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THE RELEASE OF HISTAMINE AND FORMATION OF A SLOW-REACTING SUBSTANCE (SRS-A) DURING ANAPHYLACTIC SHOCK

BY W. E. BROCKLEHURST

From the National Institute for Medical Research, Mill Hill, London, N.W. 7

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The release of histamine during anaphylaxis has been demonstrated in several species and various tissues, but it is well known that histamine alone cannot satisfactorily account for all the effects on smooth muscle observed during the anaphylactic reaction. The experiments presented in the present paper show that in addition to the release of histamine, the antigen–antibody reaction results in the formation of another smooth-muscle-stimulating substance which causes a slow and long-lasting contraction of the guinea-pig ileum, and which is resistant to anti-histamine drugs. This substance will be referred to as SRS-A.

The existence of a slow-reacting substance in the effluent collected during shock from the perfused lungs of the guinea-pig was recognized by Kellaway & Trethewie (1940). They were, however, unable to separate its effect upon the guinea-pig gut from that of histamine in the effluent, and their evidence for its presence was based on the observation that the active effluent caused a more prolonged contraction of the gut than did histamine alone. The use of anti-histamine drugs now makes it possible to study SRS-A separately.

METHODS

The experiments were performed on perfused lungs or minced tissue from sensitized animals.

Sensitization. Young albino guinea-pigs, usually male, of 200–250 g were given an intraperitoneal and a subcutaneous injection each of 100 mg dried egg albumin, as a 10% solution in saline, containing 0·5% phenol. The animals were killed with N_2O, or by dislocation of the neck, 3–5 weeks later, when they were always found to be strongly sensitized to the antigen.

Young male sandy-lop or albino rabbits were used. A primary antibody response was produced by two intramuscular injections of 25 mg of the antigen, in 1 ml. of adjuvant medium (Freund & McDermott, 1942) given 7 days apart. After 6 weeks, 5 or 6 graded doses of alum-precipitated antigen, rising from 2 to 10 mg, were given intravenously at intervals of 3 days. The animals were killed by dislocation of the neck, or bled out under pentobarbitone, 6–10 days after the last injection.

Rats of both sexes, of a hooded strain maintained at the National Institute for Medical Research, London, and weighing about 120 g, were sensitized similarly to rabbits but with

half doses (Lipton, Stone & Freund, 1956). They were killed 5–8 days after the last injection, by a blow on the head, after which the area of damage was at once isolated by passing a tight band round the neck.

Male rhesus monkeys, weighing about 2·5 kg, were sensitized by 50 mg antigen, in 2 ml. of Freund's adjuvant medium, given intramuscularly in two portions deep beneath each scapula, followed after 8 days by 80 mg of soluble antigen subcutaneously. Three weeks later the animals were bled out under pentobarbitone.

Tissue from a sensitized goat was kindly provided by Dr J. H. Humphrey. The course of sensitization was similar to that described for rabbits, and the animal was bled out under pentobarbitone 10 days after the last dose of antigen.

The lungs of a horse which had been used for the commercial production of anti-diphtheria serum were kindly provided by Dr A. C. White (Messrs Burroughs Wellcome, Beckenham). The animal had a high titre of circulating antibody, and was bled out under chloral hydrate anaesthesia.

On two occasions it was possible to obtain human lung removed from asthmatic subjects at operation. On one occasion, the tissue was immediately placed in a large volume of aerated Krebs–Henseleit (1932) solution at about 4° C. On the other, the tissue was kept wrapped in gauze at about 20° C for 1 hr before it was placed in aerated Tyrode solution at about 10° C. Both pieces of lung were obtained through the good offices of Dr A. W. Frankland of the Wright–Fleming Institute, who also provided details of the case histories and performed skin tests on the patients. Only those segments of the lobe which showed no gross evidence of disease were taken.

Lung perfusion. The intact lungs of the guinea-pig, rat, rhesus monkey and rabbit were perfused through the pulmonary artery via a flexible cannula which was passed through the right ventricle and was tied in well proximal to the bifurcation of the artery. The whole tissue was suspended by the trachea, inside a jacketed vessel which was maintained at 37° C and was lightly closed at the top. The bottom of the vessel was funnel-shaped, to collect the effluent solution. Perfusion fluid was supplied from a Marriotte bottle, and passed through a warming coil to raise its temperature to 37° C, immediately before it entered the cannula. Antigen solutions were warmed to 37° C, and injected into the perfusion fluid through a short rubber connexion between the warming coil and the cannula. In some experiments the left auricle was cannulated for the collection of the effluent, but as this gave no apparent advantage the fluid was usually allowed to flow out through an incision in the auricle. The lungs were usually air-perfused (Arunlakshana & Schild, 1950), but sometimes simply inflated through a cannula in the trachea. The rate of air perfusion was adjusted to maintain an initial pressure of 5–10 mm Hg, at the trachea; the air escaped from the alveoli through light scarifications made with a sharp triangular needle on the surface of the inflated lungs. Broncho-constriction was registered as an increased pressure difference across the lung. During the anaphylactic reaction broncho-constriction is intense and air flow becomes negligible. Air perfusion was usually stopped inmediately after injecting the antigen, or alternatively the pressure was limited to 40 mm Hg, in order to avoid interference with the flow of perfusion fluid through the blood vessels, and damage to the tissue proximal to the broncho-constriction, by over-distension with air. Unless otherwise stated, perfusion of the lungs of guinea-pigs with Tyrode solution began between 5 and 8 min after death, at a rate of about 10 ml./min. After 2 or 3 min the rate was reduced to 2 or 3 ml./min, the antigen being added to the inflow 15–20 min later. The lungs of other species were similarly flushed free from blood, and then perfused at 1–2 ml./min/g tissue. In the case of the monkey and horse, where the lung could not be perfused within 10 min of death, the tissue was either left undisturbed in the animal for a period of not more than 10 min, or was removed with minimal handling and kept in oxygenated Tyrode or Krebs–Henseleit solution at about 10° C for not longer than 1 hr. Samples of perfusate were collected into test-tubes and immediately chilled in ice. In some experiments, the collecting tube was

surrounded by ice, but this did not seem to be necessary. The samples were then centrifuged, and if not immediately used were stored at −5 to −15° C.

