Biochemical Pharmacology, 1963, Vol. 12, pp. 1037–1045. Pergamon Press Ltd Printed in Great Britain.

OBSERVATIONS ON THE CHROMATOGRAPHIC PURIFICATION OF THE SLOW REACTING SUBSTANCE OF ANAPHYLAXIS

DOREEN M. ANDERSON, P. GOADBY and W. G. SMITH

Research Laboratory in Biochemical Pharmacology,
Sunderland Technical College, Sunderland, England

(*Received* 23 *January* 1963; *accepted* 1 *May* 1963)

Abstract—Silicic acid chromatography of SRS-A (slow reacting substance of anaphylaxis) present in acid ether extracts of anaphylactic lung perfusate has established that the smooth muscle stimulant activity, as demonstrated on isolated guinea pig ileum, is found in seven separate fractions. The active components appear to possess a glycol structure and amino-nitrogen as well as a carboxyl group and may be glycosides of neuraminic acid.

ANAPHYLAXIS in guinea pig lung leads to the liberation of histamine and SRS-A (slow reacting substance of anaphylaxis.[1, 2] SRS-A has some of the chemical characteristics of a lipid soluble acid,[2, 3] but its chemical structure has never been elucidated. Smith[4, 5] has shown that Tyrode perfusates of guinea pig lungs undergoing anaphylaxis *in vitro* contain lipid in addition to histamine and SRS-A. The present report concerns an attempt to separate SRS-A from contaminating lipid by column chromatography.

EXPERIMENTAL AND RESULTS

Anaphylaxis in vitro

Guinea pigs of either sex weighing about 200 g were sensitised to commercial egg albumin (BDH) by the subcutaneous and intraperitoneal injection of 100 mg as a 5% solution in distilled water. They were fed diet 18 pellets (Oxo Ltd.) and received 50 mg ascorbic acid (BDH) each morning in drinking water contained in amber glass bottles. Overnight they received tap water.

The degree of sensitisation was determined 4 weeks later by exposing the animals to an aerosol of 1% egg albumin in distilled water according to the technique of Herxheimer.[6] Only animals showing preconvulsion times of 120 sec or less were considered sufficiently sensitive for further study.

Two to five months after the sensitising dose of antigen, the animals were killed by a blow on the back of the head. The lungs and trachea with heart attached were rapidly dissected, perfused with Tyrode solution at 37° and subjected to anaphylactic shock as described by Brocklehurst.[1] Perfusate was collected for 30 min after antigen administration, centrifuged to remove blood cells and then solvent extracted immediately. In all, twelve perfusates were used in the present study.

Solvent extraction of lipid and SRS-A from perfusate

Chakravarty[2] has shown that SRS-A can be extracted into diethyl ether from Tyrode solution, but only after acidification. This suggested a means of removing contaminating lipid from perfusate by extraction at neutral pH to remove lipids prior to extracting at acid pH to remove SRS-A.

Immediately after collection, each perfusate was examined for histamine and SRS-A by the techniques of Brocklehurst.[1] All had histamine and SRS-A within the ranges previously quoted by Smith.[5] A measured 40 ml of each perfusate was shaken with 400 ml peroxide-free diethyl ether for 10 min. The ether was separated from the aqueous layer and precipitated protein and labelled "Neutral ether extract". Concentrated hydrochloric acid (0·5 ml) was added to the aqueous layer. This precipitated more protein. A further extraction with 400 ml diethyl ether was then performed; and the separated ether layer labelled "acid either extract".

The ether extracts were concentrated to 40 ml under reduced pressure using a rotary evaporator. Aliquots (1 ml) of each were then examined for cholesterol,[7] glyceride[8] and lipid phosphorus.[9] A typical "neutral ether extract" contained 8·75 mg glyceride (calculated as tripalmitin) and 12·5 mg phospholipid. The corresponding "acid ether extract" gave negative tests for cholesterol and lipid phosphorus but a positive test for glyceride.

