ANAPHYLAXIS IN CHOPPED GUINEA PIG LUNG

I. Effect of Peptidase Substrates and Inhibitors

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Mongar and Schild have demonstrated that the anaphylactic release of histamine from chopped guinea pig lung requires calcium and a heat-labile factor, and can be inhibited by metabolic inhibitors such as iodoacetate and cyanide (1–3). The effectiveness of these and other non-specific metabolic inhibitors in preventing the *in vitro* anaphylactic reaction has been confirmed by other workers studying the release of histamine from chopped guinea pig and rat tissue (4) and the degranulation of mast cells in the mesentery of the guinea pig (5) and rat (6, 7).

Since the metabolic inhibitors used by all these workers influence a wide range of enzymes and can alter a variety of tissue metabolic processes, they offer only limited insight into the type of enzymatic processes involved. We therefore attempted to use inhibitors which were more selective in action, and most of our studies were done with competitive rather than non-competitive inhibitors. Many of the compounds studied were synthetic substrates and were selected in the hope that they would produce inhibition by competing with the natural substrate for the enzyme involved. Our first experiments were prompted by the finding of Becker (8, 9) that the first component of guinea pig complement was an esterase which could be inhibited by diisopropylfluophosphate (DFP), and by the knowledge that DFP could also prevent the anaphylactic release of histamine and slow reacting substance SRS-A (10) from perfused guinea pig lung (11). We were also guided in our initial choice of inhibitors by the finding of Cushman, Becker, and Wirtz (12) that immune hemolysis is inhibited by thiols, esters of arginine and lysine, esters or amides of tyrosine, and peptides with a terminal aromatic amino acid having a free carboxyl group.

In a preliminary report (13) we noted that amino acid ester substrates and inhibitors of chymotrypsin prevent the anaphylactic release of histamine while substrates and inhibitors of trypsin do not. The present report extends these findings. It shows that acetylation of the ester substrate so as to introduce a

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secondary peptide bond enhances inhibitory capacity, and that the relative effectiveness of the chymotrypsin substrates in inhibiting anaphylaxis differs somewhat from their relative susceptibility to hydrolysis by chymotrypsin. It demonstrates that substrates of carboxypeptidase and leucine aminopeptidase like those of trypsin are not inhibitory. It presents evidence that the phase of the anaphylactic reaction inhibited by DFP and other chymotrypsin inhibitors is in a DFP-resistant precursor state until activated by the addition of antigen, while the phase of the reaction sensitive to the thiol inhibiting agent, *N*-ethylmaleimide, is available even prior to antigen addition. As a result of these studies with inhibitors it has been possible to compare the observed characteristics of antigen-induced histamine release in guinea pig lung with those established for immune hemolysis (12, 14).

Materials

The esters, amides, and peptides were obtained mainly from Mann Research Laboratories, Inc., New York, while the amino acids and other compounds were obtained from several different sources, British Drug Houses, Ltd., Dorset, England, Hopkins and Williams, Ltd., Essex, England, and L. Light and Co., Ltd., Bucks, England. Bovine gamma globulin was supplied by Armour Corp., and albumin (egg) flake by British Drug Houses, Ltd. Soybean trypsin inhibitor was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Diisopropylfluophosphate (DFP) was obtained from Boots, Ltd., London; L-lysine ethyl ester was obtained from Dr. E. L. Becker; L-tyrosine ethyl ester, diacetyl L-lysine ethyl ester, and diacetyl L-lysine hydrazide were obtained from Dr. R. Pitt-Rivers and Mr. A. Brownstone; glycylglycylglycine and L-leucylglycylglycine were supplied by Dr. F. Sanger; and three times recrystallized egg albumin prepared by the method of Kekwick and Cannan (15) was obtained from Dr. J. H. Humphrey. The ammonium salt of 5-hydroxyindoleacetic was obtained from Roche Products, Ltd., London, and was converted to the sodium salt by cation exchange chromatography.

4-imidazole acetic acid was synthesized under the direction of Dr. J. W. Cornforth. 4-imidazole acetonitrile was prepared by the method of Dakin (16) and gave an uncorrected melting point of 136–138.5° which agrees satisfactorily with the value of 138° reported by Dakin (16). The nitrile was hydrolyzed to give 4-imidazole acetic acid. After crystallization from water, sublimation, and recrystallization the material melted with decomposition at 220–220.5° (uncorrected) which agrees satisfactorily with the reported corrected value of 222° (17).

Methods

Guinea pigs were sensitized by the injection of 10 per cent egg albumin or bovine gamma globulin in 0.5 per cent phenol, 1 ml. intraperitoneally and 1 ml. subcutaneously. Passively sensitized animals were obtained by the intrathoracic injection of serum containing 4 to 8 mg. of rabbit antiovalbumin. Approximately 3 weeks after injection of antigen for active sensitization or 48 hours after passive sensitization, the heart and lungs were excised. Visible blood was removed by immedate perfusion through the pulmonary artery for 10 minutes with Tyrode's solution at 37°C. (10). The lung tissue was then chopped into rods 0.4 x 0.4 x 5.0 mm. with a McIlwaine chopper (18). The chopped tissue was washed with Tyrode's solution at 23°C. and divided into 24 samples, each of approximately 100 mg. wet weight. These were incubated in Tyrode's solution at 37°C for 45 minutes with continuous agitation by rocking and then washed twice with Tyrode's solution at 37°C. Washing was carried out by removing the supernatant fluid through a filter formed by a nylon tuft (19) and replacing it with fresh

warm Tyrode's solution. Immediately after the second wash each sample of tissue was immersed in 3.0 ml. warm Tyrode's with or without the potential inhibitor. Ten seconds later the antigen, 1.0 mg. ovalbumin or 3.0 mg. bovine gamma globulin per ml. reaction mixture, was added. After rocking for 15 minutes at 37°C., the supernatant fluid was removed from the tissue and kept at 0°C. until assayed on the same day using the isolated ileum of the guinea pig in the presence of 2×10^{-7} M atropine. Any potential inhibitor was always studied in four replicate tubes; three of the samples received antigen and the fourth served as a control for spontaneous release of histamine. The triplicate samples gave values which fell within 10 per cent of the mean. The pH of the Tyrode's solution containing the potential inhibitor was checked before each experiment and adjusted to pH 7.6–7.8 when necessary. In general, the anaphylactic release of histamine in the control tubes containing Tyrode's solution without inhibitor ranged from 3 to 8 μg. per gm. wet lung tissue and represented 10 to 30 per cent of the total tissue histamine. The total tissue histamine was estimated by biological assay of the solution obtained by complete extraction of chopped tissue to which antigen had not been added. The tissue was boiled in 5 ml. Tyrode's solution for 5 minutes and then the tube was placed in ice for 1 hour to allow complete diffusion.

