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Release of Histamine and Slow-Reacting Substance Activities from Guinea Pig Lung.* (30212)

GERALD B. PHILLIPS† AND ELLIOTT MIDDLETON, JR.
Department of Medicine, College of Physicians and Surgeons, Columbia University

The addition of specific antigen *in vitro* to the lung of a sensitized guinea pig results in the release of histamine and a slow-reacting substance (SRS)(1,2). This release can be blocked by certain enzyme inhibitors(3,4). Cobra venom *(Naja naja)* added to the lung of a non-sensitized guinea pig also causes the release of histamine and an SRS(5). The possibility arises, therefore, that the antigen-antibody reaction activates an enzyme which is similar to one present in the cobra venom and which effects the release of histamine and SRS. That a phospholipase A might be the enzyme involved in the release by venom is suggested by the observation of Högberg and Uvnäs(6) that bee venom phospholipase A effected the release of histamine from rat mast cells. The present study, indeed, suggested that of the many enzymes in cobra venom, the phospholipase A could account for the release of histamine and SRS activities by the venom. Since phospholipase A catalyzes the hydrolysis of lecithin to lysolecithin, the effect of lysolecithin on release of histamine and SRS activities from guinea pig lung was also studied. In addition, the effect of non-specific tissue damage on release of histamine and SRS activities was evaluated.

Materials and methods. All materials to be tested were made up in Tyrode's solution, added to fresh chopped lung (or other tissues in one experiment) of non-sensitized guinea pigs in a ratio of 1 ml of solution to 0.1 g tissue, the suspension incubated at 37°C for 20 minutes unless otherwise stated, centrifuged, and the liquid phase removed and assayed for histamine and SRS activities using the guinea pig ileum as described previously (7). When any of the venom preparations were studied, this liquid phase was heated at 100°C for 15 minutes in order to inactivate the venom, which when unheated has a contractile action on the guinea pig ileum(7). At the alkaline pH of Tyrode's solution, this degree of heating has been reported to destroy all venom enzyme activity measured, while at neutral pH most of the phospholipase A activity survives(8,9). All of the venom solutions were added to the lung in a final concentration of 0.5 mg venom per ml Tyrode's solution. Samples to be compared were added to aliquots of the same guinea pig lung. A sample of the lung in plain Tyrode's solution served as a control for each run.

The hemolytic ability of various of the materials tested was estimated by making samples to a final volume of 2 ml containing a 1% suspension of human or sheep red blood cells washed 3 times with and made up in Tyrode's solution; this suspension was incubated at 37°C for one hour, centrifuged, and the supernatant diluted with water and its absorbance measured in a Klett-Summerson colorimeter using a 540 mμ filter.

Two preparations of lysolecithin, one made from beef brain lecithin and the other from beef serum lecithin, and one preparation of

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TABLE I. Effect of Heating Cobra Venom at Different pH's on Release of Histamine and Slow-Reacting Substance (SRS) Activities from Guinea Pig Lung and on Hemolytic Activity.*

Preparation†	Histamine activity‡	SRS activity§	Hemolytic activity‖
Control (no venom)	0	0	17
Unheated venom	4+	4+	335
Venom heated at pH 7.0	4+	3+	244
Venom heated at pH 5.6	2+	±	98

* Avg of 2 experiments. A third experiment using different volumes for testing gave similar results.

† Chopped guinea pig lung, 0.1 g per ml, incubated at 37°C for 20 min in Tyrode's solution containing the venom preparation at a concentration of 0.5 mg per ml (see text).

‡ Histamine activity of 0.05 ml supernatant solution tested on guinea pig ileum and graded as described in Ref 6.

§ SRS activity of 0.5 ml supernatant solution tested on guinea pig ileum and graded as described in Ref 6.

‖ Hemolytic activity of venom preparation on human red blood cells. Absorption read at 540 mμ in Klett-Summerson colorimeter (see text).

lecithin from beef brain were used. On thin-layer silicic acid chromatography the beef brain lysolecithin and lecithin preparations were pure except for a trace of material at the origin, while the beef serum lysolecithin preparation showed a trace of lecithin and sphingomyelin. The methods of preparation of the lysolecithin and lecithin have been described(10).

