Table II. Effect of proteinase inhibitors on the localized hemorrhagic SHWARTZMAN phenomenon in skin prepared with endotoxin: intravenous administration of inhibitors

Agent (dose range)	Hemorrhagic lesions			
	Area (mm^2) x intensity score (number of test areas)	Suppression (%)	2% Na_2CO_3 extract of reaction loci A_{430nm} (number of test areas)	Suppression (%)
Saline control	1626 ± 235 (6) [a]	—	11.2 ± 2.2 (6) [a]	—
Soybean trypsin inhibitor (2.4 mg)	334 ± 93 (8)	80	4.0 ± 0.7 (8)	64
Pepstatin (4.0 mg)	591 ± 240 (8)	64	3.8 ± 0.7 (8)	66

[a] Standard error of mean.

inhibitor at the doses tested effectively reduced the manifestation of the localized SHWARTZMAN phenomenon in rabbits. The suppressive effectiveness of pepstatin was less than that of soybean inhibitor if compared at the same molar concentration. This is consistent with the result of the experiments with intradermal application of inhibitors.

Prior studies have established that both platelets and granulocytes are essential to the expression of both localized and generalized SHWARTZMAN reactions[3,10]. THOMAS[4] was able to demonstrate that preparation of the skin of rabbits by intradermal injection of the granule fraction obtained from peritoneal granulocytes, followed by i.v. injection of endotoxin, resulted in a SHWARTZMAN type hemorrhagic lesion. Our analysis of the general proteolytic activity in rabbit polymorphonuclear leukocytes, as shown in Figure 1, revealed that the major activity is in the acidic range and little trypsin inhibitor-sensitive general proteolysis could be detected. The neutral and alkaline proteinases of leukocytes may therefore have strict substrate requirements with relatively limited hydrolysis, and the direct effect of such enzymes in causing tissue damage would be insignificant unless the activities of these enzymes could be linked to some activation mechanisms leading to tissue injury. The cathepsin D type acid proteinase activities of leukocytes was shown to be significantly inhibited by pepstatin but was not affected by the trypsin inhibitors at the concentrations tested.

Since the specificity of pepstatin as the inhibitor of pepsin-cathepsin D type acid proteinases has been established by numerous studies[20,23], the results of the present study may be reasonably interpreted as evidence of possible participation of cathepsin D from granulocytes in the expression of the localized SHWARTZMAN reaction. The soybean trypsin inhibitor-sensitive proteinases may well be those involved in the blood coagulation system and complement activation. Pathologic alterations of the coagulation system followed by the interaction between platelets and endotoxic lipopolysaccharide were described before[7,10]. Evidence for involvement of the complement system in the pathogenesis of the SHWARTZMAN reaction has been obtained[8]. Pepstatin has also been reported to inhibit the renin-angiotensinogen system[24,25]. The kinin-like substances potentially generated by the action of renin in blood[26] could play a role in the expression of the SHWARTZMAN phenomenon. The enzyme renin, which catalyzes these reactions at neutral pH, exhibits the characteristics of acid proteinases including pepsin and is inhibited by pepstatin[27].

Exploration of the mechanism of tissue injury by use of specific enzyme inhibitors may aid in understanding and control of this pathologic phenomenon. The system described in this communication would offer an interesting and valuable in vivo test system for evaluation of pharmacologically useful proteinase inhibitors.

Zusammenfassung. Nachweis, dass das lokale SHWARTZMAN-Phänomen in Kaninchen durch intradermale oder i.v. Gabe von Pepstatin, einem sauren Proteinase-Hemmer, oder von Sojabohnen-Trypsin-Hemmer kurz vor der auslösenden Injektion von bakteriellem Endotoxin unterdrückt werden kann.