Pharmacological activity of ether extracts

The extracts were taken to dryness under reduced pressure at 20° using a rotary evaporator. Tyrode solution (40 ml) was then added to the dry residue and the resultant solution treated under vacuum in the rotary evaporator for 10 min. This procedure was found to be necessary for the removal of volatile matter which otherwise exerted a depressant action in subsequent biological tests. (Vacuum treatment of the dry residue was surprisingly ineffective in removing the contamination.) After treatment in the rotary evaporator, the volume of solution was adjusted to 40 ml (the original volume of perfusate) with distilled water.

Neutral and acid ether extracts of perfusates were treated in this way and their SRS-A content compared with that of the perfusates from which they were derived. The comparisons were made using isolated guinea pig ileum suspended in 2 ml aerated Tyrode solution containing mepyramine 10^{-6} M and atropine 10^{-6} M. Part of a typical experiment is shown in Fig. 1.

Figure 1 shows that a neutral ether extract had no detectable mepyramine resistant smooth muscle stimulant activity. The corresponding acid ether extract, on the other hand, contained some smooth muscle stimulant activity, but less than that present in the original perfusate.

Pharmacological activity of acid ether extracts treated with silicic acid or alumina

The acid ether extracts from two perfusates were treated as follows. Each was divided into two 20 ml portions. One portion was taken to dryness at 20° using a rotary evaporator. The residue was dissolved in 20 ml Tyrode solution; treated under reduced pressure in the rotary evaporator for 10 min; and made up to volume with distilled water (acid ether extract). The second portion was stirred with 1 g Malenkrodt silicic acid for 10 min. The separated supernatant was taken to dryness *in vacuo* using a rotary evaporator, dissolved in 20 ml Tyrode solution, treated under vacuum

in the rotary evaporator for 10 min and then made up to volume with distilled water (silicic acid ether supernatant). The separated silicic acid was dried in a cold air stream and then stirred with 20 ml anhydrous methanol for 10 min. The separated methanol supernatant was taken to dryness *in vacuo* using a rotary evaporator. The residue was dissolved in 20 ml Tyrode solution, treated under vacuum in the rotary evaporator for

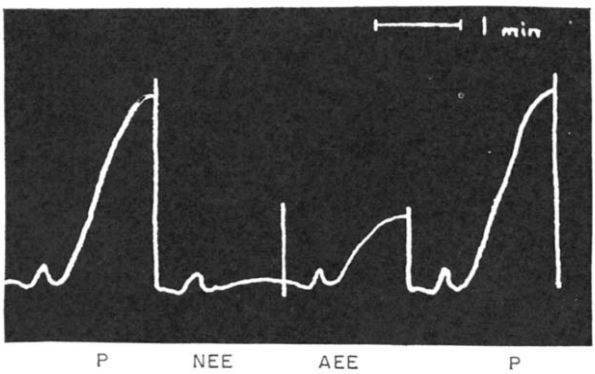

FIG. 1. The pharmacological activity of ether extracts of anaphylactic guinea pig lung perfusate. Isolated guinea pig ileum 37°. Tyrode solution containing mepyramine 10^{-6} M and atropine 10^{-6} M. 2 ml bath. Kymograph speed 16 mm/min. Drum stopped and tissue washed between responses. P = Perfusate. NEE = Neutral ether extract. AEE = Acid ether extract.