The standard dose of histamine acid phosphate used for the histamine assay on the isolated ileum ranged from 0.001 to 0.005 μg. per ml. calculated as base. The concentration of the inhibitor in the supernatant did not in general interfere with the response of the guinea pig ileum to histamine at the dilution used for assay. In those instances when interference, enhancement, or inhibition, was present a correction was made by determining the effect of the potential inhibitor on the standard histamine response.

The experimental method differs in two ways from that used by Mongar and Schild in their studies with inhibitors of anaphylaxis (1): perfused blood-free tissue was used in order to minimize any non-specific contribution from serum factors not adsorbed onto the tissue, and antigen was added ten seconds after the inhibitor so as to diminish any non-specific effects due to prolonged contact between tissue and inhibitor prior to antigen addition. We reasoned that, if the inhibitor was acting exclusively on an antigen-antibody activated enzyme sequence, there would be no advantage in adding the inhibitor 15 minutes before the antigen.

RESULTS

Time Course of the Anaphylactic Release of Histamine.—Fig. 1 shows the time course of histamine release after the addition of antigen, ovalbumin 1 mg. per ml. reaction mixture, to sensitized chopped guinea pig lung. Histamine began to appear 10 to 15 seconds after adding antigen, and two-thirds of the total amount to be released was in the supernatant fluid by the end of the 1st minute. The time course for the anaphylactic release of histamine from chopped tissue was virtually identical with that observed in perfused whole lung (10). Increasing the concentration of the antigen to 10 or even 50 mg. per ml. reaction mixture did not give greater histamine release than 1 mg. per ml. reaction mixture.

Compounds Which Fail to Inhibit the Anaphylactic Release of Histamine.—In Table I are listed compounds which are not substrates of the common peptidases and which fail to inhibit the anaphylactic release of histamine at a concentration of 20 mM. The levorotatory form of all amino acids studied, the racemic form of the aliphatic amino acids, esters of glutamic acid, glycine and glycylglycine, and a variety of peptides, polypeptides and acids all failed to inhibit the anaphylactic release of histamine.

In Table II are listed those peptidase substrates which were found to be

non-inhibitory. The first group are esters and amides or hydrazides of arginine and lysine, and are trypsin substrates (20). Neither the trypsin substrates nor the soybean trypsin inhibitor were inhibitory. The two substrates of leucine aminopeptidase tested (21), L-leucylglycylglycine and L-leucineamide, were not inhibitory at a concentration of 5 mM. L-leucineamide appeared to show slight inhibition at 20 mM, but the significance of this was difficult to interpret because at this concentration L-leucineamide released histamine in the absence of antigen. The third group are substrates of carboxypeptidase (20). When first tested

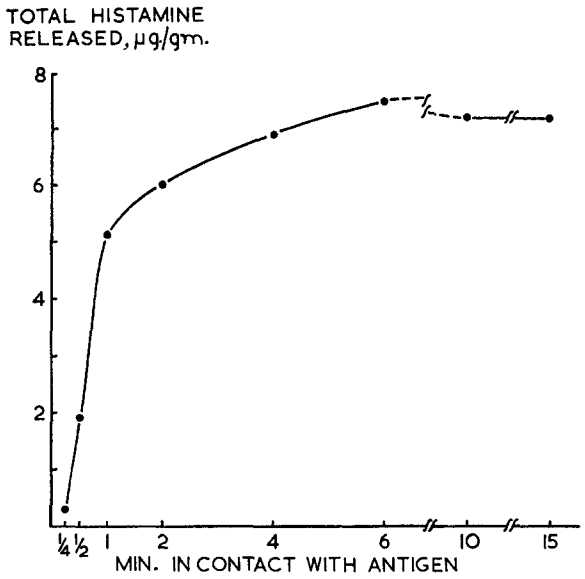

FIG. 1. Time course of histamine release after addition of antigen

these compounds appeared to be inhibitory, but inhibition was also obtained with the related peptides carbobenzoxy-L-glutamic acid, carbobenzoxyglycine, and benzoylglycylglycine which were included as controls. In order to obtain solutions of these peptides they were dissolved in 0.2 N NaOH and neutralized with 1 N HCl prior to bringing to volume with Tyrode's solution. Since this resulted in an appreciable increase in the NaCl concentration of the reaction mixture, the effect of variation in NaCl concentration on the anaphylactic reaction was investigated. It was observed that the anaphylactic release of histamine from chopped guinea pig lung was extremely sensitive to small changes in the ionic strength of the suspending medium (22). When the experiments with the substrates of carboxypeptidase were repeated so that the ionic strength of the mixture containing the peptide was no greater than that of the controls, inhibition was no longer observed.

TABLE I

*Compounds Which Fail to Inhibit the Anaphylactic Release of Histamine from Chopped, Sensitized Guinea Pig Lung**

Amino acids	Esters	Peptides, polypeptides, and other compounds
L-tryptophan	L-glutamic ethyl ester	Glycylglycine
L-tyrosine‡	Glycine ethyl ester	Glycylglycylglycine§
L-phenylalanine	Glycylglycine ethyl ester	DL-alanylglycine
L-arginine		*N*-acetylglycine
L-lysine		*N*-acetyl-L-lysine
L-leucine		*N*-acetyl-DL-leucine
DL-leucine		Glycyl-DL-leucine
L-norvaline		Carbobenzoxy-L-glutamic acid
DL-norleucine		Carbobenzoxyglycine
L-valine		Benzoylglycylglycine
DL-alanine		Soybean trypsin inhibitor‖
Glycine		Acetic acid
L-glutamic acid		Propionic acid
DL-aspartic acid		L-mandelic acid
L-histidine		Phenylsuccinic acid
L-cysteine		4-imidazole acetic acid
L-serine		Sucrose
		Glucose

* Compounds producing less than 25 per cent inhibition at a concentration of 20 mM were termed non-inhibitory.