Minced tissue. Sensitized guinea-pigs were killed by breaking the neck, and at once perfused by two routes, one cannula entering the pulmonary artery, and another entering the aorta; both auricles were cut open. Tyrode solution at 37° C, supplied at a pressure of 30–40 cm water, was used for perfusion, which continued for 10 min. Samples of various tissues were then removed, and after being lightly blotted and weighed, were placed in previously aerated Tyrode solution at 0–2° C. The samples weighed between 190 and 210 mg, except in the cases of aorta, vena cava and suprarenal cortex, where the available tissue weighed only 90–150 mg. The tissues were cut up with sharp scissors whilst still in this solution, to give particles of about 1 mm diameter, washed and incubated for 10 min at 37° C in 5 ml. Tyrode solution with oxygen bubbled through it. The Tyrode solution was then drained off and replaced by 2 ml. of fresh Tyrode solution at 37° C and incubated whilst being rocked and aerated as described by Mongar & Schild (1956). After 5 min the solution was removed, kept for assay of the spontaneous release of active substances, and at once replaced by 2 ml. of Tyrode solution containing antigen (ovalbumin) 50 μg/ml. and incubated similarly for a further 5 min, after which the solution was removed from the tissue and kept at 0° C for assay the same day.

Extraction of SRS-A from lung tissue. In some experiments SRS-A was extracted from lung tissue of the guinea-pig. Since SRS-A present in freeze-dried samples of perfusate was found to be readily soluble in 75 % aqueous ethanol, this solvent was used to make protein-free extracts of tissue. The lung was lightly blotted and weighed, then dropped into absolute ethanol chilled in ice, and cut into small pieces with sharp scissors. Absolute ethanol 3 ml./g tissue was used and later adjustments in volume were made with 75 % ethanol. The tissue was then ground up thoroughly with sand and allowed to stand at 22° C for 1 hr, after which the solution was filtered off and made up to volume through the filter. This extract was evaporated to dryness under reduced pressure, then 2 ml. distilled water and 10 ml. Tyrode solution were added before biological assay.

Biological assay. Assays for histamine and for SRS-A were routinely performed on a portion of the terminal ileum of guinea-pigs of 300–500 g. The tissue was suspended in oxygenated Tyrode solution at 37° C in an automatic apparatus, as described by Boura, Mongar & Schild (1954). A light frontal-writing lever system was used which exerted a pull of 0·5 g on the tissue, and gave a magnification of about × 5 on the kymograph.

Atropine 10^{-6} or 5×10^{-7} M was added to the Tyrode solution in the reservoir supplying the bath. Histamine acid phosphate was used to make the standard solutions, all values being expressed as histamine base. The assay for SRS-A was carried out in the presence of mepyramine 10^{-6} M. The values for SRS-A were expressed in arbitrary units, referring to 1/20 ml. of a standard sample of freeze-dried perfusate. This reference sample lost potency very slowly during storage; thus the unit is not constant throughout the whole series of experiments but permits valid comparisons within each group of experiments. A concentration of 1 unit/ml. was convenient for assays on the guinea-pig ileum.

Preparation of serum. In order to find out whether serum influences the output of histamine and SRS-A from perfused guinea-pig lung during the antigen–antibody reaction, guinea-pig serum was prepared as follows. Blood was taken by cardiac puncture, through a wide-bore needle coated with silicone, into a chilled, siliconed syringe. It was at once gently expelled into a siliconed centrifuge tube surrounded by ice, and then centrifuged at 2–6° C for 20 min at 1600 *g* or more. About two-thirds of the plasma was removed with a pipette, avoiding the plasma near the buffy coat and the uppermost portion. The plasma was allowed to clot at room temperature in a test-tube which contained a roughened glass rod. When the clot was firm (after about 15 min), the serum was expressed from the fibrin by gentle pressure between the rod and the wall of the tube. It was stored at 0° C for not more than 2 days. Serum so obtained was inactive on the guinea-pig's ileum when freshly

diluted with 3 volumes of Tyrode solution, but caused a slow contraction after being incubated with 10 volumes of Tyrode solution for 30 min.

Examination of guinea-pig blood for platelets. Some experiments were performed on the perfused lungs of guinea-pigs which had been depleted of their platelets by treatment with anti-platelet serum (Humphrey, 1955). In order to check this depletion, the blood was examined by the following procedure. 2 ml. of blood was withdrawn from the heart through a wide-bore needle coated with silicone, into a paraffin-coated syringe containing 2 ml. of 10% versene (di-sodium salt of ethylene-diaminetetraacetic acid) in normal saline, at pH 7·5, the whole having been chilled in ice. The blood and versene-saline were mixed at once, and after being transferred to a siliconed tube cooled in ice, were centrifuged at about 1600 *g* for 20 min at between 2 and 6° C. The entire buffy coat and 'carpet' of platelets was then cleanly removed with a pipette, and resuspended in cold versene-saline. A portion of this suspension was mixed with a solution containing formaldehyde and brilliant cresol blue, and examined in a Thoma haemocytometer cell.

RESULTS

Experiments on the perfused guinea-pig lung

The effluent collected from the perfused lung of a sensitized guinea-pig, after arterial injection of antigen, contained two substances which contracted the atropinized guinea-pig ileum. One substance was histamine, the other produced a much slower contraction and will be referred to as SRS-A. Figure 1 shows the assay of the effluent collected during the fifth minute after an injection of egg albumin to the perfused sensitized

