was 400 mg/kg; 42 control rats were injected with similar quantities of propandiol MERCK only. At days 1, 3, 7, 10, 14, 17 and 21, after the first dose 3 animals of each group were removed and a sample of blood taken from the retroorbital plexus. Besides haematocrit erythrocytes, reticulocytes and Heinz-bodies were counted by means of Coulter counter, methylene blue staining and nile blue staining respectively. Haemoglobin was estimated by the haemoglobin-cyanide method. The remaining animals were allowed to recover for 21 days during which time blood was taken in a similar manner as above, and the same parameters measured at similar times after stopping dosing. Another group of 5 animals were treated in the same way with AIAU in a daily overall dose of 500 mg/kg for 42 days, and on the 43rd day the thrombocytes were counted by the cocaine method and compared to 5 control animals.

Results and discussion. If rats are treated for a relatively long period with AIAU, they show marked disturbances in liver haem metabolism, which is paralleled by a EM and histologically visible pigmentation of this tissue[9], and increased urinary excretion of porphyrins and their precursors. Variations were also noted in serum enzymes and other serum parameters (RENTSCH and JOHNSTON[10]).

In our experiments we were unable to find major changes in the haematopoetic system. Erythrocytes, haematocrit and haemoglobin variations were within the margin of error of our techniques. During the experiment the reticulocytes were in the range of 21–145 cells/1000, which is within the physiological norm for the rats used. Heinz-bodies, markers of intra-erythrocyte haemoglobin destruction (RENTSCH and WITTEKIND[11]), were not found. Hence it can be seen that AIAU produces no significant alterations in the parameters measured, except for a slight elevation of the thrombocytes in the high-dose animals, a result contrary to that found in humans hypersensitive to AIAU (see Table).

Thrombocytes of rats dosed with AIAU 500 mg/kg (T) compared with those of controls (K)

Animal number	Thrombocytes per µl
K1	836×10^3
K2	772×10^3
K3	974×10^3
K4	782×10^3
K5	1102×10^3
K average	893×10^3 [a]
T1	1268×10^3
T2	1366×10^3
T3	1252×10^3
T4	1068×10^3
T5	1204×10^3
T average	1232×10^3 [b]

[a] SD: $\pm 142 \times 10^3$; [b] SD: $\pm 109 \times 10^3$; $P<0{,}01$
See text for full experimental details.

LEVIN et al.[12] suggest that AIAU exerts its pathophysiological effects for 2 reasons: 1. there is an allyl group in the molecule, and 2. the compound is converted into an active metabolite. It is also suggested that the active form is an epoxide on the allyl group. This epoxide is then thought to react with haem causing its destruction in liver.

Why then does AIAU not cause haemoglobin destruction in blood? One answer may be that red cells are unable to metabolize it and the active metabolite formed in other tissues never reaches the circulatory system. In this context it should be mentioned that other tissues besides liver can produce the active metabolite. We have found a green pigmentation in isolated microsomal preparations of lung and kidney from rats treated with AIAU[10]. This indicates a similar pattern to that in the liver where AIAU causes haem breakdown products from cytochrome P-450, colouring the microsomes.

Tissues capable of producing the active molecule probably bind it tightly and rapidly to structures near the site of its formation. This means that the effective half-life of the new compound formed must be extremely short. In the kidney, there is the possibility of an allyl group epoxide forming and being rapidly excreted due to the special nature of the tubular system. Epoxides found in rat urine after administration of AIA support this[13,14].

Zusammenfassung. Wenn Ratten längere Zeit mit 2-Allyl-2-isopropylacetylharnstoff behandelt werden, finden sich in verschiedenen Organen grün-braune Pigmente. Diese vorwiegend in Microsomen lokalisierten Produkte stammen vom Häm des Cytochroms P-450 sowie anderen Hämoproteinen. Am hämatopoetischen System konnten demgegenüber keine Veränderungen gefunden werden. Damit ist der Schluss gestattet, dass der für die Hämdenaturierung verantwortliche aktive Metabolit eine sehr kurze Halbwertzeit haben muss.

