

## A SEQUENCE OF BIOCHEMICAL EVENTS IN THE ANTIGEN-INDUCED RELEASE OF CHEMICAL MEDIATORS FROM SENSITIZED HUMAN LUNG TISSUE\*

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(Received for publication 5 July 1973)

The antigen-induced release of the chemical mediators, histamine and slow-reacting substance of anaphylaxis (SRS-A),<sup>1</sup> from sensitized human lung fragments requires divalent cations (1) and an intact glycolytic pathway (2), and is modulated by the intracellular concentrations of cyclic 3',5'-adenosine monophosphate (cyclic AMP) (2-4). These characteristics also hold for the immunologic release of histamine from guinea pig lung fragments (5, 6), human basophils in a population of peripheral leukocytes (7, 8), and rat mast cells in a peritoneal cell suspension (9, 10). Mediator release from these same tissues except for the human peripheral leukocyte suspension depends in addition upon the activation of an esterase to a form that can be inhibited by diisopropylphosphorofluoridate (DFP) (2, 11, 12).

The antigen-induced reaction in sensitized guinea pig ileal strips or lung fragments (13, 14), rat peritoneal cells (14), human leukocyte suspensions (15), or human lung fragments (1) has been separated into two phases: activation by antigen challenge in the absence of calcium and release by restoration of the calcium content without the addition of further antigen. The present studies reveal five of the steps interpolated between antigen challenge and release of histamine and SRS-A from human lung tissue passively sensitized with human IgE antibodies directed against ragweed antigens: a calcium-dependent activation of a serine esterase; a further autocatalytic activation of the esterase; an energy requirement; a second calcium-requiring, ethylenediaminetetraacetate (EDTA)-inhibitible stage; and a cyclic AMP-inhibitible phase.

### *Materials and Methods*

DL-Isoproterenol hydrochloride and 2-deoxy-D-glucose (2-DG) (Sigma Chemical Co., St. Louis, Mo.); DFP (Aldrich Chemical Co., Inc., Milwaukee, Wis.); and disodium-, tetra-

\* Supported by grants AI-07722, RR-05669, and AI-10356 from the National Institutes of Health.

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<sup>1</sup> Abbreviations used in this paper: 8-bromo cyclic GMP, 8-bromo cyclic 3',5'-guanosine monophosphate; cyclic AMP, cyclic 3',5'-adenosine monophosphate; DFP, diisopropylphosphorofluoridate; 2-DG, 2-deoxy-D-glucose; pmol/mg prot N, picomoles per milligram of protein nitrogen; SRS-A, slow-reacting substance of anaphylaxis.

sodium-, and disodium magnesium-EDTA (Fisher Scientific Co., Pittsburgh, Pa.) were obtained from the manufacturers. Antigen E was kindly supplied by the Research Resources Branch, National Institutes of Health.

Human lung tissue obtained at the time of surgery (16) was dissected free of pleura, bronchi, large blood vessels, and grossly diseased areas; sensitized by incubation in the serum of either patient E.W. (16) or patient S.A.G. (4) for 16–18 h at room temperature; washed free of serum; and placed at 37°C, unless otherwise stated, in 3 ml of Tyrode's buffer containing any agent(s) under study. The sensitized fragments were challenged with 0.2  $\mu\text{g}/\text{ml}$  antigen E 3 min later, unless denoted otherwise in the text; and the histamine and SRS-A released into the diffusate over the next 15 min were quantitated by bioassay on the isolated, atropinized guinea pig ileum (17). Residual tissue histamine was extracted by boiling the lung fragments for 8 min. All agents were prepared just before use in Tyrode's buffer modified with regard to glucose, calcium, and magnesium content as specified in the text; at the concentrations studied, these agents did not interfere with the bioassay of histamine or SRS-A or induce a nonspecific release of mediators in the absence of antigen challenge.

The experimental design (Fig. 1) for studies of the reaction sequence depended upon arresting the progression of antigen-induced mediator release by an initial manipulation, washing the tissue, and determining subsequent mediator release in a buffer that lacked antigen and was corrected as regards the initial manipulation but altered with respect to an additional condition or agent. The inhibition of mediator release by the second condition indicates that its site of action must follow the first. Conversely, failure of the second condition to influence mediator release indicates that the reaction has progressed beyond this step during the initial manipulation. The interpretation of either result requires full release of mediators after removal of the initial inhibitory condition.

The quantity of histamine or SRS-A released from the sensitized tissue by antigen challenge under the usual conditions, 15 min at 37°C in Tyrode's buffer in the absence of any agents, was taken as 100%; and the percent inhibition of mediator release by each manipulation and the percent reversibility of mediator release after correction of each manipulation (Fig. 1) was calculated on that basis.

Cyclic AMP was quantitated by employing the cyclic AMP-binding protein assay (18) as described (4).

## RESULTS

### *DFP-Sensitive Serine Esterase.—*

*Generation:* Preliminary experiments (2) that indicated that histamine but not SRS-A release could be used to study an antigen-activated serine esterase were substantiated. Treatment of sensitized human lung fragments with 6 mM DFP for 1 min before antigen challenge suppressed the release of histamine and SRS-A, while removal of DFP by washing before antigen challenge permitted release of mediators (Table I, exp. A). Exposure of the lung fragments to DFP for 3–5 min, followed by washing, resulted in progressive impairment of the antigen-induced release of SRS-A but had no effect on histamine release (Table I, exp. B). As DFP irreversibly phosphorylates serine residues in the active center of susceptible enzymes (19), the DFP-sensitive esterase involved in histamine release must be maintained in a DFP-resistant precursor form until activated by the interaction of antigen with tissue-fixed IgE antibody. In contrast, the progressive inhibition of SRS-A release by DFP unrelated to antigen challenge reveals a preformed DFP-sensitive step and does not permit determination of the presence of an antigen-activated esterase. Accordingly,