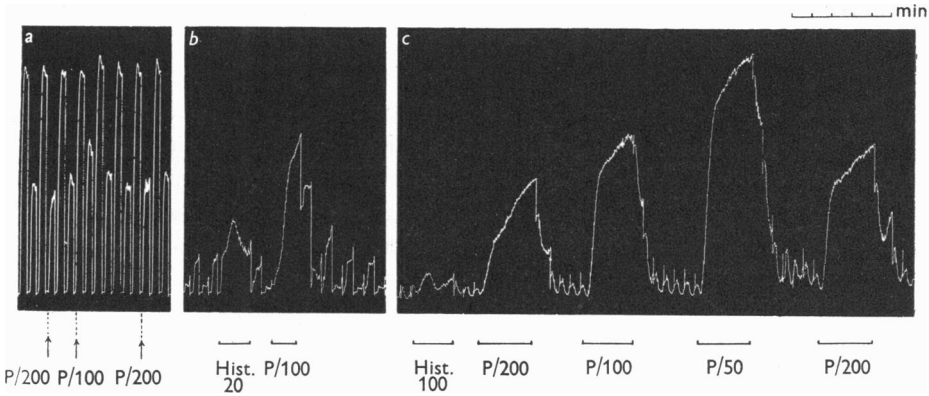

Fig. 1. The use of mepyramine to demonstrate the presence of both histamine and SRS-A in the perfusate (P) from shocked guinea-pig lung. The record shows the contractions of guinea-pig ileum suspended in Tyrode solution containing atropine 5×10^{-7} M. The concentrations of mepyramine used in (*b*) and (*c*) were present in the bath for 15 min beforehand, and at all times during the record. The kymograph began to move just before the dose of active substances was placed in the bath, and stopped when the bath was drained. *a*, Unlabelled contractions were produced by histamine 5 and 10 ng/ml. with a normal contact time (25 sec). *b*, In the presence of mepyramine 2×10^{-10} M, histamine 10 and 20 ng/ml. At 'Hist. 20' the dose was left in the bath for a longer time. *c*, In the presence of mepyramine 10^{-6} M. Movements recorded on base line are due to washing and refilling bath.

lung. This is a representative sample, containing only a trace of the antigen and avoiding most of the initial flood of histamine. Figure 1*a* shows that the histamine equivalent of the sample was approximately 1 μg/ml. When the test was repeated, in the presence of mepyramine 10^{-6} M (Fig. 1*c*), the perfusate sample caused a slow, well maintained contraction, whereas histamine alone in equivalent doses had no effect. This contraction must, therefore, be due to SRS-A, which is shown to have a rather flat dose–response slope, and to exert a prolonged effect so that the tissue becomes increasingly responsive to it. In Fig. 1*b* the effect of histamine 10 ng/ml. has been almost abolished by mepyramine 2×10^{-10} M, so as to show that the slower contraction caused by SRS-A adds on to that caused by histamine. This figure also shows that the effect of SRS-A is persistent, causing an enhanced response to histamine for several minutes following a dose of perfusate, even though the bath fluid was changed twice after each contraction. This persistence and summation of action is also seen in Fig. 1*a* and makes it probable that the histamine equivalent of the perfusate is slightly higher than its true histamine content, although at the dilutions used the histamine contraction has reached maximum before the SRS-A contraction has begun. The effect of SRS-A was not attenuated by mepyramine, since comparable contractions were produced in the presence of concentrations of 10^{-4} M and 10^{-7} M.

The presence of SRS-A in the perfusate collected during the first 2 or 3 min did not seriously interfere with the assay of histamine on the atropinized guinea-pig's gut. These samples contained upwards of 1 μg histamine/ml. and less than 50 units of SRS-A/μg histamine: in later samples the presence of a greater proportion of SRS-A tended to make the estimates of the histamine content too high. The magnitude of this interference was demonstrated when samples of perfusate were re-assayed after acid hydrolysis. The method employed (Code, 1937) destroys SRS-A but not histamine. From the results plotted in Fig. 2 it is clear that the histamine values of the first samples containing histamine 2–5 μg/ml. were practically the same whether assayed without treatment or after acid hydrolysis, but in all later samples the values obtained after acid treatment were lower. Sometimes these values were only 50 % of those obtained before treatment, whereas acid hydrolysis of comparable solutions of histamine acid phosphate (0·2 μg/ml.) entailed a loss of 10–15 % of the biological activity. The discrepancy of up to 40 % must be attributed to interference by SRS-A.

Polypeptides with smooth-muscle-stimulating activity are known to be formed after plasma has been diluted (Schachter, 1956) or kept in contact with glass (Armstrong, Jepson, Keele & Stewart, 1957). Perfusate assayed within a few seconds of leaving the lung had the same SRS-A

activity as perfusate stored for several hours at 0 or 20° C, showing that SRS-A enters the perfusion fluid during its passage through the lung, and does not develop after collection. This finding excludes the possibility that enzymes which might be released from the lung during anaphylaxis, or might become activated as a result of the dilution of tissue fluids, might then form SRS-A from some substrate in the perfusate after collection, and points to the lung tissue as the source of SRS-A.

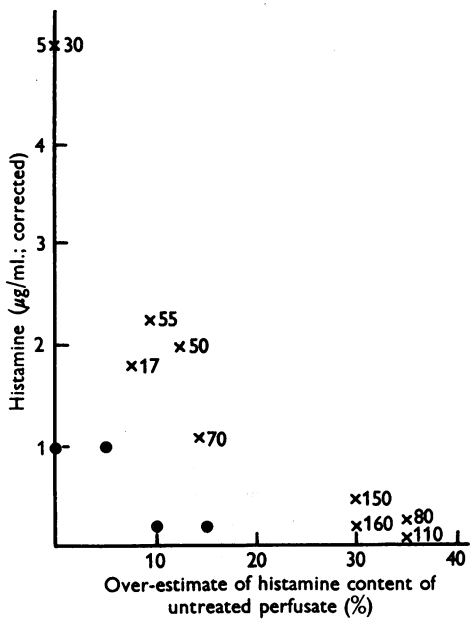

Fig. 2. The effect of SRS-A on the accuracy of the biological assay of histamine. Crosses refer to the percentage difference in the histamine assay attributable to the presence of SRS-A. The figures refer to the number of units of SRS-A, per microgram histamine, present in the various samples of perfusate. The circles ● represent the loss of biologically detectable histamine from histamine acid phosphate on acid hydrolysis. (Losses of this order have been allowed for in the estimation of the true histamine content of perfusate.)