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[23] H. IKEZAWA, T. AOYAGI T. TAKEUCHI and H. UMEZAWA, J Antibiot. *24*, 488 (1971).
[24] F. GROSS, J. LAZAR and H. ORTH, Science *175*, 656 (1972).
[25] R. P. MILLER, C. J. POPER, C. W. WILSON and E. DEVITO, Biochem. Pharmac. *21*, 2941 (1972).
[26] K. K. F. NG, in *Prostaglandins, Peptides and Amines* (Eds. P. MANTEGAZZA and E. W. HORTON; Academic Press, New York 1969) p. 9.
[27] M. OVERTURF, M. LEONARD and W. M. KIRKENDALL, Biochem. Pharmac. *23*, 671 (1974).
[28] Z. A. COHN and J. G. HIRSCH, J. exp. Med. *112*, 983 (1960).
[29] H. R. WILLIAMS and T.-Y. LIN, Biochim. biophys. Acta *250*, 603 (1971).

Effect of the Venom Sac Content of the Oriental Hornet (*Vespa orientalis*) on the Metamorphosis of the Toad Tadpole (*Bufo viridis*)

The venom of the Oriental hornet (*Vespa orientalis*) is known to contain several factors capable of preventing further development of insect larvae into which it has been injected[1]. It is reasonable to assume that the lack of development is due to impairment of the hormonal balance.

Ignoring the mechanism of action of the venom, we investigated the metamorphosis of the toad tadpole (*Bufo viridis*) under the influence of the whole content of the venom sac of the Oriental hornet, as a preliminary study. The advantage of using tadpoles is that their development is easily followed and that the venom can

Number of animals in different stages of development in normal and treated tadpoles

		Premetamorphic stage	Prometamorphic stage	Toad
End of the experiment	Normal[a]	4	10	34
	Treated[b]	17	15	10

25 animals per group at the beginning of the experiment. [a] 2 animals diet at this stage. [b] 8 animals died at this stage.

be dissolved in the water in which they are kept. Animals at two stages of development have been used in this study: the premetamorphic and prometamorphic stages[2]. Thyroid activity is absent in the first stage and increased rapidly during the second[3]. Differences in hormonal activity could induce different responses to the venom application.

Materials and methods. 100 tadpoles (*Bufo viridis*) were used in this study, 50 in the premetamorphic stage and 50 in the prometamorphic stage. The animals of each stage were divided into 2 groups of 25 each, and put into 2 separate pails, one containing 10 l of tap water (pH 6.8, temperature 22°–24 °C), the second containing tap water in which the content of 12 venom sacs were minced, which yielded approximately 6 mg venom per l. This procedure was repeated twice 3 and 5 weeks after the beginning of the experiment. The water was changed before introducing the new dose of venom. The animals were fed lettuce leaves.

The experiment was conducted for 2 months and the state of each animal recorded at the end of it. During the course of the experiment, some animals showed bleaching of the skin after venom application, they were fixed in Bouin fixative and prepared for histological examination.

Results. Two major phenomena were observed during the course of the experiment: 1. suppression of development and 2. bleaching of the skin of 11 tadpoles treated in the prometamorphic stage.

1. Development was suppressed in all of the animals treated in the premetamorphic stage and in 60% of the animals treated at the prometamorphic stage (Table).

2. Skin bleaching appeared 2 weeks after the beginning of the experiment in the gill area and spread until the whole animal acquired a grayish colour; it could be reversed by transfering the animals into clean water. The histological picture of the tail showed that the pigment granules normally present in the melanophores of the dermis disappeared from the melanophores of the treated animals, only some migrating pigmented cells were observed in the muscle layer (Figure 1 and 2).

Discussion. Our results showed that hornet venom exerts a definite suppressive effect on the metamorphosis of the tadpole. This effect clearly depends on the stage of development at which the venom has been administered; this is probably related to the stage of development of the endocrine system.