‡ Because of water insolubility L-tyrosine could not be tested at greater than 5 mM.

§ Because of limited supply this compound could not be tested at greater than 5 mM.

‖ Test concentration was 2.0 mg./ml.

TABLE II

*Substrates Which Fail to Inhibit the Anaphylactic Release of Histamine from Chopped, Sensitized Guinea Pig Lung**

Trypsin substrates	Leucine aminopeptidase substrates	Carboxypeptidase substrates
Benzoyl-L-arginine methyl ester	L-leucylglycylglycine‡	CBZ§ α-glutamyl-L-tyrosine
Benzoyl-L-arginine amide	L-leucine amide‖	CBZ glycyl-L-phenylalanine
L-lysine ethyl ester		CBZ glycyl-L-tryptophan
Diacetyl-L-lysine ethyl ester¶		Benzoylglycyl-L-phenylalanine
Diacetyl-L-lysine hydrazide¶		

* Compounds producing less than 25 per cent inhibition at a concentration of 20 mM were termed non-inhibitory.

‡ Because of limited supply this compound could not be tested at greater than 5 mM.

§ CBZ, carbobenzoxy.

‖ Because L-leucine amide released histamine in the absence of antigen, observations at a concentration of greater than 5 mM were not considered reliable.

¶ Because of limited supply these compounds could not be tested at greater than 10 mM.

Inhibition of the Anaphylactic Release of Histamine by Chymotrypsin Substrates and Inhibitors.—The compounds which consistently produced more than 50 per cent inhibition of the anaphylactic release of histamine are recorded in

TABLE III

The Inhibition of the Anaphylactic Release of Histamine from Chopped, Sensitized Guinea Pig Lung by Chymotrypsin Substrates and Inhibitors

Class of Inhibitor	Minimal concentration consistently giving greater than 50 per cent inhibition
	mM
Chymotrypsin substrates	
Glycyl-L-tyrosine amide	20
L-leucine ethyl ester	5
L-tyrosine ethyl ester	5
N-acetyl-L-tyrosine ethyl ester	4
L-phenylalanine ethyl ester	3.5
L-tryptophan ethyl ester	2
N-acetyl-L-phenylalanine ethyl ester	1
N-acetyl-L-tryptophan ethyl ester	0.5
Bifunctional chymotrypsin inhibitors	
DL-phenylalanine	20
D-phenylalanine	20
DL-tryptophan	20
D-tryptophan	20
Acetyl-DL-phenylalanine	20
Acetyl-DL tryptophan	20
Acetyl-L-tryptophan	20
Glycyl-DL-tryptophan	20
Glycyl-L-tryptophan	20
Benzoic acid	20
5-hydroxyindoleacetic acid	20
Phenylacetic acid	5
β-Phenylpropionic acid	2.5
β-Indoleacetic acid	2.5
β-Phenylcinnamic acid (trans)	2.5
β-(β-Indole)propionic acid	2.5
Monofunctional chymotrypsin inhibitors	
Nicotinamide	5
Phenol	1
Skatole	1
Indole	0.5
Organophosphorus esterase inhibitor	
Diisopropylfluophosphate	2

Table III. The first group are esters of aromatic amino acids and are typical chymotrypsin substrates (20). The observation that the esters are better inhibitors of anaphylaxis than the amide agrees with the fact that the esters are

better substrates for chymotrypsin. The finding that the acetylated ester is in each instance a better inhibitor than the non-acetylated ester is also consistent, since introduction of a secondary peptide bond makes the ester a more susceptible chymotrypsin substrate (20). On the other hand, the relative capacity of these esters to inhibit anaphylaxis is not the same as their relative susceptibility to pancreatic chymotrypsin. *N*-acetyl L-phenylalanine ethyl ester is a more potent inhibitor of the *in vitro* anaphylactic release of histamine than *N*-acetyl-L-tyrosine ethyl ester (Table III), but the latter is a better chymotrypsin substrate than the former (23). Furthermore, L-leucine ethyl ester, a poor chymotrypsin substrate (20, 24), is as potent an inhibitor of anaphylaxis as L-tyrosine ethyl ester, which is a good substrate.

These chymotrypsin substrates are termed trifunctional because they interact with chymotrypsin at three sites, the aromatic side chain, the secondary peptide bond or free amino group, and the susceptible ester bond. Whereas trifunctional compounds can be either substrates or competitive inhibitors, compounds presenting the enzyme with only two such sites, namely an aromatic residue and a polar group separated from it, may bind even more firmly and are termed bifunctional inhibitors, while compounds containing only an aromatic residue can be monofunctional inhibitors (20). It is noteworthy that 0.5 mM of the substrate *N*-acetyl-L-tryptophan ethyl ester produced 50 per cent inhibition, whereas 20 mM of the bifunctional inhibitor, *N*-acetyl-L-tryptophan, was required to achieve the same inhibition (Table III). The most active bifunctional inhibitors studied were β-phenylpropionic acid, β-phenylcinnamic acid (trans), β-indoleacetic acid, and β-(β-indole)propionic acid, and these produced more than 50 per cent inhibition of histamine release at a concentration of 2.5 mM.

Because phenylacetic acid and β-indoleacetic acid were inhibitory, 4-imidazoleacetic and 5-hydroxyindoleacetic acid, degradation products of histamine (25) and serotonin (26) respectively, were tested with the possibility in mind that they might provide a negative feedback mechanism which would make the anaphylactic reaction self-limiting. Introduction of a hydroxyl group into the five position significantly reduced the inhibtory power of β-indoleacetic acid (Table III), and 4-imidazole acetic acid had no inhibitory effect at all. The failure of 4-imidazoleacetic acid to inhibit is probably due to the fact that the imidazole ring is basic while the indole and the phenyl rings are acidic and neutral respectively. L-mandelic acid, which differs from phenylacetic acid in that the acetic acid side chain is substituted with a hydroxyl group, and phenylsuccinic acid were also not inhibitory (Table I).

The four monofunctional inhibitors (Table III) are noteworthy because the order of their relative effectiveness against *in vitro* anaphylaxis is the same as that observed by Huang and Niemann (27) against chymotrypsin. With the exception of D-tryptophan ethyl ester, indole was the most potent competitive inhibitor of chymotrypsin listed by Green and Neurath (20), and it is also one

of the two most potent inhibitors of anaphylaxis listed in Table III. The ability of phenol to inhibit the anaphylactic release of histamine was first described by Mongar and Schild (1), but the fact that phenol was a chymotrypsin inhibitor was not considered.