Time course of release of histamine and the appearance of SRS-A in the effluent. In order to estimate the concentration of histamine and SRS-A in successive samples of perfusate collected from the lung after the injection of antigen, the samples were first assayed for histamine and the assay was then repeated for SRS-A in the presence of mepyramine 10^{-6} M. Owing to the dead space of the inflow cannula and the pulmonary artery, antigen injected during perfusion at the usual rate did not reach the capillaries of the lung until about 5 sec later. This was evident when dye was injected. Since histamine and SRS-A were present in the effluent collected during

the first 30 sec after the injection of antigen, both substances must have left the tissue within 25 sec of the arrival of the antigen. Figure 3 shows that the maximal rate of histamine release was reached at about 1 min, whereas the maximal release of SRS-A occurred between the second and third minute. The figure also illustrates that the output of SRS-A decreases more slowly than that of histamine, and that appreciable amounts of SRS-A are still present 1 hr after the reaction with antigen, when histamine is no longer detectable in the effluent.

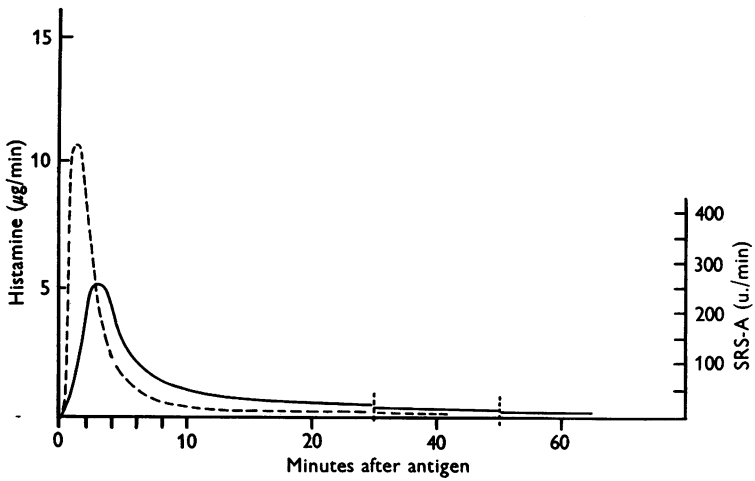

Fig. 3. Time course of the effluence of histamine and SRS-A from guinea-pig lung after the arrival of antigen: compiled from 15 experiments, each lung weighing 2–3 g. Histamine – – –, SRS-A ——. SRS-A 1 u./ml. usually caused a contraction of the guinea-pig's ileum equal to between 5 and 7 ng histamine/ml. In terms of activity in this tissue, the graph magnifies the effect of SRS-A about × 3.

The lung tissue as the source of SRS-A. The results of the following three series of experiments show that the SRS-A in the effluent is not derived from constituents of blood which might be present in the perfused lung at the time the antigen is given.

(*a*) The lungs from matched pairs of litter-mate guinea-pigs were perfused for different times or at different rates, so that one was more thoroughly freed from blood than the other. The amounts of SRS-A and histamine which were released from the two lungs by antigen, under otherwise similar conditions, were compared with each other. The presence of an appreciable amount of blood in the lung when the antigen was administered always decreased the amounts of both substances released; the yield of SRS-A was reduced rather more than that of histamine. It is thus unlikely that SRS-A was derived from the blood. When the lungs were perfused for 30 min or longer, or when the rate of perfusion exceeded

5 ml./min for 15 min or longer, the yields of both SRS-A and histamine were decreased, the decrease of SRS-A being greater than that of histamine. Optimum yields of both substances were obtained when the lungs were perfused at a rate of 2–4 ml./min for 15–20 min before the addition of the antigen. These conditions were therefore adopted in subsequent experiments.

(*b*) The addition of serum to the perfusion fluid did not affect the output of either SRS-A or histamine during anaphylactic shock. This was first shown when the lungs were perfused both before and after the addition of antigen, with Tyrode solution containing 5 or 10 % freshly diluted serum,

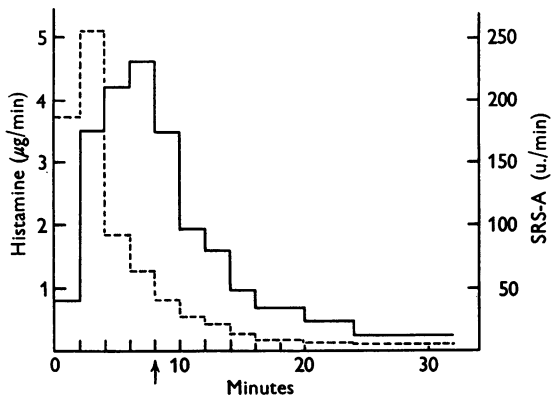

Fig. 4. The effect of adding guinea-pig serum at the arrow, to the perfusion during anaphylaxis produced by a steady level of antigen in the perfusing fluid.

and the total yields of histamine and SRS-A during 15 min compared with the yields from lungs perfused with Tyrode solution. When Ungar (1956) produced results showing that the histamine yield was increased when serum was added during the continuous administration of a low concentration of antigen, experiments based on this procedure were performed. After preliminary perfusion with Tyrode solution for 15 min, antigen in a concentration of 5 μg/ml. was added to the perfusion fluid. In the following 8 min the output of SRS-A reached its maximum. Perfusion was then continued with Tyrode solution containing 10 % of fresh serum from normal guinea-pigs, as well as the antigen. Figure 4 shows that in these experiments the output of SRS-A did not increase as a result of adding serum, but continued its normal decline.

(*c*) Substances which stimulate smooth muscle are present in platelets. To exclude the possibility that SRS-A might be derived from platelets adherent to the capillaries of perfused lungs, guinea-pigs were depleted of their plateletes by an intraperitoneal injection of 0·2 ml. of anti-platelet serum, 2 or 3 days before the lungs were used. Blood obtained by cardiac

puncture from treated animals contained 1 platelet or less/5 leucocytes, compared with more than 50 platelets/5 leucocytes in the untreated animals. Since the haematocrit was also considerably reduced, the loss of platelets per millilitre of blood was probably even higher. In spite of this great reduction in platelets, the addition of antigen to the perfusion fluid led to a substantial output of SRS-A. This was only 50–60 % of that obtained from the untreated controls, but this reduction cannot be related to the reduction in platelets. The reduction may be due to the damage which anti-platelet serum causes either directly or indirectly to capillaries throughout the lung, as was made manifest by the presence of haemorrhagic patches. These patches are probably perfused incompletely or not at all, and are therefore not subjected to the full effect of the antigen.

