dochus and duodenum of guinea pig, we confirmed our previous assumption concerning the existence in the terminal bile duct of α-receptors whose activation caused a constriction and β-receptors whose activation caused a dilatation. The contracting action of epinephrine was selectively blocked by dibenamine, while the relaxing action of isoprenaline was blocked by pronethalol.

1. Crema, A., Bertè, F., Brit. J. Pharmacol., 1963, v20, 221.
2. Benzi, G., Bertè, F., Crema, A., Frigo, G. M., ibid., 1964, v23, 101.
3. Crema, A., Benzi, G., Frigo, G. M., Bertè, F., J. Pharm. Pharmacol, in press.
4. Eisenstein, M. W., Surg. Gyn. Obst., 1949, v89, 49.

Received April 7, 1965. P.S.E.B.M., 1965, v120.

A Possible Mechanism for the Anti-Inflammatory Effects of Proteolytic Enzymes.* (30474)

W. L. GABLER†‡ AND L. S. FOSDICK (Introduced by N. M. Phatak)
Chemistry Department, Northwestern University Dental School, Chicago, Ill.

A number of investigators have reported that certain of the proteolytic enzymes possess anti-inflammatory properties(1,2,3). Innerfield(4) suggests that an enzyme may act as an anti-inflammatory agent by nature of its enzymatic properties, provided it is in an active form in the edema fluid. Among the functions of enzymes in the role of an anti-inflammatory agent, according to Innerfield, are hydrolysis of macromolecules (thus easing their removal), the lowering of the viscosity of the edema fluid and the breakdown of lymphatic blockades.

The present work was undertaken to see if enzymes injected at a point distant to the site of inflammation can be found in an active form in the edema fluid. A second phase of the work was to note the effect of a proteolytic enzyme on the flow rate and composition of lymph derived in part from the area of inflammation. The effects of an enzyme on the volume and protein composition of the lymph and edema fluid were also studied. A comparison of the changes in lymph and edema fluid under enzyme influences may shed some light on the mechanism of their anti-inflammatory activities.

The pleural space was selected as the site to induce inflammation since both edema and lymph derived from this space can be collected in quantities sufficient for analysis and relatively free from tissue contamination. Lymphatic drainage from the pleural space is channeled, in part, into the thoracic duct where it can be collected for analysis(5).

Method. Male albino rats weighing 350-390 g were used in this study. The rats were lightly anesthetized with ether; 5 ml of a solution containing 1% gum arabic in saline were injected into the right pleural space of the rat by means of a 5-mm-long #26 needle. Concomitantly, 1 ml of the test enzyme solution was injected subcutaneously into the left hind leg above the knee joint. The enzyme was dissolved in 1 ml of saline and brought to body temperature immediately prior to use. Controls received leg injections of 1 ml of saline without the enzyme.

After appropriate time intervals the rats were sacrificed with ether, pleural cavities opened and the fluid withdrawn. The volume of fluid collected was measured in a graduated centrifuge tube, allowed to clot and stored at 4°C. The clotted material was centrifuged at 2000 rpm and the supernatant was used for analysis.

To study the effect of chymotrypsin on the

* This study was supported in part by grant HF-12,422-C1, Nat. Inst. Health, U.S.P.H.S.

† Taken in part from a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree in chemistry at Northwestern University, Evanston, Ill., Aug. 1964.

‡ Present address: Biochemistry Dept., University of Oregon Dental School, Portland.

TABLE I. Activity of Enzymes in Serum and Edema Fluid in Terms of μMols CO_2 Released per Hour per ml of Fluid.

Enzyme and base	Substrate	Source of test fluid*	Time (hr) 0	½	1	2
Chymotrypsin, 10 mg	PAEE	S	0	2.35	0	0
		E	0	.77	0	0
Plasmin, 30 mg	BAEE	S	0	0	0	0
		E	0	0	0	0
Trypsin, 10 mg	"	S	0	0	0	0

* S = serum; E = edema.

flow rate and composition of lymph derived in part from the thoracic area, lymph was collected from the thoracic duct. The irritant was introduced into the pleural space as described, followed immediately by injection of 1 ml (10 mg/ml) of the chymotrypsin solution into the hind leg. Lymph was collected from control (non pleural treated), irritated and irritated-chymotrypsin treated rats approximately 4 hours after irritation. The lymph was collected by means of a micropipette and the volume of lymph collected per unit time was used as a measure of the flow rate. The rats were anesthetized prior to surgery with 42 mg/kg of sodium pentobarbital. The anesthesia was kept as light as possible. The lymph and pleural fluid were analyzed for protein by means of the micro-Kjeldahl method and the A/G ratio determined by paper electrophoresis.