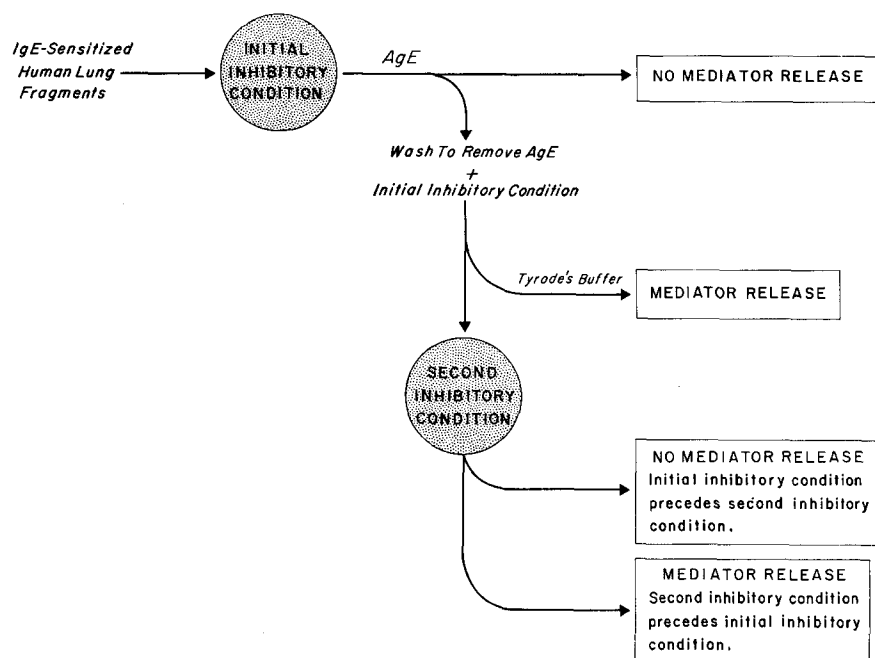


FIG. 1. Experimental design. Replicate 200-mg samples of sensitized human lung tissue fragments were challenged with antigen (AgE) in a buffer containing an initial inhibitory condition. Samples handled in parallel and washed after antigen challenge in order to remove both antigen and the initial inhibitory condition were resuspended in either Tyrode's buffer or in buffer containing a second inhibitory condition.

TABLE I  
*Effects of Preincubation with DFP on the Immunologic Release of Mediators from Human Lung Tissue*

DFP	Preincubation	Removal (3 washes)	Histamine release	SRS-A release
	<i>min</i>		%	<i>U/g</i>
Exp. A*				
—			14	500
6 mM	1	—	4	50
6 mM	1	+	10	400
Exp. B†				
—			21	500
5 mM	1	+	20	560
5 mM	3	+	20	225
5 mM	5	+	19	150

\* Sensitized human lung fragments incubated in DFP for 1 min were either challenged with antigen in the presence of DFP or washed three times and resuspended in DFP-free buffer before challenge.

† Sensitized human lung fragments were incubated with DFP for 1–5 min, washed three times, resuspended in DFP-free buffer, and challenged with antigen.

only histamine release was used in further assessment of the immunologically activated DFP-sensitive esterase.

As the DFP-sensitive esterase involved in histamine release exists in a DFP-resistant precursor form, the capacity for lung tissue challenged with antigen in the presence of DFP to release histamine after washing reflects residual proesterase activatable by tissue-bound antigen. Sensitized lung fragments were treated with DFP (5 mM), interacted with antigen 10 s later, and incubated for an additional 1–13 min at 37°C. The antigen-challenged lung fragments were removed, washed, and resuspended in Tyrode's buffer; and the amount of histamine released was compared with that from samples that had not been exposed to DFP (Fig. 2). No histamine release occurred at any interval in the presence of DFP. Removal of DFP 1 min after antigen challenge permitted nearly full release, while release declined progressively as the interval between challenge and DFP removal was extended. Thus the antigen-induced activation of the esterase to its DFP-sensitive form occurred continually over the 13 min period such that after a 13 min incubation of sensitized lung fragments with antigen in the presence of DFP, only a small portion of the esterase was still available in the precursor form for activation by tissue-bound antigen.

The experimental design used to examine the activation of the esterase at 37°C was next employed at 0°C (Fig. 3). Sensitized lung fragments incubated with antigen at 0°C for 15 and 30 min did not release histamine, but after being washed and transferred to Tyrode's buffer at 37°C yielded full histamine release as compared with replicates initially challenged with antigen at 37°C. Tissue challenged with antigen in the presence of DFP (5 mM) at 0°C for 15

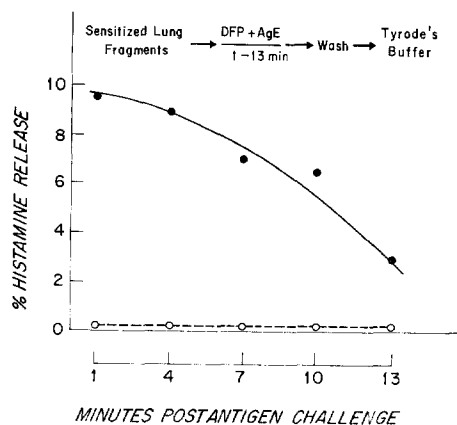


FIG. 2. Time course of the antigen-induced generation of the DFP-sensitive esterase at 37°C. (○---○), percent histamine released from sensitized lung fragments by antigen in the presence of DFP; (●—●), percent histamine released after washing and transfer of lung fragments to Tyrode's buffer. Antigen-induced histamine release from replicate samples that had never been exposed to DFP was 12.5%.

