readily with the tissue. It is difficult to ascribe the effect to removal of an inhibitory substance because it would then be necessary to postulate a corresponding loss on subsequent treatment with alumina. This increase in total activity has been consistent and the only feasible explanation may be that SRS-A after Sephadex G-50 treatment is more exposed to combine with many receptors on the test object.

As mentioned in the Introduction (p. 27) Anderson et al. (1962) attempted the purification of SRS-A using a chromatographic grade of alumina. The acid ether extract was passed through an equilibrated column and elution was carried out using lipid solvents, they used positive chemical tests for cholesterol, glycerides and lipid phosphorus as guiding factors for the biological activity. This was based on a previous work done by Smith (1962). This particular method is thorough and systematic but is lengthy, which may be the reason why the recovery obtained was very low. Biological activity was not confined to one fraction, which leads one to suspect degradation. From the evidence collected, Anderson et al. went on to conclude that the biological activity of their acid ether extracts of anaphylactic guinea-pig lung perfusate was neither a long chain fatty acid nor was it an acidic phospholipid.
The alumina method was adopted as a routine and it is therefore necessary to consider how far it meets the criteria for satisfactory purification mentioned at the beginning of this thesis.

(1) "The use of organic solvents should be limited in order to minimise the possibility of chemical changes or contamination."

In the use of the Peter Spence alumina, the only organic solvent used was ethanol, and this solvent has been shown by several workers not to have any detrimental effect on SRS-A. Ethanol removes proteins, higher molecular weight peptides, and also some phospholipids by precipitation yet retaining SRS-A activity in the mother liquor. In this purification procedure decreasing concentrations of ethanol were employed so that the active product was in a solution of about 20 per cent ethanol in water. In all the experiments performed there was never any reason to suspect contamination.

(2) "Conditions which lead to quantitative or qualitative changes in the biological activity of the material should be avoided."

Although we are unaware of the ways in which SRS-A can lose its biological activity, certain experimental procedures have been regularly associated with loss. One of these is exposure to high salt.
concentration such as may occur if freeze-drying is interrupted or if one attempts to salt out the material from an aqueous phase into an organic phase. Another hazard is organic peroxides such as are found in ether and butanol and probably many other common solvents. Loss by oxidation has often been suspected but never proved. The present purification procedure can be operated quickly and at low temperature and much of the time in vacuo or in N₂ leak. The only danger from exposure to excess salt is during the critical freeze-drying.

(3) "The purified material should retain its biological activity under convenient storage conditions for long periods."

Purified material, freeze-dried and stored at -20°C in sealed ampoules under nitrogen, retained more than 60 per cent of its biological activity after eight to twelve weeks.

(4) "The method must be adaptable to handle the quantity of purified material needed for chemical characterisation."

This method imposes no limit on the quantity of material which could be handled. Our usual procedure is to store crude perfusate at -15°C after rapid freezing with solid CO₂. When a large batch is being processed, material which has already been freeze-dried can be
stored under N₂ in the deep-freeze until all the freeze-dried material is ready. Beyond this point extraction and purification can proceed unhindered, irrespective of quantity.

**Cat Paw SRS**

When compound 43/60 was used to obtain SRS from the perfused cat's paw, the time-course of its appearance in the perfusate corresponded closely with the release of SRS-A from guinea-pig lung during anaphylaxis. The maximum rate of release occurs considerably later than that of histamine as was previously described by Chakravarty (1960). The delay was actually greater than that reported by Chakravarty and so fails either to support or negate the argument advanced by Uvnäs and his collaborators "that it is plausible to assume that the 'SRS' obtained from the cat paw either originates directly from mast cells or is secondary to their disruption". The behaviour of the perfusate from cat's paw on a column of G-50 Sephadex in the presence of low ionic buffer solution was similar to that of SRS-A perfusates from sensitised guinea-pig lungs. The SRS activity travelled with the main protein fraction in the exclusion volume of the column and was completely separated from salt and histamine. The paw 'SRS' is soluble in 80 per cent ethanol, and on passing this extract through a column of G-50
Sephadex, with alterations in the pH of the buffer, the activity was found in fractions which corresponded to those obtained when lung SRS-A was similarly treated. The paw SRS could also be eluted from a column of alumina by 20 per cent ethanol. These findings support the view that the SRS from cat's paw is essentially similar to SRS-A (Uvnäs, 1962; Anggard et al., 1965).

In Chapter VII it was stated that another source of SRS was found in the peritoneal washings of rats following local sensitisation and challenge by intra-peritoneal injection (Rapp, 1961). On investigating the fluid collected, it was discovered that saline washes released a great deal of active material irrespective of antigen-antibody reactions (this was not reported by Rapp). However, Tyrode or de Jalon solution were both found to be satisfactory giving modest control values of biological activity. Polythene receptacles were found to protect the peritoneal washings from bradykinin-destroying and -forming enzymes, and from the experience gained in the purification procedure of SRS-A on columns, siliconed glassware or polythene vessels were found to prevent the loss of the spasmogenic substances. The use of such receptacles enabled reproducible results to be obtained. Bradykinin was found to be present in the
peritoneal washings along with SRS and histamine. This was not reported by Rapp. The five minute samples did not contain much SRS (see Table 14) and it seemed possible that a larger yield of SRS would be obtained by allowing the antigen-antibody reaction to continue for a longer period.