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Inhibition of the Local Hemorrhagic Shwartzman Reaction by an Acid Proteinase Inhibitor, Pepstatin

When bacterial endotoxin is injected into rabbits intracutaneously, followed in 24 h by an i.v. injection of another endotoxin preparation, an inflammatory and necrotic reaction is manifested at the site of intracutaneous implantation of the material[1,2]. The mechanism of this local hemorrhagic SHWARTZMAN phenomenon is unclear. However, evidence indicates that a granulocyte mediated inflammatory process[3–5] is involved along with the effect of endotoxin upon the blood vessels and lymphoreticular cells[6,7]. Complement[8] as well as the blood coagulation

system[2,9–11] has also been implicated in this pathologic phenomenon. It is likely that the SHWARTZMAN reaction is a consequence of cascade reactions in which enzyme activities could play a role. The intracellular proteolytic enzymes have been suggested to be essential in the generation of this reaction[3], and trypsin was reported to potentiate it[12].

If trypsin inhibitors were injected i.v. shortly before the time of the provocative endotoxin injection, the expression of the SHWARTZMAN phenomenon was suppressed[13,14]. It was suggested that the tissue damage observed in the phenomenon was conditioned directly by or through release of proteinases, particularly the lysosomal enzymes contained in granulocytes[4]. Soybean trypsin inhibitor and bovine organ trypsin inhibitor (Trasylol) are known inhibitors of kinin generation and of the trypsin-like proteinases participating in the blood coagulation processes[15], but they do not inhibit the acid proteinases, such as cathepsin D[16], which is the major proteinase of rabbit neutrophils[17]. This class of acid proteinases has been implicated by DINGLE[18] in the pathological degradation of cartilage matrix. If the proteolytic enzymes derived from the granulocytes have indeed an essential function in eliciting the SHWARTZMAN phenomenon, the possible participation of the acid proteinases cannot be neglected.

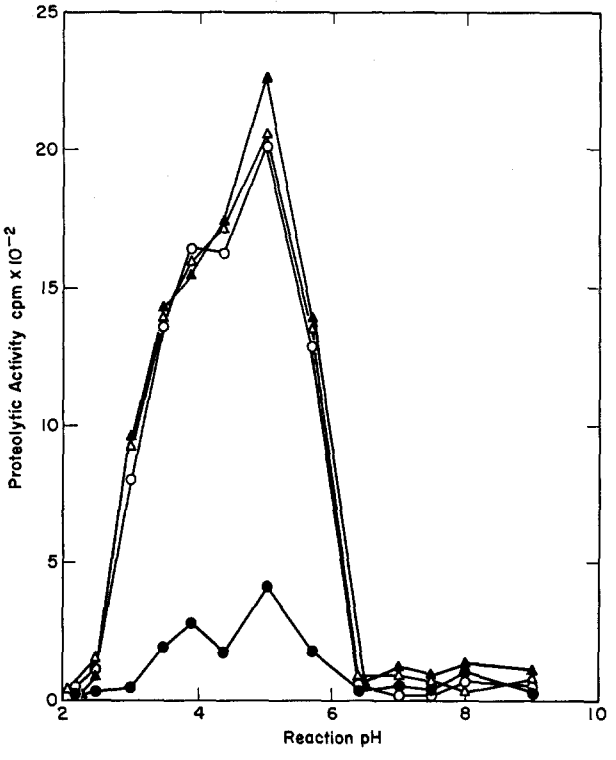

Fig. 1. pH profile of the proteolytic activity of rabbit peritoneal polymorphonuclear leukocytes. The cells (about 4×10^8), prepared according to COHN and HIRSCH[28] were homogenized with a Polytron homogenizer after freezing and thawing in 10 ml of saline solution. The material was centrifuged for 5 min at $400 \times g$, and 0.2 ml aliquots of the supernatant fraction were used for the assay. The general proteolytic activity on methyl-^{14}C-glycinated hemoglobin[29] was determined after a 2 h-incubation at 37 °C (○—○). 0.4 *M* sodium citrate buffer and 0.5 *M* *Tris*-HCl buffer were used in the pH range of 2 to 5.5 and 6 to 9, respectively. The effects of the following proteinase inhibitors were also examined in the entire pH range tested: hydroxystilbamidine at 1.9×10^{-5} *M* (▲—▲), soybean trypsin inhibitor at 0.8×10^{-6} *M* (10 μg/ml) (△—△), and pepstatin at 1.5×10^{-8} *M* (0.01 μg/ml) (●—●).