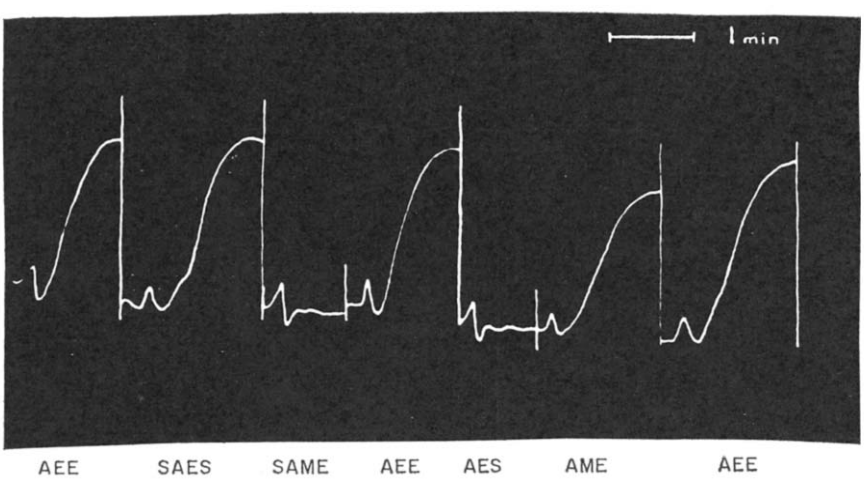

FIG. 2. The pharmacological activity of acid ether extracts of anaphylactic guinea pig lung perfusate. Isolated guinea pig ileum 37°. Tyrode solution containing mepyramine 10^{-6} M and atropine 10^{-6} M. 2 ml bath. Kymograph speed 16 mm/min. Drum stopped and tissue washed between responses. AEE = Acid ether extract. SAES = Silicic acid ether supernatant. SAME = Silicic acid methanol eluate. AES = Alumina ether supernatant. AME = Alumina methanol eluate.

10 min, and then made up to volume with distilled water (silicic acid methanol eluate). Two identical experiments were performed using 1 g chromatographic aluminium oxide instead of silicic acid to produce fractions named alumina ether supernatant and alumina methanol eluate. The pharmacological activities of these fractions are shown in Fig. 2.

It can be concluded that SRS-A was not adsorbed from solution in diethyl ether onto silicic acid. However, SRS-A was adsorbed from solution in diethyl ether by alumina, from which it could be eluted with methanol. It was, therefore, decided to attempt chromatography of SRS-A on silicic acid eluting with increasing concentrations of diethyl ether in petroleum spirit and chromatography of SRS-A on alumina using increasing concentrations of methanol in diethyl ether.

Chromatography on alumina

The acid ether extract of one perfusate was examined by chromatography on alumina. Aluminium oxide (for chromatographic analysis, BDH) was slurried in diethyl ether and poured into a glass chromatography column to form a column of alumina measuring 20 mm × 135 mm. This operation was completed immediately before the ether extractions of the perfusate. The acid ether extract was concentrated *in vacuo* using a rotary evaporator to a volume of 2 ml. It was then added dropwise to filter paper discs (6 discs of 15 mm diameter forming a wad 1 mm thick) whilst the ether was evaporated by blowing cold air from a domestic hair dryer over the discs held in surgical forceps. The dried discs were then dropped onto the top of the column of alumina. Stepwise elution was performed using 300 ml of each of the following eluants: diethyl ether, 1% methanol in diethyl ether, 2% methanol in diethyl ether, 4% methanol in diethyl ether, 8% methanol in diethyl ether, 25% methanol in diethyl ether, 100% methanol. The flow rate was adjusted to approximately 4 ml/min and the elution completed in a single 8-hr run. Using an automatic fraction collector, 20 ml fractions were collected in 100 tubes. Aliquots (1 ml) of each were examined for cholesterol,[7] glyceride[8] and lipid phosphorus.[9] The results are shown in Fig. 3.

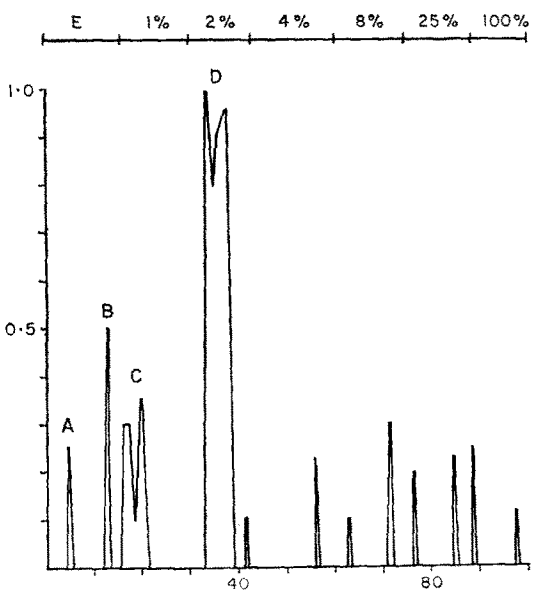

FIG. 3. The chromatography of acid ether extract of anaphylactic guinea pig lung perfusate on alumina. Ordinate = Optical density. Abscissa = Tube number. E = Diethyl ether. 1%, 2%, etc. refer to concentrations of methanol in diethyl ether. All fractions detected by glyceride reaction.[8]