The bleaching phenomenon is comparable to that described by HADLEY and GOODMAN[4] in frog skin under the influence of norepinephrine in vitro. In their work, skin bleaching results from changes in the distribution of pigment granules within the melanophores. In our case, bleaching is due to the disappearance of the pigment granules; it occurs in whole animals and is a progressive phenomenon. This suggests that bleaching is due to some physiological disturbance. The hornet venom indeed contained catecholamines[5], but during the time interval required for bleaching to occur (2 weeks) they most

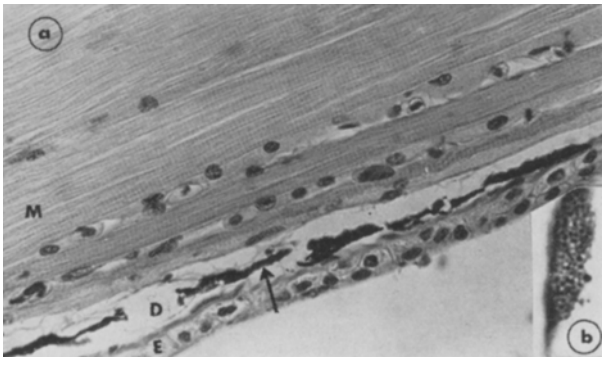

Fig. 1. a) Tail of an untreated tadpole. Hematoxylin-Eosin. Arrow: melanophore layer. ×750. b) Higher magnification of a melanophore. Note the conspicuous pigment granules. ×1990.

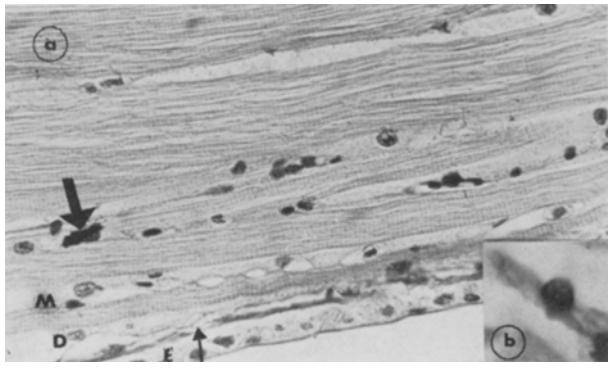

Fig. 2. a) Tail of a treated tadpole. Hematoxylin-Eosin. Thick arrow: migrating melanophore. ×750. b) Note the absence of pigment granules. ×1990. E, epidermis; D, dermis; M, muscle.

[1] J. ISHAY, unpublished observations.
[2] W. ETKIN, in *Physiology of Amphibia* (Ed. J. A. MOORE; Academic Press, New York 1964), p. 427.
[3] W. ETKIN, Science *139*, 810 (1963).
[4] M. E. HADLEY and J. M. GOLDMAN, Am. J. Physiol. *219*, 72 (1970).
[5] J. ISHAY, Z. ABRAHAM, Y. GRÜNFELD and S. GITTER, Comp. Biochem. Physiol. *48A*, 369 (1974).

probably undergo oxidation and are not active anymore. It is possible that the polypeptides contained in the hornet venom are responsible for the changes observed in the tadpoles; this will be the subject of further investigation.

Résumé. Le venin de la guêpe (*Vespa orientalis*) supprime la métamorphose du têtard de crapaud (*Bufo viridis*) chez tous les animaux traités au stade prémétamorphique et chez 60% des animaux traités au stade prométamorphique. Onze des animaux traités ont montré un éclaircissement de la peau.

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In vivo Mutagenic Interaction of Nitrite and Ethylenethiourea

Ethylenethiourea (ETU) is by itself a weakly mutagenic[1] and carcinogenic[2,3] decomposition[4] and metabolic[5] product of the fungicidal ethylene-*bis*-dithiocarbamates. Recently it has been reported that ureas and other nitrogen containing pesticides and food contaminants, after nitrosation with sodium nitrite in simulated gastric juice, become highly mutagenic[6-8] and carcinogenic[9,10] substances. Other reports have shown that the feeding of mice or rats with nitrite and amines or amides leads to the in vivo formation of the respective N-nitroso-derivatives[11] and later on in the life of the animals to tumors of various organs[12]. There have been, however, no in vivo mammalian tests for the mutagenic activity of such pesticides interacting with nitrite directly within the animal. For this reason we intended to investigate the effects of an orally given mixture of ETU and sodium nitrite on the cytology of the mouse bone marrow, because the micronucleus test is a simple, rapid, reliable mutagenicity test method[13-15] which is also easy to perform.