A systematic study of the time-course of release of SRS and the other spasmogens (Fig. 18) showed that the maximum yield of histamine was obtained at about 3 minutes, after challenge, whereas the bradykinin maximum was at 15 minutes and SRS maximum was 20 minutes. In general the biological assays for the three spasmogens were carried out on guinea-pig ileum, but the uteri from stilboestrol-treated rats were used to confirm the presence of bradykinin. The SRS-like and bradykinin-like activities were assayed before and after chymotrypsin digestion. This was to eliminate any contribution which either material may make to the contractions elicited from the guinea-pig ileum during assay. It was found that the contribution made by the bradykinin-like activity was greatest about 15 minutes after challenge but was never very great and probably of little significance.

The quantity of bradykinin varied with the purity of the antibody used. Less bradykinin was obtained when purified guinea-pig IgA containing antiovalbumin (Brocklehurst and Colquhoun, 1965) was used. The
reason for this is not known and it is beyond the scope of this investigation. It may be that antibodies other than IgA are responsible for the release of bradykinin and it may be significant that the purest sensitising globulin used contained some IgG in it.

The SRS-like material obtained from these washings strongly resembled SRS-A when assayed on the guinea-pig ileum. Furthermore there was no apparent difference when the substance was purified by the procedure used for SRS-A from guinea-pig lung and SRS from cat paw, in spite of the different methods by which the substances were originally obtained. Rapp (1961) concluded that the SRS he obtained was similar to SRS-A supplied to him by Brocklehurst. He used only simple pharmacological tests and the materials were in a very impure state. The present work strengthens the conclusion reached by Rapp.

Now that there is evidence for the release of SRS-A in conditions which do not involve antigen-antibody reactions it would seem that the suffix 'A' is unjustified. However, since it is for its role in antigen-antibody reactions that this substance is of interest and since all the present evidence suggests that it differs from a wide range of biologically active tissue products, it does not seem illogical to retain the suffix until it can be replaced on the basis of chemical knowledge.
The source of SRS-A is not yet known, although Uvnäs and his colleagues consider that the mast cells may be the source of all the slow-reacting substances under discussion. Rapp reported that when SRS was released into the rat peritoneal cavity he found no evidence of damage to mast cells. This has recently been confirmed by Rothschild (1965), who also showed that phospholipase-A did not cause mast cell damage when histamine was released. The present work has shown that SRS-A from lung and that from cat paws are identical or at least very similar. There must therefore be considerable doubt concerning the suggestion of Uvnäs (1963) that SRS-A is formed by the action of phospholipase-A on mast cells.

Different workers have reported different spectrum of biological activities for their own samples of SRS (as shown on pages 10-14). This difference clearly springs from varying degrees of purity and different methods of purification. The most important practical aspect is in respect of the behaviour of human bronchial muscle, and this has often been omitted. It is also possible that some properties of SRS-A have been obscured or that tests have been omitted because the material was excessively impure. Therefore any material purified by new techniques or methods as in the present work had to be subjected to complete biological examination. From these biological tests,
it became evident that the purest samples of SRS-A obtained in the present work gave responses in only a few preparations and that there were some differences from the results obtained with crude material. The more important effects were on the cardiovascular system, cutaneous vascular permeability and the contraction of human bronchiole muscle in vitro.

Very large doses were used with all the test preparations so that it could be safely assumed that no important response had been missed. All the preparations responded satisfactorily to small doses of other active substances.

The activity of purified SRS-A on the rabbit jejunum was very much lower than that of the crude material. This result agrees with the finding of Anggard et al. (1963) who stated that concentration of purified SRS-A necessary to contract the rabbit jejunum was 400 - 600 times greater than was required for the guinea-pig ileum.

The effect of crude SRS-A on the rabbit jejunum previously reported, must therefore be due to a second active substance. We have no evidence concerning the nature of this substance, but would tentatively suggest that it is one of the active materials which can be extracted from normal lung, e.g. one of the prosta-glandins, cerebrosides, or fatty acids.
Purified SRS-A both from lung and from paws did not affect the uteri of guinea-pig, rat and rabbit although SRS-A prepared by Berry and Collier was stated to be active upon the rat uterus at concentrations only a few times greater than those active on the guinea-pig ileum (personal communication). It had been thought that if purified SRS-A were active on the uteri of either the rat or guinea-pig, this would provide a rational explanation of the experiments performed on the rat uterus (Kellaway, 1930) and the guinea-pig uterus (Schild, 1936) from sensitised animals which were found to contract when antigen was brought in contact, even though the preparation would not respond to histamine. However, SRS-A does not contract this preparation and the most likely candidate would seem to be bradykinin. We can offer no explanation for the results of Berry and Collier whose preparation has much greater activity on uteri than we have ever found even in crude perfusates.