(*d*) In routine perfusions the heart is left attached to the lungs during perfusion and shock, and might contribute to the yields of histamine and SRS-A. When the heart alone was perfused via the coronary arteries, the average yield in three experiments was 2·5 μg histamine and 75 units SRS-A per gram of tissue. Only a fraction of this yield would be expected during lung perfusion in which the antigen makes only superficial contact with the heart.

Formation of SRS-A. The antigen–antibody reaction releases histamine from the stores of tissue histamine, but the SRS-A which appears in the effluent of the perfused lung is actually formed in the lung tissues. This is evident from experiments in which extracts of shocked and unshocked lung were tested on the mepyramine-treated guinea-pig ileum. For this purpose the right and left lungs were both perfused, but the antigen was added

TABLE 1. The histamine and SRS-A content of sensitized guinea-pig lung, and its effluent perfusate, before and after anaphylactic shock. Mean of six experiments

	Histamine (μg/g lung)		SRS-A equivalent (u./g lung)	
	Tissue	Perfusate	Tissue	Perfusate
Before antigen	17·5	0	350	0
After antigen	8·2	7·0	800	290

to only one side. Both the lungs were then extracted by grinding the tissue in ethanol, to give a final concentration of 10 ml. of 75 % alcohol/g tissue. All the extracts caused the mepyramine-treated gut to contract, but those from the shocked tissue were always more active than those from the corresponding unshocked half of the lung. In addition, the perfusate from the shocked lung contained SRS-A, whereas that from the control lung did not. The activity from the control lung was probably not due to SRS-A but to potassium and/or other normal constituents of the tissue.

The results, given in Table 1, show that SRS-A is formed within the

perfused lung by the antigen–antibody reaction, that some remains in the tissue and can be extracted from it. In contrast, the appearance of histamine in the effluent from the shocked lung is associated with a diminution of the histamine content of the tissue.

Experiments on the lungs of other species

Rabbit. Although the blood is generally considered to be the shock organ for anaphylaxis in the rabbit, the injection of antigen at the arterial inflow released both histamine and SRS-A from the perfused lungs of sensitized animals. The amounts of histamine released were relatively small, and varied in different experiments; they bore some relationship to the levels of circulating antibody, and were therefore presumed to reflect varying degrees of sensitization. Purified diphtheria toxoid, bovine serum albumin, crystalline ovalbumin, and crude egg albumin were all effective antigens. The mean yield of histamine from four strongly sensitized animals was 0·7 μg/g lung, and the corresponding yield of SRS-A was 46 units/g. The time course of histamine release differs from that observed in guinea-pig lung. The output does not reach its maximum rate until 2 or 3 min after the antigen. By the second minute the output of SRS-A is already high and remains so for upwards of 5 min, reaching a maximum 3–4 min after the injection of antigen. In one experiment SRS-A was present in the effluent 1 min before any histamine could be detected.

Rhesus monkey. The lungs of two monkeys were perfused. In each, the injection of antigen at the arterial inflow caused the appearance of both histamine and SRS-A in the effluent from the shocked lungs. The results are presented in Table 2. The amounts of histamine released were 2 and

TABLE 2. The histamine and SRS-A content of the perfusate from the isolated lungs of Rhesus monkeys during anaphylactic shock

	Experiment 1		Experiment 2	
Perfusion period (min)	Histamine (μg/min)	SRS-A (u./min)	Histamine (μg/min)	SRS-A (u./min)
Control	0	< 1	0	0
0–3	2·6	4	7·0	5
4–10	3·3	85	5·7	185
11–20	1·0	50	1·2	90
21–30	0·2	20	0·6	80
Total yield per gram lung in 30 min	2 μg	70 u.	5 μg	350 u.

The tissue was challenged with 30 mg antigen at time 0. A further 10 mg was given at the 7th minute in Expt. 1, and at the 20th minute in Expt. 2.

5 μg respectively, per gram fresh weight of lung. This is lower than the yield obtained from guinea-pig lung but represents a considerable total yield, since the lungs weigh about 20 g. The time course of histamine release

in the two experiments resembled that of rabbit. The amounts of SRS-A and histamine in the perfusate were in about the same proportion as from the guinea-pig lung. For instance, a release of 5 μg of histamine per gram would also have been associated in the guinea-pig lung with a release of SRS-A of the order of 300 units/g.

Man. On the two occasions when it was possible to perfuse segments of a lobe of lung obtained during operation, the allergens responsible for the asthma had been identified clinically. Both patients had been treated with antibiotics for tuberculosis, and although the disease was no longer active, lobectomy was performed because large cavities remained in the

TABLE 3. The histamine and SRS-A content of the perfusate from human asthmatic lung, when challenged with specific allergens

Perfusion period (min)	Histamine (μg/min)	SRS-A (u./min)
Before antigen	0	0
(→ Birch pollen)		
0–1	0·5	0
2–4	30	870
5–10	9	750
11–28	5	520
29–31	2	280
(→ Birch pollen)		
32	2	480
33–35	2	320
36–41	1·6	240
42–47	0·9	200
(→ Timothy pollen)		
48	1	200
49–51	1·5	340
52–75	0·6	165
76–78	0·3	130
Total yield	285 μg	27,000 u.

Perfusion of a 200 g segment from the middle left lobe of a woman allergic to birch tree and timothy grass pollens. 50 mg soluble allergens in 0·5 ml. saline, injected into inflow at time 0, and at the start of the 32nd and 48th minutes, as indicated.

lobe. Some segments of the removed lobe appeared to be healthy in both instances: these were dissected out and perfused with Krebs–Henseleit solution. When, after a preliminary period of perfusion, the allergens to which the patients had been sensitive were added to the perfusion fluid as it entered the lung, histamine and SRS-A appeared in the effluent. Table 3 gives the results of one of the experiments in detail. The segment was perfused 1½ hr after the lobe had been removed. During the whole of this time the tissue was stored in oxygenated Krebs–Henseleit solution at 4–10° C. A few minutes after perfusion was started the effluent became clear of blood, and the whole segment appeared to be uniformly perfused. The effluent collected before the addition of the allergen caused no contraction of the guinea-pig's ileum, but after the allergen (birch pollen) the

effluent contained considerable amounts of both histamine and SRS-A. The histamine was first detected 1 min after the allergen, but SRS-A could not be detected with certainty at that time, since the sample contained pollen solution which interfered with the assay. The sample collected during the second, third and fourth minutes contained 1·2 μg histamine and 75 units of SRS-A/ml., corresponding to an output of 30 μg/min and 900 units/min during this period. In subsequent samples the concentration of both substances decreased. When a second injection of birch pollen was given half an hour later, the output of both substances increased only slightly. The patient had also been sensitive to the pollen of timothy grass, and when a solution of this allergen was injected, a further small additional release of histamine and SRS-A occurred. The total amount of histamine released during the half hour following challenge with birch pollen was about 245 μg, and a further yield of 40 μg was obtained in the next 47 min, during which the two further doses of allergens were injected. The corresponding figures for SRS-A, expressed in arbitrary units, were 17,300 and 10,000. Since the segment of lung weighed approximately 200 g, the total release of histamine corresponded to 1·4 μg/g of tissue, and that of SRS-A to 135 units/g.