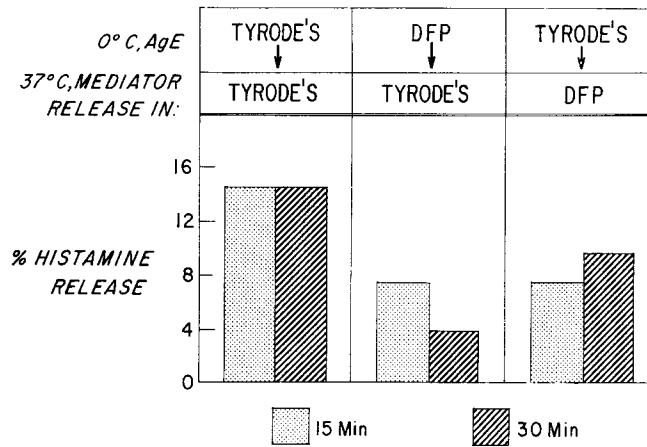


FIG. 3. Antigen-induced generation of the DFP-sensitive esterase at 0°C. Histamine release from samples challenged with antigen at 37°C was 14%.

and 30 min, washed, and transferred to Tyrode's buffer at 37°C yielded 50 and 25%, respectively, of the histamine release obtained when antigen challenge was carried out in the absence of DFP, thereby indicating that considerable activation of the esterase had occurred at 0°C. Replicate samples incubated with antigen in Tyrode's buffer for 15 and 30 min at 0°C, washed, and then transferred to DFP-containing buffer at 37°C released 50 and 75%, respectively, of the release obtained from antigen-challenged fragments transferred to buffer without DFP; thus, a significant portion of the reaction had progressed beyond the DFP-inhibitable stage even at 0°C. Whereas the histamine release observed from fragments challenged with antigen in the presence of DFP and transferred to a DFP-free buffer reflects residual activatable proesterase, that achieved after antigen challenge and transfer of the fragments to DFP-containing buffer reflects progression of the biochemical sequence beyond the active esterase stage or the capacity of the activated esterase to act upon its natural substrate more rapidly than it becomes inactivated by phosphorylation. The finding that the sum of the histamine released, 13 and 13.25%, respectively, after 15 or 30 min of antigen challenge at 0°C, under the two experimental conditions equaled that of tissue challenged directly at 37°C, 14%, supports the concept that the portions of the enzyme in precursor and active forms are being assessed.

*Calcium requirement:* The development of an experimental design that permitted an evaluation of the relative proportions of the esterase in precursor and active forms led to an analysis of the role of divalent cations in its activation (Fig. 4). Sensitized lung fragments, incubated for 10 min in Tyrode's buffer containing 1.8 mM calcium and 0.5 mM magnesium, or in buffers deficient in calcium, magnesium, or both cations, were treated with 5 mM DFP

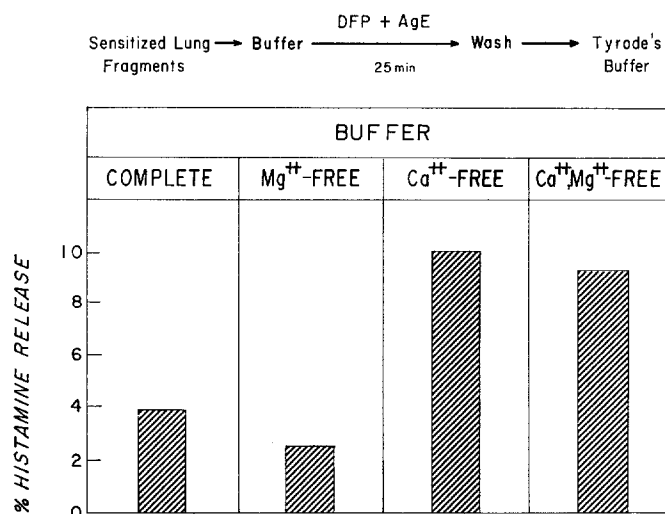


FIG. 4. Divalent cation requirement for the antigen-induced generation of the DFP-sensitive esterase. Antigen-induced histamine release from replicate samples incubated in the various buffers but never exposed to DFP was 12.5–15%.

followed in 10 s by antigen. After an additional 25 min incubation, the antigen-challenged fragments were washed and resuspended in fully constituted Tyrode's buffer, and the subsequent release of histamine was determined. No release of histamine occurred in the presence of DFP regardless of the cation content of the buffer. Antigen-challenged fragments treated with DFP in the absence of calcium released 9.25 and 10% of their histamine after being washed and transferred to Tyrode's buffer, while replicates treated in the presence of calcium released only 2.5 and 4%. Thus, in the absence of extracellular calcium, the antigen-induced conversion of the serine esterase to its active form was arrested. The fact that calcium depletion alone did not impair antigen-induced histamine release implies that prevention of esterase activation depends upon both the absence of extracellular calcium and the inactivation of any active esterase by DFP.