Inhibition of the Anaphylactic Release of Histamine by DFP.—The dose-response curve for the organophosphorus esterase inhibitor, diisopropylfluophosphate (DFP), is depicted in Fig. 2. The concentration required to produce

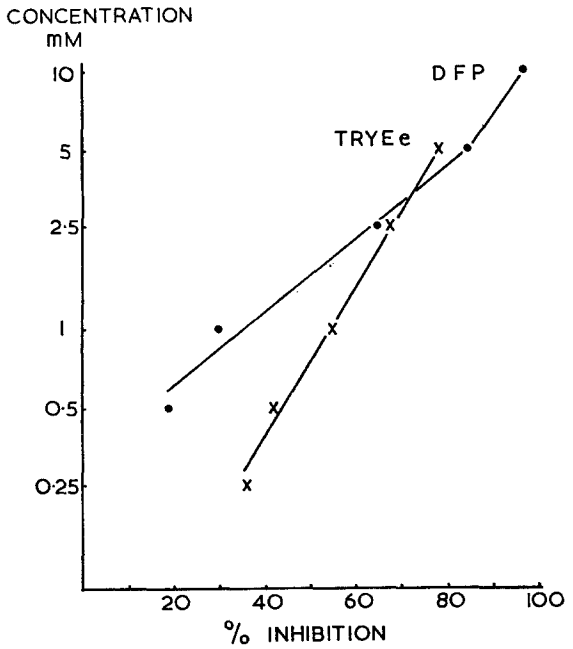

FIG. 2. Dose response curve for the inhibition of antigen induced histamine release by DFP (●——●) and L-tryptophan ethyl ester (TRYEe) (×——×).

50 per cent inhibition was approximately 2 mM. Fig. 2 also shows the dose-response curve for L-tryptophan ethyl ester. The unexpected finding that DFP, which is capable of inhibiting chymotrypsin on a 1:1 molar basis (28), required a greater concentration to produce 50 per cent inhibition than certain of the substrates and monofunctional inhibitors (Table III) can be attributed to several factors. DFP can apparently react with several different amino acid side groups, including hydroxyl, amino, and sulfhydryl groups (29). Jansen, Fellows Nutting, and Balls (30) found that the concentration of DFP needed to inhibit citrus acetylesterase could be reduced to one-tenth after further purification of the enzyme, whereas a fifty times increase in the concentration of DFP was needed when inhibition was attempted in the presence of substrate.

Similarly, when Becker (8) attempted to inhibit immune hemolysis in the presence of diluted whole complement 2.5 to 8.3 mM of DFP was required, but when DFP was used to inhibit the esterase activity of the first component of complement (C'1) on the washed red cell only 0.083 mM DFP was needed. Thus, the finding that 2 mM of DFP was needed to produce 50 per cent inhibition of the anaphylactic release of histamine from chopped lung can probably be attributed to the following: the binding of DFP at tissue sites not participating in the reaction; the very short time which DFP has to combine with the antigen-antibody activated enzyme; and the fact that DFP must do this in the presence of natural substrate.

TABLE IV

The Effect of Adding Inhibitor and Then Washing It Out before the Addition of Antigen to the Sensitized Tissue

Inhibitor	Concentration	Histamine release in μg./gm wet lung tissue		
		Inhibitor present	Inhibitor washed out*	Control‡
	mM			
A				
DFP	10	0.27	5.1	6.0
L-phenylalanine ethyl ester	20	0.6	3.5	4.1
Phenylacetic acid	20	0.54	2.4	2.7
Indole	2	0.27	3.0	2.7
B				
N-ethylmaleimide	1	3.7	0.48	9.6

* Tissue was washed four times in Tyrode's solution to remove the inhibitor before antigen was added.

‡ Tyrode's solution only.

Evidence That the Inhibitors Are Acting Exclusively on an Antigen-Antibody Activated Step.—Since DFP inhibits esterases irreversibly, it was used to ascertain whether the DFP-inhibited enzyme essential to anaphylaxis in guinea pig lung was present before or only subsequent to antigen addition. Sensitized tissue was incubated with 10 mM DFP for 15 minutes and then washed four times with Tyrode's solution before the addition of antigen. On the addition of antigen this tissue gave virtually the same histamine release as control tissue treated in a similar fashion with Tyrode's solution only (Table IV*A*). Since DFP inhibited the antigen-induced release of histamine only when present at the time of antigen addition, DFP must be acting exclusively on an antigen-antibody–activated enzyme which exists in lung tissue in a precursor form resistant to DFP until activated by the antigen-antibody interaction.

In order to be certain that none of the other classes of chymotrypsin in-

hibitors used in this work destroyed or released antibody or otherwise impaired the ability of the sensitized tissue to respond to antigen, the sensitized tissue was incubated with 20 mM L-phenylalanine ethyl ester, 20 mM phenylacetic acid, or 2 mM indole for 15 minutes and then washed four times with Tyrode's solution to remove the inhibitor before adding antigen. As shown in Table IV*A*, on the addition of antigen these tissues gave virtually the same histamine release as control tissue treated in a similar fashion with normal Tyrode's solution. In each of these experiments the quantity of inhibitor used was sufficient to give more than 80 per cent inhibition of the antigen-induced histamine

TABLE V

*The Effect of Inhibitors on the Anaphylactic Release of Histamine in Passively Sensitized Guinea Pig Tissue**

Inhibitor	Concentration	Histamine release in μg./gm. wet lung tissue		Inhibition of histamine release
		Inhibitor present	Control‡	
	mM			*per cent*
N-acetyl-L-phenylalanine ethyl ester	1	2.4	4.1	41
L-tryptophan ethyl ester	2.5	1.9	4.1	54
Indole	1	1.1	4.1	73
Phenylacetic acid§	5	1.4	2.7	48

* Guinea pigs sensitized with 4 to 8 mg. of rabbit antiovalbumin, and histamine release initiated by three times recrystallized ovalbumin, 1 mg. per ml. reaction mixture.

‡ Tyrode's solution only.

§ Data taken from another experiment.

release. This indicates that these inhibitors are probably acting on a reaction activated by the addition of antigen. Furthermore, when the tissues in which the antigen-induced histamine release had been prevented by the presence of inhibitor were washed and treated a second time with antigen, there was no histamine release. This desensitization demonstrates that the inhibitor has not prevented effective antigen-antibody interaction.