To determine if enzymes injected into the hind leg of a rat entered the serum or edema in an active form, activity assays were run on these fluids. Chymotrypsin (10 mg/ml), trypsin (10 mg/ml) or plasmin (30 mg/ml) were used as the test enzymes. The assays were all run manometrically using 4 animals per run. Chymotrypsin activity was determined according to the method of Parks and Plaut(6) using L-phenylalanine ethyl ester (PAEE) as the substrate. Plasmin and trypsin activities were run according to the method of Zeller(7) with benzoyl-L-arginine ethyl ester (BAEE) as the substrate. The activities of these enzymes in serum and edema are reported in terms of micromoles of CO_2 released per hour per ml of assay fluid.

Results and discussion. The results of the enzyme activity experiment (Table I) show that chymotrypsin at ½ hour after injection is in an active state in serum and edema fluid. This is in accord with the work done on serum by Avakian(8) and Bastian(9) who noted that trypsin is readily inactivated by potent tryptic inhibitors found in the blood while chymotryptic activity remained for some time after intramuscular injection. Plasmin activity was lacking in both the serum and edema fluid. It can be postulated that due to its larger molecular weight (MW ~ 75-100,000) it does not readily penetrate the capillaries and enter the blood. Furthermore, there are potent antiplasmin factors in the blood(10).

Finding that chymotrypsin enters the edema fluid in measurable amounts in an active form, it was therefore decided to use it for further studies on the effects of a proteolytic enzyme on the volume and protein composition of edema and lymph fluids.

The time response of the protein concentration and volume of edema fluid in irritated and irritated-chymotrypsin treated (10 mg/ml) rats is given in Table II. It will be noted that the maximum anti-inflammatory activity is seen during the first 6-8 hours of the inflammatory process. The fact that there is a reduction in both the protein concentration and volume of edema distinguishes the anti-inflammatory activity of chymotrypsin from that of salicylate, cortisone, or ACTH. Merits(11) found that these later materials bring about a reduction in the volume of edema and total protein in this fluid but a marked increase in protein concentration. Thus, it appears that the modes of action of salicylate, cortisone and ACTH differ from that of chymotrypsin. Cohen(12) established that chymotrypsin does not derive its activity from the adrenal-pituitary axis.

Lymph was collected from control, pleural irritated and pleural irritated-chymotrypsin treated rats (Table III). The pleural-irritated rats (Group B) are to be compared with the chymotrypsin-treated animals (Group C). It will be noted that there is a 41.0% increase in the lymph flow rate, 17.4% decrease in protein concentration and a 16.0% increase in the protein flow rate.

TABLE II. Time-Response for Edema Fluid in Chymotrypsin-Treated and Non-Treated Rats.

	Time, hr	No. of animals	Vol of edema, cc	Protein conc, g %	Total protein, mg
Control rats	2	5	3.3 ± .2*	1.25 ± .02*	41 ± 2 *
	4	5	4.5 ± .5	2.66 ± .30	117 ± 15
	8	4	6.8 ± .6	3.96 ± .14	270 ± 33
	16	5	7.9 ± .3	4.44 ± .25	344 ± 45
	24	5	9.0 ± .3	4.79 ± .10	433 ± 22
Chymotrypsin (10 mg)	2	4	3.5 ± .1	.69 ± .01	24 ± .5
	4	4	3.2 ± .2	1.06 ± .12	34 ± 2
	6	5	2.9 ± .3	1.28 ± .13	37 ± 3
	8	5	4.0 ± .7	1.72 ± .43	72 ± 31
	16	4	6.2 ± .6	3.11 ± .45	193 ± 30
	24	4	5.1 ± .2	4.58 ± .30	235 ± 35

* Mean ± S.E.M.

TABLE III. Comparison of Thoracic Duct Lymph in Control Edematous and Chymotrypsin-Treated Rats.

Treatment	No. of rats	Flow rate, cc kg^{-1} hr^{-1}	Conc of protein, g %	Flow rate of protein, g kg^{-1} hr^{-1}	A/G
A—Control	8	2.50 ± .51†	3.89 ± .66†	.105 ± .018†	1.10 ± .27†
B—5 cc 1% G.A.*	10	2.20 ± .21	4.02 ± .25	.088 ± .009	1.10 ± .12
C—5 cc 1% G.A., 10 mg chymotrypsin	10	3.10 ± .32	3.32 ± .50	.102 ± .16	.446 ± .17
% Change B *vs* C		+41.0	−17.4	+16.0	−59.5
Probability (B *vs* C)		P >.01	P >.01	P >.1	P >.01

* G.A. = Gum Arabic. † Mean ± S.E.M.

It is of particular importance to determine whether or not this increase in flow rate reflects an increase in lymph drainage from the pleural cavity. The pleural materials are initially high in globulin materials(5). With the onset of inflammation the capillaries become more permeable to proteins, especially the lower molecular weight albumins. Following the A/G ratio of the edema fluid over a period of time there is an increase of the A/G ratio reflecting an increase in concentration of albumins until at 16 hours the A/G ratio of the edema approaches that of blood. It will be noted (Table IV) that during the first 4 hours there is a significant increase in the A/G ratio of the enzyme-treated group as compared to the controls. This increase in A/G in the treated group can be explained on the basis of increased lymphatic drainage. The irritant (a non-protein material) can cause a flushing of globulins out of the pleural area if the lymphatics are open. This flushing initially reduces the concentration of the globulins in the pleural space. With the entrance of albumins an increased A/G ratio would be expected. In the controls there would be dilution of the globulins but because of lymph stasis they would remain in the pleural space, thus reducing the A/G ratio. The higher A/G ratio in the treated group does not

TABLE IV. Pleural Edema Fluid A/G Ratio *vs* Time.

