transferred to a scintillation vial with scintillation liquid, and the ct/min were determined in a Packard Liquid Scintillation Counter. The results are expressed as the mean ct/min/10^6 lymphocytes of triplicate cultures±SE. Student's t-test was used to estimate the significance.

Results and discussion. When low doses of α-mercaptopropionyl-glycine (50 µg/ml) were added to lymphocyte cultures of healthy subjects, a significant ($p < 0.001$) stimulation of lymphocyte blastigenesis was found (figure 1). The dose-response curve shows that ^{3}H-thymidine incorporation by lymphocytes cultured in the presence of the drug was nearly twice that of control cultures. With increasing concentrations of α-mercaptopropionyl-glycine, radioisotope incorporation progressively decreased. At doses of 100 µg/ml, a slight degree of stimulation was still noted. However, this effect subsequently diminished and at doses of 500 and 1000 µg/ml a marked inhibition of the blastic response was observed ($p < 0.001$).

Figure 2 refers to the influence of α-mercaptopropionyl-glycine on mitogen stimulated cultures. Low doses had no effect on the blastic activity, whereas higher concentrations (500 and 1000 µg/ml) caused a definite inhibition ($p < 0.001$).

α-Mercaptopropionyl-glycine potentiates enzymes that have a −SH radical, such as coenzyme A. It induces several reactions that lead to the production of succinic acid, acetic acid and other intermediate compounds, thereby furnishing cellular energy[14]. However, high doses of thiolic compounds have an antagonistic action with respect to the enzymatic activities dependent on pyridoxal-phosphate[15]; recently, this effect has been confirmed with particular regard to lymphocytes[16]. One can therefore hypothesize that α-mercaptopropionyl-glycine interferes with the receptors that prime the mechanisms preceding DNA duplication. As described above, at low or high doses respectively, activation or inhibition of the enzymes involved in DNA synthesis may be a further possibility.

* The authors wish to thank Miss Yvonne Jurcik for her help with the preparation of the manuscript.

1 T. Shiraishi, K. Tsunematsu, S. Fukaura, Y. Ishida, Y. Okuuchi and A. Ohmachi, Proc. Int. Symp. Thioly, Tokyo 1970.
2 J.C. Crawhall, E.F. Scowen and R.W. Watts, Br. med. J. *I*, 588 (1963).
3 J.S. King, Jr, Proc. Soc. exp. Biol. Med. *129*, 927 (1968).
4 G. Virella and M.F. Lopez-Virella, Clin. exp. Immunol. *7*, 85 (1970).
5 G. Realdi, G. Amadori and G. Gasparotto, Acta med. patav. *31*, 1 (1971).
6 T. Nobunaga, Y. Nakamura and S. Kondo, Jap. J. clin. exp. Med. *43*, 1427 (1966).
7 A. Koda, Proc. 2nd Int. Symp. Thiola, Montego Bay 1972.
8 L. Binderup. E. Bramm and E. Arrigoni-Martelli, Scand. J. Immunol. *7*, 259 (1978).
9 T. Igarashi, M. Okada, T. Kishimoto and A. Yamamura, J. Immunol. *118*, 1697 (1977).
10 T. Monna, T. Yamada, H. Nakazawa, Y. Mizoguchi and S. Yamamoto, Pro. 2nd Int. Symp. Thiola, Montego Bay 1972.
11 A. Böyum, Scand. J. clin. Lab. Invest. *21*, suppl. 97, 77 (1968).
12 G. Semenzato, G. Amadori, F. Tosato and G. Gasparotto, Clin. exp. Immunol. *28*, 542 (1977).
13 G. Semenzato, G. Amadori, P. Sarasin and G. Gasparotto, Immunology *34*, 721 (1978).
14 K. Kuzuya, K. Mori, H. Inoue, T. Takanashi, S. Takamori and T. Sakanata, Jap. clin. exp. Med. *41*, 1879 (1966).
15 J.G. Heddle, E.W. Mc Henry and G.H. Beaton, Can. J. Biochem. Physiol. *41*, 1215 (1963).
16 K. Krzystyniak and J. Ryeaskj, Proc. 4th Eur. Immunology Meeting, Budapest 1978, abstr. 208.