Studies of the effects of SRS-A on isolated blood vessels have been very limited, presumably because in vivo SRS-A has shown remarkably little vasomotor action. When large amounts of SRS-A were injected into intact rats, the animal died in a condition which superficially appeared to be vasomotor collapse. This led us to study more closely the effects of SRS-A on
isolated heart and blood vessel preparations. Purified SRS-A (60 units) prepared by the alumina adsorption method constricted the coronary vessels of isolated heart preparations as shown by reduction in the flow rate of Ringer solution during Langendorff perfusion. In the isolated guinea-pig heart, the amplitude of the contractions was increased and there was also some increase in rate. In the rat heart, the same dose of SRS-A caused slowing and a considerable reduction of amplitude. The effects of crude SRS-A on the isolated preparation of blood vessels was no greater than would be expected from the histamine content. However, purified material obtained from either the lung or the paw contracted the isolated guinea-pig vena cava and relaxed the swine splenic artery. It is evident therefore that SRS-A is not without activity on the cardiovascular system, and may be of some significance since the yield of SRS-A from blood vessels is relatively high. It is obvious that a very much more extensive study is required before any general conclusions can be drawn.

The contribution of SRS-A to cutaneous allergic reactions appears to be small at least in the rat and the guinea-pig since relatively large doses had to be given intradermally in order to produce blue reaction. However, this is the first time that SRS-A has been shown to produce such an effect. The effects on the
whole animal were most marked in the rat; large amounts of SRS-A were injected into rats and guinea-pigs and recordings were made with small displacement transducing devices which gave a continuous detailed record of blood pressure and respiration. In the rat there was a slow prolonged fall in arterial pressure and a rise in venous pressure usually accompanied by bradycardia. Atropinisation did not abolish the bradycardia nor did it influence the fall in blood pressure. Changes in bronchial resistance were negligible. In the guinea-pig, vascular changes, although superficially similar, were much less marked. The bronchial resistance, however, rose slowly over a period of 2 - 3 min. and was maintained for a considerable time. The whole reaction was much more prolonged than that seen with histamine; compared with the effect produced by bradykinin it was slow in onset and more rapid in recovery. Neither atropine nor mepyramine had any effect on the bronchoconstriction. At autopsy rats which had received intravenous SRS-A showed considerable oedema and engorgement of certain organs, notably the small intestine and the spleen. This effect closely resembles that reported by Parratt and West (1958) in rats subjected to challenge after active sensitisation using Bordetella pertussis as adjuvants. In guinea-pigs, there was no obvious damage to the viscera.
Bronchial muscle from both trachea and bronchioles of several species was extensively studied by Broeklehurst (1953, 1960) following the observation that human bronchiolar muscle responded vigorously in vitro to SRS-A. At that time definite responses were not obtained with any other bronchiole preparations.

SRS-A purified by both methods used in the present study was only very slightly active on isolated guinea-pig tracheal chains, but both were very active on isolated human bronchioles. The cat tracheal muscle gave no definite response with crude active perfusate, but relatively high doses (40 units/ml) of purified SRS-A produced a moderate contraction. This preparation does not respond to histamine, but responds to very small doses of 5HT. It was therefore necessary to show that bromolysergic acid diethylamide did not diminish the response to SRS-A.

The most important feature of this work is the effect on human bronchioles. The dose of SRS-A required to contract the preparation was found to be about a tenth of that which gave a threshold response on the guinea-pig preparation. Since the interest in SRS-A centres on its probable bronchoconstrictor role in human asthma, it was gratifying to find that the purified material was highly active on human tissue. Herxheimer and Stresemann (1962)
have shown that an aerosol of SRS-A causes bronchoconstriction in normal man, thus strengthening its claim as mediator in human asthmatic conditions. It has been possible to test other naturally occurring substances on the isolated human bronchides and it has been shown that large doses of substance P and irin were without effect, whilst bradykinin produced a small contraction but thereafter demonstrated tachyphylaxis. This finding helps to clarify the picture of broncho-spasm which may be produced by tissue damage in the lung.

The fact that this material was inactive on the isolated tracheal chain of the guinea-pig confronts us with some difficulty in the interpretation of the results in the whole lung. Bronchoconstriction has been observed in isolated perfused whole guinea-pig lungs and also to a moderate extent in vivo in the whole animal. We can only assume that the smaller bronchioles are more responsive than the trachea. There is some support for this in the work of Berry and Collier (1964), who have observed much stronger responses in vivo than we have been able to obtain yet produced only modest contraction of the tracheal chain with very much larger doses. There may not be an exact parallel between the work of Berry and Collier and the present work because there are variations in the pharmacology of the purified materials, and Berry and Collier have
suggested that their material may contain more than one substance. It must be remembered that their purified SRS-A was active on the rat uterus and that the bronchoconstriction produced was more rapid in onset and shorter in duration.