Since the experiments on which Tables I to IV are based involve the anaphylactic release of histamine from tissue of animals sensitized with crude ovalbumin, it seemed important to repeat some of these observations using passively sensitized tissue and tissue from animals actively sensitized with a different antigen. Some of the studies in which three times recrystallized ovalbumin was used to initiate histamine release from animals passively sensitized with rabbit antiovalbumin are noted in Table V. The concentration of the esters and of phenylacetic acid was selected so as to give 50 per cent inhibition and the concentration of indole used was expected to give about 70 per cent

inhibition. The inhibition achieved compares favourably with that anticipated from the studies with actively sensitized tissue.

To obtain comparative data with two different antigen-antibody systems in the same tissue, animals were simultaneously actively sensitized with both ovalbumin and bovine gamma globulin. As shown in Table VI, the addition of ovalbumin to the chopped tissue released 6.7 μg. of histamine per gm. of wet lung tissue and the addition of bovine gamma globulin to a different sample of the same lung tissue released 4.2 μg. histamine per gm. of wet lung tissue. L-tryptophan ethyl ester in a concentration of 2.5 mM inhibited the ovalbumin-

TABLE VI

*The Inhibition of the Anaphylactic Release of Histamine in Doubly Sensitized Guinea Pig Tissue**

Antigen used for anaphylaxis‡	Histamine release in μg./gm. wet lung tissue		Inhibition of histamine release
	L-tryptophan ethyl ester (2.5 mM)	Control§	
			per cent
Ovalbumin	1.7	6.7	75
Bovine gamma globulin	1.1	4.2	74
Ovalbumin and bovine gamma globulin	2.1	6.6	68

* Animals sensitized by simultaneous injection of ovalbumin and bovine gamma globulin.

‡ Ovalbumin used in a concentration of 1 mg. per ml. reaction mixture and bovine gamma globulin in a concentration of 3 mg. per ml. reaction mixture.

§ Tyrode's solution only.

induced histamine release 75 per cent and the bovine gamma globulin–induced histamine release 74 per cent. Thus, the inhibitor was equally effective against both antigen-antibody systems. It is of interest that virtually the same inhibition was achieved with the ester whether the two antigens were added simultaneously or individually.

The Effect of Thiols and Thiol Alkylating or Oxidizing Agents on the Anaphylactic Release of Histamine.—In view of the evidence that the intracellular peptidase, cathepsin C, splits dipeptides such as glycyl L-tyrosinamide and is inhibited by chymotrypsin substrates such as L-phenylalanine ethyl ester and L-tyrosinamide (31, 32), the possible participation of cathepsin C in the anaphylactic release of histamine was further investigated. Since cathepsin C is activated by thiols such as reduced glutathione, thioglycollic acid, and cysteine and inhibited by the thiol alkylating agent, iodoacetate (32), the effect of thiols and thiol alkylating or oxidizing agents on the anaphylactic reaction was determined. The thiols noted in Table VII released no histamine by themselves and neither enhanced nor inhibited the anaphylactic release of histamine. In

contrast, as shown in Table VII, agents which attack thiol groups inhibited histamine release.

When tissue is incubated with 1 mM of N-ethylmaleimide for 15 minutes and then washed four times to remove the inhibitor before adding antigen, there is marked inhibition (Table IV*B*). In fact, despite the washing, the 15 minute contact between the inhibitor and the tissue prior to washing and adding antigen produced more inhibition than when the antigen was added in the presence of the inhibitor. Thus, in contrast to the situation with the chymotrypsin sub-

TABLE VII

The Effect of Thiol Compounds and Thiol Inhibitors on the Anaphylactic Release of Histamine from Guinea Pig Lung

Class of inhibitor	Minimal concentration giving greater than 50 per cent inhibition
	mM
Thiols	
Cysteine	Not inhibitory*
Thioglycollic acid	Not inhibitory
γ-Glutamylcysteinylglycine (reduced glutathione)	Not inhibitory
Thiol inhibitors	
γ-glutamylcystinylglycine (oxidized glutathione)	10
γ-Glutamylcystinylvaline	5
Iodoacetate	2
N-ethylmaleimide	1

* Compounds producing less than 25 per cent inhibition at a concentration of 20 mM were considered not inhibitory.

strates and inhibitors, including DFP, the phase of the *in vitro* anaphylactic reaction sensitive to the thiol inhibitor is available in the absence of antigen.

DISCUSSION

The observation that the anaphylactic release of histamine from chopped guinea pig lung is prevented by chymotrypsin substrates and inhibitors (Table III), but not by trypsin, carboxypeptidase, or leucine aminopeptidase substrates (Table II) implies that activation of a chymotrypsin-like enzyme is a necessary condition for this reaction. The ability of chymotrypsin substrates to inhibit the anaphylactic release of histamine is not limited to tissue from animals actively sensitized with ovalbumin, but can also be demonstrated with tissue from animals passively sensitized with rabbit antiovalbumin (Table V) or actively sensitized with bovine gamma globulin (Table VI). Since DFP inhibited the anaphylactic release of histamine only when present at the time of antigen addition (Table IV*A*), the DFP-sensitive enzyme must exist in lung

tissue in a DFP-resistant precursor form until activated by the antigen-antibody interaction. This would be consistent with the fact that chymotrypsinogen and trypsinogen are resistant to DFP, while chymotrypsin and trypsin are inhibited (28). That DFP, the other chymotrypsin inhibitors, and the chymotrypsin substrates are acting on an antigen-antibody activated step is indicated by the failure of any of these substances to inhibit if removed from the tissue by washing prior to antigen addition (Table IV*A*), and by the complete desensitization of the tissue without histamine release when the antigen is added in the presence of the inhibitor.