Four fractions of glyceride positive material were eluted (A, B, C and D). These were found in tubes 5, 12, 16–22 and 33–39 respectively. Parts of fractions C and D also gave cholesterol positive reactions. The numerous small peaks of glyceride positive material which followed fraction D were interpreted as an indication that SRS-A was undergoing decomposition and fractions A to D were not examined for pharmacological activity.

Chromatography on silicic acid

The acid ether extracts of four perfusates were examined by silicic acid chromatography. Malenkdrodt silicic acid (100 mesh) was suspended in 4 vols. distilled water. After standing for 10 min, most of the water (and suspended fine particles) was decanted off. The process was repeated twice more, and the washed silicic acid then filtered and oven dried at 110° overnight.[10] The oven-dried material was used to prepare 20 × 130 mm columns and dehydrated by the solvent washes recommended by Hirsch and Ahrens.[11] Conditioning of the prepared column was completed immediately before the ether extractions of the perfusate. The acid ether extract of perfusate was concentrated under reduced pressure with a rotary evaporator and then added dropwise to filter paper discs (6 discs measuring 15 mm in diameter and forming a wad 1 mm thick). The ether was evaporated by blowing cold air over the discs held in surgical forceps. The dried discs were dropped onto the top of the silicic acid column and stepwise elution performed using the following solvents:

1% diethyl ether in petroleum spirit (60–80)	360 ml
4% diethyl ether in petroleum spirit (60–80)	300 ml
8% diethyl ether in petroleum spirit (60–80)	660 ml
25% diethyl ether in petroleum spirit (60–80)	660 ml
100% diethyl ether	200 ml
100% methanol	200 ml

Except for minor adjustments in the volumes of eluant used, this was the technique of Hirsch and Ahrens for separating neutral lipids.[10] The flow rate was adjusted to about 4 ml/min, so that elution was started about noon, stopped at the end of the 8% ether and restarted on the following morning. Using an automatic fraction collector, a total of 140 fractions of 20 ml was collected. Aliquots (1 ml) from each tube were examined for cholesterol,[7] and glyceride.[8] In addition, aliquots (1 ml) from tubes 100 to 124 were examined for lipid phosphorus.[9] The results of one column are shown in Fig. 4.

Several distinct fractions (I to IX) of glyceride positive material were obtained. These were located respectively in tubes 6, 34, 39, 55, 60, 112, 123 and 129. Fraction 80 gave a yellow colour with the cholesterol reagent instead of the expected pink colour. There were no positive reactions for lipid phosphorus.

Pharmacological activity of fractions I to IX from silicic acid chromatography

Immediately after their detection and approximately 1 hr after their elution from silicic acid, fractions I to IX were examined for pharmacological activity. Organic solvent was removed under reduced pressure using a rotary evaporator. The dry residue was dissolved in 10 ml Tyrode solution; treated in the rotary evaporator under

reduced pressure for 10 min to remove the last traces of organic solvent; made up to 10 ml with distilled water; and then examined for SRS-A activity on isolated guinea pig ileum suspended in 2 ml aerated Tyrode solution containing atropine 10^{-6} M and mepyramine 10^{-6} M. Estimates of the number of units of SRS-A activity contained in each fraction were made by comparing the responses obtained with those elicited