These observations call for a reappraisal of the statement made by Brocklehurst (1962), "On the basis of in vitro studies the dose of SRS-A necessary to produce a threshold contraction on guinea-pig bronchioles must be at least ten times larger than that which contracts the corresponding human tissue. It therefore follows that the anaphylactic bronchospasm of the guinea-pig is an imperfect model of human asthma." The present study bears out the first part of Brocklehurst's contention. However, the behaviour of the anaesthetised rat as compared to the anaesthetised guinea-pig partially justifies the second part of his contention. Berry and Collier (1964) disagreed with Brocklehurst's suggestion that the anaphylactic bronchospasm of the guinea-pig is an imperfect model of human asthma. More work must be done before any firm conclusions can be drawn.

Recently large amounts of information have become available concerning the chemistry of the prostaglandins but the pharmacology is still relatively sketchy. The situation is complicated further by the possibility that some metabolites may possess biological activity.
At present even the chemistry of these metabolites is incomplete. Some prostaglandins have been obtained from lungs (Anggard and Bergstrom, 1963) and it has also been reported that PGF₂α will increase the bronchial resistance of perfused guinea-pig lungs. It is therefore likely that gross damage will release prostaglandins from the tissue and this would certainly complicate the picture when SRS-A is obtained from chopped tissue. However, all the prostaglandins so far studied have activity on the isolated rat uterus, hamster colon and the rabbit duodenum and most of them are relatively inactive on guinea-pig ileum (Linn et al., 1961; Anggard and Bergstrom, 1961). This clearly distinguishes the prostaglandins from SRS-A but it might be well to bear in mind that some of the prostaglandin metabolites may possess unexpected pharmacological properties.

Irin has pharmacological properties which are similar to the prostaglandins and present evidence suggests that it is closely related chemically (Ambache, 1963). This substance has been clearly differentiated from SRS-A. Both purified guinea-pig SRS-A and cat paw SRS in doses up to 50 units/ml have failed to elicit any response from hamster colon and ileum. Neither does such material contract the fowl rectal caecum although crude material is slightly active on this tissue.
Only one difference has been observed between purified SRS-A obtained from paws and that from lung. The chick crop (Bowman and Everett, 1964) is much more reactive to the material from paws. This shows incidentally that the method of purification is still imperfect. We have no means of knowing where this second kind of activity comes from, and we have not investigated it further because it is the product of 48/80 reaction not anaphylaxis and it does not seem to modify the responses of any of the test tissues.

Work on the chemical identity of SRS-A is continuing and is far from complete but there are some new facts which should be discussed in relation to other recently published work. Recently Smith and co-workers have made two suggestions that SRS-A is based on neuraminic acid. The first envisaged SRS-A as a mixture of neuraminic acid glycosides resulting from partial hydrolysis of lung mucoproteins (Anderson, Goadby and Smith, 1963; Smith, 1962), but from our own work no such active products have been obtained in this way, and the chemical evidence probably does not refer to SRS-A at all because the material used was impure. The second suggestion was that SRS-A is a ganglioside (Smith, 1966), which maintains the presence of neuraminic acid as did the first suggestion, in the SRS-A molecule. Biological activity of this type of glycolipid has been reported by Bogoch and co-workers...
(1962) who obtained samples of gangliosides by methanol/chloroform extraction of cerebral hemispheres. Smith has used SRS-A and a sample of lipid extracted from lung by the procedure used by Bogoch. Chromatographic separation occurred on silicic acid during gradient elution, using increasing proportions of methanol in chloroform. He found a roughly comparable distribution of neuraminyl derivatives and lipid phosphorus in those fractions corresponding to 60 - 100 per cent methanol content, but did not report where biological activity was located. Using a similar system Anggard et al. (1963) eluted the active SRS-A with 30 per cent methanol in chloroform. They found this to be optimum conditions required under this system; but it must be remembered that the movement of the biological activity depends greatly upon the type of lipid present. In the present work, using both n-propanol:water:ammonia (as Smith originally used) and chloroform:methanol:acetic acid:water (65:25:8:4) on thin layer plates of Silica-G, biological activity was obtained in Tyrode eluates from the origin of both the chloroform extracted SRS-A and the untreated alumina purified material. The active region gave little if any colour reaction for phosphatide, although with the chloroform extract spots giving positive reactions for phosphatides were present which had Rf values corresponding to sphingomyelin, phosphatidyl choline
and a slight colour for phosphatidal ethanolamine. All these were found to be biologically inactive.