Acetylation of the ester substrate, so as to introduce a secondary peptide bond, enhances effectiveness both as an inhibitor of *in vitro* anaphylaxis (Table III) and as a substrate of chymotrypsin (20). However, the relative potency of these acetylated and non-acetylated aromatic esters as inhibitors of *in vitro* anaphylaxis (Table III) differs from their relative susceptibility to pancreatic chymotrypsin (23). Furthermore, leucine ethyl ester inhibits antigen-induced histamine release (Table III), although it is a very poor chymotrypsin substrate (20, 24) in comparison to the aromatic amino acid esters. This inhibitory activity of L-leucine ethyl ester cannot be used as evidence for the participation of leucine aminopeptidase in the anaphylactic reaction because L-leucine ethyl ester is neither a substrate nor an inhibitor of leucine aminopeptidase (21, 33). The relative ability of substrates to act as inhibitors of an enzyme does not necessarily parallel their susceptibility as substrates, and so the studies on inhibition of *in vitro* anaphylaxis are only presumptive evidence that the chymotrypsin-like enzyme required for histamine release differs from pancreatic chymotrypsin in substrate specificity. The fact that pancreatic chymotrypsin failed to release histamine from chopped guinea pig lung (13) may mean that these possible differences in substrate specificity are significant, or may simply mean that extracellular chymotrypsin of pancreatic origin is not an adequate substitute for the antigen-antibody–activated chymotrypsin-like enzyme which presumably is activated near its natural substrate.

The finding that *N*-ethylmaleimide inhibited the anaphylactic reaction even when the tissue was washed repeatedly prior to antigen addition, shows that *N*-ethylmaleimide is not necessarily acting on an antigen-antibody–activated step. Either that part of the anaphylactic reaction sensitive to *N*-ethylmalemide is susceptible to inactivation prior to the antigen-antibody interaction, or the thiol inhibiting agent is preventing histamine release in some non-specific way. In either case the situation with the thiol inhibitor is in striking contrast to that with DFP which is not inhibitory unless present at the time of antigen addition and is clearly acting on an antigen-antibody activated step.

All the conclusions in the present report are based on the release of histamine as an index of the *in vitro* anaphylactic reaction, but some observations have also been made on the effect of these inhibitors on the other known product of

anaphylaxis in guinea pig tissue, slow reacting substance SRS-A (10). DFP inhibits the anaphylactic release of both histamine and SRS-A from perfused guinea pig lung (11), and L-tryptophan ethyl ester inhibits the anaphylactic release of both histamine and SRS-A from chopped guinea pig lung (34). In both instances the inhibition of histamine and SRS-A release are similar in degree, indicating that activation of the chymotrypsin-like enzyme is a necessary step in the release of both these pharmacologically active agents.

The steps leading to the anaphylactic release of histamine and SRS-A from guinea pig tissue are presumably multiple and there is no reason to assume that the characteristics of this reaction are properties of a single enzyme. In fact, despite the initial perfusion and subsequent washing of the chopped tissue, it is still possible that adsorbed serum factors are present and perhaps even necessary for the *in vitro* anaphylactic reaction. In view of the probable complexity of this reaction it would be unreasonable to expect an exact parallel with other biological systems to which it may nevertheless be related. Attention will, however, be drawn to the similarities and differences which emerge from a comparison of the anaphylactic reaction with immune hemolysis. In addition, the relevance of certain intracellular enzymes will be considered.

The mast cell enzymes are of interest because much of the tissue histamine is located in mast cells (35), and because the mast cells in the mesentery of the sensitized guinea pig (5) or rat (6) are degranulated on the addition of antigen. Benditt and Arase (36) observed that suspensions of isolated intact rat mast cells exhibit chymotrypsin-like activity which is inhibited by DFP. Although such mast cell activity could be the source of the chymotrypsin-like enzyme implicated in the anaphylactic release of histamine from guinea pig lung, there is one important discrepancy. The rat mast cell activity was demonstrated in non-sensitized animals without prior activation by an antigen-antibody reaction. Hence, unless the rat mast cell enzyme was activated during isolation or assay, it follows that the rat mast cells do not contain the inactive precursor form which seems to exist in guinea pig lung.

The intracellular enzyme, cathepsin C, like the anaphylactic reaction in guinea pig lung, is inhibited by chymotrypsin substrates (31) and by iodoacetate (32). However, cathepsin C is resistant to 1 mM DFP and is activated by thiols (32), whereas the anaphylactic reaction is inhibited by DFP (Table III) and is not affected by the thiols (Table VII). Potassium cyanide also activates cathepsin C (32), but inhibits the anaphylactic release of histamine in guinea pig lung (1, 37). Thus, cathepsin C can not be involved in the anaphylactic release of histamine unless these discrepancies are attributed to other participating factors which have not yet been characterized.

Since bifunctional chymotrypsin inhibitors are effective inhibitors of carboxypeptidase (20, 38), enzymes of the latter group might also be implicated in *in vitro* anaphylaxis in guinea pig. The fact that carboxypeptidase is DFP-

resistant does not exclude it from consideration in a multiple step system. On the other hand, the failure of substrates of carboxypeptidase to inhibit (Table II) suggests that such activity may not be involved. The finding that monobasic fatty acids inhibit anaphylaxis (34) could also be used as evidence for the participation of a carboxypeptidase-like enzyme, but carboxypeptidase is most effectively inhibited by short chain fatty acids like valeric (38), while the *in vitro* anaphylactic reaction is poorly inhibited by valeric in comparison to the effect of the longer fatty acids like decanoic or dodecanoic (34).

A comparison of hemolysis of the sensitized red cell by guinea pig complement with the anaphylactic release of histamine from guinea pig lung is of interest, because complement has been implicated in the *in vitro* anaphylactic release of histamine from rabbit platelets (39) and in the production of passive cutaneous anaphylaxis in the rat (40). DFP, an inhibitor of the first component of complement (C'1) (8), and *N*-acetyl-L-tyrosine ethyl ester, a substrate of C'1 (9), inhibit both immune hemolysis (8, 12) and the anaphylactic release of histamine (Table III) from guinea pig lung. The heat-labile, calcium-requiring factor necessary for the *in vitro* anaphylactic reaction (2, 3) could also be cited as evidence for the participation of C'1, although the anaphylactic mechanism is heat-labile at 45°C. while C'1 is labile at 56°C. Evidence to involve the third component of complement (C'3) is also available. The sensitivity of the anaphylactic release of histamine from chopped guinea pig lung to changes in the salt concentration of Tyrode's solution (22) is remarkably similar to the effect of variation in salt concentration on lysis of the sensitized red cell—carrying the first, fourth, and second components of complement (EAC'1,4,2)—by the addition of C'3 (41). Furthermore, this reaction between the EAC'1,4,2 cell and C'3 is inhibited by salicylaldoxime (42), and so is the anaphylactic release of histamine (22); 0.5 mM salicylaldoxime causing 50 per cent inhibition in each case. Although magnesium is required for immune hemolysis (14), but not for the *in vitro* anaphylactic reaction in guinea pig lung (3), it is possible to discount this difference by assuming that the tissue supplies the small amount of magnesium necessary, when none is present in the suspending medium. In experiments in which anaphylaxis has been prevented by chelation of calcium and magnesium (3, 43) with ethylenediaminetetraacetate, the restoration of the anaphylactic reaction by the addition of calcium cannot be attributed solely to a calcium requirement, because Ca^{++} displaces Mg^{++} from the chelating agent.