In this communication, we present evidence indicating that by proper administration route and amount, a specific inhibitor of acid proteinase, pepstatin[19], can suppress the SHWARTZMAN phenomenon. This suggests the involvement of steps catalyzed by acid proteinases in the reaction.

Materials and methods. Adult female white rabbits (New Zealand), weighing 1.5–2.5 kg, were partially depilated of the abdominal hair with the aid of clippers and a depilatory agent. The proteinase inhibitors employed were crystalline soybean trypsin inhibitor (Calbiochem, California), pepstatin (a generous gift of Dr. H. UMEZAWA, Institute of Microbial Chemistry, Tokyo), hydroxystilbamidine isethionate (May & Baker, New York), and Compound A [sodium-2-(N-methyl-4-octylbenzenesulfonic) ethanesulfonate] (General Aniline & Film, New York). The inhibitors were either made up in a solution or a fine suspension in pyrogen free saline or in 50% sterile dimethylsulfoxide (DMSO) in saline, and were administered intradermally 30 min before the provocative injection of endotoxin. For evaluation of inhibitors applied intradermally, 4 test sites on each animal were used for various inhibitors and a control. In the experiment using i.v. injection of inhibitors in 20% DMSO-sterile saline, 2 reaction sites were prepared on each animal. In all cases the preparative dose of endotoxic lipopolysaccharide (Lipopolysaccharide W *E. coli* 026:B6, Difco Laboratories, Michigan) was 50 μg, given intradermally in a total volume of 0.5 ml of saline solution. The provocative dose was 100 or 250 μg of endotoxin in 1 ml of pyrogen free saline solution, given i.v. 22 to 24 h following the preparative dose. The extent of hemorrhagic lesions was determined by the measurement of a hemorrhagic area size in millimeters, by scoring of the apparent intensity of hemorrhage and by estimation of the absorbances at 430 nm of the 2% sodium carbonate extract of a 40×40 mm excized test area of skins. These determinations were made either at 5 or 20 h following the provocative injection of endotoxin. In a positive response, the skin sites prepared developed typical hemorrhagic lesions within 5 h.

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Results and discussion. The results of the intradermal application of proteinase inhibitors are shown in Table I. Each of the inhibitors examined caused suppression of the SHWARTZMAN reaction to varying degrees when compared to the controls. However, a dose response relationship was difficult to establish in the dose range tested. For example, soybean trypsin inhibitor given in amounts of 0.5, 1.0 and 2.0 mg per site was suppressive in each case, but no correlation could be observed between the dose given and the extent of lesions. This is due partially to the insensitivity of quantitative evaluation of the phenomenon, but may also be due to the step which is sensitive to the proteinase inhibitors occurring at the early stage of the series of cascade reactions.

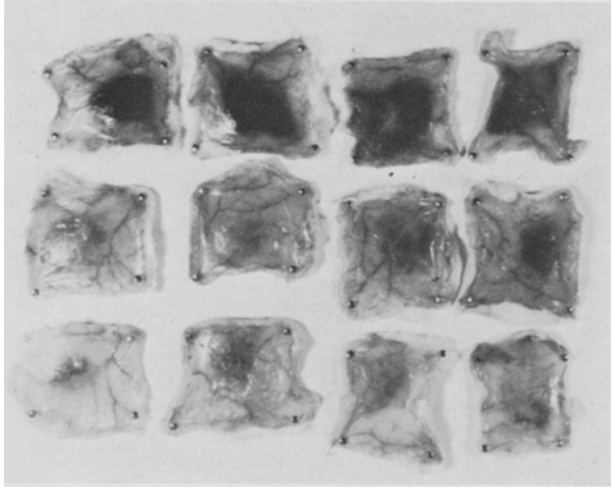

Fig. 2. Control. The excized skins (the dermis side) from the rabbits given intradermal injection of 50 μg of endotoxin followed 24 h later by 250 μg of endotoxin in 1 ml of saline i.v. 30 min prior to the provocative endotoxin injection animals were given 4 ml of 20% DMSO in saline i.v. Soybean trypsin inhibitor. The excized skins from rabbits subjected to the same treatment as the control group but given 2.4 mg of soybean trypsin inhibitor in 20% DMSO-saline i.v. prior to the provocative injection. Pepstatin. The excized skins from the animals with the same treatment of the controls but given 4 mg of pepstatin in 20% DMSO-saline, i.v. prior to the provocative endotoxin injection.