From the above evidence it appears that SRS-A is unlikely to be a phosphatide, but could still be a polar phospholipid. The first part of this statement confirms the work done by Anggard and co-workers (1963) but in this case the SRS-A was found to be fairly soluble in chloroform. The present work leaves the chemical identity of SRS-A still unknown. Now that it is possible to move some of the contaminants away from the biological activity and still retain the characteristic activity (i.e. slow contraction of the atropinised and neopyraminised guinea-pig ileum without tachyphylaxis), more work on its chemical identity is envisaged. As mentioned, SRS-A could still be a polar phospholipid, but if so it must have very high potency. Until we have some idea of its true potency, we have no clue to the degree of purity of any sample. We are thus faced with the dilemma of what results are meaningful and which tests should be undertaken first. The decisions have to take account of

(i) the cost and labour of producing the sample in question - this is always considerable.

(ii) the chance of recovering the sample, or going on to a second test.

(iii) the value of a negative answer, with the probability that this is due to too little material.
(iv) the reliability and usefulness of a positive reaction or test - initially the reaction must be rather general, or the chances of getting a 'positive' are small: later, specificity matters most.

It is evident that the results, if they really refer to SRS-A, will converge towards the truth and that the tempo of achievement will quicken as the field narrows. We cannot at present see any real convergence, and feel that our best course at present is to assume that our purest samples are reasonably pure, and to investigate their physical properties (e.g. U.V. and I.R. absorption spectra) in the hope of getting a lead.
### Summary of Pharmacological Properties of SRS-A

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Perfusate (SRS-A)</th>
<th>Guinea pig Purified SRS-A</th>
<th>Cat paw Purified SRS-A (Alumina)</th>
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<td>0( 20)</td>
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<td>+(5)</td>
<td>+(5)</td>
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<tr>
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TABLE 20 (contd.)
Summary of Pharmacological Properties of SRS-A

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<th>Cat paw Purified SRS-A (Alumina)</th>
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<td>0(20)</td>
<td>+(10)</td>
</tr>
<tr>
<td>Rat arterial pressure</td>
<td>0(60)</td>
<td>-(20)</td>
<td>-(30)</td>
</tr>
<tr>
<td>venous pressure</td>
<td>0(60)</td>
<td>-(20)</td>
<td>-(30)</td>
</tr>
<tr>
<td>bronchial resistance</td>
<td>0(60)</td>
<td>+(20)</td>
<td>+(30)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>arterial pressure</td>
<td>0(60)</td>
<td>-(30)</td>
<td></td>
</tr>
<tr>
<td>venous pressure</td>
<td>0(60)</td>
<td>+(30)</td>
<td></td>
</tr>
<tr>
<td>bronchial resistance</td>
<td>0(60)</td>
<td>+(30)</td>
<td></td>
</tr>
<tr>
<td>Frog rectum</td>
<td>+(15)</td>
<td>+(15)</td>
<td></td>
</tr>
<tr>
<td>Swine splenic artery</td>
<td></td>
<td>-(20)</td>
<td>-(20)</td>
</tr>
<tr>
<td>Human bronchioles</td>
<td>+(5)</td>
<td>+(3)</td>
<td>+(3)</td>
</tr>
</tbody>
</table>

Figures in brackets indicate effective dose level or maximum dose tried, expressed as units/ml, or units/animal.

+ = contraction, rise of arterial or venous pressure, increased bronchial resistance
- = relaxation, fall of arterial or venous pressure, reduced bronchial resistance
0 = no response
Section I

Preparation of antigen

Except where mentioned, egg albumin was used as the antigen throughout the present experiments.

Crude egg albumin

A freshly made 10 per cent solution of commercial dried egg albumin in normal saline containing 0.5 per cent phenol was used. After the phenol was added, the mixture was left at room temperature for an hour and any precipitate discarded.

Crystalline egg albumin

This material (Eastman x 5 recrystallised) was used in concentrations of 1 mg/ml in Tyrode solution and kept at -10°C whenever storage was necessary.

Alum-adsorbed egg albumin

4.6 ml of N sodium bicarbonate was added to 10 ml of 10 per cent solution of crude egg albumin in normal saline. A 10 per cent aqueous solution of potash alum was added dropwise whilst stirring, until the pH came to 6.8 (B.D.H. capillator or Pye direct reading meter). Evolution of CO₂ ceased as this point approached. The mixture was kept at 4°C for six hours and centrifuged. The precipitate was washed twice in distilled water and finally was suspended in 10 ml of 0.154 M saline. The protein concentration of the
suspension was measured by adding acid until precipitate dissolved and measuring the optical density at 280 μm.

Assay Methods

Histamine.—The biological assay was performed on a portion of the terminal ileum of guinea-pigs of 250 to 300 g bodyweight using the matching technique. Histamine 5 μg/ml.

The tissue was suspended in oxygenated Tyrode solution at 35°C containing atropine 3·10⁻⁷ g/ml. A light frontal writing lever system was used which exerted a pull of 0.5 g on the tissue and gave a magnification of x 8 on the kymograph.