The cobra *(Naja naja)* and Russell viper *(Vipera russelli)* venoms were obtained from Ross Allen's Reptile Institute, Inc., Silver Springs, Fla. The phospholipase D, a preparation from cabbage, was a product of General Biochemicals, Chagrin Falls, Ohio. Heparin Sodium, U.S.P., containing 168,991 units per g, was obtained from Fisher Scientific Co., N. Y.

Results. Effect of heat and heparin on ability of cobra venom to release histamine and SRS activities. When cobra venom is heated at 100°C for 15 minutes in a neutral or acid solution, all of the known enzymes present are apparently destroyed except for the phospholipase A(8,9). Two solutions of the cobra venom, one in distilled water and the other in distilled water brought to pH 5.6 with HCl, thus were heated at 100°C for 15 minutes, made to isotonicity with hypertonic Tyrode's solution such that the final concentration of venom was 0.5 mg per ml, and tested on the chopped guinea pig lung along with an unheated venom solution of the same concentration (Table I). All 3 preparations caused release of histamine and SRS activities; with respect to the SRS release, the unheated solution was the most active, the venom heated at pH 5.6 was the least active and the venom heated at pH 7.0 was of intermediate activity. All of the preparations hemolyzed human but not sheep red blood cells, the unheated venom again being the most active, the venom heated at pH 5.6 being about one-fourth as active, and the venom heated at neutrality being about three-fourths as active as the unheated venom. The histamine- and SRS-releasing activity of the preparations, therefore, appeared to correlate with the phospholipase A activity as determined by degree of hemolysis(11). The SRS formed, moreover, was found to be dialyzable, as is the SRS released by the action of the unheated cobra venom(7). Fig. 1 illustrates the SRS activities in one experiment as recorded kymographically. When the above experiment was repeated using a different batch of cobra venom, similar results were obtained. These observations suggested

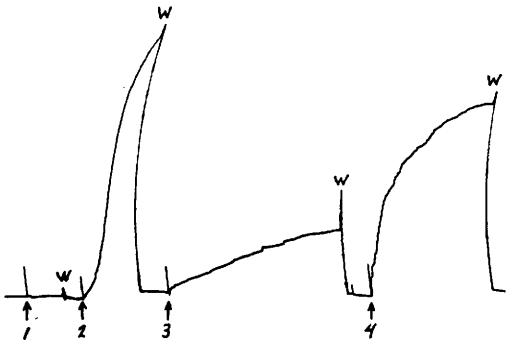

FIG. 1. Kymograph record of slow-reacting substance activity in supernatants of chopped guinea pig lung treated with venom preparations (see text). W, washout of tissue bath; 1, 0.5 ml control (no venom); 2, 0.5 ml unheated venom; 3, 0.5 ml venom heated at pH 5.6; 4, 0.5 ml venom heated at pH 7.0. The 2.5 ml tissue bath bathing the ileum at 37°C contained 1.5×10^{-6} M atropine and 3×10^{-7} M pyrilamine.

that the phospholipase A of the venom was responsible for the release of histamine and SRS activities. Heating the venom appeared to cause some reduction in activity of the phospholipase A, the reduction being greater at pH 5.6.