There are, at least, six differences between *in vitro* anaphylaxis in guinea pig lung and immune hemolysis. (*a*) Thiols such as cysteine, thioglycollic acid, and reduced glutathione inhibit immune hemolysis (12) and the esterase activity of the first component of complement (44), but do not inhibit anaphylaxis (Table VII). (*b*) Iodoacetate does not inhibit immune hemolysis (45), but iodoacetate and several thiol oxidizing agents produce irreversible changes which prevent

anaphylaxis (Table IV*B*, Table VII). (*c*) Esters of basic amino acids such as arginine and lysine inhibit immune hemolysis (12), but do not inhibit anaphylaxis (Table II). (*d*) Peptides with a terminal aromatic amino acid exhibiting a free carboxyl group, *i.e.* carboxypeptidase substrates, inhibit immune hemolysis (12), but do not prevent anaphylaxis (Table II). (*e*) Potassium cyanide does not prevent immune hemolysis (45), but does inhibit the anaphylactic release of histamine (1, 37). (*f*) Phenylacetic acid does not inhibit immune hemolysis at a concentration of 20 mM (12), but 5 mM is sufficient to produce 50 per cent inhibition of antigen-induced histamine release (Table III).

Despite these differences it can still be reasoned that complement participates merely at one stage in the *in vitro* anaphylactic reaction, and that the remaining steps differ in the two phenomena and are responsible for the discrepancies between immune hemolysis and antigen-induced histamine release. The final decision as to whether or not complement is involved must await further progress in the understanding of the *in vitro* anaphylactic reaction and its dissection into individual steps as is being done with immune hemolysis (14). If this can be accomplished, a detailed comparison including studies with inhibitors and substitution of the complement components into the anaphylactic sequence will reveal whether or not complement is required for anaphylaxis in guinea pig lung.

SUMMARY

The quantitative release of histamine by specific antigen from perfused, chopped, sensitized guinea pig lung has been used to study the effect of peptidase substrates and inhibitors on the anaphylactic reaction.

The anaphylactic release of histamine is prevented by chymotrypsin substrates and inhibitors but not by trypsin, carboxypeptidase, or leucine aminopeptidase substrates or the soybean trypsin inhibitor. The chymotrypsin substrates and inhibitors appear to be acting on an antigen-antibody–activated step because these substances fail to inhibit if the tissue is washed free of them prior to antigen addition, and because there is complete desensitization of the tissue without histamine release when the antigen is added in the presence of these inhibitors. The inhibitors work equally well in tissue from passively sensitized animals or in tissue from animals actively sensitized with either ovalbumin or bovine gamma globulin. These observations suggest that activation of a chymotrypsin-like enzyme is a necessary condition for the anaphylactic release of histamine in guinea pig lung.

Diisopropylfluophosphate is inhibitory when present at the time of antigen addition but not when the tissue is washed free of unfixed diisopropylfluophosphate prior to adding antigen. This indicates that diisopropylfluophosphate must be acting exclusively on an enzyme which exists in lung tissue in a precursor form resistant to diisopropylfluophosphate until activated by the antigen-antibody interaction.

Thiol alkylating or oxidizing agents also prevent the anaphylactic release of histamine, but in contrast to the situation with diisopropylfluophosphate and the other chymotrypsin inhibitors, the phase of the anaphylactic reaction inhibited by *N*-ethylmaleimide is available prior to the antigen-antibody interaction.

The similarities and differences between immune hemolysis and anaphylaxis in chopped guinea pig lung are considered in detail.

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BIBLIOGRAPHY

1. Mongar, J. L., and Schild, H. O., Inhibition of the anaphylactic reaction, *J. Physiol.*, 1957, **135**, 301.
2. Mongar, J. L., and Schild, H. O., Effect of temperature on the anaphylactic reaction, *J. Physiol.*, 1957, **135**, 320.
3. Mongar, J. L., and Schild, H. O., The effect of calcium and pH on the anaphylactic reaction, *J. Physiol.*, 1958, **140**, 272.
4. Chakravarty, N., The mechanism of histamine release in anaphylactic reaction in guinea pig and rat, *Acta Physiol. Scand.*, 1960, **48**, 146.
5. Mota, I., Effect of antigen and octylamine on mast cells and histamine content of sensitized guinea-pig tissues, *J. Physiol.*, 1959, **147**, 425.
6. Mota, I., Action of anaphylactic shock and anaphylatoxin on mast cells and histamine in rats, *Brit. J. Pharmacol.*, 1957, **12**, 453.
7. Hogberg, B. and Uvnas, B., Further observations on the disruption of rat mesentery mast cells caused by compound 48/80, antigen-antibody reaction, lecithinase A, and decylamine, *Acta Physiol. Scand.*, 1960, **48**, 133.
8. Becker, E. L., Concerning the mechanism of complement action. I. Inhibition of complement activity by diisopropylfluophosphate, *J. Immunol.*, 1956, **77**, 462.
9. Becker, E. L., Concerning the mechanism of complement action. II. The nature of the first component of guinea pig complement, *J. Immunol.*, 1956, **77**, 469.
10. Brocklehurst, W. E., The release of histamine and formation of a slow-reacting substance (SRS-A) during anaphylactic shock, *J. Physiol.*, 1960, **151**, 416.
11. Becker, E. L., *In vitro* models for the allergic reaction, *in* Mechanisms of Hypersensitivity, (J. H. Shaffer, G. A. Logrippo, and M. W. Chase, editors), Boston, Little, Brown, & Company, 1959, 305).
12. Cushman, W. F., Becker, E. L., and Wirtz, G., Concerning the mechanism of complement action. III. Inhibitors of complement activity, *J. Immunol.*, 1957, **79**, 80.
13. Austen, K. F., and Brocklehurst, W. E., Inhibition of the anaphylactic release of histamine from chopped guinea pig lung by chymotrypsin substrates and inhibitors, *Nature*, 1960, **186**, 866.
14. Mayer, M. M., Studies on the mechanism of hemolysis by antibody and complement, *Progr. Allergy*, 1956, **5**, 215.
15. Kekwick, R. A., and Cannan, R. K., The hydrogen ion dissociation curve of the crystalline albumin of the hen's egg, *Biochem. J.*, 1936, **30**, 227.
16. Dakin, H. D., The oxidation of amino-acids to cyanides, *Biochem. J.*, 1916, **10**, 319.