Slow reacting substance (SRS-A).—This was usually performed on the same ileum as was used for the histamine assay. The tissue was bathed in oxygenated Tyrode solution containing atropine 3·10⁻⁷ and mepyramine 10⁻⁶ g/ml. The standard SRS-A values were expressed in arbitrary units referring to the laboratory standard freeze-dried crude perfusate (1 unit being equivalent to 0.5 mg of laboratory standard which gave a contraction of about 20 mm on the smoked drum (i.e. 10 - 15% shortening of the tissue). A typical assay of histamine and SRS-A is shown in Figures 2(a) and (b).

Gel filtration of perfusate

G-50 Sephadex: preparation, packing and regeneration.—Medium G-50 Sephadex (water regain 5.0 g/g dry gel: No. 9860 C) was obtained from A. B. Pharmacia
<table>
<thead>
<tr>
<th>NaCl</th>
<th>NaH₂PO₄·2H₂O</th>
<th>Na₂HPO₄·12H₂O</th>
<th>pH</th>
<th>Specific conductivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>moles/l</td>
<td>g/l</td>
<td>g/l</td>
<td></td>
<td>m. mho. cm⁻¹</td>
</tr>
<tr>
<td>0.01</td>
<td>0.585</td>
<td>0.5</td>
<td>6.02</td>
<td>7.40</td>
</tr>
<tr>
<td>0.10</td>
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<td>0.5</td>
<td>6.02</td>
<td>7.35</td>
</tr>
<tr>
<td>0.18</td>
<td>10.52</td>
<td>0.5</td>
<td>6.02</td>
<td>7.20</td>
</tr>
</tbody>
</table>

*25°C*
It was allowed to swell in buffer for several hours. Fine particles were removed by sucking off the supernatant after the bulk of the particles had settled. When this process had been repeated several times the supernatant was clear. The slurry was then exposed to low pressures to remove dissolved air.

The delivery tube of the siliconed glass column was packed with a skein of polythene turnings and an extension tube was fitted to the tap. The slurry was poured and the column packed in the way described by Flodin (1962). The upper layer was stirred to ensure a flat surface but no disc was added.

The perfusate or alcoholic extract was introduced through a 1 mm bore polythene tube close to the top of the gel: 10 ml of perfusate could be layered on this way within 5 - 10 minutes without disturbance of the gel or mixing with the buffer.

After use the column was dismantled and the process of sedimentation in buffer was repeated. The gel was stored in 2 M salt at 4°C.

Sodium phosphate buffers

The composition of the buffers used for the columns of 0-50, DEAE-Sephadex is shown in Table 21.
DEAE-Sephadex: preparation, packing and regeneration

DEAE-Sephadex, A-25 or A-50 medium (water regain = 5.0 g/g, anion capacity $3.5 \pm 0.5$ meq/g) was obtained from Pharmacia.

The gel was equilibrated with buffer, fine particles were washed away, and dissolved air was removed in the same way as the G-50 Sephadex. The slurry was poured and the column was packed. Buffer was passed until the pH of the eluate was identical with that of the buffer applied.

After use the gel was stirred with 2 volumes of 0.5 N NaOH and transferred to a No. 54 Whatman filter paper in a Buchner funnel. It was then washed with distilled water under suction. It was next stirred with 2 volumes of 0.5 N HCl, and finally washed with several litres of distilled water followed by buffer. This process appeared to produce more fine particles but the subsequent behaviour of the rest of the gel was not altered.

Measurements of protein concentration

The optical density of solutions to light of wavelength 275 mp was determined using a Unicam SP 500 Spectrophotometer. This was taken as an approximate measure of the protein concentration. A standard calibration curve was prepared with ovalbumin (5 x crystallised) in an initial concentration of 10 mg/ml.
This standard was used for the measurement of protein concentrations in the fractions from the DEAE-Sephadex, G-50 Sephadex columns.

**Measurement of Specific Conductivity**

All determinations were made on a Phillips conductivity bridge (Type PR 9500) at 1,000 c.p.s.

Measurements for the location of the electrolyte peak in the eluate from a column were made at room temperature using a micro-cell (constant = 0.70).

More accurate measurements were made to check the composition of the buffers used in elution. For this purpose the samples were brought to 25 ± 0.5° in a water bath and larger cells were used (constant = 1.33).

**Preparation of Antisera**

(a) **Rabbit anti-BSA sera.**- Rabbits were injected intramuscularly with 10 mg BSA in 0.5 or 1.0 ml of complete Freund's adjuvant of the following composition:- protein solution in 0.15 M saline 4 vol., light liquid paraffin B.P. + mycobacteria 4 vol., Crill K-16 (sorbitan mono oleate (Croda Ltd., Goole, Yorks) 1 vol. The light liquid paraffin contained 4 mg/ml of heat killed mycobacteria (M. tuberculosis human type. Stains C, DT and PN mixed Central Veterinary Laboratory, Weybridge, Surrey). After 1 week the same dose was given I.M. in a different site and after another week subcutaneously in 4 sites. After a 6 week interval
booster doses of 1, 2, 2 and 4 mg of aluminium hydroxide adsorbed BSA were given intravenously at 2 day intervals. The animals were bled one week after the last injection.