An alkaline protease in the delayed hypersensitivity skin lesions in the guinea-pigs[1]

T. Kambara and M. Hashimoto

Department of Pathophysiology, Toxicology Institute, Kumamoto University Medical School, Kumamoto 860 (Japan), 24 October 1978

Summary. An alkaline hemoglobinolytic protease was extracted from the delayed hypersensitivity skin lesions induced by bovine γ-globulin as an antigen in the guinea-pig. The enzyme was heat-labile and inhibited by thiol-blocking reagents. The mol.wt was more than 100,000 and optimal pH around 9.

The mechanisms of delayed hypersensitivity reactions (DHR) are the focus of much current interest. However, the underlying biochemical reaction is still unknown. While lymphokines are thought to be putative soluble mediators of DHR[2,3], their relevance to in vivo events in cellular hypersenitivity is still unclear.

In a series of experiments to elucidate chemical agents in vivo sites of DHR, we have reported on the presence of macrophage-chemotactic factors in DHR skin sites in guinea-pig skin[4-6]. The present report adds to these reports the presence of an alkaline protease (ALPS) in the skin lesions of DHR.

Materials and methods. As previously reported[5,6], Hartley guinea-pigs, 300–500 g of both sexes, were sensitized by injecting into 4 foot pads 10 µg of bovine γ-globulin (BGG, Armour, Kankakee, Illinois, USA) emulsified in complete Freund's adjuvant. 7 days later, the animals were injected intradermally with 10 µg/site of BGG in the flanks of the animals in 20 sites. Precipitating serum antibody to BGG could not be demonstrated in any of the animals tested at this time. Inflamed skin sites, 24-h-old, were excised and dried in acetone according to the method of Hayashi et al.[7]. The extract, obtained from the skin acetone powder with 67 mM phosphate buffer, pH 7.4 (1 g/10 ml) for 4 h, was precipitated by ammonium sulfate (0–80% saturation) and the precipitate was redissolved in 10 mM phosphate-buffered 0.15 M NaCl, pH 7.4, and dialyzed against the same buffer for 16 h.

Proteolytic activity was measured utilizing ^{3}H-acetylated hemoglobin as a substrate[8]. Gel filtration was performed using Sephadex G-100 (Pharmacia, Uppsala, Sweden).

Results and discussion. 2 pH optima of the proteolytic activities were obtained at acidity and alkalinity in the inflamed skin extract (figure 1). It was of interest to note that the proteolytic activity in extracts of inflamed skin sites was 3 times as high as that of extracts from control unchallenged skin in sensitized animals at pH 9. The extract was found to contain mainly heat-labile and thiol protease active at pH 9 (table).

The extracts were subjected to Sephadex G-100 filtration. Main ALPS activity eluted in the void volume and was separated from acid protease activity which was retarded. The mol.wt of ALPS was thus estimated to be more than 100,000. The ALPS had optimal activity around pH 9. As

demonstrated in the table, the enzyme was heat-labile. Thiol-specific reagents, p-chloromercuribenzoic acid (1 mM) and p-chloromercuriphenylsulfonic acid (1 mM) were inhibitory, while thiol-reducing agent, dithiothreitol (5 mM), partially activated the enzyme. Activity was little affected by phenylmethylsulfonylfluoride (1 mM), soybean trypsin inhibitor (10^{-1} mM), disodium ethylene diaminetetra-acetate (5 mM) and pepstatin (5 mM). Trasylol (250 units) was inhibitory. These observations suggested that ALPS fraction contained mainly a thiol protease. In a preliminary experiment, a mixed mononuclear-polymorphonuclear infiltrate was obtained within 3 h of injection of this enzyme fraction into normal guinea-pig skin, suggesting its participation in cellular events in the inflammation. The role this enzyme plays in the expression of DHR, if any, such as production of macrophage-chemotactic factors[5,6] remains to be elucidated. Furthermore, the similarity of the present ALPS to lymphocyte neutral protease[9] raises an interesting question as to identification, difference or relation between them. The biological significance of proteases receives growing attention. However, literature on the distribution of neutral and alkaline thiol protease in the normal and pathological skin is scarce[10]. Hopsu-Havu and his group[10-14] recorded cathepsin B-like enzyme in the skin extracts of several animal species. The pH optimum of the enzyme is around 6. It is activated by thiol reagents and ethylene diaminetetra-acetic acid and has the mol. wt of 27,000–28,400. Accordingly, this enzyme is distinct from the present ALPS. Hayashi et al.[15,16] extracted 2 neutral proteases from rabbit skin Arthus reaction and thermal injury. These hydrolyzed casein optimally at pH 7.1 and hemoglobin at pH 6–7, and have mol. wts of 200,000 and 14,000. These enzymes seem to be different from the present ALPS because the pH optimum of the latter is around 9. In the absence of satisfactorily purified preparations, it is not at present possible to go beyond this statement.