Time, hr	No. of rats	A/G
	Controls*	
2	4	.280 ± .005‡
4	4	.446 ± .054
8	5	1.22 ± .10
16	5	1.36 ± .11
	Chymotrypsin treated†	
2	4	.739 ± .040
4	4	.893 ± .058
8	4	1.25 ± .16
16	4	1.24 ± .07
	Test of significance	
2	Probability	P > .01
4		P > .01
8		P =1 (N.S.)
16		P > .2 (N.S.)

* Controls—5 cc of an irritant (1.0% gum arabic in saline) injected into pleural space.

† Same irritant as controls with 10 mg of chymotrypsin injected into leg.

‡ Mean ± S.E.M.

result from an increase in capillary permeability since total protein concentration in this group is markedly reduced.

Furthermore, if there is initially an increase in flow of globulins from the pleural space in the chymotrypsin-treated rats this should result in a change in the A/G ratio of the lymph. The drop in the A/G ratio of the thoracic duct lymph in the enzyme-treated group (Table III) further supports the contention that chymotrypsin increases lymphatic drainage from the inflamed pleural space. If chymotrypsin or some substance released by chymotrypsin caused a generalized increase in lymphatic drainage, we would expect to find an elevated A/G ratio since it would be expected that there would be an increase in the smaller, more abundant albumin materials in the lymph. The drop in protein concentrations of the thoracic duct lymph (Table III) is likewise explainable on the basis of increased lymph drainage from the pleural space. Since the irritant was non-protein in nature it would dilute the proteins in the lymph if it could enter the lymph.

These results indicate that chymotrypsin or some substance released by chymotrypsin increased lymph drainage from the inflamed pleural space without a concomitant increase in capillary permeability. This could result from enzymatic hydrolysis of the proteins which caused lymph stasis in the irritated animals.

Summary. The activity of chymotrypsin, plasmin and trypsin in serum and edema fluid was determined manometrically. It was found that only chymotrypsin, of the enzymes tested, could be found in an active form in these fluids. The volume of edema and its protein concentration were compared in pleural irritated and irritated-chymotrypsin treated rats. Flow rate and protein composition of thoracic duct lymph were also compared between both groups. Changes in lymph and edema in the 2 groups indicate that chymotrypsin increases the rate of flow of lymph from the pleural space without a concomitant increase in capillary permeability. No attempt was made to describe the mechanism responsible for this increase in lymph flow.

1. Martin, J. C., Brendel, R., Beeter, J. H., Proc. Soc. Exp. Biol. and Med., 1954, v86, 636.

2. Hechter, O., Ann. N. Y. Acad. Sci., 1950, v52, 1028.

3. DeSalva, S. J., Evans, R. A., Arch. Intern. Pharmacodyn., 1962, v137, 383.

4. Innerfield, I., Ann. N. Y. Acad. Sci., 1957, v68, 167.

5. Gabler, W. L., Fosdick, L. S., Proc. Soc. Exp. Biol. and Med., 1964, v115, 915.

6. Parks, R. E., Plaut, G. W. E., J. Biol. Chem., 1953, v203, 755.

7. Zeller, E. A., Devi, A., Carbon, J. A., Fed. Proc., 1956, v15, 1278.

8. Avakian, S., New Eng. J. Med., 1961, v264, 764.

9. Bastian, J. W., Hill, R. G., Ercoli, N., Proc. Soc. Exp. Biol. and Med., 1956, v92, 800.

10. Norman, P. S., J. Exp. Med., 1958, v108, 53.

11. Merits, J., Ph.D. Dissertation, Northwestern Univ., Evanston, Ill., 1955.

12. Cohen, H., Graff, M., Kleinberg, W., Proc. Soc. Exp. Biol. and Med., 1955, v88, 517.

Received June 8, 1965. P.S.E.B.M., 1965, v120.

Responses of Cell Cultures to Insecticides. I. Acute Toxicity to Human Cells.* (30475)

JANIS GABLIKS AND LEO FRIEDMAN†

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge

The increased use of agricultural pesticides poses a continuous challenge to scientists concerned with setting of residue tolerances that will be consistent with the safety of the food supply. Although the toxic manifestations of

* Contribution No. 657 from Dept. of Nutrition and Food Science. Supported in part by NIH Grant CA-06988-01 and in part by U. S. Army Contract DA-49-193-MD-2533.

† With technical assistance of Elio Goffi.