(b) *Guinea-pig anti-ovalbumin sera.*-- Guinea-pigs were given three injections of 5 mg ovalbumin in 0.5 ml complete Freund's adjuvant, described above, at weekly intervals, the first two being I.M., the third S.C. After 6 weeks aluminium hydroxide adsorbed ovalbumin was given I.P., every two days for 4 doses, increasing from 0.2 ml to 1.0 ml. The animals were bled one week after the last injection.

**Purification of Proteins**

**Preparative electrophoresis**

Preparative electrophoresis was performed using polyvinyl chloride - polyvinyl acetate co-polymer particles (Pevikon C-870, Guest Industrials Ltd., see Muller-Eberhard, 1960), using a method developed by Colquhoun (1964).

After washing the particles with barbitone buffer (pH 8.6, 0.09 M), a suspension in this buffer was poured into a perspex frame 36 cm x 18 cm x 1.5 cm lined with thin polythene sheet. The block was 6.5 cm above the level of the buffer in the electrode baths. Connections were made with three thicknesses of lint. In the experiments the serum samples were concentrated before
applying to the Pevikon, usually 20 ml of serum were concentrated to 5 - 8 ml using Biodryex (Lövdalens Industri AB, Stockholm). After cooling the block in a cold room at 1°C the sample was applied by cutting a slot about 1 cm wide 5 to 7 cm from the cathode end of the block. The slot ended about 2 cm from each edge of the frame and a short distance from the bottom. The Pevikon removed to form the slot was blotted on lint and mixed with the serum to form a slurry which was poured carefully back into the slot. After smoothing the surface with a palette knife it was covered with polythene and electrophoresis was performed using a potential gradient of 3.5 to 4.5 V cm⁻¹. Electrophoresis continued until the \( \beta \)-globulins approached the anode end: this took about 40 hours. Haemoglobin is a convenient marker for the \( \beta \)-globulin region. When the ambient temperature in the cold room was 1°C, the steady state temperature in the block was about 11°C. At the end of the run the block was cut into 1 cm wide sections each of which was suspended in 10 ml of NaCl 0.154 g/100 ml \( \mu = 0.8932 \) and after 5 minutes the eluate was separated on a sintered glass filter (porosity 1 or 2) leading to a container with a side arm attached to a water pump. The particles were resuspended and the process was repeated until 30 ml of eluate had been collected. A control experiment showed that the first three elutions
removed most of the protein, and a fourth elution produced an increment of less than 5 per cent. The eluates from each section were analysed for protein content and concentrated by pressure dialysis at 1°C.

All sections cathodal to the first coloured sections (= haemoglobin) were taken as "total γ-globulin". The pooled concentrated eluates were filtered through "Millipore" cellulose acetate membrane filters in an all glass assembly to remove very fine particles of Pevikon.

(2) **DEAE cellulose chromatography**

Columns were made from diethylaminoethyl (DEAE) cellulose prepared using 100 - 200 mesh Solka Floc cellulose according to Peterson and Sober (1956), or bought from Kodak Ltd. (Kirkby Trading Estate, Liverpool). All columns and samples were equilibrated with 0.01 M pH 8.2 sodium phosphate buffer.

To separate the total γ-globulins obtained by preparative electrophoresis the samples were concentrated to a volume of 3 to 5 ml using Biodryex, and then dialysed against 0.01 M sodium phosphate buffer pH 8.2. The concentrated samples were applied to columns 1.5 cm diameter and 35 to 40 cm height. The eluting buffer was the same as that used to equilibrate both the sample and the column, the pressure being adjusted to give a flow of 0.6 ml/min. After the first protein peak had been
eluted, a gradient of rising ionic strength was started using a device of the sort described by Peterson and Sober (1959). The same buffer was used throughout, and the ionic strength was increased by the addition of sodium chloride to a maximum concentration of 0.30 M. Fractions of about 10 ml were collected and scanned for protein concentration by measuring their optical density at 280 nm, they were concentrated by pressure dialysis at 1°C against 0.154 M saline.
(1) McEwan’s solution contains in 1 litre
(McEwan, 1956; Hukovic, Rand and Vanov, 1965)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
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</tr>
<tr>
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<tr>
<td>calcium chloride</td>
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</tr>
<tr>
<td>sodium di-hydrogen phosphate</td>
<td>0.146 g</td>
</tr>
<tr>
<td>sodium bicarbonate</td>
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<tr>
<td>glucose</td>
<td>2.0 g</td>
</tr>
<tr>
<td>sucrose</td>
<td>4.0 g</td>
</tr>
</tbody>
</table>