*Energy-Requiring Stage.*—Antigen challenge of sensitized lung fragments in buffer devoid of glucose did not impair mediator release, while the introduction of 2-deoxyglucose (2-DG), a competitive inhibitor of phosphoglucoisomerase (20), produced a dose-dependent inhibition of the release of histamine and SRS-A (2). Inhibition of mediator release by 2-DG required a 3 min preincubation period before antigen challenge (Fig. 5). Reversibility of the inhibition of mediator release after antigen challenge in the presence of 2-DG, as assessed by washing and transfer of the challenged fragments to glucose-containing medium, was apparent for less than 3 min. In order to determine if the antigen-induced activation of the DFP-sensitive esterase preceded the labile energy-

requiring stage, the serine esterase was maintained in its precursor form by combining 2-DG treatment of sensitized fragments with antigen challenge in calcium-free buffer containing DFP (Fig. 6). Sensitized lung fragments were incubated in buffer deficient in calcium, magnesium, and glucose for 5 min; 5 mM DFP, 10 mM 2-DG, or both inhibitors were added, followed in 3 min by antigen; 1–25 min later the fragments were removed, washed, and resuspended in fully constituted Tyrode's buffer. While the absence of divalent cations and glucose from the incubation medium had no effect upon the amount of histamine

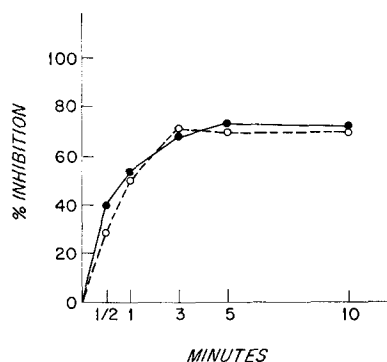


FIG. 5. Time course of the inhibition of mediator release by 2-deoxyglucose. Sensitized lung fragments were incubated with 10 mM 2-DG in glucose-free buffer for 0.5–10 min before antigen challenge. (●—●), histamine released; (○---○), SRS-A released. Release of histamine and SRS-A from replicate samples challenged in the absence of 2-DG was 20% and 250 U/g, respectively.

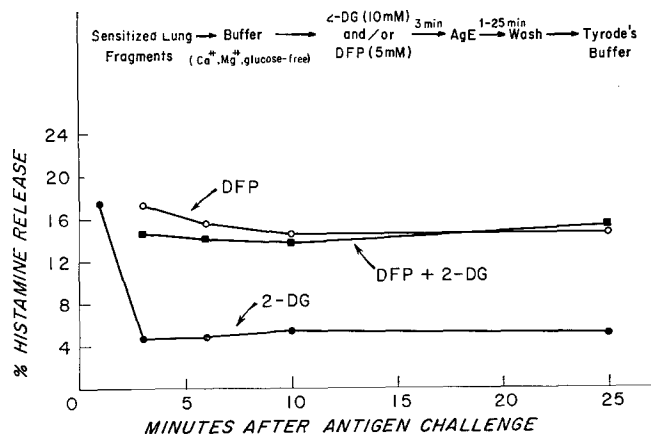


FIG. 6. Capacity to prevent the occurrence of the labile energy-requiring stage in mediator release by maintaining the DFP-sensitive esterase in its precursor state. Antigen-induced histamine release from replicate samples incubated in calcium-, magnesium-, and glucose-free buffer in the absence of inhibitors was 22%.

released from the fragments by antigen challenge, the presence of either DFP or 2-DG throughout the reaction totally suppressed mediator release. The inhibition of the immunologic release of histamine by 2-DG was reversible for only 1 min after antigen challenge. In contrast, antigen challenge in the presence of 2-DG plus DFP or DFP alone permitted 15–17% of the total histamine to be released when the fragments were washed and transferred to Tyrode's buffer 3–25 min later. The finding that DFP treatment of the sensitized lung fragments in calcium- and glucose-free buffer prevented the appearance of the labile energy-requiring step apparent with antigen challenge in the absence of DFP indicates that activation of the esterase precedes the energy-requiring step.

*Second Calcium-Requiring, EDTA-Inhibitible Step.*—EDTA produced a dose-related inhibition of the antigen-induced release of histamine and SRS-A (1) with an  $ID_{50}$  of 0.3 mM and  $ID_{100}$  of 2 mM having been observed in the present experiments. Although the inhibition by EDTA was reversible if the antigen-treated tissue was washed and transferred to fully constituted Tyrode's buffer (1), the reversibility was progressively lost beginning 3 min after antigen challenge (Fig. 7). Suppression of the immunologic release of mediators by EDTA occurred immediately after its introduction and, as shown in Table II, was reproduced by magnesium EDTA and reversed by calcium and not by magnesium.

Sensitized lung fragments incubated with antigen at 0°C for 30 min, washed, and transferred to DFP-containing buffer gave full mediator release while replicate fragments transferred to calcium-free, EDTA-containing buffer were

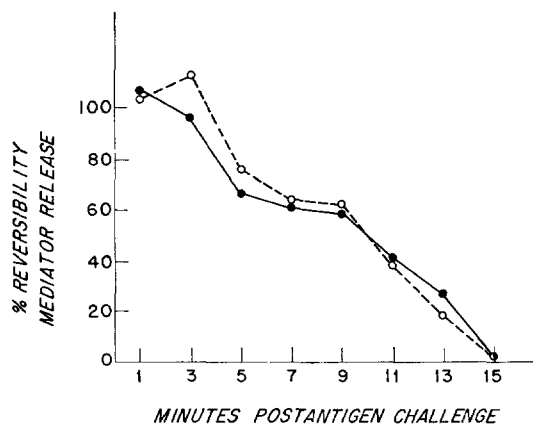


FIG. 7. Time course of the reversibility of EDTA inhibition of the antigen-induced release of mediators. Sensitized lung fragments were incubated in calcium- and magnesium-free buffer containing 5 mM EDTA for 1 min, challenged with antigen, washed, and transferred to Tyrode's buffer from 1 to 15 min later. (●—●), histamine released; (○—○), SRS-A released. Antigen-induced release of histamine and SRS-A from replicate samples in calcium- and magnesium-free buffer in the absence of EDTA was 49% and 785 U/g, respectively. No antigen-induced release of mediators occurred in the presence of EDTA.



markedly inhibited (Table III). Thus, EDTA inhibition of mediator release occurs at a step after the antigen-induced calcium-dependent activation of the serine esterase.