We began our experiments with preliminary in vitro studies, in which the *Salmonella typhimurium* tester strains, kindly provided by Prof. B. N. AMES (University of California, Berkeley), were used. The results displayed in Table I show the large increase in the relative mutagenicity when a N-nitrosated ETU was placed onto the test plates.

For our main task, young female ICR mice obtained from the Tierzuchtinstitut of the University of Zürich were given the various test compounds either orally or i.p. Two dosages of each compound were applied. For the i.p. application, the compounds were dissolved in sterile physiological saline, cantaining 3% DMSO. The concentration of each compound was calculated in such a way that a 30 g animal received the proper dose in a volume of 0.50 ml. Orally given compounds were dissolved in a 2% gum Arab solution and were applied with means of a stomach tube. 4 mice were used for each concentration and they received the test substances twice 24 h apart. 6 h after the second application, the mice were sacrificed and the bone marrow smears prepared and stained as described by SCHMID[13].

To differentiate clearly the effects of the ETU-nitrite interaction from those of the single components, we applied them at the same dose levels as used in the mixture. Also included in this test were control animals, which received no treatment, and a positive control with the known mutagen Trenimon (2, 3, 5-*tris*-ethyleneiminobenzoquinone-(1,4).

Chromosome breakage, as an expression of mutational events, leads to the formation of small, separate micronuclei in blood cells. They are easiest to recognize in

[1] J. P. SEILER, Mutation Res. *26*, 189 (1974).
[2] J. R. M. INNES, B. M. ULLAND, M. G. VALERIO, L. PETRUCELLI, L. FISHBEIN, E. R. HART, A. J. PALLOTTA, R. R. BATES, H. L. FALK, J. J. GAST, M. KLEIN, I. MITCHELL and J. PETERS, J. natn. Cancer Inst. *42*, 1101 (1969).
[3] B. M. ULLAND, J. H. WEISBURGER, E. K. WEISBURGER, J. M. RICE and R. CYPHER, J. natn. Cancer Inst. *49*, 583 (1972).
[4] W. H. NEWSOME and G. W. LAVER, Bull. envir. Contam. Toxic. *10*, 151 (1973).
[5] T. SATO and C. TOMIZAWA, Bull. natn. Inst. agric. Sci., Tokyo, Ser. C, *12*, 181 (1960).
[6] R. ELESPURU, W. LIJINSKY and J. K. SETLOW, Nature, Lond. *247*, 386 (1974).
[7] H. ENDO and K. TAKAHASHI, Nature, Lond. *245*, 325 (1973).
[8] R. K. ELESPURU and W. LIJINSKY, Fd. Cosmet. Toxicol. *11*, 807 (1973).
[9] D. SIEBERT and G. EISENBRAND, Mutation Res. *22*, 121 (1974).
[10] O. UNGERER, G. EISENBRAND and R. PREUSSMANN, J. Krebsforsch. *81*, 217 (1974).
[11] G. EISENBRAND, O. UNGERER and R. PREUSSMANN, Fd. Cosmet. Toxicol. *12*, 229 (1974).
[12] W. LIJINSKY, H. W. TAYLOR, C. SNYDER and P. NETTESHEIM, Nature, Lond. *244*, 176 (1973).
[13] K. BOLLER and W. SCHMID, Humangenetik *11*, 35 (1970).
[14] W. SCHMID, Agents Actions *3*, 77 (1973).
[15] M. VON LEDEBUR and W. SCHMID, Mutation Res. *19*, 109 (1973).

Table I. Relative mutagenic activities of various compounds on *Salmonella typhimurium* strain his G 46

Compound	Concentration (m*M*)	Relative mutagenicity	Comments
None	—	1.0	Spontaneous mutations
ETU	1	2.5	Dissolved in DMSO
$NaNO_2$	5	1.5	Dissolved in acetate buffer (pH 4.5)
ETU + $NaNO_2$	1 + 5	14.0	Dissolved in acetate buffer (pH 4.5)
2-Aminopurine	0.5	10.0	Positive control, dissolved in water

The plate test was performed as outlined by AMES[16] and modified by us[1]. Relative mutagenicity is defined as the quotient from the mean number of colonies on the test plates divided by the mean number of spontaneously arizing colonies.