A small amount of tissue from the same lobe was used to test the reaction to the two allergens by the diffusion method (Mongar & Schild, 1956). When particles of chopped tissue were suspended in oxygenated Tyrode solution containing the allergen, both histamine and SRS-A were released (W. E. Brocklehurst & J. L. Mongar, unpublished).

Two of the allergens to which the second specimen of lung was sensitive were grass pollen and cat scurf. The perfusion of this lung specimen was incomplete and the tissue contained clotted blood, since storage after the lobectomy had been unsatisfactory. Grass pollen was tested first; it caused the release of both histamine and SRS-A. The subsequent injection of cat scurf led to a renewed output of histamine and SRS-A in the effluent, showing that the grass pollen had not desensitized the tissue against cat scurf. From a part of this specimen which was not used for perfusion bronchioles were dissected and chains of rings set up as isolated tissues, in Krebs–Henseleit solution. The addition of specific allergens to the bath caused contraction of the smooth muscle of the bronchioles, and both histamine and SRS-A were found in the bath fluid, when tested on the guinea-pig ileum.

Rat. The lungs of rats sensitized to crystalline ovalbumin or to human serum albumin were perfused. An injection at the arterial inflow of 10 mg of the antigen did not cause the appearance in the venous effluent of either histamine or SRS-A. This was not due to inadequate sensitization of the animals, since at the time of the experiment the blood contained between

1 and 6 mg of antibodies/ml., and intradermal injections of the antigen regularly produced local anaphylactic reactions in animals of the same group. Furthermore, the final intravenous dose of antigen given in the course of sensitization had in all cases produced prostration characteristic of anaphylactic shock. In a few experiments in which the liver or hind quarters of sensitized rats were similarly perfused, the addition of the antigen likewise failed to cause the appearance of histamine or SRS-A in the venous effluent.

Goat. The lungs of one goat, sensitized to rabbit serum albumin, were perfused after the animal had been bled out under pentobarbitone anaesthesia. No histamine or SRS-A appeared in the venous effluent after the addition to the perfusion fluid of 10 μg of the antigen/ml. At the time of the experiment the blood contained 5 mg circulating antibodies/ml., and mild anaphylaxis had been observed during the course of sensitization.

Horse. Two separate segments of the lungs from a hyper-immunized horse were perfused free from blood shortly after death. In both perfusions the injection of 2 ml of the antigen solution (a formolized culture of *Corynebacterium diphtheriae*) at the arterial inflow failed to cause the appearance of histamine or SRS-A in the venous effluent.

Experiments on minced tissues of the guinea-pig

The diffusion technique of Mongar & Schild (1956) was used to show that, in various tissues of the guinea-pig, the antigen–antibody reaction leads to the appearance of SRS-A in the Tyrode solution in which the tissue particles are suspended. The results are given in Table 4. The experimental procedure differed slightly from that of Mongar & Schild in that the tissues were perfused *in situ* to remove blood before being minced. This was necessary in order to prevent undue interference with the assay of SRS-A by plasma kinins and other smooth-muscle-contracting substances derived from the blood. The histamine released into the bathing fluid was also assayed, and the results are included in the Table.

The control values were obtained by incubation of the washed tissue fragments for 5 min in Tyrode solution without the addition of antigen. During this period some histamine was released into the bathing fluid from most tissues; the same result had been obtained by Mongar & Schild. With the exception of the lung and submaxillary gland, no substance resembling SRS-A was found in this bathing fluid, and in both these cases the amount was very small. After the Tyrode solution was removed from the tissue, the fragments were resuspended in Tyrode solution containing the antigen (ovalbumin, 50 μg/ml.), and again incubated for 5 min. With the exception of striated muscle fibres, liver and cerebral hemisphere, all tissues released histamine into the bathing fluid. The largest amounts of

histamine were released from the lung tissue, then in descending order, from vena cava, aorta, ileum, trachea and suprarenals. The histamine released from the sample of diaphragm did not originate from the muscle, because when muscle freed from pleura and peritoneum was minced and incubated with the antigen no histamine was released.

The quantities of histamine and of SRS-A appeared to be related in a few tissues, but this was not general. By far the highest amounts of both substances were obtained from the lung tissue. The vena cava, which released about 14% of the amount of histamine released by the lung,

TABLE 4. The amounts of histamine and SRS-A obtained by diffusion from various tissues of a sensitized guinea-pig. All tissues were perfused to remove blood before being cut into uniform fragments

		Before antigen		Antigen present	
Tissue	No. of expts.	Histamine (ng/g)	SRS-A (u./g)	Histamine (ng/g)	SRS-A (u./g)
Lung	7	200	< 10	7000	300
Trachea	5	30	0	500	0–25*
Ileum	5	60	0	600	0
Ileum without mucosa	2	100	0	500	0
Uterus	3	20	0	200	20
Uterus without endometrium	1	30	0	100	10
Aorta	7	60	0	800	40
Great veins	3	150	0	1000	40
Diaphragm	2	30	0	300	20
Diaphragm muscle only	2	0	0	0	0
Striped muscle (sartorius)	1	0	0	0	0
Skin	3	50	0	70	0
Liver	3	< 10	0	< 10	0
Spleen	6	0	0	200	0–5
Pancreas	4	< 10	0	250	10
Suprarenal	2	100†	0	500†	?5†
Suprarenal cortex	1	100	0	100	0
Submaxillary gland	2	10	<2	80	8
Cerebral hemisphere	1	0	0	0	0

* Variable. † Inhibitor substance (?adrenaline) present.

yielded about 13% of the SRS-A found in lung samples; figures for aorta were comparable. On the other hand, the bath fluid of ileum and trachea usually contained no detectable SRS-A, although relatively large amounts of histamine were released. A number of other tissues, which included salivary gland, pancreas and uterus, released relatively little histamine, yet the bath fluid always contained measurable amounts of SRS-A. The bath fluid from those tissues which released no histamine (liver, brain, striated muscle) did not contain SRS-A. Skin released only small amounts of histamine, and no SRS-A could be detected.