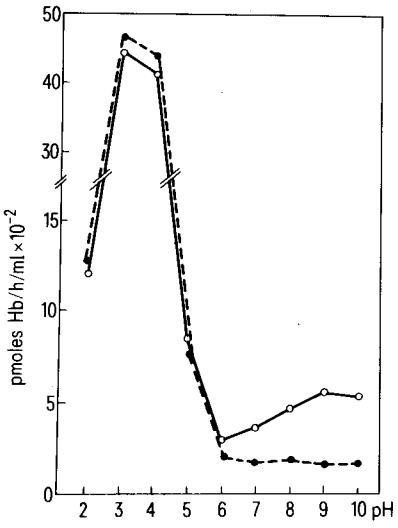

Fig. 1. pH curve of the proteolytic activities in the extracts of delayed hypersensitivity skin sites (O——O) and control unchallenged skin (● – – – ●) in guinea-pigs. The proteolytic activities at various pH were assayed in mixtures containing 50 µg of ^{3}H-acetylated hemoglobin (Hb), 50 µl buffer, and 50 µl sample. After 60 min incubation at 37 °C in a shaker, 50 µl of cold 2.5% Hb were added, followed by 100 µl of 50% trichloroacetic acid. The radioactivity of 200 µl acid-soluble material was determined by a Packard Tri-Carb scintillation spectrometer (Model 2450, Packard, New York, USA). Appropriate blanks consisting of 0.15 M NaCl or heated enzyme preparation (100 °C for 10 min) were incubated simultaneously. All assays were conducted in duplicate, and activity is expressed as pmoles Hb degraded per h per ml of the extract.

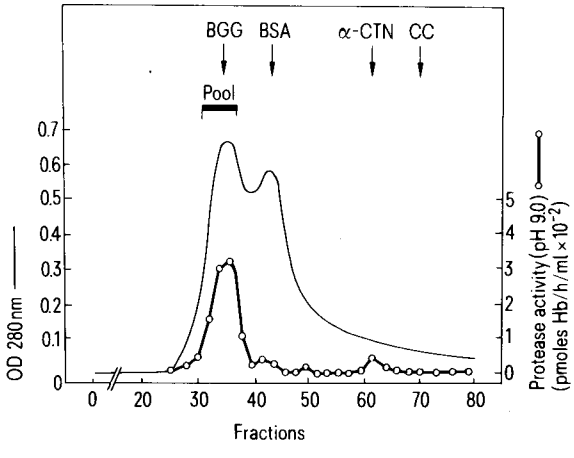

Fig. 2. Spehadex G-100 chromatography of ALPS. The extract was eluted on Sephadex G-100 equilibrated with 10 mM phosphate-buffered 0.15 M NaCl. Fractions (4 ml) were collected and assayed for absorbance at 280 nm (———) and pH 9.0 protease activities (O——O). BGG, bovine γ-globulin (mol. wt 150,000 Sigma, St. Louis, USA); BSA, bovine serum albumin (mol. wt 67,000, Sigma); α-CTN, α-chymotrypsinogen (mol. wt 25,000, Sigma); CC, cytchrom C (mol. wt 12,500, Sigma).