Attention was next directed towards determining whether this second cal-

TABLE II  
*Reversal of EDTA Inhibition of the Immunologic Release of Chemical Mediators*

Antigen challenge in	Wash	Divalent cation content of 2nd buffer	Histamine release	SRS-A release
			%	U/g
Calcium- and magnesium-free buffer	—	—	11	375
EDTA	—	—	0	0
EDTA	+	Mg <sup>++</sup>	0	0
EDTA	+	Ca <sup>++</sup>	9	375
MgEDTA	—	—	0	0
MgEDTA	+	Ca <sup>++</sup>	10	375
Tyrode's buffer	—	—	11	375

Sensitized human lung fragments were incubated for 1 min in calcium- and magnesium-free buffer containing 5 mM EDTA or 5 mM magnesium-EDTA and were challenged with antigen. Replicate samples in EDTA or magnesium-EDTA-containing buffer were washed three times in calcium- and magnesium-free buffer 3 min after antigen challenge and transferred to buffer containing calcium or magnesium.

TABLE III  
*Capacity for DFP or EDTA to Inhibit the Immunologic Release of Histamine after Antigen Challenge at 0°C*

Second incubation medium	Histamine release
	%
Tyrode's buffer	27
5 mM EDTA in calcium- and magnesium-free buffer	6
5 mM DFP in Tyrode's buffer	20

Sensitized human lung fragments were challenged with antigen in Tyrode's buffer at 0°C, incubated at 0°C for 30 min, washed, and transferred to a second incubation medium at 37°C. No antigen-induced mediator release occurred at 0°C. Replicate samples challenged by antigen in Tyrode's buffer at 37°C released 27% of their total histamine content.

cium-requiring, EDTA-inhibitable step preceded or followed the energy-dependent phase (Fig. 8). Sensitized lung fragments were incubated in buffer deficient in divalent cations and containing EDTA (5 mM) or deficient in glucose and containing 2-DG (10 mM) for 3 min, challenged with antigen, washed 3 min later, and resuspended in fully constituted Tyrode's buffer. Replicate samples in EDTA-containing buffer were transferred 1 min after antigen challenge to buffer devoid of calcium, magnesium, and glucose and

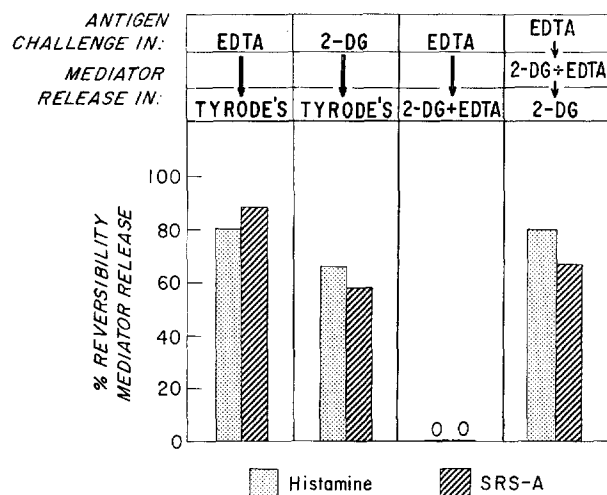


FIG. 8. Failure of 2-deoxyglucose to prevent cation reversal of EDTA inhibition of mediator release. Antigen-induced histamine and SRS-A release from replicate samples incubated in either calcium- and magnesium-free or glucose-free buffer was 23–24% and 750 U/g, respectively, in both instances.

containing both EDTA and 2-DG; one-half the samples were maintained in this buffer while the others were transferred 3 min later to glucose-free buffer containing 2-DG in the presence of calcium and magnesium. Sensitized lung fragments challenged with antigen in the presence of EDTA or 2-DG released no mediators, but after being washed and transferred to normal Tyrode's buffer yielded approximately 80 and 60%, respectively, of the histamine and SRS-A released from replicate fragments challenged with antigen but never exposed to either inhibitor. Transfer of antigen-challenged lung fragments from buffer containing EDTA to buffer containing both EDTA and 2-DG yielded no mediator release; in contrast, subsequent transfer of replicate fragments to buffer containing 2-DG was accompanied by mediator release comparable to that observed from fragments challenged in the presence of EDTA and transferred directly to fully constituted Tyrode's buffer. The failure of 2-DG to prevent cation reversal of EDTA inhibition indicates that the energy-requiring stage cannot follow the calcium-requiring, EDTA-inhibitable step.

EDTA should therefore be capable of preventing mediator release after glucose reversal of 2-DG inhibition (Fig. 9). Sensitized lung fragments, incubated in buffer devoid of divalent cations and containing EDTA or deficient in glucose and containing 2-DG for 3 min, were challenged with antigen, incubated for 3 min, washed, and transferred to normal Tyrode's buffer. Replicate samples in 2-DG were transferred 3 min after antigen challenge to calcium- and magnesium-free buffer containing EDTA; half the fragments

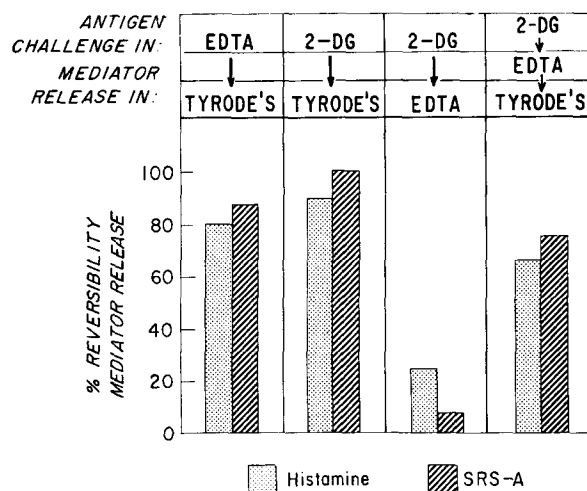


FIG. 9. Capacity of EDTA to prevent glucose reversal of 2-deoxyglucose inhibition of mediator release. Antigen-induced histamine and SRS-A release in calcium- and magnesium-free or glucose-free buffer was 18% and 500 U/g, respectively.