Condrea, de Vries, and Mager(11), extending the work of others(12,13), demonstrated that the phospholipase A of cobra venom when isolated was not by itself hemolytic to human red blood cells but was hemolytic when mixed with another protein fraction of the venom which they called direct lytic factor (DLF). The possibility arose, therefore, that release of histamine and SRS activities by the phospholipase A might also require the DLF of the venom, as the DLF was reported to be stable to heating at 100°C for 15 minutes at pH 5.5(11). To test this possibility the effect of unheated and heated cobra venom with the DLF removed and of Russell viper venom, which has been reported to contain a phospholipase A but no DLF (11), on release of histamine and SRS activities from the guinea pig lung was observed. The DLF was removed from cobra venom by precipitation with heparin as follows(11): 0.1 ml of 10% venom in water was added to 0.1 ml of 10% heparin in water, mixed, allowed to stand 20 minutes at room temperature, centrifuged, and the supernatant added to 20 ml of Tyrode's solution. The unheated venom treated in this way was about one-seventh as hemolytic to human red blood cells as untreated unheated venom, while the heparin-treated heated venom was not hemolytic at all. The Russell viper venom was very slightly if at all hemolytic. The heated and unheated heparin-treated cobra venom and the Russell viper venom released both histamine and SRS activities while the control solution containing heparin alone did not. The SRS-releasing ability of the Russell viper venom, however, was slight as compared to the cobra venom. These results suggested that the DLF of the cobra venom was not necessary for release of histamine and SRS activities.

Lack of release of histamine and SRS activities by a phospholipase D. A preparation of phospholipase D which had essentially no phospholipase A activity was tested on the chopped guinea pig lung. Although this enzyme also splits lecithin, but at a different position from phospholipase A, the release of histamine or SRS activity was not detected after its addition, suggesting some degree of specificity for phospholipase A in this release.

Release of histamine and SRS activities by lysolecithin. Since the phospholipase A of cobra venom catalyzes the hydrolysis of lecithin to lysolecithin, the release of histamine and SRS activities from chopped guinea pig lung by lysolecithin was studied. Concentrations of lysolecithin, and of lecithin as a control, ranging from 0.013 to 0.50 μM/ml were incubated with chopped guinea pig lung at 37°C for 20 minutes. Although the release of histamine and SRS activities was evident in 7 out of 8 experiments and occurred with the lowest lysolecithin concentration used (0.013 μM), the degree of activity was slight ($\pm$ - +, see Ref. 7) except in 2 instances where strong activity (4+, see Ref. 7) appeared. No dose-response relationship was evident. The histamine and SRS activities released, however, may have been greater than measured in this assay system, as lysolecithin was shown previously to inhibit the spasmogenic action of histamine and anaphylactic SRS on the guinea pig ileum(14). The lysolecithin-induced SRS contractions of the guinea pig ileum, like those produced by anaphylactic and venom-induced SRS, were delayed in onset and slow in progression and relaxation. The lysolecithin or lecithin in Tyrode's solution had no contractile action on the assay muscle. In one experiment, lysolecithin when incubated with guinea pig muscle at concentrations of 0.032 and 0.064 μM/ml and with guinea pig heart, liver, spleen, small intestine, diaphragm, or brain at a concentration of 0.10 μM/ml did not appear to release SRS activity. Following incubation of the guinea pig lung with lysolecithin, the fluid phase was lyophilized and extracted twice with 80% ethanol (1 vol 80% ethanol per 5 vol of original fluid phase per extraction); this extract was acidified to pH$<$4 with 1 N HCl and reextracted with ether, which was then taken to dryness; the residue when taken up in Tyrode's solution

TABLE II. Effect of Temperature on Release of Histamine and Slow-Reacting Substance (SRS) Activities from Guinea Pig Lung by Non-Specific Tissue Damage.

Treatment of* lung tissue	Histamine activity†	SRS activity†
Lyophilized		
Incubated at 37°C	4+	4+
0°C	4+	0
Homogenized		
Incubated at 37°C	4+	4+
0°C	4+	0
Heated at 100°C and incubated at 37°C	4+	0
Incubated at 37°C and heated at 100°C	4+	4+

* Following treatment (lyophilization or homogenization) tissue was suspended in 1 ml Tyrode's solution per 0.1 g original tissue and kept at specified temperature for 20 min. Homogenization was done at 0°C (see text).

† Figures grading the activity of histamine and SRS were obtained as described in Ref 6. 0.5 ml or less of the supernatant solutions was tested; equal volumes were tested in any given experiment.

and tested showed SRS activity. The solubility properties of lysolecithin-induced SRS were, therefore, similar to those of anaphylactic and venom-induced SRS(7). When the 80% alcohol extract was evaporated and the residue taken up in Tyrode's solution, the SRS activity was found to be dialyzable. Anaphylactic(2) and venom-induced SRS(7) are also dialyzable after such treatment. When a solution of the lysolecithin itself was acidified to pH$<$4 with 1 N HCl, extracted with ether, the ether dried, and the residue taken up in Tyrode's solution and tested, no SRS activity was detected.