Properties of an alkaline protease of the delayed hypersensitivity skin lesions in guinea-pigs. The skin extract and Sephadex G-100 eluate of the enzyme was incubated with the compounds indicated at the final concentration designated for 20 min at room temperature before the substrate was added. Data are given as relative activity in duplicate assays

Treatment of enzyme	Final concentration (mM)	Relative activity (%) Extract	Sephadex G-100 eluate
None		100	100
Phenylmethylsulfonylfluoride	1	86	93
Soybean trypsin inhibitor	10^{-1}	87	85
p-Chloromercuribenzoic acid	1	27	46
p-Chloromercuriphenylsulfonic acid	1	25	47
Dithiothreitol	5	127	118
Disodium ethylenediaminetetraacetate	5	83	95
Pepstatin	5	103	97
Trasylol	250 (units)	70	61
Heating, 56 °C for 30 min		53	24

1 We thank Prof. Hideo Hayashi and Yoshimasa Morino, Kumamoto University, for valuable discussions. This work was supported in part by grants from the Ministry of Education in Japan, the Naito Research Grant, Tokyo, and the Mitsukoshi Foundation, Tokyo.

2 E. Pick and J.L. Turk, Clin. exp. Immunol. *10*, 1 (1972).
3 J.R. David, in: Progress in Immunology, p.339. Ed. B. Amos. Academic Press, New York 1971.
4 T. Kambara, H. Katsuya and S. Maeda, Acta path. jap. *22*, 465 (1972).
5 T. Kambara, K. Ueda and S. Maeda, Am. J. Path. *87*, 359 (1977).
6 K. Ueda and T. Kambara, Am. J. Path. *92*, 241 (1978).
7 H. Hayashi, H. Miyoshi, R. Nitta and K. Udaka, Br. J. exp. Path. *43*, 564 (1962).
8 M.B. Hille, A.J. Barrett, J.T. Dingle and H.B. Fell, Exp. Cell Res. *61*, 470 (1970).
9 T. Kambara, M. Hirata and T. Hiraoka, Experientia *34*, 526 (1978).

10 V.K. Hopsu-Havu, J.E. Fräki and M. Jarvinen, in: Proteinases in Mammalian Cells and Tissues, p. 545. Ed. A.J. Barrett. North-Holland, Amsterdam 1977.
11 C.T. Jansén and V.K. Hopsu-Havu, Acta derm.-vener., Stockh. *49*, 468 (1969).
12 J.E. Fräki and V.K. Hopsu-Havu, Arch. derm. Forsch. *243*, 52 (1972).
13 M. Järvinen and V.K. Hopsu-Havu, Acta chem. scand. *B29*, 671 (1975).
14 M. Järvinen and V.K. Hopsu-Havu, Acta chem. scand. *B29*, 772 (1975).
15 H. Hayashi, Int. Rev. Cytol. *40*, 101 (1975).
16 M. Koono, M. Muto and H. Hayashi, Tohoku J. exp. Med. *94*, 231 (1968).

Relationship between the pentose phosphate shunt and methemoglobin reductase activity in human erythrocytes: Effect of aging on methemoglobin reductase activity

C. Ioppolo, D.L. Currell[1], L. Civalleri and E. Antonini

Institute of Chemistry, Faculty of Medicine, University of Rome, C.N.R. Center of Molecular Biology, Roma (Italy), 23 October 1978

Summary. The increase in methemoglobin reductase activity in human erythrocytes upon incubation with inosine, phosphate, pyruvate occurs only in the presence of methylene blue. No difference in activity of the methemoglobin reductases was observed between enzyme extracts of fresh cells and aged cells.

It was reported earlier[2] that human erythrocytes incubated with inosine (10 mM), pyruvate (10 mM) and phosphate (5 mM) (IPP) showed an increase in methylene blue catalyzed methemoglobin reductase activity which was somewhat more prominent in fresh cells than in those stored for 16-30 days in ACD. Inosine is believed to influence red cell metabolism by providing substrate for the pentose phosphate shunt which in turn increases the flow of substrate into the glycolytic pathway[3].