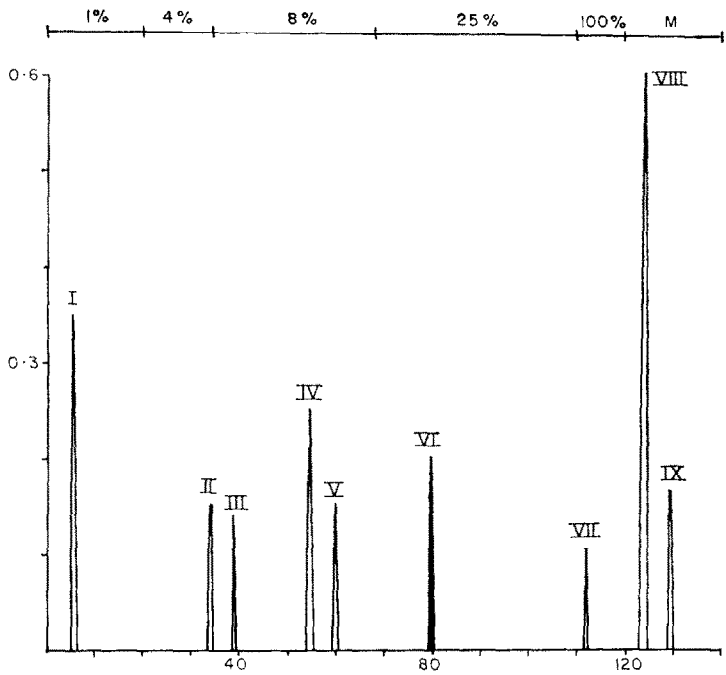

FIG. 4. The chromatography of acid ether extract of anaphylactic guinea pig lung perfusate on silicic acid. Ordinate = Optical density. Abscissa = Tube number. M = Methanol. 1%, 4%, etc. refer to concentrations of diethyl ether in petroleum spirit (60–80). All fractions detected by glyceride reaction except VI which was detected by cholesterol reaction.

by a laboratory standard preparation of SRS-A prepared as described by Brocklehurst.[1] At a concentration of 1 unit/ml this standard produced contractions of unsensitised guinea pig ileum in Tyrode solution equivalent to those elicited by histamine in a concentration of 10 mg/ml. The activity of the column fractions is shown in Figs. 5 and 6.

In all, this separation of an acid ether extract of anaphylactic guinea pig lung perfusate by chromatography on silicic acid has been performed on four occasions. That reported in Figs. 4–6 is the fourth. On each occasion, a perfusate from a single guinea pig lung was used and the same seven active fractions were obtained. The relative amounts of each varied widely from experiment to experiment and on some occasions additional peaks of glycerol positive but pharmacologically inactive material were noted.

When the fourth separation (Fig. 4) had been completed and the pharmacological activity of the detected fractions had been established, all the remaining tubes which

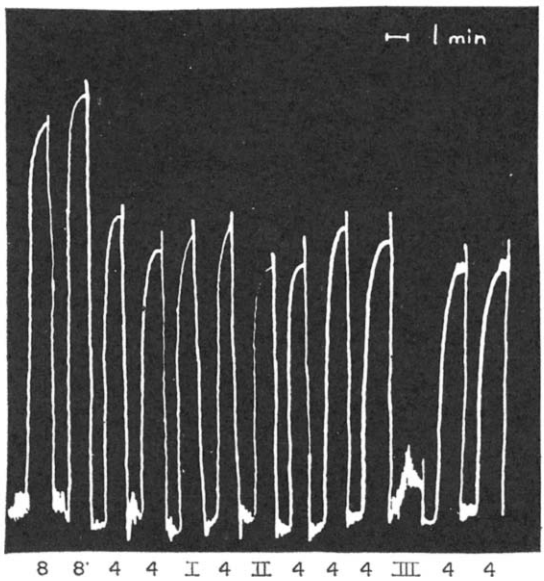

FIG. 5. The pharmacological activity of fractions I to III. Isolated guinea pig ileum 37°. Tyrode solution containing mepyramine 10^{-6} M and atropine 10^{-6} M. 2 ml bath. Kymograph speed = 4 mm/min. Drum stopped and tissue washed between responses. 4 and 8 refer to units/ml standard SRS-A. I, II and III refer to fractions.