Under the experimental conditions, soybean trypsin inhibitor reduced the hemorrhagic lesions due to the SHWARTZMAN phenomenon on an average of about 65% with respect to the control. The acid proteinase inhibitor, pepstatin (mol. wt. 685.9), administered in a suspension of saline or 50% DMSO at 0.5, 1.0, and 2.0 mg dose per site, partially suppressed the manifestation of the localized SHWARTZMAN reaction. However, the extent of inhibition by pepstatin, on an overall average, was not as remarkable as the protective effect of soybean trypsin inhibitor (mol. wt. 21,500), which was compared at less than 1/20 the molar quantity of pepstatin. Since the dissociation constant (K_i) for cathepsin D-pepstatin complex is in the order of 10^{-8} to 10^{-9} M[20,21], 2 mg (about 3 μmoles) of pepstatin per site could be expected to effectively inhibit the acid proteinases potentially sequestered at the reaction loci. Thus, the steps which possibly involve acid proteinases appeared to be less critical to the development of the localized SHWARTZMAN reaction when compared with those involving the soybean trypsin inhibitor-sensitive proteinases. Nevertheless, the inhibitory effect of pepstatin was reproducible, and moreover, other oligopeptides, which inhibit cathepsin D and pepsin with K_i values of 10^{-7} to 10^{-8} M, showed a significant reduction in both the severity and incidence of hemorrhagic necrosis, while the non-inhibitor peptides with similar amino acid sequences did not (unpublished results). Compound A, which inhibits in vitro hemoglobinolysis by cathepsin D at pH 4 and by trypsin at pH 7, did not give a conclusive result, mainly because this reagent itself caused a direct inflammatory reaction of the skin. In one experiment, hydroxystilbamidine, a potent trypsin inhibitor in vitro (K_i, 6.2×10^{-6} M)[22], failed to suppress the SHWARTZMAN reaction.

The activity of proteinase inhibitors to reduce the localized hemorrhagic SHWARTZMAN reaction was also demonstrated by i.v. administration of inhibitors prior to the provoking intravenous endotoxin injection (Table II, Figure 2). Both pepstatin and soybean trypsin

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Table I. Suppression of the SHWARTZMAN reaction by proteinase inhibitors in skin prepared with endotoxin: intradermal administration of inhibitors[a]

Agent (dose range)	Hemorrhagic lesions			
	Area (mm^2) × intensity score (number of animals)	Suppression (%)	2% Na_2CO_3 extract of reaction loci[b] A_{430nm} (number of animals)	Suppression (%)
Saline control	764 ± 29.6[c] (23)[d]	—	10 ± 0.8[c] (10)	—
Soybean trypsin inhibitor (0.5–1 mg)	266 ± 23.9 (17)	65	6.1 ± 0.2 (9)	39
Pepstatin (0.5–2 mg)	410 ± 29.3 (17)	46	5.7 ± 0.9 (12)	43
Compound A (0.6–15 mg)	670 ± 106 (18)	12	5.9 ± 0.5 (10)	41

[a] The results of 5 separate experiments with different amounts of inhibitors are summarized by normalization of all other experimental data to the control level in the first experiment. The normalization factors ranged from 1.5 to 5.5. [b] A 16 cm^2 area of the reaction loci was extracted with 10 ml of 2% Na_2CO_3. The extract after filtration through glass wool was subjected to determination of A_{430nm} and protein concentration. [c] Standard error of mean. [d] About 74% of the animals showed a positive SHWARTZMAN reaction with an intensity score (1 to 4) of 3. The mortality rate in the control group was about 7% in 5 experiments.

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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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