(2) Krebs (1950) solution as used by Brocklehurst in 1955 contains in 1 litre

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>sodium chloride</td>
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</tr>
<tr>
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<tr>
<td>calcium chloride</td>
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<tr>
<td>potassium di-hydrogen phosphate</td>
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<tr>
<td>magnesium sulphate</td>
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<tr>
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<tr>
<td>glucose</td>
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</tr>
<tr>
<td>sodium pyruvate (or l-lactate)</td>
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<tr>
<td>sodium fumarate</td>
<td>0.9 g</td>
</tr>
<tr>
<td>sodium glutamate</td>
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</tr>
</tbody>
</table>

brought to pH 8.0 with sodium bicarbonate

Aerated with O₂ + 5% CO₂ before use.
Section II

Preparation of Egg Phospholipids

Extraction from yolk

Fertile eggs, obtained from hens, and less than 24 hours old, were used throughout the work. The whites of the eggs were separated and the yolks freeze-dried. After thorough mixing in a blender the dried material was packed, in vacuo and stored at -30°C. Extraction of the dried yolk was carried out according to the scheme of Rhodes and Lea (1956). The solvents were de-oxygenated by bubbling with CO₂.

Dried yolk from 12 eggs extracted in blender with 250 ml of acetone at 0-4°C and filtered. Repeat extraction. Extracts combined (AS1)

Residue extracted by shaking for 30 min. at 0-4°C with 150 ml of MeOH-CHCl₃ (1:1, v/v), filtered. Repeat extraction.

Residue discarded

Extracts evaporated to dryness (E1). Dissolved in 50 ml light petroleum, pptd. with 400 ml of acetone at 0°C, centrifuged, decanted. Repeat extraction/precipitation twice.

Extract (AS2)

Precipitate dissolved in 50 ml of light petroleum, stored for 16 hr at 0°C, centrifuged, residue washed.

Residue P1 Extract evaporated to dryness (CP)

In the main most of the phospholipids (about 93%) was recovered in the cold methanol-CHCl₃ extract (E1).
Section II (contd.)

Lipid extraction of lung tissue

The problem of extracting lipids from tissue and other sources is dealt with by Hanahan (1960) and Entenman (1957). The extraction was done by either homogenising or shaking the freeze-dried lungs with 17 vols of HCl₃:MeOH (2:1 v/v). After 4 hours, in Quickfit tubes in the dark at room temperature, the extract was filtered through glass wool. The residue was collected and re-extracted for 1 hour with 3 vols of CHCl₃:MeOH (2:1 v/v). The extract was added to the original 17 vols of extracts. The total extract was then washed with 0.2 vols of 0.5 per cent NaCl to remove contaminants. The purpose of the cation is to prevent the loss of acidic phospholipids into the aqueous phase. The aqueous phase was removed by suction through Pasteur pipette and in some determinations collected in a trap for total phosphorus estimation. Any interfacial "fluff" was also removed. The interface was then washed three times with small amounts of "pure solvents upper phase" which consisted of CHCl₃:MeOH (45:48 v/v) and then a small quantity of MeOH was added to create one phase. The extract was then dried overnight with anhydrous Na₂SO₄. This was then spun off on a centrifuge and the lipid extracts reduced to a very small volume in a tube in a stream of nitrogen. The extracts were never taken to dryness.
The lipids were chromatographed as soon as possible after preparation and stored at -15°C in the dark in stoppered tubes under oxygen-free nitrogen.

Qualitative examination for lipid constituents present in various fractions of SRS-A obtained from G-50 and alumina columns, lung lipids and egg phospholipids

**Total cholesterol**

This was determined by the method of Hamel and Dam (1955). 1 ml of zinc chloride reagent and 1 ml acetyl chloride were added to one aliquot of sample in a graduated tube. The tubes were heated at 55°C for 20 minutes and then cooled. After making up to 4 ml with chloroform, the optical density at 528 μ was determined in a spectrophotometer. 0.1 mg/ml pure cholesterol was used as standard.

**Results.**—The lung extracts gave rosy pink colorations and the test samples gave readings of cholesterol content equivalent to 40 ng, whereas the fractions of SRS-A obtained from both columns (i.e. G-50 and alumina) gave no pink coloration.

**Total phosphorus**

A modification of the method of Shin (1962), a sensitive modification of the method of Fiske and Subbarow (1925), was used. The sample was digested
with 0.2 ml 18N H₂SO₄ for 15 minutes. The digestion was carried out in Pyrex tubes to prevent bumping.

After the initial digestion a certain degree of charring had occurred due to organic material in the sample. To remove the colour due to charring, 1 drop of H₂O₂ (100 vols) was added and the digestion continued until all the H₂O₂ had been removed. If this is not done a yellow oxidation product of ammonium molybdate is formed when this is added during the determination. The acid in the digestion tube was made up to 2.0 ml and 0.4 ml. 5 per cent ammonium molybdate was added, followed by 0.1 ml of Fiske-Subbarow reagent. After mixing, the tubes were placed in a boiling water bath for 5 minutes and then cooled in ice. The blue colour produced was read at 830 µ after 15 minutes.

Lecithin should be used as standard, but as it is very unstable under storage conditions, sodium hydrogen orthophosphate in a concentration of 10 µg/ml was used.
REFERENCES

Brocklehurst, W.E. (1953). J. Physiol. 120, 16P.


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