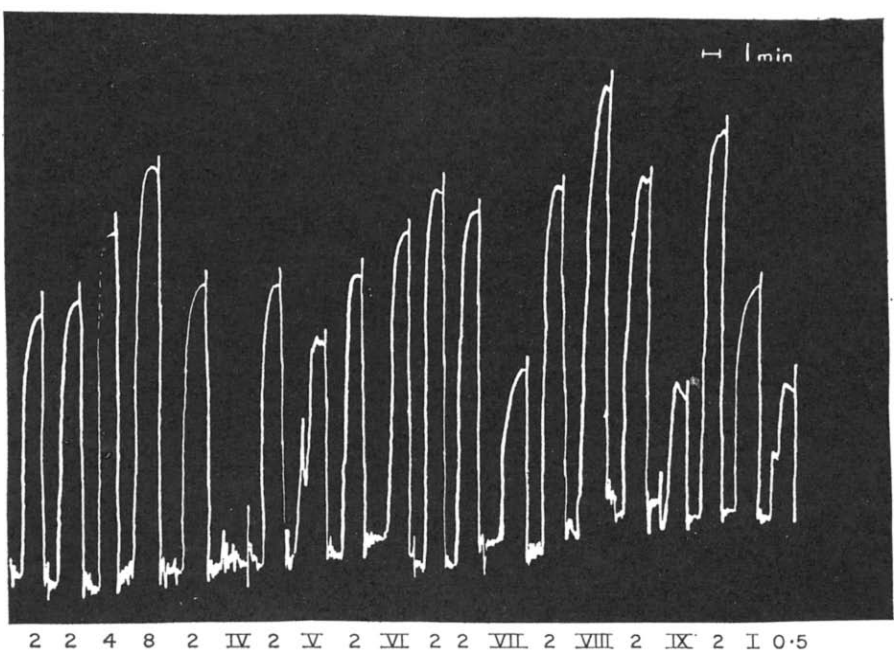

FIG. 6. The pharmacological activity of fractions IV to IX. Isolated guinea pig ileum 37°. Tyrode solution containing mepyramine 10^{-6} M and atropine 10^{-6} M. 2 ml bath. Kymograph speed = 4 mm/min. Drum stopped and tissue washed between responses. 2, 4, 8, etc. refer to units/ml standard SRS-A. IV, V, etc. refer to fractions.

had failed to exhibit chemical reactions were examined for pharmacological activity. None could be detected in any of the tubes, with the exception of tube 81 (which immediately followed fraction VI). This tube contained 5 units of SRS-A and was considered to represent a tail of fraction VI.

Tests for amino-nitrogen were applied to all the fractions exhibiting evidence of glycol structure isolated during the fourth separation, i.e. those shown in Figs. 4–6. All seven active fractions gave positive tests for amino-nitrogen. None was detected in the two inactive fractions. The relative chemical data on each of the active fractions is given in Table 1.

TABLE 1. CHEMICAL ANALYSIS OF PHARMACOLOGICALLY ACTIVE FRACTIONS

Fraction	μmole NH_2	μmole CH_2OH	$—CH_2OH/—NH_2$	Activity per fraction	Total activity (%)
I	0·038	0·116	2·94	37	17·8
II	0·013	0·041	3·14	25	12·0
V	0·038	0·106	3·05	11	5·3
VI	0·20	—	—	26	12·5
VII	0·043	0·045	1·04	8	3·8
VIII	0·028	0·206	7·35	95	45·6
IX	0·026	0·049	1·89	6	2·9
				208	

Column load = 1140 units.
Recovery = 208 units = 18·2 per cent.