were maintained in this buffer while the other half were transferred 3 min later to fully constituted Tyrode's buffer. Lung tissue replicates challenged with antigen in the presence of EDTA or 2-DG released no mediators, but upon transfer to normal buffer yielded 85 and 90%, respectively, of the histamine and SRS-A released by replicates challenged with antigen but never exposed to either inhibitor. Transfer of antigen-treated lung fragments from 2-DG-containing buffer to EDTA-containing medium maintained the inhibition of mediator release despite the absence of 2-DG and presence of glucose, while replicate samples transferred to divalent cation-containing buffer were reconstituted in terms of mediator release. Thus, the energy-requiring stage must precede the second calcium-requiring, EDTA-inhibitable step in the antigen-induced reaction.

*Cyclic AMP-Inhibitable Stage.*—In order to determine the site at which cyclic AMP modulates the immunologic release of chemical mediators, it was necessary to investigate the effects on cyclic AMP levels of the various maneuvers employed to develop the sequence. As shown in Table IV, stimulation of fragments in Tyrode's buffer by 1  $\mu$ M isoproterenol increased the tissue concentrations of cyclic AMP from 6.4 pmol/mg protein nitrogen (pmol/mg prot N) to 223 pmol/mg prot N, and the results in calcium- and magnesium-free buffer, glucose-free buffer containing 2-DG, and Tyrode's buffer containing DFP were comparable. EDTA in calcium- and magnesium-free buffer increased baseline cyclic AMP to 41 pmol/mg prot N and exhibited a synergistic effect when combined with isoproterenol, the tissue level of cyclic AMP rising to 520 pmol/mg prot N.

As EDTA alone increased cyclic AMP concentrations and acted synergistically with isoproterenol, the capacity of isoproterenol to prevent calcium reversal of EDTA inhibition of the immunologic release of mediators was determined (Fig. 10). Sensitized lung fragments suspended in calcium- and magnesium-

TABLE IV  
Effect of Various Inhibitors of Mediator Release upon the Capacity of Isoproterenol to Increase the Intracellular Levels of Cyclic AMP

Agent	Isoproterenol	Cyclic AMP	
		<i>pmol/mg prot N</i>	(SEM)
Tyrode's buffer	—	6.4	(2.3)
“ “	+	223.0	(18.6)
Calcium- and magnesium-free buffer	—	6.3	(1.8)
“ “ “ “	+	248.6	(26.0)
EDTA	—	41.2	(3.4)
“	+	520.2	(28.9)
2-DG	—	6.5	(2.1)
“	+	193.7	(16.4)
DFP	—	10.2	(1.6)
“	+	207.6	(14.3)

The cyclic AMP content of human lung fragments was determined after a 5 min incubation at 37°C in Tyrode's buffer, calcium- and magnesium-free buffer, calcium- and magnesium-free buffer containing 5 mM EDTA, glucose-free buffer containing 10 mM 2-DG, or Tyrode's buffer containing 5 mM DFP. Replicate samples were incubated in these same buffers containing 1  $\mu$ M isoproterenol.

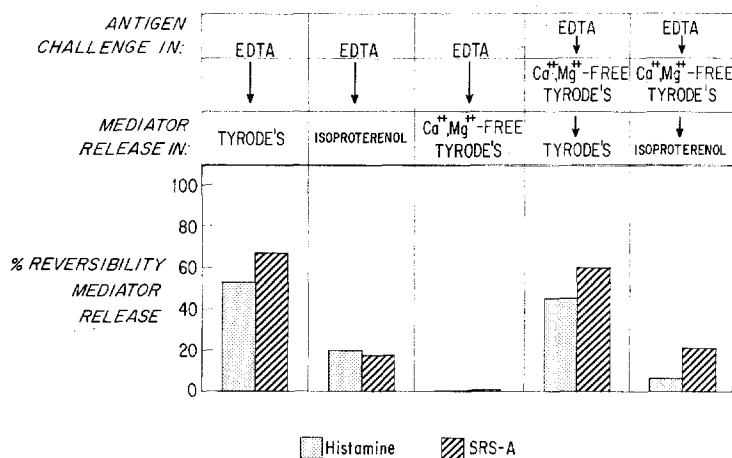


FIG. 10. Capacity of isoproterenol to prevent cation reversal of EDTA inhibition of mediator release. Antigen-induced histamine and SRS-A release in calcium- and magnesium-free buffer in the absence of EDTA was 16% and 150 U/g, respectively. No antigen-induced release of mediators occurred in the presence of either EDTA or isoproterenol.

free buffer containing 5mM EDTA were challenged with antigen, incubated for 3 min, washed, and transferred to Tyrode's buffer, either alone or containing 0.5  $\mu$ M isoproterenol. Samples transferred to isoproterenol yielded less than 40% of the release of mediators obtained from fragments transferred to buffer alone. In order to eliminate the effects of EDTA on the tissue levels of cyclic AMP, another set of replicate samples was washed 3 min after antigen challenge in the presence of EDTA, transferred to calcium- and magnesium-free Tyrode's buffer for an additional 5 min, and then placed in Tyrode's buffer with and without isoproterenol. Isoproterenol again prevented cation reversal of the EDTA-dependent inhibition, placing the inhibitory action of cyclic AMP coincident with or subsequent to the second calcium-requiring, EDTA-inhibitable step. These data were supported by the demonstration that sensitized tissue challenged by antigen in glucose-free buffer containing 2-DG, washed, and transferred to normal Tyrode's buffer released histamine and SRS-A, while replicate samples transferred to buffer containing 5  $\mu$ M isoproterenol did not.