Release of histamine and SRS activities by tissue damage. Since lysolecithin is damaging to tissue (unpublished observations), its ability to release histamine and SRS activities might be non-specific. To test this hypothesis, the lung tissue was injured in other ways and the release of histamine and SRS activities observed (Table II). Lyophilization or homogenization of the chopped guinea pig lung released considerable histamine activity on suspension in Tyrode's solution. Moreover, acetone extracted histamine activity from the chopped lung. If the lung was first heated at 100°C for 15 minutes and then homogenized, histamine was again released. These observations confirm the finding that histamine is present pre-formed in the lung and can be easily leached out by non-enzymatic, non-specific tissue damaging procedures(3,4). Such, however, was not the case for SRS activity. Lyophilization or homogenization did not immediately release appreciable SRS activity. When chopped guinea pig lung was lyophilized and kept in Tyrode's solution at 0°C or homogenized at 0°C and kept in Tyrode's solution at that temperature, SRS release was not observed, but when the suspension of lyophilized or homogenized material was then incubated at 37°C for 20 minutes, typical SRS activity was seen. This activity appeared to be dialyzable. If the tissue was heated at 100°C for 15 minutes and then homogenized and incubated, or if it was heated immediately after homogenization and then incubated, the release of SRS activity was not demonstrable. If the tissue was homogenized, incubated, and then heated, SRS activity was present. Chopped lung treated with cobra venom likewise showed no SRS activity when kept at 0°C but showed typical activity when incubated at 37°C for 20 minutes.

These results suggest that the SRS activity, unlike the histamine activity, is not present pre-formed in the lung, but is formed, presumably enzymatically, on disruption of the cells. An alternative possibility is that the SRS is bound in the cells and is released by enzymatic action following disruption of the cells.

Discussion. Considerable evidence has accumulated to indicate that the reaction of antigen and antibody results in activation of certain enzymes, although no such enzyme has yet been identified positively(3,4). The release of histamine and a slow-reacting substance (SRS) from guinea pig lung *in vitro* by both antigen-antibody reaction(1,2) and cobra venom(5), and the parallel effectiveness of the latter substances in releasing the former activities in a variety of guinea pig tissues(7) suggests that the venom may contain an enzyme similar to one activated by the antigen-antibody reaction. The identity of any enzyme in venom responsible for this release, therefore, is of interest. The present

study suggests that the phospholipase A activity of the venom, which can be isolated from many if not all of the other venom enzyme activities by virtue of its unusual property of withstanding a temperature of 100°C for 15 minutes at neutral or acid pH(8,9), can effect this release. Moreover, the phospholipase A of cobra venom appeared to be effective without the presence of the direct lytic factor of the venom, a factor which has been reported to be necessary for the hemolysis of human red blood cells by the venom phospholipase A(11). The possibility arises, therefore, that an enzyme with activity similar to that of a phospholipase A is involved in antigen-antibody reactions. Indeed, this theory has been proposed previously by Högberg and Uvnäs(6) who reported that of the many enzymes tested, only the phospholipase A had a significant disrupting and histamine-releasing activity on rat mast cells; further evidence, also indirect, was presented to suggest the hypothesis that the antigen-antibody reaction causes the activation in the membrane of the rat mast cell of a phospholipase A which in turn causes disruption of this cell and triggers the release of histamine(6,15). Keller(16) furthermore, has reported the formation of lysolecithin in rat mast cells during antigen-antibody reaction *in vitro*. Using a serum system, Fischer and Haupt (17) have also reported the formation of lysolecithin during antigen-antibody-complement reaction *in vitro,* but evidence has been presented to the contrary(10).