DISCUSSION

These experiments provide evidence that the activity of acid ether extracts of anaphylactic guinea pig lung perfusate is not due to the presence in them of a long chain fatty acid or an acidic phospholipid, both of which would have a different chromatographic behaviour from that of the active fractions obtained. Long chain fatty acids are eluted from silicic acid under these chromatographic conditions by 8% diethyl ether in light petroleum.[11] In these experiments they would be expected to be present in tubes 32–40. Of the two peaks eluted in this region, that in tube 34 (fraction II) represented only 12 per cent of the total pharmacological activity eluted from the column, whilst that in tube 39 had no pharmacological activity. An acidic phospholipid would be eluted under these experimental conditions by methanol.[11] However, neither the material in tube 123 (fraction VIII) or tube 129 (fraction IX) gave reactions for lipid phosphorus. Thus whilst there is a possibility that fraction II owed its activity to a long chain fatty acid, the remaining six active fractions cannot possess such a structure.

The test for glyceride used in these experiments involved alkaline hydrolysis of glyceride to fatty acid and glycerol, followed by periodate oxidation of the glycerol so formed. The formaldehyde derived from the two $—CH_2OH$ groups was then coupled to chromotrophic acid and estimated colorimetrically. This test thus detects $—CH_2OH$ groups in a glycol structure and it was noted that the chromatographic fractions exhibiting positive reactions in this test also did so without preliminary

alkaline hydrolysis. Since the test is also capable of detecting —CH_2OH groups in amino-glycols, the test for amino-nitrogen was applied to all fractions giving a positive glyceride reaction. Six of the seven fractions showing pharmacological activity gave positive reactions for both —CH_2OH and —NH_2 groups.

Since the chemical reactivity was confined to tubes which contained pharmacologically active material, there are two possible explanations. Either the structure responsible for the activity was consistently contaminated with material of amino-glycol character, or the active material itself contained both a glycol structure and amino-nitrogen. Since SRS-A partitions into diethyl ether from an aqueous phase only at acid pH, it appears to possess a carboxyl group and has been described as a lipid soluble acid.[2, 3] The activity eluted from silicic acid in these experiments may thus be due to a mixture of related substances, each of which could be described as a polyhydroxyamino carboxylic acid. A possible but not certain exception to this might be fraction VI which failed to exhibit a reaction for glycol —CH_2OH. (This material may not possess a glycol structure with terminal —CH_2OH, or it may have contained either a terminal —CH_2OH which was itself substituted, or a glycol structure was substituted in the position adjacent to the terminal —CH_2OH.) The apparent abundance of terminal —CH_2OH groups in most of the active fractions, especially fraction VIII requires the active structure in these fractions to possess several branches of glycol character. Alternatively it might indicate that the active fractions are glycosides containing a variable number of sugar molecules. Each aldohexose molecule attached by a glycoside link would, for instance, account for one —CH_2OH group.

This particular interpretation of the results is compatible with the suggestion that SRS-A is a mixture of glycosides of neuraminic acid.[12]

The quantities of material isolated in these experiments were insufficient for a reliable study of the structure or structures responsible for the pharmacological activity. In view of the very low recovery (18·2 per cent) of pharmacological activity from silicic acid columns under the experimental conditions reported here, it appears that the isolation of SRS-A in sufficient quantity to establish its structure unequivocally requires the development of alternative separation techniques.

REFERENCES

1. W. E. Brocklehurst, *J. Physiol.* **151**, 416 (1960).
2. N. Chakravarty, *Acta physiol. Scand.* **48**, 167 (1960).
3. W. Vogt, *Pharmacol. Rev.* **10**, 407 (1958).
4. W. G. Smith, *Biochem. Pharmacol.* **8**, 168 (1961).
5. W. G. Smith, *Biochem. Pharmacol.* **11**, 183 (1962).
6. H. Herxheimer, *J. Physiol.* **117**, 251 (1952).
7. H. K. Hanel and H. Dam, *Acta chem. Scand.* **9**, 677 (1955).
8. E. Van Handel and Zilversmit, *J. Lab. Clin. Med.* **50**, 152 (1957).
9. G. R. Bartlett, *J. Biol. Chem.* **234**, 466 (1959).
10. E. O. Titus, Personal Communication (1960).
11. J. Hirsch and E. H. Ahrens, *J. Biol. Chem.* **233**, 211 (1960).
12. W. G. Smith, *Life Sciences* **1**, 133 (1962).