The bath fluid from some shocked tissues caused the mepyramine-treated gut to contract in a manner not characteristic of SRS-A. The type of contractions produced suggested that the active substance released from

skin may have been potassium, and that from the ileum might have been 5-hydroxytryptamine. The amounts were small. Samples from the suprarenals contained inhibitory substances, probably sympathomimetic amines, and produced contractions which resembled those due to SRS-A to which a trace of adrenaline had been added.

The absence of SRS-A in the bath fluid from some minced tissues incubated with antigen is not absolute proof that anaphylaxis does not normally lead to the formation of SRS-A in these tissues, because the method is less suitable for the detection of SRS-A than the method of perfusing whole organs. This was shown for the ileum. In three experiments the entire ileum from a sensitized guinea-pig was perfused through its artery, and the venous effluent was collected, precautions being taken not to collect fluid coming from the intestinal lumen. The effluent collected after the addition of antigen to the perfusion fluid contained readily detectable amounts of SRS-A, as well as histamine, but no 5-HT was found.

TABLE 5. The amounts of histamine and SRS-A obtained from guinea-pig ileum during anaphylaxis *in vitro*, by perfusion or diffusion

	Time (min)	Histamine (ng/g)	SRS-A (u./g)
Perfusion	0–10	50	3
	0–10	140	2
	0–5	250	6
	6–15	10	5
Diffusion	0–10	160	$< \frac{1}{2}$
	0–10	150	$< \frac{1}{2}$
	0–5	640	< 1
	0–5	600	$?1\frac{1}{2}$

Antigen 10^{-4} g/ml. present continuously from time 0.

DISCUSSION

The present experiments on perfused and minced tissues of sensitized animals show that the antigen–antibody reaction leads not only to the release of histamine but also to the formation of a slow-reacting smooth-muscle-stimulating substance. Release of histamine and appearance of SRS-A, however, do not run parallel in all tissues.

Although the presence of a slow-reacting substance in the perfusate from sensitized lungs of guinea-pigs had previously been recognized by Kellaway & Trethewie (1940), they were unable to separate its effect from that of histamine, since at that time anti-histamine drugs were not available. In the present experiments mepyramine has been used to abolish the effect of histamine and reveals that of SRS-A.

SRS-A appeared as early as histamine in the effluent from the perfused lungs, although the maximum rate of output occurred about $1\frac{1}{2}$ min later

than the maximum output of histamine. This is in contrast to the conclusion reached by Kellaway & Trethewie, who stated that the slow-reacting substance was present only in later fractions of the effluent. Samples of effluent collected shortly after the antigen contain large amounts of histamine in comparison with the amounts of SRS-A, so that when they are tested on the guinea-pig's ileum, without the use of an antihistamine drug, it is necessary to dilute them very greatly, and the effect of SRS-A then becomes relatively insignificant. With the weakly sensitized animals used by Kellaway & Trethewie this would be particularly likely, and offers a reasonable explanation for their failure to detect SRS-A in early samples.

The amount of SRS-A formed during the anaphylactic reaction varies greatly with the kind of tissue, and is probably determined by the availability of the necessary substrate and the amount of enzyme activated during the reaction. The largest amounts were formed in lung tissue, and in tissue from arteries and veins, hence vascular tissue is a particularly good source.

In the guinea-pig the lung was found to be the major source of SRS-A; it is therefore the organ upon which experiments regarding the mode of formation can best be performed. The amount of SRS-A produced by the lungs is probably greater than that produced by all the rest of the body, and the local concentration produced in this tissue during anaphylaxis will exceed that reached anywhere else.

In the study of SRS-A the use of perfused organs has many advantages. Not only is the yield of SRS-A greater than that from minced tissue, but the resulting active solution is relatively free from protein, salts and other constituents of the tissue. This greatly increases the reliability of biological tests. In addition, the reaction takes place in tissue which has suffered no damage, has remained at or near 37° C, and can be aerated adequately during the perfusion; anaphylaxis therefore occurs under conditions resembling those which exist *in vivo*, with the sole exception that blood is absent.

The title SRS-A refers only to a principle possessing characteristic biological activity, and does not imply that this is necessarily a single substance. Nevertheless, this is probably so, since the biological activity is located in a single band after being submitted to electrophoresis (Charlwood & Gordon, 1958). SRS-A differs in its pharmacological properties from bradykinin, 5-hydroxytryptamine, substance P, and many other substances (Brocklehurst, 1953) and appears to be a principle not previously studied.

In the absence of more specific tests the identification as SRS-A of the biologically active substance released from tissues other than guinea-pig lung and human lung has been based on pharmacological criteria such as

the characteristic prolonged contraction and slow relaxation, the persisting stimulation made apparent by a following dose of histamine, the entire absence of tachyphylaxis, and the relation of dose to response when tested on the guinea-pig ileum.

The finding that SRS-A is formed in the course of the antigen–antibody reaction strongly suggests an enzymic mechanism. According to Mongar & Schild (1957) the release of histamine is also the result of an enzymic process. However, it is not likely that the enzymes are identical, because SRS-A formation is prolonged, whereas the release of histamine is 'explosive'. The release of histamine occurs suddenly and declines quickly, even when only part of the releasable histamine is freed; the enzyme responsible for this must therefore act only for a very brief period, whereas that which forms SRS-A remains active for some time.