The method used to estimate methemoglobin reductase activity involved following the rate of reduction of methemoglobin in the presence of methylene blue and either lactate or glucose as substrate after oxidation of the red cells with nitrite[4]. There is considerable evidence that the catalytic effect of methylene blue involves an NADPH-dependent methemoglobin reducing system[5,6]. Since methylene blue is known to increase pentose phosphate shunt activity which in turn supplies NADPH the work reported here was undertaken to explore further the relationships between inosine and methylene blue stimulation of methemoglobin reductase activity.

The lack of effect of IPP incubation on methemoglobin reductase activity in the absence of methylene blue shown in figure 1A and the large increase in activity in the presence of methylene blue (figure 1B) is consistent with the idea that the effects of both inosine and methylene blue are operative through the pentose phosphate shunt. The reduction of methylene blue by the NADPH produced by IPP incubation is known to be more rapid than the reduction of methemoglobin directly[5]. The reduced methylene blue can also reduce the methemoglobin nonenzymatically as has been shown by Beutler and Baluda[5] and Sass et al.[6].

Earlier work[7] with rabbit erythrocytes indicated that the loss of methemoglobin reductase activity through hemolysis was much less when the hemolysis was carried out in the presence of nicotinamide and NADPH. The results in figures 2A and 2B show that the low methemoglobin reductase activity in 26-day-old erythrocytes and their hemolysates is increased markedly for cells incubated with IPP and their hemolysates. Thus if the increased reductase activity upon incubation is due to production of NADPH

Comparison of methemoglobin reductase activity of 'crude enzyme extract' in fresh and aged erythrocytes

Extract	Pyridine nucleotide	$\frac{\mu\text{moles DCIP min}^{-1}}{\text{mg protein}} \times 10^3$
Red cells 30 days old	NADH	376
	NADPH	156
Fresh red cells	NADH	333
	NADPH	143

Assay performed by method of Sugita et al.[12]; NADPH dependent activity was determined in the absence of KCl.

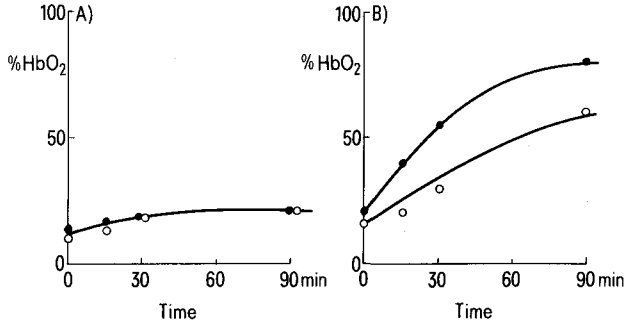

Fig. 1. Effect of incubation of human erythrocytes (stored 6 days in ACD buffer) with inosine (10 mM), pyruvate (10 mM), and phosphate (5 mM) on the rate of reduction of methemoglobin in 0.05 Tris HCl buffer, pH 7.4, 0.1 M NaCl, T = 37 °C: *A* in presence of glucose 20 mM; *B* glucose 20 mM, methylene blue, 5×10^{-5} M; ●, red cells incubated with IPP; ○, red cells without incubation.

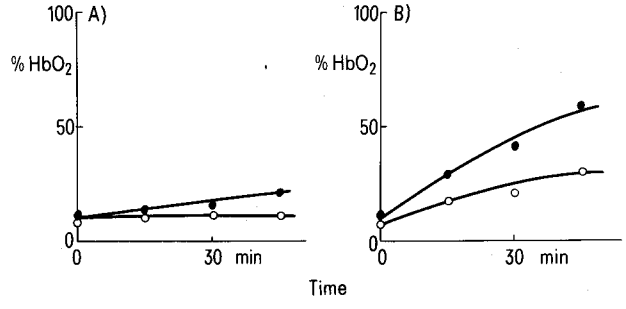

Fig. 2. Rate of reduction of methemoglobin in red cells from blood stored 26 days in ACD at 4 °C and their hemolysates: *A* before incubation of the cells with IPP; *B* after incubation with IPP. Conditions: glucose 20 mM, methylene blue 5×10^{-5} M, 0.05 Tris HCl buffer, pH 7.4, NaCl 0.1 M, T = 37 °C. ●, intact erythrocytes; ○, after hemolysis in 10 vol distilled water, centrifugation and resuspension in Tris buffer.