Since venom phospholipase A catalyzes the hydrolysis of lecithin to lysolecithin, lysolecithin was tested and also found to cause release of histamine and SRS activities, although the latter activity was less than with venom. Lysolecithin has also been reported to cause the disruption of rat mast cells(6).

But whether the SRS resulting from antigen-antibody reaction is the same as that released by venom and lysolecithin cannot be stated. One difference is that the SRS produced in the former reaction was not dialyzable(2) whereas that produced from venom was(7). This difference, however, could be explained by the observation that the SRS released by the antigen-antibody reaction appears to be bound to a protein, from which it can be easily dissociated by alcohol(2), and that the protein binding of the venom-induced SRS is broken by the proteolytic enzymes of the venom(2). The antigen-antibody-released SRS following alcohol extraction, moreover, appeared to have similar solubility properties to the venom- and lysolecithin-induced SRS. In the present study, however, the venom phospholipase A-induced SRS was found to be dialyzable even though the other venom enzymes were apparently inactivated. One possible explanation is that the venom phospholipase A released tissue enzymes which split an SRS-protein bond and rendered the SRS dialyzable. In support of this explanation was the finding that the SRS formed on homogenization or lyophilization of the guinea pig lung also appeared to be dialyzable.

The release of histamine and SRS activities from the guinea pig lung by lysolecithin, however, may have been due to non-specific damage to the cells by lysolecithin, as homogenization or lyophilization of the lung tissue followed by incubation in Tyrode's solution also resulted in formation of SRS activity. When the homogenized or lyophilized tissue in Tyrode's solution was kept at 0°C, however, SRS activity was not released, but such activity did appear when these preparations were then incubated at 37°C. These results suggest that the SRS activity, unlike the histamine activity, was not present preformed in the lung tissue but was formed enzymatically on the release of tissue enzymes. Anaphylactic SRS likewise appears to be formed in the lung tissue after addition of the antigen(18,19). An alternative explanation is that the SRS was present preformed in the tissue bound to another molecule, as a protein, and that the enzymes released caused its liberation. The lysolecithin, therefore, either specifically or non-specifically, could have caused the release of tissue enzymes, which in turn catalyzed the formation and/or release of an SRS.

Although the evidence is indirect, the possible participation of a phospholipase A and/or lysolecithin in the events following anti-

gen-antibody reaction is, therefore, suggested and warrants further study.

Summary. A phospholipase A preparation from cobra venom *(Naja naja)*, made by heating the venom in neutral or acid solution at 100°C for 15 minutes, released histamine and SRS activities from chopped guinea pig lung, as did the unheated venom. The direct lytic factor of the venom which is required for venom hemolysis did not appear to be necessary for this release. Lysolecithin, which is formed by the action of phospholipase A on lecithin, also released histamine and SRS activities from chopped guinea pig lung. Suspensions of homogenized or lyophilized guinea pig lung did not release SRS activity when kept at 0°C but did release this activity when incubated at 37°C.

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Quantitative Microdetermination of Enzymes in Sweat Gland III. Succinic Dehydrogenase in Cystic Fibrosis.* (30213)

G. E. GIBBS AND K. REIMER

Department of Pediatrics, University of Nebraska College of Medicine, Omaha

Various metabolic enzymes in sweat glands are being assayed in an attempt to discover the basic defect in cystic fibrosis. Manifestations of this disease include an excessive concentration of sodium chloride in sweat, representing failure to achieve the normal hypotonicity with respect to extracellular fluid. The abnormality is not understood, but may involve failure of a "sodium pump" mechanism concerned with retrieval of sodium and chloride.

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Alkaline phosphatase, acid phosphatase, adenosine triphosphatase, cholinesterases, and isocitric, glucose-6-phosphate, lactic, and malic dehydrogenases have been explored in the present series(1,2). This communication concerns succinic dehydrogenase (SDH). This enzyme is of special interest due to its association with various absorptive and secretory organs. It represents an analytical difficulty, in that it is easily destroyed in the process of freezing-drying. Chamness *et al* reported that "enzymes in hydrogen transport are damaged by drying" as indicated by failure of frozen-dried skin to demon-