#### DISCUSSION

Five sequential steps interspaced between the antigen-induced activation of human lung fragments sensitized with IgE and the release of chemical mediators have been delineated. The experimental design (Fig. 1) that permits this analysis is based upon the capacity to maintain the serine esterase essential to mediator release in its DFP-resistant precursor state despite antigen challenge and upon the ability to arrest reversibly the reaction sequence by various manipulations. The former permitted determination of those conditions essential to activation of the serine esterase. The latter permitted various inhibitory maneuvers to be sequenced; reversal of the initial arresting condition with restoration of mediator release in the presence of a second inhibitor of different character indicated that the new agent exerted its inhibitory effect before the step at which the reaction had been arrested, while continued inhibition indicated that the new condition acted subsequently.

As the DFP-sensitive serine esterase involved in histamine release exists in a DFP-resistant precursor form (Table I, exp. A), the capacity for lung tissue fragments, challenged with antigen in the presence of DFP, to release histamine after being washed reflects residual proesterase activatable by tissue-bound antigen. Varying the time interval between immunologic activation in the presence of DFP and transfer of the lung fragments to Tyrode's buffer reveals progressive depletion of the residual activatable proesterase at 37°C (Fig. 2) and even at 0°C (Fig. 3). That this depletion at 0°C is due to the formation of the active esterase in the presence of DFP is revealed by parallel studies in which DFP, introduced 15 and 30 min after antigen challenge at 0°C, permits the release of histamine either because the reaction has progressed beyond the esterase or because the active esterase has a greater reactivity with its natural

substrate than with the phosphorylating agent. The finding that the sum of the histamine released by the residual activatable esterase, after transfer of the tissue challenged with antigen in the presence of DFP, and by the active esterase, after transfer of tissue challenged with antigen to buffer containing DFP, is identical to the amount released from tissue directly challenged in the absence of DFP supports the view that these two maneuvers assess the total tissue content of the precursor and active esterase (Fig. 3).

Whereas antigen challenge of sensitized tissue in the presence of DFP at both 37° and 0°C (Figs. 2 and 3) permitted depletion of the proesterase by activation to its esterase form, this was not the case when calcium was omitted from the DFP-containing buffer (Fig. 4). Sensitized lung tissue fragments challenged at 37°C in calcium-free buffer containing DFP released no histamine until washed and transferred to Tyrode's buffer; full histamine release was then observed (Fig. 4), indicating preservation of the proesterase state of the enzyme until transfer. Since calcium depletion alone does not prevent antigen-induced histamine release at 37°C (Table II), the inability of antigen to activate the proesterase depends upon both the absence of extracellular calcium and the presence of DFP. The full mediator release by antigen challenge in the absence of extracellular calcium, in contradistinction to maintenance of the proesterase state when DFP is present in calcium-free buffer, indicates a minimal calcium requirement for initial antigen-induced activation of the proesterase followed by autocatalytic activation by the activated esterase.

Both EDTA and DFP inhibited the reaction when added simultaneously with antigen, while 2-DG required a 3 min period of preincubation with the tissue in glucose-free buffer in order to suppress the immunologic release of mediators (Fig. 5). Further, the antigen-activated stage blocked by the presence of 2-DG was labile and could be reversed, as assessed by subsequent mediator release, only if the tissue was washed and restored to normal buffer within 1-3 min of antigen challenge (Fig. 6). The capacity of the combination of DFP and depletion of calcium to prevent activation of the serine esterase (Fig. 4) should preclude the reaction from reaching the 2-DG-inhibitible but labile step if the esterase activation occurs earlier than the energy step in the reaction sequence, and this indeed was the case (Fig. 6).

EDTA could inhibit mediator release either by preventing the calcium-dependent activation of the proesterase or by acting at an additional divalent cation-requiring step. The divalent cation requirement for both esterase activation (Fig. 4) and reversal of EDTA inhibition (Table II) is met by calcium and not by magnesium. Reversibility of the inhibition of mediator release by EDTA is accomplished completely by washing the tissue and replenishing calcium within 3 min after antigen challenge; it falls off entirely over the next 12 min (Fig. 7). The finding that the antigen-induced reaction sequence progresses over 30 min at 0°C to a point where transfer of the tissue to DFP-containing buffer permits mediator release (Fig. 3, Table III), while transfer to EDTA-

containing buffer does not (Table III), places the second calcium-requiring, EDTA-inhibitable step subsequent to the appearance of the activated esterase. That it is also subsequent to the energy-requiring, 2-DG-inhibitable step is indicated by the inhibition of mediator release by EDTA after glucose reversal of 2-DG inhibition (Fig. 9). Conversely, the finding that 2-DG does not prevent calcium reversal of EDTA inhibition established that the energy step precedes this second calcium-requiring, EDTA-inhibitable stage (Fig. 8).

The finding that isoproterenol prevents calcium reversal of EDTA inhibition of mediator release could mean that increased concentrations of cyclic AMP act either coincident with or subsequent to the second calcium-requiring, EDTA-inhibitable step (Fig. 10). A coincident site of action might be in terms of the synergistic effect of EDTA and isoproterenol on cyclic AMP levels (Table IV), an action possibly related to the inhibitory effect of calcium on adenylate cyclase (21). The capacity of isoproterenol to inhibit mediator release after the tissue has been transferred from EDTA-containing buffer to calcium- and magnesium-free buffer, a maneuver designed to eliminate the synergism between isoproterenol and EDTA, favors discrete sites of action (Fig. 10). Further, the immediate reversibility of EDTA inhibition by calcium as opposed to the persistent inhibition by isoproterenol as assessed by washing and transferring the fragments to Tyrode's buffer from 1 to 15 min after antigen challenge again favors individual sites of action. An attractive possibility would be a role for intracellular calcium in the activation of a contractile protein that is inhibited through phosphorylation by a cyclic AMP-dependent kinase.