17. Pyman, F. L., A new synthesis of 4(or 5)-β-aminoethylglyoxaline, one of the active principles of ergot, *J. Chem. Soc.*, 1911, **99,** 668.
18. McIlwaine, H., and Buddle, H. L., Techniques in tissue metabolism. I. A mechanical chopper, *Biochem. J.*, 1953, **53,** 412.
19. Brocklehurst, W. E., Humphrey, J. H., and Perry, W. L. M., *In vitro* uptake of rabbit antibody by chopped guinea pig lung and its relationship to anaphylactic sensitization, *Immunology*, in press.
20. Green, N.M., and Neurath, H., Proteolytic enzymes *in* The Proteins, (H. Nuerath, and K. Baily, editors), New York, Academic Press, Inc., 1954, 2*B*, 1057.
21. Smith, E. L., and Spackman, D. H., Leucine aminopeptidase V. Activation, specificity, and mechanism of action, *J. Biol. Chem.*, 1955, **212,** 271.
22. Austen, K. F., and Brocklehurst, W. E., The influence of ionic strength on the anaphylactic reaction in guinea-pig lung, *J. Physiol.*, 1960, **153,** 66P.
23. Neurath, H., and Schwert, G. W., The mode of action of the crystalline pancreatic proteolytic enzymes, *Chem. Rev.*, 1950, **46,** 69.
24. Goldenberg, H., Goldenberg, V., and McLaren, A. D., An inquiry into the enzyme-catalyzed hydrolysis of leucine ethyl ester by chymotrypsin, *Biochim. et Biophysica Acta*, 1951, **7,** 110.
25. Schayer, R. W., Catabolism of physiological quantities of histamine *in vivo*, *Physiol. Rev.*, 1959, **39,** 116.
26. Udenfriend, S., Titus, E., Weissbach, H., and Peterson, R. E., Biogenesis and metabolism of 5-hydroxyindole compounds, *J. Biol. Chem.*, 1956, **219,** 335.
27. Huang, H. T., and Niemann, C., The inhibition of the alpha chymotrypsin catalyzed hydrolysis of nicotinyl-L-tryptophanamide and of methyl hippurate by indole, *J. Am. Chem. Soc.*, 1953, **75,** 1395.
28. Jansen, E. F., Fellows Nutting, M. D., Jang, R., and Balls, A. K., Inhibition of the proteinase and esterase activities of trypsin and chymotrypsin by diisopropylfluophosphate: crystallization of inhibited chymotrypsin, *J. Biol. Chem.*, 1949, **179,** 189.
29. Heinicke, R. M., and Mori, R., Effect of diisopropylfluophosphate on sulfhydryl proteases, *Science*, 1959, **129,** 1678.
30. Jansen, E. F., Fellows Nutting, M. D. and Balls, A. K., The reversible inhibition of acetylesterase by diisopropylfluophosphate and tetraethyl pyrophosphate, *J. Biol. Chem.*, 1948, **175,** 975.
31. Izumiya, N., and Fruton, J. S., Specificity of cathepsin, C, *J. Biol. Chem.*, 1956, **218,** 59.
32. Fruton, J. S., and Mycek, M. J., Studies on beef spleen cathepsin C, *Arch. Biochem. and Biophysica*, 1956, **65,** 11.
33. Smith, E. L., personal communication.
34. Austen, K. F., and Brocklehurst, W. E., Anaphylaxis in chopped guinea pig lung. II. Enhancement of the anaphylactic release of histamine and slow reacting substance by certain dibasic aliphatic acids and inhibition by monobasic fatty acids, *J. Exp. Med.*, 1961, **113,** 541.
35. Riley, J. F., Pharmacology and functions of mast cells, *Pharmacol. Rev.*, 1955, **7,** 267.
36. Benditt, E. P., and Arase, M., An enzyme in mast cells with properties like chymotrypsin, *J. Exp. Med.*, 1959, **110,** 451.

37. Austen, K. F., and Brocklehurst, W. E., The failure of inhibitors of aerobic metabolism to prevent the anaphylactic release of histamine from guinea pig lung, *J. Physiol.*, 1960, **154,** 11P.
38. Smith, E. L., Lumry, R., and Polglase, W. J., The van der Waals factor in carboxypeptidase interaction with inhibitors and substrates, *J. Physic. and Colloid Chem.*, 1951, **55,** 125.
39. Humphrey, J. H. and Jaques, R., The release of histamine and 5-hydroxytryptamine (serotonin) from platelets by antigen-antibody reactions *in vitro*, *J. Physiol.*, 1955, **128,** 9.
40. Osler, A. G., Hawrisiak, M. M., Ovary, Z., Siqueira, M., and Bier, O. G., Studies on the mechanism of hypersensitivity phenomena II. The participation of complement in passive cutaneous anaphylaxis of the albino rat, *J. Exp. Med.*, 1957, **106,** 811.
41. Becker, E. L., and Wirtz, G. H., The salt-sensitive step in immune hemolysis, *Biochim. et Biophysica Acta*, 1959, **35,** 291.
42. Mills, S. E., and Levine, L., The inhibition of immune hemolysis by salicylaldoxime, *Immunology*, 1959, **2,** 368.
43. Chakravarty, N., and Uvnas, B., Histamine and a lipid-soluble smooth-muscle stimulating principle ('SRS') in anaphylactic reaction, *Acta Physiol. Scand.*, 1959, **48,** 1.
44. Ratnoff, O. D. and Lepow, I. H., Some properties of an esterase derived from preparations of the first component of complement, *J. Exp. Med.*, 1957, **106,** 327.
45. Yenson, M., The nature of complement, *Istanbul Univ. Tib Falultesi Mecmuasi*, 1945, **8,** 4240.