In all species in which the antigen–antibody reaction in the lung resulted in release of histamine it also led to the formation of SRS-A, but there was no quantitative correlation between the two. The degree of sensitization affected the two phenomena to a different degree. Weakly sensitized guinea-pig tissue usually produced little SRS-A, and moderate yields of histamine (5–10% of tissue content), whereas strongly sensitized tissue yielded up to 5 times as much histamine, and often 20 times as much SRS-A. Further, the release of the same amount of histamine was accompanied by the formation of larger amounts of SRS-A in the two specimens of human lung than was usual in the lung of the guinea-pig.

According to Ungar (1956) the presence of serum enhances the release of histamine by antigen. This finding was not confirmed. The guinea-pigs used in the present experiments were more strongly sensitized than those used by Ungar, and particular care was taken to ensure that the serum used was not contaminated by products from the blood cells, and also that the proteolytic activity which develops as a result of clotting remained minimal. These modifications might be the reason for the discrepancy between the results; it is likely that the release of histamine during a low-level anaphylactic reaction is augmented by the addition of traces of proteolytic enzymes.

Rocha e Silva (1950) found that the perfused liver of the sensitized dog released only traces of histamine, when challenged by the antigen, unless the perfusion fluid consisted of whole blood without an anticoagulant. He attributed the release of histamine to the break-down of aggregates of platelets and leucocytes trapped in the capillaries. The role of blood would appear to vary greatly between species, since in the present experiments on the perfused lung of man, monkey and guinea-pig substantial yields of histamine and SRS-A were obtained from tissue which was virtually free from blood. In some experiments with guinea-pig lungs, blood was

deliberately left in the tissue, or the platelets were destroyed previously by an antiserum, but this had no commensurate influence on the output of histamine or of SRS-A. Therefore, at least as far as the guinea-pig is concerned, blood residues and platelets appear to play no essential part in the release of histamine, nor can they be considered as the source of SRS-A.

The present results are incompatible with the view of Rocha e Silva, Bier & Aronson (1951) that anaphylatoxin contributes significantly to the release of histamine in the antigen–antibody reaction. This view was based on their finding that anaphylatoxin released between 14·3 and 32·6 μg histamine from the perfused lungs of a guinea-pig, whereas Bartosch, Feldberg & Nagel (1932) had obtained a release of only 0·5–4 μg, and Daly, Peat & Schild (1935) a release of 0·17–12 μg, from perfused lungs during anaphylaxis. However, the lungs from strongly sensitized guinea-pigs used in the present experiments regularly yielded 6–12 μg histamine per gram of tissue, with occasional yields of 15 μg/g representing 40 μg or more per lung, in response to the antigen, without the participation of blood elements, and therefore without anaphylatoxin formation. Moreover, in these experiments histamine release began in less than 30 sec, and reached a maximum rate about 1 min after addition of the antigen, which is comparable with the latency observed in anaphylactic shock in the intact guinea-pig. This suggests that *in vivo*, as well as *in vitro*, the direct effect of antigen on the sensitized tissue is primarily responsible for the release of histamine. A mechanism based on anaphylatoxin formation would appear to require a longer time, because it would be necessary for blood proteases to be activated, forming anaphylatoxin, and for this to leave the blood stream before releasing histamine in the tissues.

The occurrence of SRS-A following the antigen–antibody reaction is not dependent upon the kind of antigen used; ovalbumin, bovine and human serum albumin, diphtheria toxoid, pollen extract, cat and horse scurf, and pneumococcus polysaccharide (SSS III) all released histamine and caused the appearance of SRS-A. The fact that Schild, Hawkins, Mongar & Herxheimer (1951) did not detect any substance other than histamine in the bath fluid in which human asthmatic tissue had been shown to contract to pollen and other antigens does not conflict with this conclusion, because their experiments were designed primarily to prove that histamine was released. The methods they used to assay the bath fluid would not be expected to reveal the presence of SRS-A. In the present experiments solutions which had bathed human bronchial rings during anaphylactic contraction were found to contain SRS-A when tested at concentrations higher than those suitable for histamine assay.

The release of histamine from rabbit lung has not been described

previously. In the present experiments substantial amounts were only released when the tissue was highly sensitized, and even then the release did not result in bronchomotor or vasomotor responses of the lung. Thus, the underlying biochemical mechanism appears to be the same in the rabbit as in the guinea-pig, but the smooth muscle of the rabbit's lung is less sensitive to histamine, and the tissue as a whole reacts less vigorously to the antigen. The finding that neither histamine nor SRS-A appeared in the effluent from perfused lungs of sensitized rats, shows that either the perfusion conditions which are suitable for the guinea-pig and rabbit are not satisfactory for the rat, or that there is some fundamental difference such as the ability of the lung tissue to fix antibody. The few experiments with tissue from the horse and goat suggest that in these species also the lung may not be involved in anaphylaxis, or that blood may be essential to the reaction.

The proven existence of a second smooth-muscle-stimulating substance in anaphylaxis, particularly in lung, does not detract from the importance of histamine in anaphylaxis, but offers an explanation for some smooth-muscle reactions which cannot readily be attributed to histamine.

SUMMARY

1. When antigen is injected at the arterial inflow of the perfused lung of a sensitized guinea-pig, the effluent contains histamine and a slow-reacting smooth-muscle-stimulating substance (SRS-A).

2. SRS-A was found to be resistant to mepyramine, and could therefore be assayed on the mepyramine-treated guinea-pig ileum.

3. The time course of the output of SRS-A from the perfused lung of sensitized guinea-pigs, after the addition of antigen, is slower in onset and more prolonged than that of histamine.

4. SRS-A originates from the lung tissue; platelets and other constituents of blood are not the source of it, and are not necessary for its occurrence.

5. Whereas histamine is released from pre-existing stores in the tissue, SRS-A is formed as a result of the antigen–antibody reaction in the lung.

6. SRS-A was present in the effluent, after the antigen had been added to the perfused lungs of sensitized rabbits and monkeys, and of asthmatic human patients.

7. No SRS-A or histamine was detected in the effluent from perfused lungs of rats, on the addition of antigen, although by other criteria the animals were sensitized.

8. When formation of SRS-A was studied in particles of tissue from various organs of sensitized guinea-pigs, it was found to occur chiefly in

lung and vascular tissue. Some formation occurred in particles of salivary gland, spleen, pancreas and uterus, but little or none was detected in particles of ileum, trachea and skin.

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