It was not possible to study the site at which 8-bromo cyclic 3',5'-guanosine monophosphate (8-bromo cyclic GMP) acts to enhance the immunologic release of chemical mediators (4) as the experimental design used to sequence the reactions relies on calcium depletion at two discrete stages, and the augmentation of mediator release by 8-bromo cyclic GMP is not observed in the absence of extracellular calcium.

The sequence of biochemical events initiated by the interaction of ragweed antigen with tissue-fixed antiragweed IgE antibodies in human lung tissue (Fig. 11) proceeds from the calcium-dependent activation of a DFP-sensitive serine esterase; the further autocatalytic activation of the esterase; a 2-DG-inhibitable energy requirement; a second calcium-requiring, EDTA-inhibitable stage; and a cyclic AMP-suppressible step to the release of histamine and SRS-A. Although the scheme depicts SRS-A release as involving activation of a DFP-sensitive esterase, as has been firmly established for histamine, this point in fact could not be studied due to the inhibition of SRS-A release by preincubation with DFP before antigen challenge (Table I, exp. B). Three of these steps have also been recognized in the release of histamine from the isolated rat peritoneal mast cell treated with band 2 protein derived from rabbit leukocytes (22). Histamine release induced by this cationic protein involves the activation of a DFP-sensitive esterase from a DFP-resistant precursor form, an energy-

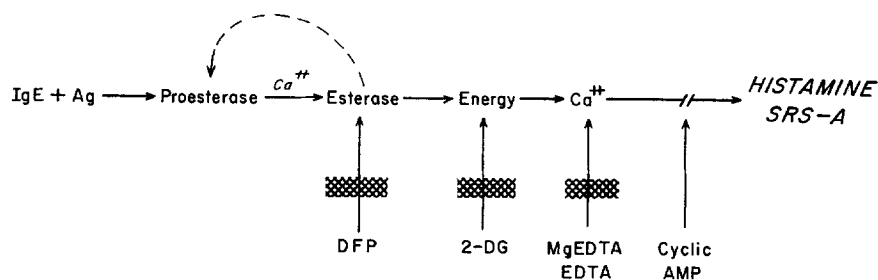


FIG. 11. Schematic diagram of the sequence of biochemical events in the antigen-induced release of chemical mediators from human lung tissue sensitized with IgE.

requiring step inhibitable by dinitrophenol or iodoacetate in glucose-free medium, and an EDTA-inhibitable stage recognized only by initiating the reaction at 0°C and then transferring the cells to 37°C buffer with EDTA. A calcium requirement was not established for either esterase activation or reversal of EDTA inhibition. The division of antigen-induced histamine release from mixed cell populations of human leukocytes into a calcium-independent activation stage and a calcium-dependent release phase (15) has been accompanied by evidence that inhibition by isoproterenol and 2-DG occur in the initial and final phases, respectively (23). Such a sequence might be explicable if calcium deficiency alone prevented esterase activation in this system and in addition had a synergistic effect with isoproterenol.

#### SUMMARY

Five sequential steps interspaced between the antigen activation of human lung fragments sensitized with IgE and the release of the chemical mediators, histamine and slow-reacting substance of anaphylaxis (SRS-A), have been delineated. The experimental design that permits this analysis is based upon the capacity to maintain the serine esterase essential to mediator release in its diisopropylphosphofluoridate (DFP)-resistant precursor state despite antigen challenge and upon the ability to arrest reversibly the reaction sequence by various manipulations. When sensitized lung fragments are challenged with antigen in the presence of DFP, a serine esterase is converted to its active DFP-inhibitable state; this conversion is prevented if antigen challenge in the presence of DFP occurs in calcium-free buffer indicating that immunologic activation of the esterase requires extracellular calcium. The fact that calcium depletion alone does not impair antigen-induced histamine release implies that prevention of esterase activation depends upon both the absence of extracellular calcium and the inactivation of any active esterase by DFP to prevent an autocatalytic feedback activation. Arresting the antigen-induced activation of the serine esterase by the combination of DFP in calcium-free buffer precludes the sequence from reaching the labile, 2-deoxyglucose (2-DG)-



inhibitable, energy-requiring step, indicating that proesterase activation precedes this energy-requiring stage. The 2-DG-inhibitable step precedes a second calcium-requiring, EDTA-inhibitable stage, as EDTA prevents glucose reversal of 2-DG inhibition of antigen-challenged tissue, while the presence of 2-DG does not prevent calcium reversal of EDTA inhibition. The finding that isoproterenol prevents calcium reversal of EDTA inhibition of mediator release suggests that the inhibitory site of action of increased concentrations of cyclic AMP is coincident with or subsequent to the second calcium-requiring, EDTA-inhibitable step. Therefore, the sequence of biochemical events initiated by the interaction of antigen with tissue-fixed IgE antibodies appears to proceed from the calcium-requiring activation of a DFP-sensitive serine esterase; the further autocatalytic activation of the esterase; a 2-DG-inhibitable energy requirement; a second calcium-requiring, EDTA-inhibitable stage; and a cyclic AMP-inhibitable step to the release of histamine and SRS-A.

The authors gratefully acknowledge the skilled technical assistance of Ms. Nancy Timmerman.

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